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Antioxidant Evaluation and Polyphenol Contents of Hydro Ethanolic Extract's Fractions from *Ephedra nebrodensis*

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ABSTRACT

Ephedra nebrodensis, is a medicinal plant, traditionally in Algeria used for the cure of various ailments such as hepatic disorders. On this basis in present study the phytochemical screening and *in vitro* antioxidant activity of various fractions (n-hexane, chloroform, ethyl acetate, *n*-butanol and water) of ethanol extract obtained by maceration from aerial parts of *E. nebrodensis* were investigated. Different fractions were accessed for their yield percentage, total polyphenol content (TPC), total flavonoid content (TFC), total tannins content (TTC) and antioxidant potential (DPPH - 2,2-diphenyl-1-picrylhydrazyl; ABTS - 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid), reducing power assay (FRAP - ferric reducing antioxidant power activity),and bleaching of β-carotene test . Results showed that ethyl acetate fraction (EF) represents the highest amount of total polyphenols, flavonoids and tannins with 269.669 ± 0.031 µg gallic acid equivalent/mg of DE, 44.507 ± 0.003 µg quercetin equivalents/mg of DE and 228.487 ± 1.362 µg tannic acid equivalents/mg of DE, respectively. In antioxidant assays, ethyl acetate fractions (EF) showed the strongest DPPH and ABTS antioxidant (IC_{50}: 0.009 and 0.004 µg/mL) and FRAP potential (EC_{50}: 0.005 ± 0.000 µg/mL), which significantly correlate to its high content of polyphenolics, flavonoids and tannins. Further, fractions were rich in phenolic compounds. The present results support the possible use of the ethyl acetate fraction from *E. nebrodensis* in pharmaceutical industries as a natural antioxidant.

Keywords: *Ephedra nebrodensis*, polyphenols, flavonoids, tannins, antioxidant capacity.

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1. INTRODUCTION

Oxidative stress is defined as an imbalance between the generation of free radicals and the body's capacity to neutralize their adverse effects or detoxify them by neutralization by antioxidants. Since free radicals are very reactive species, their surproduction during oxidation stress can cause damage to DNA, proteins and lipids, which lead to cell damage, and following the manifestation of pathology such as inflammatory diseases, cardiovascular disease and diabetes [1].

In the last few years, intensive research has been performed out on antioxidants to exploit their capacity to neutralize free radicals [2,3]. Although many synthetic components, including butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propylgallate (PG) with significant antioxidant potential are widely available, but It has also been shown to have some side effects [4]. Plant it has been documented that the extracts have several health benefits, due to the large diversity of free radical scavenging phytocomponents [5]. Medicinal plants are the main source of a variety of biochemicals, i.e. phenolic compounds, flavonoids, terpenoids, and vitamins which are important free radical scavengers [6,7,8].

The genus Ephedra (Ephedraceae) is the source of different classes of natural products such as alkaloids, flavonoids, tannins and polysaccharides [9]. With biological activities, including anti-proliferative [10], anti-asthmatic [11], weight reduction [12,13], anti-inflammatory [14], and hypoglycaemic properties [15, 16].

The aerial parts of *E. nebrodensis*, is among the cardio-protective plants [17]. In addition short term and low dose consumption of the hydro-ethanolic extract of *E. major*, it has a protective effect in cirrhotic patients [18]. Many investigations indicated that *Ephedra nebrodensis* have

exhibited some biological activities such as antihistaminic, adaptogenic, antinociceptive, anti-inflammatory and antioxidant activities [19]. Moreover, research report by [20] suggests that the ethanol: acetone extract of *E. nebrodensis* has a preventive effect against the cardiotoxic effects induced by doxorubicin. In this respect, Amakura et al. [21] reported that the phytochemical profile from the aerial parts of *Ephedra nebrodensis* revealed presence of two phenolic glycosides (nebrodenside A and nebrodenside B) and *o*-coumaric acid glucoside, epicatechin and ephedrine.

The present research has been carried out to investigate the fractions of ethanol extract from aerial parts of *Ephedra nebrodensis* for its TPC, TFC ant TT content and *in vitro* antioxidant. To the best of our knowledge, this is the first study related to comparison between the fractions of aerial parts of *Ephedra nebrodensis* from Algeria.

2. MATERIAL AND METHODS

2.1. Plant collection and extraction

Ephedra nebrodensis was collected in April 2017, from Nafla region, Wilaya of Batna in the East of Algeria. Then the aerial parts of the plant were dried in shadow and ground to a fine powder. The ethanolic extract was obtained by maceration in water/ethanol mixture (30:70) for 3 days. The resultant extract was filtered through Wattman paper n°3 and the solvent was removed by rotary evaporator under reduced pressure at 45°C. The resulting crude extract (CE) was then stored at -4 °C until further analysis [22]. The fractionation of the CE is carried out using a series of solvents of increasing polarity. The crude extract is first mixed with hexane (V / V) to remove lipids, and after separation, the upper organic phase is recovered. This step is repeated several times. The hexane is then evaporated and the resulting fraction is considered the hexane fraction (HF). The lower aqueous phase was further fractionated with chloroform to give the chloroform fraction (CF), then with ethyl acetate to give the ethyl acetate fraction (FE), another solvent *n*-butanol to give the butanol fraction (BF), and finally the aqueous fraction (AF). All fractions were evaporated, dried and stored at -4°C until use.

2.2. Total polyphenol content

The total polyphenols content was determined by the Folin–Ciocalteu method as described by Li et al. [23] with slight modification. In brief, 0.1 mL of fractions was mixed with 0.5 mL of Folin-Ciocalteu reagent (diluted 10 times). After 4 min, 0.4 mL of 7.5 % sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 90 min in dark at room temperature. The absorbance of all samples was measured at 760 nm and the results are expressed in microgram of gallic acid equivalents per milligram dried extract (µg GAE/mg DE).

2.3. Total flavonoids content

The total flavonoids content of each extract was determined by a colorimetric method as described by mouffouk et al. [24]. 1 mL of fractions was mixed with 1 mL of aluminium chloride ($AlCl_3$) solution (2%) and allowed to stand for 10 min. the absorbance of the mixture was then determined at 430 nm versus prepared methanol blank. Results were expressed as micrograms quercetin equivalent per milligram dried extract (µg QE /mg DE).

2.4. Total tannins content

The capacity to precipitate haemoglobin was determined by using bovine fresh blood according to the method described by Gharzouli et al. [25]. Briefly, a volume of samples was mixed with an equal volume of hemolysed bovine blood (absorbance = 1.6). After 20 min, the mixture was centrifuged at 4000 rpm for 10 min, and the absorbance of the supernatant was measured at 756 nm. Results were expressed as micrograms tannic acid equivalent per milligram of dried extract (µg TAE/mg DE).

2.5. Antioxidant activity

2.5.1. DPPH radical scavenging assay

The free radical scavenging activity of the fractions was measured by 2,2- diphenyl-1-picrylhydrazyl (DPPH) assay [26]. After dissolving the CE and fractions in methanol, the solution of DPPH in methanol (0.04 mg/mL) was prepared and 1250 µL of this solution was added to 50 µL of fractions solution at different concentrations. The mixture was shaken vigorously and then kept in the dark for 30 minutes at room temperature. Then, the absorbance was measured at 517 nm. BHT was used as standards. Radical scavenging activity was calculated using the following equation:

Radical scavenging activity (%) = $(A_{blank} - A_{sample} / A_{blank}) \times 100$

A_{blank}: Absorbance of the control. A_{sample}: Absorbance of the reagent with extract.

2.5.2. ABTS radical scavenging assay

ABTS+ radical cation was generated by a reaction of 7 mM ABTS with 2.45 mM potassium persulfate. The reaction mixture was allowed to stand in the dark for 16 h at room temperature. The solution was then diluted by mixing ABTS solution with methanol to obtain an absorbance of 0.70 ± 0.02 units at 734 nm. Then, 50 µL of the sample was mixed with 1ml of ABTS+ solution and kept for 10 min at room temperature. The absorbance of reaction mixture was measured at 734 nm using spectrophotometer [27].

% ABTS radical scavenging activity = $(A_{control} - A_{sample} / A_{control}) \times 100$

A_{blank}: absorbance of ABTS radical and ethanol. A_{sample}: Absorbance of the ABTS with extract or standards.

2.5.3. Ferric reducing antioxidant power (FRAP) assay

The reducing power activity of fractions from *E. nebrodensis* is determined as described by Bouaziz et al [28], with slight modification. Briefly, 100 µL of different concentrations of the samples were mixed with 100 µL of sodium phosphate buffer (pH: 6.6) and 100 µL of potassium ferricyanide (1 %), the mixture was incubated at 50 ° C for 20 minutes. Thereafter, 250 µL tricloroacetic acid (TCA) (10%) were added, the mixture was centrifuged at 3000 rpm during 10 min. After that, 250 µL of upper layer was mixed with 500 µL ferric chloride $FeCl_3$ (0.1%) and 250 µL of distilled water. The absorbance of the mixture was measured at 700 nm. The EC_{50} value (µg extract / mL) is the effective concentration obtained from a linear regression curve. BHT was used as positive standard.

2.5.4. β-Carotene Bleaching Assay

In this test, the antioxidant capacity of the CE and fractions was determined according to the method of Kartal et al. [29]. Briefly, a stock solution of β-carotene-linoleic acid mixture was prepared as follows: 0.5 mg β-carotene was dissolved in 1 mL of chloroform and 25 Ml linoleic acid and 200 mg Tween 40. Chloroform was completely evaporated, and then, 100 ml distilled water saturated with oxygen (30 min, 100 mL/min) was added with vigorous shaking. 2500 µL of this reaction mixture were dispensed into the test tube and 350 µl of samples, prepared at 2 mg /ml concentrations, were added and the emulsion system was incubated for 48 h at

room temperature. The same procedure was repeated with synthetic antioxidant BHT as positive control, and blanks (MeOH and H_2O). The absorbance of the mixture was measured at 490 nm after; 0, 1, 2, 4, 6 and 24 hours. The antioxidant activity (AA) was measured in terms of successful bleaching of β -carotene by using the following equation:

AA % = $A_{sample} / A_{BHT} \times 100$.

A_{sample}: Absorbance in the presence of the extract; A_{BHT}: Absorbance in the presence of positive control BHT.

2.6. Statistical analysis

Results have been represented in the form of mean ± standard deviation (SD). One way ANOVA (analysis of variance) with Duncan's multiple range tests was used to determine the significance of obtained results. The probability (p) value ≤ 0.05 was considered significant for ANOVA and marked correlations among different assays. Values of IC_{50} were calculated through non-linear regression analysis (sigmoidal fitting with variable slope) using Graph Pad Prism v. 5.0

3. RESULTS

The yield percentage of fractions was found to vary from 3.20 to 13.73 %. The highest yield percentage was obtained in *n*-butanol fraction (BF; 13.73 %) followed by aqueous fraction (AF; 11.20 %), (CF; 9.73 %), (EF; 5.80 %) and *n*-hexane fraction (HF; 3.20 %), respectively (table 1).

Table 1: Fractions yield percentage, TPC, TFC and TTC of different fractions obtained from ethanol extract of aerial parts of *Ephedra nebrodensis*.

Fractions [0,25mg]	Yield (%)	TPC (µg GAE/mg DE)	TFC (µg QE/mg DE)	TTC (µg TAE/mg DE)
HF	3.20	17.491 ± 0.049	22.936 ± 0.001	50.756 ± 0.990
CF	9.73	104.420 ± 0.026	33.643 ± 0.046	143.917 ± 0.120
EF	5.80	269.669 ± 0.031	44.507 ± 0.003	228.487 ± 1.362
BF	13.73	29.984 ± 0.001	26.281 ± 0.002	135.567 ± 0.456
AF	11.20	153.554 ± 0.004	34.226 ± 0.004	144.358 ± 3.082

Values expressed are means ± SD. of three parallel measurements. TPC: Total phenolic content. TFC: Total flavonoids content. TTC: Total tannins content. GAE: Gallic acid equivalent. QE: Quercetin equivalent. TAE: Tannic acid equivalent. HF: Hexane fraction; CF: chloroform fraction; EF: Ethyl Acetate fraction extract; BF: *n*-butanol fraction; AF: Aqueous fraction.

The present evaluation of *E. nebrodensis* have shown the polyphenol content varied from 17.491 ± 0.046 - 269.669 ± 0.031 µg GAE/mg DE and flavonoid content from 22.936 ± 0.001 - 44.507 ± 0.003 µg QE/mg DE. Data depicts the presence of higher TPC and TFC in EF (269.669 ± 0.031 µg GAE/mg DE; 44.507 ± 0.003 µg QE/mg DE) followed by AF (153.554 ± 0.004 µg GAE/mg DE; 34.226 ± 0.004 µg QE/mg DE), CF (104.420 ± 0.026 µg GAE/mg DE; 33.643 ± 0.046 µg QE/mg DE), BF (29.984 ± 0.001 µg QE/mg DE; 26.281 ± 0.002 µg QE/mg DE) and HF (17.491 ± 0.049 µg QE/mg DE; 22.936 ± 0.001 µg QE/mg DE) and are listed in Table 1.

The results also enregistered, that EF contained the highest amount of tannins (228.487 ± 1.362 µg TAE/mg DE), followed by AF (144.358 ± 3.082 µg TAE/mg DE), CF (143.917 ± 0.120 µg TAE/mg DE), BF (135.567 ± 0.456 µg TAE/mg DE) and the HF (50.756 ± 0.990 µg TAE/mg DE).

The free radical scavenging activity of five fractions has been studied with IC_{50} and inhibition percentage % values and listed in Table 2. Percentage DPPH scavenging activities varied from 63.627 ± 3.023 % – 96.635 ± 3.016% following a descending order: EF > BF > AF > CF > HF.

For the calculation of IC_{50} values, different concentration of samples were tested against DPPH radicals and it was found that EF (IC_{50}: 0.009 µg/mL), AF (IC_{50}: 0.016 ± 0.000 µg/mL), and CF (IC_{50}: 0.045 ± 0.000 µg/mL) are most potent among all the samples and in comparison with BHT, these fractions are the most potent.

Table 2: DPPH and ABTS assays of different fractions obtained from ethanol extract of areal part of *Ephedra nebrodensis*.

FRACTIONS	% inhibition	IC_{50} (µg/mL)
DPPH		
HF (20 mg/ml)	93.627 ± 3.023*	0.303 ± 0.024***
CF (4 mg/ml)	93.358 ± 0.154*	0.045 ± 0.000
EF (2 mg/ml)	96.635 ± 3.016**	0.009 ± 0.000
BF (10 mg/ml)	95.721 ± 0.715**	0.151 ± 0.011***
AF (2 mg/ml)	95.426 ± 0.871**	0.016 ± 0.000
BHT (2 mg/ml)	88.148 ± 0.900	0.023 ± 0.001
ABTS		
HF (10 mg/ml)	96.424 ± 0.171***	0.071 ± 0.003***
CF (2 mg/ml)	99.746 ± 0.071	0.011 ± 0.000
EF (1 mg/ml)	99.453 ± 0.255	0.004 ± 0.002
BF (4 mg/ml)	99.226 ± 0.156	0.029 ±0.000*
AF (1 mg/ml)	98.884 ± 0.331*	0.011 ± 0.001
BHT (1 mg/ml)	98.672 ± 0.241	0.008 ± 0.001

IC_{50} and % inhibition values represent the means ± SD of three parallel measurements (p ≤0.05); BHT: was used as standards; HF: Hexane fraction; CF: Chloroform fraction; EF: Ethyl acetat fraction; BF: Butanol fraction; AF: aqueous fraction (* P < 0.05, * P < 0.01, *** P < 0.001).

In the present study, ABTS scavenging activity of five fractions were found to vary from 96.424 ± 0.171 % - 99.746 ± 0.071 % (Table 2). BHT has been used as a positive control having compelling ability to scavenge ABTS free radicals with % inhibition of 98.672 ± 0.241 at 100 µg/mL which is similar to the EF (99.453 ± 0.255 %), and AF (98.884 ± 0.331 %). The results revealed that the EF exhibited the highest radical scavenging activity with IC_{50} (0.004 ± 0.002 µg/ml), which was significantly near to that of BHT (0.008 ± 0.001 µg/mL), followed by AF and CF (IC_{50}: 0.011 ± 0.001 µg/mL), than BF (IC_{50}: µg/mL) finally, HF (IC_{50}: 0.071 ± 0.003 µg/mL).

Values for FRAP assay were observed to range from 0.005 ± 0.000 – 0.279 ± 0.022 µg/mL (Fig. 1). FRAP activity of EF, AF and CF has been found to outperform other samples with FRAP activity of 0.005 ± 0.000, 0.016 ± 0.000 and 0.025 ± 0.001 µg/mL respectively, in comparison to BF and HF (0.088 ± 0.002 and 0.279 ± 0.022 µg/mL) respectively. These results are found in good agreement with TPC, TFC, TTC values, and free radical activities (DPPH and ABTS).

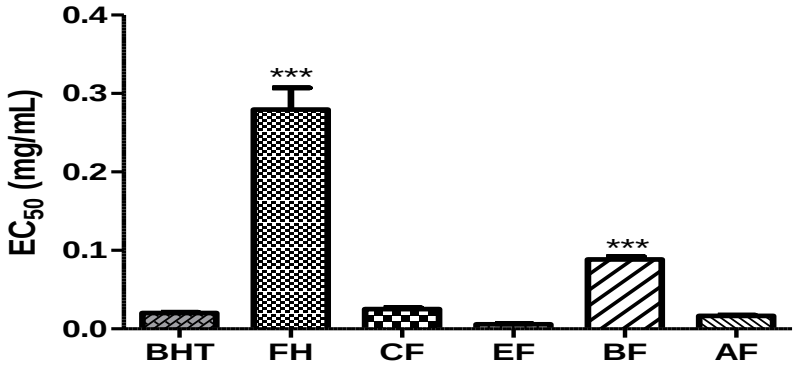

Figure 1: A comparison between different fractions in reducing power assay. Data were presented as EC_{50}.means ± SD (n = 3). (*** p <0.001) compared to BHT as standards.

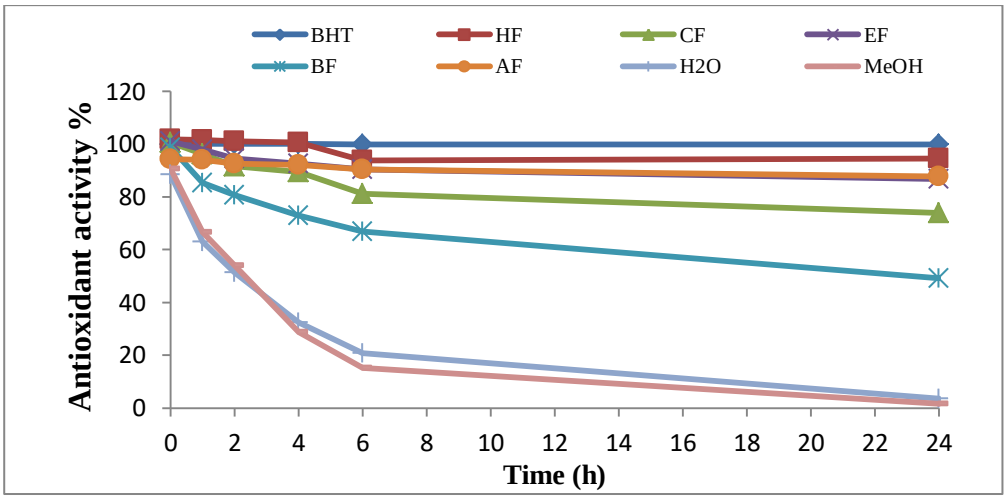

Figure 2: Kinetics of β-carotene bleaching in the presence of HF, CF, EF, BF, AF, water, methanol and BHT during 24h. HF: Hexane fraction; CF: chloroform fraction; EF: Ethyl Acetate fraction extract; BF: *n*-butanol fraction; AF: Aqueous fraction; BHT: butylated hydroxytoluene; MeOH: methanol; H_2O: water.

The β-carotene/linoleic acid test was used to evaluate the antioxidant effect of *E. nebrodensis* fractions. As shown in Fig. 2, the inhibition percentage % values were found to be in the following order: BHT < HF < EF < AF < CF < BF (Fig. 2).

4. DISCUSSION

In present study, ethanol extract of aerial part of *Ephedra nebrodensis* was fractionated by solvent-solvent partitioning to obtain five fractions with progressive polarity (*n*-hexane, chloroform, ethyl acetate, *n*-butanol and water). The *n*-butanol fraction and AF have a significantly higher yield contributing as the maximum among the fractions obtained. However, a significant difference between yield percentage of BF and EF has also been observed (Table 1). According to the results obtained, the most polar fractions give a high yield. A similar course of results was found in case of *Codonopsis clematidea* [30] where the polar fraction (BF) has maximum yield than other fractions. On the other hand according to the results obtained by Nsimba et al. [31], they found that the non-polar fraction (HF) of *Chenopodium quinoa* and *Amaranthus cruentus* has a maximum yield compared to other extracts.

The phenols are major plant system constituents that are dispersed as secondary metabolites, playing a key role as antioxidants, stress protection, plant growth and pigment synthesis. Flavonoids are considered to play a crucial role in preventing biological systems from the adverse effects of oxidative processes on macromolecules [32,33,34]. The difference between the polyphenol and flavonoid content of the various fractions can be explained by the separation of

the crude extract using different solvents that concentrate the main active phytocompounds according to their polarity. Higher EF values confirm the presence of active components of medium polarity. This suggests that antioxidant activity could be the attribute of the phenolic and flavonoid content of these fractions. An identical trend was observed in the case of *Achillea millefolium*, *Foeniculum vulgare*, *Lavandula latifolia* [35] and *Codonopsis clematidea* [30] where EF showed a higher value than the other fractions. In addition, the authors reported such a relationship between the antioxidant with PTC and TFC [36].

Tannins are also important compounds in Ephedra, which exist mainly in the form of condensation. Principally, proanthocyanidins have been shown to be present in many Ephedra plants (*E. przewalskii*, *E. alata*, *E. distachya*, *E. fragilis*, and *E. intermedia*; *E. californica*, *E. nevadensis*, *E. fasciculata*, *E. trifurca*, *E. torreyana*, and *E. viridis*) [37]. Currently, condensed tannins of procyanidin A are the most common in Ephedra [38], containing dimers, trimers, and tetramers [9].

The results of DPPH assay are in good accordance with polyphenolic, flavonoids and tannins content, where a similar trend was observed. Lowest effect on DPPH radical was shown by HF (0.3034 ± 0.0247 %), which has the minimal yield percentage. Although, % DPPH scavenging activity of EF (96.635 ± 3.016 %) and AF (95.426 ± 0.871 %) at 2 mg/mL is higher to BHT (88.148 ± 0.900 %). This suggests that the strongest radical scavengers found in *Ephedra nebrodensis* are of mid-polar in nature. However, this may be attributed due to condensing the active compounds in these two fractions and removal of undesirable components. An equivalent result was reported in case of *Lysimachia foenum-graecum* [34] and *Codonopsis clematidea* [30] where EF has shown better IC_{50} in comparison to the other fractions.

ABTS assay is a pH-independent assay, in which reduction of free radicals increases with an increase in antioxidant molecules [39]. The ABTS scavenging results has been correlation with the TPC, TFC and TTC values similar to as found with DPPH assay. The result suggests that the effects shown by EF, AF and BF on ABTS radicals could prevent or ameliorate the oxidative damages. The observations of our study corroborates with the study on *Codonopsis clematidea and Ficus microcarpa* L. which revealed an identical performance pattern of EF among all the other fractions [30,40]. The results of FRAP assay coincide very well with earlier reports by other authors where FRAP assay was observed to show analogous results to DPPH and ABTS [30,41].

The obtained results for β-carotene bleaching assay can be due to the presence of polyphenols and flavonoids which have the major contribution to the antioxidant potential of the aerial part fractions. In fact, the literature has demonstrated that a good correlation has been found between antioxidant activity and the content of polyphenols and flavonoids [42,43].

5. CONCLUSION

The study clearly marked the EF as most effective in antioxidant activity which may be an attribute of its higher TPC, TFC and TTC values, further, fractions of *Ephedra nebrodensis* can be proposed as a valuable antioxidant natural source with immense utility in development of health promoting nutraceutical products. However, there is a need to further investigate the fractions in a more elaborated way to understand the composition of the phenolic, flavonoid, and other biologically active compounds. However, preclinical *in vivo* safety studies are strongly warranted before these fractions can be used for product development related applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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