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Original Article

Antioxidant activity of aqueous extracts from *Crataegus oxyacantha* leaves

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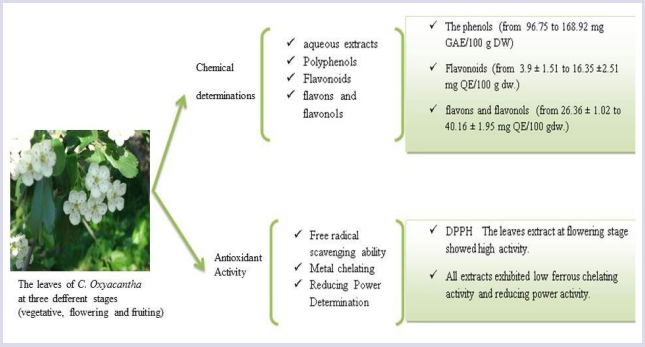
ABSTRACT

Background: *Crataegus (Rosaceae)* genus comprises more than 200 species worldwide but few species have been used medicinally. In the Mediterranean region, the predominant species of this genus is *C. oxyacantha* syn. *Monogyna* (known as Hawthorn) which is protective against the treatment of mild heart diseases. **Methods:** The leaves were collected at three different stages (vegetative, flowering and fruiting). The aim of this work was to determine total phenol, flavonoid, flavon and flavonol contents, as well as antioxidant activity of hawthorn leaves aqueous extracts. Chemical determinations were carried out using spectrophotometric methods, whereas antioxidant activity was assessed according to 1,1 diphenyl-2-picrylhydrazyl(DPPH), iron chelating ability and reducing power. **Results:** Total phenol content varied from 96.75 to 168.92 mg gallic acid equivalent (GAE)/100 g of dry weight; flavonoids ranged from 3.9 ± 1.51 to 16.35 ± 2.51 mg of quercetin equivalent (QE)/100 g of dry weight from the extract of leaves at flowering, vegetative and fruiting stages respectively; and the flavons and flavonols ranged from 26.36 ± 1.02 to 40.16 ± 1.95 mg of quercetin equivalent (QE)/100 g of dry weight. The leaves extract at flowering stage showed high scavenging activity against the free radical DPPH with an IC50 value of 45.23 ± 0.07 µg/ml. The leaves extract from all the stages exhibited low ferrous chelating activity and reducing power activity (IC_{50} between 262.26 ± 1.32 and 506.04 ± 1.95 µg/ml) compared to BHA (IC_{50} 41.41 ± 0.51 µg/ml).

Key words: Antioxidant activity, *C. oxyacantha*, Hawthorn, Phenolic content, *Rosaceae*.

SUMMARY

- The leaves at the flowering stage showed the highest polyphenol and flavonoid content followed by those at the vegetative andfruiting stages.
- The extract of leaves in the vegetative stage shows the higher content of flavones and flavones.
- The leaves extract at floweringstage showed high scavenging activity against the free radical DPPH.
- The leaves extract from all the stagesexhibited low ferrous chelating activity and reducing power activity.



PICTORIAL ABSTRACT

Abbreviations used: DPPH (1,1-diphenyl-2-picrylhydrazyl), BHA (butylated hydroxyanisol), Na_2co_3 (Sodium carbonate), $Alcl_3$ (Aluminium chloride), EDTA (Ethylenediaminetetraacetic acid), QE (quercetin equivalent), GAE (gallic acid equivalent).

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INTRODUCTION

Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism. The oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS).[1] ROS, which include free radicals such as superoxide anion radicals ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$) and non-free radical species such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are various forms of activated oxygen. The interaction of these species with molecules of a lipid produces new radicals (hydro peroxides and peroxides). In response to a variety of factors including tobacco smoke, pollutants, ionizing radiations, alcohol, synthetic pesticides and solvent, their production increases.[2] ROS can cause oxidative damage to proteins, lipids, enzymes and DNA and have also been linked to pathogenesis of oxidative diseases.[3] Living organisms possess a number of protective mechanisms against the oxidative stress and toxic effects of ROS. Antioxidants regulate various oxidative reactions naturally occurring in tissues and are evaluated as potential anti-aging agents.[4] Hence, antioxidants can terminate or retard the oxidation process by scavenging free radicals, chelating free catalytic metals and also by acting as electron donors. Antioxidant compounds can scavenge free radicals and increase shelf life by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food and pharmaceutical products during processing and storage. Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods.[5,6]

Hawthorn (*Crataegus spp.*), belonging to the Rosaceae family, consists of small trees and shrubs. Common names for hawthorns may include mayblossom, quick thorn, whitethorn, haw hazels; gazelle, halves; hawthorn, and bread and cheese tree.[7] They are native to the Mediterranean region, North Africa, Europe and Central Asia. There are more than 200 species worldwide, but very few have been tested and used for medicinal purposes. These include *C. oxycantha*, *C. laevigata*, *C. monogyna*, *C. orientalis* and *C. pinnatifida*.[8] The medicinal use of extracts or tinctures prepared from leaves, flowers and/or fruits dates back to ancient times. They are now officially listed as herbal drugs in pharmacopoeias in countries such as Germany, France, China and England.[9,10]

In this study, three different aqueous extracts leaves at three developmen-

tal stages were prepared and their antioxidant activities *in vitro* were determined, including free radical scavenging effects, chelating ability and reducing power in order to evaluate its natural antioxidant properties.

MATERIALS AND METHODS

Plant material and chemicals

Leaves of *C. oxyacantha* were collected from Beni Fouda, Algeria in three phases (vegetative, flowering and fruiting phases). The plant was taxonomically identified in department of Ecology and Vegetal Biology, Faculty of Nature and Life Sciences, University F.A. Setif-1. The plant samples were cut into small pieces and air dried at room temperature.

1,1-diphenyl-2-picrylhydrazyl (DPPH), quercetine, gallic acid, ferrozine, potassium ferricyanide, butylated hydroxyanisol (BHA) were obtained from Sigma Chemical (St. Louis, MO, USA). All other chemicals and solvents were obtained from Fluka.

Aqueous extracts

For the preparation of water extracts, leaves (10 g) were extracted with boiling water (250 ml) for 10min. The plant materials remained in the warm water for 15 min and were then filtered. The filtrates were concentrated in a rotary evaporator (Büchi) to reduce the volume and kept in dark until use.[11]

Total phenolic content determination

Total phenolic content of *C. oxyacantha* extracts was determined according to Folin-Ciocalteu spectrophotometric method.[12] A volume of 0.5 mL of 50% Folin-Ciocalteu reagent was mixed in a test tube containing 0.1 mL of extract. The resultant mixture was allowed to react for 1 min and 1.5 mL of 20% Na_2CO_3 was added. It was mixed thoroughly and placed in the dark for 2 h and then the absorbance was recorded at 760 nm using a visible light spectrophotometer (Spectronic 20 genesys TM). A gallic acid standard curve was obtained for the calculation of phenolic content.

Total flavonoid content determination

Total flavonoid content was determined using a spectrophotometric method based on the formation of flavonoid complex with aluminum. A volume of 1 ml of 2% $AlCl_3$ methanol solution was added to 1 ml of sample solution at room temperature. After 30 min incubation the absorbance was measured at 430 nm using a visible light spectrophotometer. Yellow color indicated that the extracts contained flavonoids. Quercetin was used as standard for calibration. Total flavonoid content was calculated as quercetin equivalent (QE).[13]

Determination of flavons and flavonols

Flavons and flavonols in extracts were expressed as quercetine equivalent. Quercetine was used to make the calibration curve. The standard solutions or extracts (0.5 mL) were mixed with 1.5 mL 95% ethanol (V/V), 0.1 mL 10% $AlCl_3$ (m/V), 0.1 mL (1M) potassium acetate and 2.8 mL water. The volume of 10% $AlCl_3$ was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured spectrophotometry at 415 nm.[13]

Free radical scavenging ability

Antiradical scavenging activity was evaluated using 1,1-diphenyl-2-picrylhydrazil (DPPH) radical (0.004% in methanol), according to the method described by Kulsic.[14] The extracts were dissolved in distillated water. BHA was used as the standard.

1mL of the DPPH solution was mixed with 1mL of the sample solution or the standard and then kept in the dark for 30 min. The optical density (DO) was measured using a Spectrophotometer at 515 nm against methanol. The blank consist of 1mL of methanol and 1mL of DPPH solution (0.004%).

All determinations were performed in triplicate. The optical density was recorded and percent of inhibition (PI) was calculated as follows: PI% = $[(A_0 - At)/A_0]$ 100, where

A_0 is optical density of the blank and A_t is optical density in the presence of extracts.

Metal chelating

Ferrous ions chelating activity of aqueous extracts of *C. oxycantha* and commercial antioxidants was determined according to Geckil *et al* (2005)[15] method. Into tubes containing 1.7 ml distilled water and 50 µl of 0.2 mM $FeCl_2$, 50 µl of sample solution was added and the mixture was left at room temperature for 5 min. To this mixture, 0.1 ml ferrozine (5 mM) was added and final color was monitored spectrophotometrically at 562 after 10 min incubation. The metal chelating efficiency of sample was determined by comparing with the chelating activity of Ethyl Diamine Tetra-acetic Acid (EDTA). The inhibition percentage of ferrozine-Fe^{2+} complex formation against blanks containing $FeCl_2$ and ferrozine was calculated by the formula;

% inhibition=[(A_0- A_1)/ Ac] x100

Where, A_0 indicates the absorbance of the control and A_1 the absorbance in the presence of *C. oxyacantha* extracts, commercial antioxidant or EDTA.

Reducing Power Determination

The reducing power of leaves extracts of *C. oxyacantha* was determined according to Lim *et al.*(2007),[16] 0.1 ml of each extracts in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_2Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of tri-chloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured spectrophotometrically at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. BHA was used as positive control.

RESULTS AND DISCUSSION

Total polyphenols, flavonoids, flavons and flavonols content were evaluated in the *C. oxyacantha* leaves aqueous extract obtained during three different stages by Folin Ciocalteu and $AlCl_3$ methods (Table 1). Total phenolic content of the leaves at the flowering and vegetative stages were 168.92 mg GAE/100 g DW and 165.35 mg GAE/100 g DW respectively, whereas at the

Table 1: Content of total polyphenols, total flavonoids and flavons and flavonols determined in the *C. oxyacantha* leaves aqueous extracts obtained at different stages

Leaves Extracts	Total polyphenols[1]	flavonoids[2]	flavons and flavonols[2]
Vegetative	165.35 ± 0.0	9.65 ± 2.18	40.16 ± 1.95
Flowerings	168.92 ± 0.0	16.35 ± 2.51	26.36 ± 1.02
Fruiting	96.75 ± 0.0	3.9 ± 1.5	30.39 ± 0.6

1) Mg of GAE/ 100 g dw; 2) Mg of QE/100 g dw; 3) Each value in the table is represented as mean ± SD (n=3).

Table 2: Antioxidant activity of C. oxyacantha leaves extracts at vegetative, flowering and fruity stages and positive control (BHA, EDTA)

	IC_{50} (ug/ml)[3]		
	DPPH	R.P. [1]	M.C[2]
Vegetative	64.79 ± 1.07	506.04 ± 1.95	1400.71 ± 4.09
Flowering	45.23 ± 0.07	262.26 ± 1.32	1257.84 ± 0.0
Fruity	72.51 ± 2.	379.07 ± 2	1500 ± 0.0
BHA	14.12 ± 0.0	41.41 ± 0.51	-
EDTA	-	-	43.53 ± 1.35

1) Reducing Power; 2) Metal Chelating.; 3) Each value in the table is represented as mean ± SD (n=3).

fruiting stage it was 96.75 mg GAE/100 g DW. These results show that the leaves can make a rich source of polyphenols when compared to the fruit content, the part of the plant most investigated. In comparison, Kosatic[17] found that the polyphenol content ranged from 2.12 ± 0.12 to 30.63 ± 2.56 mg GAE /g of fresh fruit when extracted with organic solvents.

The leaves at the flowering stage showed the highest flavonoid content (16.35 ± 2.51 mg QE/100 g DW), followed by those at the vegetative and fruiting stages. The extract of leaves in the vegetative stage shows the higher content of flavons and flavonols (40.16 ± 1.95 mg QE/100 g DW). Total polyphenol and total flavonoid varies in different leaf extracts.

DPPH radical scavenging activity

The free radical scavenging activities were evaluated using the DPPH assay (Table 2). DPPH possesses a proton free radical having characteristic absorption, which decreases on exposure to radical scavengers.[18] The method is widely used to predict the ability of flavonoids to transfer H atoms to radicals is based on the free radical, 1, 1-diphenyl-2-picrylhydrazyl in the DPPH assay. The antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. In this study, DPPH radical scavenging method was used to assess the determination of potential radical scavenging activities of aqueous extract of leaves at vegetative, flowering and fruiting stages. The reduction ability of DPPH radicals' formation was determined by the decrease in its absorbance at 515 nm induced by antioxidants. Free radical scavenging effects results were defined as the amount of antioxidant necessary to decrease the initial DPPH radical concentration by 50% in 30 minutes (IC_{50}). The leaves at flowering stage exerted the highest antiradical activity with an IC_{50} value of 45.23 ± 0.07 µg /ml, followed by leaves at vegetative stage (64.79 ± 1.07 µg/ml) and leaves in fruiting stage (72.51 ± 2 µg/ml). However, these activities are very low comparatively to that of BHA with an IC_{50} value of 14.12 µg/ml (Table 2). Generally, leaves extract at flowering stage have the highest free radical scavenging activity. Our results suggested that phenolic content increased the free radical scavenging activities.

Metal chelating

An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which possess the ability to catalyze hydroperoxide decomposition and Fenton-type reactions.[19] Chelating agents may also serve as secondary antioxidants since they reduce redox potential, thereby stabilizing the oxidized forms of metal species.[20] Therefore, the ion (II) chelating capacities of the extracts were screened. The iron–ferrozine complex has maximum absorbance at 562 nm and a large decrease in absorbance indicates strong chelating power by forming a stable iron (II) chelate. In the presence of chelating agents, complex formation between ferrozine and Fe^{2+} is disrupted, resulting in reduction in the red colour of the complex. Measurement of the color decrease therefore allows estimation of the chelating activity of the co-existing chelator.[21,22] In this study, the aqueous extracts of three stages of leaves and reference compounds (EDTA) were found to have interfered with the formation of the ferrous and ferrozine complex, indicating that they contained chelating activity and were able to capture ferrous before ferrozine.

The metal chelating capacities of the leaves at vegetative, flowering and fruiting stages water extracts are shown in Table 2. The metal inhibition capacities increased with increasing extract concentrations, indicating that chelating compounds were present in these plant extracts. The metal chelating activity of the extracts was strongly dependent on the stage of collection due to the various antioxidant potentials of the compounds. Among these, the flowering stage extract possessed higher metal chelating activities compared to the other extracts.

Transition metals, especially iron (II), found in the biological systems may act as pro-oxidants. A pro-oxidant does not act as harmful agent for bio molecules directly, but facilitates production of such species which may cause damage to bio-molecules. Polyphenolic compounds, thus by binding with Fe (II), do not allow them to act as pro-oxidant and thus add to an indirect defense mechanism against potentially harmful radical species.

Reducing Power

Different studies have indicated that the electron donation capacity, reflecting the reducing power, of bioactive compounds is associated with antioxidant activity.[23] The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.[24,25] There are a number of assays designed to measure overall antioxidant activity or reducing potential, as an indication of host total capacity to withstand free radical stress.[26] In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power of leaves was excellent compared to that of the BHA standard (Table 2); the reducing power of leaves at flowering stage (262.26 ± 1.32 µg/ml) was higher than extracts of leaves at fruiting and vegetative stages (379.07 ± 2 and 506.04 ± 1.95 µg/ml respectively). Reducing power of BHA 41.41 ± 0.5 µg/ml.

CONCLUSION

The replacement of synthetic with natural antioxidants (because of implications for human health) may be advantageous. In the present study analysis of total phenolic and flavonoid content and antioxidant activities showed that the leaves of *C. oxyacantha* collected in different stages can be a potent source of natural antioxidants.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in this study.

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