Original Article

Isolation and structure elucidation of antioxidant compounds from leaves of *Laurus nobilis* and *Emex spinosus*

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ABSTRACT: In recent years, there has been increasing interest in finding naturally occurring antioxidants from plants for use in food and medicinal materials to replace synthetic antioxidants since such antioxidants are being restricted due to their side effects like carcinogenicity. The aim of this work was to examine the *in vitro* antioxidant activity of *Laurus nobilis* and *Emex spinosus* leaves and to isolate and structurally elucidate the active compounds in those leaves. The aqueous ethanolic extracts (70%) of *Laurus nobilis* and *Emex spinosus* leaves exhibited free radical scavenging action against 1,1-diphenyl-2-picrylhydrazyl (DPPH). Their concentrations of 50% inhibition (IC_{50}) were 25.3 and 20.73 µg/mL, respectively. Activity-guided separation of these extracts using a combination of different chromatographic methods (TLC and column chromatography) resulted in the isolation of five chromatographically pure compounds (three from *Laurus nobilis* and two from *Emex spinosus* leaves). Spectroscopic methods (^1H, ^{13}C-NMR, UV and MS) and chemical methods (detection tests and acidic hydrolysis) revealed the isolated antioxidant compounds to be flavonoid substances that were identified as kaempferol, kaempferol-3-rhamnopyranoside, and kaempferol-3,7-dirhamnopyranoside from *Laurus nobilis* extract and luteolin and rutin from *Emex spinosus* extract. The five flavonoids had varying ability to inhibit DPPH radicals (IC_{50} from 4 to 35.8 µg/mL). Luteolin and rutin had strong scavenging action with an IC_{50} of 4 and 4.6 µg/mL, respectively, and this action was stronger than that of synthetic antioxidant BHA, *i.e.,* butylated hydroxyanisole (IC_{50} = 5.6 µg/mL).

Keywords: Plant antioxidants, flavonoids, *Laurus nobilis*, *Emex spinosus*, DPPH

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1. Introduction

Free radical reactions occur in the human body and food systems. Reactive oxygen/nitrogen species (ROS and RNS) are produced as a part of normal metabolic processes. The imbalance between production of these species and the capacity of normal detoxification systems in favor of the oxidant leads to oxidative stress, which causes cell damage as a result of the interaction of reactive species with cellular constituents. This then leads to the development of various acute and chronic human diseases such as cancer, cataracts, and heart disease (*1*). Since antioxidants block the oxidation process that produces free radicals, they may be used as a way to prevent chronic diseases and health problems.

In addition to the adverse health effects of reactive species, the oxidative deterioration of components in foods is responsible for rancid odors and flavors. These odors and flavors decrease the organoleptic and nutritional quality of processed foods. The addition of synthetic antioxidants such as butylated hydroxyanisol (BHA) occurs widely in the food industry. However, the use of these synthetic antioxidants has been questioned due to their potential risks and toxicity (*2*).

Many antioxidant compounds that naturally occur from plant sources have been identified as free radical or active oxygen scavengers (*3*).

Recently, interest has increased considerably in finding natural antioxidants from plant material for use in food or medicinal materials to replace synthetic antioxidants. In addition, natural antioxidants have the capacity to improve food quality and stability and can also act as nutraceuticals to terminate free radical chain reactions in biological systems and thus may provide additional health benefits to consumers (*4*).

Laurus nobilis is an evergreen tree. It grows spontaneously in scrubland and woods in Europe and around the Mediterranean. It is also popular as an ornamental tree in gardens. It has long been used to flavor food. It has medicinal uses as a stimulant of gastric secretion, a diaphoretic, and is used to treat rheumatic complaints (*5*).

Emex spinosus is a common wild herbaceous plant known as dirs el-agooz in Egypt, where its leaves

are used in traditional medicine to relieve stomach disorders and to stimulate appetite (*6*).

The aim of this work was to examine the antioxidant activity of *Laurus nobilis* and *Emex spinosus* leaves and to also isolate and structurally identify the active constituent(s) responsible for this activity.

2. Materials and Methods

2.1. *Plant materials*

Leaves of *Emex spinosus* (Polygonaceae) and *Laurus nobilis* (Lauraceae) were collected at the flowering stage in April 2008 from the Experimental Farm of the Faculty of Agriculture, Fayoum University and El Shorouk Farm, located on the Cairo-Alexandria Desert Road 72 km north of Cairo, respectively. Species were authenticated by the Botany Department, Faculty of Science, Cairo University. Vouchered specimens of both plants (ES_{10}, LN_{12}, respectively) were deposited in the herbarium of the Biochemistry Department, Faculty of Agriculture, Fayoum University.

2.2. *Extraction of bioactive constituent(s)*

Ground, air-dried leaves of *Laurus nobilis* and *Emex spinosus* (350 g each) were extracted three times with 700 mL each of EtOH/H_2O (7:3, v/v) at room temperature (28 ± 2°C). After filtration, the combined extracts were evaporated under reduced pressure to yield 37.5 g and 42.3 g, respectively. The residue of aqueous ethanolic extract of each plant (35 g and 40 g, respectively) was suspended in water (150 mL) and extracted with $CHCl_3$ (3 × 100 mL) to yield $CHCl_3$ soluble components (Fraction A). The aqueous layer was freeze-dried and then extracted with EtOAc (3 × 150 mL) to yield EtOAc soluble components (Fraction B) and aqueous soluble components (Fraction C). The three fractions of chloroform, ethyl acetate, and water were concentrated to yield 8.8 g (Fraction A), 7.2 g (Fraction B), and 18.4 g (Fraction C) from *Laurus nobilis* extract and 10.2 g (Fraction A), 8.1 g (Fraction B), and 21.1 g (Fraction C) from *Emex spinosus* extract. These fractions were tested for their free radical scavenging activity.

2.3. *Antioxidant activity*

The antioxidant activity of the aqueous ethanolic extracts, fractions, BHA, and isolated compounds was assessed by measuring free-radical scavenging activity *via* the decoloration of a methanol solution of the free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described elsewhere (*7*) as follows: two mL of methanol solution of each test material at various concentrations (2-50 µg/mL) were added to a 2 mL solution of DPPH (25 mg/L) in methanol, and the reaction mixture was shaken vigorously.

After incubation at room temperature for 30 min, the absorbance (A) of DPPH was determined with a spectrophotometer at 517 nm, and the radical scavenging activity of each sample was expressed as percentage inhibition:

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

IC_{50} (sample concentration required for 50% inhibition) was obtained by linear regression analysis of the dose response curve, plotted as the % inhibition and concentration (µg/mL). Butylated hydroxyanisole (BHA), which is a well-known antioxidant, was used as a positive control. The mean values were obtained from triplicate analysis.

2.4. *Isolation of antioxidant compound(s)*

Isolation of antioxidant compounds was done as follows using the most abundant active fraction B (EtOAc fraction) from each plant.

First, *Laurus nobilis* 7.0 g was subjected to column chromatography (CC) over Sephadex LH-20 (40 g) and eluted with methanol to yield 40 fractions of 10 mL each. Based on the differences in composition indicated by thin-layer chromatography (TLC) ($CH_3Cl/CH_3OH/H_2O$, 75:25:2, v/v), six fractions designated I, II, III, IV, V, and VI were obtained and then tested for free radical scavenging activity. The active fraction VI (928 mg) was chromatographed over a silica gel column (20 g; 230-400 mesh, Merck) and eluted with 100 mL of the following solvent mixtures of $CH_3Cl/CH_3OH/H_2O$ (90:10:0, v/v; 80:20:2, v/v; and 75:25:2, v/v) for each eluent. In accordance with the differences in composition revealed by TLC, five fractions designated VIa, VIb, VIc, VId, and VIe were obtained. The fractions VIa (180 mg) eluted with $CH_3Cl/CH_3OH/H_2O$ (90:10:0, v/v, between 0-50 mL), VId (245 mg) eluted with $CH_3Cl/CH_3OH/H_2O$ (80:20:2, v/v, between 60-100 mL), and VIe (228 mg) eluted with $CH_3Cl/CH_3OH/H_2O$ (75:25:2, v/v, between 0-100 mL) that contained the major compounds were further purified by preparative TLC using $CH_3Cl/CH_3OH/H_2O$ (90:10:0, v/v; 80:20:2, v/v; and 75:25:2, v/v, respectively) to yield the pure compounds A (126 mg), B (190 mg), and C (132 mg), respectively.

Second, *Emex spinosus* 8.0 g was chromatographed over a silica gel column (200 g; 230-400 mesh, Merck) and eluted with the solvent mixtures of $CH_3Cl/CH_3OH/H_2O$ (70:30:1, v/v, and 50:50:2, v/v; 650 mL each eluent). Thirty-two fractions of each eluent were collected. The eluates were combined on the basis of similarity of TLC profiles to yield 8 fractions designated 1 to 8 and then tested for free radical scavenging activity. The active fractions 2 (1.38 g; eluted with 70:30:1, v/v, between 80-340 mL) and 5 (1.57 g; eluted with 50:50:2,

v/v, between 70-160 mL) were further purified several times over Sephadex LH-20 and silica gel columns as shown in Figure 1 to yield two active compounds, D (283 mg) and E (312 mg).

2.5. *Analytical and preparative TLC*

Analytical and preparative TLC were carried out on Merck precoated silica gel plates (F_{254} thickness: 0.25 mm and 2.0 mm, respectively) using the following solvent systems: 1) *n*-butanol-acetic acid-water (4:1:5, v/v, upper layer), 2) ethyl acetate-acetic acid-formic acid-water (100:11:11:27, v/v), 3) dichloromethane-methanol-water (50:25:5, v/v), 4) chloroform-acetone (50:6, v/v), 5) chloroform-methanol (90:10, v/v, and 80:20, v/v), and 6) chloroform-methanol-water (80:20:2, v/v; 75:25:2, v/v; 70:30:1, v/v; 50:50:2, v/v; and 70:30:5, v/v).

Spots on TLC were detected under UV light (254 and 365 nm) and by spraying with concentrated H_2SO_4 followed by heating at 105°C for 5 min and or by 5% $AlCl_3$. Sugars were detected by spraying with naphthoresorcinol-phosphoric acid followed by heating at 105°C for l0 min.

2.6. *Structure identification of antioxidant compounds*

Antioxidant compounds were characterized by chemical investigation (detection tests and acid hydrolysis) and spectroscopic methods.

2.6.1. *Detection tests*

Isolated compounds were detected according to methods described elsewhere (*8*).

2.6.2. *Acid hydrolysis*

Acid hydrolysis was performed in a sealed tube at 100°C for 4 h with 2 mg of the isolated compound in 2 mL of 10% HCl. The aglycon moiety was extracted with Et_2O and analyzed by TLC with system 4. The aqueous layer was neutralized with *N,N*-dioctylamine (10% in $CHCl_3$). After evaporation to dryness, the sugars were identified by TLC with system 3 by comparison with authentic samples.

2.6.3. *Spectroscopic methods*

Nuclear magnetic resonance (*MMR*) *spectroscopy* – ^1H and ^{13}C-NMR spectra of the isolated compounds were recorded in CD_3OD on a Varian Mercury VXR-300 spectrometer at the Central Laboratory, Faculty of Science, Cairo University, Egypt. Chemical shifts (ppm) were related to that of the solvent.

Mass spectrometry (MS) – Mass spectra were recorded on a GC-MS QP1000 EX Shimadzu mass spectrometer at the Micro Analytical Laboratory, Faculty of Science, Cairo University, Egypt.

Ultra violet spectrometry (UV) – UV spectra were recorded on a Cecil Series 3000 spectrophotometer in accordance with the method of Mabry *et al.* (*13*).

3. Results and Discussion

The antioxidant activity of the aqueous ethanolic extracts (70%) and isolated compounds of *Laurus nobilis* and *Emex spinosus* leaves is shown in Table 1. The aqueous ethanolic extract of both types of leaves exhibited free radical scavenging action against

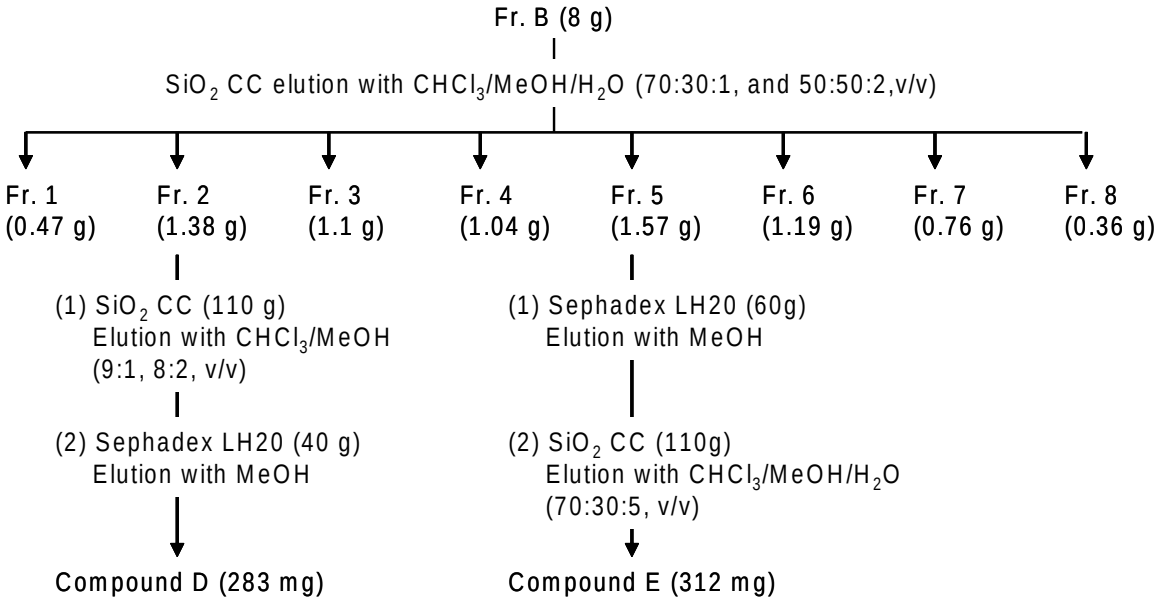


Figure 1. Flow diagram for the isolation of antioxidant compounds D and E from *Emex spinosus*.

1,1-diphenyl-2-picrylhydrazyl (DPPH). As is evident, EtOH extract of *Emex spinosus* was more potent as an antioxidant than EtOH extract of *Laurus nobilis* since its IC_{50} value was lower than those of the *Laurus* extract (IC_{50} = 20.73 and 25.30 µg/mL, respectively). IC_{50} was defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. A lower IC_{50} value reflects better DPPH radical scavenging activity (*9*). The variation in the antioxidant effect of the two active extracts may be due to the differences in their secondary constituents (*10-12*).

Activity-guided separation of these extracts as described previously resulted in the isolation of five chromatographically pure compounds designated A, B, and C from *Laurus nobilis* and D and E from *Emex spinosus* extracts. The results in Table 1 indicate that the five compounds A, B, C, D, and E had varying IC_{50} values ranging from 4 (compound D) to 35.8 µg/mL (compound C). Among the three isolated free radical scavengers from *Laurus nobilis* extract, *i.e.,* A, B, and C, compound A was the most potent (IC_{50} = 7.7 µg/mL). However, the lowest effective compound was C (IC_{50} = 35.8 µg/mL). This variation in potency is probably due to structural differences.

In contrast, compounds D and E isolated from *Emex spinosus* extract had the highest level of scavenging action with an IC_{50} of 4 and 4.6 µg/mL, respectively. Thus, the isolated compounds from each plant were in part responsible for the antioxidant activity of the aqueous ethanolic extracts of *Laurus nobilis* and *Emex spinosus*. The results in Table 1 also show that compounds D and E had stronger scavenging action than did the positive control BHA (butylated hydroxyanisole), which is known to be a very efficient synthetic antioxidant agent (IC_{50} = 5.6 µg/mL).

Table 1. Antioxidant activity of aqueous ethanolic extracts (70%) and isolated compounds (A to E) from the leaves of *Laurus nobilis* and *Emex spinosus* on DPPH free radical

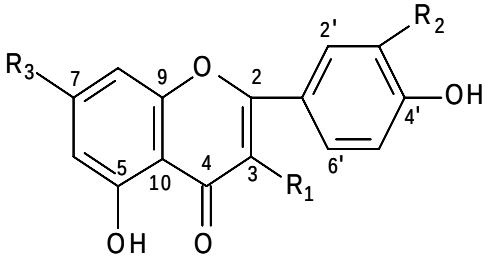
Test materials	IC_{50} (µg/mL)[a]
Aqueous EtOH extract of *Laurus nobilis*	25.30
Aqueous EtOH extract of *Emex spinosa*	20.73
Compound A	7.70
Compound B	20.87
Compound C	35.80
Compound D	4.00
Compound E	4.60
Butylated hydroxyanisole (BHA)	5.60

[a]: Amount required for 50% reduction of DPPH free radicals after 30 min.

The structures of the five active isolated compounds (A to E, Figure 2) were identified as kaempferol (A), kaempferol-3-*O*-α-L-rhamnoside (B), kaempferol 3,7-di-*O*-α-L-rhamnoside (C), luteolin (D), and rutin (E) from the results obtained from chemical and spectroscopic methods as well as by comparing the spectroscopic data (Table 2) with data in the literature (*13-17*).

The differences in structure for the five isolated compounds explain the variation in the antioxidant effect of these compounds. The structure-activity relationship revealed that a free OH group at C-3 (like kaempferol) and 2 hydroxyl groups *ortho* to each other on ring B (like luteolin and rutin) enhanced antioxidant activity whereas *O*-glycosylation at C-3 and C-7 (like kaempferol glycosides B and C) decreased such activity. This relationship has previously been reported (*18,19*).

The present results revealed that the five isolated antioxidant compounds are flavonoids or rather are the most common and abundant classes of flavonoids, *i.e.,* flavones (luteolin, D), flavonols (kaempferol, A), and flavonol glycosides (kaempferol-3-rhamnopyranoside, kaempferol-3,7-dirhamnopyranoside and rutin B, C, and E, respectively). These classes of flavonoids seem to be the most potent at protecting the human body against reactive oxygen species (*20*). Body cells and tissues are continuously threatened by the damage caused by free radical and reactive oxygen



Compounds	R_1	R_2	R_3	$[M]^+$	MF	UVλ(nm)/MeOH
A	OH	H	OH	286	$C_{15}H_{10}O_6$	265, 365
B	*O*-rhamnosyl	H	OH	432	$C_{21}H_{20}O_{10}$	265, 350
C	*O*-rhamnosyl	H	*O*-rhamnosyl	578	$C_{27}H_{30}O_{14}$	265, 350
D	H	OH	OH	286	$C_{15}H_{10}O_6$	250, 350
E	*O*-rutinosyl	OH	OH	610	$C_{27}H_{30}O_{16}$	265, 360

Figure 2. Structural formula of the isolated compounds.

Table 2. ^{1}H, ^{13}C-NMR chemical shifts (ppm) of the five isolated compounds in CD_3OD

Atom	A		B		C		D		E	
	δC	δH	δC	δH	δC	δH	δC	δH	δC	δH
Aglycon										
2	156.2	–	161.3	–	161.6	–	166.1	–	158.4	–
3	130.9	–	133.8	–	134.1	–	103.2	6.5 s	135.6	–
4	178.8	–	179.1	–	178.9	–	183.8	–	179.3	–
5	162.1	–	162.9	–	162.6	–	163.2	–	162.8	–
6	100.1	6.14 d *J* = 2.1	100.7	6.19 d *J* = 2.1	101.8	6.31 d *J* = 2.1	100.5	6.20 d *J* = 2.2	99.9	6.19 d *J* = 2.1
7	168.2	–	168.7	–	167.2	–	166.3	–	165.9	–
8	94.9	6.36 d *J* = 2.4	95.4	6.35 d *J* = 2.1	96.8	6.49 d *J* = 2.1	95.00	6.41 d *J* = 1.9	94.9	6.39 d *J* = 2.1
9	158.4	–	158.7	–	158.5	–	160.1	–	159.3	–
10	105.6	–	105.4	–	105.7	–	105.1	–	105.6	–
1′	122.1	–	122.6	–	122.8	–	123.7	–	123.6	–
2′	130.9	7.7 d *J* = 8.7	131.2	7.8 d *J* = 8.4	131.6	7.6 d *J* = 8.4	114.2	7.4 d *J* = 6.0	116.2	7.9 d *J* = 2.1
3′	115.4	6.7 d *J* = 8.4	115.9	6.8 d *J* = 8.1	116.0	6.8 d *J* = 8.2	147.2	–	145.7	–
4′	161.6	–	161.7	–	161.5	–	151.2	–	149.7	–
5′	116.5	6.7 d *J* = 8.4	116.8	6.8 d *J* = 8.1	116.8	6.8 d *J* = 8.2	120.32	6.9 d *J* = 8.1	123.1	6.9 d *J* = 8.7
6′	131.6	7.7 d *J* = 8.7	131.9	7.8 d *J* = 8.4	131.6	7.6 d *J* = 8.4	117.2	7.4 d *J* = 6.0	117.7	7.63 dd *J* = 2.1, 8.7
Rha										
1	–	–	101.6	5.7 d *J* = 1.8	101.4	5.3 d *J* = 1.5	–	–	104.7	4.5 d *J* = 1.5
2	–	–	69.8	3.3-3.6 m	70.1	3.2-3.7 m	–	–	72.0	3.3-3.7 m
3	–	–	71.9	3.3-3.6 m	71.4	3.2-3.7 m	–	–	72.2	3.3-3.7 m
4	–	–	72.8	3.3-3.6 m	72.7	3.2-3.7 m	–	–	73.9	3.3-3.7 m
5	–	–	68.5	3.3-3.6 m	68.9	3.2-3.7 m	–	–	69.6	3.3-3.7 m
6	–	–	17.5	0.9 d *J* = 6	17.4	0.81 d *J* = 6.4	–	–	17.8	1.1 d *J* = 6.2
Glc or Rha										
1	–	–	–	–	102.1	5.2 d *J* = 1.4	–	–	102.3	5.1 d *J* = 7.2
2	–	–	–	–	70.3	3.2-3.7 m	–	–	75.6	3.3-3.7 m
3	–	–	–	–	71.8	3.2-3.7 m	–	–	78.1	3.3-3.7 m
4	–	–	–	–	72.6	3.2-3.7 m	–	–	71.3	3.3-3.7 m
5	–	–	–	–	69.1	3.2-3.7 m	–	–	77.1	3.3-3.7 m
6	–	–	–	–	17.7	0.87 d *J* = 6.1	–	–	68.6	3.48, 3.83 d

Abbreviations: Glc, glucose; Rha, Rhamnose.

species (ROS) that are produced during normal oxygen metabolism or are induced by exogenous damage (*21*). Flavonoids cannot be produced by the human body and have thus to be taken in mainly through one's daily diet. They have been found in dietary components, included fruits, vegetables, olive oil, tea, and red wine (*22*). Several beneficial properties have been attributed to these dietary compounds, including anticarcinogenic, anti-inflammatory, and antiviral action (*18*). In addition, they inhibit lipid peroxidation, platelet aggregation, and the activity of enzyme systems like the lipoxygenase enzyme system. The flavonoids display these types of action as antioxidants, chelators of divalent cations, and free radical scavengers and thus may be involved in preventing free radical mediated cytotoxicity and lipid peroxidation, both of which are associated with cell aging and chronic diseases such as atherosclerosis (*23,24*). Their remarkable antioxidant properties are due to three aspects. First is the hydrogen donating-substituents (hydroxyl groups) attached to the aromatic ring structures of flavonoids, allowing flavonoids to undergo a redox reaction that helps them to scavenge free radicals more easily. Second is a stable delocalization system consisting of aromatic and hetero cyclic rings as well as multiple unsaturated bonds that helps to delocalize the resulting free radicals. Third is the presence of certain structural groups that are capable of forming transition metal-chelating complexes that can regulate the production of ROS such as $OH^{\bullet}$ and O_2^{-2} (*25*). Because flavonoids are widely distributed in edible plants and beverages and have previously been used in traditional medicine, they are likely to have minimal toxicity (*26*). Moreover, the leaves of *Laurus nobilis* have long been used to flavor food (*5*) while the leaves of *Emex spinosus* are used in traditional medicine to relieve stomach disorders and to stimulate appetite (*6*).

4. Conclusion

In this study, it was found that *Laurus nobilis* and *Emex spinosus* leaves were potential sources of antioxidant components that would help to increase the overall antioxidant capacity of an organism and protect it against lipid peroxidation induced by oxidative stress or used as food additive to delay the oxidative deterioration of foods.

The aqueous ethanolic of *Emex spinosus* showed higher activity than *Laurus nobilis* leaves and its strong antioxidant activity could be related to the activity of falvonoids compounds found in this extract. Further studies are needed to investigate the *in vivo* pharmacological and toxicological properties of *Emex spinosus* extract, since the high activity could be considered as a new antioxidant ingredient for the nutraceutical or functional food market.

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