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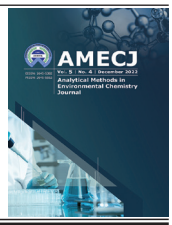

Evaluating the effect of ethanol foliar feeding on the essential oil, phenolic content, and antioxidant activities of Ducrosia anethifolia

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ABSTRACT

Ducrosia anethifolia (Apiaceae) is a medicinal aromatic plant distributed in Iran and Afghanistan. This research aims to investigate the composition of the plant essential oil, determine the total flavonoid and phenolic contents, and evaluate its antioxidant activities after ethanol foliar feeding. For this purpose, 0, 10, 20, 40, and 80% v/v of aqueous ethanol solutions were sprayed on different batches of the plants. Then, the essential oils were obtained using water distillation. Compounds were analyzed by Gas chromatography-mass spectrometry technique(GC-MS) using a validated method. The method was validated as per the ICH guidelines for linearity, precision, accuracy, robustness, LOD, and LOQ. The total contents of phenols and flavonoids were measured using spectrophotometric methods. The antioxidant activity was evaluated using DPPH and FRAP assays. The results showed that n-decanal, cis-verbenyl acetate and dodecanal were the major compounds in all treatments. However, alcohol could cause significant differences in the essential oils qualitatively and quantitatively. The results showed that 40% ethanol could increase the number of phenolics and flavonoids and consequently the antioxidant activity. Thus, ethanol foliar feeding can be used as an appropriate approach to increase the essential oil of D. anethifolia as well as its phenolic and flavonoid contents.

1. Introduction

Ducrosia anethifolia (DC.) Boiss. (Asteraceae) is an aromatic herbaceous and biennial plant with a height of 10–30 cm. The stems are glabrous and branched mostly from the base. The leaves are ovate-oblong, 2–6 cm long, and branched, with a petiole length of 5–18 cm. The edges of the petals are jagged and slightly shaggy and the compound umbel inflorescence has white flowers. In Iran, the plant is known as Moshgak, Mushk Boo, Darvishan Ginger, Reshgak, Khorkhundai, Gavarshkh, and mount Coriander [1]. The genus Ducrosia has three species in Iran including D. anethifolia, D. assadii Alava, and D. flabellifolia Boiss. Ducrosia anethifolia grows wildly in mountainous and plain areas on sandy soils in

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different regions of Iran such as Kerman, Khorasan, Zanjan, Shushtar, Behbahan, Shiraz, Kazerun, Borazjan, Noorabad, Farashband, Firoozabad, Jahrom, Darab, and Sistan Baluchestan [2]. It also grows natively in countries from the Mediterranean range to W. Pakistan such as Afghanistan, Pakistan, Iraq, Syria, Lebanon, and some Arab countries [3]. Ducrosia assadii is endemic to Iran and D. flabellifolia is native to Syria, W. Iran, and Arabian Peninsula [4]. Essential oils are one of the most pharmacologically important constituents in plants belonging to Ducrosia. Terpene compounds are reported to be responsible for many medicinal activities of the oil of this medicinal plant. The volatile compounds of this aromatic plant are used as a flavoring additive in various beverages and desserts. Besides, Moshgak is also used as an edible vegetable [5, 6]. Several studies have investigated the essential oil composition of D. anethifolia revealing that the long chain oxygenated hydrocarbons such as decanal, dodecanal, and decanol constitute the major compounds in the essential oil. In addition, monoterpenes including α-pinene, α-thujene, linalool, cis-citronellyl acetate, and oxygenated sesquiterpenes such as chrysanthenyl acetate have been reported as the main constituents of the essential oil [7]. In Iranian traditional medicine, the aerial parts of D. anethifolia have been used to relieve various pains such as headaches, back pain, and colic pain. It has been also used for the treatment of seizures, insomnia, heartburn, cataract, inflammation of the inner wall of the nose, and colds. Pharmacological studies show that the plants belonging to this genus have muscle relaxant, CNS depressant, and anti-depressant properties. In addition, the essential oils obtained from D. anethifolia have antimicrobial properties against gram-positive bacteria, yeasts, and some dermatophytes. The essential oil of D. anethifolia is reported to have antifungal properties by preventing the growth of parasitic fungi such as Candida albicans on the skin [8-10]. It could also improve kidney function and lower the lipid levels of the blood. Alpha-pinene as one of the major compounds in essential oil is probably responsible for the anti-anxiety effect of the plant. Myrcene, as another main component of the plant, has several pharmacological activities including anti-radical, inhibitory effects, anti-cancer, and anti-tumor properties [11-13]. The use of methanol and ethanol foliar feeding is one of the most important approaches for increasing plant growth and harvest yield. Research has shown that ethanol becomes acetaldehyde after penetration into the plant tissue. Acetaldehyde is transformed into acetate (acetic acid) by the acetaldehyde dehydrogenase enzyme. Acetic acid also converts to acetyl coenzyme A, which eventually turns into carbon dioxide and dioxide. Methanol, ethanol, and other alcohols are non-toxic to plants and can simply penetrate the membrane of plant cells. The absorption rate directly depends on the density of alcohol. Therefore, the application of methanol and ethanol foliar feeding on the aerial parts of C3 plants (the plants that only use the standard method of carbon dioxide fixation by the enzyme Rubisco) [14], in which their light breathing is large can compensate for part of the stabilized carbon losses and in this way, increase pure photosynthesis and dry matter production per unit area. As a result, some studies conducted in the field of agronomic C3 plants have shown that methanol could affect the performance of these plants positively [15]. Thus, the aim of this study was to evaluate the effect of ethanol foliar feeding on the essential oil composition and yields of D. anethifolia. In addition, the antioxidant activities of the plant extract were investigated and the total phenol and flavonoid contents of the plant were measured.

2. Material and Methods

2.1. Planting and harvesting

This study was performed at the research farm of the research center for medicinal and ornamental plants of Sistan and Baluchestan University with a latitude of 29°27'N and a longitude of 60°51'E at an altitude of 1410 m from sea level. In this experiment, seeds of the plant samples were planted in 7 rows of binary, each row was about 80 centimeters and the distance between every two rows was 20

centimeters. The plants were divided into five treatment groups including control (distilled water) and four concentrations of ethanol solution (10, 20, 40, and 80%). The ethanol spraying was performed before the flowering stage of the plants every three days 6 times. The spraying process began in early May and the plants were harvested in early June (spring 2016). After harvesting, the aerial parts of the plants were dried in the shade and stored until use. After collecting, the plants were transferred to the laboratory and dried in the shade. Then, the plants were milled, powdered, and prepared for essential oil.

2.2. Extraction Procedure

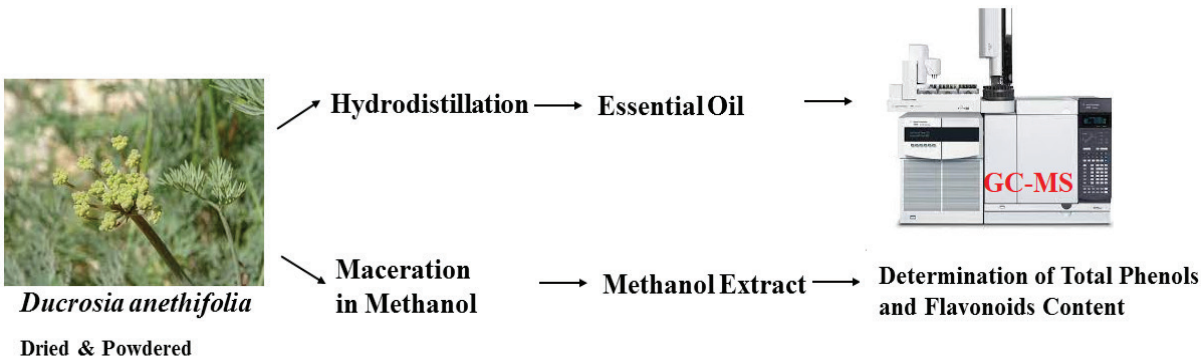
The dried aerial parts of the plants for each treatment were powdered separately with an electric mill. Then, the powdered samples were subjected to extraction. The essential oil of the samples was obtained by water distillation. For this purpose, 50 g of each dried plant sample was subjected to hydrodistillation for 3 h using a Clevenger-type apparatus (1000 ml water). The essential oils were collected in separate glass vials and were dehydrated with aid of sodium sulfate and magnesium sulfate salts. In order to obtain the extracts of the samples, the maceration method was used. About 5.0 g of the powdered samples were weighed accurately and added to separate Erlenmeyer flasks containing 50 ml methanol (covered with aluminum sheets) and placed on a magnetic stirrer for 24 hours. Then, the extracts were filtered with Watman filter paper and the solvent was removed by a rotary evaporator to obtain dry extracts (Schema 1).

2.3. GC-MS Analysis

In the next step, the essential oils were subjected to the GC-MS analysis to separate the chemical constituents and identify them according to their mass characteristics and retention times. The analysis of the essential oils was carried out using an Agilent system equipped with an HP-5S column (30 m × 250 µm, film thickness 0.25 µm) interfaced with a quadrupole mass detector (MS 5977A). Oven temperature 50-250°C (3°C per minute), injector temperature 250°C, injection volume: 0.1 µL, split injection with a split ratio of 1:50 helium as the carrier gas with flow rate 1 mL min^{-1}, ion source: 70 eV, ionization current: 150 µA, and scan range: 35-465. The method was validated as per the ICH guidelines for linearity, precision, accuracy, robustness, LOD, and LOQ according to our previous study [16]. Identification of the chemical constituents of the essential oil was carried out using AMDIS software (www.amdis.net) and identified by its retention indices with reference to the n-alkanes series (C6-C20), comparison of their retention time, mass spectra, and computer matching with the Wiley 7 nL and NISt library database.

2.4. Determination of total phenolic content

Total phenol content was determined by the Folin-Ciocalteu reagent. A dilute solution of the extracts (0.05:1 g mL^{-1}) or gallic acid (standard phenolic compound) was mixed with the Folin-Ciocalteu reagent (2.5 ml, 1:10 diluted with distilled water) and aqueous Na_2CO_3 (2 ml, 5%). The mixture was allowed to stand for 30 min and the phenolic contents were determined by colorimetry at 765 nm. The total phenolic content was determined as mg of gallic



Schema 1. Procedure for determination of flavonoid, phenolic contents in Ducrosia anethifolia

acid equivalent using an equation obtained from the standard gallic acid calibration curve [17].

2.5. Determination of total flavonoid content

The flavonoid contents of the extracts were measured by aluminum chloride coloration using quercetin as standard [18]. To extract flavonoids, 0.1 g of each extract was solved in 10 mL ethanol 80%. Then, 100 µL of the solution was added to a test tube, and 100 µL of 10% $AlCl_3$, 100 µL of 1 M sodium acetate, 1.5 mL of ethanol 96%, and 3.2 mL of distilled water were added and vortexed for 1 minute. The control treatment included 3.4 mL of distilled water, 100 µL of 1 M sodium acetate, and 1.5 mL ethanol 96%. After 30 minutes, adsorption was read at 415 nm.

2.6. DPPH free radical scavenging activity

This spectrophotometric method was used to evaluate the antioxidant activity of the extracts. DPPH is a reagent that measures free radical scavenging activity [19]. Zero, 0.01, 0.02, 0.03, 0.04, 0.05 mL of concentration 2000 mg L^{-1} of the extracts and positive control (ascorbic acid) were added to 1.0 mL of 0.1 mM solution of DPPH (Sigma, St Louis) in methanol. The reaction mixture was shaken and then incubated for 30 min at room temperature. The remaining amount of DPPH was determined at 517 nm against a blank using a spectrophotometer (Milton Roy Company Spectronic 2OD). All tests were carried out five times.

2.7. Ferric-reducing antioxidant power (FRAP) assay

The antioxidant capacity of the plant extracts was done by Iron reduction (FRAP assay) according to Sadeghi et al [17]. For this purpose, 300 mM acetate buffer (pH 3.610) mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$-$6H_2O$ solution were mixed for the preparation of stock. FRAP reagent was prepared right away before analysis by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$-$6H_2O$ solution. Plant extracts (1000 µg mL^{-1}) were prepared. 200 µg mL^{-1} of the extracts was mixed with 1.8 mL of the FRAP reagent and was incubated at 37 ºC for 30 min in the dark condition before being used. Then, readings of the colored products (ferrous tripyridyltriazine complex) were determined at 595 nm against a distilled water blank. $FeSO_4$-$7H_2O$ (100-1000 µM) was used for calibration. Ascorbic acid was used as a positive control. Results are expressed as mM Fe^{2+} per mg sample [17].

3. Results

3.1. Chemical composition of the essential oils

Table 1 shows the major components of the essential oil in the different treatments and control along with the percentage of each compound. The results revealed that ethanol spraying had a significant effect on the amount and yield of the essential oils. The yield of the essential oil was enhanced by increasing the amount of alcohol from 10% to 40% treatment with a decline in 80% alcohol-treated samples. The chemical composition of the essential oil of the control and that of the 10% treatment were to some extent similar. Interestingly, while in the two treatments 20% and 40%, the chemical composition is rather the same, their chemical composition is different from the blank and 10% treatment. Although the chemical composition of the 80% treatment was similar to 20% and 40%, there were some differences. For instance, compound 2-isopropyl-5-methyl-3-cyclohexene-1-one was only identified in the 80% treatment. Decanal, cis-verbenyl acetate, and dodecanal were major components in all samples with variations in different treatments.

Table 2 shows that monoterpenes and other compounds including alkanes are dominated in the essential oil samples. The number of oxygenated monoterpenes is increased in 20%, 40%, and 80% treatments while hydrocarbon monoterpenes are decreased in these samples compared to the control. Hydrocarbon monoterpenes are absent in the 20% sample.

The number of oxygenated sesquiterpenes is almost the same in all treatments with a small increase of 20% and 40%. The highest number of hydrocarbon sesquiterpenes were observed for 20% sample.

3.2. Determination of total flavonoid content

The highest and the lowest amount of total phenol per 1g of the plant powder were observed for 40%

Table 1. The major compounds identified in the essential oil of the different treatments.

[a]Rt	[b]RI	Compound	Control	10%	20%	40%	80%
4.936	939	α-Pinene	5.272	4.678	-	-	0.564
5.52	975	Sabinene	0.936	0.876	-	-	-
5.589	979	β-Pinene	0.251	0.223	-	-	-
5.737	990	β-Myrcene	1.428	1.341	-	-	-
6.287	1024	ρ-Cymene	4.217	3.593	-	-	0.330
6.355	1029	d-Limonene	5.178	4.575	-	-	0.348
7.271	1088	Terpinolene	0.343	0.329	-	0.523	-
7.46	1100	Nonanal	0.849	1.018	0.425	-	1.057
8.135	1137	cis-Verbenol	0.809	0.682	0.779	-	0.927
8.204	1140	Citronellal	0.801	0.902	-	-	-
8.364	1150	trans-Verbenol	0.247	0.210	0.962	0.607	0.253
8.432	1169	1-Nonanol	0.732	0.783	-	-	0.710
8.713	1179	ρ-Cymen-8-ol	0.458	0.419	-	-	0.620
8.776	1185	Cryptone	0.606	0.696	-	-	-
8.965	1200	Decanal	20.044	20.681	9.098	10.596	13.571
9.274	1250	3,7-Dimethyl-2-octen-1-ol	1.709	1.479	-	-	0.353
9.754	1274	[c]IMC Hexane	-	-	-	-	10.191
9.817	1282	cis-Verbenyl acetate	20.598	18.151	36.274	42.415	21.766
9.88	1286	5-Undecanol	1.816	1.560	1.038	0.778	1.311
10.155	1290	Lavandulyl acetate	0.915	0.828	1.562	1.315	0.623
10.367	1298	trans-Pinocarvyl acetate	-	-	0.420	1.093	-
10.395	1306	Undecanal	1.008	1.183	-	-	1.131
10.498	1352	Citronellyl acetate	0.659	0.634	2.058	2.255	1.019
11.408	1381	Geranyl acetate	1.387	1.467	0.958	1.243	0.331
11.677	1408	Z-Caryophyllene	0.451	0.575	0.490	-	0.243
11.757	1410	Dodecanal	10.768	11.953	6.777	6.431	11.053
12.072	1420	β-Caryophyllene	1.666	1.731	1.359	1.308	0.351
12.501	1436	γ-Elemene	3.458	3.246	22.994	1.055	2.038
13.25	1510	[d]CPCP	-	-	-	18.913	16.705
14.029	1578	Spathulenol	1.898	2.096	2.287	1.577	1.417
14.12	1583	Caryophyllene oxide	1.568	1.709	1.304	0.796	1.161
14.252	1612	Tetradecanal	0.342	0.452	0.467	0.425	0.814
14.515	1620	Unknown	2.536	2.689	1.287	1.165	0.604
14.652	1632	γ-Eudesmol	0.264	0.266	2.008	3.832	1.287
14.738	1677	Z-Nerolidyl acetate	0.778	0.833	0.724	0.462	0.225
14.887	1680	β-Eudesmol	0.506	0.576	1.168	-	1.723
14.939	1685	*n*-Tetradecanol	1.092	1.334	0.990	0.665	1.582
Total			73.176	90.761	95.429	97.454	94.287

[a]Retention time; [b]Kovats Index
[c]MC Hexane: 2-Isopropyl-5-methyl-3-cyclohexen-1-one
[d]CPCP: 2-Cyclopentylidene cyclopentanone

Table 2. The amount of different volatile compounds in different treatments.

Compounds	Control	10%	20%	40%	80%
Oxygenated Monoterpenes	28.189	25.468	43.013	59.524	36.083
Hydrocarbon Monoterpenes	17.625	16.453	-	0.523	1.272
Oxygenated Sesquiterpenes	5.014	5.480	7.491	6.667	5.813
Hydrocarbon Sesquiterpenes	5.575	5.552	24.843	2.363	4.670
Other Compounds	25.883	38.964	18.795	37.808	47.934

and 80% ethanol samples, respectively (Fig. 1). The highest and the lowest amount of total phenol per 1.0 g of the plant extract were observed for 40% and 10% ethanol samples, respectively (Fig. 2).

3.3. Determination of total flavonoid content

As shown in Figure 3, the 40% ethanol treatment showed the highest amount of flavonoid compared to the rest of the treatments.

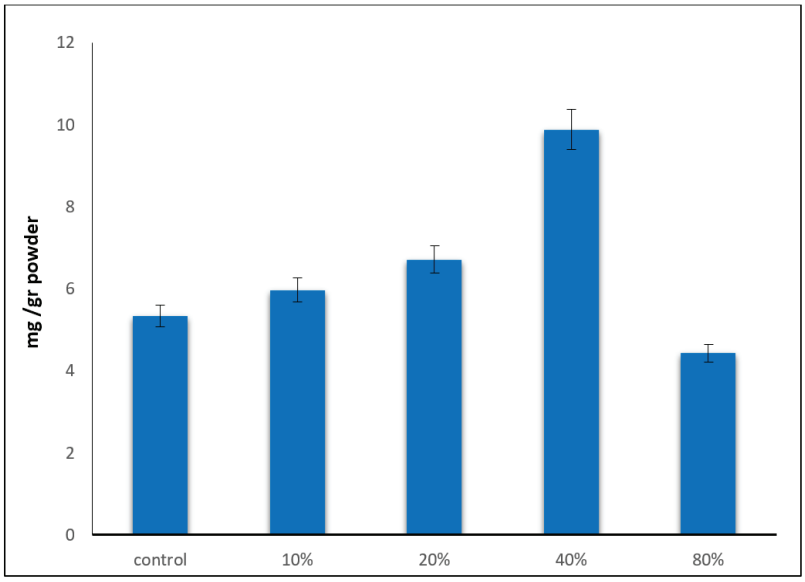


Fig. 1. The total phenol content in the plant powder of different treatments.

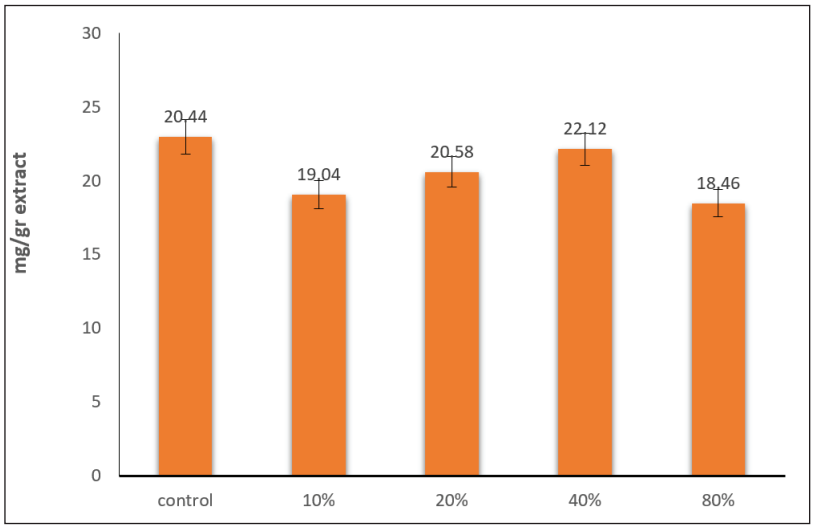


Fig. 2. The total phenol content of the extracts from different treatments.

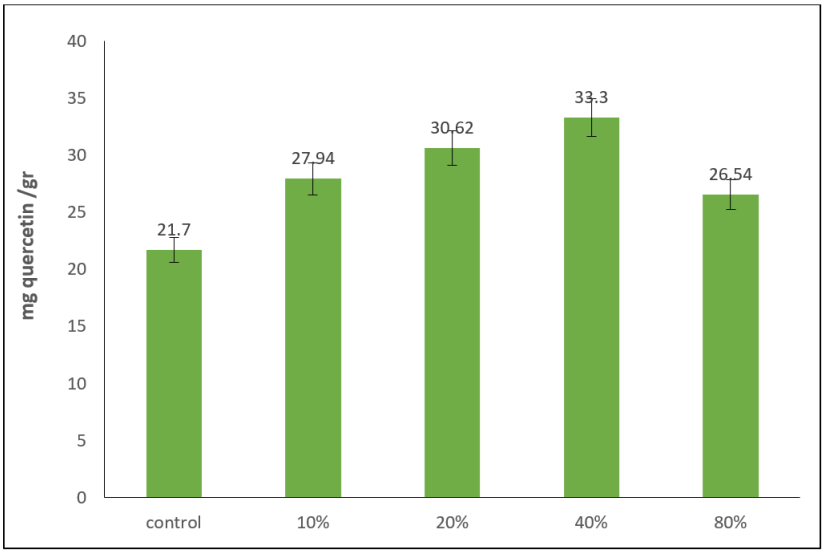


Fig. 3. The total flavonoid content of the extracts from different treatments.

3.4. DPPH free radical scavenging activity

The results of DPPH radical-scavenging activity assay are shown in Figure 4. Considering the large variation of IC_{50}, the lowest antioxidant activity was observed for 80% treatment compared to the control. Treatments with 10%, 20%, and 40% showed almost the same antioxidant activity, all higher than that of the control.

3.5. FRAP assay

All analyzed extracts demonstrated significant antioxidant capacities with FRAP test. The 40% treatment showed 53.00 $mMFe^{2+}$/mg sample with the highest antioxidant activity compared to reducing power of ascorbic acid (69.00 $mMFe^{2+}$/mg sample) (Fig. 5).

Totally, the results from the determination of phenolic and flavonoid contents of the samples as well as DPPH and FRAP assays revealed that the amount of these compounds and the antioxidant capacity of the plant samples were influenced by the use of ethanol spraying which was shown in Table 3. By increasing the concentration of alcohol from 10% to 40%, the phenol and flavonoid contents and consequently the antioxidant activity reached their maximum rate.

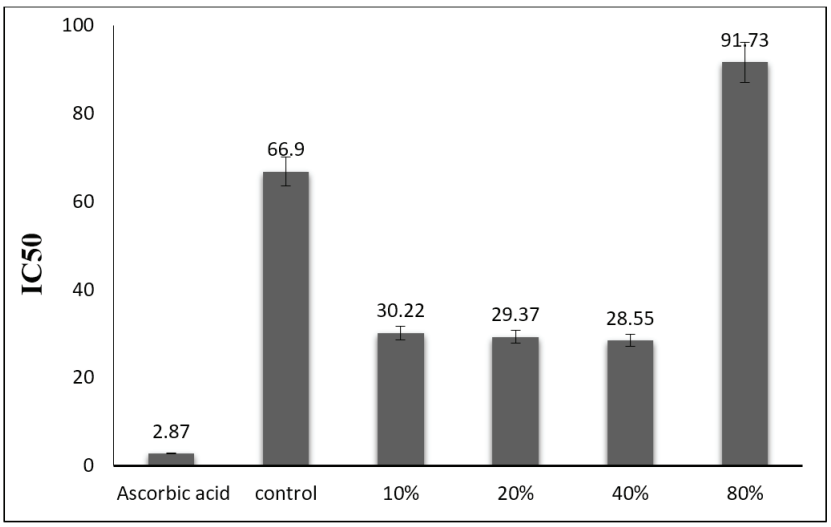


Fig. 4. DPPH free radical scavenging activities observed for different treatments compared to ascorbic acid as positive control

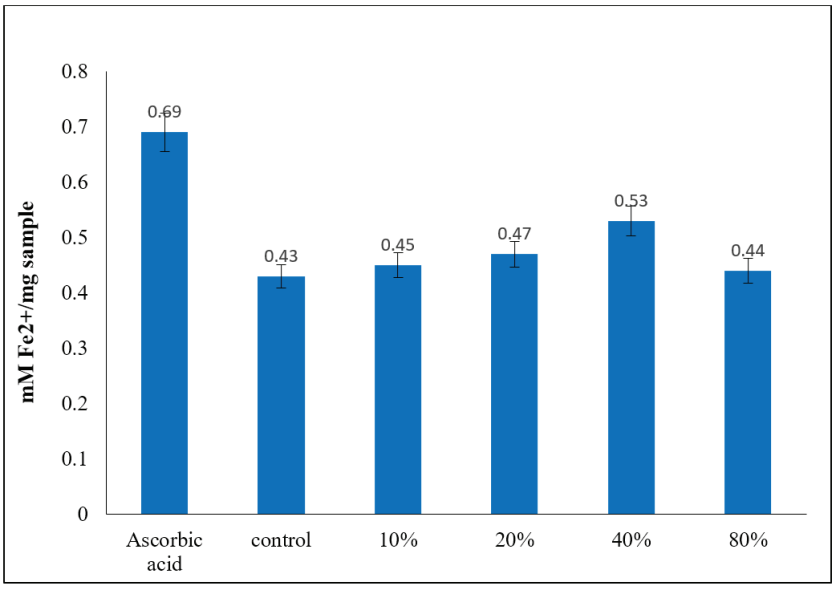


Fig. 5. The antioxidant capacity of different treatments compared to ascorbic acid as a positive control

Table 3. Comparing the phenolic and flavonoid contents as well as antioxidant activities of the samples.

Plant samples	IC_{50}	Fe^{2+} mM/ mg sample	mg total phenol/ 1 gr powder	mg total phenol/ 1 gr extract	mg quercetin/ 1 gr extract
Control	2.87	0.69	5.33	20.44	21.7
10%	66.9	0.43	5.97	19.04	27.94
20%	30.22	0.45	6.71	20.58	30.62
40%	28.55	0.47	9.88	22.12	33.3
80%	91.73	0.44	4.43	18.46	26.54

4. Discussion

Foliar feeding is a technique of feeding plants by applying liquid fertilizer directly to the leaves. Due to the increased rate of absorption through the aerial parts of plants, it is an excellent method to deliver food and elements required for plants much faster [19]. Studies show that using alcohols with different concentrations would exert different effects on different plant species. The most important role for methanol operating in C_3 plants is to prevent light respiration, probably due to increased CO_2 concentration in leaves. If the concentration of CO_2 increases in leaves, ribulose 1,5-bisphosphate will react with CO_2 instead of O_2, and the carboxylation function will occur. Therefore, the alcohol-induced biomass increase of the C_3 plants might cause the plant to use methanol as a direct source of carbon for serine biosynthesis and reduce carbon waste through light respiration [15]. There are several reports investigating the effect of alcohol on the function of different plants. For instance, Zbiec and Podsiad (2003) investigated the effect of alcohol spraying and reported the increasing quantitative and qualitative yield of this technique on plants such as geranium, wheat, turnip, and sugar beet [21]. Iqbal Makhdum et al. studied the effect of methanol spraying on cotton plants and observed that 30% methanol treatment has been able to increase plant function compared with the control treatment [22]. In another research, performed by Safarzadeh Vishkaei who studied the effect of methanol on peanuts, 30% methanol treatment could increase the height of plant and grain function. Methanol and ethanol (30%) spraying could increase plant growth and the essential oil amount of peppermint [23]. The application of alcohol foliar feeding

would induce increasing the production of cytokinin and plant growth [24]. In addition, foliar feeding of alcohols in plants might induce increasing the plant metabolites including essential oils. Studies show that plants exposed to environmental stresses might increase the production of their specialized metabolites to confront the stimulant leading to more metabolite synthesis.

The current study showed that alcohol foliar feeding had effects on the amount of essential oil of the medicinal plant D. anethifolia and its composition. Our results showed that monoterpenes and alkanes such as n-decanal were the major components in the essential oil of D. anethifolia. In most of the studies investigating the essential oil composition of this medicinal plant, n-decanal was reported as the main constituent [25,26]. For example, Salari et al. have reported n-decanal (22.29%) as the most abundant constituent of D. anethifolia essential oil (Kerman), followed by alkanes decanol (22.18%) and dodecanol (11/79%) [27]. There are also some studies in which decanal has not been reported as the major compound [3,28,29]. The results of Arbabi et al. showed that cis-chrysanthenyl acetate with an average amount of 44.77% was the main compound of the essential oil of D. anethifolia from Sistan & Baluchistan [2].

Besides, our results revealed that this technique had beneficial effects on the amount of phenolic and flavonoid contents and consequently on the antioxidant activity of the plant. In total, the best efficiency was observed for 40% ethanol treatment. This can be due to the nutritional role of ethanol as a carbon source, stimulus, and active substance in metabolic reactions. In addition, ethanol plays an essential role in the biosynthetic pathway for the production of terpenoids. The opposite effect observed in the higher percentages of alcohol might be due to the plant poisoning leading to the decreased level of specialized metabolites and antioxidant capacity. The results of this experiment showed that the use of the hydroalcohol spraying could increase essential oil production, so it is suggested to use of hydroalcoholic foliar feeding to increase D. anethifolia metabolites in future research.

5. Conclusion

Taken together, the results of this experiment showed that ethanol spraying could increase the production of essential oil as well as phenolic and flavonoid compounds in D. anethifolia. Since the plant is an important medicinal plant, this might increase its efficacy. Thus, hydroalcoholic foliar feeding can be a valuable strategy to increase the specialized metabolites of D. anethifolia in future research.

6. Acknowledgements

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