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Antioxidant activity of *Echinophora platyloba* DC essential oil: a comparative study on four different methods

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Keywords

Echinophora platyloba, ABTS, DPPH, FRAP, β-carotene bleaching test

Abstract

The present study is conducted in order to investigate the antioxidant capacity of *Echinophora platyloba* DC (Duncan) essential oil (EO), growing wild in west Azerbaijan, Iran, with four different assays. The aerial parts of *Echinophora platyloba* DC were provided from Maraghe city district, northwest of Iran and its phytochemicals were determined using GC-MS analysis. In order to evaluate and compare the antioxidant activity of the EO, various concentrations (1, 2.5, 5 and 10 mg.ml-1) of the oil and reference antioxidants (ascorbic acid and BHT) were analyzed by four different assays such as 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric reducing antioxidant power (FRAP) and β-carotene bleaching test (BCBT). The oil exhibited moderate antioxidant activity with a dose-response in DPPH and ABTS assays and it exhibited strong antioxidant activity in FRAP and BCBT assays. It is concluded that FRAP and BCBT assays are more suitable spectrometric assays for antioxidant capacity evaluation of *Echinophora platyloba*.

Abbreviations

E. platyloba: *Echinophora platyloba*
EO: Essential Oil
BHT: Butylated Hydroxytoluene
DPPH: 2,2-Diphenyl-1-Picrylhydrazyl
ABTS: 2,2'-Azino-Bis 3-ethylbenzothiazoline-6-Sulphonic acid
FRAP: Ferric Reducing Antioxidant Power
BCBT: β-Carotene Bleaching Test
SD: Standard Deviation
DC: Duncan

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Introduction

The genus *Echinophora* (family Umbelliferae, subfamily Apioideae, tribe Echinophoreae), is represented in the flora of Iran by four species two of which are endemics including *Echinophora platyloba* DC and *Echinophora cinerea* (Boiss). *E. platyloba* DC (with the Persian name "Khosharizeh", "Khosharouzeh" or "Tigh Toragh") that is a midsummer plant, i.e. one of the most fascinating species that wildly grows and is well known for its favorable aromatic properties. Fresh and dried aerial parts of this species are used as a seasoning and anti-mold agent in pickled cauliflower, gherkin, and native dairy products like cheese, yoghurt and doogh (a savory yogurt-based beverage popular in Iran) in the west and northwest of Iran (Saei-Dehkordi et al., 2012).

Different assays, including a wide range of spectrophotometric assays have been employed to measure the antioxidant capacity of EOs and their extracts. There is a certain principle in most of these assays. This principle is that a colored radical or redox-active compound is generated; then the ability of a sample for scavenging the synthetic radical or reducing the redox-active compound is monitored using a spectrophotometer and an appropriate standard such as ascorbic acid is applied to quantify antioxidant capacity (Floegel et al., 2011). The most frequently used assays are 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assays as well as β-carotene bleaching test (BCBT) and ferric reducing antioxidant power (FRAP) (Ou et al., 2002; Thaipong et al., 2006).

Application of chemical antioxidants in food could result in different negative health effects such as hepatic damages, toxicities, and cancer. Therefore, a global interest is emerging in antioxidant characteristics of EOs (Rohani et al., 2011; Saei-Dehkordi et al., 2010; Saei-Dehkordi et al., 2012). Antioxidants that have originated from plants have biological effects by scavenging free radicals and inhibiting oxidation. Application of these natural compounds with antioxidant properties can prevent oxidation and extend the shelf life of foods (Ani et al., 2006). On the other hand, consumption of foods containing natural antioxidant may transmit these bioactive compounds to the body to play a role in the inhibition of oxidative damage due to free radicals formed in the human cells (Sharafati-chaleshtori et al., 2012). To our best knowledge, there is no comprehensive work focusing on antioxidant capacity of *E. platyloba* DC. EO. Moreover, no data have been published on the antioxidant capacity of the oil of this plant grown in the northwest of Iran as one of the most geographically important regions of this plant's growth due to its commonly conventional consumption in various foods. Therefore, the present study is conducted to assess and compare the antioxidant properties of *E. platyloba* EO grown in the northwest of Iran using four routine assays including DPPH, ABTS, BCBT and FRAP.

Materials and Methods

Plant material

The aerial parts of *E. platyloba* DC were harvested at the flowering stage (10th June to 15th August 2010) from wild grown plants in the Maragheh city district of East Azerbaijan province, Iran. A voucher specimen (no. 6502) was deposited in the Herbarium of West Azerbaijan Agricultural and Natural Resource Center, Urmia, Iran.

Extraction and analysis of *E.platyloba* were provided according to the method recommended by the European Pharmacopeia (Ahmad et al., 1999). The obtained chemical composition of *E.platyloba* EO components were described by Hashemi et al., (2016).

DPPH radical-scavenging assay

This assay was performed as previously described with minor modification (Erkan et al., 2008). 50 µL of various concentrations (1, 2.5, 5 and 10 mg.ml-1) of the *E. platyloba* EO and reference antioxidants, ascorbic acid and BHT (Merck , India), in methanol were mixed with 2 ml of methanolic DPPH solution (24 µg.mL-1). After an incubation period of 60 min at room temperature in a dark place, the absorbance at 517 nm, the wavelength of maximum absorbance of DPPH, were measured as ASample, using a spectrophotometer (LKB Novaspec II; Pharmacia, Sweden). A solution without the test material was also carried out applying the same procedure and the absorbance was recorded as ABlank. The free radical-scavenging activity was calculated according to the following equation:

Radical scavenging activity (%) = 100 × (ABlank – ASample)/ABlank

β-carotene bleaching test (BCBT)

β-carotene bleaching test was carried out as previously described with minor modifications (Miraliakbari and Shahidi, 2008). A solution of 5 mg β-carotene in 10 ml chloroform was prepared and 1 mL of this solution was added into a 100 ml round bottom flask. Chloroform was removed under vacuum using a rotary evaporator (Heidolph laborta 4003, SchwaBach, Germany) at 40°C.Then 25 µL linoleic acid, 400 mg Tween 40 emulsifier and 100 ml of aerated distilled water were added to the flask and shaken vigorously. Aliquots of 2.5 ml of this emulsion were transferred into a series of test tubes containing 350 µL of various concentrations (1, 2.5, 5 and 10 mg.ml-1) of the *E. platyloba* EO and reference antioxidants (ascorbic acid and BHT). The absorbance of each tube was measured at 470 nm immediately at time zero and subsequently over a two-hour period at 20 min intervals while the tubes were kept in a water bath at 50°C. The capacity of the *E. platyloba* EO to protect against oxidation of β -carotene was determined as shown in the following equation:

I % = (Aβ-carotene after 2h assay / AInitial β-carotene) ×100

Table 1
DPPH radical scavenging activity of *Echinophora platyloba* DC essential oil

Sample	C 1 (mg/ml)	C 2.5 (mg/ml)	C 5 (mg/ml)	C 10 (mg/ml)
Essential oil	16.70 ± 3.17 ^{aA}	29.70 ± 1.40 ^{bB}	36.36 ± 1.25 ^{cC}	41.43 ± 2.81 ^{dD}
Ascorbic acid	99.99 ± 0.00 ^{aA}	99.99 ± 0.00 ^{aA}	99.99 ± 0.00 ^{aA}	99.99 ± 0.00 ^{aA}
BHT	99.98 ± 0.00 ^{aA}	99.98 ± 0.00 ^{aA}	99.99 ± 0.00 ^{aA}	99.99 ± 0.00 ^{aA}

Same uppercase and lowercase letters indicate no significant differences within a row and column, respectively ($p > 0.05$).

Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was performed followed by the method previously described with some modifications (Benzie, 1996). The working FRAP reagent was prepared by mixing 300 mM acetate buffer, pH 3.6, (3.1 g sodium acetate and 16 ml glacial acetic acid made up to 1 L with distilled water); 10mM 2,4,6-tri-2-pyridyl- 1,3,5-triazin (TPTZ) in 40 mM HCL; 20 mM ferric chloride at 10:1:1. Aliquots of 4.5 ml of the mixture were added into the tubes containing 150 µL of various concentrations (1, 2.5, 5 and 10 mg/ml) of the *E. platyloba* EO in methanol and reference antioxidants (ascorbic acid and BHT) and kept at 37°C for 8 min. The absorbance of each tube was measured at 593 nm using a spectrophotometer (LKB Novaspec II; Pharmacia, Sweden) against working FRAP reagent without any antioxidant agent as blank. Data were expressed as µmolFe+2. mgEO -1 relative to the values obtained with a standard curve prepared using known concentrations of Fe+2 solutions.

ABTS radical scavenging assay

The free radical scavenging activity of the *E. platyloba* EO against radical cation (ABTS+) was measured according to the previously reported procedure (Ye et al., 2013). The ABTS solution (7 mmol/l) and potassium persulphate solution (2.45 mmol/l) in distilled water were separately prepared and reacted together to produce ABTS radicals. The mixture was kept in the dark at room temperature for 16 h. In the moment of use, the ABTS+ solution was diluted with phosphate buffer saline (PBS) to an absorbance of 0.70 at 734 nm. Aliquots of 200 µL of various concentrations (1, 2.5, 5 and 10 mg/ml) of the *E. platyloba* EO in methanol and reference antioxidants (ascorbic acid and BHT) were added to 2 mL of ABTS+ solution and mixed vigorously. After reaction at room temperature for 6 min, the absorbance at 734 nm was measured and the ABTS+ scavenging effect was calculated by using the following formula:

Radical scavenging activity (%) = [1- (ASample / ABlank)]×100

Statistical analysis

All assays were performed in triplicates and results were expressed as a mean ± standard deviation (SD). The data were statistically analyzed in one-way ANOVA using SPSS (16.0) softwar*E.* Post hoc tests were applied for pair wise comparison of the means which is indicated by subscript letters (for different concentrations c1-c10) or stars (for different samples: EO, AA, and BHT).

Results

The major compounds of the EO were p-cis-Ocimene (26.51%), 2,3 Dimethyl-1,3-cyclohexadiene (9.87%), Alphapinene (7.96%), gamma-dodecalactone (5.84%) and Nerolidol (5.66%), representing 93.29% of the total content of the oil (Hashemi et al., 2016).

Antioxidant activity

DPPH: Results of DPPH assay of *E. platyloba* EO are presented in Table 1, which is in the order: ascorbic acid> BHT> *E. platyloba* EO. The same concentrations of ascorbic acid and BHT were used for comparison. Scavenging of the DPPH radicals by the oil was dose dependent and increased with increasing the EO concentration.

BCBT

As it can be seen in Table 2, *E. platyloba* EO had a strong activity in maintenance of β-carotene molecules, especially in C10 (78.25%), which was higher than ascorbic acid but it was lower than BHT ($p<0.05$). The same concentration of ascorbic acid and BHT were used as standards to compare the values.

FRAP

The antioxidant efficiency of the *E. platyloba* EO using the FRAP method was calculated with reference to the reaction formula, given by a Fe+2 solution of known concentration. The same concentration of ascorbic acid and BHT were used for comparison. As it is shown in Table 3 the oil was stronger than the standards to reduce the Fe+3 to Fe+2.

ABTS

Table 4 lists the results of the ABTS test of *E. platyloba* DC EO. The ABTS radical scavenging of the oil was dose dependent and increased with the increase of the *E. platyloba* EO concentration. The same concentration of ascorbic acid and BHT were used for comparison.

Discussion

Table 2
BCBT antioxidant power of *Echinophora platyloba* DC essential oil

Sample	C 1 (mg/ml)	C 2.5 (mg/ml)	C 5 (mg/ml)	C 10 (mg/ml)
Essential oil	32.28 ± 1.28 ^{aA}	44.50 ± 1.89 ^{bB}	56.85 ± 1.42 ^{cC}	78.25 ± 2.15 ^{aD}
Ascorbic acid	9.52 ± 2.20 ^{bA}	12.33 ± 3.11 ^{aB}	17.94 ± 2.45 ^{aC}	27.51 ± 3.58 ^{bD}
BHT	82.34 ± 0.74 ^{aA}	88.53 ± 0.43 ^{cB}	95.83 ± 0.52 ^{bC}	99.99 ± 0.00 ^{cD}

Same uppercase and lowercase letters indicate no significant differences within a row and column, respectively ($p > 0.05$).

There are various analytical methods for evaluation of antioxidant capacity as follows: spectrometric techniques, electrochemical techniques, biosensors method, and chromatographic methods (Pisoschi and Negulescu, 2012). Regarding the complexity of the analytical instruments, the photometric methods are the simplest in comparison with other techniques. DPPH, ABTS, BCBT and FRAP assays are based on the reaction of a radical with an antioxidant molecule, donating a hydrogen atom (Pisoschi et al., 2009). The DPPH assay has gained high popularity over the last decade due to its rapidity, simplicity and sensitivity (Aliakbarlu et al., 2013).

Although another study has been previously carried out by Saei-Dehkordi et al., (2012) on the antioxidative activity of *E. platyloba* DC EO, using the oil of the plant grown in the southwest of Iran and using two antioxidant evaluation methods (DPPH and BCBT), the major differences and strengths of the present study with it are the different geographical region of the plant that is an important factor affecting phytochemicals and other characteristics of the oils, and using more different antioxidant evaluation. Saei-Dehkordi et al., (2012) have reported higher antioxidative capacity of the oil in comparison with the current study, which can be due to different components of the *E. platyloba* oil. Either oxygenated monoterpenes or monoterpene hydrocarbons could be the main antioxidant components of the plants. In addition, oxygenated sesquiterpenes and sesquiterpene hydrocarbons are attributed to their weak antioxidant capacity. Surely, the presence of phenolic compounds (high amount of thymol + carvacrol) in the *E. platyloba* EO composition of their study could affect the total antioxidant capacity. It should be considered that differences in the geographical region, climatic conditions stage of maturity, distillation conditions and other factors are already known as the major causes of different components of EOs (Moradi et al., 2014).

In BCBT assay linoleic acid is used as a model lipid substrate in an emulsified form. In fact, this assay is somewhere between assays using only model substrates (*E*.g. ABTS or DPPH) and those employing real lipids (Koleva et al., 2002). Regarding the basis of this assay, the yellowish color of β-carotene disappears in BCBT assay due to its reaction with radicals derived by linoleic acid oxidation and antioxidants prevent its oxidation and delay bleaching of β-carotene resulting in maintenance of β-carotene (Saei-Dehkordi et al., 2012). The BCBT results in the present study are completely consistent with other studies which shows higher antioxidant activity of different EOs compared with ascorbic acid using BCBT (Kulisic et al., 2004; Prakash et al., 2012). Ruberto and Baratta (2000) (Ruberto and Baratta, 2000) reported that the strongly activated methylene groups in monoterpene hydrocarbons are the major reason for their antioxidant capacity especially in BCBT assay. In the present study, ocimene which belongs to monoterpene hydrocarbons, was the major constituent of the *E. platyloba* EO (26.51%) and this could be the reason for the strong activity of the oil in maintenance of β-carotene molecules.

Table 3
FRAP reducing power of *Echinophora platyloba* DC essential oil

sample	C 1 (mg/ml)	C 2.5 (mg/ml)	C 5 (mg/ml)	C 10 (mg/ml)
Essential oil	204.77 ± 1.20 ^{aA}	305.68 ± 1.82 ^{bB}	332.12 ± 2.11 ^{aC}	+
Ascorbic acid	101.89 ± 1.80 ^{bA}	214.88 ± 2.05 ^{aB}	266.72 ± 3.21 ^{bC}	+
BHT	170.97 ± 3.52 ^{cA}	281.78 ± 2.65 ^{cB}	328.67 ± 1.85 ^{cC}	+

+ This concentration was not detectable by the spectrophotometer due to intense color.
Same uppercase and lowercase letters indicate no significant differences within a row and column, respectively ($p > 0.05$).

Table 4
ABTS radical scavenging activity of *Echinophora platyloba* DC essential oil

Sample	C 1 (mg/ml)	C 2.5 (mg/ml)	C 5 (mg/ml)	C 10 (mg/ml)
Essential oil	13.05 ± 0.39 ^{aA}	25.76 ± 1.86 ^{aB}	37.28 ± 4.22 ^{aC}	63.53 ± 2.45 ^{aD}
Ascorbic acid	98.40- 0.09 ^{bA}	98.75 ± 0.00 ^{bB}	99.16 ± 0.01 ^{bC}	99.65 ± 0.09 ^{bD}
BHT	98.19 ± 0.19 ^{bA}	98.68 ± 0.09 ^{bB}	99.37 ± 0.09 ^{cC}	99.72 ± 0.19 ^{bD}

Same uppercase and lowercase letters indicate no significant differences within a row and column, respectively ($p > 0.05$).

Use of FRAP assay is a versatile method and can be readily applied to both EOs and extracts (aqueous, alcoholic and acetone extracts) of plants. The ability of any antioxidant to reduce Fe+3 (TPTZ) complexes (ferric iron) to the intensely blue-colored Fe+2 (TPTZ) complexes (ferrous iron) at acidic pH, is the basis of this method (Bhatt et al., 2012). The *E. platyloba* EO showed stronger antioxidant activity than ascorbic acid and BHT using FRAP assay in this study. Pirbalouti et al.,(2013) evaluated the antioxidant activity of methanol extracts of Heracleum lasiopetalum Boiss, Kelussia odoratissima Mozaff., and *E.platyloba* DC. by three assays, including DPPH, FRAP, and TEAC. From all three assays, *E. platyloba* had the highest antioxidant activity. They showed that FRAP assay increased in the order of BHT ≥ *E. platyloba*. Different components of *E.platyloba* EOs explains the dissimilar results of FRAP assay. Wong et al (2006) divided antioxidant powers in four groups as : very low FRAP (<10 µmol Fe(II)/g), low FRAP (10–50 µmol Fe (II)/g), good FRAP (50–100 µmol Fe(II)/g) and high FRAP (100–500 µmol Fe(II)/g). *E. platyloba* EO of current study also showed high FRAP amount.

The ABTS radical is also commonly used to evaluate the in vitro antioxidant activity of different substrates based on scavenging and discoloration of intense green ABTS radicals. This method measures the antioxidant activity of both water-soluble and lipid-soluble antioxidants (Erkan et al., 2008). To the best of our knowledge, this is the first study providing data on antioxidant activities of *E. platyloba* EO for scavenging ABTS radicals. The results revealed that the concentration responses of the oil to ABTS assay were quite similar to the DPPH reaction. However, there is an important limitation for interpreting the role of hydrophilic antioxidants, because DPPH can only be dissolved in organic solvents such as ethanol or methanol. This can be the reason for lower scavenging activity of the oil in the DPPH results of this study than he ABTS results (Miliauskas et al., 2004; Zeng et al., 2011).

Remarkable differences were observed for the antioxidant ability of *E. platyloba* EO using different methods, which was a weaker antioxidant in DPPH and ABTS assays, while it was a strong antioxidant in BCBT and FRAP assays. The antioxidant power of the oil in different methods was in the following order: DPPH < ABTS < BCBT< FRAP. The observed differences could be due to different polarities of solvent and pH in the reaction media (Koleva et al., 2002).

Conclusion

The results of this study revealed that the antioxidant activities of plant EO and its extracts should be evaluated by different assays. Indeed, due to various different chemical aspects of phytochemicals, using at least two assays with completely different basis is recommended to determine antioxidant activity and establish authenticity. According to the results, FRAP and BCBT assays are more suitable spectrometric assays for antioxidant capacity evaluation of *Echinophora platyloba*.

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فعالیت آنتی اکسیدانی روغن فرار اکینوفورا پلیتی لوبا: یک مطالعه ی مقایسه ای برروی چهار روش متفاوت

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چکیده

مطالعه حاضر به منظور بررسی جامع قابلیت آنتی‌اکسیدانی عصاره روغنی *اکینوفورا پلیتی لوبا*، که به صورت وحشی در آذربایجان غربی، ایران رشد میکند، انجام شد. بخش‌های هوایی *اکینوفورا پلیتی لوبا* از ناحیه شهرستان مراغه، شمال غرب ایران فراهم شد و فیتوکمیکال‌های آن با روش آنالیز GC-MS تعیین گردید. به منظور ارزیابی و مقایسه فعالیت آنتی‌اکسیدانی روغن ضروری، غلظت‌های متفاوتی (۱، ۵/۲، ۵ و ۱۰ میلی گرم/میلی لیتر) از روغن و آنتی‌اکسیدان‌های مرجع با چهار روش ۲و۲- آنیزو- بیس ۳- اتیل بنزوتیازولین – ۶- سولفونیک اسید (ABTS)، ۲و ۲- دیفنیل ۱- ۱- پیکریل هیدرازیل (DPPH)، توان آنتی - اکسیدانی کاهنده فریک (FRAP) و آزمون بیرنگ کننده بتاکاروتن (BCBT) آزمایش شد. روغن ضروری *اکینوفورا پلیتی لوبا* فعالیت آنتی‌اکسیدانی متوسط وابسته به دوز در روش DPPH و ABTS و فعالیت آنتی‌اکسیدانی قوی در روش FRAP و BCBT از خود نشان داد. روش های FRAP و BCBT روش های کدورت سنجی مناسبی برای ارزیابی قابلیت آنتی‌اکسیدانی *اکینوفورا پلیتی لوبا* می‌باشند.

واژگان کلیدی: *اکینوفورا پلیتی لوبا*، ABTST، DPPH، FRAP، آزمون بیرنگ‌کننده بتاکاروتن