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In vitro antioxidant properties of flavonoid fractions from *Pistacia atlantica* Desf. subsp. atlantica fruit using five techniques

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Abstract
The purpose of this study is to evaluate the antioxidant activity of the flavonoid fractions of *Pistacia atlantica* fruit using two different solvent: ethyl acetate and butanol. Antioxidant activity was measured by five methods: total antioxidant capacity, reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, hydroxyl (OH) radical scavenging, and β-carotene bleaching activity. The results showed that, the ethyl acetate fraction has a better reducing capacity (EC_{50} = 0.055 ± 0.002 mg/mL) than that of ascorbic acid (0.063 ± 0.002 mg/ml), a strong scavenging activity of radicals $DPPH^{\bullet}$ (EC_{50} = 14.64 mg Antioxidant/g DPPH) and $OH^{\bullet}$ (EC_{50} = 0.27 mg/mL) in comparison with butylated hydroxyl toluene (BHT) (EC_{50} = 13.47 mg/g) and butylated hydroxyanisole (BHA) (EC_{50} = 0.30 mg/ml), respectively. It also exhibited strong β- carotene bleaching (EC_{50} = 3.52 mg/mL) and total antioxidant capacity (31.20 ±1.87 mg EAA/g) compared to butanolic fraction. The highest antioxidant activity of ethyl acetate fraction may be attributed to the presence of flavonodal constituents such as flavonols and phenolic acids, which confirmed by TLC in both fractions. These results suggest that fruits of *P. atlantica* can be used as a source of natural antioxidant in food and pharmaceutical industry.

Keywords: Antioxidant activity, Fruits, *Pistacia atlantica*, Flavonoids.

1. Introduction
The Reactive Oxygen Species (ROS) naturally produced in our body are highly reactive molecules responsible for the deterioration of macromolecules in cell (proteins, lipids, glucides, DNA). Under normal conditions, the damage is controlled by the antioxidant defence system which includes enzymatic (superoxide dismutase, catalase and glutathione peroxidase), non-enzymatic factors (vitamins: ascorbic acid, tocopherols, carotenoids) and other antioxidant micronutrients present in fruits, vegetables and plant [1, 2].
The oxidative stress appears in case of excessive concentration in ROS. To enhance the organism defence against oxidative stress, exogenous antioxidants are required. These molecules are naturally occurring in plant [3]. For instance, the polyphenols are natural antioxidants, with a wide range of biological effects, such as antimutagenic, anticarcinogenic, antiallergenic, antiplatelet, anti-ischemic and anti-inflammatory effects [4]. The polyphenols, because of their redox-properties, react directly with the ROS. The phenolic function is also able to inhibit enzyme and to chelate trace elements responsible for production of ROS via singulet dioxygene [5].
The polyphenol and other molecules with antioxidant activity can be extracted from plant with solvents. The yield in extracted material depends on both the solvent and the extraction method [6, 7]. The extraction method must allow complete extraction of the compounds of interest, and avoid their modification [8]. Generally, water, aqueous ethanol, methanol, acetone, ethyl acetate and petroleum ether are used for plants extraction.
Pistacia atlantica Desf. Subsp *atlantica* is a one of *Pistacia* species widely distributed in arid and semiarid regions of Algeria. *Pistacia* species are known for their potential antioxidant properties [9, 10, 11, 12], and also for their antimicrobial [13], anti-inflammatory and cytotoxic activities [14, 15]. In Algeria, the fruit, called Elkhodiri, have a high level in oil (39.80%) and in protein (10.39 %). Locally, the oil, mixed with crushed date and whey, is eaten at any hour of the day [16]. In folk medicine, the fruit is used locally as an antidiarrheal [17].
The present investigation was undertaken to study the antioxidant potential of two flavonoid fractions of the fruit of *P. atlantica* using 5 different methods such as total antioxidant capacity, reducing power, DPPH

radical scavenging, hydroxyl radical scavenging and β –carotene bleaching effect. These antioxidant activities were compared to those of standard synthetic antioxidants such as BHA, BHT, Trolox, and ascorbic acid.

2. Materials and methods

2.1. Plant material

Fruits of *P. atlantica* Desf. were collected in Mai 2007 in the area of Ain Fezza, near Tlemcen, in the northern part of Algeria. The plant material identification was carried out at the laboratory of botany (University of Tlemcen), where a voucher specimen (No. 1784) has been deposited. The identification was done according to the New Flora of Algeria [18]. The fruits were dried in a shadowy place at room temperature, packed in paper bags and stored for future uses at the Laboratory of Natural Products (Department of Biology, Faculty of Sciences, University of Tlemcen, Algeria).

2.2. Extraction procedure

Ethyl acetate and butan-1-ol fractions were prepared according to the Bekkara *et al.* [19] as follows: The power (1 g) of *P. atlantica* fruits was macerated with 20 ml of methanol and gently stirred for 48 hours. After filtration through Whatman n°1 filter paper, the solvent was eliminated under reduced pressure in a rotary evaporator at 60 °C. The residue (crude extract) was suspended in boiling water (10 mL) and the suspension successively extracted with ethyl acetate (10 mL) and with butan-1-ol (10 mL). The solvents were evaporated under reduced pressure to dryness. The dried fractions was weighed and dissolved in 3 mL of methanol for further analysis.

2.3. Total antioxidant capacity

The total antioxidant capacity (TAC) of the plant fractions was evaluated by the phosphomolybdenum method of Prieto *et al*. [20]. A 0.3 mL aliquot of the plant flavonoid fractions was mixed with 3 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated at 95°C for 90 min. After, the samples had cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity of the samples was expressed as milligrams of ascorbic acid equivalents per gram of dry matter (mg AAE/ g DM).

2.4. Reducing power

The reducing power of the fractions was determined according to the method of Oyaizu [21]. Various concentrations of the flavonoid fractions (mg/mL) in distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and 1% of potassium ferricyanide water solution (2.5 mL, $K_3[Fe(CN)_6]$). The mixture was incubated at 50°C for 20 min. Aliquots of trichloracetic acid (2.5 mL, 10%) were added to the mixture which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and a freshly prepared $FeCl_3$ solution (0.5 mL, 0.1%). The absorbance was measured at 700 nm. An increased absorbance of the reaction mixture was taken to mean an increased reducing power. Ascorbic acid was used as a positive control.

2.5. Free radical scavenging activity, DPPH assay

The free radical scavenging activity was measured by a modified $DPPH^{\bullet}$ assay [22]. A solution of the flavonoid fractions prepared as describe above (50 µL) was added to 1950 µL of methanolic DPPH solution (0.025 g/L). The decreasing absorbance at 515 nm was monitored in order to reach constant values. The $DPPH^{\bullet}$ concentration in the reaction medium was calculated from the following calibration curve, determined by linear regression:

$$\mathbf{A_{515nm} = 24.41 \times [DPPH^{\bullet}]t + 0.0022 \qquad R^2 = 0.999}$$

Where $[DPPH^{\bullet}]t$ was expressed as mg/mL at *t* time.

The percentage of the remaining $DPPH^{\bullet}$ (% $DPPH^{\bullet}_{REM}$) at the steady state was calculated as follows:

$$\mathbf{\% \ DPPH^{\bullet}_{REM} = 100 \times [DPPH^{\bullet}]_t / [DPPH^{\bullet}]_{t=0}}$$

where $[DPPH^{\bullet}]t=0$ and $[DPPH^{\bullet}]t$ are concentrations of $DPPH^{\bullet}$ at t=0 and t= t, respectively.

Using various antioxidant concentrations, it was possible to determine the amount of antioxidant necessary to halve the initial $DPPH^{\bullet}$ concentration (EC_{50}).

EC_{50} is expressed in mg of dry extract per g of DPPH. The time needed to reach the EC_{50} concentration, noted T_{EC50}, was graphically determined. The antiradical efficiency (AE) was calculated as follows:

$$\mathbf{AE = 1/ (EC_{50} \times T_{EC50})}$$

2.6. Hydroxyl radical scavenging assay

The effect of flavonoid fractions on hydroxyl radicals was assayed by using the deoxyribose method [23]. The reaction mixture contains the following reagents: 0.4 mL of phosphate buffer saline (50 mmol/L, pH 7.4), 0.1 mL of extracts solution, 0.1 mL of EDTA (1.04 mmol/L), 0.1 mL of $FeCl_3$ (1 mmol/L) and 0.1 mL of 2-deoxyribose (60 mmol/L). The reaction was started by the addition of 0.1 mL of ascorbic acid (2 mmol/L) and 0.1 mL of H_2O_2 (10 mmol/L). After incubation at 37°C for 1 h, the reaction was stopped by adding 1 mL of thiobarbutiric acid (TBA) 10 g/L follow-up by 1 mL of chlorhydric acid (HCl) (25%), then heating the tubes in a boiling water bath for 15 min. The contents were cooled and absorbance of the mixture was measured at 532 nm against reagent blank. Decreased absorbance of the reaction mixture indicates decreased oxidation of deoxyribose. The percentage inhibition of deoxyribose oxidation was calculated using the following equation:

$$\textbf{Hydroxyl radical scavenging activity \%) = } [A_0 - (A_1 - A_2)] \times 100 / A_0$$

where A_0 was the absorbance of the control (without extract) and A_1 was the absorbance in the presence of the extract and the deoxyribose, A_2 was the absorbance in the presence of the extract without deoxyribose.

2.7. β-carotene bleaching method

The antioxidant activity of flavonoid fractions was evaluated using β-carotene-linoleate model system, as described by Moure *et al.* [24]. Two milligrams of β-carotene were dissolved in 10 mL chloroform and 1 mL β-carotene solution was mixed with 20 µL of purified linoleic acid and 200 mg of Tween 40 emulsifier. After evaporation of chloroform under vacuum, oxygenated distilled water (100 mL) were added by vigorous shaking. To an aliquot of 4 mL of this emulsion, 200 µL of extracts or the BHA were added and mixed well. The absorbance at 470 nm, which was regarded as t =0 min, was measured, immediately, against a blank consisting of the emulsion without β-carotene. The capped tubes were placed in a water bath at 50°C for a period of 2 h. Thereafter, the absorbance of each sample was measured at 470 nm (A_{120}). For the positive control, sample was replaced with BHA. A negative control consisted of 200 µL methanol instead of extract or BHA. The antioxidant activity (AA) was calculated according to the following equation:

$$AA = [(A_{A(120)} - A_{C(120)}) / (A_{C(0)} - A_{C(120)}] \times 100$$

where $A_{A(120)}$ is the absorbance of the sample at t = 120 min; $A_{C(120)}$ is the absorbance of the control at t = 120 min and $A_{C(0)}$ is the absorbance of the control at t = 0 min.

2.8. Thin-layer chromatography (TLC) analysis

The ethyl acetate and the butan-1-ol fractions were analysed by thin-layer chromatography (Silica gel GF_{254}, Merck), using three mobile phase systems, (a) Toluene-ethyl acetate-formic acid (36:12:05), (b) Toluene-acetone-formic acid (38:10:05) and (c) chloroform-methanol-water-acetic acid (100:15:0.5:03). UV light fluorescence at 254 and 365 nm was used to detect flavonoids.

2.9. Statistical analysis

Variables expressed as mean values and standard deviations (SD) were calculated using Excel. Correlation coefficient of antioxidant activity was determined using Microcal Origin 6.

3. Results and discussion

Several methods are available to measure the antioxidant activity of food and biological systems [25]. In the present study, we combined five complementary assays: total antioxidant capacity, reducing power, scavenging activity on DPPH and hydroxyl radicals and inhibition of β-carotene bleaching to study the antioxidant activities of selected plant extracts.

3.1. Total antioxidant capacity

The phosphomolybdenum method was used to evaluate the total antioxidant activity of the fruit fractions from P. atlantica. In the presence of extracts, Mo (VI) is reduced to Mo (V) and forms a green coloured phosphomolybdenum V complex at acidic pH, which shows a maximum absorbance at 695 nm [20]. The results for total antioxidant capacity are given in Table 1 and are expressed as mg ascorbic acid equivalents (AAE)/g of dry matter (DM). The ethyl acetate fraction (31.20 ± 1.87 mg/g) showed a strong antioxidant capacity, while butanolic fraction showed the least (20.21 ± 1.02 mg AAE/g DM). It is noted that a higher absorbance value of the reaction mixture indicates stronger total antioxidant activity. According to our literature survey, this is the first report on total antioxidant capacity of the fruits by the phosphomolybdenum complex.

Table 1. Antioxidant properties of the flavonoid fractions on reducing power, hydroxyl radical scavenging assay, β-carotene-linoleic acid assay and total antioxidant capacity.

Samples	TAC (mg AAE/ g DM)[a]	EC_{50} (mg/mL)		
		Reducing power	Hydroxyl radical scavenging assay	β-Carotene-linoleic acid assay
Ethyl acetate fraction	31.20 ± 1.87	0.05 ± 0.00	0.27 ± 0.00	3.52 ± 0.07
Butanolic fraction	20.21 ± 1.02	0.14 ± 0.01	0.38 ± 0.00	37.81 ± 4.43
Ascorbic acid		0.06 ± 0.002		
BHA			0.30 ± 0.05	0.03 ± 0.005

[a] mg ascorbic acid equivalent/ g dry matter.

3.2. Reducing power property

Fe (III) reduction is often used as an indicator of electron donation as well as one of the major mechanisms of actions of antioxidants. This test is a simple, reproducible, rapid, and inexpensive method that measures reductive ability of antiradical and it is evaluated by measuring the Fe^{+3}/ferric cyanide complex to the ferrous transformation in the presence of *P. atlantica* fruit fractions. Increasing absorbance at 700 nm indicates an increase in reducing ability. The results for ferric reducing power activity of flavonoid fractions with compared to ascorbic acid are reported in Figure 1. It was found that the reducing power increased with the concentration of each extract. However, the best reducing power of the ethyl acetate fraction rose from 0.47 at 0.05 mg/mL to 2.29 at 0.25 mg/mL. This activity is interesting while comparing with ascorbic acid (2.36) and butanolic fraction (0.89) at 0.25 mg/mL. EC_{50} value was calculated, it was about 0.05 ± 0.00 mg/mL for ethyl acetate fraction and was comparatively more effective than ascorbic acid (0.06 ± 0.002 mg/mL) and butanolic fraction (0.14 ± 0.01 mg/ mL) (Table1). Compared to the literature, Benhammou *et al*. [26] investigated the reducing power activity of leaf ethanolic extracts of *P. lentiscu*s and *P. atlantica* and found lower results to ours.

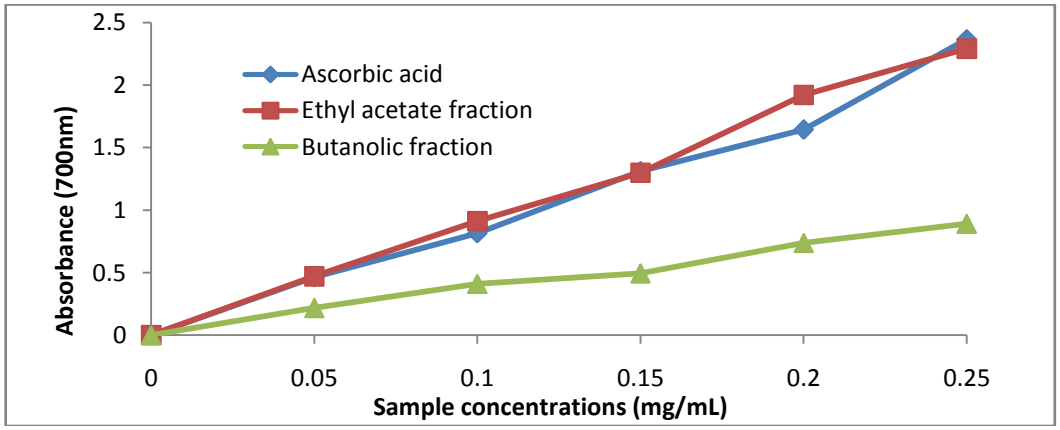

Fig.1. Reducing power of ethyl acetate and butanolic fractions and standard ascorbic acid. Each value is expressed as a mean ± S.D.

3.3. DPPH radical-scavenging activity

In this study, the ethyl acetate and butanolic fractions were applied to a DPPH• free radical reaction system to determine the scavenging activity and kinetics [27]. In the presence of a hydrogen-donating antioxidant, DPPH radical with purple color is reduced to yellow-colored diphenyl picryl hydrazine. Figure 2 (A, B) is time dependent plots of residual DPPH• for various concentrations of plant fractions. The high concentration leads to decrease the amount of remaining DPPH• and therefore the free radical scavenging activity is higher.

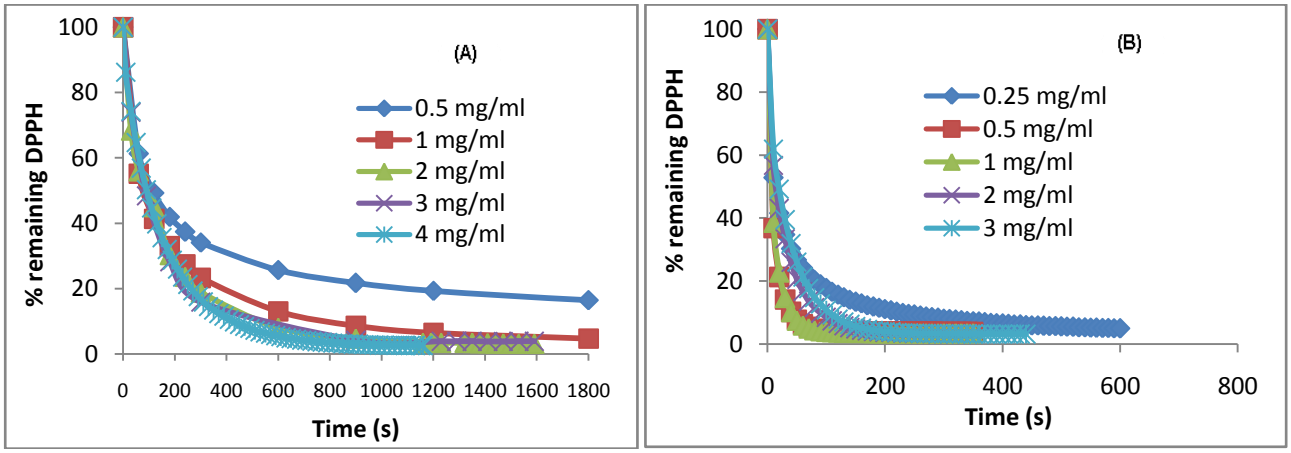

Fig. 2. Reaction kinetics of flavonoid fractions to quench free radical. (A) butanolic fraction; (B) ethyl acetate fraction. Each value is expressed as a mean ± S.D.

Table 2 shows the kinetic behaviour obtained for the both fractions and standard antioxidants at different concentrations. At 3 mg/mL, the percentages of DPPH remaining were 3.17% at 6.16 min for ethyl acetate fraction and 3.85% at 20 min for butanolic fraction.

Table 2. Scavenging effect of flavonoid fractions and standard antioxidants on DPPH$^{\bullet}$.

Samples	Concentrations (mg/mL)	Concentrations (mg Antioxidant/ g DPPH$^{\bullet}$)	Time (min) at the steady state	Remaining DPPH$^{\bullet}$ at the steady state (%)
Ethyl acetate fraction	0.25	100	16.16	4.11
	0.5	200	4.83	3.87
	1	400	5.16	3.45
	2	800	5.33	3.25
	3	1200	6.16	3.17
Butanolic fraction	0.5	200	119	8.31
	1	400	65	4.29
	2	800	25.5	3.12
	3	1200	20	3.85
	4	1600	18.16	2.60
Ascorbic acid	0.062	24.8	1.5	49.03
	0.12	48	0.83	43.64
	0.14	56	2.75	47.88
	0.19	76	3.41	27.49
	0.22	88	2.91	7.98
	0.24	96	1.16	7.18
BHT	1	400	66	8.11
	2.5	1000	26	8.80
	4	1600	20	9.22
	5	2000	16	7.90
	6	2400	18	8.30
Trolox	0.25	100	6.16	26.93
	0.5	200	4.33	5.03
	1	400	2	4.76
	2	800	1.08	4.22
	3	1200	1.25	4.31

From the analysis of EC_{50} values, which obtained from the plot of the fractions of residual DPPH at the steady state against the concentrations (Table 3), the DPPH radical scavenging activity of the ethyl acetate fraction (14.64 mg Antioxidant/g DPPH) was found to be higher followed by butanolic extract (49.21 mg/g). The EC_{50}

value for ethyl acetate fraction was close to that of BHT (13.47 mg/g), while the butanolic fraction showed comparable levels of free radical scavenging activity with trolox used as positive control (49.21 mg/g).
The parameter, antiradical efficiency (AE) which is more discriminatory than EC_{50} according to Sanchez-Moreno *et al.* [22]. It was used to define the antioxidant capacity of flavonoid fraction of fruits using the both EC_{50} and the time needed to reach the steady state to EC_{50} concentration (T_{EC50}). Table 3 shows that ascorbic acid was more powerful antioxidant than the flavonoid fractions and the standard antioxidants (trolox and BHT). The ethyl acetate fraction (29.34 $\times 10^{-5}$) exhibited a good antiradical capacity, which was about three times that the butanolic fraction (10.12 $\times 10^{-5}$). The AE of *P. atlantica* fruits from ethyl acetate fraction is of interest as compared to that of *P. atlantica* leaves (13.8×10^{-5}) and comparable to that of *P. lentiscus* (32.8×10^{-5}) [28].

Table 3. 50% radical scavenging activity concentration of antioxidants and their antiradical efficiencies (AE).

Samples	EC_{50} (mg Antioxidant/ g DPPH	T_{EC50} (min)	AE $\times 10^{-5}$
Ethvl acetate fraction	14.64	232.75	29.34
Butanolic fraction	49.21	200.69	10.12
Ascorbic acid	39.53	0.60	4174.46
BHT	13.47	161.52	45.97
Trolox	49.21	7.80	260.42

3.4. Hydroxyl radical scavenging activity
Scavenging of $OH^{\bullet}$ is an important antioxidant activity because of the very high reactivity of the $OH^{\bullet}$, which enables it to react with a wide range of molecules, such as sugars, amino acids, lipids, and nucleotides, found in living cells [29]. Thus, removing $OH^{\bullet}$ is very important for the protection of living systems. As shown in Figure 3, both fractions and standard BHA registered good hydroxyl radical scavenging activity in a concentration-dependent manner (0.5-4 mg/mL).

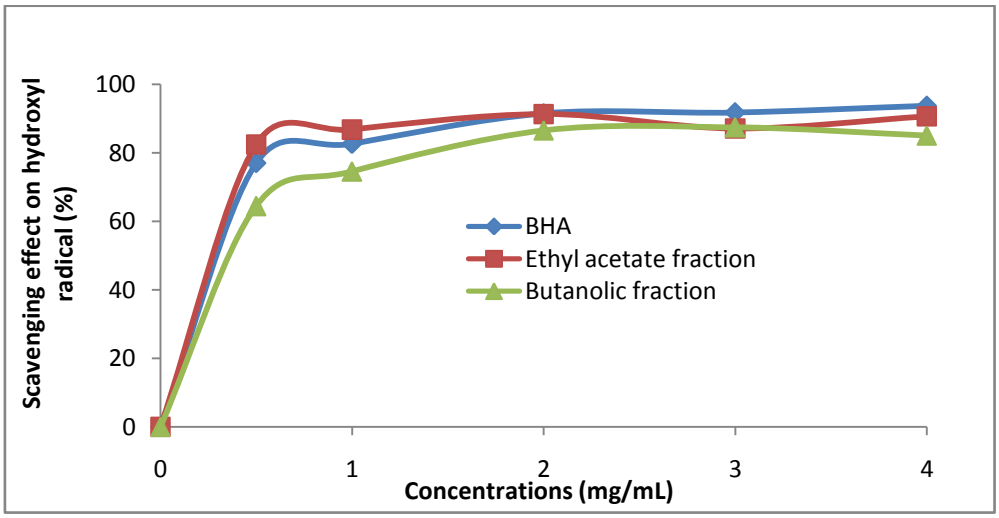

Fig. 3. Hydroxyl radical scavenging activity of ethyl acetate and butanolic fractions and standard BHA. Each value is expressed as a mean ± S.D.

The maximal inhibitory percentages exceeded 60% at 0.5 mg/mL and were 85.04%, 90.61 and 93.69% in the presence of butanolic fraction, ethyl acetate fraction and BHA at 4 mg/mL), respectively. The $OH^{\bullet}$ scavenging ability of the ethyl acetate fraction (EC_{50} value of 0.27 ± 0.00 mg/mL) was found to be higher than that of the butanolic fraction (EC_{50} = 0.38 ± 0.00 mg/mL) (Table 1). The ability of *P. atlantica* fractions to scavenge hydroxyl radicals appears to directly relate to the inhibition of lipid peroxidation, and acts as scavengers of active oxygen species by breaking free radical chains.

3.5. β-Carotene bleaching assay

This method is based on the discoloration of β-carotene owing to its reaction with linoleic acid-generated free radicals in an emulsion system. The addition of the antioxidant prevented the bleaching of β-carotene. This process was monitored spectrophotometrically. β-carotene bleaching assay showed the dose dependent response curve for both fractions at concentrations ranging from 0.5 to 4 mg/mL (Figure 3). At 4 mg/mL, the bleaching inhibitions were 16.12% for butanolic fraction and 60.73% for ethyl acetate fraction. With respect to BHA, the percentage was 97.59% at 2.5 mg/mL. The EC_{50} values of for ethyl acetate fraction was 3.52 ± 0.07 mg/mL and was followed by BHA 0.03 ± 0.005 mg/mL and butanolic fraction 37.81 ± 4.43 g/ml, respectively (Table 1). This data suggested that ethyl acetate fraction of fruit which contains nonpolar components has strong ability to neutralize the linoleate-free radical and to convert them into more stable non-reactive species.

Our data demonstrate the excellent antioxidant activity of ethyl acetate and butanolic fractions of *P. atlantica* fruits that could be explained by the presence of phenolic acids and flavonols, which are the major class of flavonoids, contributors to the antioxidant and free radical scavenging activities. This group of natural products exhibit many biological and pharmacological activities. Indeed, the TLC confirmed the presence of gallic acid in both fractions. Our findings are in good agreement with the results of a previous investigations that were indicated the occurrence of phenolic compounds, such as gallic acid and its derivatives, quercetin and myricetin derivatives in *Pistacia* species [11, 14, 25, 30, 31].

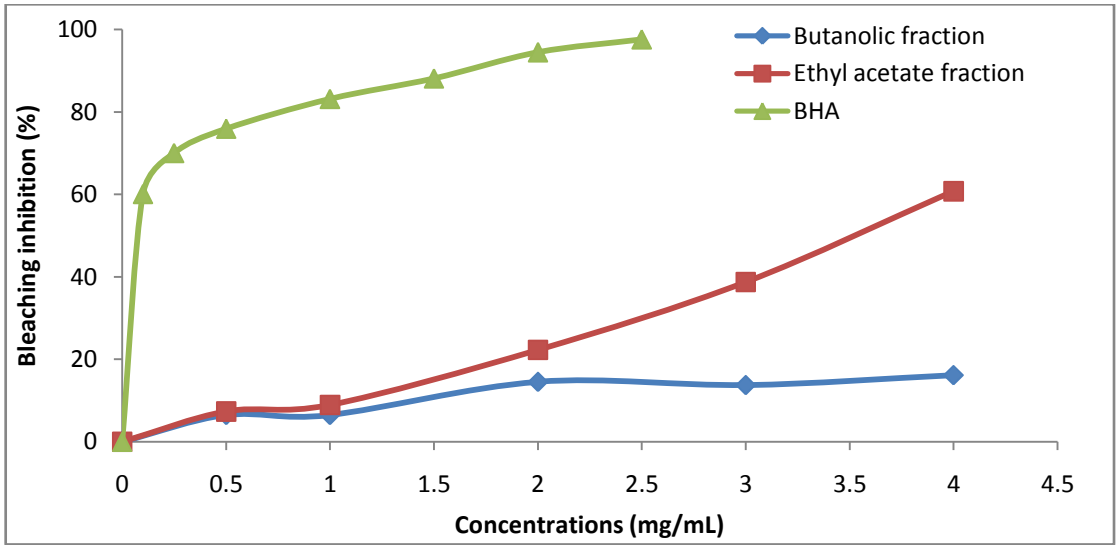

Fig. 4. Inhibition (%) of lipid peroxidation of ethyl acetate and butanolic fractions and standard BHA by the β-carotene bleaching method. Each value is expressed as a mean ± S.D.

Conclusion

In this study, we report for the first time, the antioxidant activity of fruit flavonoid fractions of *P. atlantica*. The ethyl acetate fraction exhibited the highest results in all the *in vitro* models of antioxidant assays studied. The results from various free radical scavenging systems revealed that the fruits of this plant had significant antioxidant activity and free radical scavenging activity. These findings confirm the consumption of these fruits as a foodstuff by certain Algerian populations. Further studies are needed to confirm if these *in vitro* activities can be replicated *in vivo*, to identify the active principles from these fractions and to elucidate their different antioxidant mechanisms responsible for the therapeutic efficacy.

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