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Methanolic extract of *Eryngium creticum* Lam leaves, flowers and roots: Quantification and qualification of phenolic contents, antitumor effect and antioxidant capacity

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ABSTRACT: Natural products are potential sources of prospective antitumor agents. The phenolic and flavonoid contents of leaves, flowers, and roots of *Eryngium creticum* were quantified using liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS). The antioxidant capacity and antitumor activity of the three extracts were tested on MCF-7 and Hep2. The average total phenolic contents of *E. creticum* leaves, flowers, and roots were 12.43 ± 0.24, 10.09 ± 0.20, and 4.78 ± 0.10 mg of GAE/g of dw, respectively. The average total flavonoids were 4.77 ± 0.10, 6.23 ± 0.12, and 5.62 ± 0.12 mg of QE/g of dw, respectively. The extracts showed considerable DPPH$^{\cdot}$ scavenger activity with average IC_{50} values of 1.41 ± 0.06, 2.70 ± 0.06, and 3.03 ± 0.05 μg/mL, respectively. The average IC_{50} values of scavenging capacity of NO$^{\cdot}$ radicals were 0.11 ± 0.03, 0.69 ± 0.04, and 0.44 ± 0.01 μg/mL, respectively. The average IC_{50} values in MCF-7 were 1.25 ± 0.50, 3.75 ± 0.48, and 1.88 ± 0.28 mg/mL, respectively. In Hep2, the average IC_{50} values were 1.16 ± 0.58, 3.75 ± 0.79, and 1.31 ± 0.47 μg/mL, respectively. Phenolic content varied between the different extracts; leaves showed the highest levels of gallic acid (3.60 μg/mL), querciti-3-*O*-galactoside (0.82 μg/mL), cyanin chloride (1.52 μg/mL), and rosmarinic acid (4.65 μg/mL). Roots showed the lowest quantities of the measured phenolics, except for chlorogenic acid (6.00 μg/mL) and rosmarinic acid (1.86 μg/mL). The three extracts of *E. creticum* possess *in vitro* antitumor activities, which could be utilized as an adjunct with chemotherapy.

KEYWORDS: Eryngium; plant extract; phenolic compounds; cytotoxicity; breast cancer; antitumor agents

1. INTRODUCTION

Natural products are potential sources of prospective antitumor that could be used in adjunct with chemotherapy. There is a growing application of complementary and alternative medicine (CAM), particularly, herbal medicine in cancer; it is used as palliative care for cancer patients with significantly reduced adverse effects of chemotherapy such as vomiting, nausea, and decreased peripheral blood leukocytes [1].

E. creticum Lam; Superdivision: Spermatophyta; Division: Angiospermae; Class: Dicotyledoneae; Family: Umbelliferae (Apiaceae); Genus: Eryngium. Common name: Eryngo (see Figure 1). *E. creticum* is a wild edible plant, mainly used in salads, that belongs to the genus Eryngium L. which comprises about 250 species that grow worldwide [2]. The plant is a spiny perennial or sometimes a biennial or annual, reaching up to 50 cm in height with an erect branched stem [2]. The leaves are sessile and palmately divided into 3-8 prickly lobes [2]. *E. creticum* Lam is found only in Jordan, Palestine, Lebanon and Syria [3]. The Arabs use this

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plant as a traditional remedy for snakebites, diabetes, kidney stones, tumors; stomach ulcers, and cataracts [2]. They also use it to treat liver diseases, poisoning, anemia, and infertility problems [4]. In Islamic medicine, the roots in many recipes are used to treat edema and inflammations, like sinusitis and urinary tract infections [2]. These traditional recipes need scientific validation for their *in vitro* and *in vivo* effects.

There is a number of researches that have scientifically proven the claimed traditional remedies of *E. creticum* [4]. For example, hypoglycemic effect [5-7], anti-nociceptive [8], anti-inflammatory [9], anti-bacterial activity [10], *in vitro* antagonistic effect on scorpion venom [11], antioxidant activity [3, 12], anti-hemolytic activity [13], anti-mutagenic effect [14], cytotoxic activity [15].

In this context, the current research is investigating the phenolic and flavonoid contents of a methanolic extract of *E. creticum*. The contents were analyzed using LC-ESI-MS/MS. The antioxidant capacity was determined by measuring the capacity of scavenging NO radicals and reducing DPPH radicals by the extracts of *E. creticum* leaves, flowers, and roots. In addition, two different cell lines were selected to test the antitumor activity of the three methanolic extracts against cancer cell lines. The cells were human breast cancer cells (MCF-7), and human epithelial type 2 (Hep-2).

In vitro **analysis:**
Total phenolics
Total flavonoids
Antioxidant activity
Antitumor activity

		Methanolic extract of *E. creticum* (M ± SD)		
		Leaves	Flowers	Roots
Biological active compounds	Total phenolics (mg of GAE/g of dw)	12.43 ±0.24	10.09 ±0.20	4.78 ±0.10
	Total flavonoids (mg of QE/g of dw)	4.77 ±0.10	6.23 ±0.12	5.62 ±0.12
Antioxidant activity	$IC_{50}DPPH^{\cdot}$ (µg/ml) BHT = 6.32 ± 0.06 µg/ml	1.41± 0.06	2.70± 0.06	3.03± 0.05
	$NO^{\cdot}$ (µg/ml) BHT = 9.59 ± 0.08 µg/ml	0.11 ± 0.03	0.69 ± 0.04	0.44 ± 0.01
Antitumoractivity	IC_{50}in MCF-7 (µg/ml)	1.25 ± 0.50	3.75 ± 0.48	1.88 ± 0.28
	IC_{50}in HEp2 (µg/ml)	1.16 ± 0.58	3.75 ± 0.79	1.31 ± 0.47

Figure 1. Graphical abstract of the experiment including a photo of *E. creticum* green leaves (top), green flowers (middle), and light brown roots (bottom). LC-ESI-MS/MS analysis and the major phenolic compounds in the extract of each part of the plant. *In vitro* analysis of the biological, antioxidant, and antitumor activities.

2. RESULTS AND DISCUSSION

2.1. Determination of total phenolic contents (TPC) and total flavonoid contents (TFC)

Phenolic contents are potent natural antioxidants, anti-inflammatory, antimicrobial, and antitumor compounds [16]. In the current research, the total phenolics and total flavonoids were measured in the methanolic extracts of *E. creticum* leaves, flowers, and roots. Chromatographic analysis was conducted using LC-ESI-MS/MS to qualify and quantify 24 secondary metabolites, mostly phenolics in the three different parts of the plant. Further, antioxidant capacities and antitumor activities of the three extracts were determined.

The extracts of *E. creticum* leaves, flowers, and roots were analyzed to calculate their total phenolic and total flavonoid contents. These compounds possess antioxidant activities. The average quantities of phenolics in the methanolic extracts of leaves, flowers, and roots were 12.43 ± 0.24, 10.09 ± 0.20, and 4.78 ± 0.10 mg of GAE/g of dw, respectively (Figure 1). The average levels of TPC in the three different parts of the plant were

ordered from greatest to least as leaves > flowers > roots. The average TFC levels were 4.77 ± 0.10, 6.23 ± 0.12, and 5.62 ± 0.12 mg of QE/g of dw in *E. creticum* leaves, flowers and roots, respectively (Figure 1). TFC order was as flowers > roots> leaves.

The total phenolics in this study as well as the flavonoid contents of leaves, flowers, and roots were found to be similar to values obtained in a previous study of 16.7 and 9 mg CAE per 100 g fresh weight of Lebanese *E. creticum* leaves and stems, respectively [3]. Variability in these levels is expected between studies due to different extraction methods, solvents, duration, and surrounding environments. Furthermore, it was reported that prolonged exposure of crud extracts to light and oxygen would lead to oxidation of their phenolic contents and lower their phenolic yield [12]. Despite having these factors, our results are in harmony with the previous analysis.

2.2. Reduction of DPPH$^{\cdot}$ radicals and NO$^{\cdot}$ scavenger capacity

Flavonoids and phenolics have high antioxidant activities. Antioxidants are substances that delay oxidation reactions and inhibit the polymerization chain initiated by free radicals [3]. The current assay illustrates that the plant extracts may reduce/inhibit the production of free radicals by donating hydrogen atoms, producing the de-colorization in the DPPH assays [17]. The three extracts of leaves, flowers and roots showed considerable DPPH$^{\cdot}$ scavenger activity with average IC_{50} values of 1.41 ± 0.06, 2.70 ± 0.06, and 3.03 ± 0.05 μg/mL, respectively (Figure 2). The values were still significantly higher ($p < 0.05$) than the IC_{50} of synthetic antioxidant BHT (IC_{50} = 15.32 ± 0.09 μg/mL. The highest activity was recorded for leaves extract then for flowers extract, while the lowest activity was for roots extract.

Nitric oxide (NO) is produced by the enzyme nitric oxide synthase. It has cytoprotective effects at low concentrations and cytotoxic properties at high concentrations. Under physiological conditions, a low level of NO is produced by constitutive nitric oxide synthase (cNOS). In pathological conditions, high levels of NO is produced by inducible nitric oxide synthase (iNOS), which is pro-inflammatory and cytotoxic [18]. Per our study, the average IC_{50} values of the scavenging capacity of NO$^{\cdot}$ radicals were 0.11 ± 0.03, 0.69 ± 0.04, and 0.44 ± 0.01 μg/mL respectively for leaves, flowers and roots (Figure 1). These values were significantly higher ($p < 0.05$) than the IC_{50} of synthetic antioxidant BHT (IC_{50} = 9.59 ± 0.08 μg/mL). The highest capacity was recorded for leaves extract, while the lowest capacity was for flowers extract.

In total, methanolic extracts of *E. creticum* leaves, flowers, and roots exhibited very good inhibition of iNOS. Such antioxidant capacity was due to the phenolic contents in the selected extracts. It was reported that methanolic extracts and rosmarinic acid analogs exhibited different levels of antioxidant activity with weak radical scavenging activity in *in vitro* assays [19]. Similar findings were reported in a previous study on the aqueous and ethanolic extracts of *E. creticum* (leaves, stems, roots, and the whole plant) using DPPH and superoxide radical scavenging. Findings revealed a dose-dependent antioxidant activity up to 77%, 89%, and 70% at the concentration of 0.5 mg/mL of leaves, stems and roots, respectively, for the first harvest, while, it reached 73%, 59% and 34% at the same concentration, respectively for the second harvest [20]. In addition, various *in vitro* studies confirmed the remarkable antioxidant potential of *Eryngium* species [16]. As evident, the current plant extract possesses antioxidant activities. Since oxidative stress is important for the pathogenesis of various human diseases, discovering natural antioxidants can lead to the development of new natural drugs to prevent or treat various human disorders.

2.3. Antitumor activity of *E. creticum* leaves, flowers and roots extracts

Flavonoids and polyphenols were proven to possess antioxidant and free radical scavenging activities, which induce effects against tumors, heart diseases, AIDS, and other pathologies [21]. The prepared extracts had antitumor activities indicated by the cytotoxic effect on MCF-7 breast cancer cells and Hep2 Hela contaminant carcinoma. Leaves extract inhibited the growth of 50% of the cultured cells at 1.25 μg/mL after 24 hours incubation. Related studies had variable reports of antitumor activities due to different extraction methods, different selected plant parts, and growth conditions of the plant. For example, a study found that 5 μg/mL methanolic extract of *E. creticum* leaves decreased the number of MCF-7 by 28% after 24 hours incubation contrary to studies on the same plant but from an altitude, 300 m reported that the leaves at 0.5 mg/ml exerted an antioxidant activity of 90 % [22]. Methanolic, aqueous, and ethyl acetate extracts of *E. creticum* were found cytotoxic on MCF-7 as the stems extracts inhibited cell growth by 68% and leaves extract by 72% [2]. Among 15 tested plant species, aqueous extract of *E. creticum* showed the highest antitumor effect by 84.30% inhibition [16]. Other studies reported that the plant saponin compounds exhibited moderate or

weak antitumor effects on the human pancreas, prostate, lung, colon, and leukemia cancer cells [19]. Regardless of the variable reports, *E. creticum* possesses antitumor activities, which is promising for the future development of anticancer drugs.

Figure 2 presents the antitumor activities of the plant leaves, flowers, and roots extracts expressed as half-maximal inhibition concentration (IC_{50}). The average IC_{50} ± SD in MCF-7 were 1.25 ± 0.50, 3.75 ± 0.48, and 1.88 ± 0.28 μg/mL, respectively. In Hep2, the average IC_{50} ± SD were 1.16 ± 0.58, 3.75 ± 0.79, and 1.31 ± 0.47 μg/mL, respectively. The highest antitumor activity in both cell lines was for leaves than for roots, and the least was for flowers extract. Data is also shown in Figure 1 for connecting extracts to their averages.

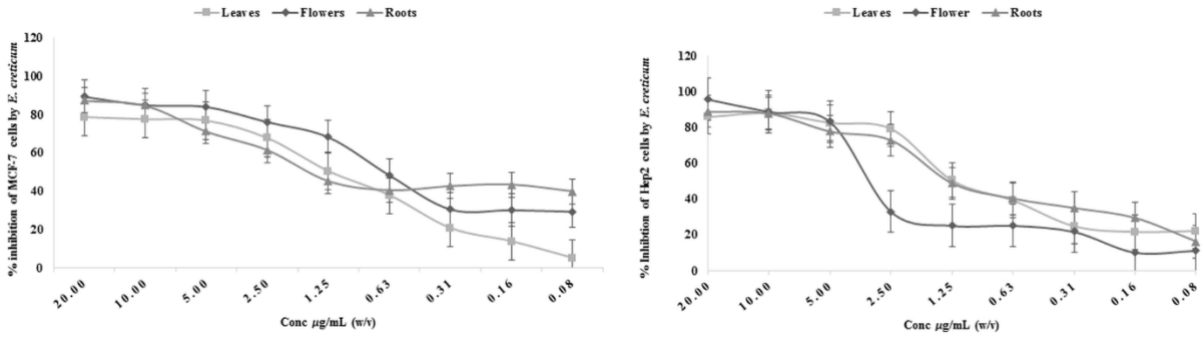

Figure 2. Inhibition (%) of MCF-7 and Hep2 by *E. creticum* leaves, flowers, and roots at different concentrations (μg/mL). Whiskers are representing error bars.

2.4. Liquid Chromatography-Electrospray Tandem Mass Spectrometry (LC-ESI-MS/MS) analysis of *E. creticum* leaves, flowers, and roots

The methanolic extracts of the leaves, flowers, and roots of *E. creticum* were analyzed using an LC-ESI-MS/MS method and an in-house library of 24 secondary metabolites, mostly phenolics (Table 1). The identified compounds (Table 2) varied between the different plant part extracts, leaves, flowers, and roots. Some were not detected in any part of the plant such as catechol, epicatechin, and epigallocatechin. Others varied quantitively between the different plant parts such as rosmarinic acid (4.65, 0.016, and 1.86 μg/mL, respectively). Leaves extracts had the highest levels of gallic acid, querciti-3-*O*-galactoside, cyanin chloride, and rosmarinic acid (3.60, 0.82, 1.52, and 4.65 μg/mL, respectively). Generally, roots recorded the lowest quantities of the measured compounds except chlorogenic (6.00 μg/mL) and rosmarinic acids (1.86 μg/mL). Flowers had the highest levels of myrtillin, caffeic acid, hydroxybenzoic acid, *p*-cumaric acid, *trans*-cinnamaldehyde, and vanillic acid (0.131, 0.173, 0.257, 0.067, 0.118, and 0.357 μg/mL, respectively).

The quantification of the selected compounds in the leaves, flowers, and roots of *E. creticum* revealed variable quantities between these plant parts. Leaves recorded the highest levels of different compounds. For example, gallic acid in leaves was around 10 times higher than in roots, while it was not detected in flowers. Gallic acid has superoxide anions scavenging activity and has the capacity to inhibit myeloperoxidase release and activity as well as interfere with the assembly of active NADPH-oxidase [23]. In fact, DPPH scavenging activity was significantly correlated with TPC (r > 0.99) [24]. These findings would explain the highest antioxidant and antitumor activities recorded in our study for leaf extracts relative to flower and root extracts. Furthermore, apigenin in leaves was around 17 times the quantity in roots and 70 times that in flowers. Cyanin chloride in leaves was around 17 times the level in flowers, and 38 times level in the roots. The isoquercitin level in leaves was 1.5 times higher than in flowers, and 15 times the level in roots. Such contents would also contribute to the superior performance of the current leaves extract compared to the extracts from the remaining plant parts.

Table 1. A library of 24 reference standard compounds using LC-ESI-MS/MS analysis.

Compound ID	Retention time (min)	Mwt (g/mol)	Precursor ion $[M-1]^-$	Product ions
Apigenin	8.63	270.24	269	151, 117
Caffeic acid	6.35	180.16	134.2	134.2, 106.4, 89.1
Catechol		110.11	109	109
Chlorogenic acid		354.31	353	191
p-cumaric acid	7.05	164.16	118.8	92.7, 118.8
Cyanin chloride	8.24	647.0	287.1	213, 137.2
Epicatechin		290.30	289.1	108.8
Epigallocatechin gallate		458.37	475	168.8
Ellagic acid	8.25	302.20	149.7	149.7, 150.6
Ferulic acid	7.17	194.18	133.9	133.9, 177.8
Gallic acid	1.74	170.12	168.5, 169	124.6
Hydroxybenzoic acid	6.35	138.12	88.4	88.4, 106.8
Hypericin		504.45	503	405
Isoquercitrin	7.33	464.40	464.9	300
Luteolin	8.21	286.24	285	217, 199, 175, 151
Myrtillin	7.21	500.80	463	301
Procyanidin B2		578.52	577.1	125, 289, 407, 425, 451
Quercetin	8.27	302.20	301	273, 243, 179
Quercetin 3-*O*-galactoside	7.23	464.38	463	301
Rosmarinic acid	7.66	360.32	160.7	160.7, 132.7
Rutin	7.07	610.52	609	301
Syringic acid		198.17	107.8	107.8, 151.2
trans-Cinamaldehyde	8.05	132.16	103.7	103.7
Vanillic acid	6.63	168.15	107.8	107.8, 151.2

Worth to mention that flowers recorded the highest levels of caffeic acid, myrtillin, hydroxybenzoic acid, *trans*-cinnamaldehyde, and vanillic acid. Vanillic acid is an oxidized form of vanillin and is a flavoring agent found in plants and fruits. Recently, phenolic contents have been used for their antioxidant, antimicrobial, and chemo-preventive effects. However, their mechanisms *in vivo* are not completely understood [25].

Interestingly, roots recorded the highest levels of chlorogenic acid while it was not detected in leaves and flowers. Roots also had high levels of rosmarinic acid compared to other plant parts. Similar findings were found in previous studies. The spectrophotometric analysis of *E. creticum* stems and leaves recorded polyphenolics ranging from 9 mg to 16.7 mg of caffeic acid [3]. This is in harmony with the current report of caffeic acid quantity in leaves. Worth to mention that rosmarinic acid consists of two phenylpropanoids: caffeic acid and 3-(3,4-dihydroxyphenyl)lactic acid [26]. At higher concentrations, caffeic acid was a more effective antioxidant than chlorogenic acids during autoxidation of triacylglycerols of sunflower oil at 100°C [27]. These reports would justify the antioxidant and antitumor activities of the current root extract.

Other studies reported different contents. For example, an analysis after hydrodistillation of Iranian *E. creticum* stems using gas chromatography coupled to mass spectrometry recorded the presence of bornyl acetate, camphor, *a*-pinene, germacrene D, borneol, and *a*-thujene [28].

Table 2. The concentration of 24 phenolic compounds in *E. creticum* leaves, flowers, and roots using LC-ESI-MS/MS analysis.

Compound	Concentration (µg/ml)		
	Leaves	**Flowers**	**Roots**
Apigenin	0.330	0.004	0.019
Caffeic acid	0.009	0.173	ND
Chlorogenic acid	ND	ND	6.000
p-Cumaric acid	0.049	0.067	0.016
Cyanin chloride	1.520	0.086	0.040
Ellagic acid	0.245	0.024	0.007
Ferulic acid	0.029	0.036	0.023
Gallic acid	3.600	ND	0.310
Hydroxy benzoic acid	0.044	0.257	0.024
Isoquercitrin	0.371	0.233	0.024
Luteolin	0.171	ND	ND
Myrtillin	0.105	0.131	0.002
Quercetin	0.126	0.006	0.006
Querciti-3-*O*-galactoside	0.819	0.384	0.031
Rosmarinic acid	4.650	0.016	1.860
Rutin	0.129	0.068	0.020
trans-Cinnamaldehyde	0.022	0.118	0.018
Vanilic acid	0.038	0.357	0.047

N.A.: Not detected

There are other studies that analyzed related species. For example, essential oil from *E. maritimum* was reported to contain deltione, marmesine, quercitol, 3-(*β*-D-glucopyranosyloxymethyl)-2,4,4-trimethyl-2,5-cyclohexa-dien-1-one, *β*-sitosterol, *β*-sitosterol-*β*-D-glucopyranose, mannitol and dulcitol [29]. *E. alpinum* contained caryophyllene oxide, bicyclogermacrene and germacrene D [30]. A study from Poland showed that *E. creticum* contained several chemical constituents, mostly sesquiterpenes, monoterpenes, sitosterols, sugars, aldehydes, and coumarins [2]. The latter two compounds were similarly reported in the current plant phenolic profile. Generally, our analysis found leaves the richest in secondary metabolites compared to flowers and roots. This is in harmony with a previous analysis that compared *E. creticum* leaves to stems [22]. Consequently, *E. creticum* could present medical importance due to the presence of phenolics and flavonoids that possess antitumor and antioxidant properties.

2. CONCLUSION

This study is the first to evaluate total phenolics and total flavonoids in methanolic extracts of leaves, flowers, and roots of *E. creticum* grown in Jordan. Further, this is the first report of 24 secondary metabolites, mostly phenolic quantities using LC-ESI-MS/MS. The work is the first to investigate the flowers antitumor and antioxidant activities compared to leaves and roots extracts. The chromatographic analysis identified the major phenolic constituents responsible for the antioxidant activities of the selected plant extracts as follows: gallic acid, querciti-3-O-galactoside, cyanin chloride, and rosmarinic acid1 in leaves extract; caffiec acid, mytrillin, hydroxybenzoic acid, transcinnamaldehyde, and vanillic acid in flowers extract; chlorogenic acid and rosmarinic acid in roots extract. *E. creticum* is a potential natural antioxidant source for the food industry and pharmaceutical products. It possesses antitumor properties that could be used as an adjunct to chemotherapy and should be investigated in future studies.

3. MATERIAL AND METHODS

3.1. Collection and preparation of *E. creticum*

A total of 5 kg of the fresh plant was obtained from a local herbal shop in Jordan, during May and June 2018. This plant sample was collected from Ajloun, Irbid and Mafraq. The plant was identified by the Botany expert, Professor Jamil Lahham, in the Department of Biology at Yarmouk University. A voucher specimen was deposited in the Faculty of Pharmacy at Jordan University of Science and Technology. The plant was divided into three parts; leaves (spines), flowers, and roots. The three parts were washed with tap water, kept to dry in the shade on paper in a clean, ventilated room with an average humidity range of 35%, and a room temperature of 27 ± 2 °C. Three weeks later of frequent stirring and turning, the plant was dry and ready to be grounded until powder using a grinder (HR 2924, Philips, Netherlands). The powder was stored away from sunlight in dry, cool conditions for later extraction.

3.2. Extraction of *E. creticum*

The extraction method was performed according to a previously in-house method [31]. A total of 200 g of the plant powder from leaves, flowers, and roots was dissolved separately in a solution of methanol/deionized water (80:20, v/v). Deionized water was obtained from a Milli-Q Element water purification system (Millipore, Bedford, USA). The mixture was shaken for 24 hours at 140 rpm using an orbital shaker (SSL1, Stuart, Staffordshire, UK). The mixture was sonicated at 60 % amplitude for 30 minutes (ON: 20 sec; OFF: 5 sec) using an ultrasonic processor (Sonics and Materials Inc., USA). Then the mixture was vacuum filtrated in a laboratory constructed filtration unit composed of a Labconco 117 rotary vane dual stage mechanical vacuum pump Rebuilt (Ideal Vacuum Products LLC., New Mexico); 1 x 1m length of heavy walled rubber tubing; 2 x cable ties; 1 x 250 ml Pyrex Buchner flask; 1 x set of filter cones; 1 x porcelain Buchner funnel to fit 7 cm filter papers; and 1 x retort stand set. Then, the filtrate was vaporized using a rotary evaporator (Rotavapour Model Hahn Vapor HS-2005S-N, Korea) under 700mmHg in a water bath at 40 °C. The evaporate was cooled with – 10 ˚C water that was injected into a condenser coil with a speed of 80 L/hr. Afterward, the methanolic layer was evaporated (80% of the distillate), then the remaining 20% of the distillate was vacuum filtrated as described before. Finally, the resulting extract was freeze-dried in a laboratory freeze dryer (LyoQuest-85, Telstar technologies, Spain) at – 80 ˚C for 48 hours. Then, a cycle of freezing/evaporation/vacuum at – 80 °C for 48 hours, followed by another cycle at 30 °C for 12 hours was run for further dryness. The resulting viscous green extract was stored at – 80 for later analysis. The yields of crude extract of *E. creticum* were 14.9% for leaves, 10.7% for flowers, and 26.7% (w/w) for roots.

3.3. Determination of total phenolic contents (TPC)

The total phenolic contents were performed according to Beara *et al*. [32] with slight modifications for testing in 96-well microplates [32]. Briefly, the extracts of *E. creticum* leaves, flowers and roots were prepared in concentrations of 20.0, 10.0, 5, 2.5, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04, and 0.02 mg/mL. The standard gallic acid concentrations were 2.0, 1.0, 0.5, 0.25, 0.125, 0.062, 0.031, 0.016, 0.008, 0.004, and 0.002 mg/mL. A total of 30 μL of each extract or standard concentration was added to 150 μL of 0.1 moL/L Folin-Ciolcateu (FC). Ten minutes later, 120 μL of 7.5% sodium bicarbonate was added. Two hours later, the absorbencies were measured at 760 nm. The Phenolic contents were determined on the basis of the standard calibration curve of gallic acid. All samples were measured in triplicates and the total phenolics were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (dw).

3.4. Determination of total flavonoid contents (TFC)

The aluminum chloride colorimetric method used for the 96-well microplate was adopted from Beara *et al.* and optimized for the different extract concentrations to determine TFC. Samples from leaves, flowers and roots extracts were prepared in concentrations of 20.00, 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04 and 0.02 mg/mL. The standard was quercetin solution which was prepared in the range of 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04 and 0.01 mg/mL. A total of 30 μL of the *E. creticum* leaves, flowers and roots extract as well as from the standard solutions were diluted with 90 μL of methanol, 6 µl of 10% aluminum chloride (substituted with distilled water in blank probe), 6 μL of 1 moL/L potassium acetate and 170 μL distilled water.

Thirty minutes later, absorbencies were measured at 415 nm. The samples were tested in triplicates, the values of TFC were calculated according to the standard calibration curve, and the means of TFC were recorded in mg of quercetin equivalents (QE) per g of dw.

3.5. Reduction of DPPH radicals

The scavenging capacity of the *E. creticum* extracts of leaves, flowers and roots was measured in 96-well microplates [33]. A total of 10 μL of each of the following freshly prepared extract concentrations 20.00, 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04 and 0.02 μg/mL was added to 100 μL of 90 μmoL DPPH solution in methanol. The mixture was then diluted in 190 μL methanol. The standard BHT was prepared as the following concentrations 40.00, 20.00, 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08 and 0.04 μg/mL. The control contained only the solvent while the blank contained 290 μL methanol and 10 μL extract. Absorbencies were measured after one hour at 515 nm. The antioxidant capacity was expressed as IC_{50}, which represents the extract concentration that inhibited DPPH radical formation by 50%.

3.6. NO radical scavenger capacity

The test of nitric oxide (NO) radical scavenging capacity was based on the Beara *et al.* method [33]. The reaction mixture (0.5 mL of 10 mmoL/L sodium nitroprusside, 0.5 mL of phosphate buffer at pH 7.4, and 30 μL of the standard BHT or leaves, flowers, and roots extracts with a concentration range of 20.00, 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, 0.08, and 0.04 μg/mL were incubated at 25^{o}C for 90 minutes. The blank was prepared by adding 1 mL buffer to 30 µL of the extract. Then, 0.2% N-(1-naphthyl)ethylenediamine dihydrochloride was added to 1 mL of solution (equal amounts of 2% sulfanilamide in 4% phosphoric acid) and was allowed to stand for 3 minutes. The absorbance was measured at 546 nm against blanks. All samples were tested in triplicates. The resulting IC_{50} values were compared to the IC_{50} of BHT which was used as the positive control standard.

3.7. Antitumor activity of *E. creticum* leaves, flowers and roots extracts

The following two cell lines were used to assess the antitumor activity of *E. creticum* extracts (Leaves, flowers, and roots) using the standard MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) colorimetric assay: MCF-7 (human mammary gland adenocarcinoma, ATCC HTB-22) and Hep2 (human HeLa contaminant carcinoma, ATCC CCL-23).

The tested cells number was initially adjusted to be 1 x 10^5 cell/mL in complete DMEM media supplemented with 10% FBS, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. Then 100 μL cell suspension cells aliquot was added to a 96-well culture plate (1 x 10^4 cell/well). Four wells were filled with 100 μL complete media alone (without cells as control "Blank"). Cells were incubated in a CO_2 incubator at 37 ^{o}C in the presence of 5% CO_2 for 24 hours. 20 μg/mL leaves, flowers, and roots extracts were prepared in 100% DMSO and were filtered using 0.2-µm syringe filters before use. Extracts were serially diluted 1:1 in complete media for MCF-7 to form 20.00, 10.00, 5.00, 2.50, 1.25, 0.63, 0.31, 0.16, and 0.08 μg/mL. Same serial dilutions were prepared in complete media for Hep2 using the three extracts. A total of 100 μL stock and diluted extracts were added (in duplicates) to the cells. Eight wells of media alone were used as controls by the addition of 100 μL media per well. Cells were incubated in a CO_2 incubator at 37 ^{o}C in the presence of 5% CO_2 for 24 hours. 20 μL of 0.2μm filtered MTT/PBS solution (M2128, 5 mg/mL, 98% Thiazolyl Blue Tetrazolium Bromide, Sigma) were added to each well. Plates were incubated for 4 hours in the CO_2 incubator. Later the culture media was removed from wells (about 200 μL) and 150 μL MTT solvent (40 mM HCL and 0.1% Tween 20 in isopropanol) were added to dissolve formazan crystals. Finally, plates were incubated at room temperature with shaking for 15 minutes. Absorbencies were measured at 560 nm using GloMax Explorer Multimode Microplate Reader (GM3500, Promega, USA).

3.8. Liquid Chromatography-Electrospray Tandem Mass Spectrometry (LC-ESI-MS/MS) analysis of phenolics of *E. creticum* leaves, flowers, and roots

Chromatographic separations of phenolic standards (Table 1) and crude extracts of the three parts of *E. creticum* (leaves, flowers, and roots) were carried out using a liquid chromatography system from Agilent Technologies (API 3200, Applied Biosystems/MDS Sciex, USA) equipped with a triple-quadruple mass

spectrometer (MS). The LC system includes a G1379A on-line degasser, a G1311A binary pump, a G1367B high-performance autosampler. The analytical column was Zorbax SB-C_{18} (150 mm × 4.6 mm ID, 5 μm; Agilent Technologies, Wilmington, DE, USA). The mobile phase was a mixture of solvent A (0.1% formic acid) and solvent B (acetonitrile) in a 9:1 ratio, and it was eluted using an isocratic system at a flow rate of 1 mL/min. Solvent gradients (A:B) at 0, 3, 8, 11, 13, and 15 min were 90:10, 90:10, 20:80, 20:80, 90:10, and 90:10, respectively. The solvents were filtered using 0.45-μm syringe filters (ALWSCI Group, China). The column temperature was maintained at 25°C. Stock solutions of crude methanolic extract of *E. creticum* were prepared (1 mg/mL) in HPLC grade methanol (Fisher Scientific, UK). The sample was then injected into LC-ESI-MS/MS and ran with the same solvent system. The sample injection volume was 15 μL. A library of 24 reference standard compounds using dynamic MRM in the negative mode was constructed (Table 1). The concentration of the detected compounds in plant samples was determined from the peak areas using the linear regression equation obtained from the standards calibration curves. The sources of the standards were from Sigma, except vanillic acid (Sigma-Aldrich), and ferulic acid (Fluka). Processing of data to calculate concentrations and to investigate the sub-fractions of the chromatogram was performed using Acquisition Method Analyst software (Version 1.6.3, SCIEX, USA).

3.9. Statistical analysis

The results were expressed as Mean ± Standard Deviation (M ± SD). The data were analyzed using ANOVA in the package 'rcdmr' of the statistical R-software version 3.4.2 (R Development Core Team, 2020). Tukey's contrast analysis was utilized to determine significant differences between and within groups. The value $p < 0.05$ was considered significant.

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