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Original Research Article

Effect of Different Ethanol Concentrations, Using Different Extraction Techniques, on the Antioxidant Capacity of Lebanese *Eryngium creticum*

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ABSTRACT

This study aims to investigate the effects of different percentages of ethanol (40%, 80%, and 100%) and extraction times (2h, 4h, 12h for reflux), by using conventional (maceration and reflux) and non-conventional (microwave assisted extraction) extraction techniques, on the bioactive phenolic compounds and the antioxidant activity of the Lebanese *Eryngium creticum*. The quantification of these extracts showed that this plant is rich in secondary metabolites. Moreover, the non-conventional microwave assisted extraction technique was more effective (1.718 g of extract compared to the other used methods) along with a reduced extraction time and less used solvent.
The antioxidant activity was evaluated using the DPPH radical scavenging assay and ferric reducing antioxidant power. Results showed that extracts, using 40% ethanol, possessed the highest iron chelating activity (87.92%). On the other hand, the extracts had the highest DPPH scavenging activity (89.92%) after using 80% ethanol. All these results prove that *E. creticum* is a good source of different antioxidant and bioactive compounds.

Keyword: *Eryngium creticum*; ethanol-water binary solvent; antioxidant activity; extraction conditions.

INTRODUCTION

Fossil records date the human use of plants as a medicine, at least to the Middle Paleolithic age, since 60,000 years ago [1]. Since that time, the development of traditional medical systems, that have incorporated plants as means oftherapy, can be traced back merely as far as

recorded documents of their likeness. However, the value of these systems is much more important than a significant anthropological orarcheological fact. Its value is as a methodology of medicinal agents, in which, according tothe World Health Organization (WHO), almost 65% of the world's population has incorporated into their primary modality of health care [2].

The goals for using plants as sources of therapeutic agents are many. First, it is a source of novel bioactive compounds, or known structures, that are used for the semi-synthesis or production of patentable entities of higher activity and/or lower toxicity, such as metformin, nabilone, oxycodone and other narcoticanalgesics, which are agents used as pharmacologic tools. Moreover, the whole plant, or even a part of it, can be used as a herbal remedy (cranberry, garlic, ginkgo biloba….). Most importantly, plants are used mainly to extract bioactive compounds for the direct use as drugs, such as taxol or vinblastine that have medicinal values (anti-inflammatory, antiviral and antioxidant capacities) [3].

Antioxidants are considered important nutraceuticals. They are defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate. The concept of antioxidant capacity originated first from chemistry, and was later studied in biology, medicine, epidemiology, plant biotechnology and nutrition.

Bioactive constituents with antioxidant activities have been found in high concentrations in many plants [4]. However, they are known to present potential health risks and toxicity, the reason why some synthetic antioxidants should be replaced by natural antioxidants [5]. Therefore, there is a great interest in the replacement of synthetic antioxidants with natural sources, especially from plant materials [6]. Plants have been studied chemically from the perspective of active constituents. Several studies have been conducted in order to determine the best parameters and extraction techniques that are able to give the highest yields of antioxidant extracts in the lowest costs. Presently, there is an increasing interest, both in the industrial field and in the scientific research field for medicinal herbs due to its strong biological properties. These properties are characterized by the presence of many biological substances, including some vitamins, flavonoids, terpenoids, carotenoids, phytoestrogens, and minerals. Phenolic substances have shown the highest antioxidant activity of plant among the other compounds [7].

Eryngium creticum is a well-known plant in Lebanon, Palestine, Jordan and in scattered localities in Europe. It is widely grown in some mountain regions. The traditional use of this plant is mainly focused on culinary usage as crudity and main salad ingredient. It was, as well, used traditionally as a laxative and for the treatment of skin infections, tumors, and poisoning, suggesting its potential antioxidant capacities. For this reason, it is very interesting and necessary to conduct more recent scientific studies on this endemic plant.

The main objective of this study was to determine the effect of ethanol concentrations on the antioxidant capacity via *in vitro* tests (DPPH, FRAP) for *E. creticum* extracts using different extraction techniques.

MATERIAL AND METHODS

Plant collection and preparation of powders

Fresh plants were gathered from Beirut during the winter season in February 2014. Before extraction, the plant's materials were well-cleaned and washed under the running tap water, then extended by ground in one layer, and left to dry in the shade inside the limit at room temperature, away from sun light. During the drying process, the plant was turned over to

allow homogeneous drying. After this period, the dried plant should be grinded by a grinder to obtain a powder form and then preserved in a container away from light, heat, and moisture for later use.

All of the chemicals used were of analytical grade. Methanol and ethanol were purchased from BDH, England. Ferrozine and DPPH were purchased from sigma Aldrich.

Sterilization of the samples wasn't performed for the purpose of determining the antioxidant activity at their raw state.

Extraction techniques

Extraction, as the term is used pharmaceutically, involves the separation of medicinally active portions of plant or animal tissues from the inactive or inert components by using selective solvents in standard extraction procedures. The products obtained from plants are relatively impure liquids, semisolids, or powders intended only for the oral or the external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts, and powdered extracts. Such preparations popularly have been called galenicals (named after Galen, the second century Greek physician). The purposes of standardized extraction procedures for crude drugs are to attain the therapeutically desired portion and to eliminate the inert material by treatment with a selective solvent.

In this study ethanol-water binary solvent, of three different concentrations 40%, 80%, and 100% ethanol, was added respectively to the dried powder.

Methods of extraction of medicinal plants

Maceration

5 g of *Eryngium creticum* powder was placed in a beaker with 250 ml of the three different percentages of ethanol (40%, 80% and 100%). The solution is macerated under room temperature for 48 hours with agitation of 360 rpm, until the soluble matter has dissolved. After maceration, the solution was clarified by filtration under vacuum, using the filter paper Whatman (0.45 µ filter units), and then was concentrated by a rotary evaporator (under 40°C then 60°C under reduced pressure). Finally, the obtained extracts were weighed and stored in the refrigerator for later analysis [8].

Reflux method

5 g of *E. creticum* powder was placed in a round bottom flask with 250 ml ethanol of three different percentages (40%, 80% and 100%). Each of the solutions obtained were then refluxed under heat, for 2 hours at 60°C, 4 hours at 40°C, and 12 hours at 60°C. After reflux, the solutions were filtered under vacuum using filter paper Whatman (0.45 µ filter units) and concentrated by a rotary evaporator, at 40°C and 60°C respectively under reduced pressure. Then, the obtained extracts were weighed and stored in the refrigerator for later analysis [8].

Microwave Assisted Extraction (MAE)

A domestic microwave oven (KOG-3767, DAEWOO) of total capacity of 850 W was used.1 g of *Eryngium creticum* powder was placed in threaded round bottom top PFA vials with 50 ml ethanol of different concentrations (40%, 80% and 100%). The vials were inserted into the microwave oven into PFA beaker. The resulting mixtures were irradiated with microwaves (750 powers). The irradiation is achieved for 2 min with 45 s power on, 30 s power off, then 15 s power on. After the irradiation, the samples were allowed to cool at room temperature, and then filtrated under vacuum using filter paper Whatman (0.45 µ filter units) and concentrated by a rotary evaporator under 40°C and 60°C under reduced pressure. The obtained extracts were weighed and stored in the refrigerator for later analysis [8].

Evaluation of the antioxidant activity

DPPH radical scavenging activity

The method of Farhan et al. [7] has been used for the scavenging ability of DPPH antioxidant test. 1 mL of different concentrations of all diluted extracts in methanol, obtained from the different extraction techniques using several concentrations of ethanol (40% 80% 100%), was added to 1mL of DPPH (0.15 mM in methanol). At the same time, a control consisting of 1 mL DPPH with 1 mL methanol was prepared. The mixture was shaken very well by hand and then incubated in the dark at room temperature for 30 min. The absorbance was then measured at 517 nm by a Gene Quant 1300 UV- Vis spectrophotometer. The ascorbic acid was used as a positive control and the methanol was used as blank.

The DPPH scavenging ability of plant extracts was calculated using the following equation:

% ScavengingActivity= [(Abs control −Abs sample)]/ (Abs control)] ×100

The Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample. Also, three controls have been prepared.

Chelating effects on ferrous ions

The method of Farhan et al. [7] has been used to estimate the chelating effect on ferrous ions. 0.5 ml of various concentrations of all extracts was mixed with 0.5 ml of FeSO4 (0.12 mM), and with 0.5 ml of Ferrozine (0.6 mM). The mixtures were allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured by Gene Quant 1300 UV- Vis spectro-photometrically at 562 nm. Ultra-pure water of the sample solution was used as a control without the extracts; the ultra-pure water was used as a blank. EDTA-Na2 was used as a standardizedreference. All measurements were performed in Triplicates. The Ferrozine solution (3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine- 4,4´-disulfonic acid Na-salt) (0.6 mM) was prepared in Ultra-pure water and stored in the dark at room temperature.

The ability of the sample to chelate ferrous ion relative to the control (consisting of iron and Ferrozine only) was calculated using the formula:

Ferrous ion-chelating ability (%) = [(A control − A sample) / A control] ×100

RESULTS AND DISCUSSION

Antioxidants' and phenols' yields from natural sources, was strongly associated with various extraction parameters, such as solvent concentration, extraction time and temperature [9]. Thus the first part of this study aimed to identify the extraction technique that provides the best yield of extracts, while the second part of this study aimed to identify the optimal conditions for these three extraction parameters. For each extraction parameter, the extracts were subjected to two essays: DPPH and FRAP, as a marker to identify the optimal conditions for the extraction parameter. One-at-a-time design was performed to determine the optimal parameters of ethanol/water in the primary solvent, as well as the time and the temperatures for extraction. As shown in the table 1, the mass of phenolic extracts varied based upon the used extraction technique. Among the two used conventional extraction techniques, maceration and reflux (for the same solvent),and after comparing the means of the extracted amounts, it was shown that there is an insignificant difference (they yielded approximately the same results with 0.229 mg, 0.381 mg and 0.525 mg). It should be noted that the reflux method for 12 hours gave the lowest yield (0.024 mg). This could be explained by takinginto consideration the thermal sensitivity of the plant material, that could have led to its denaturation, or destruction, as proven in the study conducted on Biomolecules in Binary Solvents [10], where it showed that heat, acids and bases, reducing

Table 1: Mass (in mg) of the phenolic extracts based upon the used extraction technique

Extraction technique	Maceration			Reflux 2 h			Reflux 4 h			Reflux 12 h			Microwave assisted extraction (MAE)		
Mass of the extract (g)	Eth 100 %	Eth 80%	Eth 40%	Eth 100 %	Eth 80%	Eth 40%	Eth 100 %	Eth 80%	Eth 40%	Eth 100 %	Eth 80%	Eth 40%	Eth 100 %	Eth 80%	Eth 40 %
	0.229	1.097	1.346	0.525	0.976	1.624	0.381	1.062	1.442	0.024	1.039	1.250	0.239	1.230	1.718

agents, and alcohol influences the protein structure and thereby the function. However, among the non-conventional technique and the conventional extraction techniques, a significant difference was noticed for the same solvent. MAE gave an average yield of 11% to 18% higher than that obtained in maceration and reflux.

Therefore, we can say that among the conventional extraction techniques, both maceration and reflux gave similar results. Thus, both techniques can be used for the phenolic compounds extraction, with a preference for the reflux technique that is less time consuming.

As a comparison between conventional and nonconventional extraction techniques, MAE gave the highest values (Fig. 1). Thus, it is a more effective extraction technique than both, the reflux and the maceration, techniques. These findings are in agreement with the study on Pterocarpus marsupium ROXB, where the extraction yield was highest with MAE [11].

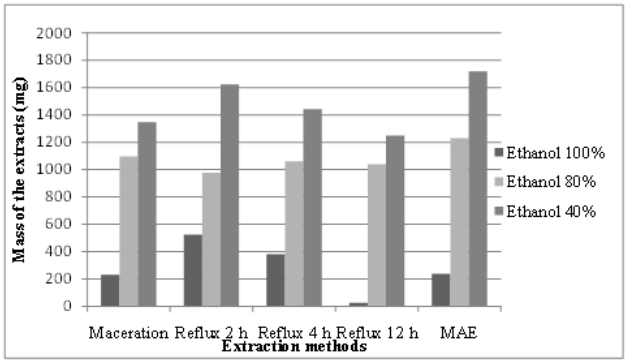


Fig. 1: Mass of extracts (mg) obtained by diffrent extraction methods

The effect of ethanol concentration on the TPC extracts level

Optimized extraction conditions are known to achieve the highest level of antioxidants in plant extracts. Although optimization of extraction parameters for *E. creticum* using ethanol-water binary solvent has not been reported, our work aims to determine it. Therefore, three different ethanol concentrations where first used in both, the conventional and nonconventional extraction methods, in order to determine the most efficient solvent composition able to give the highest antioxidants yields. After examining the different results obtained from the previous experiments, results showed that among the different percentages of ethanol used to extract phenolic compounds from *E. creticum*, 40% ethanol yielded the highest levels of TPC. It is in agreement with the study on mengkudu (*Morindaci trifolia)* [12] that were extracted using 40% ethanol. According to the principle of "like dissolve like", solvents would only extract those compounds that have similar polarity with the solvents [13]. In other word, the phenolic compounds extracted from *E. creticum* would have the same polarity with the

extraction solvent. Based on the results, other ethanol concentration (80% ethanol) was found to give similar values, to some degree. Hence, we suggested that *E. creticum* consisted of diverse phenolic compounds with different polarities. However, based on the result of the extractions, it was optimized at 40%, and thus we suggested that most of the phenolic compounds presented in *E. creticum* had a moderate polar characteristic. Increasing of ethanol concentration up to 80% was associated with an increase in the amounts of crude extract. Based on these experimental results, it was believed that highly active phenolic compounds presented in *E. creticum* were moderately polar. However, a further increase in the ethanol concentration to 100% caused a significant decrement in the extracted mass. The reason of this result was believed to be due to an extraction of phenolic compounds having different molecular weights. Previous studies have reported that DPPH assay is more favorable to react with low molecular weight phenolic compounds [14]; therefore, DPPH will be performed in order to evaluate the antioxidant capacities of the crude extracts and the effect of different concentrations of water-ethanol binary solvent on it. Hence, we proposed that 100% ethanol was less efficient to extract low molecular weight phenolic compounds with high antioxidant capacity from *E. creticum*, as compared to 80% ethanol (Fig. 2).

Effect of Time on the extraction of phenolic compounds:

Results showed (Fig. 3) that antioxidants that were extracted from *E. creticum* with reflux for 2 hours for 40% and 100% Ethanol and 4 hours for 80% ethanol as extraction time possessed the highest yield. It is known that the binding of antioxidants to the plant matrices shows various degrees, hence prolonged extraction may allow extend exposure of antioxidant to the light and O_2 which will lead to rapid degradation of the antioxidant. In addition to that, temperature is known to weaken the viscosity and the surface of the plant cell walls as it increases during extraction or when long exposures occur [10]. Which is mentioned by a previous study made on lemon grass, galangal, holy basil and rosemary [15] that reported that prolonged extraction would lead to a decrease in the phenolic content of crude extract as oxidation of phenolic compounds was possible to be occurred by prolonging the exposure to environment factors such as light and oxygen. Based upon those results we found that 2 to 4 hours as extraction time under heat is one of the optimal conditions to obtain the highest yields with *E. creticum*.

DPPH radical scavenging activity

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a kind of stable organic radical. The capacity of biological reagents to scavenge DPPH radical can be expressed as its magnitude of antioxidant ability. For this reason, this essay is used to quantify radical-scavenging capacity. Thus, the antioxidant activity of the plant extracts, and the standard, were assessed on the basis of free radical scavenging effect of the stable DPPH free radical activity [16]. The results are expressed as percentage of antioxidant scavenging activity (AS%), and later as IC50 value, which is the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%. In the present study, crude extracts (by 80% ethanol of *E. creticum*), especially via 2 hours reflux, have shown a high antioxidant activity which can be attributed to its high TPC. However, 12 hours reflux ethanolic extracts (100% ethanol), has lower radical scavenging activity which might be exactly opposite to its TPC value since it is not the lowest. According to the study made on the kinetics and mechanism of antioxidants using the DPPH method [17], the reaction of DPPH with certain phenols is reversible, which results

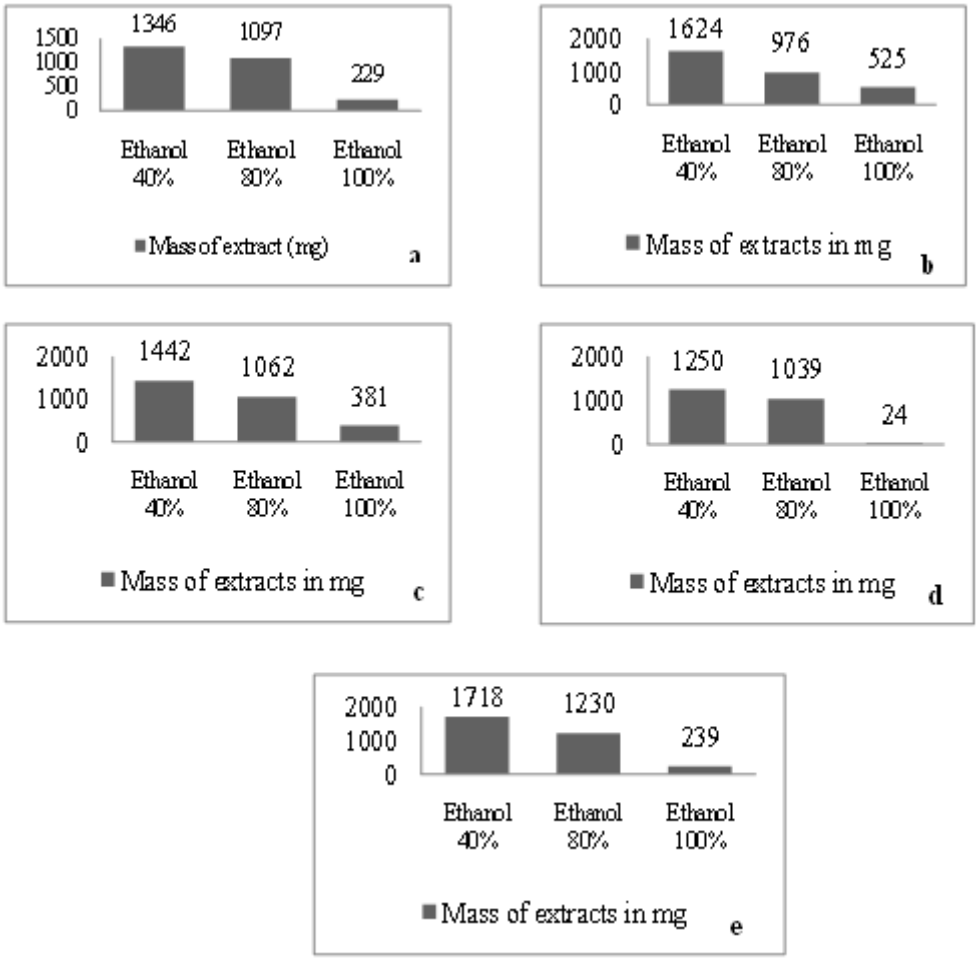


Fig. 2: Amounts of extract obtained by using different ethanol concentrations (a- maceration, b- reflux 2 h, c-reflux 4 h, d-reflux 12 h, e-MAE)

in low readings for the antioxidant activity. Potent property of scavenging free radicals is higher with 80% ethanol (as a binary solvent), and with using reflux for 2 hours as an extraction method, based upon the results of the DPPH free radical scavenging assay. Thus, it can be used perhaps as a potent source for cancer therapy. A change in the free radical scavenging ability of ethanolic extracts of *E. creticum*, on the basis of percent inhibition, has been detected. It is evident from Fig. 4, that the ethanolic extracts after 2 hours reflux, with 80% ethanol, had the highest free radical scavenging potential compared to that after 4 hours reflux, with 100% ethanol, which was the lowest, among the studied ethanolic extracts.

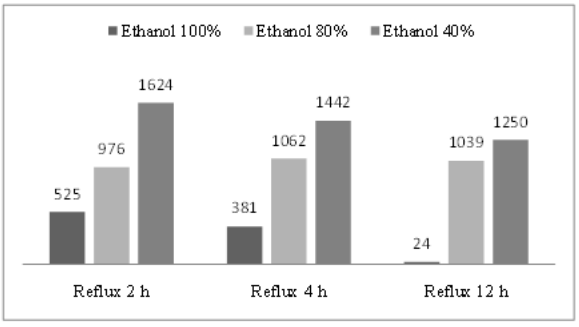
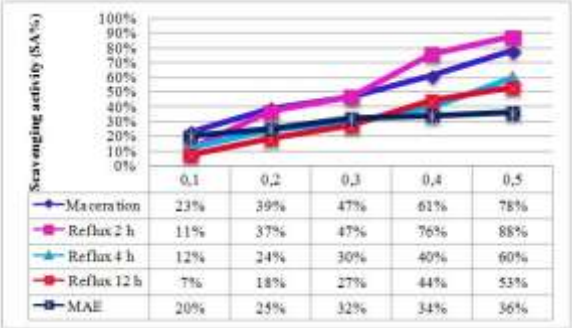


Fig. 3: Mass of extracts (mg) obtained by reflux for different extraction time

In order to confirm the antioxidant power of these extracts from the different extraction methods and for the same concentration of the ethanolic solvent (80% ethanol), with an exception of 12 hours reflux for which 100% ethanol was used, DPPH assay was applied. The obtained results showed that 0.5 mg/mL of the extracts significantly increased the percentage of scavenger activity by 78% and 88% respectively for maceration and 2 hours reflux, as shown in Fig. 4. These results show that by using 80% of ethanol as a solvent, a higher antioxidant activity was shown at different studied concentrations, as shown in the tables listed above. On the other hand, 5 mg/mL of the binary solvent extracts from *E. creticum* significantly increased the percentage of scavenger activity. Thereafter, DPPH scavenging ability increased with the concentration of the extracts.



	0,1	0,2	0,3	0,4	0,5
Maceration	23%	39%	47%	61%	78%
Reflux 2 h	11%	37%	47%	76%	88%
Reflux 4 h	12%	24%	30%	40%	60%
Reflux 12 h	7%	18%	27%	44%	53%
MAE	20%	25%	32%	34%	36%

Fig.4: Positive correlation between the concentration of ethanol by different extraction methods and the DPPH test

The DPPH test provides information about the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH gives a strong absorption band at 517 nm in visible spectroscopy (deep violet color) [18]. The efficacies of antioxidants are often associated with their ability to scavenge stable free radicals. In the present study, 80% of aqueous ethanol extracts exhibited, for 0.5 mg/ml of the extracts, a DPPH radical scavenger activity of 78% and 88% respectively for maceration and 2 hours reflux, with low IC50 values respectively in comparison to 40% and 100% ethanol extracts, whereby DPPH radical scavenger activity was lower by 11% to 49%. FRAP assay measures the reducing ability of antioxidants against oxidative effects of reactive oxygen species. Electron donating antioxidants can be described as reductants, and the inactivation of oxidants by reductants can be described as redox reactions. Total antioxidant power may be referred analogously to total reducing power. In the current study methanol and aqueous methanol extracts exhibited, for 0.5 mg/mL of 40% ethanol extracts, the highest ferrous ion-chelating ability ≈70-88% followed by 80% and 100% ethanol. Even though the scavenging activity (%) for the DPPH assay are high for 80% ethanol extracts, the low antioxidant activities assessed using FRAP assay may not show that the 80% ethanol extracts are not potent antioxidants. As for the mechanism of reaction for DPPH assay is different from FRAP assay [19], low antioxidant activity for the samples is not questionable. *E. creticum* is a potential source of functional food, for it possesses natural antioxidants with strong scavenging activity, and its extracts can be used as natural reducing agents too.

CONCLUSION

Our obtained results demonstrated that in the Lebanese *Eryngium creticum*, the content of active extracted molecules was different among the different extraction techniques, the binary solvent concentration, the temperature, and the used extraction time. The optimal extraction conditions for *E. creticum* were 2 hours extraction time at 60 °C, using 40% ethanol via reflux conventional extraction technique, and 40% ethanol via MAE non-conventional extraction technique. The optimal extraction conditions have shown an important DPPH scavenging activity with 80% ethanol, and exhibited a high ferrous ion-chelating ability with 40% ethanol. *E. creticum* is a potential natural antioxidant source for food industry or

pharmaceutical applications, and may, as well, possess an important anti-carcinogenic potential that should be investigated in future studies.

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CONFLICT OF INTEREST STATEMENT

None Declared

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