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Antioxidant properties, proximate content and cytotoxic activity of *Echinophora tournefortii* Jaub. & Spach

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Abstract

This work was designed to evaluate the phenolic, flavonoid content and biological activities (antioxidant and cytotoxic) of *E. tournefortii* extracts (methanol, acetone and water) as well as to determined proximate parameters (ash, fat, protein and carbohydrate). Among the three different extracts of *E. tournefortii* evaluated, the methanol extract, showed the highest amount of antioxidant activities (β-carotene, 88.62%). There were statistical differences among the radical scavenging (DPPH and ABTS) and antioxidant (phosphomolybdenum) activities of the different extracts of *E. tournefortii*. In the metal chelating and reducing power activities, the water extract exhibited the highest chelating and reducing capacity (29.88% and 0.206mg/mL respectively). All extracts of *E. tournefortii* exhibited cytotoxic activities and this plant possesses nutrients. These findings will provide addition information for the further investigation of this plant, for understanding the efficacy of *E. tournefortii* as a food ingredient, as well as for preventing oxidative stress mediated disorders.

Keywords: *Echinophora tournefortii*; antioxidant; cytotoxic; proximate compound.

Practical Application: *Echinophora tournefortii* may be considered an alternative source of antioxidant and cytotoxic agents for pharmacological applications and for food industry.

1 Introduction

As the life source for living organisms, oxygen produces oxygen radicals with physical and chemical events. For living organisms, especially those that metabolize oxygen, reactive oxygen types are formed through enzyme-catalyzed metabolic pathways, by various biological functions, by exposure to ultraviolet light, or when foreign substances are taken up in the body. Reactive oxygen species cause damage to the cell by altering the structure, resulting in several mutations such as base modifications, base deletions, and chain breaks on the DNA and RNA. As a result of these mutations, the protein synthesis mechanisms that begin with the transcription of nucleic acids, change and thus, damage can occur in many of the enzyme-catalyzed metabolic pathways. Major DNA damage cannot be repaired and can, cause cell death or range of cancers (Nordberg & Arner, 2001; McCord, 2000). In these cases, antioxidants that, convert reactive oxygen species to non-toxic products and stop or eliminate the adverse effects of reactive oxygen species, prevent some disorders, such as cardiovascular diseases, cataracts, diabetes and infections. It is acknowledged that consumers believe any medicine derived from plant sources is safer and healthier than synthetic ones and hence there is more focus on the replacement of synthetic antioxidants with natural additives. For this reason, in order to find new and effective sources, studies on the screening of medicinal plants containing functional compounds that provide antioxidant properties, have become very important in recent years (Al-Dabbas, 2017).

Echinophora tournefortii Jaub. & Spach belongs to the genus *Echinophora* and locally it is called 'dikenli çörtük' in Turkish. This plant is a perennial and 20-40 cm high (Baytop, 1994). The genus *Echinophora* belongs to the Apiaceae family, which comprises approximately 10 species, and is distributed through the Mediterranean and Middle East regions (Rechinger, 1987). In the Flora of Turkey, this genus is represented by six species, three of which are endemic (Davis, 1972). Members of this genus are used for imparting flavor to foods such as cheese, yogurt (Delazar et al., 2015) and in folk medicine it is used to treat diseases such as gastric ulcers and wounds (Gokbulut et al., 2013). Various *Echinophora* plants have also previously been investigated for the antioxidant activities of the essential oils (Delazar et al., 2015; Mileski et al., 2014; Gokbulut et al., 2013; Gholivand et al., 2011) and various extracts (Mileski et al., 2014; Gholivand et al., 2011). Nevertheless, there is no report that has studied the phenolic composition and biological activities of various extracts of *E. tournefortii*. The aim of this study is to examine the following three items: (1) the antioxidant capacities of methanol, acetone and water extracts using six complementary methods, radical scavenging (DPPH and ABTS), the antioxidant (β-carotene/linoleic acid test system, phosphomolybdenum), reducing power and metal chelating assays (2) total phenolic and flavonoid content (3) proximate composition and (4) cytotoxic activity.

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2 Materials and methods

2.1 Plant materials and preparation of plant extracts

Echinophora tournefortii was collected at the flowering stage, from Kınıklı, Denizli, Turkey, in August 2017. The plant material was identified and stored with voucher specimens (*Echinophora tournefortii*; Herbarium No: 2017-200) at the private herbarium of Dr. Mehmet Cicek, plant taxonomist from the Biology Department of the Arts and Science Faculty, Pamukkale University, Denizli, Turkey. The aerial parts of *E. tournefortii* were air-dried and powdered in our laboratory. The extractions were performed by mixing the sample (30 g) with 300 mL of solvents with varying polarities (methanol, acetone and water) and shaking at 50 °C for 6 h in a temperature controlled shaker. The extracts were filtered twice with filter paper (Whatman No.1) and evaporated using a rotary evaporator (IKA RV10D, Staufen, Germany) under vacuum at 40-50 °C. The samples were lyophilized (Labconco FreeZone, Kansas City, MO) and kept at -20 °C until tested. All the experiments were done in triplicates.

2.2 Chemicals

β-carotene, Linoleic acid, 2,2-Diphenyl-1-picryl hydrazyl radical (DPPH), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), Phosphate buffer, Iron (III) Chloride, Quercetin, Sodium phosphate, Gallic acid, methanol, ethanol and acetone were purchased from Sigma-Aldrich. Butylated hydroxy toluene (BHT), Folin-Ciocalteu reagent and Tween 20 were purchased from Merck (Darmstadt, Germany). Other chemicals and solvents were of analytical grade.

2.3 Determination of total antioxidant activities

β-carotene/linoleic acid method

Using this method, antioxidant activity was carried out, in accordance with the method of Ismail & Tan (2002). The β-carotene stock solution was prepared as follows: 0.2 mg β-carotene was dissolved in chloroform and 0.02 mL of linoleic acid and 0.2 mL of 100% Tween 20 was added. The chloroform was evaporated using a rotary evaporator and 100 mL of distilled water was added to the remaining residue. The extracts (1 mg/mL) were mixed with this emulsion (24 mL) and the initial absorbances were immediately measured with a spectrophotometer at 470 nm. The reaction mixture was incubated at 50 °C for 2 hours and then the absorbance of this mixture was measured again. The BHT was used as a positive control.

Phosphomolybdenum method

The antioxidant capacities of the *E. tournefortii* extracts were evaluated according to Prieto et al. (1999). The reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate was prepared and the reagent solution (3 mL) and 0.3 mL extract were mixed. The reaction mixture were incubated at 95 °C for 90 min. The absorbances of the mixtures were measured at 695 nm using a spectrophotometer. The antioxidant activity of the extracts was denoted as equivalence of ascorbic acid.

2.4 Evaluation of radical scavenging

Free radical scavenging activity (DPPH)

The DPPH radical scavenging activity of the *E. tournefortii* extracts was studied using DPPH as described by Meriga et al. (2012) with slight modification.. One milliliter of the different concentrations of the extracts (0.05-0.25 mg/mL) were mixed with 4 mL of methanolic DPPH solution. After 30 minutes, the decrease in absorbance of each extract and/or control (BHT) was measured at 517 nm. The results were assessed as IC_{50} values.

ABTS radical cation scavenging activity

The radical scavenging activity of *E. tournefortii* was determined according to the procedure of Shalaby & Shanab (2013) with slight modifications. ABTS (7mM) and potassium persulfate (2.45 mM) solutions were mixed and stored in a dark room for 12-16 h before to use. Before the analysis, the ABTS solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm. Following the addition of 4.5 mL of the ABTS reaction mixture to the various concentrations (50-250 μg/mL) of the extracts (1 mg/mL), the reaction mixture was vortexed. After keeping at room temperature for 15 min, the absorbance of the samples was read at 734 nm. The results were assessed as IC_{50} values.

Metal chelating activity

The metal chelating power of the *E. tournefortii* extracts was determined according to the method described by Karpagasundari & Kulothungan (2014) with slight modifications. The sample (1 mL) and 3.2 mL of ddH_2O was mixed with 2 mM $FeCl_2$ (0.1 mL) solution. After 30 s, 5 mM of ferrozine (0.2 mL) was added. The reaction was activated by adding ferrozine and then the reaction mixture was incubation at room temperature about 10 min. The absorbance of the solutions was read at 562 nm.

Reducing power activity

The reducing power of the *E. tournefortii* extracts was estimated using the method described by Oyaizu (1986) with slight modifications. Different concentrations of the sample was mixed with the same volume of 0.2M phosphate buffer and 1% potassium ferricyanide. The mixture was kept at 50 °C for 20 min. Trichloroacetic acid (10%) was added to reaction mixture. The aliquot of the upper layer (1.5 mL) was combined with the same volume of the ddH_2O and 0.1% ferric chloride. After keeping at room temperature for 10 min, the absorbance of the samples was read at at 700 nm. Ascorbic acid was used as a positive control.

2.5 Determination of total phenolic and flavonoid content

Total phenolic content

Total phenolic content was evaluated using the Folin-Ciocalteu method (Slinkard & Singleton, 1977). In this method, the extract (1 mg/mL) was mixed with 1 mL Folin-Ciocalteu reagent and 46 mL distilled water. After 3 min, 3mL of 2% sodium carbonate (Na_2CO_3) solution was added. After keeping in the dark at room

temperature for 2h, the absorbance of the samples was read at at 760 nm. The outcomes were shown as the equivalents of Gallic acid (mg GAE g^{-1} extract).

Total flavonoid content

The total flavonoid content was determined using the method of Arvouet-Grand et al. (1994). One milliliter $AlCl_3$ (2%) was mixed with the 2 mg/mL of extract solution (1 mL). The absorbance of the reaction mixtures was measured at 415 nm after 10 min incubation at room temperature. The flavonoid content was evaluated as equivalents as quercetin (mg QEs/g extract).

Proximate composition

The *E. tournefortii* plant samples were analyzed to determine proximate parameters (proteins, fat, carbohydrates, ash and energy) according to the protocols mentioned in Association of Official Analytical Chemists (1995). The crude fat of samples was evaluated by extracting a powdered sample with petroleum ether, using a Soxhlet apparatus. The crude protein content was determined through the macro-Kjeldahl method. The ash was estimated by incineration at 650 ± 15 °C. Total carbohydrates were determined by difference. Energy was calculated according to Energy (kilocalorie)=4×(g protein+g carbohydrate)+ 9×(g fat).

Cytotoxic activity

Possible cytotoxic activities were measured using the brine shrimp lethality test (Meyer et al., 1982). *Artemia salina* is a simple marine organism that can be used to determine toxicity through the prediction of the medium lethal concentration (LC_{50}). This method is an alternative method for screening toxicity, which is cheap, effective, simple and rapid (Kanwar, 2007). The *Artemia salina* eggs were left to incubate under artificial light for 48 h at 28 °C in artificial seawater (38 g sea salt was dissolved in 1 L water). After incubation for 48 h, the nauplii were attracted to one side of the beaker using a light source and collected with a Pasteur pipette. The tubes containing ten nauplii, 0.5mL different concentration of plant extract (1000, 500, 100, 50 and 10 ppm) and 4.5 mL of brine solution as well as control tubes were maintained under artificial light for 24 h at 28 °C. After 24 h, the number of surviving nauplii was counted for each concentration of the extracts and controls. The larvae were considered dead if no movement of the appendage was observed within 10 sec. The EPA Probit Analysis Program was used for data analysis (Finney, 1971).

2.6 Statistical analysis

The MINITAB Statistical Package program were used to analyze the results. Variations between the different extracts were tested using Analyses of Variance (ANOVA) and a Tukey test was conducted to see how the groups differed from each other (P<0.05). The results were presented as mean ± SE (standard Error). The various groups were shown with different letters in the same column.

3 Results and discussion

Antioxidant capacity can also contain different mechanisms, such as radical scavenging, reducing power and chelating activities (Jabri-Karoui et al., 2012). For this reason, we applied various antioxidant methods (DPPH, phosphomolybdenum and chelating activity etc.) to evaluate true antioxidant potential of the *E. tournefortii* extracts.

3.1 Total antioxidant activity (β-Carotene-linoleic acid and Phosphomolybdenum methods)

In the present study, the potential of the plant to inhibit linoleic acid oxidation was evaluated using the β-Carotene/linoleic acid test system. The results exhibit that among the extracts of *E. tournefortii* evaluated, the methanol extract, showed stronger antioxidant activity. Although, the capacities of all these extracts were less than that BHT (93.71%), they exhibited strong antioxidant properties (Table 1).

There were no differences (p>0.05) between the methanol and water extracts, but acetone extract was found to be statistically different than the other extracts ($F_{3,28}$= 1640.13 p<0.001). The antioxidant capacity for methanol extract determined in present study was higher than that reported by Gholivand et al. (2011) in the polar and non-polar sub-fraction of the methanol extract from *E. platyloba*. Antioxidants minimize oxidation of the lipid components in the cell membranes as well as inhibit linoleic acid oxidation (Tepe et al., 2007). The capability of the methanol, acetone and water extracts of *E. tournefortii*, to inhibit the oxidation determined in present study by this test also reveals the antioxidant property and protective capacity of the all extracts of plant on a the cellular basis. All of the *E. tournefortii* extracts analyzed were found to effectively inhibit linoleic acid oxidation, thus demonstrating that they possess strong antioxidant capacities.

The total antioxidant activities determination of the *E. tournefortii* extracts using the Phosphomolybdenum method is based on the formation of green phosphate /Mo (V) complex

Table 1. Antioxidant properties of *E. tournefortii* extracts.

Sample	DPPH (IC_{50}, µg/mL)*	ABTS (IC_{50}, µg/mL)*	β-carotene/ linoleic acid (%)*	Phosphomolybdenum (µg/mg)*	Power reducing (mg/mL)*
Methanol	124.63 ± 9.02 c	110.44 ± 4.73 b	88.62 ± 0.92 b	34.99 ± 0.45 b	0.098 ± 0.008 b
Acetone	222.24 ± 6.45 a	191.46 ± 10.7 a	34.29 ± 0.67 c	54.07 ± 1.56 a	0.198 ± 0.02 a
Water	175.32 ± 2.02 b	55.51 ± 2.52 c	86.76 ± 0.74 b	28.13 ± 2.12 c	0.206 ± 0.01 a
BHT	31.76 ± 1.72 d	12.89 ± 1.20 d	93.71 ± 0.23 a	nt	nt

BHT: Standard antioxidant; nt: not tested; DPPH: 2,2-Diphenyl-1-picryl hydrazyl radical; ABTS: 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid; *Values are given as the mean of three measurements (n=3) ± standard error. Mean values followed by different letters in a column are significantly different (p<0.05).

resulting from the reduction of Mo (VI) to Mo (V) in the acidic medium by extract (Prieto et al.,1999). The results of the phosphomolybdenum assay presented in Table 1 indicate that acetone, methanol and water extracts possess antioxidant capacities. The antioxidant activities were found to be statistically different among all extracts ($F_{2,21}$=76.32 p<0.001). The appearance of different antioxidant activities in the plant extracts that were obtained from the different solvents may result from the polarity of the solvents used. In addition, to the best of our knowledge, this is the first study to date on total antioxidant activities with β-carotene/linoleic acid and Phosphomolybdenum method in extracts from *E. tournefortii*.

3.2 Radical scavenging activity (ABTS and DPPH)

The ABTS scavenging capacity of the methanol, acetone and water extracts from *E. tournefortii* were determined and the results are shown in Table 1. The values of IC_{50} were in the following order: BHT < water< methanol<acetone. Although standard (BHT) showed the highest radical scavenging activity (over 80%), the methanol and water extracts as effective as the standard. The methanol and water extracts appear to scavenge the radicals and this is an important issue in pharmacological applications, as exposure to free radicals that are capable of oxidizing biomolecules cause cell damage and result of this cell damage may increase the risk of several diseases, such as cancer and diabetes (Lobo et al., 2010).

According to Meriga et al. (2012) the free radical scavenging potential of extracts and BHT were also tested on DPPH radicals. The results of the radical scavenging capability were calculated to be a concentration, 50% of which was scavenged by DPPH (IC_{50}). The low IC_{50} value shows the high radical scavenging property. The free radical scavenging capacity of extracts from this plant is between 124.63-222.24 μg/mL (Table 1) and there were statistically differences among the radical scavenging activities of different extracts of *E. tournefortii* and BHT ($F_{3,28}$=204.50 p<0.001). Similar to our findings, Mileski et al. (2014) found that the free radical scavenging activities varied according to the solvents used. Khazai et al. (2011) have shown that in *E. platyloba* the methanolic extract had a higher radical scavenging activity than in the water extract, which were similar to our results. The DPPH radical scavenging activities for the methanol extract determined in this study were higher than in the non-polar sub-fraction and lower than in the polar sub-fraction of the methanol extract from *E. platyloba* as reported by Gholivand et al. (2011). Antioxidants prohibit free radical damage by scavenging radicals or preventing radical formation. There is search for natural food compounds with high antioxidative activity in recent years, due to health concerns resulting from the use of synthetic antioxidants. Our body contains several enzyme systems that scavenge free radicals. Our food diets from plant-derived antioxidants can be an alternative supply for those enzymes. Therefore, the higher intake of foods that include high level of antioxidants is gaining importance (Al-Dabbas, 2017; Lobo et al., 2010). The present study reveals that the *E. tournefortii* extracts could serve as strong radical scavengers, and due to this property, to prevent free radical mediated disorders, they may be used as a food ingredient, as well as for pharmacological applications.

3.3 Metal chelating property

Transition metals such as Fe^{+2} ions, are catalysts in the formation of radicals that induce damage to living cells. Chelating agents existing in plant extracts have the ability to reduce radical formation and lipid peroxidation (Al-Dabbas, 2017). The Fe^{+2} chelating ability of *E. tournefortii* extracts were determined by measuring the iron-ferrozine complex and the results were compared with EDTA (Figure 1). Metal chelating activity of the acetone, methanol and water extracts were 26.13 ± 1.73, 24.20 ± 3.21 and 29.88 ±4 .14% respectively. Although the chelating activity of all *E. tournefortii* extracts was lower than EDTA (76.41 ± 0.20%), all the extracts of *E. tournefortii* are capable of chelating Fe^{+2} ions and the formation of iron-ferrozine complexes were hindered in the presence of the extracts, indicating that *E. tournefortii* extracts chelate the iron and prevent the completion of the reaction.

3.4 Reducing power activity

The reducing ability of the methanol, acetone and water extracts from *E. tournefortii* were measured in this study and the results of the determination of ferric reducing activity are shown in Table 1. The results exhibit that water extract showed stronger power reducing antioxidant activity. In addition, there were no differences (p>0.05) between the acetone and water extracts, but methanol extract was found to be statistically different than the other extracts ($F_{2,21}$ = 14.88 p<0.001). According to these results all extracts of *E. tournefortii* exhibited potential power reducing antioxidant capacity.

3.5 Total phenolic and flavonoid content

Total phenolic and flavonoid contents in the methanol, water and acetone extracts from *E. tournefortii* were ascertained in present study. Of the total phenolic contents of the *E. tournefortii* extracts, the methanol extract showed the highest total phenolic contents with 159.05 ± 3.42 mgGAE/g, followed by the water and acetone extracts with 92.06 ± 2.44 and 69.84 ± 1.29 mgGAE/g,

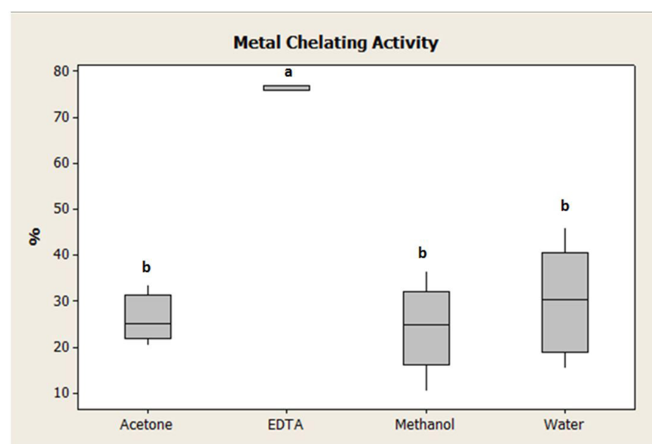

Figure 1. Metal chelating activity of different extracts of *E. tournefortii*. EDTA: Standard antioxidant (different groups were shown with different letters on each boxplot).

respectively. Among all the extracts, the total phenolic contents were found to be statistically different ($F_{2,24}$ = 335.09 p<0.001). As with our analysis, in previous studies, it has also been established that the total phenolic content was highest in the methanolic extract of *E. platyloba* (Khazai et al., 2011) and *E. sibthorpiana* Guss (Mileski et al., 2014). The highest flavonoid content was found for the acetone extract (66.28 ± 0 mgQEs/g) of *E. tournefortii* and this was followed by the methanol and water extracts with 44 ± 0.29 and 19.63 ± 0.43 mgQEs/g, respectively. There were statistically differences ($F_{2,21}$=6037.66 p<0.001) in the total flavonoid contents of the various extracts of *E. tournefortii*. In the present study, the volume of total phenolic and flavonoid content in the extracts differed according to the solvent. Similar to our findings, Khazai et al. (2011) and Mileski et al. (2014) found that the total phenolic and flavonoid contents varied according to the solvents used. Many researchers have reported that the antioxidant properties of plants are directly related to their contents of phenolics and flavonoids, which have a tendency to chelate metals and scavenge active oxygen species (Al-Dabbas, 2017; Jung et al., 2003). According to the results of the present study, the phenolic and flavonoid contents were found to be considerable in all extracts investigated. These results shows us that the all extracts studied have antioxidant capacities.

3.6 Proximate analysis

In the present study, the analysis of crude protein, crude fat, ash, carbohydrate and energy as a proximate content of *E. tournefortii* were determined and are presented in Table 2. In comparison with previous studies, the fat and carbohydrate content of *E. tournefortii* was found to be lower than *Daucus carota* as reported by Ozcan & Chalchat (2007) and *Coriandrum sativum* as reported by Hussain et al. (2009). The energy value of the *E. tournefortii* was higher than *Apium graveolens* (Caunii et al., 2010) but lower than *Cuminum cyminum* (Singh et al., 2017) and . *Coriandrum sativum* (Hussain et al., 2009). In addition, the ash and protein content of *E. tourneforti* 8.50 and 8.47 g/100 g dw respectively. Our findings are in accordance with the results of previous studies in which the ash content ranged from 4.33% to 20.7% and the protein content ranged from 5.11% to 25.19% in some plants belonging to Apiaceae family (Tunçturk & Ozgokce, 2015; Hussain et al., 2009; Ozcan & Chalchat, 2007). The proximate composition of plants provides valuable information with regard to their its nutritional quality. Some medicinal plants that used as food source have nutritional significance. Their further investigation can help us to understand the importance of these medicinal plants (Pandey et al., 2006).

Table 2. Proximate analysis of air dried aerial parts of *E. tournefortii.*

Constituents	Aerial parts
Ash (g/100 g dw)	8.50 ± 0.78
Carbohydrate (g/100 g dw)	19.98 ± 0.97
Proteins (g/100 g dw)	8.47 ± 0.32
Fat (g/100 g dw)	0.05 ± 0.10
Energy (kcal/100 g dw)	114.25
dw: dry weight	

3.7 Cytotoxic activity

Brine shrimp is a practical and economic preliminary cytotoxicity method for the investigation and assessment of toxicity, antifungal and antiparasitic properties. In the toxicity evaluation of plant extracts using the brine shrimp lethality test, LC_{50} values lower than 1000µg/mL are considered to be bioactive (Meyer et al., 1982). The lethality of the methanol, water and acetone extracts of *E. tournefortii* were 272.84, 151.084 and 133.458 µg/mL, respectively. All extracts of *E. tournefortii* showed cytotoxic activity in brine shrimp. This significant lethality of *E. tournefortii* extracts can be the source of potential cytotoxic components in this species which needs to be further investigated.

4 Conclusion

In recent years, although many pharmaceutical studies are being conducted, the biological activities of many medicinal and aromatic plants are not yet completely understood. It is therefore necessary to carry out further investigations on the biological activities of medicinal plants, such as their antioxidant capacity and cytotoxic properties. Due to their medicinal properties, *Echinophora* plants have long been used in folk medicine and the present study has been conducted in order to evaluate their antioxidant capacity and cytotoxic activities, as well as to determine the total phenolic and flavonoid content of the acetone, methanol and water extracts of *E. tournefortii*. In this study, although the values of antioxidant activities exhibited differences according to model system used, all the extracts generally showed strong antioxidant activities. The *E. tournefortii* extracts also possessed rich phenolic and flavonoid content together with cytotoxic activity. In addition, our current study on proximate evaluation of *E. tournefortii* has revealed that this plant possesses nutrients and can contribute greatly towards nutritional requirements. In brief, E. tournefortii extracts contain antioxidative and cytotoxic compounds. In addition, studies should be conducted on the isolation and identification of these components in the extracts. Nevertheless, by paying attention to the results obtained, *E tournefortii* may be considered an alternative source of antioxidant and cytotoxic agents for pharmacological applications and these results obtained may provide additional information on the potential use of this plant for food additive. The data from this study could provide useful information for the prevention and treatment of various human diseases and for the potential use of this plant in our diet, however, further research would be required before such uses could be proposed with confidence.

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