

Phenolic compounds from *Foeniculum vulgare* (Subsp. *Piperitum*) (Apiaceae) herb and evaluation of hepatoprotective antioxidant activity

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ABSTRACT

Objective: The study was designed to evaluate the antioxidant and hepatoprotective activities of the 80% methanolic extract as well as the ethyl acetate (EtOAc) and butanol (BuOH) fractions of the wild fennel (*Foeniculum vulgare* (Subsp; *Piperitum*)) and cultivated fennel (*F. vulgare* var. *azoricum*). In addition, quantification of the total phenolic content in the 80% methanol extract of fennel wild and cultivated herbs is measured. **Materials and Methods:** An amount of 400 g of air dried powdered herb of wild and cultivated fennel were sonicated with aqueous methanol (80%), successively extracted with Hexane, EtOAc, and n-BuOH. The EtOAc and n-BuOH were subjected to repeated column chromatography on silica gel and Sephadex LH-20. The antioxidant effect was determined *in vitro* using 1,1-diphenyl-2-picrylhydrazyl (DPPH). Hepatoprotective activity was carried out using a Wistar male rat (250–300 g). Total phenolic and flavonoid contents were determined as chlorogenic acid and rutin equivalents, respectively. **Results:** Two phenolic compounds, i.e., 3,4-dihydroxy-phenethylalcohol-6-O-caffeoyl-β-D-glucopyranoside and 3',8'-binaringenin were isolated from the fennel wild herb, their structures were elucidated by spectral methods including 1D NMR, 2D NMR, and UV. The EtOAc and BuOH fractions of wild fennel were found to exhibit a radical scavenging activity higher than those of cultivated fennel. An *in vitro* method of rat hepatocytes monolayer culture was used for the investigation of hepatotoxic effects of the 80% methanol extract on the wild and cultivated fennel, which were > 1000 and 1000 μg/mL, respectively. As well as, their hepatoprotective effect against the toxic effect of paracetamol (25 mM) was exerted at 12.5 μg/mL concentration. **Conclusions:** Fennel (*F. Vulgare*) is a widespread plant species commonly used as a spice and flavoring. The results obtained in this study indicated that the fennel (*F. vulgare*) herb is a potential source of natural antioxidant. Two phenolic compounds, i.e. 3,4-dihydroxy-phenethylalcohol-6-O-caffeoyl-β-D-glucopyranoside (**A**) and 3',8'-binaringenin (**B**) were isolated from the fennel wild herb for the first time.

Key words: Antioxidant (Apiaceae), azoricum, binaringenin, *Foeniculum vulgare*, hepatoprotection, phenolic compounds, *piperitum*

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INTRODUCTION

Fennel (*Foeniculum vulgare* Miller, Apiaceae) is one of the most widespread plants of the Apiaceae family (formerly the Umbelliferae). It is a perennial herb and a well-

known Mediterranean aromatic plant,^[1,2] but nowadays it can be found in several temperate areas of the world, both as wild and cultivated species, commonly used as a spice and flavoring and in folk medicine as a carminative, digestive, lactagogue, and diuretic.^[3] Fennel has been used in the folk medicine for centuries to cure digestion and respiratory disorders, and its herbal drug preparations are used for dyspeptic complaints such as mild, spasmodic gastrointestinal complaints, bloating, and flatulence.^[4] Fennel is also used for catarrh of the upper respiratory tract. Despite being widely studied for its essential oils by

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gas chromatography-mass spectrometry (GC-MS),^[5] little information is available on the nonvolatile constituents of the fennel. Polyphenolic compounds are associated with the prevention of disease assumed to be induced by oxidative stress, such as cardiovascular diseases, cancer, and inflammation. The possible protective effects reported are generally associated with the antioxidant activity of the polyphenolics.^[6] The purpose of this study was to evaluate the hepatoprotective and antioxidant activities of the 80% of methanol extracts of the fennel herb and to elucidate their antioxidative actions. In this report, we describe the isolation and structure elucidation of two phenolic compounds: 3,4-dihydroxy-phenethylalcohol-6-*O*-caffeoyl- β -D-glucopyranoside and 3',8'-binaringenin which were isolated for the first time from *F. vulgare* subsp. *Piperitum*. The structural elucidation has been performed by spectral analysis, including 1D NMR, 2D NMR, and UV techniques.

MATERIALS AND METHODS

Plant material

Wild fennel (*F. vulgare* subsp. *Piperitum*) were collected during the flowering period (March 2009) from Marsa-Matroh area, Egypt. The cultivated fennel (*F. vulgare* var. *azoricum*) was collected from the Sekem Company Plantation, Bilbase city, Egypt (March 2009). The plants were kindly authenticated by Prof. Dr. Ibrahim El-Garf, Department of Botany, Faculty of Science, Cairo University. The herb was dried in oven at 40 °C, finely powdered and kept for phytochemicals and biological studies.

Extraction and isolation

First, 400 g of air dried powdered herb of wild and cultivated fennel were sonicated with aqueous methanol (80%) (3 × 1 L). The extracts were combined and evaporated under reduced pressure at 45 °C to yield a dark greenish black residue (about 72.5 g).

The residue was dissolved in 200 mL dist. H₂O and was successively extracted with hexane, ethyl acetate (EtOAc) and n-butanol (n-BuOH) (3 × 500 mL for each). Fractions were evaporated under reduced pressure. The fractions were examined by thin-layer chromatography (TLC) using the solvent system: ethyl acetate:formic acid:acetic Acid:H₂O (30:1.2:0.8:8). It was found that both EtOAc and n-BuOH contain the same compounds on TLC. Therefore, they were collected together (EB). About 10 g of the EB fraction was subjected to column chromatography using silica gel. The elution was carried out firstly with 100% hexane, followed by methylene dichloride/hexane in the ratio of 1:1, 100% CH₂Cl₂ and then increasing the polarity by 10% stepwise using MeOH until reaches 100% MeOH.

The similar fractions were collected together, concentrated under reduced pressure at temperature 45 °C.

The fractions were monitored by TLC on silica gel plates (Merck) (Darmstadt, Germany) developed with the solvent system: EtOAc–HCOOH–AcOH–H₂O (30:1.2:0.8:8). Spots were detected using Neu's spray reagent (1% diphenylboric acidethanolamine complex in methanol)^[7] and visualized under UV light at 254 and 365 nm. The fractions eluted with CH₂Cl₂/MeOH (80:20) and MeOH were collected together and subjected to Sephadex LH-20 CC using H₂O, H₂O/EtOH, and EtOH as eluent. The fraction eluted with H₂O/EtOH and EtOH were collected together and subjected again to Sephadex LH-20 CC using MeOH as eluent to afford compounds A and B.

Determination of total phenolic content

Eighty percent of the methanolic extract was prepared using 5 g of the dried powder herb of both wild and cultivated fennel. The yield of 80% methanolic extract was completed to 100 mL with methanol in a measuring flask. Total phenolic content (TPC) were measured as chlorogenic acid equivalents using (UV/VIS, 2041 spectrophotometer). The Folin–Ciocalteu method was used to determine the TPC according to the method described by Meda *et al.*^[8] From each extract, 1 mL was mixed with 0.5 mL of 0.2 N Folin–Ciocalteu reagent for 5 min and 1 mL of saturated solution of Na₂CO₃ (40 g/100 mL) was then added. After incubation at room temperature for 30 min, the reaction mixtures were diluted to 10 mL with deionized water. Absorbance of the clear solution was measured at 725 nm. Concentration of the total phenolic content was determined from a standard calibration curve using chlorogenic acid as standard.

Determination of total flavonoid content

Total flavonoid content (TFC) was determined using a method adapted by Meda *et al.*^[8] According to that method, 3 mL of 2% AlCl₃ in methanol and 2 mL methanol were mixed separately with 3 mL of 80% alcoholic extract and absorption reading at 415 nm (UV/VIS, 2041 spectrophotometer) was taken after 30 min against a blank sample consisting of a 3 mL of AlCl₃ with 3 mL of methanol.

Free radical scavenging activity (DPPH)

The sample was measured in terms of hydrogen-donating or radical-scavenging ability using the stable radical DPPH•. Briefly, 1.5 mL of a DPPH methanolic solution (20 mg/L) was added to 0.75 mL of extract, at different concentrations ranging from 10 to 200 μ L/mL (methanol for the control). The absorbance was measured at 517 nm after 20 min of reaction. The percent of DPPH of the sample was calculated according to the following equation:

% decolorization $[1 - (\text{Abs. sample}/\text{Abs. control}) \times 100]$. The decoloration was plotted against the sample extract concentration, and a logarithmic regression curve was established to calculate the IC_{50} , which is the amount of sample necessary to decrease by 50% the absorbance of DPPH.^[9]

Rat hepatocytes monolayer culture

Isolation and preparation of rat hepatocytes monolayer culture

A primary culture of rat hepatocytes was prepared according to the Seglen method,^[10] which was modified by Kiso *et al.*,^[11] using a Wistar male rat (250–300 g). The rat was obtained from the animal house of the NRC (National Research Center, Cairo). Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals.^[12]

IC_{50} determination on rat hepatocytes monolayer culture

After 22–24 h, the rat hepatocyte monolayer was washed twice with phosphate buffer saline (PBS). In order to determine IC_{50} , different concentrations were prepared for each sample (100–1000 $\mu\text{g mL}^{-1}$). After 2 h of cells incubation with the extract, cell viability was determined using the MTT assay. The assay was performed according to the method of Mosmann,^[13] modified by Carmichael *et al.*^[14] Absorbance of formazan crystals produced by viable cells was read at 540 and 630 nm dual wavelength using the Automatic Kinetic Microplate Reader (Labsystems Multiskan RC reader). Each experiment was repeated three times, and the mean absorption of each concentration was calculated. A graph plotted with x -axis showing the different concentrations of the extract used and the y -axis showing the absorbance percentage of viable cells. The IC_{50} was graphically determined from the concentration that yielded an absorption coinciding with the 50% of cells that received no extract.

Evaluation of hepatoprotective activity

The primary rat hepatocyte monolayer was prepared as in Section (*Rat hepatocytes monolayer culture*). Different concentrations were prepared from the 80% methanol (12.5–100 $\mu\text{g/mL}$) using the serial dilutions technique by dissolving in DMSO (1% maximum concentration). For each concentration, three replicates were carried out; in addition to positive control, that was 50 $\mu\text{g/mL}$ Silymarin. The plate was incubated for 2 h at 37°C and 5% CO_2 , then washed twice with PBS. A 200 μL of 25 mM paracetamol was added to each well. After 1 h of cells incubation with the paracetamol, cell viability was determined using the MTT assay. The concentration of the extract that was able to protect the cells from the hepatotoxic effect of

paracetamol by 100% was considered hepatoprotective.

RESULTS

Spectrometric identification of compounds A and B

Compounds **A** and **B** were identified as the 3,4-dihydroxy-phenethylalcohol-6-*O*-caffeoyl- β -D-glucopyranoside and 3',8'-binaringenin depend on the spectral data and compared with the published data.^[15,16]

Quantitative estimation of TPC and TFC

TPC

TPC of both 80% methanol extracts expressed as mg of chlorogenic acid equivalents/1 g herb could be calculated from the following equation

$$Y = 6.4X + 0.065$$

where Y is the absorbance and X is the corresponding concentration mg/mL. The wild fennel contains 2.4% and the cultivated one contains 3.1%.

TFC

TFC was determined using a calibration curve with rutin as standard. TFC of 80% alcoholic extract was expressed as mg of rutin equivalents/1 g of herb could be calculated from the following equation:

$$Y = 6.24X - 0.01$$

Flavonoid content of wild is 1.2% and cultivated fennel is 1.6%.

Hepatotoxicity

The assay was applied with a broad range of concentrations of the studied extracts (from 125 to 1000 $\mu\text{g/mL}$) on the monolayer of rat hepatocytes. It revealed that the 80% methanolic extract of the wild and cultivated fennel had IC_{50} effects at a concentration of >1000 and 1000 $\mu\text{g/mL}$, respectively [Figure 1].

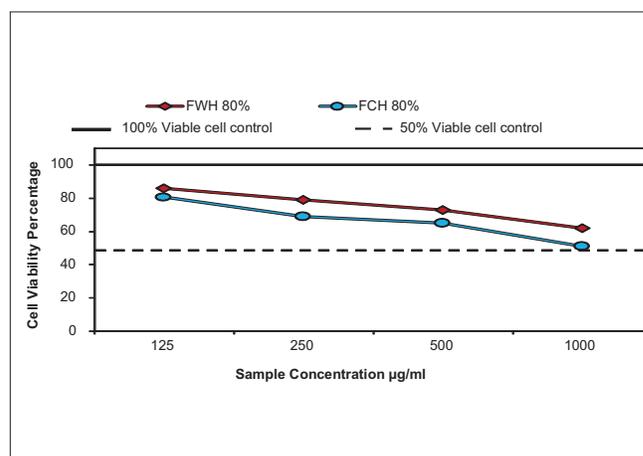


Figure 1: Viability of monolayer of rat hepatocytes after 2 h treatment with different concentrations of the extracts using the MTT calorimetric assay

Evaluation of hepatoprotective activity

The hepatoprotective effects of both 80% methanolic extracts of wild and cultivated fennel herb against the toxic effect of 25 mM paracetamol on the monolayer hepatocyte cells was 12.5 µg/mL.

From the results of the hepatoprotective and hepatotoxic effect of the methanolic extract of wild and cultivated fennel, we can conclude that they showed a safety margin, as the hepatotoxicity dose is 80 folds that of the hepatoprotection dose [Figure 2].

Free radical scavenging activity (DPPH)

Radical scavenging activity (expressed as absorbance percentage) of *F. vulgare* subsp. *piperitum* (wild) and *F. vulgare* subsp. *azoricum* (cultivated) extracts [Table 1 and Figure 3] as well the two isolated compounds [Table 2].

DISCUSSION

Two compounds **A** and **B** were isolated for the first time from the wild fennel and identified as 3,4-dihydroxyphenethylalcohol-6-*O*-caffeoyl-β-D-glucopyranoside and 3',8'-binaringenin, respectively. Their structures were elucidated by spectral methods including 1D NMR,

2D NMR and UV. The total phenolic and flavonoid contents of the wild fennel and cultivated fennel were determined according to the Meda method.^[8] It revealed that the cultivated fennel had more phenolic and flavonoid contents (3.1% and 1.6%, respectively) than that found in the wild fennel (2.4% and 1.2%; respectively). The 80% methanolic extract of the wild and cultivated fennel showed a hepatoprotective effect at a concentration of 12.5 µg/mL and a hepatotoxic effect at a concentration of 1000 µg/mL. From this we can conclude that they both show a safety margin, as the hepatotoxicity dose is 80 folds that of the hepatoprotective dose.

The antioxidant activity was measured as free radical scavenging activity (DPPH). The ethyl acetate and butanol fractions of the wild fennel were found to exhibit a radical scavenging activity higher than those of the cultivated fennel. At the same time, compounds **A** showed a very high radical scavenging activity comparing with that of trolox while compound **B** gave moderate activity.

There is little information available on the nonvolatile constituents of fennel and the polyphenolic compounds

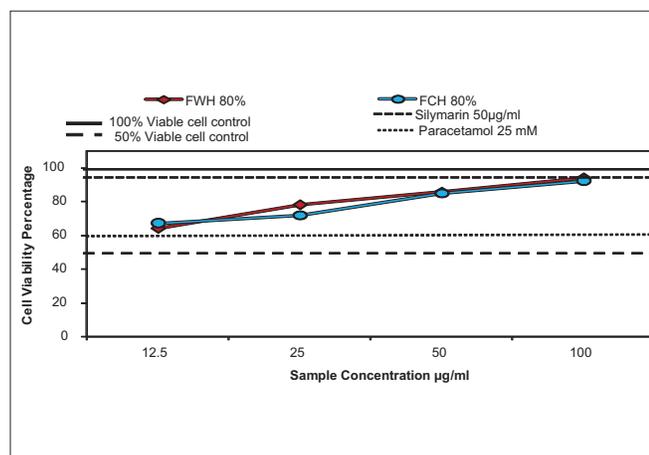


Figure 2: Viability of monolayer of rat hepatocytes after 2 h treatment with different concentrations of the extracts followed by treatment with 25 mM paracetamol for 1 h in comparison with 50 µg silymarin as control using the MTT calorimetric assay.

Table 2: Radical scavenging activity (expressed as absorbance percentage) of the two isolated compounds from *Foeniculum vulgare* subsp. *piperitum* (wild)

Antioxidant activity (%)	Compound
95.19	Compound A
88.03	Compound B
96.16	Trolox

Table 1: Radical scavenging activity (expressed as absorbance percentage) of *Foeniculum vulgare* subsp. *piperitum* (wild) extracts and *Foeniculum vulgare* subsp. *azoricum* (cultivated)

Cultivated fennel (antioxidant activity %)	Wild fennel (antioxidant activity %)	Fractions
93.34	93.71	80% MeOH
87.36	94.08	EtOAc
88.37	93.95	BuOH
96.15	96.15	Trolox

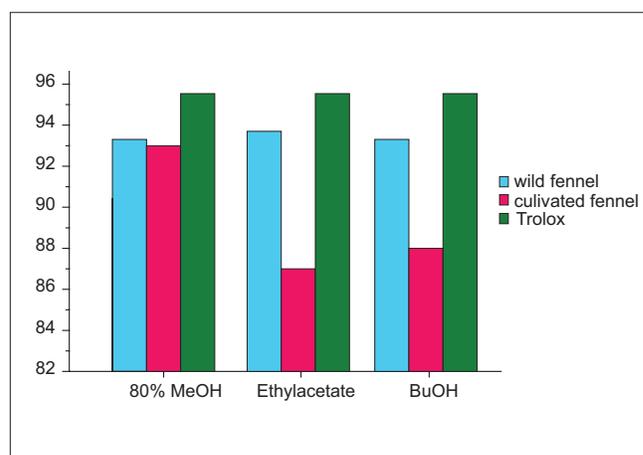
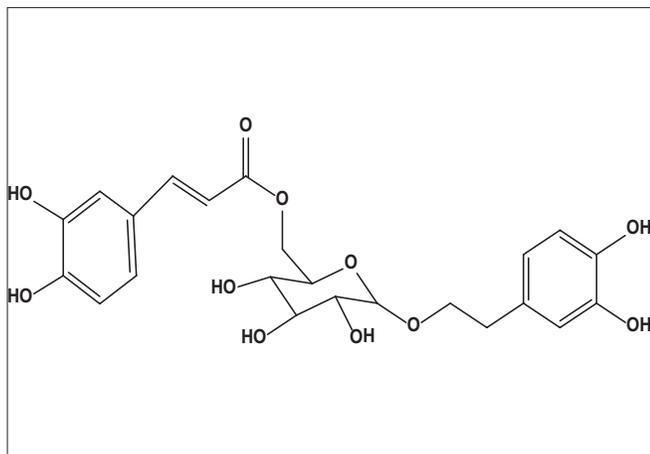
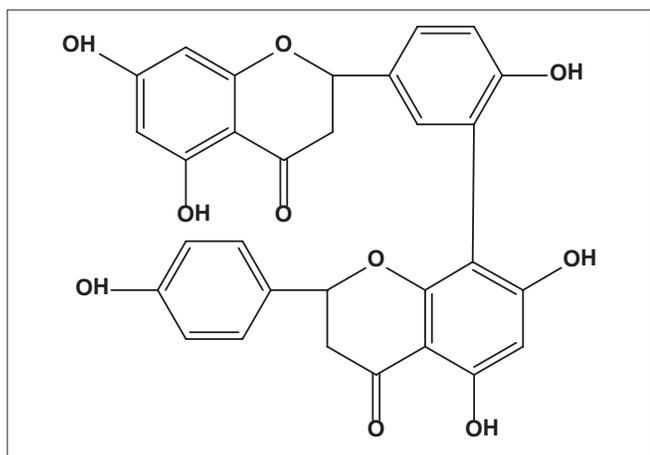


Figure 3: Radical scavenging activity (expressed as absorbance percentage) of *Foeniculum vulgare* subsp. *piperitum* (wild) extracts and *Foeniculum vulgare* subsp. *azoricum* (cultivated) in comparison with Trolox.



Compound A: 3, 4-dihydroxy-phenethylalcohol-6-O-caffeoyl- β -D-glucopyranoside



Compound B: (3', 8'')-Binaringenin

are associated with the prevention of disease thought to be induced by oxidative stress, such as cardiovascular diseases, cancer, and inflammation. In this study, we aimed to highlight on the antioxidant and hepatic-activity of phenolic compounds of the wild and cultivated fennel.

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