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Free radical scavenging action of phenolic compounds from *Limonium Bondueli* (Plumbaginaceae)

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ABSTRACT

Antioxidant activities of ethyl acetate extract from Limonium bonduelli and its constituents : eriodictyol, luteolin, apigenin and 4-hydroxy-3-methoxy benzoic acid , were studied in vitro. The phenolic and flavonoid contents were determined and inhibition of malondialdehyde formation and the scavenging of DPPH were assayed. The extract and pure flavonoids, eriodictyol and luteolin showed a high antioxidant effect, especially scavenging of DPPH anions and inhibition of lipid peroxidation compared to vitamin C. The total content of phenolic compounds and total flavonoids content of ethyl acetate extract of Limonium bonduelli were 353 µg of gallic acid equivalents/mg extract and 170.5 µg of quercetin equivalents/mg extract respectively. eriodictyol, luteolin, apigenin and 4-hydroxy-3-methoxy benzoic acid are described for the first time for Limonium Bonduelli.

Key words: eriodictyol; luteolin; apigenin; *Limonium bonduelli*; Lipid peroxidation; DPPH; Free radical scavenging capacity.

INTRODUCTION

The natural phenolic compounds and flavonoids have received increasing interest in the last years [1]. They may reduce the risk of development of several diseases caused by oxidative stress, including neurodegenerative, cardiovascular diseases, cancer, diabetes and others [2]. Their protective effects stem for the ability to inhibit lipid peroxidation, chelat redox-active metals (by binding iron), and attenuate other processes involving reactive oxygen species (such as through radical scavenging) [3-5]. The excellent antioxidant activity of flavonoids depends on their chemical structure: the presence of hydroxyl groups in positions 3' and 4' of the B ring, double bound between carbon C-2 and C-3 of the ring C, additionally free hydroxyl groups in position 3 of ring C and in position 5 in ring A, together with the carbonyl group in position 4, are also important for the antioxidant activity of these compounds [6].
In continuation of our phytochemical and biological work on Saharian plants [7-23], we report here the antioxydant activity of the flavonoids and phenolic constituents of *Limonium Bonduelli* (Lestib.) Sauv. Et Vindt an endemic Saharian species which was not previously investigated.

MATERIALS AND METHODS

Plant material

The genus *Limonium*, formerly known as Statice, is a member of the Plumbaginaceae family and involves 150 wild species. These species are widely distributed in coastal regions and plains throughout the world in both tropical and

temperate zones such as Europe, Middle East, Latin America, Africa, [24]. This genus is represented by about 23 species in Algeria [25].

Limonium bonduelli is a half-hardy annual plant with winged panicles of bright yellow flowers (Figure 1) which is endemic of the septentrional Sahara. It's considered as a subspecies of the Mediterranean species *Limonium sinuatum* [25]. Aerial parts of this species were collected on April 2011 at Mogheul near Bechar in the South West of Algeria. The voucher specimen was identified by Prof. Mohamed Kaabeche from University of Setif and was deposited at the Research Unity VARENBIOMOL under the reference : LB/236/04-11.

Figure 1: *Limonium Bonduelli.*

Extraction and isolation

The dried aerial parts of *L. Bonduelli* (1500 g) were macerated with EtOH-H_2O (70:30 v/v) for 24 hr three times; the residue was filtered, concentrated then successively extracted with $CHCl_3$, EtOAc and *n*-BuOH. A 13 g portion of the EtOAc extract was chromatographed on silica gel (230-400 mesh) column using $CHCl_3$/CH_3COCH_3 as eluent, to yield 20 fractions from which the four described compounds **1-4** were isolated and purified on silica gel TLC plates eluted with a mixture of $CHCl_3$/ EtOH (8,5 /1,5).

Determination of total phenolic compounds

The content of total phenolic compounds in ethyl acetate extract from *Limonium bonduelli* was determined using Folin–Ciocalteu reagent according to the method of Singleton et al. [26]. 20 µl of ethyl acetate extract from *Limonium bonduelli* (1 mg/ml) was mixed with 100 µl Folin–Ciocalteu reagent and 1580 µl of distilled water, followed by 300 µl 20 % sodium carbonate (Na_2CO_3) three min later. The mixture was shaken for 2 h at room temperature and absorbance was measured at 765 nm. All tests were performed in triplicate. Gallic acid was used as a standard. The standard curve was prepared using 0, 50, 100, 150, 200, 250, 500 mg /L solutions of gallic acid in methanol: water (10:90, v/v). The concentration of total phenolic compounds in ethyl acetate extract from *Limonium bonduelli* was determined as µg of gallic acid equivalents (GAE) per 1mg of extract using the following equation obtained from a standard gallic acid graph ($R2$ = 0.991). Absorbance = 0.01 × Gallic acid (µg).

Determination of total flavonoid content

Total flavonoid content was estimated according to the method described by Wang et al. [27]. Briefly, to 0.5 ml of sample, 0.5 ml of 2% $AlCl_3$ methanol solution was added. After 1 h incubation at room temperature, the absorbance was measured at 420 nm. Total flavonoid content was calculated as µg of quercetin equivalents (QE) per 1 mg of extract using the following equation obtained from a standard quercetin graph ($R2$ = 0.983)**.**

$$\text{Absorbance} = 0.34 \times \text{Quercetin } (\mu g) + 0.015.$$

DPPH radical-scavenging activity assay

The hydrogen donating ability of test samples (ethyl acetate fraction from *Limonium bonduelli* and isolated flavonoids : eriodictyol, luteolin apigenin, was examined in the presence of DPPH (1,1-diphenyl-2-picrylhydrazyl) radical using the method described by Braca et al. [28]. 3 ml of methanol DPPH solution (0.004%) was added to various concentrations (1, 2.5, 5, 10, 15, 20, 30, 50, 75 µg/ml) of the test samples in methanol. After 30 min incubation at room temperature absorbance was measured at 517 nm**.** Inhibition of free radical DPPH in percent (I %) was calculated as follows;

I% = (A control – A sample/A control) ×100;

Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted of inhibition percentage against extract concentration. Tests were carried out in triplicate. Ascorbic acid was used as the control.

Assays of lipid peroxidation using *vitellose*

Lipid peroxidation assay was performed according to modified protocol of Cao and Ikeda [29], to measure the lipid peroxide formed, using egg *vitellose* homogenates as lipid-rich media. Egg homogenate (0.5 ml of 10%, v/v) were incubated with samples or vitamin C (10, 20, 30, 40, 50, 60, 80, 100 µl/ml) in the presence of 50 µl of $FeSO_4$ (0.07 M) at 37°C for 30 min. Then 1 ml trichloroacetic acid (TCA 20%), and 1.5 ml thiobarbituric acid (TBA 1%) was added in succession, and the resulting mixture was vortex and then heated at 95°C for 15 min. After centrifugation at 4000 rpm for 20 min to remove precipitated protein the color of the complex was detected at 532 nm. The lipid peroxidation scavenging activity (%) was calculated by the following equation:

I% = (A control – A sample/A control) ×100.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The statistical significance of differences between results obtained for samples and standard antioxidant was evaluated by student test using SPSS program (version, 12.0).

RESULTS AND DISCUSSION

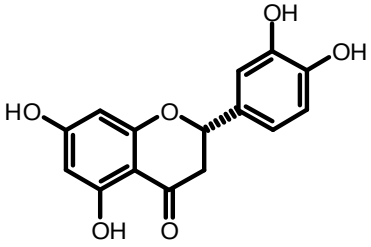
Isolated and identified compounds

Compound 1: $C_{15}H_{12}O_6$. yellow powder, Mass spectrum (EI, 70 eV, *m/z* (*I*rel %): 288[$M^{+\bullet}$] (95), 287[M-H]$^+$(43.7). ^{1}H NMR(500 MHz, CD_3OD, δ, ppm, *J*/Hz): 6.93 (1H, br s, H-2'), 6.82 (2H, m, H-5', H-6'), 5.94 (1H, d, *J*=2.0, H-8), 5.91 (1H, d, *J*=2.0, H-6), 5.29 (1H, dd, *J*=12.7; 3.1, H-2), 3.06 (1H, dd, *J*=17.1; 12.7, H-3$_\alpha$), 2.69 (1H, dd, *J*=17.1; 3.1, H-3$_\beta$), ^{13}C NMR (500 MHz, δ, ppm): 197.8 (C-4, C=O), 168.4 (C-7), 165.4 (C-5), 164.9 (C-9), 146.9 (C-3'), 146.5 (C-5'), 131.8 (C-4'), 119.3 (C-1'), 116.3 (C-2'), 114.8 (C-6'), 103.4 (C-10), 97.1 (C-6), 96.2 (C-8), 80.5 (C-2), 44.1 (C-3). These assignments were completed on the basis of the results of HSQC, HMBC and COSY experiments. This compound was characterized as 5, 7, 3', 4'-tetrahydroxyflavanone (Eriodictyol).

Compound 2: $C_{15}H_{10}O_5$, yellow needles, mp.348-350 °C, UV (λ$_{max}$, MeOH, nm): 269, 335; +NaOH: 274, 324, 392; +$AlCl_3$: 274, 348, 382; $AlCl_3$/HCl: 274, 334,380; +NaOAc: 276, 306, 380. ^{1}H NMR(400 MHz, CD_3OD, δ, ppm, *J*/Hz): 7.77 (2H, d, *J*=8.5; H-2', H6'); 6.84 (2H, d, *J*=8.5; H-3', H5'); 6.49 (1H, s, H-3); 6.36 (1H, d, *J*=2.2; H-8); 6.11 (1H, d, *J*=2.2; H-6). Mass spectrum (EI, 70 eV, *m/z* (*I*rel %): 270[$M^{+\bullet}$] (12.3), 271[M+H]$^+$(47.7). This compound was characterized as 5,7,4'-trihydroxyflavone (Apigenin).

Compound 3: $C_{15}H_{10}O_6$, yellow needles, mp.329-331 °C, UV (λ$_{max}$, MeOH, nm): 264, 350; +NaOH: 267, 329, 402; +$AlCl_3$: 273, 303, 422; $AlCl_3$/HCl: 274, 297,385; NaOAc: 254, 269, 352. ^{1}H NMR (400 MHz, CD_3OD, δ, ppm, *J*/Hz): 7.52 (1H, d, *J*=2.1; H-2'); 7.49 (1H, dd, *J*=8.4; 2.1, H-6'); 7.02 (1H, d, *J*=8.4; H-5'); 6.62 (1H, s, H-3); 6.55 (1H, d, *J*=2.1; H-8); 6.26(1H, d, *J*=2.1; H-6). The spectral data led to the structure of 5,7,3',4'-tetrahydroxy- flavone (Luteolin).

Compound 4: $C_8H_8O_4$, ^{1}H NMR (400 MHz, CD_3OD, δ, ppm, *J*/Hz): 7.60 (1H, d, *J*=1.6; H-2); 7.51 (1H, dd, *J*=8.2; 1.6, H-6); 6.77 (1H, d, *J*=8.2; H-5); 3.95 (3H, s, OCH-3); Mass spectrum (EI, 70 eV, *m/z* (*I*rel %): 168[$M^{+\bullet}$] (60.3), 153[M-15]$^+$(33.7), 149[M+H-18]$^+$(100). These spectral data led to the structure of 4-hydroxy-3-methoxy- benzoic acid . The structure of these compounds were elucidated by extensive UV, MS and NMR spectroscopic analysis as well as by comparing their spectroscopic data with those reported in the literature [30-35].



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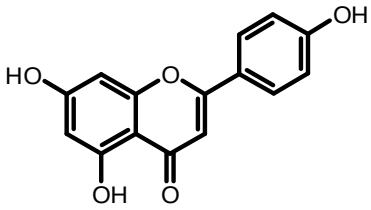

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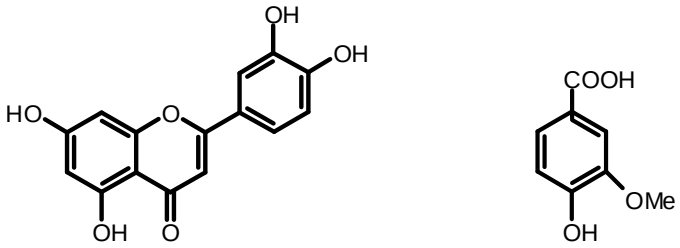

Figure 2: structures of the compounds 1-4

Total phenolic compounds

Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups [36]. The total amount of phenolic compounds in the plant extract was determined as microgram of gallic acid equivalent by using an equation that was obtained from standard gallic acid graph. Ethyl acetate extract of *Limonium bonduelli* exhibited higher phenolic content (353 µg of gallic acid equivalents/mg extract) (Table 1).

Table 1. The phenolic and flavonoid contents of ethyl acetate extract of *Limonium bonduelli*

Species	Extract/Fraction	µg GAE/mg extract	µg QE /mg extract
Limonium bonduelli	Ethyl acetate	353±1.28	170.5±0.41

Total flavonoid content

Flavonoids as one of the most diverse and widespread group of natural compounds are probably the most important natural phenols. These compounds possess a board spectrum of chemical and biological activities including radical scavenging properties [37]. Ethyl acetate extract of *Limonium bonduelli* contained higher flavonoid content (170.5µg quercetin equivalent/mg extract) (Table 1).

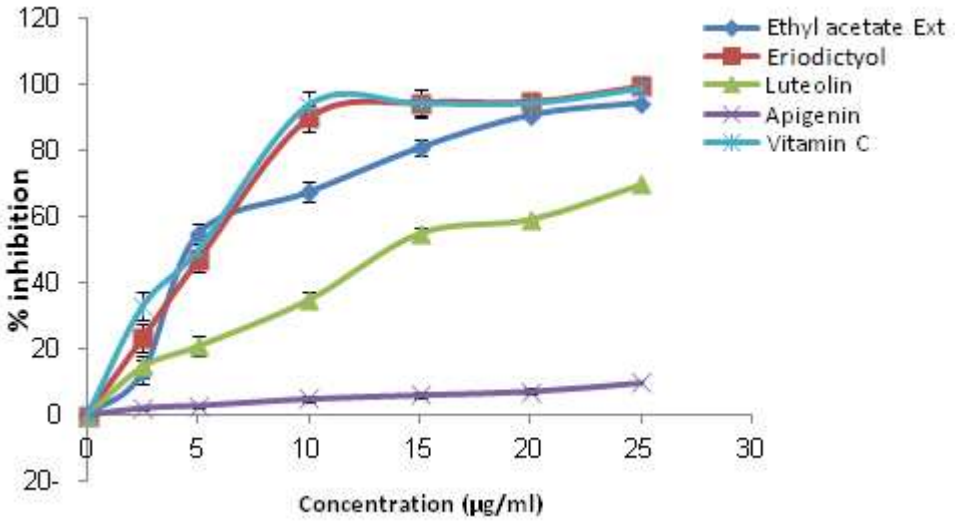

Figure 3: DPPH Scavenging activities of ethyl acetate extract of *Limonium bonduelli*, isolated compounds and Vitamin C. Values are mean ± SD of three samples analyzed individually in triplicate.

DPPH radical scavenging activity

In the present study, the free radical scavenging activity of ethyl acetate extract of *Limonium bonduelli*, three pure flavonoids and Vitamin C were evaluated through their ability to quench 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. Ethyl acetate extract of *Limonium bonduelli* and isolated flavonoids have DPPH radical scavenging activities at various degrees. Figure 3 and table 2 illustrated a significant decrease of DPPH radical due to the scavenging ability of tested samples which correlates with a dose effect dependent. Scavenging activity differs between tested samples. It is shown that chemical structure has an important impact on radical scavenging activity. The higher antioxidant activity depends on their molecular structure, and the number and position of the hydroxyl groups. Two hydroxyl groups in B ring are required to stronger antioxidant potential; lack of them (apigenin) significantly reduces this activity [38]. The scavenging effect of ethyl acetate extract of *Limonium bonduelli*, pure flavonoids and

Vitamin C on the DPPH radical decreased in the order of Vitamin C > Eriodictyol > Ethyl acetate extract > Luteolin > Apigenin at the concentration of 20 µg/ml, the resulting inhibitions were 96%, 95%, 91%, 59.3%, 7.3% respectively. Comparing IC_{50} values of tested samples, we observed that eriodictyol and ethyl acetate extract are the most effective antioxidants. Only 5.4 µg/ml and 7.7 µg/ml respectively needed to eliminate 50% of DPPH radical, which nearly comparable to the reference control vitamin C (5µg/ml). The value for luteolin was 15.3 µg/ml. Apigenin showed the weakest IC_{50} radical scavenging potential against DPPH (324.1 µg/ml).

Table 2: IC_{50} values of ethyl acetate extract of *Limonium bonduelli* and isolated compounds in relation to DPPH inhibition

Extract/Compound	IC_{50} (µg/ml)
Ethyl acetate extract of *L. bonduelli*	7.7
Eriodictyol	5.4
Luteolin	15.3
Apigenin	324.1
Vitamin C	5

Inhibition of lipid peroxidation

In the present study, we measured the potential of ethyl acetate extract of *Limonium bonduelli* and eriodictyol to inhibit non-enzymatic lipid peroxidation in egg *vitellose* homogenate, induced by the $FeSO_4$ system. Inhibitions of lipid peroxidation (Figure 4) by tested samples were observed to be lower in magnitude in comparison to standard vitamin C. On the other hand, ethyl acetate extract of *Limonium bonduelli*, isolated compound (eriodictyol) and vitamin C were different in their degree of lipid peroxidation prevention in egg *vitellose* homogenate. The percentage inhibition of lipid peroxidation by 100 µg/ml ethyl acetate extract of *Limonium bonduelli* and eriodictyol were found to be 58% and 69.25% respectively. The ratio at this concentration for vitamin C was found to be 86.95% inhibition of lipid peroxidation. The IC_{50} of ethyl acetate extract of *Limonium bonduelli*, eriodictyol and vitamin C (standard) were 20.88, 22 and 20 µg/ml respectively (Table 3). Decrease in lipid peroxidation by ethyl acetate extract of *Limonium bonduelli* and eriodictyol may be a result of it scavenging free radicals produced by $FeSO_4$ in the reaction system. Structure-activity analysis revealed that the flavonoid molecule with polyhydroxylated substitutions on rings A and B and a 4-keto moiety would confer upon the compound potent anti-peroxidative properties [39].

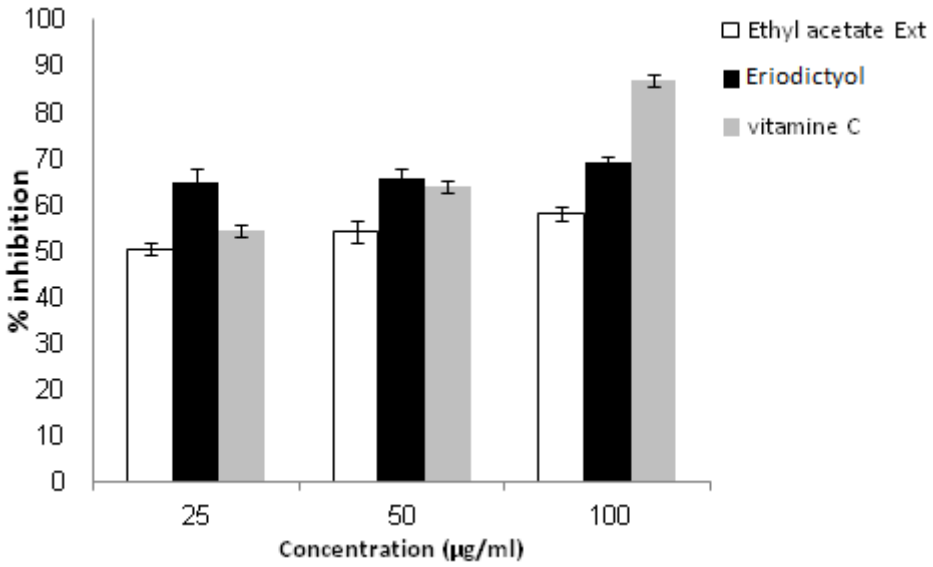

Figure 4 : The effect of ethyl acetate extract of *Limonium bonduelli*, isolated compound (Eriodictyol) and Vitamin C on inhibition of FeSO4 induced lipid peroxidation of egg *vitellose*. Values are expressed in terms of mean ± SD. for three observations.

Table 3: IC_{50} values of ethyl acetate extract of *Limonium bonduelli* and isolated compound (Eriodictyol) in relation to lipid peroxidation inhibition

Extract/Compound	IC_{50} (µg/ml)
Ethyl acetate extract of *L. bonduelli*	20.88
Eriodictyol	22
Vitamin C	20

CONCLUSION

The results clearly indicated high antioxidant potential of ethyl acetate extract of *Limonium bonduelli* and isolated flavonoids. Eriodictyol exhibited the highest level of radical scavenging properties at all concentrations among tested samples followed by ethyl acetate extract of *Limonium bonduelli*, luteolin and apigenin.

This study demonstrated that DPPH radical scavenging activity and inhibition of lipid peroxidation depend on the number and order of OH groups in the flavonoids structure. In addition, the potent antioxidative activity of ethyl acetate extract of *Limonium bonduelli* might result from its high contents of polyphenolic compounds and flavonoids such as eriodictyol and luteolin. Eriodictyol, apigenin, luteolin and 4-hydroxy-3-methoxybenzoic acid are described for the first time from *Limonium Bonduelli*.

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