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Secondary Metabolites and Antioxidant Activity of *Limonium duriusculum* (de Girard) Kuntze Extracts

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No reports are available in literature on phytochemical investigation of chloroform, ethyl acetate and *n*-butanol soluble parts of the aqueous-MeOH extract of aerial parts of *Limonium duriusculum*. This report identify from the extracts two new flavanones: 3β,5,6,7,8,3',4'-heptahydroxyflavanone named duriusculin A (**8**) slightly contamined by its epimer, 3α,5,6,7,8,3',4'-heptahydroxyflavanone (less than 10 %), named duriusculin B (**9**), together with, β-sitosterol (**1**); apigenin (**2**); methyl gallate 4-methyl ether (**3**); 4-*O*-methylgallic acid (**4**); methyl gallate (**5**); vanillic acid (**6**); pinoresinol (**7**) and apigenin 7-*O*-β-*D*-(6''-methylglucuronide) (**10**). The antioxidant properties of the *n*-butanol extract (BEL) were investigated using three assay systems: (2,2-diphenyl-1-picrylhydrazyl radical scavenging (DPPH), lipid peroxidation (LPO) inhibition, and ferrous ion chelating (FIC) methods. This extract showed significant antioxidant activity in the lipid peroxidation inhibition and the ferrous ion chelating methods compared with standards. Compounds **2** and **10** are strongly accumulated by this plant, more than 1.6 g and 1.1 g per kg of air-dried plant material, respectively.

Keywords: Duriusculin A and B, Flavonoids, Phenolic compounds, *Limonium duriusculum*, Plumbaginaceae, Antioxidant activity.

INTRODUCTION

Currently, the phytochemical investigations of plants represent a potential for the discovery of new bioactive chemical substances. As a part of our on-going program in this field on Algerian plants [1-3], we investigated *Limonium duriusculum* (de Girard) Kuntze. The genus *Limonium,* formerly known as *Statice*, (Plumbaginaceae) involves 150 wild species distributed in coastal regions and plains throughout the world in both tropical and temperate zones [4,5]. This genus is represented by about 23 species in Algeria [6]. Many *Limonium* species are used in folk and modern medicine and are rich sources of bioactive compounds [7-10]. Some species are reported as crop wild relatives [11]. This genus is reported to possess anti-inflammatory, anticancer and antioxidant activities [12,13]. *Limonium duriusculum* (de Girard) Kuntze was collected from the area of Mila in the North-East Algeria, where the mixture "powdered flowers-pure honey" is consumed by local populations to treat allergies. This species grows in the western area of the Mediterranean. A literature survey indicated no previously reported chemical nor biological studies on it. Thus, the purpose of this work is the research and the structural elucidation of new compound with potential biological activity from the chloroform, ethyl acetate and *n*-butanol soluble parts of the MeOH-H_2O extract of the aerial parts of this plant, as well as the evaluation of the antioxidant properties of its *n*-butanol extract (BEL), using different assay systems.

EXPERIMENTAL

General procedure: Ultraviolet spectra were recorded using a Shimadzu model UV-1700 spectrophotometer. NMR spectra were obtained by Bruker model Avance 400 and AMX-500 spectrometers with standard pulse sequences, operating at 400 and 500 MHz for ^{1}H and 100 and 125 MHz for ^{13}C, respectively. MeOH-d_4, DMSO-d_6, acetone-d_6, pyridine-d_5 or $CDCl_3$ were used as solvents with TMS as internal standard. EIMS and HR-EIMS spectra were taken on a Micromass model Autospec (70 eV) spectrometer. HR-ESIMS spectra were performed with a LCT Premier XE Micromass Waters spectrometer

in positive ionization mode (Waters Corporation). Column chromatography (CC) was carried out with Si gel Fluka (cat. 60737) (40-63 μm), and column fractions were monitored by TLC Si gel 60 F_{254}, 0.2 mm, Macherey Nagel (cat. 818-333) by detection with a spraying reagent ($CH_3CO_2H/H_2O/H_2SO_4$; 80:16:4) followed by heating at 100 °C. Preparative TLC was carried out on Si gel 60 $PF_{254+366}$ (20 × 20 cm, 1 mm thickness, Analtech cat. 02014).

Aerial parts of *Limonium duriusculum (*de Girard) Kuntze were collected in the flowering stage on May 2010 from the area of Mila in the North-East Algeria and authenticated by Professor Hocine Laouer (Ferhat Abbas University, Setif, Algeria) according to Quezel and Santa [6]. A voucher specimen (LDP0510-MIL-ALG-66) has been deposited in the Herbarium of the VARENBIOMOL research unit, Frères Mentouri University, Constantine 1.

Extraction and isolation: Air-dried aerial parts (leaves and flowers, 5 kg) were macerated at room temperature with MeOH–H_2O (70:30, v/v) for 24 h, three times. The filtrates were combined, concentrated under reduced pressure (up to 35 °C) and dissolved in H_2O (2 L) under magnetic stirring and maintained at 4 °C for one night to precipitate a maximum amount of chlorophylls. After filtration, the resulting solution was successively extracted with $CHCl_3$, EtOAc and *n*-BuOH. The organic phases were dried with Na_2SO_4, filtered using common filter paper and concentrated in vacuum at room temperature to obtain the following extracts: $CHCl_3$ (5.47 g), EtOAc (50.06 g) and *n*-BuOH (63.64 g). The chloroform extract was fractioned by column chromatography (silica gel; petroleum ether/diethyl ether) step gradients to yield 5 fractions (F_1-F_5) obtained by combining the eluates on the basis of TLC analysis. Fraction F_4 (80:20) in which a white precipitate was formed, gave after concentration and washing with methanol, β-sitosterol (**1**, 80 mg) [14]. Fraction F_6 (75:25, 70:30, 65:35 and 60:40) gave after concentration a yellowish powder which was washed with acetone to obtain apigenin (**2**, 108 mg) [15,16]. A portion of the EtOAc extract (22 g) was fractionated by column chromatography (silica gel; *n*-hexane/EtOAc) step gradients, to yield 28 fractions (F_1-F_{28}) obtained by combining the eluates on the basis of TLC analysis. Fraction F_3 (346 mg) (*n*-hexane/EtOAc, 95:05), was rechromatographed on a silica gel column using an isocratic system of hexane/EtOAC (60:40) as eluent to obtain 4 subfractions. Subfraction 1 (65 mg) was submitted to preparative plates of silica gel (hexane/EtOAc, 60:40) to give methyl gallate 4-methyl ether (**3**, 27 mg) as a white powder [17]. Fractions F_4 (95:5; 274.6 mg) and F_5 (90:10; 87 mg) which had a similar composition after concentration, were combined and rechromatographed on a silica gel column eluted with the isocratic system CH_2Cl_2/MeOH (90:10) to obtain 8 subfractions. Subfraction 1 (45 mg) was submitted to TLC on preparative plates of silica gel (CH_2Cl_2/MeOH; 90:10) to give 4-*O*-methylgallic acid **4** (38 mg) as white powder [18]. Fraction F_7 (90:10; 720 mg) was rechromatographed on a silica gel column eluted with the isocratic system hexane/EtOAc (40:60) to obtain 7 subfractions. Subfraction 1 (295 mg) and subfraction 7 (210 mg) were submitted to TLC on preparative plates of silica gel (hexane/EtOAc; 40:60) to give methyl gallate (**5**, 225 mg) [19] and vanillic acid (**6**, 108 mg) [20] as white powders respectively. Fractions 10 to 24 (85:15 to 60:40) in which a yellowish precipitate was formed, were combined and filtered. The obtained precipitate was washed with MeOH to give apigenin (2.900 g) **2**. A part of the *n*-BuOH extract (22 g) was fractionated by column chromatography (silica gel; EtOAc/AcOH/H_2O; 90:10:10) to yield 25 fractions (F_1-F_{25}) according to their TLC behaviour. Fractions F_2 to F_5 in which a yellowish precipitate was formed, were combined and evaporated. The obtained solid was washed with MeOH to give apigenin **2** (178 mg). Fractions F_6 (1100 mg), F_7 (1270 mg), F_8 (1280 mg), F_9 (800 mg), F_{10} (1232 mg) and F_{11} (1300 mg) which had similar composition after concentration were combined and rechromatographed on a silica gel column using the system CH_2Cl_2/MeOH, with increasing polarity, resulting in the obtaining of 7 subfractions, according to TLC monitoring. Subfraction 2 (98:2 to 80:20) gave after evaporation and washing with MeOH, apigenin (**2**, 243.2 mg). Subfraction 4 (70:30) gave after purification on preparative plates of silica gel (CH_2Cl_2: MeOH; 70:30), pinoresinol (**7**, 223.5 mg) [21,22]. Subfraction 6 (40:60), gave after purification on preparative plates of silica gel (CH_2Cl_2:MeOH; 40:60) the new compound, 3β,5,6,7,8,3',4'-heptahydroxyflavanone named duriusculin A (**8**, 98.5 mg) slightly contamined by its epimer, 3α,5,6,7,8,3',4'-heptahydroxyflavanone named duriusculin B **9**. Fractions F_{12} to F_{22} which showed the presence of a major component (TLC analysis) were combined and evaporated to give a solid residue of 8.48 g. This residue gave after dissolving in acetone on warming, followed by cooling, a precipitate which was filtered and washed with cold acetone to obtain apigenin 7-*O*-β-(6''-methylglucuronide) **10** (1.900 g) [23-25] (Fig. 1).

Determination of antioxidant activity

Determination of DPPH radical scavenging activity: The ability to scavenge the stable free radical 1,1-diphenyl-2-picrylhydrazyl ($DPPH^{\bullet}$) was determined on the basis of the method of Magalhaes *et al.* [26] with minor modifications. A solution of 0.2 mM DPPH in MeOH was prepared and 1 mL of this solution was mixed with 1 mL of extract in MeOH. After 0.5 h, the absorbance of the mixture was measured spectrophotometrically at 517 nm. Trolox and quercetin were used as references. Results were expressed as percentage of inhibition of the DPPH radical according to the following equation:

$$\text{Inhibition of DPPH}^{\bullet}\ (\%) = 1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Ferrous-chelating ability: The chelating of ferrous ions by BEL was estimated by the method of Dinis *et al*. [27]. Briefly, 50 μL of 2 mM $FeCl_2$ was added to the extract (1 mL). The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance was measured spectrophotometrically at 562 nm. EDTA was used as reference.

Inhibition of lipid peroxide (LPO) formation induced by Fe^{2+}/ascorbic acid system: The reaction mixture containing rat liver homogenate (0.1 mL, 25 % w/v) in Tris-HCl (30 mM), ferrous ammonium sulfate (0.16 mM), ascorbic acid (0.06 mM) and different concentrations of the extract in a final volume of

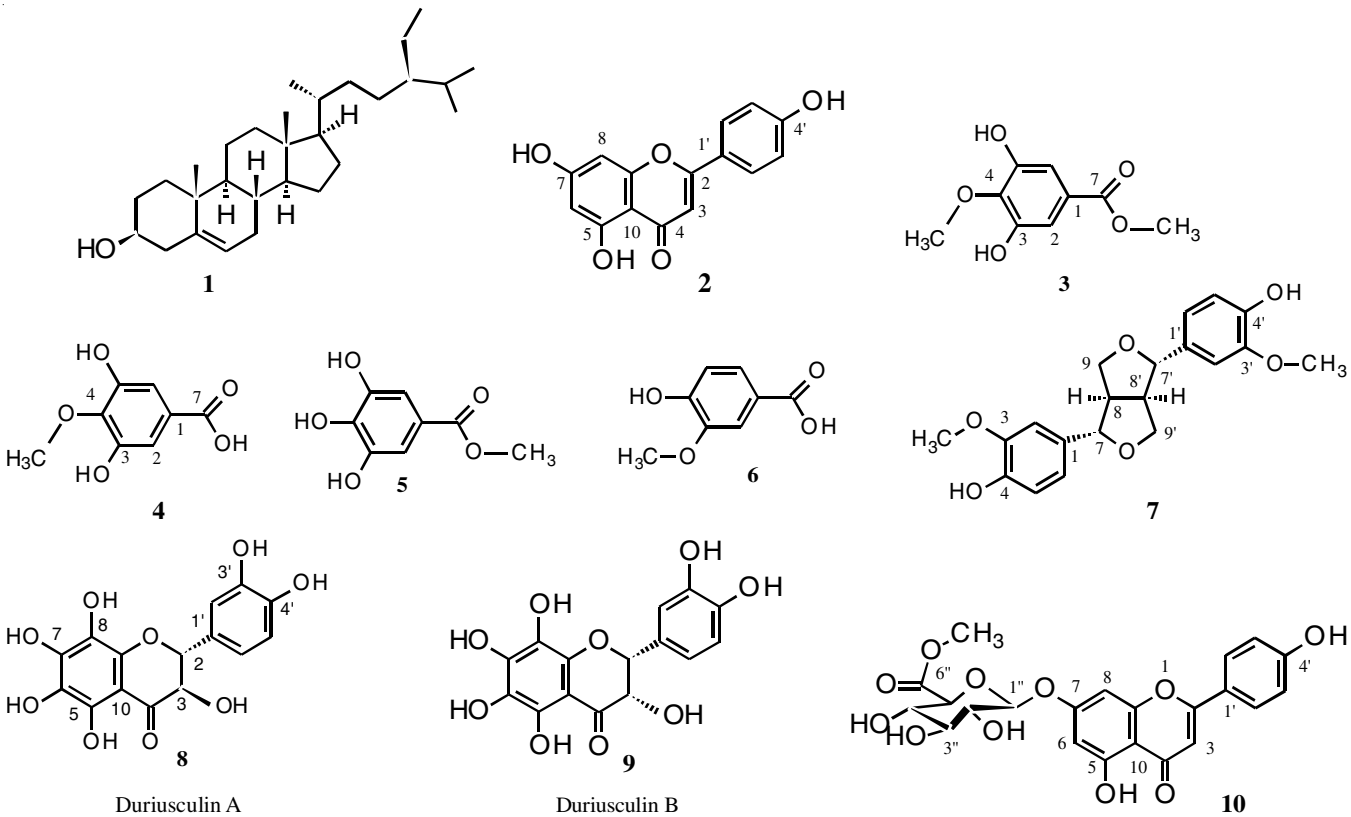

Fig. 1. Structures of the identified compounds (**1**-**10**) from *L. duriusculum*

0.5 mL was incubated for 1 h at 37 °C and the resulting thiobarbituric reacting substance (TBARS) was measured at 532 nm) [28].

All the assays were carried out in triplicates and the results expressed as means ± standard deviation. IC_{50} value (µg extract/mL) is the effective concentration, which proves 50 % of activity, was calculated for each assay. Statistical comparisons were done with Student's test. Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Structure elucidation: The identified compounds are reported in Fig. 1. Compound **8** was isolated as yellow amorphous powder. The HR-EIMS spectrum presented accurate mass of $[M]^{+\cdot}$ ion at *m/z* 336.048135 according to the molecular formula $C_{15}H_{12}O_9$. The ^{1}H MNR spectrum recorded in MeOH-d_4 (Table-1), showed an ABM system (δ_H 6.96, d, J = 1.9 Hz; 6.84, dd, J = 8.1, 1.9 Hz; 6.81, d, J = 8.1 Hz) typical of the three coupled protons H-2', H-6' and H-5' of ring B of a flavonoid skeleton. The same spectrum showed also a one-proton doublet (δ_H 4.49, d, J = 11.1 Hz) assigned to H-3 (δ_C 72.28 ppm, HSQC experiment spectrum) of a 3β-hydroxylated flavanone on the basis of its correlation in the COSY spectrum, with another proton (H-2) of which the signal at δ 4.89 (δ_C 83.71 ppm, HSQC experiment spectrum) was partially overlapped by that of the water of the used solvent (MeOH-d_4). These assignments were confirmed by the ^{1}H NMR spectrum recorded in the same solvent at 65 °C, in which an AM system is clearly observed at δ 4.82 and 4.37 ppm, J = 11.1 Hz and by the ROESY interactions between these two protons and H-2' and H-6'. The β-orientation of the hydroxyl group was deduced from the value of the coupling constant (11.1 Hz). Analysis of the ^{13}C NMR, HSQC and HMBC spectra confirmed these results and led to the assignment of C-4 (δ 196.92), C-7 (δ 167.62), C-4' (δ 147.70), C-3' (δ 144.92), C-1' (δ 128.52), C-6' (δ 119.52), C-5' (δ 114.79), C-2' (δ 114.57), C-10 (δ 100.39). All these data led to 3β,5,6,7,8,3',4'-heptahydroxyflavanone. This compound was new, we named it duriuculin A. The ^{1}H NMR spectrum of this compound also showed small signals of an AX system at δ_H 5.27 and 4.19 ppm (J = 2.8 Hz) and an ABM system at δ_H 6.99 (d, J = 1.9 Hz), 6.84 (dd, J = 8.1; 1.9 Hz) and 6.75 (d, J = 8.1 Hz) which were assigned to H-2, H-3, H-2', H-6' and H-5' respectivement, of the epimer 3α,5,6,7,8,3',4'-heptahydroxyflavanone that we named duriusculin B. This observation is supported by the value of the coupling constant raised in the signals of H-2 and H-3. The intensities of these signals were very low compared to those of duriusculin A (less than 10 %).

β-Sitosterol (1): White crystals, m.p. 135-137 °C; ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm) = 5.36 (br d, 1H, J = 6.4 Hz, H-6), 3.53 (m, 1H, H-3), 1.01 (s, 3H, H_3-29), 0.93 (d, 3H, J = 6.5 Hz, H_3-19), 0.84 (t, 3H, J = 7.2 Hz, H_3-24), 0.83 (d, 3H, J = 6.4 Hz, H_3-26), 0.81 (d, 3H, J = 6.4 Hz, H_3-27) 0.68 (s, 3H, H_3-28); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) = 140.77 (C, C-5), 121.66 (CH, C-6), 71.79 (CH, C-3), 56.79 (CH, C-14), 56.12 (CH, C-17), 50.19 (CH, C-9), 45.89 (CH, C-22), 42.34 (C, C-13), 42.32 (CH_2, C-4), 39.81 (CH_2, C-12), 37.28 (CH_2, C-1), 36.52 (C, C-10), 36.14 (CH, C-18), 33.99 (CH_2, C-20), 31.92 (CH_2 and CH , C-7 and C-8), 31.67 (CH_2, C-2), 29.24 (CH, C-25), 28.23 (CH_2, C-16), 26.20 (CH_2, C-21), 24.30 (CH_2, C-15), 23.11 (CH_2, C-23), 21.10 (CH_2, C-11), 19.79

TABLE-1
^{13}C NMR AND ^{1}H NMR DATA AT 125/500 MHz RESP. OF DURIUSCULIN A (**8**) AND B (**9**) IN MeOH-d_4, δ IN ppm, *J* (Hz), IN PARENTHESES

C	Compound **8**			Compound **9**	
	δ_C	δ_H	δ_H*	δ_H	δ_H*
2	83.71	4.89[a]	4.82 d (11.1)	5.27 d (2.8)	5.19 d (2.8)
3	72.28	4.49 d (11.1)	4.37 d (11.1)	4.19 d (2.8)	4.16 d (2.8)
4	196.92	–	–	–	–
5	nd	–	–	–	–
6	nd	–	–	–	–
7	167.62	–	–	–	–
8	nd	–	–	–	–
9	nd	–	–	–	–
10	100.39	–	–	–	–
1'	128.52	–	–	–	–
2'	114.57	6.96 d (1.9)	6.88 d (1.9)	6.99 d (1.9)	6.91 d (1.9)
3'	144.92	–	–	–	–
4'	147.70	–	–	–	–
5'	114.79	6.81 d (8.1)	6.71 d (8.1)	6.75 d (8.1)	6.67 d (8.1)
6'	119.52	6.84 dd (8.1, 1.9)	6.76 dd (8.1; 1.9)	6.84 dd (8.1; 1.9)	6.76 dd (8.1; 1.9)

*Recorded at 65 °C. nd: not detected; [a]Partially overlapped by the water signal of MeOH-d_4 assigned from ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC

(CH_3, C-28), 19.37 (CH_3, C-27), 19.06 (CH_3, C=26), 18.78 (CH_3, C-19), 11.98 (CH_3, C-24), 11.86 (CH_3, C-29).

Apigenin (2): Yellowish powder, HR-EIMS (+): *m/z* 270.0526 $[M]^{+\cdot}$ (calculated for $C_{15}H_{10}O_5$: 270.0528), formula $C_{15}H_{10}O_5$; ^{1}H NMR (400 MHz, pyridine-d_5): δ (ppm) = 13.63 (1H, br s, 5-OH), 7.99 (2H, d, *J* = 8.7 Hz, H-2', H-6'), 7.08 (2H, d, *J* = 8.7 Hz, H-3', H-5'), 6.78 (1H, s, H-3), 6.69 (1H, d, *J* = 2.0 Hz, H-8), 6.61 (1H, d, *J* = 2.0 Hz, H-6), ^{13}C NMR (100 MHz, pyridine-d_5): δ (ppm) = 183.74 (C, C-4), 166.75 (C, C-7), 165.50 (C, C-2), 163.88 (C, C-5), 163.59 (C, C-4'), 159.52 (C, C-9), 129.94 (CH, C-2', C-6'), 123.35 (C, C-1'), 117.81 (CH, C-3', C-5'), 106.01 (C, C-10), 104.99 (CH, C-3), 100.94 (CH, C-6), 95.86 (CH, C-8).

Methyl gallate 4-methyl ether (3): White powder, HR-EIMS (+): *m/z* 198.0520 $[M]^{+\cdot}$ (calculated for $C_9H_{10}O_5$: 198.0528.0372), formula $C_9H_{10}O_5$; ^{1}H NMR (400 MHz, acetone-d_6): δ (ppm)= 7.08 (2H, s, H-2, H-6), 3.85 (3H, s, 4-OCH_3), 3.80 (3H, s, 7-OCH_3); ^{13}C NMR (100 MHz, acetone-d_6): δ (ppm) = 165.78 (C, C-7), 150.11 (C, C-3, C-5), 139,26 (C, C-4), 125.29 (C, C-1), 108.65 (CH, C-2, C-6), 59.49 (CH_3, 4-OCH_3), 51.01 (CH_3, 7-OCH_3.

4-*O*-Methylgallic acid (4): White powder, ^{1}H NMR (400 MHz, acetone-d_6): δ (ppm) = 7.14 (2H, s, H-2, H-6), 3.89 (3H, s, 4-OCH_3); ^{13}C NMR (100 MHz, acetone-d_6): δ (ppm) = 166.52 (C, C-7), 150.23 (C, C-3, C-5), 139,39 (C, C-4), 125.90 (C, C-1), 109.14 (CH, C-2, C-6), 59.69 (CH_3, 4-OCH_3).

Methyl gallate (5): White powder, HR-EIMS (+): *m/z* 184.0376 $[M]^{+\cdot}$ (calculated for $C_8H_8O_5$:184.0372), formula $C_8H_8O_5$; ^{1}H NMR (400 MHz, acetone-d_6): δ (ppm) = 8.23 (3H, br s, 3-OH, 4-OH, 5-OH), 7.15 (2H, s, H-2, H-6), 3.81 (3H, s, 7-OCH_3); ^{13}C NMR (100 MHz, acetone-d_6): δ (ppm) = 166.21 (C, C-7), 144.89 (C, C-3, C-5), 137,62 (C, C-4), 120.65 (C, C-1), 108.76 (CH, C-2, C-6), 50.90 (CH_3, 7-OCH_3).

Vanillic acid (6): White powder, HR-EIMS (+): *m/z* 168.0428 $[M]^{+\cdot}$ (calculated for $C_8H_8O_4$: 168.0423), formula $C_8H_8O_4$; ^{1}H NMR (400 MHz, acetone-d_6): δ (ppm) = 8.40 (1H, br s, 4-OH), 7.62 (1H, dd, *J* = 8.2; 1.8 Hz, H-6), 7.59 (1H, d, *J* = 1.8 Hz, H-2), 6.93 (1H, d, *J* = 8.2 Hz, H-5), 3.94 (3H, s, 3-OCH_3); ^{13}C NMR (100 MHz, acetone-d_6): δ (ppm) =168.89 (C, C-7), 151.19 (C, C-4), 147,15 (C, C-3), 123.00 (CH, C-6), 121.89 (C, C-1), 114.64 (CH, C-5), 112.55 (CH, C-2), 55.39 (CH_3, 3-OCH_3).

Pinoresinol (7): White powder, HR-EIMS (+): *m/z* 358.1425 $[M]^{+\cdot}$ (calculated for $C_{20}H_{22}O_6$: 358.1416), formula $C_{20}H_{22}O_6$; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 6.82 (2H, d, *J* = 2.0 Hz, H-2, H-2'), 6.80 (2H, d, *J* = 8.2 Hz, H-5, H-5'), 6.74 (2H, dd, *J* = 8.2; 2.0 Hz, H-6, H-6'), 4.66 (2H, d, *J* = 4.4 Hz, H-7, H-7'), 4.17 (2H, dd, *J* = 9.1; 6.9 Hz, H-9a, H-9'a), 3.82 (6H, s, 3-OCH_3, 3'-OCH_3), 3.80 (2H, dd, *J* = 9.1; 3.8 Hz, H-9b, H-9'b), 3.03 (2H, m, H-8, H-8'); ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) = 146.79 (C, C-3, C-3'), 145.30 (C, C-4, C-4'), 132.87(C, C-1, C-1'), 118.97 (CH, C-6, C-6'), 114.36 (CH, C-5, C-5'), 108.73 (CH, C-2, C-2'), 85.90 (CH, C-7, C-7'), 71.66 (CH_2, C-9, C-9'), 55.97 (CH_3, s, 3-OCH_3, 3'-OCH_3), 54.14 (CH, C-8, C-8').

Apigenin 7-*O*-β-(6''-methylglucuronide) (10): White powder, HR-ESIMS (+): *m/z* 483.0896 (100 %) $[M+Na]^+$ (calculated for $C_{22}H_{20}O_{11}Na$: 483.0903), formula $C_{22}H_{20}O_{11}$; ^{1}H NMR (500 MHz, DMSO-d_6), δ (ppm)= 12.98 (1H, br s, 5-OH), 7.96 (2H, d, *J* = 8.9 Hz, H-2', H-6'), 6.95 (2H, d, *J* = 8.9 Hz, H-3', H-5'), 6.86 (1H, s, H-3), 6.85 (1H, d, *J* = 2.2 Hz, H-8), 6.48 (1H, d, *J* = 2.2 Hz, H-6), 5.56 (1H, br s, 2''-OH), 5.47(1H, br s, 4''-OH), 5.32 (1H, d, *J* = 7.4 Hz, H-1''), 5.30 (1H, br s, 3''-OH), 4.21 (1H, d, *J* = 9.4 Hz, H-5''), 3.68 (3H, s, 6''-OCH_3), 3.43 (1H, br t, *J* = 9.4 Hz, H-4''), 3.34 (2H, m*, H-2'', H-3''). *: Partially overlapped by the signal of water solvent; ^{13}C NMR (125 MHz, DMSO-d_6): δ (ppm) =182.47 (C, C-4), 169.67 (C, C-6''), 164.82 (C, C-2), 162.92 (C, C-7), 161.89 (C, C-5), 161.70 (C, C-4'), 157.45 (C, C-9), 129.10 (CH, C-2', C-6'), 121,51 (C, C-1'), 116.51 (CH, C-3', C-5'), 105.99 (C, C-10), 103.65 (CH, C-3), 99.82 (CH, C-6), 99.61 (CH, C-1''), 95.14 (CH, C-8), 75.93 (CH, C-3''), 75.67 (CH, C-5''), 73.23 (CH,C-2''), 71.81 (CH, C-4''), 52.46 (CH_3, 6''-OCH_3).

Antioxidant activity: The beneficial effect of flavonoids is mainly associated with different antioxidative mechanisms

which act as enzymatic inhibitory, reducing agents, by donating hydrogen, by quenching singlet oxygen, by acting as chelator and by trapping free radical [29].

Scavenging effect on DPPH radical: DPPH• scavenging assay has been widely used to provide basic information on the antioxidant ability. The DPPH• scavenging activity is measured as the reactive decrease in absorbance of DPPH as it reacts with antioxidant compounds [26]. The results (Fig. 2) showed that the BEL exhibited a moderate free radical scavenging activity, and dose dependently scavenged DPPH radical. BEL required 150-200 µg/mL to reach 71 % DPPH• scavenging effect whereas the reference substance (quercetin) to achieve 77 % of response only needed 10 µg/mL. On the basis of IC_{50} values, quercetin (2.17 ± 0.14 µg/mL) was found to be 22 times of that of BEL (48.83 µg/mL) (Table-2). The great activity of gap between the BEL and quercetin may be due to the position and the less hydroxylation degree of the flavonoid skeleton of apigenin and apigenin 7-*O*-β-(6''-methylglucuronide) which were the major products contained in BEL.

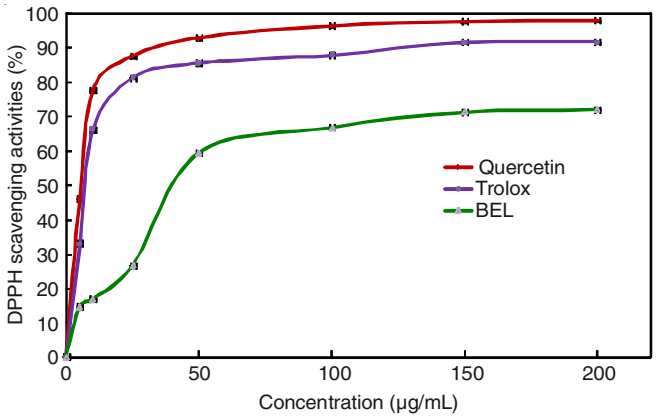

Fig. 2. DPPH radical-scavenging activities of BEL and standards. Each value represents a mean ± SD (n = 3), P < 0.05

TABLE-2
IC_{50} VALUES OF ANTIOXIDANT ACTIVITIES (µg/mL)

Extract and standards	DPPH•	Lipid peroxidation	Ferrous-chelating
BEL	48.83 ± 0.16	85.30 ± 3.79	62.95 ± 8.07
Quercetin	2.17 ± 0.14	28.85 ± 2.63	–
Trolox	5.73 ± 0.26	69.54 ± 0.55	–
EDTA	–	–	33.31 ± 6.89

Ferrous-chelating ability: Among transition metals, iron is known as the most important lipid oxidation, pro-oxidant due to its high reactivity [30]. The iron accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and aloxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation [31]. Chelating agents may inactivate metal ions and potentially inhibit the metal dependant process [32]. The iron-chelating capacity assay measures the ability of antioxidant to compete with ferrozine in chelating ferrous ions [27]. As depicted in Fig. 3, the BEL (71-75 %) exhibited a high ferrous chelating activity at 200-400 µg/mL, which was dose response curve. IC_{50} value of BEL (62.95 ± 8.07 µg/mL) was 2-fold less than that of EDTA (33.31 µg/mL) (Table-2). The good ferrous chelating activity could result from the interaction of the biomolecules present in BEL.

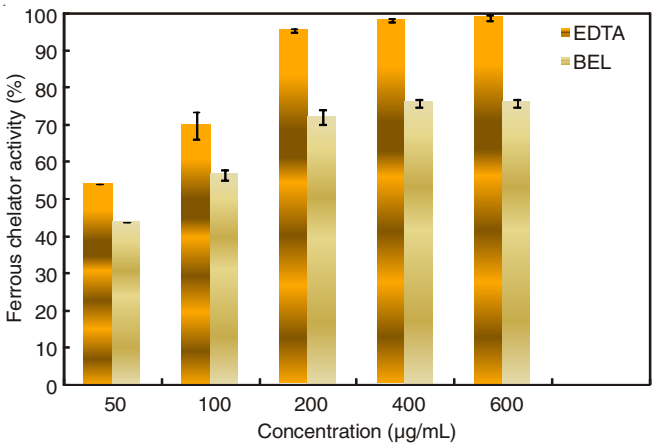

Fig. 3. Ferrous ion chelating activity of BEL and standards. Each value represents a mean ± SD (n = 3), P < 0.05

Inhibition of lipid peroxide (LPO) formation: Lipid peroxidation is regarded as one of the based mechanisms of tissue damage caused by free radicals. Increasing lipid peroxide is generally believed to be an important underling cause of the initiation of oxidative stress related various tissue injuries, cell death, and the progression of many acute and chronic diseases [33]. In foods, lipid oxidation occurs when oxygen reacts with lipid in a series of free radical chain reactions that lead to complex changes which causes quality loses [34]. Ferrous ion stimulates lipid peroxide through various mechanisms such as decomposition of lipid peroxide, the generation of OH•, or by forming perferryl or ferryl species [35]. Fig. 4 showed that BEL dose dependently inhibited the amount of MDA generated by Fe^{2+}-ascorbate system in liver homogenates. At 200 µg/mL, BEL and Trolox gave the same response (70 %). The IC_{50} values followed the order of: quercetin (28.85 ± 2.63 µg/mL) > Trolox (69.54 ± 0.55 µg/mL) > BEL (85.30 ± 3.79 µg/mL) (Table-2). The substances termed antioxidant can influence the peroxidation process through either a simple or complex mechanisms. According to the phytochemical investigation, the BEL was found to contain phenolics such as gallic acid derivatives and pinoresinol, and afforded different flavonoid profiles such as apigenin and apigenin 7-*O*-β-*D*-(6''-methyl-glucuronide) which were the predominant components. Some studies reported that apigenin have been proved to be effective inhibitor of lipid peroxidation [36,37].

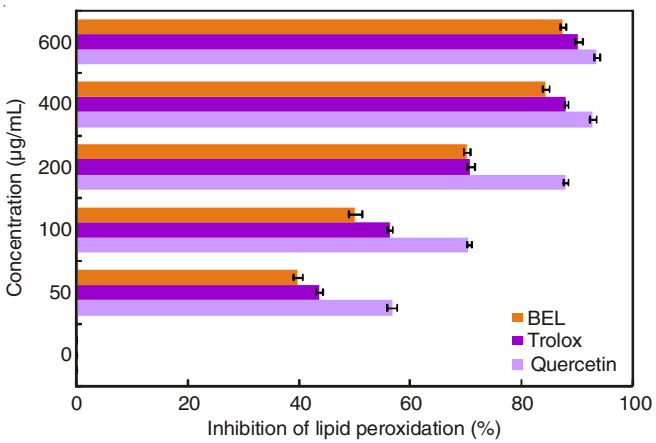

Fig. 4. Inhibition of lipid peroxidation activities of BEL and standards. Each value represents a mean ± SD (n = 3), P < 0.05

Conclusion

The identified compounds (**1-10**) are reported in Fig. 1. It is important to note that to the best of our knowledge, the flavanones **8** and **9** are new, we named them duriusculin A and duriusculin B, respectively. Moreover, this study showed that apigenin **2** and apigenin 7-*O*-β-*D*-(6''-methylglucuronide) **10** are strongly accumulated by this plant, more than 1.6 g and 1.1 g per kg of air-dried aerial parts respectively. These results revealed an important wealth in these flavonoids, evidenced that this species might be developed industrially for its rich content of these bioactive components. For this reason, this plant could be a good candidate for culture as a crop. Regarding the antioxidant activity, the *n*-butanol extract showed significant antioxidant activity in the lipid peroxide inhibition and the FIC methods compared with standards, which could be due to the major compounds apigenin and apigenin 7-*O*-β-*D*- (6''-methylglucuronide) and the possible synergism of these flavonoids with other compounds present in the studied extract [38].

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