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COMPARATIVE STUDY ON THE COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF FENNEL HYDROLATES

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Abstract

The aim of this study was to investigate the composition and biological properties of hydrolates from fennel (Foeniculum vulgare L.*) seeds and their usefulness in food and biomedical applications. Hydrolates were obtained by steam- and hydro-distillation of fennel seeds followed by essential oil separation. GC-MS analysis indicated the presence of volatile compounds in the hydrodistilled extract. HPLC analysis of fennel hydrolates showed a higher concentration of phenolic compounds in the steam distilled extract. The antioxidant activity, determined as Trolox equivalent antioxidant capacity (TEAC) and cupric reducing antioxidant capacity (CUPRAC), was correlated to the total phenolic content of fennel hydrolates, being higher in the steam distilled extract. Thus, besides volatiles, the phenolic compounds could significantly increase antioxidant activity. In turn, higher inhibition of bacterial growth was found for the hydrodistilled extract, due to the significant number of volatile compounds. In conclusion, fennel hydrolates represent a waste of significant interest for valorization within the circular bioeconomy and further application in the food and biomedical industry.*

Key words*: Foeniculum vulgare, by-product, phenolics, antioxidant activity, antimicrobial activity.*

INTRODUCTION

Fennel (*Foeniculum vulgare* L.) is a member of the Apiaceae family, native to the Mediterranean area, but globally widespread due to increased demand for fennel essential oil. Traditionally, the fennel seed oil is used for culinary purposes, due to its flavouring properties, being also a valuable ingredient of cosmetic and pharmaceutical products (Badgujar et al., 2014; Dahmani et al., 2022). Moreover, fennel essential oil has demonstrated insecticidal effects against different species of aphids (Digilio et al., 2008; Pavela, 2018; Dunan et al., 2021), suggesting its potential use as a biopesticide for the protection of plants in culture.

During essential oil production by hydrodistillation or steam distillation, large quantities of floral water residues or hydrolates are generated, but they are generally discarded. However, hydrolates have been reported to contain important quantities of bioactive compounds, such as water-soluble volatile substances (oxygenated monoterpenes, sesquiterpenes, hydrocarbon derivatives) (Acimovic et al., 2020; Gaspar-Pintiliescu et al., 2022a). These compounds are responsible for the antimicrobial activity of fennel hydrolates. Previous studies have reported that fennel hydrolates have exerted strong inhibition on the growth of the *Aspergillus parasiticus* NRRL 2999 strain (Ozcan, 2005). Also, fennel hydrolates were 50 times concentrated using solid phase extraction and exhibited significant antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Candida albicans* strains, but high resistance in the case of several strains of Arcobacter-like bacteria (Silha et al., 2020). Moreover, the biofilm formation was significantly reduced by the concentrated hydrolate. Another study has reported no antimicrobial activity of fennel hydrolate tested in various Gram-positive and Gram-negative bacterial strains (Sagdic and Ozcan, 2003).

From safety point of view, fennel hydrolate exhibited no cytotoxicity effect in human A549 lung carcinoma cells, suggesting their applicability in the food and biomedical industry (Silha et al., 2020).

Regarding the antioxidant activity of fennel hydrolate, there is scarce information. A study mentioned its ferric reducing antioxidant power (FRAP), due to the volatile compounds content (Lado et al., 2004). No studies on the polyphenolic composition of fennel hydrolates and their valuable bioactivity that could modulate the biological properties were found.

In this context, the aim of this paper was to investigate the chemical composition of Romanian fennel seeds hydrolates obtained by steam- and hydro-distillation and the correlation of both monoterpenes and polyphenols content to their antioxidant and antimicrobial activity, to improve their food and biomedical applications.

MATERIALS AND METHODS

Plant material and chemicals

Fennel (*Foeniculum vulgare* L.) seeds were harvested from ecological cultures of HOFIGAL Export-Import S.A. Romania. They were dried, ground, and stored in a cool and dry place, until the extraction process. All used reagents were of analytical grade and purchased from Sigma-Aldrich (Germany), unless otherwise specified.

Extraction of fennel hydrolates

The hydrolates were obtained from fennel seeds by distillation using a Clevenger-type extraction apparatus (J.P. Selecta, Spain). The ground material (25 g) was moistened in ultrapure water. Two different extraction methods were applied, one based on steam distillation using distilled water as a steam source (Hay et al., 2015) and the other on hydrodistillation (Silha et al., 2020) using ultrapure water (750 mL), at 100°C, for 2 h. At the end of the distillation process, the essential oil was separated from the aqueous fraction, representing the fennel hydrolates, based on density difference. The steam distilled and hydrodistilled fennel hydrolates were filtered and stored in the dark, at 4°C, until analysis. The extraction yield was determined as a percentage of initial dry weight (d.w.).

Gas-chromatography/mass-spectrometry (GC-MS) analysis

Fennel hydrolates were analyzed by GC-MS using Focus GS-type equipment coupled to a mass spectrometer DSQ II (Thermo Electron Corporation, USA), as previously described (Mihai et al., 2021). Briefly, the separation was performed on Macrogol 20000 R capillary column (30 m x 0.25 mm i.d. and 0.25 µm film thickness). The carrier gas was helium at a flow rate of 1 mL/min. The mass spectrometer was operated at 70 eV with a scan interval of 0.5 s and scan range between 40-1000 m/z. The identification of the main constituents was performed by comparing the spectra with NIST mass spectral database.

HPLC analysis

Fennel hydrolates were investigated by HPLC analysis on a reverse phase column C18 Zorbax Eclipse XDB (150 x 4.6 i.d. mm) using an Agilent 1200 HPLC system consisting of a quaternary pump, thermostated autosampler, and diode array detector (Agilent, Germany). A sample (10 µL) was injected and then eluted using mobile phase A consisting of 2 mM sodium acetate, pH 3, and mobile phase B, acetonitrile, using the following gradient: 2-20% B, 0-30 min; 20-30% B, 30-40 min; 30% B, 40-50 min; 30-2% B, 50-60 min (Craciunescu et al. 2012). of Compounds identification was performed by comparison to the retention time of standard phenolic acids (gallic acid, chlorogenic acid, caffeic acid, ferulic acid) and flavonoids (rutin, quercetin 3-O-glucoside (isoquercetin), quercetin, luteolin, apigenin, kaempferol) (Sigma-Aldrich, Germany). The identified compounds were quantified by peak area integration.

Determination of total phenolic and flavonoid content

Total phenolic content was determined by the Folin-Ciocalteu method, as previously described (Gaspar-Pintiliescu et al. 2022b). Briefly, the sample was mixed with Folin-Ciocalteu reagent (1:5, v/v) and incubated in the dark, for 5 min. Then, 2 mL of 12% sodium carbonate were added and the mixture was incubated at room temperature, for 30 min. The optical density (OD) was measured at 765 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). The standard curve was built using different concentrations of caffeic acid in

the range of concentrations 0-500 µg/mL. The results were expressed as caffeic acid equivalents (CAE).

Total flavonoid content was determined by the aluminum chloride method, as previously described (Gaspar-Pintiliescu et al., 2022b). Briefly, the sample was mixed with methanol (1: 3, v/v), and then, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water were added, and incubation was performed at room temperature, for 30 min. The OD was read at 415 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). The standard curve was built using different concentrations of quercetin in the range of 0-150 µg/mL. The results were expressed as quercetin equivalents (QE).

Determination of Trolox equivalent antioxidant capacity (TEAC)

The TEAC assay was performed according to the protocol of Hay et al. (2015) with minor modifications. Briefly, a stock solution was prepared by mixing solutions of 7 mM 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2.45 mM potassium persulfate (1:1, v/v) and then, incubated at room temperature, in the dark, for 16 h. Before the experiments, the stock solution was diluted to an OD of 0.70 ± 0.02 at 734 nm. Different concentrations of the sample were mixed with ABTS solution (1:10, v/v) and incubated at room temperature, in the dark, for 10 min. The OD was read at 734 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). The standard curve was built using Trolox, an analog of vitamin E, in the range of concentrations 0-250 µM. The results were expressed as Trolox equivalents (TE).

Determination of cupric ion reducing antioxidant capacity (CUPRAC)

The CUPRAC assay was performed according to an adapted protocol of Georgiev et al. (2019). Briefly, 1 mL of 10 mM $CuCl_2$ solution was mixed with 1 mL of 7.5 mM neocuproine and 1 mL of 1 M ammonium acetate buffer, pH 7. The mixture was vortexed and incubated at room temperature, for 10 min, to develop the complex. Then, 100 µL of sample and 1 mL of distilled water were added and incubation continued at room temperature, for 1 h. The OD was read at 450 nm using an UV-VIS spectrophotometer (V-650, Jasco, Japan). A blank was prepared by sample replacement with distilled water. The standard curve was built using Trolox in the range of concentrations 0.1-1.0 mM. The results were expressed as TE.

Antibacterial activity evaluation by microdilution assay

The antibacterial activity of fennel hydrolates was tested in *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 9027) strains by serial microdilution assay, as previously described (Saviuc et al. 2017). Briefly, bacterial cultures were grown in brain heart infusion broth at 35°C, for 24 h, and the working culture was adjusted at a concentration of 10^8 UFC/mL to 0.5 McFarland standard. Then, serial dilutions of the sample (0.1-7 mg/mL) were incubated with microbial suspension in the wells of a 96-well microplate, at 35°C, for 24 h. The OD was read at 600 nm using a SPEC-TROstar Nano microplate reader (BMG Labtech, Germany). A bacitracin solution served as positive control. The minimum inhibitory concentration (MIC) was determined as the minimum concentration that inhibited microbial growth, compared to the control.

Statistical analysis

The experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD) (n = 3). Statistical analysis was performed by two-tailed, two-sample equal variance Student *t*-test on control-sample pairs of interest. Differences were considered statistically significant at $p<0.05$.

RESULTS AND DISCUSSION

Preparation of fennel hydrolates

Fennel hydrolates were obtained during the steam- and hydro-distillation process of essential oil preparation, as colored solutions with a pleasant odor. These methods gave extraction yields of 12.33±0.89% (w/w) and 17.68±1.54% (w/w), respectively.

Volatile compounds composition

GC-MS analysis of fennel hydrolates has shown that the extract obtained by hydrodistillation contained volatile substances, while the steam distilled extract presented only traces (not determined). The retention time of the identified compounds and their relative percentage are presented in Table 1.

Table 1. Composition of volatile compounds determined by GC-MS of the hydrolate obtained from fennel seeds by hydrodistillation

No.	Compound	Retention time	% of total peak area
	Monoterpenes hydrocarbons		
1.	α-pinene	6.23	5.16
2.	β-pinene	9.61	0.36
3.	β-phellandrene	10.43	0.11
4.	δ-3-carene	11.84	0.15
5.	α-phellandrene	12.76	4.46
6.	myrcene	13.06	0.47
7.	limonene	14.68	1.50
8.	trans-β-ocimene	17.27	0.30
9.	α-terpinene	17.51	0.53
10.	o-cymene	19.01	0.48
	Oxygenated monoterpenes		
11.	fenchone	26.49	8.09
12.	camphor	33.44	0.09
13.	linalool	36.57	0.12
14.	estragole	43.02	1.36
15.	trans-anethole	51.66	76.82
	Monoterpenes hydrocarbons		**13.52**
	Oxygenated monoterpenes		**86.48**

Fennel hydrolate obtained by hydrodistillation contained 15 volatile compounds, from which monoterpenes hydrocarbons represented 13.52% and their oxygenated derivatives represented 86.48%. The most abundant constituents were trans-anethole (76.82%), fenchone (8.09%), and estragole (1.36%), but also α-pinene (5.16%), α-phellandrene (4.46%) and limonene (1.5%) were present.

Previous studies have reported that trans-anethole, fenchone, estragole, and limonene were the major compounds found in fennel essential oils (Anwar et al., 2009; Damayanti and Setyawan, 2012; Ahmed et al., 2019; Kalleli et al., 2019). However, their proportion varied according to plant maturation stage, harvesting season, climate, and geographical conditions (Telci et al., 2009; Ahmed et al., 2019). Fenchone was proposed as a biomarker of various commercial formulations of essential oil and extracts of *F. vulgare* seeds (Alam et al., 2019). Fennel hydrolates usually contain low quantities of volatile compounds, regardless of the used extraction method. Thus, the hydrolates of Czech fennel seeds obtained by steam- and hydro-distillation were rich in the oxygenated monoterpenes estragole and fenchone (Silha et al., 2020), which were also predominantly found in the composition of the hydrodistilled fennel hydrolate.

Polyphenolic compounds composition

Several polyphenolic compounds were identified and quantified in both fennel hydrolates by HPLC analysis (Table 2).

Table 2. Concentration of polyphenolic compounds in fennel seeds hydrolates obtained by steam- and hydro-distillation, determined by HPLC analysis. The results are expressed as mean ± standard deviation (n = 3)

No.	Compound	Fennel hydrolate obtained by steam distillation (μg/g d.w.)	Fennel hydrolate obtained by hydrodistillation (μg/g d.w.)
	Phenolic acids		
1.	Gallic acid	ND	ND
2.	Chlorogenic acid	6.85 ± 0.31	1.97 ± 0.07
3.	Caffeic acid	10.95 ± 0.45	9.40 ± 0.34
4.	Ferulic acid	8.56 ± 0.38	6.76 ± 0.26
	Flavonoids		
5.	Rutin	ND	ND
6.	Isoquercetin (Quercetin 3-glucoside)	3.20 ± 0.16	2.52 ± 0.10
7.	Quercetin	1.53 ± 0.07	ND
8.	Luteolin	ND	ND
9.	Apigenin	ND	ND
10.	Kaempferol	ND	ND

ND - not determined.

The results have shown that chlorogenic, caffeic, and ferulic acid were the major compounds identified in both fennel hydrolates, but higher quantities were found in the steam distilled extract. Quercetin and its glucoside form, isoquercetin were the main flavonoids detected in the steam distilled extract. Previous studies have also indicated the presence of chlorogenic, caffeic, and ferulic acids, and quercetin in alcoholic extracts of fennel seeds (Roby et al., 2013; Odeh and Allaf, 2017). No HPLC studies on fennel hydrolates were found.

The total phenolic and flavonoid content of fennel hydrolates is presented in Table 3. The results have shown that the values of total phenolic (11.15 mg/100 g d.w.) and flavonoid (2.17 mg/100 g d.w.) content of fennel hydrolate obtained by steam distillation were ~2-fold higher than those found in the hydrodistilled extract. Previous studies have reported values of total phenolic content between 250-4224 mg/100 g for fennel seed ethanolic extracts, according to the geographical area, solvent, temperature, and extraction method (Ahmed et al., 2019; Kalleli et al., 2019; Malin et al., 2022). No reports were found for fennel hydrolates.

Table 3. Total phenolic and flavonoid content and the antioxidant activity of fennel hydrolates. The results are expressed as mean ± standard deviation (n = 3)

Sample	Total phenolic content (mg CAE/ 100 g d.w.)	Total flavonoid content (mg QE/ 100 g d.w.)	TEAC (mM TE/ g d.w.)	CUPRAC (mM TE/ g d.w.)
Fennel hydrolate by steam distillation	11.15 ± 0.39	2.17 ± 0.02	626.12 ± 35.79	365.50 ± 14.48
Fennel hydrolate by hydro-distillation	6.49 ± 0.24	0.79 ± 0.01	375.99 ± 20.79	320.07 ± 23.93

Antioxidant activity of fennel hydrolates

The antioxidant activity of fennel hydrolates was assessed by two different methods, i.e., TEAC assay based on hydrogen atom transfer (HAT) mechanism of free radicals scavenging and CUPRAC assay based on single electron transfer (SET). The results are presented in Table 3. The TEAC of fennel hydrolate obtained by steam distillation (626.12 mM TE/g d.w.) was 1.67 higher than that of the hydrodistilled extract (375.99 mM TE/g d.w.). The CUPRAC of the steam distilled hydrolate (365.50 mM TE/g d.w.) was close to that registered for the hydrodistilled extract (320.07 mM TE/g d.w.).

These data have shown that the antioxidant activity of fennel hydrolates varied directly proportional to the total phenolic content. The volatile compounds, in particular anethole and phellandrene, might be also involved in the antioxidant capacity of hydrolates, as previously reported, due to their ring structure with delocalized electrons (Lado et al., 2004).

Antimicrobial activity of fennel hydrolates

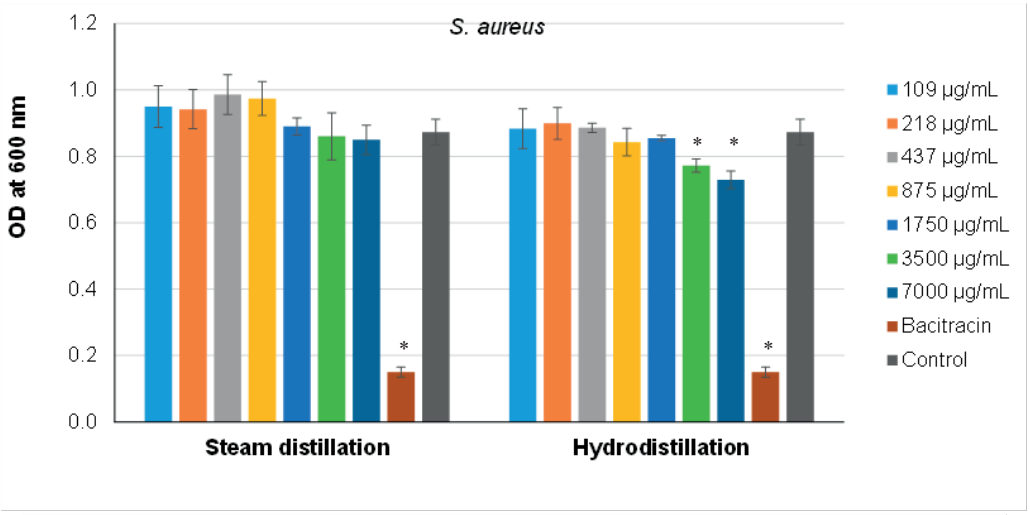
The antimicrobial activity of fennel hydrolates was evaluated in the cultures of the Gram-positive *S. aureus* bacterial strain and the Gram-negative *P. aeruginosa* strain. The results are presented in Figure 1.

The results showed that the steam distilled hydrolate had no antimicrobial activity against any of the tested bacterial strains. The hydrolate obtained by hydrodistillation could inhibit the bacterial growth of *S. aureus* and *P. aeruginosa* strains at a MIC of 3500 µg/mL. A previous study on hydrodistilled fennel hydrolate has shown *S. aureus* inhibition, to a similar extent to fennel essential oil or extract (Silha et al., 2020). This activity was mostly attributable to the volatile compounds present in the fennel hydrolate obtained by hydrodistillation. The compounds containing several functional groups were reported to exert higher antimicrobial activity, due to their affinity to bind the bacterial cell membrane (Basavegowda and Baek, 2021). Thus, oxygenated monoterpenes, such as linalool, eucalyptol, menthol, and thymol, and the monoterpene hydrocarbons pinene, terpinene, and limonene showed the capacity to inhibit bacterial growth (Badawy et al., 2019). In addition, a study on fennel essential oil showed its antifungal activity against *Aspergillus niger* and *Penicillium expansum*, at doses between 14-19 µg/mL (Olaru &Popa, 2019).

CONCLUSIONS

All these data have shown that significant quantities of water-soluble bioactive polyphenolic and volatile compounds were present in fennel hydrolates. The fennel hydrolate obtained by steam distillation had higher total phenolic content, compared to that of the hydrodistilled extract, and good antioxidant activity. The hydrodistilled fennel hydrolate exerted antimicrobial activity, while the steam distilled extract had no activity in *S. aureus* and *P. aeruginosa* cultures. This activity could be correlated to the higher content of volatile compounds found in the hydrodistilled extract. In conclusion, it was demonstrated that the composition of fennel

hydrolates varied with the distillation method and provided different antioxidant and antimicrobial activity. This study provided valuable information on fennel hydrolates for further use in food or biomedical applications.



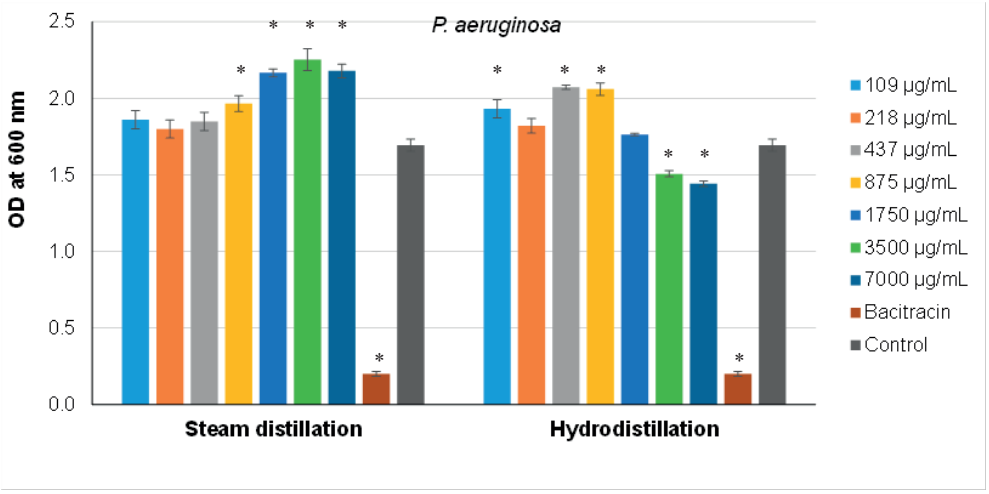


Figure 1. Bacterial growth inhibition of *S. aureus* and *P. aeruginosa* in the presence of fennel seeds hydrolates, after 24 h. The results are expressed as mean ± SD (n = 3). *p<0.05, compared to control.

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