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Phytochemical screening and antioxidant properties of methanolic extract and different fractions of *Crataegus azarolus* leaves and flowers from Algeria

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Abstract

The aim of the present study is to investigate the antioxidant activities of crude methanolic extract and its different fractions obtained from Algerian *Crataegus azarolus* leaves and flowers using different tests such as DPPH (1, 1-diphenyl-2-picrylhydrazyl radical), reducing power and β-carotene assays. Furthermore, a preliminary phytochemical screening was performed using standard protocols. Total phenolics and total flavonoïds contents of the extracts were measured by Folin Ciocalteu and Aluminium chloride methods respectively. The obtained results indicated that ethyl acetate fraction (EAF) showed the highest DPPH radical scavenging activity with an IC_{50} value of 9.72±0.102 µg/ml, followed by crude methanolic extract (CME) and diethyl ether fraction (DEF) with an IC50 value of 20.96±0.340 and 68.69±2.490 µg/ml, respectively. However, the ethyl acetate fraction showed a good reducing power (EC_{50}= 30.96±0.563 µg/ml) as compared to other fractions. In β-carotene/linoleic acid assay, the best inhibition was found in chloroform fraction (CHF) with an IC_{50} of 65.45±1.027 µg/ml. The phytochemical screening showed the presence of alkaloids, tannin, anthraquinones, steroids and triterpenes. Total phenolics varied from 2.83 mg to 111.96 GAE/g in each fraction. The ethyl acetate fraction contained the highest amount of flavonoïds (5.87±0.255 mg QE/g of fraction) compared to other fractions. As a conclusion, the results of the present study indicate that the aerial part extracts of *Crataegus azarolus* is a good source of natural antioxidant constituents.

Introduction

Reactive Oxygen Species (ROS) including free radicals, are well known to induce oxidative damage by reacting with various biomolecules like lipids, DNA and proteins, causing cellular injury and death (Steenkamp *et al.*, 2005). These reactive species play important roles in several chronic human diseases and aging process such as: cancer, diabetes, hypertension, atherosclerosis and Parkinson's diseases (Gagliardi *et al*., 2009; Bangou *et al*., 2011). ROS can cause lipid peroxidation in foods, which leads to their deterioration (Miller *et al*., 1995; Sasaki *et al*., 1996). Thus, antioxidant agents that can prevent or inhibit the oxidation process in human body and food products by removing free radical intermediates are desired. Many antioxidant compounds, naturally occurring from plant sources such as flavonoïds, tannins and phenolic acids, have been identified as free radical or active oxygen scavengers (Yen and Duh, 1994; Duh, 1998).

Crataegus azarolus is a medicinal plant belonging to the Rosaceae family, known as Hawthorns. It was originally derived from the East of Algeria. This plant is a shrub with 5-10 m height. Their leaves are obviate and deeply lobed, and it has yellow fruits. Traditionally, hawthorns are used to treat diabetes, cardiovascular diseases, hypotensive, cancer and other diseases (AI-Khazarji *et al*., 1993; Nawash and Al-Horani, 2011). Many scientific investigations have indicated that *Crataegu*s leaves, flowers and volatile oils are rich in triterpene acids and phenolic compounds including flavonoïds and phenolic acids which exhibited important biological effects, such as anti-inflammatory, antioxidant and antimicrobial activities (Ammon and Haendel, 1998; Kris-Etherton *et al.*, 2002; Svedstrom *et al.*, 2006; Shatoor *et al.*, 2012).

The aim of the present study was: (i) to evaluate the antioxidant activity of the crude methanolic extract and its different fractions of *Crataegus azarolus* leaves and flowers, using three different methods: DPPH, reducing power and β-carotene bleaching methods, (ii) to screen their phytochemical

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compounds and (iii) to estimate their total phenolics and flavonoïds.

Materials and Methods

Chemicals

Gallic acid, quercetin, linoleic acid, butylated hydroxytoluene (BHT), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH•), Folin–Ciocalteu, sodium carbonate, aluminium chloride, potassium ferricyanide [K3Fe(CN)6], ferric chloride (FeCl3), Ascorbic acid, β-carotene, Tween 40, methanol, chloroform, diethyl ether and ethyl acetate were obtained from Sigma (Sigma-Aldrich, Germany).

Plant material

Leaves and flowers of *Crataegus azarolus* were collected in March 2012, in Hamma area in Constantine, East of Algeria. The collected plant was identified and authenticated by botany department, USTHB University, Algeria.

Extraction and fractionation

The air-dried powdered leaves and flowers of *Crataegus azarolus* (20 g) were extracted, three times, with 100% methanol (600 ml) for 48 h at room temperature. After filtration through whatman N°1 filter paper, the extract was evaporated in rotary evaporator. The methanolic extract was resuspended in 200 ml of distilled water and partitioned sequentially with chloroform (300 ml), diethyl ether (300 ml) and ethyl acetate (300 ml) respectively. These three organic fractions and crude methanolic extract were obtained after evaporating the solvent and kept in the dark at 4°C until testing.

Total phenolic content

The total phenolic content of the extract was estimated using the Folin-Ciocalteu method adapted from Singleton and Rossi (1965). To 0.25 ml of diluted extract, 3.5 ml of distilled water was added followed by 0.25 ml of Folin-Ciocalteu solution. After 3 minutes, 1 ml sodium carbonate (20% w/v) was added. The whole mixture was incubated at 40°C for 40 minutes, and the absorbance of the resultant solution was read at 685 nm. A standard curve was prepared using gallic acid with various concentrations (2, 4, 6, 8, 10 µg/ml). Total phenolic content was expressed as mg gallic acid equivalents (GAE)/g of samples.

Total flavonoïds content

The total flavonoïds content was determined according to the aluminum chloride colorimetric method described by Bahorun *et al.* (1996). 1ml of the extract was added to 1ml of 2% aluminium trichloride ($AlCl_3$). After 10 min, the absorbance of the solution was read at 430 nm. Quercetin was used for the standard calibration curve with different concentrations (0.4, 0.8, 1.2, 1.6, 1.8, 2.2 µg/ml). Flavonoïds contents are expressed as mg quercetin equivalents (QE)/g of samples.

DPPH radical scavenging activity

The DPPH free radical activity was performed according to the procedure described by Musa *et al*. (2011). Briefly, 1 ml of different concentration of samples (10, 20, 100, 200, 300, 400 µg/ml, final concentration) were mixed with 1 ml of methanol solution of DPPH (0.04 %). Thirty minutes later, the absorbance of each sample was measured at 517 nm against blank samples. BHT and Ascorbic acid were used as reference standards. The percentage of inhibition activity was calculated using the following equation:

$$\% \text{ Inhibition} = (Ac - As/Ac) \times 100$$

Where: Ac is the absorbance of the control and As is the absorbance of the sample solution. The antiradical activity of samples was expressed as IC_{50} value, which represented the effective concentration of sample required to scavenge 50% of DPPH radicals.

Reducing power

The reducing power was determined according to the procedure described by Yen and Chen (1995). Different concentrations of extracts (10, 50, 100, 150 µg/ml) in 1 ml distilled water were mixed with 2.50 ml of a 0.2 M sodium phosphate buffer (pH = 6.6) and 2.50 ml of 1% potassium ferricyanide ($K_3Fe(CN)_6$), and incubated in a water bath at 50°C for 20 min. Then, 2.50 ml of 10% trichloroacetic acid were added to the mixture that was centrifuged at 3000 rpm for 10 min. The supernatant (2.50 ml) was then mixed with 2.50 ml distilled water and 0.50 ml of 0.1% ferric chloride solution and the absorbance was measured at 700 nm. BHT and Ascorbic acid were used as reference standards. The reduction power of extracts was expressed as EC_{50} value, which represented the concentration at which the absorbance being 0.5.

Determination of antioxidant activity with the β-carotene bleaching assay

Determination of antioxidant activity with the β-carotene bleaching assay was performed according

to the procedure described by Miraliakbari and Shahidi (2008). A mixture of β-carotene and linoleic acid was prepared with 0.5 mg of β-carotene in 1 ml chloroform, 25 µl of linoleic, acid and 200 mg Tween 40. The chloroform was evaporated under vacuum and 100 ml of oxygenated distilled water were then added to the residue. The samples (1mg/ml) were dissolved in methanol and 350 µl of each sample solution were added to 2.5 ml of the above mixture in test tubes. The test tubes were incubated in a hot water bath at 50°C for 2 h. Absorbance of the sample was measured every 30 min for 2 h at 470 nm. The percentage inhibition of the samples was calculated using the following equation (Bamoniri *et al*., 2010):

I% = (A β-carotene after 2-h assay/A initial β-carotene) × 100

Where a β-carotene after 2-h assay is the absorbance values of β-carotene after 2 h assay remaining in the samples and A initial β- carotene is the absorbance value of β-carotene at the beginning of the experiments.

Phytochemical screening

Phytochemical screening of various fractions of *Crataegus azarolus* leaves and flowers was performed for the presence of a number of chemical groups such as alkaloids, terpenoids, steroids, saponins, tannins, anthraquinones and anthocyanins. This phytochemical study was qualitatively expressed as positive (+) or negative (-).

Alkaloids

About 0.2 g of each sample was warmed with 2% H_2SO_4 for two minutes. The reaction were filtered and a few drops of Dragendroff,s reagent were added to each filtrate. Orange red precipitate indicates the presence of alkaloids moiety (Kam *et al*., 2001).

Tannins

1 ml of the sample was added to 2 ml of water and 2-3 drops of diluted solution of ferric chloride. A dark green solution indicates the presence of tannins (Rizk, 1982).

Anthraquinone

About 0.5 g of each extract was boiled with 10 % HCL for few minutes on water bath. The reaction mixture was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to each filtrate. Few drops of 10 % ammonia was added to each mixture and heated. Formation of rose-pink color indicates the presence of anthraquinones (Odebeyi and Sofowora, 1978).

Sterols and triterpenes

All samples are diluted in methanol, then, 0.5 ml of both acetic anhydride and chloroform were added. After that, 1 ml of concentrated sulphuric acid was added (Liebermann-Burchard reaction). At the contact zone of the two liquids a brownish red ring was formed denoting the presence of sterols and triterpenes (Harbone, 1976).

Saponins

The extract with 20 ml of distilled water was agitated in a graduated cylinder for 15 minutes. The formation of 1cm layer of foam indicated the presence of saponins (Kumar *et al.*, 2009).

Anthocyanin

2 ml of each sample, dissolved in distillated water, was added to 2 ml of HCl (2N) and 2 ml of NH3 (2N). The appearance of pink red turns blue violet indicates presence of Anthocyanin (Lebreton *et al.,* 1967).

Statistical analysis

Data are given as the mean ± standard deviation (SD) of three measurements. Statistical analysis was performed by ANOVA procedures using Graph pad prism software and $P<0.05$ was considered to be statistically significant.

Results and Discussion

Total phenolics and flavonoïds content

The total phenolics and flavonoïds contents in the crude extract and fractions of leaves and flowers of *Crataegus azarolus* are presented in Table 1. Total phenolics compounds (TPC) in the different fractions of *Crataegus azarolus*, as determined by Folin Ciocalteu assay, was expressed as Gallic acid equivalents by reference to a standard curve ($y = 0.099x + 0.142$, $R^2 = 0.99$). The TPC varied from 2.83 to 111.96 mg GAE/g of fraction. The results showed that ethyl acetate (EAF) has the highest phenolic concentration of *Crataegus azarolus* (111.96 ± 1.661 mg GAE/g of fraction), followed by crude methanolic extract (CME) (67.68 ± 2.241 mg GAE/g of extract). However, chloroform fraction (CHF) presented the lowest level of TPC (2.03± 0.065 mg GAE/g fraction).

The content of the total phenolics in the fractions of *Crataegus azarolus* decreased in the following order: EAF > CME > DEF > CHF. The total flavonoïds content (TFC) of various fractions

Table 1. Total phenolic and flavonoïds contents of the crude methanolic extract and fractions from leaves and flowers of *Crataegus azarolus*

	Total phenolics (mg GAE/g of fraction)	Total flavonoïds (mg QE/g of fraction)
CME	67.68 ± 2.241	5.18 ± 0.123
EAF	111.96 ± 1.661	5.87 ± 0.255
DEF	26.35 ± 1.316	2.82 ± 0.221
CHF	2.83 ± 0.065	0.35 ± 0.041

Each value is expressed as means of three replicates ± standard.

of *Crataegus azarolus*, was determined using spectrophetometric method with Aluminum chloride, and is expressed as quercetin equivalent (the standard curve equation y= 0.476 x + 0.003, $R^2 = 0.99$). The TFC of each fraction varied from 0.35 to 5.87 mg QE/g of fraction. The ethyl acetate fraction contained the highest amounts of flavonoïds (5.87±0.255 mg QE/g of fraction) compared to other fractions. The flavonoïds content in the fractions decreased in the order of EAF > CME > DEF > CHF.

The concentration of total phenolic and flavonoïd contents in different extracts depends on the polarity of solvents used in the extract preparation (Marinova *et al*., 1997). According to this study, the riches of crude extract and its fractions of *Crataegus azarolus* in flavonoids and mainly in phenolics can explain their antioxidant activity. It appears that the genus *Crataegus* is very rich in phenolics and flavonoids. Consequently, Simirgiotis (2013) showed that the methanolic extract of the aerial parts of *Crataegus monogyna* contains large amounts of phenolics and flavonoids. Phenolic compounds such as flavonoïds, tannin and phenolic acids are plant secondary metabolites and they are very important in plants. These compounds contain hydroxyl groups which are responsible for the radical scavenging effect (Choi *et al*., 2002; Kim *et al*., 2003; Valentão *et al*., 2003; Apak *et al*., 2007; Dai and Mumper, 2010).

Phytochemical screening

The phytochemical screening and qualitative estimation of the crude methanolic extract and different fractions of *Crataegus azarolus* leaves and flowers revealed the presence of alkaloids, tannin, anthraquinones, steroids and triterpenes (Table 2). These compounds varied within the fractions. Anthraquinones and saponin were absent in all tested fractions. The existence of tannins, terpenes and alkaloids in *Crataegus azarolus* may contribute to its

Table 2. Phytochemical screening of the crude methanolic extract and fractions of leaves and flowers of *Crataegus azarolus*

	CME	EAF	DEF	CHF
Alkaloids	+	+	+	++
Tannin gallic acid	-	+++	++	-
Tannin catechique	+	-	-	+
Anthraquinones	+	++++	+++	+
Anthracyanines	-	-	-	-
Steroids	++	-	-	+++
Triterpenes	++	+++	++	-
Saponin	-	-	-	-

+++: Strong positive test; ++: Low positive test; +: Weak positive test; –: Negative test.

antioxidant and anti-inflammatory activities and can be used to give human resistance against parasites and bacteria. The presence of quinones is probably responsible for a number of pharmacological activities such as radical scavenging activity.

DPPH radical scavenging activity

The DPPH scavenging activity has been widely used for screening antioxidant activity of plant extracts. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al*., 1997). In this assay, the presence of antioxidants in the extract reduced DPPH free radical, by accepting an electron from the antioxidant (Bondet *et al*., 1997; Mohammad *et al*., 2009). The reduction capability of DPPH radical was determined by the decrease in its absorbance at 517 nm.

Figure 1 shows the concentration dependent response curve of DPPH scavenging activities of the crude methanolic extract and fractions of leaves and flowers of *Crataegus azarolus*. All the fractions were compared with ascorbic acid and BHT as standard references. The results showed that the various fractions of *Crataegus azarolus* had a good scavenging effect. Ethyl acetate fractions (EAF) showed the highest DPPH radical scavenging activity with an IC_{50} value of 9.72±0.102 µg/ml, followed by crude methanolic extract (CME) and diethyl ether fractions (DEF) with IC_{50} values of 20.96±0.340 and 68.69±2.490 µg/ml, respectively. The IC_{50} values of BHT and ascorbic acid were found to be 19.54±0.320 and 1.17±0.005 µg/ml, respectively. The Chloroform

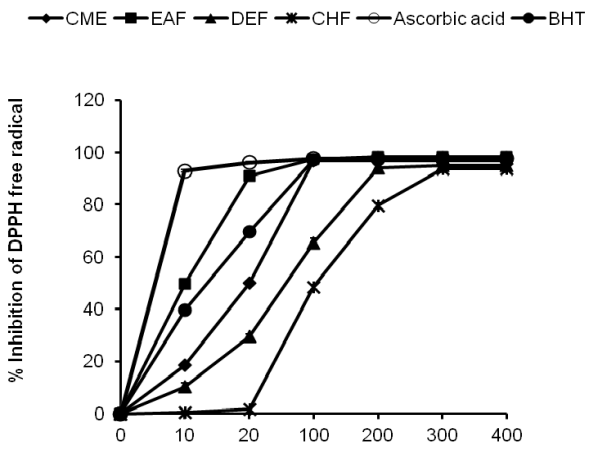

Figure 1. Free radical scavenging effect of the crude methanolic extract and fractions of leaves and flowers of *Crataegus azarolus* by DPPH

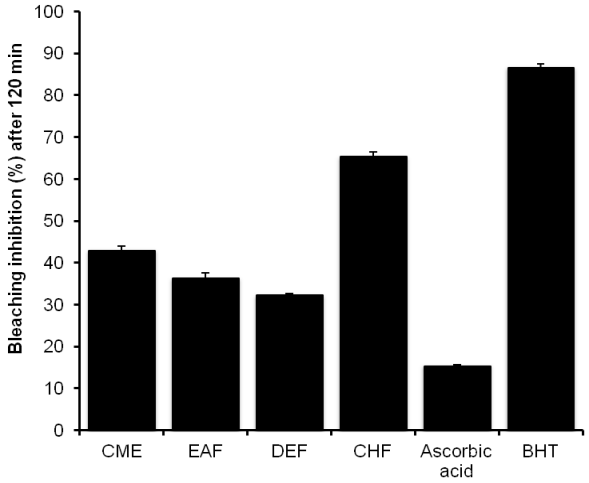

Figure 2. β-carotene bleaching activities of the crude methanolic extract and fractions of leaves and flowers of *Crataegus azarolus*

fractions exhibited the lowest free radical scavenging activity with an IC_{50} of 139.87µg/ml. This result was confirmed by other studies, where the highest antioxidant activity, was obtained with ethyl acetate. Experiments carried out *in vitro* by Mohammedi and Atik (2011), demonstrated that the flavonoid extract from the leaves and flowers of *Crataegus oxyacantha* has a strong antioxidant potential (IC_{50} 2.74µg / ml). Moreover, Dastmalchi and colleagues (2008) found that the ethanol extract of hawthorn is capable of trapping the radical DPPH in a dose-dependent manner.

Based on the results of this study, the high phenolics and flavonoïds contents in *Crataegus azarolus* extracts can explain its high free radical scavenging capacity. It has been recognized that phenolics and flavonoïds contents of plants, can increase cellular defenses and diminished malignant transformation of cells. These phytochemicals can inhibit and scavenge the free radicals by donating hydrogen atom and exhibit important role as potential antioxidant (Kähkönen *et al*., 1999; Proestos *et al*., 2006).

Reducing power

In the reducing power method, the presence of antioxidants in the test compounds or extract reduction of Fe^{3+}/ ferricyanid complex to the ferrous form Fe^{+2}, was determined by measuring absorbance of the Perl's Prussian blue at 700 nm . This method is based on the presence of reductones, which exert the antioxidant action by breaking the free radical chain by donating a hydrogen atom (Nabavi *et al*., 2009a). The reducing power had been used in the model system to investigate the potential antioxidant capacity of several natural compounds (Beckman *et al.*, 1990).

Reducing power of the crude methanolic extract and different fractions of *Crataegus azarolus* increased with increasing concentration (Table.3). Among the fractions, the ethyl acetate fraction (EAF) of *Crataegus azarolus* showed the highest reducing power ability (EC_{50}= 30.96±0.563 µg/ml) as compared to other studied fractions (EC_{50} values of CME and DEF are 45.33±0.110 and 67.79±4.720 µg/ml, respectively), while chloroform fraction (CHF) had considerably less effective reducing power (EC_{50} = 642.66±3.214).

Ascorbic acid and BHT showed high reducing power with EC_{50} value of 5.94±0.047 and 12.14±0.128 µg/ml, respectively. Bouaziz *et al.* (2014) studied the antioxidant activity of four extracts prepared from *Crataegus azarolus* leaves: FAE, CME, CHF, and EQE. Their results indicated that FAE exhibited the better activity followed by CME, CHF and EQE. Numerous investigations, have confirmed the high linear correlation between total phenolic contents and antioxidant activity of plant extracts (Zheng and Wang, 2001; Cai *et al*., 2004).

Determination of antioxidant activity with the β-carotene bleaching assay

In β-carotene blanching assay, oxidation of linoleic acid produces hydroperoxides as free radicals during incubation at 50°C, which attack the β-carotene, resulting in a bleaching of the reaction emulsion. Figure 2, shows the antioxidant activity of leaves and flowers of *Crataegus azarolus* extracts as measured by the blanching of β-carotene. The obtained results showed that all extracts of Crataegus exerted a moderate antioxidant activity, in the

Table 3. EC_{50} values (µg/ml) of the crude methanolic extract and fractions of leaves and flowers of *Crataegus azarolus* in reducing power assay

	EC 50
CME	45.33 ±0.11
EAF	31.04 ± 2.02
DEF	67.79± 4.72
CHF	642.66 ± 3.21
Asorbic acid	5.9 ± 0.04
BHT	12.14 ± 0.12

comparison with BHT.

The inhibition ration of the chloroform (CHF), crude methanolic extracts (CME), ethyle acetate fraction (EAF), diethyl ether fraction (DEF) of *Crataegus azarolus* after 120 min reaction time were 65.45±1.027%, 43.00±0.974%, 36.37±1.170%, 32.33±0.304% respectively. BHT and chloroform extracts were the strongest antioxidants (80.53±0.770%, 65.45±1.027% respectively), while Ascorbic acid showed no antioxidant activity (15.51±0.223%). The presence of antioxidants in extracts will minimize the extent of β-carotene yellow color by neutralizing the hydroperoxide formed in the system (Jayaprakasha *et al*., 2001). Our study showed similar results as those obtained by Bor *et al.* (2012) for the ethanolic extract from the leaves of *Crataegus orientalis* (42.37%).

Conclusion

The present study concluded that the crude methanolic extract and its various fractions obtained from the leaves and flowers of *Crataegus azarolus* exhibit interesting antioxidant capacity. The obtained results show that the methanolic extract and ethyl acetate fraction contained the highest amount of flavonoïds and phenolics compounds and exhibited great antioxidant activities, when compared to other solvent fractions. It can also be concluded that *Crataegus azarolus* extract can be used as a good source of natural antioxidant as well as in pharmaceutical applications.

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