Total Phenol, Antioxidant and Antibacterial Activity of the Essential Oil and Extracts of *Ferulago angulata* ssp. *angulata*

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Received: 15 Sep. 2012 **Accepted:** 24 Oct. 2012

Abstract

Background: In recent years, plants natural products have gained considerable attention as natural preservers for use in different industries. Due to their free radical scavenging activities, phenolic compounds can prevent a wide range of diseases such as cancers and cardiovascular and neurodegenerative diseases. For a long time, different *Ferulago* species have been in use as medicinal plants worldwide.

Objective: The aim of this research was to investigate total phenolics and the antioxidant and antibacterial activities of essential oil and different extracts from *F. angulata* growing wild in Iran.

Methods: Antioxidant activity of our samples was examined by DPPH assay and their phenolic content was determined using the Folin – Ciocalteau method. Moreover, their inhibitory effects against five gram-negative and gram-positive bacteria including *Shigella boidii, Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus and Enterococcus faecalis* were tested by using the disk diffusion method.

Results: Ethyl acetate fraction and methanol extract contained the highest levels of phenolic compounds (229.2 and 202.9 µg/mg galic acid) in this study. Also, essential oil of this plant exhibited low free radical scavenging activity (IC_{50}= 36129.43µg/ml). On the other hand, ethyl acetate and aqueous fractions had the highest antioxidant activity (IC_{50} values of 20.153 and 28.28 µg/ml, respectively). Finally, studied samples had no significant antibacterial activities against tested organisms.

Conclusion: The essential oil of this plant can be used as a flavoring agent while extracts prepared from it have the potential to be used as natural antioxidants in relevant industries.

Keywords: ***Ferulago angulata* ssp. *angulata*, Antioxidant activity, Antibacterial activity, Total phenolics**

Introduction

Due to having single electrons, free radicals are very reactive and damage body biomolecules such as proteins, non-saturated amino acids, nucleic acids and carbohydrates [1]. Two defensive systems including enzymatic and non-enzymatic (vitamin E, caretonoids, ascorbic acid, uric acid, glutathione, hormones such as angiotansin and estrogen, phenolic compounds, etc.) are present and reduce deleterious effects of free radicals on the human body with prevention of formation of free radicals, relieving injuries from these compounds, increasing excretion of affected molecules and minimizing cell mutations [1 – 3]. Considering deleterious effects of free radicals and keep in mind that these molecules can cause more than one hundred diseases, therefore, use of antioxidant compounds is very important [3]. With respect to potential side effects of synthetic antioxidants and regarding increasing use of canned foods due to changes in life style in recent years, discovery of natural antioxidant has great importance. Plants essential oils and extracts are known as natural food preservers [4]. Since these compounds are mostly safe and acceptable by consumers, they have great importance [5]. *Ferulago angulata* ssp. *angulata* is a perennial member of Apiaceae family and grows wild in mountainous area of west of Iran [6]. Previous studies have been shown that aerial parts of *F. angulata* plant including stems, leaves, flowers and seeds contain essential oil [7, 8]. Some biological activities of these essential oils have been reported and their chemical constituents revealed [8]. *Ferulago* species are used as the flavouring agents and for their sedative, tonic, digestive and anti-parasitic properties [9, 10]. Considering potential of plant essential oils and extracts for substituting synthetic antioxidants and preservers, the study of potential of *F. angulata* ssp. *angulata* can be useful for industrial application of this plant as a natural preserver. Therefore, the present study was done to investigate total phenolics, antioxidant and antibacterial activity of extracts and essential oil of *F. angulata* ssp. *angulata.*

Material and Methods

Inflorescences of *F. angulata* ssp. *angulata* were collected at the early stages of flower opening from Besry village (Markazi province) at altitudes between 2600 - 2800 in April 2011. Plant materials were identified at the Research Institute of Forests and Rangelands Herbarium. A voucher specimen (No. 3151) has been kept at the Markazi Province Agriculture and Natural Recourses Research Center Herbarium.

Chemicals

Methanol, n-hexane, dichloromethane, ethyl acetate, Folin reagent and BHT (Merck, Germany), DPPH (Sigma-Aldrich, USA), Alborz distilled water (Pouya, Arak), sodium carbonate, gallic acid, Mueller Hinton agar medium (High Media Co.) and blank disc (Padtanteb, Iran).

Isolation of essential oil

Plant materials (50 grams) were powdered using an electric grinder. Afterwards, plant sample was added to a 1 liter balloon containing 500 cc distilled water. Essential oil was isolated using a Clevenger-type apparatus for 3 h. After isolation, essential oil was dried over anhydrous sodium sulphate and kept in a dark vial before use. The yield of the essential oil was calculated to be 3.75% (w/w).

Preparation of plant extracts

To prepare plant extract using maceration method, plant materials (300 grams, 100 grams in each Erlenmeyer) were added to three 1000 Erlenmeyer containing 200 ml pure methanol. Afterwards, Erlenmeyer were transferred on a shaker with 130 rpm for 48 h, after which methanol solutions were filtered using a filter paper. Then, 50 cc methanol were added to each Erlenmeyer and they were shaked for a further 24 h and filtered solutions were added to previous extract. Finally, obtained extract was concentrated using a rotary-evaporator apparatus at 30 rpm at 50^{o} C. The extract yield was 7.1% on the base of dry weight of plant materials. A part of obtained extract was kept in a dark vial as methanol extract and remaining was added to an Erlenmeyer containing 200 cc distilled water. To precipitate wax and resins, aqueous solution was incubated at 3^{o} C for 24 h. Afterwards, this solution was filtered using a filter paper and used for preparation of different fractions according to the method described in Fig. 1 [11]. Methanol extract of studied plant contained only negligible amount of *n*-hexane fraction and, therefore, it was excluded from further studies.

Separated fractions were again concentrated using a rotary-evaporator apparatus, transferred to 20 ml glasses and incubated in an incubator at 50 ^{o} C for 48 h. Dried extracts were finally subjected to antioxidant, total phenolics and antibacterial experiments.

Preparation of aqueous extract

To prepare aqueous extract, powdered plant materials (50 grams) were soaked in water and boiled for 30 min. Obtained solution was cooled, filtered, concentrated, and finally dried in an incubator for 24 h at 60 ^{o} C. Prepared extract was kept at 4 ^{o} C until use.

Antioxidant activity assay

In this study, antioxidant activities of the essential oil and different extracts of *F. angulata* ssp. *angulata* were assessed using DPPH method. The method is on the base of transfer of hydrogen atom to DPPH and scavenging capacity of free radicals (DPPH) [12]. To do this, different concentrations of each extract were prepared using methanol. Afterwards, 1 ml of each plant extract was mixed with 2 ml of methanol solution of DPPH and the experiment was repeated three times [13]. After 20 min, the absorbance of all solutions and control sample was measured at 517 nm using a spectrophotometer. The following equation was served to calculate RSC percentage:

$RSC\ (\%) = 100 \times [(A_{control} - A_{sample})/A_{control}]$

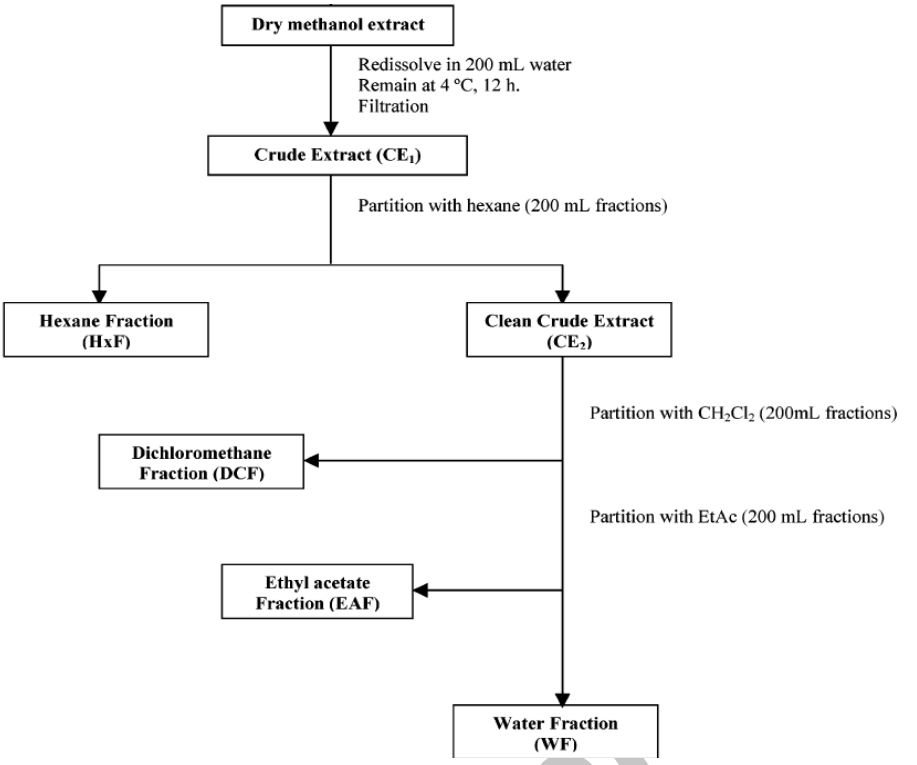

Fig. 1- Parejo et al. (2002) Fractionation of methanol extract of *F. angulata* ssp. *angulata*

Where $A_{control}$ and A_{sample} are the absorbance of control and tested solutions, respectively. Obtained results were compared with BHT as the positive control.

Total phenolics of plant extracts

To measure total phenolics of each plant the method described by Gao et al. (2000) was served in which Gallic acid as standard and Folin-Ciocalteu's reagent are used. To do this, 100 µl of each plant extract were mixed by 500 µl of 10% Folin solution. After 3 min, 400 µl of 7.5% sodium carbonate solution were added to the mentioned solution at the dark. All samples were incubated at the dark at the room temperature. The blank sample was prepared in the same way with methanol instead of plant extracts. Afterwards, absorbance of all prepared samples was measured at 765 nm using a spectrophotometer. Gallic acid standard curve was used to calculate total phenolics of each sample. Different concentrations of gallic acid were 0, 25, 50, 75 and 100 mg/l. To prepare these concentrations, a solution of 100 µg/ml gallic acid using 50% methanol was prepared at first and obtained solution was diluted. Above-mentioned procedure was performed to measure different concentrations of gallic acid. The blank sample was prepared in the same way with 50% methanol instead of gallic acid. Absorbance of different concentrations of gallic acid was recorded at 765 nm. Finally, gallic acid standard curve was prepared using absorbance of different concentrations of this compound. Results were expressed as µg/ mg gallic acid.

Antimicrobial assay

Antibacterial activity of different extracts of *F. angulata* ssp. *angulata* was examined by using disk diffusion method (kirby bauer disk diffusion method) and *Shigella boidii*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus and Enterococcus faecalis*. These gram-positive and gram-negative bacteria were obtained from Pasteur Institute of Iran. In brief, several colonies of freshly cultured bacteria (18 h) on Mueller-Hinton agar were suspend to 5 ml sterile physiological serum and concentration of inoculate was checked with 0.5 McFarland standard tube. Afterwards, plates containing Mueller-Hinton agars were inoculated with inoculate solutions using sterile swabs. The essential oil and extracts prepared from plant were sterilized by passing through 0.45 µm filters. 20 µl of the essential oil and each extract (10%w/v) were added to sterile disks and they were placed on plates. Finally, plates were incubated at 35^{o} C for 24 h and then diameter of inhibition zone measured. Blank disk was containing of solution for every one of tested material. And every test was repeated for 7 times.

Statistical analysis

The experiment was repeated three times and obtained data were analyzed usin one-way ANOVA. Means were compared using Duncan's multiple range tests at the significant level of 0.05 and curves were drawn in Microsoft Office Excel program.

Results

Total phenolics

There were significant differences ($p<0.05$) among total phenolics of the essential oil, extracts and fractions; these results are presented in Fig. 2. As can be seen, ethyl acetate fraction, methanol extract, aqueous extract and aqueous fraction had the highest level of phenolics compounds (229.2, 202.9, 131.6 and 81.92 µg/mg galic acid, respectively). The level of phenolics compounds in dichloromethane fraction (81.7 µg/mg galic acid) and aqueous fraction (81.92 µg/mg galic acid) was not significantly different and these compounds amounted 37.3 µg/mg galic acid in the essential oil (Fig. 2).

Free radical scavenging activity

In this study, significant differences ($p<0.05$) were observed among free radical scavenging activity of tested samples (Fig. 3). Ethyl acetate fraction exhibited the highest activity with the IC_{50} value of 20.2 µg/ml. Considering IC_{50} value of the essential oil (36129.4 µg/ml), it can be concluded that the essential oil of this plant has no antioxidant activity. Similarly, the low level of antioxidant activity was observed in the dichloromethane fraction (122.4 µg/ml). Differences among free radical scavenging activity of methanol and aqueous extracts and the aqueous fraction were not significant. After ethyl acetate fraction, these extracts had the highest level of antioxidant activities. The IC_{50} value of BHT was 5.9 µg/ml (Fig. 3).

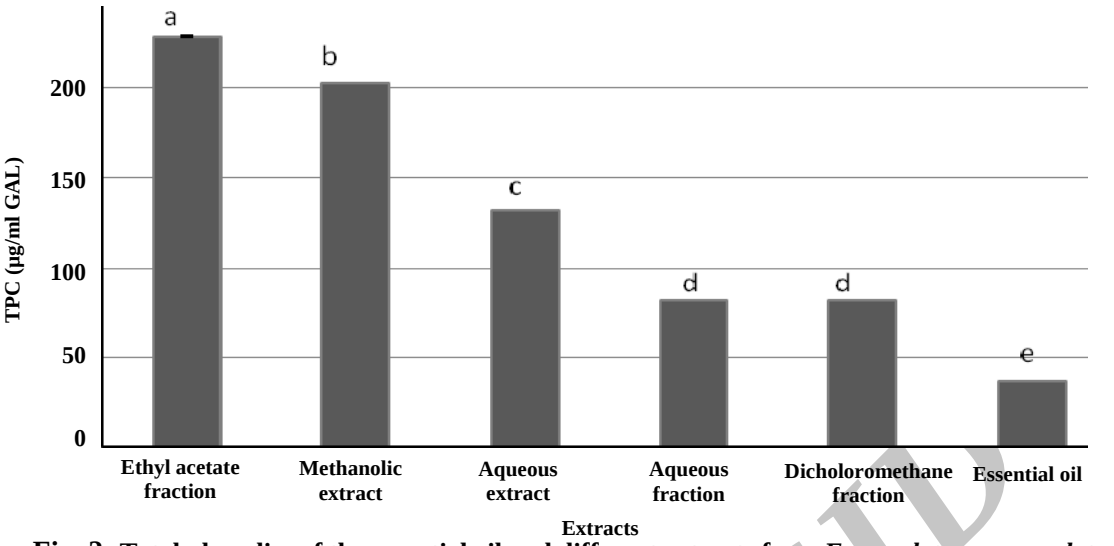

Fig. 2- Total phenolics of the essential oil and different extracts from *F. angulata* ssp. *angulata*
Means with at least one similar letter are not significantly different at the significant level of p<0.05

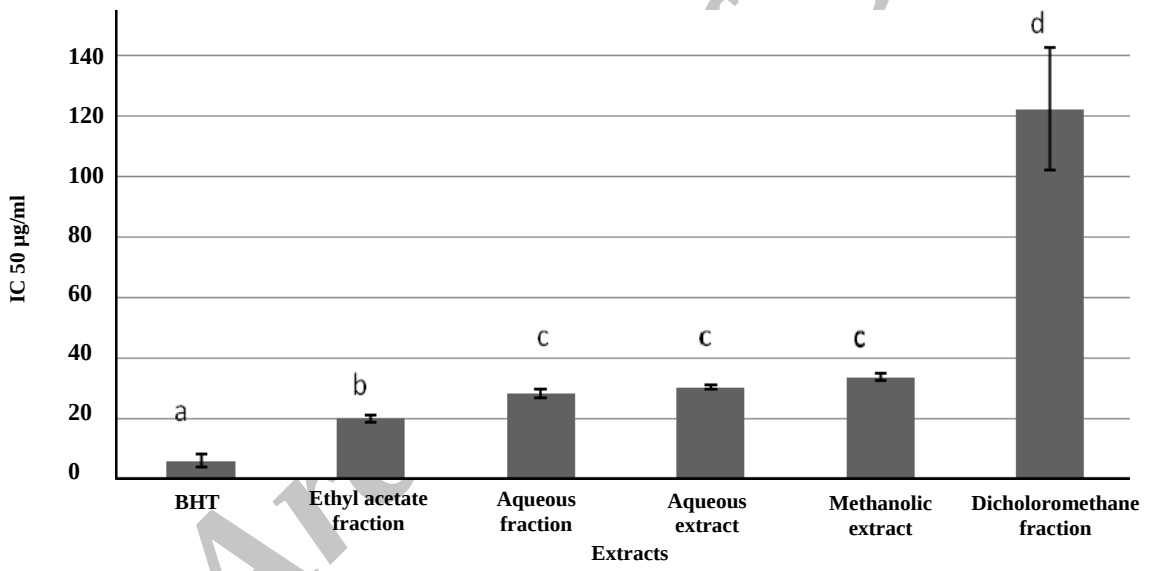

Fig. 3. IC_{50} values of BHT and different extracts from *F. angulata* ssp. *angulata*
Means with at least one similar letter are not significantly different at the significant level of P<0.05

Antimicrobial activity

According to results of in vitro antimicrobial activities using the disk diffusion agar method, the essential oil and extracts of *F. angulata* ssp. *angulata* have not possess any inhibition activity against tested microorganisms even in the high concentrations (10% w/v).

Discussion

Antioxidant activity of plant essential oils and extracts is due to their active components

content. A linear relationship between antioxidant activity of plant extracts and their phenolic content has been reported [15]. On the other hand, phenolic compounds have the antioxidant activity and protect cells against oxidative reactions [16]. In this study, it has been shown that the essential oil of *F. angulata* ssp. *angulata* possesses the low level of antioxidant activity (36129.4 µg/ml) and it has the lowest amount of phenolic compounds among tested samples (37.2 µg/ml). It seems that the low level of antioxidant activity of *F. angulata* ssp. *angulata* essential oil is due to absence of phenolic compounds. In a study by Ruberto et al. (1999) it has been revealed that phenolic compounds such as thymol and carvacrol have the highest antioxidant activity among other component of essential oil. After these compounds, the highest antioxidant activity was observed in γ-terpinene and terpinolene. According to previous studies, α-pinene (16.93 - 32.74%), (Z)-β-ocimene (13.47-20.85%), allo-ocimene (5.82 - 8.07%), bornyl acetate (2.3 - 11.97%) and γ-terpinene (0.93 - 12.95%) are the main components of essential oil of this plant which belong to group of monoterpene compounds [18]. Since these compounds except γ-terpinene possess insignificant antioxidant activity, the low level of antioxidant activity of essential oil in this plant is obvious.

The highest free radical scavenging activity of ethyl acetate fraction (229.2 µg/ml) among tested samples can be related to its high content of phenolic compounds (IC_{50}=20.2 µg/ml). In this study, no positive relationship was observed between antioxidant activity of methanol extract and aqueous fraction of *F. angulata* ssp. *angulata* and their phenolics content. Variation among the antioxidant activity of different extracts is due to differences in the antioxidant activity of their constituents which differentially extract with the application of solvents with different polarity [19]. According to previous studies, polar extracts have the higher free radical scavenging activity than non-polar ones which can be related to the presence of phenolic acids and flavonoids [20]. This finding is in agreement with the results of the present study on the antioxidant activity of aqueous extract and ethyl acetate fraction. In agreement to the results of previous studies, it has been shown in this study that the level of phenolic compounds is higher in extracts and fractions such as ethyl acetate and aqueous fractions and methanol extract which have more polarity. In this study, no significant differences were observed between total phenolics of dichloromethane and aqueous fractions. As mentioned, polarity of solvents is determinant on the extraction of polyphenols and, therefore, the level of phenolic compounds in extracts and fractions with the higher degree of polarity was more. Differences in antioxidant activity of different extracts may be related to the level and type of phenolic compounds. In addition, the presence of other antioxidants such as vitamins C and A and β-carotene is determinant in this respect [21 – 23]. The positive relation between TPC and DPPH, which was observed in this study, suggests that phenolic compounds are potent free radical

scavenging agents. Since active substances are mostly present in the filamentous parts of plants that allows dissolve better in water, aqueous extracts exhibit the higher antioxidant activity than organic ones [24].

Scavenging activity of DPPH and, as the result, antioxidant activity is in relation to the concentration of phenolic compounds and degree of their hydroxylation [25]. In other words, polyphenols are more effective in scavenging of free radicals in living and non-living systems. In the present study, the highest free radical scavenging activity was observed in ethyl acetate fraction (IC_{50}=20.153 µg/ml) which is considerable in compare to BHT (IC_{50}=5.89 µg/ml). In contrast, the essential oil and dichloromethane fraction exhibited the lowest levels of free radical scavenging activity (Fig. 2).

In this study, Folin-Ciocalteu's reagent method was used which because of its simplicity, sensitivity and accuracy is widely used [26]. Due to the presence of compounds such as sugars, aromatic amines and ascorbic acid that can be rapidly oxidized, in this method the level of phenolic compounds may be estimated more than that of actual amount [27]. The lower antioxidant activity of methanol extract despite its higher content of phenolic compounds may be related to this fact. Considering absence of phenolic compounds such as thymol and carvacrol in the essential oil of this plant is the reason for its insignificant antibacterial activity.

So far, antibacterial activity of several medicinal plants has been investigated in several studies. In a study by Khaleghi-Sigariodi et al. (2005), the essential oil of *F. Bernardii* exhibited a low level of antibacterial activity against *Basillus subtilis*, *E. coli* and *Staphylococus aureus* and *Candida albicans* and no activity against *Pseudomonas aeruginosa* [28]. Although, Taran et al. (2009) reported antimicrobial effects of essential oils obtained from aerial parts and seeds of *F. angulata subsp. carduchorum* against tested bacteria and fungi, however, in our study, essential oils and extracts of *Ferulago angulata ssp. angulata* exhibited no considerable biological activities.

Conclusion

With respect to the considerable antioxidant activity of *F. angulata* ssp. *angulata* extracts that may be due to the presence of phenolic and especially polyphenolic compounds, the extracts have the potential to be used as a natural antioxidant agent in food and pharmaceutical industries. On the other hand, the essential oil of this plant has no significant antibacterial and antioxidant activities and can be used only as a flavouring agent.

Acknowledgment

Authors wish to thank Arak University Research Department for financial supports.

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