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ELEMENTAL ANALYSIS AND BIOACTIVITIES OF *ECHINOPS ECHINATUS* ROXB. (GLOBE THISTLE) VIA SPECTROSCOPIC TECHNIQUES

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Abstract

Echinops echinatus Roxb., belonging to family Asteraceae is traditionally used as appetizer, carminative, liver tonic, to treat jaundice, diabetes, and heart diseases. It has been reported to be a rich source of flavonoids with significant pharmacological activities. This study aimed to quantify 28 mineral elements, total phenolic and flavonoid contents, antioxidant, and anti-cholinesterase activities in its leaves, stem, flowers and achene extracts. In elemental analysis, the concentrations of Ca (9199.3 to 63975.6 µg/g) and K (7866.1 to 42700.6 µg/g) were comparatively high. Among micro elements, Sr and Zn were high in the analyzed samples. The concentrations of toxic elements were within the safe ranges established by WHO. Furthermore, ethyl acetate extract was the most enriched extract with phenolic and flavonoid contents as well as the most potent free radical scavenger (DPPH IC_{50} 9.54 µg/mL, ABTS IC_{50} 5.88 µg/mL). In anti-diabetic activity, leaves and stem methanol extract was the strong inhibitor of α-glucosidase enzyme with IC_{50} values of 371.4 and 368.6 µg/mL, respectively. Methanol and ethyl acetate extracts also showed dual cholinesterase enzymes inhibition. Thus, *E. echinatus* could act as a potential source of natural antioxidant and does not pose any threats on consumption.

Key words: *Echinops echinatus*, Micro elements, Toxic elements, Anti-diabetic, Anticholinesterase.

Introduction

The genus *Echinops* Roxb. belonging to family Asteraceae, is commonly known as globe thistle and distributed throughout Southern Europe, Tropical and North Africa and Asia. *Echinops echinatus* (*E. echinatus*) is a xerophytic, erect, and spiny herb of about 100 cm height native of Pakistan, India, and Sri Lanka (Radulović, & Denić, 2013). The stem has white cottony hairs, basal leaves ± petiolate, oblanceolate, deeply pinnatifid, upper lanceolate, and white to purple colour flowers (Maurya *et al*., 2015). The plant is used as appetizer, carminative, to relieve asthma, decreased urination, strangury and urinary discharge, a liver tonic, aphrodisiac, general and nerve tonic, to treat hysteria, dyspepsia, jaundice, diabetes, leucorrhea, round worms, colds, whooping cough in children, fever, brain disease, migraine, heart diseases, joint pains, hemorrhoids, wounds, and eczema in folklore medicines (Desta, 1993; Dadhich *et al*., 2010; Sarvaiya *et al*., 2017). The previous phytochemical analysis reported isolation of several types of flavonoids with potent pharmacological activities (Singh *et al*., 1991; Singh & Pandey, 1994; Singh *et al*., 2006; Maurya *et al*., 2015). Furthermore, its pharmacological evaluation reported its significant antioxidant, antimicrobial, analgesic, hepatoprotective, diuretic, hypoglycemic, antispasmodic, antifertility, anti-androgenic, anti-inflammatory, and antipyretic activities (Sharma & Mehta, 1989; Padashetty & Mishra, 2007; Somashekar & Mishra, 2007; Patel *et al*., 2011; Agrawal *et al*., 2012). Plants have a wide range of pharmacological activities, as previously, some literature also reported cardiac, pulmonary, hepatic, hematological, renal, and endocrinal toxicities from the continuous use of herbs and herbal drugs. For example, a regular consumption of herbal tea, which contained high levels of pyrrolizidine alkaloids caused a veno-occlusive disease in 18-month-old baby (Sperl *et al*., 1995; Chen & Huo, 2010). Similarly, several other teas such as *Illicium anisatum* (star anise), *Aconitum carmichaelii* and *Aconitum kusnezoffii* teas were adulterated with some toxic compounds (Wang *et al*., 2014; Shen *et al*., 2012). In addition, *Ginkgo biloba* and *Silybum marianum* products, which are used as anti-Alzheimer's and hepatoprotective agents, respectively are reported to cause bleeding, hemorrhage and hepatic coma (WHO, 1999). Indian and Chinese traditional medicines between 1991 and 1995, the lead, arsenic and mercury poising arising with herbal remedies was reported. Likewise, among 2080 traditional Chinese medicines, 42 products contained the content of heavy metals exceeding the permissible limits (Saeed *et al*., 2010; Shen *et al*., 2012).

In countries like Pakistan, there is no authentic health safety and adulterants evaluation knowledge on several medicinal herbs and their products, which might increases the chances of fraud, consumers' uncertainty and lethal effects on human health. Therefore, it is necessary to develop research methods and to standardize the safety, toxicity and pharmacological importance of medicinal herbs and their products.

The previous studies on *E. echinatus* showed that there was no detailed report on the nutritional, safety and toxicity evaluation. Hence, to determine the nutritional and pharmacological values, and to ensure the consumers from any threats by the use of *E. echinatus*, the study was designed to analyze the contents of twenty-eight elements, total phenolic and flavonoid contents, antioxidant, antidiabetic, and anticholinesterase activities in leaves, stem, flowers and achenes of *E. echinatus.* Elements including aluminium (Al), calcium (Ca), iron (Fe), potassium (K), magnesium (Mg), sodium (Na), phosphorus (P), sulphur (S), zinc (Zn), lithium (Li), beryllium (Be), vanadium (V), chromium (Cr), cobalt (Co), nickel (Ni), copper (Cu), gallium (Ga), selenium (Se), rubidium (Rb), strontium (Sr), cesium (Cs), barium (Ba), uranium (U), thallium (Tl),

indium (In), arsenic (As), cadmium (Cd) and lead (Pb) were analyzed using advanced analytical techniques of inductively coupled plasma-optical emission spectroscopy (ICP-OES) and ICP-mass spectrometry (ICP-MS). The analytical techniques applied were validated using several validation and assurance parameters, which include limits of detection (LOD), limits of quantification (LOQ), precision (coefficient of variance, CV%), spiking recovery tests, and analyzing the certified reference materials (NIST-1570a), Spinach leaves. The total phenolic and flavonoid contents, antioxidant, and anticholinesterase (acetylcholinesterase, AChE and butyrylcholinesterase, BChE) activities were determined using UV spectrophotometer and microplate reader. To the best of the authors' knowledge, up to now, there are no reports on the elemental analysis and cholinesterase inhibitory activity of *E. echinatus* different parts. Herein, the study describes the detailed elemental and anticholinesterase activity assessment of four different parts of *E. echinatus* for the first time.

Material and Methods

Samples collection: *E. echinatus* was collected from district Swabi, Khyber Pakhtunkhwa, Pakistan and identified by a plant taxonomist. A voucher specimen was deposited in the Herbaium, Department of Botany, Kohat University of Science & Technology, Kohat. The plant parts were carefully separated, and dried at room temperature in the shade. To obtain various extracts, the different parts were grinded to powder and extracted with different solvents using Soxhlet extractor. To quantify elements, the leaves, stem, flowers and achenes were microwave acid digested using nitric acid and hydrogen peroxide.

Chemicals and instrumentation: The solvents and chemicals used were either analytical reagent (AR) or HPLC grade. Chemicals used in the elemental analysis included nitric acid (HNO_3), hydrogen peroxide (H_2O_2), ultra-pure deionized water, and the multi-element standards. Ultrapure deionized water (>18.0 MΩ.cm), HNO_3 and H_2O_2 were purchased from a Millipore (Bedford, MA, USA) and Dongwoo, Fine-Chem (Korea). Multi-element standard solutions containing macro, micro and trace elements at 1000 mg/L and 10 mg/L, and a standard reference material (SRM-1570a), spinach leaves were purchased from Perkin Elmer (CT, USA) and National Institute of Standards and Technology; NIST (Gaithersburg MD, USA), respectively. Glass and plastic ware were cleaned by soaking in 10% HNO_3 solution overnight and then rinsing several times with deionized water. Chemicals used in the analysis of phenolic and flavonoid contents, antioxidant, and anticholinesterase activities were purchased from Sigma-Aldrich and Merck (USA and Germany). For elements analysis, the samples were prepared by an advanced technique of nitric acid digestion using a microwave digestion system (Analytikjena Topwave 3000, Austria) and subjected to ICP-OES (Optima 8000) and ICP-MS (300D) analysis. Total phenolic and flavonoid contents, antioxidant, and anticholinesterase activities were analyzed by UV spectrophotometer (UV-180, Shimadzu, Japan) and a Tecan Infinite 200 Pro Microplate spectrometer.

Samples preparation for elemental analysis: For elemental analysis, the leaves, stem, flowers and achenes samples were prepared by nitric acid assisted microwave digestion and decomposition procedures as used by Khan *et al*. (2014). Approximately, 0.5 g of the dried powdered samples (in triplicate) were taken and transferred into microwave highly tight and stable digestion vessels made up of polytetrafluoroethylene (PTFE) polymer. Then, to digest and decompose the samples and finally to obtain clear samples, 1.0 mL of H_2O_2 (30%, v/v) and 7.0 mL of HNO_3 (70%) were added to PTFE vessels. The microwave digestion system was operated at a power of 1000 W using various increased temperatures, and then the power at 0 W for cooling purposes. The temperature program was set as: 80°C for 5 min, 120°C for 5 min, 150°C for 5 min, 180°C for 20 min, and the cooling was set at 40°C. After combustion and decomposition, the contents obtained from the digestion were diluted to the volume of 20.0 g using deionized water, and subjected to elemental analysis by ICP-OES and ICP-MS techniques.

Elemental analysis (macro, micro and trace elements): Macro, and micro, trace essential, trace non-toxic and trace toxic elements were analyzed by ICP-OES and ICP-MS techniques, respectively. Data quantitation and drift correction of the analyzed elements was achieved by analyzing the multi-element standards at the intervals of every ten analyses.

Validation and quality assurance for elemental analysis: For calibration curve, six different concentrations of working standard solutions were prepared. The quality parameters for validation including correlation coefficient (R^2), LODs, LOQs, CV%, and accuracy were evaluated according to the methods of Khan *et al*. (2014). Analytical qualities of the data were also checked by analyzing (NIST-1570a), spinach leaves.

Extraction procedure: Shade dried powdered leaves, stem, flowers and seeds were extracted by Soxhlet extraction method. The filtered extracts were evaporated with rotary evaporator. Aqueous extracts were obtained by sonication procedure. Different extracts were then subjected for the total phenolic and flavonoid contents determination, and antioxidant and anticholinesterase activities using UV spectrophotometer and microplate reader.

Evaluation of total phenolic (TPC) and flavonoid contents (TFC): TPC of the *E. echinatus* leaves, stem, flowers and seeds extracts was determined using slightly modified procedure of Thaipong *et al*. (2006) Folin-Ciocalteu's phenol reagent assay (Jamila *et al*., 2014). For TPC, gallic acid was taken as a standard and the results were expressed as micromole gallic acid equivalents per gram of extracts (µM GAE/g). TFC of the extracts was evaluated using colourimetric method (Zhishen *et al*., 1999). Rutin and quercetin were the standard compounds and the results were represented as micromole rutin and quercetin equivalents per gram of extracts (µM RE/g and µM QE/g).

Antioxidant activity: Antioxidant activity of the extracts was analyzed using free radical scavenging of DPPH and ABTS, and ferric ion reducing antioxidant (FRAP) assays (Jamila *et al*., 2014).The calibration curves were achieved by analyzing the standard compounds; trolox, gallic acid and ascorbic acid. The scavenging percentage of the DPPH and ABTS radicals were calculated using the formula given below.

$$\% \text{ Scavenging} = [(1-(A_{sample}/A_{control})] \times 100$$

The results of radicals inhibition were given as IC_{50} while that of FRAP assay as µM TE/g.

Antidiabetic activity: Antidiabetic activity of *E. echinatus* was evaluated by the inhibition of *α*-glucosidase and *α*-amylase. In *α*-glucosidase inhibitory activity, a starch solution as a substrate, and plant extracts were incubated with 0.2 M Tris buffer (pH 8.0) for 5-10 min at 37°C. Then, 1 mL of *α*-glucosidase enzyme (1U/mL) was added and incubated for further 10 min. In order to stop the reaction, the reaction mixture was subjected to heating on boiling water bath for 3 min. The amount of liberated glucose was then recorded (Matsui *et al*., 2001; Andrade-Cetto *et al*., 2008). *α*-amylase enzyme inhibitory activity was determined by mixing and incubating 300 µL of extracts as well as standard drug in the concentration range of 100 to 1000 µg/mL with 300 µL α-amylase (0.5 mg/mL) solution in 0.0002 M phosphate buffer (pH of 6.9) for 10 min at 25°C. Then, 300 µL of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added and incubated at 25°C for further 10 min. After this, to stop the reaction, 1.0 mL of 3, 5 dinitrosalicylic acid colour reagent was added. All the tubes were then incubated in a boiling water bath for 5 min, and cooled to room temperature. After cooling, the reaction mixture was diluted with 10 mL deionized water and absorbance was recorded at a wavelength of 540 nm (Heidari *et al*., 2005; Xiao *et al*., 2006). The *α*- glucosidase and *α*-amylase enzymes activity was recorded as %inhibition using the eq. 1 % relative enzyme activity = enzyme activity of test/enzyme activity of control)*100 – (1)

Cholinesterase inhibitory activity: The potential of cholinesterases (AChE and BChE) inhibition of the different extracts of leaves, stem, flowers and achenes were analyzed by the slightly modified method of Ellman's assay (Ellman *et al*., 1961; Jamila *et al*., 2015). The obtained results were represented in terms of IC_{50}. Physostigmine and galanthamine were used as reference standards.

Statistical analyses

All the obtained results were expressed as means ± standard deviations (n = 3). The mean significant differences (represented by superscript letters) between the assayed values were analyzed using ANOVA (one-way analysis of variance) along with Tukey's HSD test using an SPSS software, version 20.0 (SPSS Inc., Chicago, USA) and the IC_{50} values were calculated with the help of GraphPad Prism 7 (GraphPad Software Inc., La Jolla, USA). The linear correlation coefficients between TPC and the antioxidant assays of the extracts of four different parts of the *E. echinatus* using Pearson's test were also calculated.

Results and Discussion

Validation and quality assurance for elemental analysis: Table 1 describes the quality parameters utilized for the analysis of macro, micro and trace elements by ICP-OES and ICP-MS, respectively. Linearity (R^2) values for macro elements by ICP-OES were 0.99735–0.99994, for micro, trace essential, trace non-toxic and trace toxic elements by ICP-MS, the values were 0.99959–0.99994, 0.99989–0.99994, 0.99991–0.99999, and 0.99986–0.99997. Precision (CV%) values were 0.522–2.195% (< 3%), and spike recovery (%) was 91.3–104.8%. Recoveries for the reference standard material were 92.3–104.1% for ICP-OES and 93.0–102.5% for ICP-MS (Table 2), and the mean recovery values of the analytes were within the interval of confidence ($p < 0.05$) calculated for the certified values, which further confirmed the quality of the methods used. The validation results of ICP-MS analysis for each of the quality parameter were more precise, and CV% and recovery values (%) were similar. All the estimated quality parameter values fulfilled the required criteria of Association of Official Analytical Chemists.

Concentrations of the analyzed elements: Depending on the quantity of the elements present in or required by the body are classified into several groups such as macro, micro, trace essential, and trace toxic elements. The requirement of human body of these elements/ nutrients is up to 100 mg per day, for example, sodium (Na), potassium (K), magnesium (Mg), and calcium (Ca) are named as macronutrients or macro elements. Those, such as zinc (Zn), copper (Cu), chromium (Cr), iron (Fe), which are needed in a quantity less than 100 mg per day are called micro nutrients. Some other elements, which are called trace elements, have concentrations less than 100 µg/g. There are certain elements, which cause lethal effects and can be toxic even when present at very low concentrations. These toxic elements include arsenic (As), indium (In), uranium (U), beryllium (Be), cadmium (Cd), lead (Pb), and mercury (Hg) (Khan *et al*., 2014). These toxic elements are the key hazardous environmental contaminants and adulterants, which are absorbed and accumulated by the plants and vegetables from the soil and enter in our body through the consumption of those plants and food. The excessive intake and accumulation of these toxic elements in human body may cause serious health toxicities and problems. Therefore, it is crucial to determine the human health risks associated with the presence and excess accumulation of the toxic elements in food, food products, plants, herbs, and herbal medicinal drugs.

Table 1. Analytical methods validation parameters (R^2, limits of detection and quantification, and spike recovery) for macro, micro, and trace elements.

Element	Correlation coefficient (R^2)	Limits of detection (ng/g)	Limits of quantification (ng/g)	Coefficient of variance (CV%)	Spike recovery† (%)
Macro elements					
Al	0.99994	8.512	28.089	1.212	104.1
Ca	0.99735	9.124	30.109	1.423	91.3
Fe	0.99979	0.449	1.4817	1.148	91.3
K	0.99768	12.224	40.339	1.192	91.7
Mg	0.99782	8.112	26.769	1.138	94.2
Na	0.99838	12.986	42.853	2.195	94.1
P	0.99981	7.569	24.977	1.179	98.6
S	0.99991	11.115	36.679	2.155	94.7
Micro elements					
Cu	0.99959	0.051	0.1683	1.150	101.1
Ni	0.99988	0.253	0.8349	1.031	104.2
Rb	0.99990	0.058	0.1914	1.137	97.4
Sr	0.99986	0.474	1.5642	1.163	94.2
Zn	0.99980	0.215	0.7095	1.364	94.2
Trace essential elements					
Co	0.99989	0.044	0.1452	0.891	93.4
Cr	0.99994	0.099	0.3267	0.997	97.3
Se	0.99992	0.241	0.7953	1.151	103.7
V	0.99992	0.032	0.1056	1.650	96.2
Trace non-toxic elements					
Ba	0.99999	0.076	0.2508	0.926	96.6
Be	0.99997	0.067	0.2211	0.551	91.4
Ga	0.99991	0.048	0.1584	0.522	96.4
Li	0.99997	0.071	0.2343	0.979	98.6
Trace toxic elements					
As	0.99997	0.067	0.2211	1.404	98.4
Cd	0.99997	0.083	0.2739	1.097	104.2
In	0.99996	0.038	0.1254	0.939	94.2
Pb	0.99994	0.052	0.1716	0.882	104.8
Tl	0.99986	0.037	0.1221	1.114	94.7
U	0.99994	0.008	0.012	1.54	102.0

†Macro elements were spiked at 3,000 µg/kg, while micro and trace elements were spiked at 50 µg/kg

The concentrations (µg/g) of the analyzed elements in the various parts of *E. echinatus* are recorded as mean ± standard deviation on dry weight basis (Table 3). Profiling the macro elements in *E. echinatus*, these elements were present in the decreasing order of Ca > K > Na > P > Mg > Al > Fe > S (leaves), K > Ca > Na > P > Mg > S > Fe > Al (stem), K > Ca > P > Mg > S > Na > Fe > Al (flowers), and Ca > P > K > Mg > Na > S > Fe > Al (achenes). In concentration terms, Ca, K, Na and P were present in the high levels followed by Mg, S, and Fe (Table 3). Concerning the contents of the analyzed macro elements in each part of *E. echinatus*, Ca showed the highest concentration (63,975.6 µg/g, leaves), whereas iron had the lowest level (61.6 µg/g, stem). The contents of macro elements were significantly different ($p < 0.05$) among different parts of *E. echinatus*.

Along with bioactive compounds, herbs are also the important source of essential micro elements. The mean concentrations of micro elements exhibited the decreasing order of: Sr > Zn > Rb > Ni > Cu in leaves, Sr > Rb > Zn > Ni > Cu in stem and flowers, and Zn > Sr > Rb > Cu > Ni in achenes (Table 3). Micro elements among the different parts were significantly different. For example, Sr had the highest content (219.5 µg/g) and Cu showed the lowest levels (1.23 µg/g) in leaves.

Essential trace elements including Co, Cr, Mo, Se, and V are the key cofactors during metabolic processes. Therefore, *E. echinatus*, an important medicinal herb was also quantified for the content of these essential elements. Among these elements, Cr showed the highest concentrations (1.80 to 0.684 µg/g) in all the analyzed samples, whereas Co was the lowest in leaves and stem. Se showed lower values in flowers and seed samples.

Table 2. Analysis of certified reference material (NIST-1570a), Spinach leaves by ICP-OES and ICP-MS.

Element	Certified values (µg/g)	Observed values (µg/g)	Recovery (%)
ICP-OES			
Ca	1.527 ± 0.041	1.408 ± 0.002	92.3
K	2.903 ± 0.052	2.775 ± 0.007	95.6
Mg	0.89	0.928 ± 0.010	95.9
Na	1.818 ± 0.043	1.779 ± 0.004	102.1
P	0.518 ± 0.011	0.498 ±	104.1
ICP-MS			
As	0.068 ± 0.012	0.069± 0.012	101.4
Cd	2.89 ± 0.07	2.69 ± 0.06	93.0
Co	0.39 ± 0.05	0.40 ± 0.07	102.5
Cu	12.2 ± 0.6	11.7 ± 0.24	95.9
Mn	75.9 ±1.9	74.2 ± 0.09	97.7
Ni	2.14 ± 0.10	2.04 ± 0.02	95.3
Se	0.117 ± 0.0009	0.116 ± 0.005	99.1
V	0.57 ± 0.03	0.557 ± 0.03	97.7
Zn	82.0 ± 3	81.4 ± 0.59	99.2

Data are represented as mean ± standard deviation (n = 5)

Table 3. Mean concentrations (µg/g) of macro, micro, and trace essential and toxic elements in the *E. echinatus* leaves, stem, flowers and achenes

Elements	Leaves	Stem	Flowers	Seeds
Macro elements				
Al	$1086.1^{c} \pm 259.6$	$23.2^{a} \pm 7.19$	$81.2^{a} \pm 6.09$	$58.9^{b} \pm 1.04$
Ca	$63975.6^{d} \pm 1365.7$	$11955.6^{b} \pm 232.1$	$20963.2^{c} \pm 925.2$	$9199.3^{a} \pm 318.3$
Fe	$995.9^{c} \pm 25.8$	$61.6^{a} \pm 6.7$	$147.3^{b} \pm 11.0$	$153.3^{b} \pm 14.8$
K	$39406.6^{c} \pm 923.2$	$42700.6^{d} \pm 1659.9$	$25480.8^{b} \pm 740.4$	$7866.1^{a} \pm 130.4$
Mg	$4128.8^{d} \pm 67.0$	$1142.6^{a} \pm 15.3$	$2012.2^{b} \pm 146.5$	$3772.8^{c} \pm 23.9$
Na	$10359.2^{d} \pm 164.4$	$8227.4^{c} \pm 229.9$	$334.2^{a} \pm 11.3$	$620.1^{b} \pm 49.6$
P	$2299.3^{d} \pm 46.4$	$1334.7^{a} \pm 52.7$	$5554.9^{b} \pm 183.8$	$8003.1^{c} \pm 90.2$
S	$411.0^{b} \pm 13.8$	$115.9^{a} \pm 4.35$	$500.0^{c} \pm 7.74$	$438.2^{b} \pm 5.62$
Micro elements				
Cu^{63}	$1.23^{a} \pm 0.125$	$1.40^{a} \pm 0.022$	$1.94^{b} \pm 0.049$	$5.36^{c} \pm 0.125$
Ni^{60}	$4.64^{b} \pm 0.181$	$2.00^{a} \pm 0.180$	$4.00^{b} \pm 0.043$	$4.83^{b} \pm 0.219$
Rb^{85}	$53.9^{b} \pm 4.14$	$67.9^{c} \pm 4.00$	$82.9^{d} \pm 9.08$	$8.02^{a} \pm 0.207$
Sr^{87}	$219.5^{d} \pm 19.8$	$135.2^{b} \pm 4.51$	$161.5^{c} \pm 11.1$	$13.6^{a} \pm 0.871$
Zn	$69.4^{c} \pm 1.93$	$28.5^{a} \pm 4.20$	$50.7^{b} \pm 4.20$	$113.3^{d} \pm 1.35$
Trace essential elements				
V^{51}	$0.904^{c} \pm 0.064$	$0.122^{a} \pm 0.009$	$0.250^{b} \pm 0.015$	$0.229^{b} \pm 0.030$
Cr^{52}	$1.43^{b} \pm 0.185$	$0.790^{a} \pm 0.051$	$0.770^{a} \pm 0.029$	$1.80^{b} \pm 0.067$
Cr^{53}	$1.56^{d} \pm 0.205$	$0.843^{b} \pm 0.049$	$0.684^{a} \pm 0.034$	$1.30^{c} \pm 0.080$
Co^{59}	$0.296^{b} \pm 0.033$	$0.101^{a} \pm 0.0008$	$0.359^{c} \pm 0.035$	$0.234^{b} \pm 0.025$
Se^{82}	$0.398^{c} \pm 0.48$	$0.150^{b} \pm 0.009$	$0.156^{b} \pm 0.005$	$0.054^{a} \pm 0.008$
Trace non-toxic elements				
Li^{7}	$1.98^{d} \pm 0.373$	$0.917^{c} \pm 0.017$	$0.462^{b} \pm 0.018$	$0.293^{a} \pm 0.048$
Be^{9}	$0.007^{c} \pm 0.003$	$0.0007^{a} \pm 0.0001$	$0.002^{b} \pm 0.0002$	$0.002^{b} \pm 0.0004$
Ga^{69}	$0.419^{c} \pm 0.070$	$0.336^{b} \pm 0.017$	$0.252^{b} \pm 0.011$	$0.127^{a} \pm 0.012$
Cs^{133}	$0.105^{c} \pm 0.005$	$0.020^{a} \pm 0.002$	$0.038^{b} \pm 0.002$	$0.023^{a} \pm 0.003$
Ba^{138}	$287.7^{d} \pm 18.7$	$191.7^{c} \pm 4.79$	$135.1^{b} \pm 6.25$	$8.65^{a} \pm 1.25$
Trace toxic elements				
U^{238}	$0.033^{d} \pm 0.003$	$0.006^{b} \pm 0.0005$	$0.0005^{a} \pm 0.0002$	$0.008^{c} \pm 0.002$
Tl^{205}	$0.012^{d} \pm 0.0003$	$0.006^{c} \pm 0.0002$	$0.003^{b} \pm 0.0003$	$0.001^{a} \pm 0.0003$
In^{115}	$0.0005^{c} \pm 0.0001$	$0.0002^{b} \pm 0.00006$	$0.00005^{a} \pm 0.000008$	$0.0002^{b} \pm 0.00007$
Pb^{206}	$0.579^{d} \pm 0.063$	$0.085^{a} \pm 0.007$	$0.163^{c} \pm 0.009$	$0.120^{b} \pm 0.013$
Pb^{208}	$0.572^{d} \pm 0.062$	$0.086^{a} \pm 0.004$	$0.160^{c} \pm 0.008$	$0.119^{b} \pm 0.013$
As^{75}	$0.418^{d} \pm 0.029$	$0.096^{b} \pm 0.0009$	$0.146^{c} \pm 0.009$	$0.048^{a} \pm 0.009$
Cd^{111}	$0.022^{b} \pm 0.003$	$0.014^{a} \pm 0.001$	$0.021^{b} \pm 0.001$	$0.025^{b} \pm 0.002$

Lowercase superscripts (a-d) in a row represent significant differences at $p < 0.05$

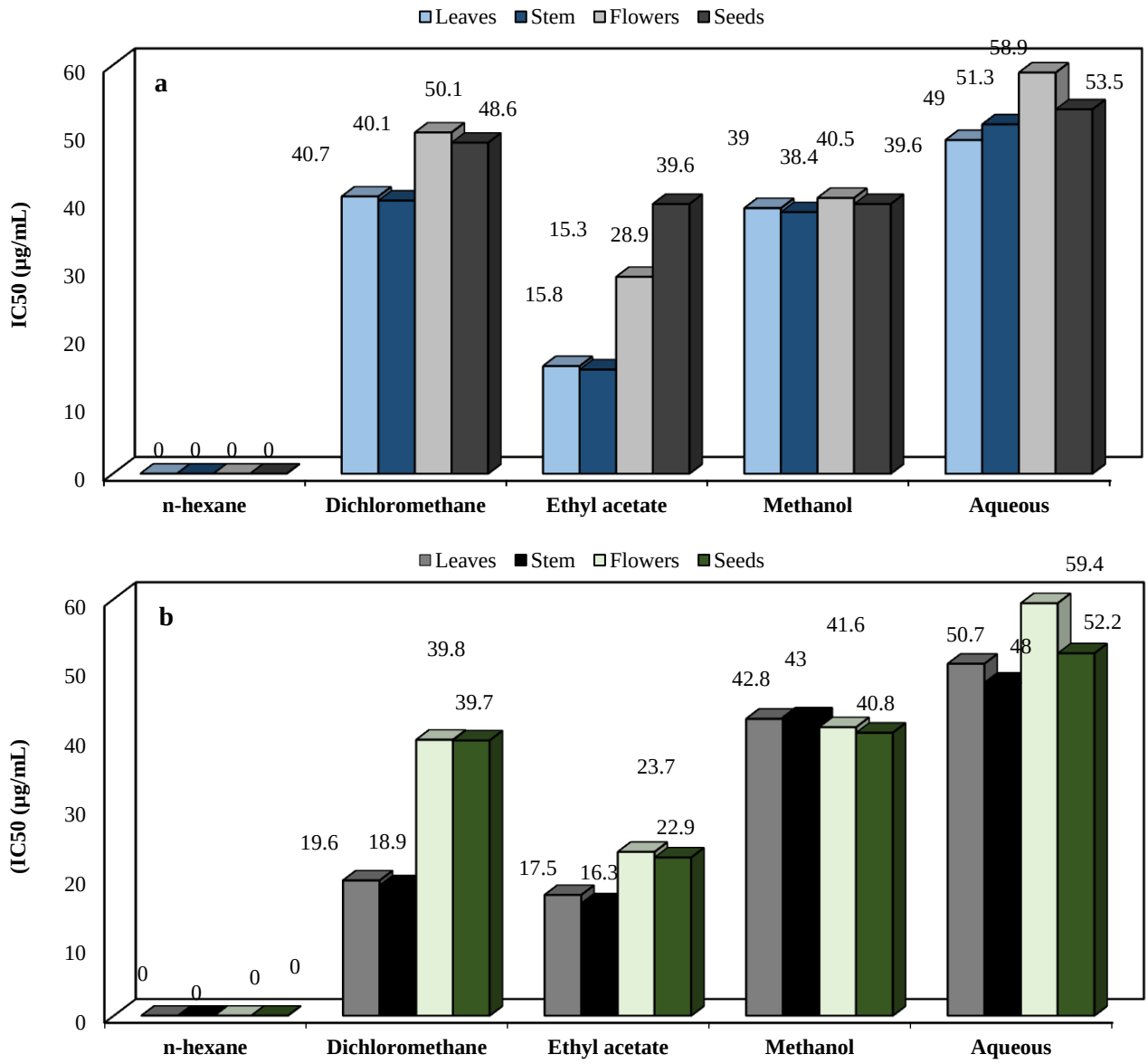

Fig. 1. Plot of IC_{50} values obtained in (a) acetylcholinesterase and (b) butyrylcholinesterase enzymes inhibitory activity of leaves, stem, flowers and achenes of *E. echinatus*.

Regarding the content levels of non-toxic and toxic trace elements (Table 3), Ba was comparatively high in all the parts of *E. echinatus* with the highest (287.7 µg/g) in leaves, followed by stem (191.7 µg/g) and flowers (135.1 µg/g). All the trace non-toxic elements were found high in the leaves. Trace toxic elements among the samples (Tale 3) showing decreasing order of the concentration levels were found as Pb > As > U > Cd > Tl > In in leaves, Pb > As > Cd > U and Tl > In in stem, Pb > As > Cd > Tl > U > In in flowers, and Pb >As > Cd > U > Tl > In in seeds. The concentration of Pb was the highest (0.579 µg/g) in leaves followed by flowers (0.163 µg/g). Overall, the content of toxic elements analyzed in the current study are within the safe limits and below the provisional tolerable weekly intake (PTWI) values given by World Health Organization (WHO 1996 &1999).

Total phenolic and flavonoid content: Plant phenolics are the important constituents with antioxidant, anticancer, anti-inflammatory and antimicrobial potentials. In antioxidant activity, the presence of hydroxyl groups due to donation of hydrogen, is responsible for reducing the free radicals or ferric ions, hence, preventing the radical chain reactions. In the current study, phenolic content in *E. echinatus* determined by Folin-Ciocalteu method showed that ethyl acetate extract of the leaves, stem, flowers and achenes has the highest content of phenolics followed by methanol extracts. The ethyl acetate extract of stem has shown the highest content of phenolics with 2410.5 µMGAE/g, followed by that of the leaves (2395.4 µMGAE/g) (Table 4). Furthermore, ethyl acetate extract of flowers and methanol extract of leaves have shown equal content of phenolics with 2106.5 and 2152.7 µMGAE/g, respectively.

Flavonoids, a class of phenolics, have been recognized as potent antioxidants, anti-inflammatory and anticancer. In the present study, similar to the results of phenolic content, total flavonoid content were high in the stem extracts followed by that of the leaves. The ethyl acetate extracts of stem and leaves respectively exhibited flavonoidal content with 4184.5 µMRE/g, 4357.9 µMQE/g, and 4017.8 µMRE/g, 4183.6 µMQE/g (Table 4). The results showed that the polar extracts (ethyl

acetate, methanol) of all the parts had higher content than the less polar dichloromethane and *n*-hexane extracts.

Antioxidant activity: Antioxidant activity of the plants or food is attributed to the presence of phenolic and flavonoidal constituents. In the current study, antioxidant activity of *E. echinatus* leaves, stem, flowers and seeds extracts was evaluated by DPPH, ABTS and FRAP assays (Table 5). From the results, it was found that the decreasing order of antioxidant capacity is: ethyl acetate > methanol > aqueous > dichloromethane > *n*-hexane. In all the performed assays, ethyl acetate extracts of the analyzed parts were more potent than the other corresponding extracts. In DPPH assay, ethyl acetate extract of the stem was equipotent to the standard antioxidant; Trolox (IC_{50} 9.53 µg/mL) showing IC_{50} value of 9.54 µg/mL (Table 5). *n*-hexane and dichloromethane extracts showed IC_{50} values of more than 50 µg/mL. Regarding the ABTS assay, *E. echinatus* was more potent inhibitor of ABTS radical. However, the potency of the various extracts was in the same pattern of the DPPH assay. The ethyl acetate extract of stem displayed ABTS inhibition with the IC_{50} of 5.88 µg/mL followed by ethyl acetate extract of leaves (7.19 µg/mL) and methanolic extract of stem (8.83 µg/mL). In this assay, all the values were higher than the standards; ascorbic acid, trolox and gallic acid (Table 5). Concerning the iron reducing abilities of the *E. echinatus*, the stem ethyl acetate extract showed a FRAP value of 5930.3 µMTE/g (Table 5). In the correlation analyses study, it was found that TPC of extracts of *E. echinatus* exhibited strong correlations with TFC, DPPH and ABTS. The Pearson's correlation (r) values between TPC and DPPH for ethyl acetate extracts of leaves, stem, flowers and seeds were: 0.891, 0.911, 0.889, and 0.890 at $p < 0.01$, respectively. Furthermore, the correlation coefficients for TPC and ABTS were greater 0.900. Because the reaction of phenolic compounds with Folin-Ciocalteu's reagent and ABTS assays are based on the same electron transfer reaction (Karamać *et al*., 2018), high correlation coefficients were expected. Likewise, the correlation coefficients of TFC with DPPH, ABTS and FRAP were greater than 0.900. From the current finding, it can be concluded that *E. echinatus* extracts contained some phenolic compounds that were active in the antioxidant assays.

Antidiabetic activity: In α-amylase enzyme activity, the inhibition was dose-dependent and increased with increasing concentration of the extracts. Among the extracts at 100µg/mL, leaves, stem, flower and seeds ethyl acetate and methanol extracts were the strong inhibitors of α-amylase enzyme. Ethyl acetate extract inhibited with 39.8%, 41.5%, 31.7%, and 29.2%, which was increased to 75.5%, 78.4%, 67.3%, and 61.8% at 1000 µg/mL, respectively. The IC_{50} values calculated from the concentrations verses %inhibition were 516.9 µg/mL, 489.1 µg/mL, 592.8 µg/mL, and 619.3 µg/mL, respectively as compared to the reference standard; acarbose with the IC_{50} of 307.3 µg/mL. Methanol extract of leaves, stem, flowers and achenes showed IC_{50} values of 571.3 µg/mL, 473.4 µg/mL, 627.9 µg/mL, and 699.5 µg/mL, respectively. All the other extracts of the four *E. echinatus* parts showed IC_{50} values higher than 800 µg/mL, which could be weak inhibitors of α-amylase enzyme. In this activity, methanol and ethyl acetate extracts were the most potent extracts. In α-glucosidase enzyme inhibitory activity, methanol extract of leaves and stem exhibited a significant inhibitory potential with IC_{50} values of 371.4 µg/mL and 368.6 µg/mL, respectively compared to the standard acarbose (211.7 µg/mL).

Anticholinesterase activity: The results (IC_{50}values) of various extracts of different part of *E. echinatus* (Fig. 1) showed that the ethyl acetate and methanol extracts are the strong AChE and BChE inhibitors. The stem and leaves ethyl acetate extracts inhibited AChE strongly with IC_{50}15.3 µg/mL and 15.8 µg/mL, respectively compared to the reference standards; physostigmine and galanthamine having IC_{50} values 0.05 and 2.1 µM/mL. In BChE inhibition, ethyl acetate extract of leaves and stem was the strongest inhibitor with IC_{50} 17.5 and 16.3 µg/mL compared to physostigmine and galanthamine (IC_{50} 0.08 and 19.3 µM/mL). The other extracts moderately inhibited both the AChE and BChE enzymes. Both the enzymes are involved in the breaking down of the acetylcholine (ACh), therefore, the dual inhibitors, which inhibit both the enzymes could increase level of ACh and ultimately provide greater clinical efficacy (Jamila *et al*., 2015). This is the first report on the cholinesterase inhibitory activity of *E. echinatus* samples.

Conclusions

In the analysis of concentrations of elements, among the macro elements, Ca and K were present at high levels. Furthermore, Sr and Zn elements were high in all the samples. Regarding the trace toxic elements, all the values were within the safe ranges established by WHO. Concerning the phenolic and flavonoid content, the ethyl acetate extract of the leaves, stem, flowers and seeds of *E. echinatus* was enriched with these contents. The samples also displayed promising antioxidant, anti-diabetic and anticholinesterase activities. From the results of the current study, it is concluded that the traditional uses of *E. echinatus* might be due to the presence of anti-oxidative phenolic and flavonoidal constituents. Furthermore, from the toxic elements result, it could be concluded that its consumption might not pose any threats to the human health.

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