



PHENOLIC PROFILE, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES FROM THE Apiaceae FAMILY (DRY SEEDS)

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ABSTRACT

Objective: In the present study, a comparative evaluation of the phenolic compounds antioxidant capacity and antibacterial activity to several kinds of seeds from the Apiaceae family, to which belong: *Foeniculum vulgare* (Fennel), *Anethum graveolens* (Dill), *Pimpinella anisum* (Anise), *Carum carvi* (Caraway) and *Coriandrum sativum* (Coriander) were carried out. **Methods:** The total phenolic content of seeds was measured spectrophotometrically by using the Folin-Ciocalteu assay, the total flavonoids was measured spectrophotometrically by using the aluminum chloride colorimetric. Antioxidant capacity was analysed by 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging effect of the seeds and was determined also spectrophotometrically. Antibacterial activity was analysed by ISO standards. **Results:** The total phenolic and flavonoid contents of the *Foeniculum vulgare* (Fennel) varied between 115.96 mg GAE/100g and 68.10 mg CE/100g. The *Coriandrum sativum* (Coriander) content is lower (from 17.04 mg GAE/100g to 11.10 mg CE/100g, respectively). The highest radical scavenging effect was observed in the *Foeniculum vulgare* (Fennel) with IC₅₀ of 113.19 ml/L. In our study, the methanol extract of seeds didn't have any antimicrobial activity. **Conclusions:** An original data for total phenolic and total flavonoid contents are present in this study. They are a basis for assessment of the role of Apiaceae family dry seeds against free radicals effect and antibacterial activity. The results show that methanolic extract has the highest of total phenolic and total flavonoid contents, high potential of antioxidant activity of dry seeds from Apiaceae family, to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*). From all results, the solvent extracts of dry seeds are rich in phytochemical contents, which possessed high antioxidant and antimicrobial activities. Therefore the data found in this work might be used for further study of extracts of dry seeds from Apiaceae family on various applications such as health supplement and pharmaceutical benefits.

Keywords: *Anethum graveolens* (Dill), *Carum carvi* (Caraway), *Coriandrum sativum* (Coriander), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), E.coli, *Foeniculum vulgare* (Fennel), *Pimpinella anisum* (Anise), *Pseudomonas aeruginosa*, *Salmonella* species, *S.aureus*, Total flavonoids, Total phenolics, Yeasts and Moulds.

INTRODUCTION

Plants are known as a large source of natural phytochemicals which contain biological activities [1-3]. Several studies have shown that plants phytochemical could be used as therapeutical benefit for treatment of diseases [4]. The plants contain many bioactive substances such as flavonoids (vitexin, isovitexin, catechin, epicatechin, orientin and isorientin), anthraquinones especially in roots (emodin and chrysophanol), quinones, carotenoids, vitamins (especially vitamin C, α -tocopherol, β -carotene and β -tocopherol), proteins, essential oils, lipids, carbohydrates, reducing sugars, phenols, tannins, saponins, triterpenoids and organic acids [5-7]. The therapeutic effects of many plant spices and herbs suggest the presence of antioxidative and antimicrobial constituents in their tissues [8]. Antioxidants can be used to reverse the harmful and pathological effect of the free radicals, such as flavonoids, phenolic acids and diterpenes [8, 9]. The necessity of new antimicrobial agents and strategies for their use in the treatment of serious gram-negative and gram-positive infections is evident and is greater than ever because of emergence or multidrug-resistance in common pathogens, the rapid emergence of new infections, and the potential for use of multidrug-resistant agents in bioweapons [9, 10]. Plants from the family Apiaceae are commonly used as food, flavoring of foods and for medical purposes [11]. In particular, the seeds from family Apiaceae are known to be used as a household remedy for complications such as hypertension [11, 12]. Additionally other seed extracts from Apiaceae family have been observed for their health beneficial effects. Essential seed oils form *Foeniculum vulgare* (Fennel) such as anethole and limonene are used for medicinal purposes, and the seeds are also used as tranquilizers and tonics [11, 13]. Aqueous extracts of fennel seeds have hypotensive effects in a dose related manner [11, 13]. *Anethum graveolens* (Dill) seed extract has been used to treat diarrhea, ingestion and common colds [11, 14], and Dill is also fed to cows and goats for improving milk production [11, 15]. *Coriandrum sativum* (Coriander) seeds are often used as food flavoring agent and to treat ulcers [11, 14].

Carum carvi (Caraway) plant is beneficial in treatment and management of type II diabetes and cardiovascular diseases, and evokes beneficial effects on elevation of lipids in the bloodstream [11, 16, 39, 40]. *Pimpinella anisum* (Anise) belongs to the Middle East region, where it is used as an aromatic spice and to help indigestion [11, 17].

The aim of this study is evaluation the total phenolic and total flavonoid contents and antioxidant activities of methanolic extract of dry seeds from Apiaceae family to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*) from Bulgaria, and also their antibacterial activity is done.

MATERIALS AND METHODS

Plant materia

The study covered some varieties of species from different regions of Bulgaria: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*). The sapling lasted one year according to the seasonality of harvesting for individual species. The dried seeds were kept in a dry place until further use.

Chemical reagents: Methanol HPLC; gallic acid, (+)-catechin; Folin-Ciocalteu's phenol reagent; Sodium carbonate Na₂CO₃; Sodium Nitrite NaNO₂; Aluminium (III) chloride AlCl₃; Sodium hydroxide NaOH; 2,2-diphenyl-1-picrylhydrazyl (DPPH-); Ascorbic acid.. All other chemicals were of analytical grade (Sigma Chem. Co).

Sample preparation A ground dried sample of 0.5 g was weighted and phenolic and flavonoid compounds were extracted with 50 mL 80% aqueous methanol on an ultrasonic bath for 20 minutes. An aliquot (2 mL) of the extracts was ultracentrifuged for 5 minutes at 14 000 rpm [18].

Determination of total phenolic assay The total phenolic content of dry herbs was determined by using the Folin-Ciocalteu assay. An aliquot (1 mL) of extracts or standard solution of gallic acid (20, 40, 60, 80 and 100 mg/L) was added to 25 mL volumetric flask, containing 9 mL of distilled deionized water (dd water). Reagent blank using dd water was prepared. One milliliter of Folin-Ciocalteu's phenol reagent was added to the mixture and shaken. After 5 minutes, 10 mL of 7% Sodium carbonate solution was added to the mixture. The solution was diluted to volume (25 mL) with dd water and mixed. After incubation for 90 minutes at room temperature, the absorbance against prepared reagent blank was determined at 750 nm with an UV-VIS Spectrophotometer Perkin Elmer Lambda 5. Data of total phenolic contents of white birch leaves are expressed as milligrams of gallic acid equivalents (GAE) per 100 grammes dry weight (mg GAE/100g dw). All samples were analyzed in triplicate [18].

Determination of total flavonoid assay: Total flavonoid content was measured by aluminum chloride colorimetric assay. An aliquot (1 ml) of extracts or standard solution of (+)-catechin (20, 40, 60, 80 and 100 mg/L) was added to 10 mL volumetric flask, containing 4 mL of dd water. To the flask was added 0.3 mL 5% sodium nitrite. After 5 minutes, 0.3 mL 10% aluminium (III) chloride was added. At sixth minutes, 2 mL 1 M sodium hydroxide was added and the total volume was made up to 10 mL with dd water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm with an UV-VIS Spectrophotometer Perkin Elmer Lambda 5. Data of total flavonoid contents of dry herbs are expressed as milligrams of (+)-catechin equivalents (CE) per 100 grammes dry weight (mg CE/100g dw). All samples were analyzed in triplicate [18].

DPPH assay

The most commonly used antioxidant methods are ABTS and DPPH. Both of them are characterized by excellent reproducibility under certain assay conditions, but they also show significant differences in their response to antioxidants. The DPPH free radical (DPPH \cdot) does not require any special preparation, while the ABTS radical cation (ABTS \cdot^+) must be generated by enzymes or chemical reactions [19]. In the DPPH \cdot free radical method antioxidant efficiency is measured at ambient temperature and thus eliminates the risk of thermal degradation of the molecules tested [20]. The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-colored methanol solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH). This spectrophotometric assay uses the stable radical DPPH as a reagent. One thousand microlitres of various concentrations of the extracts in ethanol were added to 4 ml of 0.004% methanol solution of DPPH. After a 60 minutes incubation period at room temperature, the absorbance was read against a blank at 517 nm. All spectrophotometric data were acquired using a Helios β UV-Vis spectrophotometer (Unicam Spectrophotometer, Great Britain). Disposable cuvettes (1 cm x 1 cm x 4.5cm) from Ratiolab (Dreieich, Germany) were used for visible absorbance measurements.

Calculations

Inhibition of free radical by DPPH in percent (I %) was calculated in following way:

$$I = \frac{(A_{blank} - A_{sample})}{A_{blank}} \times 100, \%$$

where

A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound); A_{sample} is the absorbance of the test compound.

Extract concentration providing 50% inhibition (IC50%) was calculated from the graph plotting inhibition percentage against extract concentration [21, 22]. The objectives of this study were to evaluate and compare total antioxidant capacity to dry seeds from

Apiaceae family to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*).

Antibacterial activity

Sample preparation

ISO 6887 – 4 Microbiology of food and animal feeding stuffs – Preparation of test samples, initial suspension and decimal dilutions for microbiological examination – Part 4: specific rules for the preparation of products other than milk and milk products, meat and meat products, and fish and fishery products [23].

Tested microorganisms

ISO 4833 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of microorganisms – Colony-count technique at 30° C [23].

ISO 4831 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *coliforms* – Most probable number technique [23].

ISO 7251 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of presumptive *Escherichia coli* - Most probable number technique [23].

ISO 6579 Microbiology of food and animal feeding stuffs – Horizontal method for the detection of *Salmonella spp.* [23].

ISO 6888-3 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) – Part 3: detection and MPN technique for low number [23].

ISO 7954 Microbiology of food and animal feeding stuffs – General guidance for the enumeration of yeasts and moulds - Colony-count technique at 25° C [23].

Statistical Analysis

All experiments were performed in either duplicates or triplicates. Analysis at every time point from each experiment was carried out in triplicate. The statistical parameters are calculated in terms of the reproducibility of the experimental data using a statistical package universal ANOVA.

RESULTS

Different phytochemicals have various protective and therapeutic effects which are essential to prevent diseases and maintain a state of well being. Methanolic extract of Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*) were analyzed for its phytoconstituents. The quantitative estimation of the phytochemical constituents of Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*) show that the seeds are rich in total phenols and total flavonoids (data shown in the Table 1). The presence of this phenolic profile in dry seeds is a significant finding in this present study. The content for total phenolics and total flavonoids of Fennel (*Foeniculum vulgare*) varied between 115.96 mg GAE/100g and 68.10 mg CE/100g and it was found to be much higher than the content of Coriander (*Coriandrum sativum*) (ranged between 17.04 mg GAE/100g and 11.10 mg CE/100g), as is shown in Table 1 with gallic acid and catechin as standards. These results indicate that a higher antioxidant activity of the Fennel (*Foeniculum vulgare*) methanol extract compared to the Coriander (*Coriandrum sativum*) methanol extract may be in correlation to the phenolic and flavonoid content of respective plant extract. Polyphenolic compounds have been found to protect erythrocytes from oxidative stress or increase their resistance to damage caused by oxidants [24-27]. They are able to act as antioxidants in a number of ways, mainly as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelating agents [27-29].

Table 1: Total phenolics and total flavonoids in the studied Bulgarian dry seeds

Bulgarian dry seeds	Total phenolics, (mg GAE /100g dw)	Total flavonoids, (mg CE /100g dw)
<i>Foeniculum vulgare</i> (Fennel)	115.96 (RSD 5.7; n=3)	68.10 (RSD 5.8; n=3)
<i>Anethum graveolens</i> (Dill)	69.87 (RSD 5.7; n=3)	49.10 (RSD 7.5; n=3)
<i>Pimpinella anisum</i> (Anise)	46.17 (RSD 7.7; n=3)	17.43 (RSD 8.6; n=3)
<i>Carum carvi</i> (Caraway)	25.96 (RSD 8.1; n=3)	11.77 (RSD 8.9; n=3)
<i>Coriandrum sativu</i> (Coriander)	17.04 (RSD 8.6; n=3)	11.10 (RSD 8.8; n=3)

The methanolic extract was subjected to screening for their possible antioxidant activity. The DPPH[•] assay provided information on the reactivity of test compounds with a stable free radical. Because its odd electron DPPH[•] gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger, the absorption vanishes, and the resulting decolorization is stoichiometric with respect to the number of electrons taken up. To evaluate the scavenging effect of DPPH[•]

on methanolic extracts of dry seeds from Apiaceae family to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*), DPPH[•] inhibition was investigated and these results are shown as relative activities against control.

The graph in Figure 1 (Inhibition/Concentration) presents the results obtained to the method described above. The values of IC50% of the analyzed extracts are shown in Table 2.

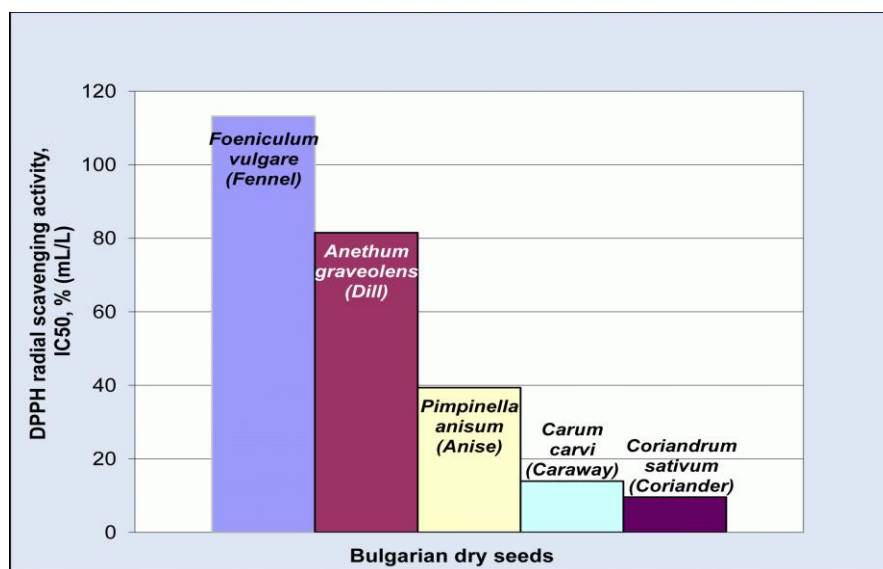


Figure 1: Free radical scavenging capacity of the extracts measured in DPPH assay

Table 2: DPPH radical scavenging activity of Bulgarian dry seeds

Bulgarian dry seeds	DPPH radical scavenging activity, IC50% (mL/L)
<i>Foeniculum vulgare</i> (Fennel)	113.19 RSD 4.4% (n=3)
<i>Anethum graveolens</i> (Dill)	81.52 RSD 5.2% (n=3)
<i>Pimpinella anisum</i> (Anise)	39.36 RSD 4.6% (n=3)
<i>Carum carvi</i> (Caraway)	13.94 RSD 8.7% (n=3)
<i>Coriandrum sativum</i> (Coriander)	9.56 RSD 8.9% (n=3)

As mentioned above, the IC50% is a parameter representing the seeds concentration, able to inhibit 50% of used DPPH amount. It was determined by drawing a graph with simple concentration on the abscissa and free radical inhibition capacity IC(%) as ordinate. A series of samples were prepared as already described. The initial seed sample was diluted in a manner to obtain a linear graph with lies in the zone of 0 to 50% radical scavenging capacity. The sample concentration with reduces 50% of free radicals can be calculated by using graph equation. All concentration studied showed free radical scavenging activity. It is

evident that the 50% of inhibition value for Fennel (*Foeniculum vulgare*) methanol extract seems to be fairly significant when compared to the methanol extract of Coriander (*Coriandrum sativu*). (IC50% = 113.19 ml/L methanolic extract of Fennel (*Foeniculum vulgare*) was necessary to obtain 50% of DPPH degradation). IC50% values of the extracts were compared to IC50% value of a "standard" antioxidant, in this case ascorbic acid (AA), obtained by the same procedure. The ratios (IC50%)AA/(IC50%) extract are shown in Table 3. We compare the antioxidant capacity of our samples with this of Vitamin C (Ascorbic Acid). The same procedure was applied to vitamin C, and its IC50 value

was determined. They represent the ascorbic acid equivalent of the extracts antioxidant capacity (AOCE_{AA}), i.e. the amount of

ascorbic acid in grams equivalent to one liter extract.

Table 3: Antioxidant capacity of tested in Apiaceae dry seeds

Apiaceae dry seeds	Ascorbic acid equivalent of the extracts antioxidant capacity (AOCE _{AA}), (g/L)
Fennel (<i>Foeniculum vulgare</i>)	0.136; RSD 5.4% (n=3)
Dill (<i>Anethum graveolens</i>)	0.188; RSD 5.1% (n=3)
Anise (<i>Pimpinella anisum</i>)	0.390; RSD 4.5% (n=3)
Caraway (<i>Carum carvi</i>)	1.102; RSD 3.9% (n=3)
Coriander (<i>Coriandrum sativu</i>)	1.607; RSD 3.4% (n=3)

Antibacterial activity on methanolic extracts of dry seeds from Apiaceae family to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway

(*Carum carvi*) and Coriander (*Coriandrum sativum*) are showed in the Table 4. They show the microbial limits or the absence of specified microorganisms in seeds.

Table 4: Microbiological tests

Samples	Aerobic bacteria: Total plate count, CfU/g	Coliforms <i>E.coli</i> MPN /1 g	<i>Pseudomonas aeruginosa</i>	<i>Salmonella</i> species in 25.0 g	<i>S.aureus</i>	Yeasts CfU/g	Moulds CfU/g
Fennel (<i>Foeniculum vulgare</i>)	< 10	< 0.30	Absent in 1,0 g	Absent in 25.0 g	Absent in 1.0 g	2.0x10 ¹	< 10
Dill (<i>Anethum graveolens</i>)	< 10	< 0.30	Absent in 1,0 g	Absent in 25.0 g	Absent in 1.0 g	< 10	< 10
Anise (<i>Pimpinella anisum</i>)	< 10	< 0.30	Absent in 1,0 g	Absent in 25.0 g	Absent in 1.0 g	2.0x10 ¹	< 10
Caraway (<i>Carum carvi</i>)	< 10	< 0.30	Absent in 1,0 g	Absent in 25.0 g	Absent in 1.0 g	< 10	< 10
Coriander (<i>Coriandrum sativu</i>)	< 10	< 0.30	Absent in 1,0 g	Absent in 25.0 g	Absent in 1.0 g	< 10	< 10

DISCUSSION

Polyphenolic compounds have been found to protect erythrocytes from oxidative stress or increase their resistance to damage caused by oxidants [24-27]. They are able to act as antioxidants in a number of ways, mainly as reducing agents, hydrogen donors, singlet oxygen quenchers, and metal chelating agents [27-29].

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH⁺) has been widely used to evaluate the antioxidant capacity (AOC) of natural antioxidants. DPPH⁺ is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.

The AOC of tested samples was determined photometrically using the DPPH⁺ method [20]. 1 ml diluted extract sample was added to 4 ml 0.004 % solution of DPPH⁺ (SIGMA[®]) in methanol (0.004 mg/100 ml). After 60 min solution retention in the dark, the light absorption was measured in 1 cm standard cuvette at 517 nm with UV-VIS-spectrophotometer (UNICAM[®]-Helios β). The reference liquid was methanol. The free radical inhibition (scavenging) was calculated by the expression

$$IC(\%) = \frac{(A_0 - A_s)}{A_0} \times 100$$

where A₀ is absorption of the reference liquid not containing extracts (it's 0.004 % solution of DPPH⁺ in methanol), A_s is absorption of the extract containing sample. The DPPH⁺ solution was freshly prepared daily, stored in a flask covered with aluminium foil, and kept in the dark at 4^oC before measurements.

As mentioned above, the IC₅₀ is a parameter representing the extract concentration, able to inhibit 50 % of the used DPPH⁺ amount. It was determined by drawing a graph with sample concentration C_w (ml extract/L solvent) on the abscissa and free radical inhibition capacity I [%] as ordinate. A series of samples was prepared as already described. The initial extract sample was diluted in a manner to obtain a linear graph which lies in the zone

of 0 to over 50 % radical scavenging capacity. The extract concentration which reduces 50% of free radicals can be calculated by using Figure 1 equation or simply determined from the graph. Each sample was analyzed at least three times, and a mean value was calculated [30].

High correlation was reported between the antioxidant capacity and total phenol and flavonoids contents of plants [31, 32]. Besides antioxidant capacity, phenolic compounds exhibit a wide range of biological activities, including anti-carcinogenic, anti-inflammatory, anti-viral, anti-allergic, estrogenic, immune-stimulating agents, antiallergenic, anti-atherogenic, anti-inflammatory, anti-microbial, antithrombotic, anti-stress, anti-hyperglycemia, cardio protective and vasodilatory effects [33]. It is well known that plant phenolics, in general, are the highly effective free radical scavenging and antioxidants.

Antibacterial activity on methanolic extracts of dry seeds from Apiaceae family to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*). The total aerobic microbial count and the total yeast and mould count (presented as colony-forming units per gram CFU/g per dry seed), the absence of *Salmonella* species, and Gram-negative. Concerning the moulds and yeasts, significant differences between the different dry seeds were not found. In our study, the methanol extract of seeds did not have any antimicrobial activity against *E. coli* strains but it did have antimicrobial activity against *P. aeruginosa*. All samples showed negative results for toxigenic species. This result suggested that the antibacterial activity of the extracts was affected by different kinds of phytochemical composed in each stages of the fruit development. It is well known that the active compounds called phytochemicals were produced for plant against microbial pathogens which were considered to be potent source of novel compounds with having biological activities such as antioxidant and antimicrobial activities [34, 35]. The development of drug from natural medicinal plants instead of commercial antimicrobial drugs has been focused in recent years [36]. The obtained results from this work indicated that alcoholic

extracts of dry seeds are effective against the selected bacteria which slightly differed in types of bacteria and efficacies. This result might be caused from the characteristics of each bacterial cell wall [37]. With previous reports, many bioactive produced by plants have been found to protect plants against bacteria, fungi and pests [36, 38]. Therefore, it is no surprise that the extracts of dry seeds were composed of antibacterial activity. The results of total phenolic and total flavonoid contents found to relate directly on antioxidant and antibacterial activities of the extracts. However, other active compounds such as steroids, alkaloids or tannins may be involved in the tested biological activities which should be further performed.

CONCLUSIONS

In this paper original data for total phenolic and total flavonoid contents are a basis for assessment of the role of Apiaceae family dry seeds against free radicals effect and antibacterial activity. The results can be concluded that methanolic extract has the highest of total phenolic and total flavonoid contents, high potential of antioxidant activity of dry seeds from Apiaceae family to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*). From all of results, the solvent extracts of dry seeds are rich in phytochemical contents which possessed high antioxidant and antimicrobial activities. Therefore the data found in this work might be used for further study of dry seeds from Apiaceae family to which belong: Fennel (*Foeniculum vulgare*), Dill (*Anethum graveolens*), Anise (*Pimpinella anisum*), Caraway (*Carum carvi*) and Coriander (*Coriandrum sativum*) extracts on various applications such as health supplement and pharmaceutical benefits.

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