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Phytochemical Analysis, Antioxidant Activity And Hypotensive Effect Of Algerian Azarole(*Crataegus azarolus* L.) Leaves Extracts.

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ABSTRACT

The present study was undertaken to investigate the hypotensive and the *in vitro* antioxidant activities of *Crataegus azarolus*L. (Rosaceae) , a plant widely used as natural remedy for hypertension in folk medicine. The antioxidant potential of methanolic extract (ME) and its three fractions of Chloroform (CHE), ethyl acetate (EAE)and water (AqE) have been investigated using several assays. Total phenolic, total flavonoid and tannins contents of the extracts were estimated, whereas EAE was also subjected to analysis by different chromatographic methods. Chemical analysis of EAE revealed to the isolation of two flavonoids; new favanol, (+)-catechin (1), and a previously identified flavonol, hyperoside (2) from C.azarolus leaves. EAE extract showed the highest polyphenolic and flavonoids contents (396.04 ± 1.20 mg GAE/g of dry extract and 32.73 ± 0.03mg QE/g of dry extract) respectively. Similarly, this extract possessed the highest antioxidant activity in all antioxidant models except for its ferrous ion chelating capacity whereas AqE and ME are the most active extracts. Intravenous administration of ME and EAE decreased mean arterial blood pressure, systolic and diastolic blood pressure in anesthetized rats dose-dependently, at the dose range of 0.4 to 12 mg/kg. In conclusion, the present study supported the significant potential to use C. *azarolus* by-products as a source of natural antioxidants and provides scientific justification for its traditional uses as cardioprotective and antihypertensive remedy.

Keywords:*Crataegus azarolus*, polyphenols, antioxidant, free radicals, hypertension.

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality worldwide and hypertension is one of the most important risk factors for CVD [1]. Hypertension affects more than 600 million populations and results in 13% of total deaths globally [2], and it is estimated that there will be 29% of the world's adult with hypertension by 2025 [3].

Intracellular oxidative stress is mainly caused by an imbalance between the activity of endogenous pro-oxidative enzymes and antioxidant enzymes [4, 5], and thus disturb vascular cell functions and alter the release of vasodilator mediators including nitric oxide (NO) leading to the pathogenesis of hypertension mainly by mediating aberrant contractility [1, 6]. Recent findings clearly show that the consumption of plant-derived polyphenolic compounds and natural antioxidant supplements may be used to protect the body against various diseases, including cancer, cardiovascular, and neurodegenerative diseases. Natural antioxidants help the endogenous antioxidant system to reverse oxidative damage or protect oxidative stress induced deterioration [7, 8].

Phenolic compounds and flavonoids have been found to have therapeutic applications against different diseases caused by oxidative stress, and recently several researchers demonstrated the correlation between polyphenolic compounds and the antioxidant activity of plant extracts [9].

Crataegus species (Rosaceae), known as "Hawthorn" are widely distributed throughout the northern temperate region of the world with approximately 280 species [10]. Extensive animal as well as human research studies have shown the most important features of *Crataegus* extracts in their positive isotropic, vasodilator, diutetic [11], and hypotensive effects [12]. The phytochemical studies revealed that the major components in hawthorn are polyphenols [13, 14, 15,16]. Many of these phenolic compounds have been shown to be cytoprotective by reducing oxidative stress thereby giving a solid basis to the proposal that the antioxidant content of *Crataegus* could account for its cardioprotective properties [17].

Several ethnopharmacological surveys on the therapeutic use of *Crataegus* azarolus, the predominant species which populates the mountains of the Mediterranean basin revealed its use *in* the Arab traditional medicine to treat cardiovascular diseases, as well as cancer, diabetes and sexual weakness [18]. Furthermore, the fruit and flowers of this plant have a hypotensive effect as well as acting as a direct and mild heart tonic [19]. However, the phytochemical profile and the pharmacological activity of *C. azarolus* leaves have not been investigated thoroughly. Thus, the present study was undertaken to analyze *C. azarolus* leaves chemically and to evaluate its *in vivo* hypotensive effect in anesthetized rats. Also, the antioxidant activity *using* different *in vitro* models and the total phenolic , flavonoids and tannins contents in the various leaves extract*s* were estimated to determine the relationship between free radical scavenging activity and polyphenolic contents.

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MATERIALS AND METHODS

Chemicals

Linoleic acid, ammonium thiocyanate, β-carotene, butylated hydroxyl toluene (BHT), were purchased from Fluka Chemical Co. (Buchs, Switzerland). 2, 2-diphenyl-1-picryl-hydrazyl (DPPH), ethylene diamine tetra acetic acid (EDTA), gallic acid, 6-hydroxy-2,5,7,8-tetramethyl chroman-2-carboxylic acid (trolox), 2.2´-Azino-bis(3-ethylbenzenothiazoline 6-sulfonic acid) (ABTS), Folin-Ciocalteu reagent were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium persulphate, potassium ferricyanide, trichloroacetic acid (TCA), thiobarbituric acid (TBA), Ferrozine, ferrous and ferric chloride were obtained from Merck. All other reagents were of analytical grade.

Animals

Healthy male adult albino rats, weighing 200–300 g were used. Animals were housed in an air-conditioned animal room, with 12 h/12 h light/dark photoperiod, and maintained with free access to water and ad libitum feeding.

Collection of plant material and preparation of extracts

The leaves of *C. azarolus* were collected from Setif region in eastern Algeria during June, 2012. The plant was identified by Pr. Hocine Louar from the laboratory of Botany, Faculty of Natural and Life Sciences, University, Setif, Algeria. The leaves were separated, dried under shadow and powdered. For preparation of dry leaves extracts, the extraction procedure for phenolic compounds was conducted as described by Markham [20]. The powdered plant material (100g) was extracted with methanol (85% and 50%), at room temperature for 5 days. Then, the suspension was filtered through a Buchner funnel and concentrated under reduced pressure on a rotary evaporator to give crude methanolic extract (ME). The aqueous solution was successively extracted with hexane, chloroform and ethyl acetate. Each fraction was evaporated to dryness under reduced pressure to give hexane (HE), chloroform (CHE), ethyl acetate (EAE), and the remaining aqueous (AqE) extracts. The extracts were stored at -20 °C until use.

Phytochemical analysis

The grounded plant material (1kg) was extracted by maceration with 5 L methanol (85%) at room temperature for 5 days. The filtered solvent was evaporated under vacuum and lyophilized to afford a crude methanolic extract (ME) (144 g). Using the same extraction protocol as above, the residue (100 g) was dissolved in 1L H_2O– MeOH mixture (9:1) and was then partitioned by successive extractions with different solvents of increasing polarity to yield CHE, EAE and AqE partitions. The EAE (28 g) was fractionated on column chromatography using silica gel S (0.063-0.2 mm, Riedel-Dehaen, 120g). The column was first eluted with chloroform, and then with chloroform- methanol mixtures of increasing polarity. Totally, 38 fractions (250 ml for each) were obtained and similar fractions were combined according to their thin-layer chromatographic plates (TLC), using different solvent systems.The resulting eight fractions were then purified by preparative thin-layer

chromatography and the structures of the isolated compounds were determined by mass spectrometry and ^{1}H and ^{13}C NMR spectra.

Determination of total polyphenols content

The total polyphenols content was determined by the Folin–Ciocalteu method as described by Li et al [21] with slight modification. In brief, 0.1ml of *C. azarolus* extracts were mixed with 0.5 ml of Folin-Ciocalteu reagent (diluted 10 times). After 4 min, 0.4 ml of 7.5% sodium carbonate (Na_2CO_3) solution was added. The final mixture was shaken and then incubated for 90 min in dark at room temperature. The absorbance of all samples was measured at 760 nm and the results are expressed in milligrams of gallic acid equivalents per gram dried weight (mg GAE/g DW).

Determination of total flavonoids content

The total flavonoids content of each extract was determined by a colorimetric method as described by Bahorun et al [22]. Each sample (1 ml) was mixed with 1 ml of aluminium chloride ($AlCl_3$) solution (2%) and allowed to stand for 10 min. Absorbance of the mixture was then determined at 430 nm versus prepared methanol blank. Results were expressed as quercetin equivalent per gram dried weight (mg QE /g DW).

Determination of tannins content

The capacity to precipitate haemoglobin was determined by using bovine fresh blood according to the method described by Batesmith (1973) [23]. Briefly, a volume of each plant extract was mixed with an equal volume of hemolysed bovine blood (absorbance = 1.6). After 20 min, the mixture was centrifuged at 4000 rpm for 10 min, and the absorbance of the supernatant was measured at 756nm. Results were expressed as mg equivalent tannic acid per gram dried weight (mg TAE/g DW).

Evaluation of antioxidant activity

DPPH radical scavenging assay

Free radical scavenging activity against 2, 2-diphenyl- 1-picrylhydrazyl (DPPH) radical was measured using the method described by Burits and Bucar [24]. 50 µl of different dilutions of the extracts were added to 5 ml of a 0.004% methanolic solution of DPPH. After 30 min at room temperature, the absorbance was measured at 517 nm. BHT, rutin, quercetin and gallic acid were used as standards. Inhibition of free radical DPPH in percent (I %) was calculated in following way:

$$I\% = 100 (A_{control} - A_{sample}) / A_{control}$$

Where A control is the absorbance of the blank solution (containing all reagents except the test compound), and A sample is the absorbance in the presence of the test compound. Extract concentration providing 50 % inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

ABTS radical scavenging activity assay

The spectrophotometric analysis of $ABTS^+$ radical scavenging activity was determined according to method of Re et al [25] with some modifications. This method is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of trolox, a water-soluble a-tocopherol analogue. The $ABTS^+$ solution was produced by the reaction of 7 mM of ABTS solution in 2.45 mM potassium persulfate (final concentration). The mixture was kept in the dark at room temperature for 24 h before use. The solution was diluted with methanol and equilibrated at room temperature to give an absorbance of 0.70±0.02 at 734 nm. Then, 50 µl of sample was mixed with 1ml of $ABTS^+$ solution and kept for 30 min at room temperature; the absorbance of reaction mixture was measured at 734 nm. Trolox was used as positive control. The $ABTS^+$ radical scavenging ability was calculated according to the same equation as that in the DPPH assay.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity was assayed according to the method of Li et al [26] with slight modification. In this system, hydroxyl radicals were generated by the Fenton reaction. Hydroxyl radicals could oxidize Fe^{2+} into Fe^{3+}, and only Fe^{2+} could combined with 1,10-phenanthroline to form a red compound (1,10-phenanthroline-Fe^{2+}) with the maximum absorbance at 536 nm. The concentration of hydroxyl radical was reflected by the degree of decolorization of the reaction solution. Briefly, A mixture of 100 µl of 1,10-phenanthroline (5.0 mM), 100 µl of FeSO4 (5.0 mM) and 100 µl of EDTA (15 mM) were mixed with 70 µl of sodium phosphate buffer (0.2 M, pH 7.4). Then, 100 µl of sample (1 mg/ml) and 140 µl of H_2O_2 (0.01%) were added. The mixture was incubated at 37 °C for 60 min, and the absorbance was measured at 536 nm. Vitamin C was used as a positive control. To calculate hydroxyl radical scavenging, the following equation was used:

$$\text{Hydroxyl radical scavenging activity (\%)} = (A_s - A_0) \times 100/(A_c - A_0)$$

Where: As is the absorbance of the sample ; A_0 is the absorbance of the blank solution using distilled water instead of sample; and Ac is the absorbance of a control solution in the absence of H_2O_2.

Ferrous Ion Chelating Activity

Ferrous ion chelating activity was measured by inhibition of the formation of Fe^{2+}-ferrozine complex after treatment of various concentration of *C. azarolus* fractions with Fe^{2+}, following the method of Decker and Welch [27]. The reaction mixture (1.50 ml) contained 500 µl test material or EDTA, 100 µl $FeCl_2$ (0.6 mM in water) and 900 µl MeOH. The control contained all the reaction reagents except the extract and EDTA. The mixture was shaken well and allowed to react at room temperature for 5 min; 100 µL of ferrozine (5 mM in methanol) was then added. The absorbance of the Fe^{2+}-ferrozine complex was measured at 562 nm. The chelating effect was calculated as a percentage, using the same equation as that in the DPPH assay. IC_{50} value defined as the effective concentration of test material that is required to chelate 50% of iron ions.

Reducing power

Fe^{+3}reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action [28]. The reducing power of the *C. azarolus* extracts was determined according to the method of Chung et al [29]. A 0.1 ml aliquot of different concentrations of each fraction, were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min, to reduce ferricyanide into ferrocyanide. After that, 0.25 ml of 10% trichloroacetic acid was added to the mixture to stop the reaction and centrifuged at 2790 g for 10 min. The supernatant (0. 25 ml) was added to distilled water (0.25 ml) and 0.1% ferric chloride (0.5 ml) and then the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. BHT was used as a positive control.

β-Carotene Bleaching Assay

In this assay, antioxidant capacity is determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation [30]. A stock solution of *β*-carotene-linoleic acid mixture was prepared as follows: 0.5 mg *β*-carotene was dissolved in 1 ml of chloroform and 25 µl linoleic acid and 200 mg Tween 40. Chloroform was completely evaporated, and then, 100 ml distilled water saturated with oxygen (30 min, 100 ml/min) were added with vigorous shaking. 2500 µl of this reaction mixture were dispensed into test tubes and 350 µl of the various extracts, prepared at 2 mg /ml concentrations, were added and the emulsion system was incubated for 48 h at room temperature. The same procedure was repeated with synthetic antioxidant BHT as positive control, and blanks (MeOH and H_2O). The absorbance of the mixture was measured at 490 nm after; 0, 1, 2, 4, 6 and 24 hour. The antioxidant activity (AA) was measured in terms of successful bleaching of *β* -carotene by using the following equation:

AA% = A sample / A BHT × 100.

A sample: Absorbance in the presence of the extract; A BHT: Absorbance in the presence of positive control BHT.

Ferric thiocyanate (FTC) method

The antioxidant activity of plant extracts on the inhibition of linoleic acid peroxidation was assayed with a modified thiocyanate method described by Yen et al [31]. FTC method was used to determine the amount of peroxide at the initial stage of lipid peroxidation. The peroxide reacts with ferrous chloride ($FeCl_2$) to form a reddish ferric chloride ($FeCl_3$) pigment. In this method, the concentration of peroxide decreases as the antioxidant activity increases. A sample solution (0.5 ml) of plant fractions was mixed with 2.5 ml of 0.02 M linoleic acid emulsion at pH 7.0 and 2 ml of 0.2 M phosphate buffer at pH 7.0. The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier, and 50 ml of phosphate buffer. The reaction mixture was incubated at 37 °C for 5 days. To 0.1 ml of the reaction mixture at 24 h intervals was added 75% EtOH (4.7 ml), 30% ammonium thiocyanate (0.1 ml), 0.02 M ferrous chloride in 3.5% HCl (0.1 ml).

Three minutes after the addition of ferrous chloride to the reaction mixture, the absorbance was measured at 500 nm. The measurement was taken every 24 hours until the absorbance of the control reached its maximum value. BHT and vitamin C were used as positive controls.

Thiobarbituric Acid (TBA) Method

The TBA test was conducted on the final day of FTC according to the method described by Kikuzaki and Nakatani [32] to determine the malonaldehyde (MDA) formation from linoleic acid peroxidation. The same sample preparation method as described in the FTC method was used. To 1 ml of sample solution, 20% trichloroacetic acid (2 ml) and thiobarbituric acid solution (2 ml) were added. The mixture was placed in a boiling water bath for 10 minutes. After cooling, it was then centrifuged at 3000 rpm for 20 minutes. Absorbance of the supernatant was measured at 532 nm. Antioxidant activity was recorded based on the absorbance of the final day of the FTC assay. Both methods (FTC and TBA) described antioxidant activity by percent inhibition:

$$\% \text{ inhibition} = 100 - [(A \text{ sample} / A\text{control}) \times 100]$$

Where A control and A sample are the absorbances of the control (without sample) and the experimental (with sample) reactions, respectively.

Blood pressure measurements in anesthetized rats

The blood pressure of the anesthetized rats was recorded by method described by Abdalla et al [33]. Male albino rats were anesthetized with sodium thiopental (50 mg/Kg body weight; i.p). The right common carotid artery was exposed and a catheter was introduced for the recording of blood pressure using P23AA Statham pressure transducer situated at the level of the heart and connected to a Gilson polygraph. The right femoral vein was also catheterized for the intravenous injection of ME and EAE. After a steady baseline of blood pressure was obtained (about 15 minutes), ME and EAE was injected in doses of 0.04, 0.12, 0.4, 1.2, 4, and 12 mg/kg body weight. Blood pressure was allowed to return to the resting level before every next dosing. The changes in systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial blood pressure (MABP) were recorded and expressed as percent of their respective control values obtained before drugs administration. Mean arterial blood pressure (MABP) was calculated by adding the values of DBP and one-third of pulse pressure.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism (version 5.01 for Windows). All *in vitro* results were calculated as mean ± standard deviation (SD) and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnet's test. The pharmacological results were presented as mean ± standard error of mean (S.E.M.) of six experiments. Values were compared by using paired Student's *t*-test. In all cases, The *P*-values less than 0.05 were considered statistically significant.

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RESULTS AND DISCUSSION

Phytochemical Analysis

The structure elucidation of the isolated bioactive compounds from the EAE fraction of *C. azarolus* was carried out by spectral techniques; ^{1}H NMR, ^{13}C NMR, DEPT 135 and mass spectra. The structures of two compounds were: hyperoside (Quercetin-3-O-galactoside) and (+)-catechin (Fig.1). The chemical structure of this two flavonoids was identified in comparison of NMR and mass spectral results of this study data with the literature values [34, 35].

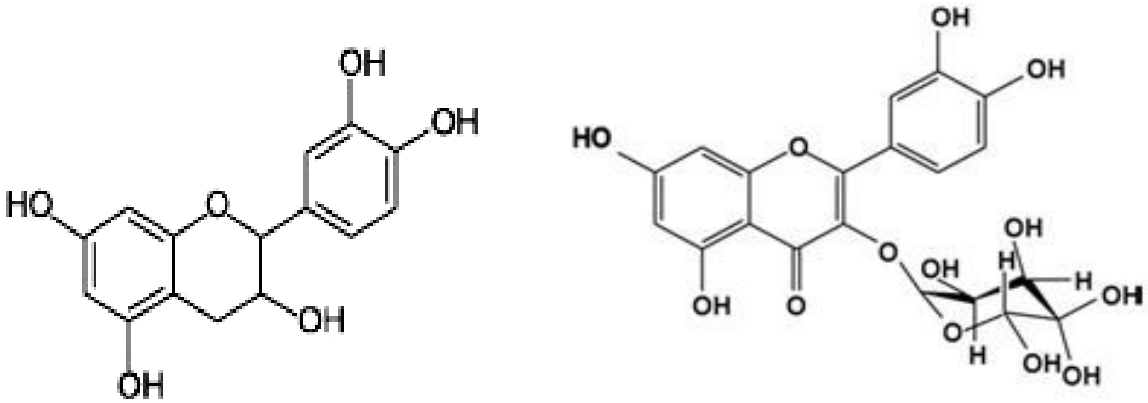

Fig.1. Bioactive compounds isolated from of *C.* azaolus leaves
(+)- Catechin(1) and hyperoside(2)

(+)-Catechin

The mother liquor of fraction [5] gave one major spot which was purified using preparative TLC eluting with chloroform /methanol (80:20) to give a red–orange powder identified as (+)-catechin**(1).**

^{1}H-NMR (DMSO-d_6) δ: 2.49 (1H,m,H-4 α), 2.69 (1H,dd,J=4.1,16,2Hz,H-4 β), 4,01(1H,br .s,H-3), 6.68 (2H,m, H-5',H-6'), 4.68(1H,br .s,H-2), 6.9 (1H,s, H-2') , 5.74(1H,d,J=1.8Hz,H-6), 5.92(1H,d,J=1,8Hz, H-8).
^{13}C-NMR (DMSO-d_6) δ: 28.7 (C-4), 99.2 (C-6), 65.4 (C-3), 78.5 (C-2), 94.6 (C-6),95.6 (C-8),99.0(C-10),115.5(C-2' and C-5'),118.4 (C-6'), 131.1 (C-1'), 144.9 (C-3 'and C-4'), 156.1 (C-7), 156.7 (C-5), 157.0(C-9), 76.3 (C-5), 102.2 (C-1).

Quercetin-3-O-glucoside (hyperoside)

Treatment of fraction (6-12) with methanol afforded a pure yellowish solid , which was identified as quercetin-3-O-galacoside **(2)**.

The ^{1}H-NMR (DMSO-d_6) δ: 3.15-5.65 (sugar protons), 5.85(1H, d, J= 7.7 Hz ,anomeric). 6.19(1H, d, J= 2Hz, H-8), 6.8(1H, d, J = 8.5 Hz, H-5'), 7,48 (1H, d, J = 2,2 Hz, H-2'), 7.62 (1H,dd,J =2,2 and 8,5Hz, H-6')

The ^{13}C-NMR (DMSO-d_6) δ: 94.0 (C-8), 99.2 (C-6), 104.4 (C-10), 115,7(C-2'), 116.4 (C-5'), 121.5 (C-6' and C-1'), 133.9 (C-3), 145.3 (C-3'), 149.0 (C-4'),156,7(C-2 and C-9),161.7(C-5),164.6 (C-7), 178 (C-4).galactose signals: 60.6 (C-6), 68.4 (C-4),71.7 (C-2), 73.6(C-3),76.3(C-5), 102.2 (C-1).

Crataegus species has shown to be a rich source of polyphenols. In particular, dihydroflavonoids, glucosides and galactosides[13]. Well-known naturally occurring flavonoids, i.e., hyperoside, catechin were previously reported from several *crataegus* species. Hyperoside was isolated from the leaves [36] and flowers of *C. azarolus*[37], C*. aronia*[38], *C. tanacetifolia*[39], *C. monogyna*[40], *C. pentagyna*, and C*. laevigata*[38]. Catechin was extracted from *C. pinnatifida*[41], C. *monogyna*[42], and *C. azarolus* flowers [37]. Nevertheless, as far as we know, this is the first report for the isolation of (+)-catechin in *C. azarolus* leaves.

Total polyphenols, flavonoids and tannins contents

Plant phenolics, tannins and flavanoids represent major groups of plant constituents that work predominantly as powerful antioxidants or scavenger of free radicals. They play beneficial role in human health and cure or prevent ailments such as inflammatory disorders, cardiovascular diseases, cancer and diabetes which occur due to the deregulation of free radicals generation in the cells [43]. In the present study, total phenolics, flavonoids and tannins contents among the different extracts are presented in Table 1.The total phenolic content in *C. azarolus* extracts was determined by the Folin–Ciocalteu method, which is considered the best method for total phenolic content (including tannins) determination. The total polyphenols content varied in the different extracts and ranged from 38.25 to 396.04 mg GAE /g DW, and their contents were in the following order: EAE > ME > CHE >AqE .

Table 1: Total polyphenols, flavonoids and tannins contents in *C .azarolus* extracts.

Extracts	Total phenolic content (mg GAE/g)	Total flavonoids (mg QE/g)	Total tannins (mg TAE/g)
ME	188.91±0,633	21.03±0.810	115.33±1.191
CHE	149.08± 0.416	24.46±0 .166	126.53±1.045
EAE	396.04±1.204	32.62±0.033	90.26±0 .834
AqE	38.25± 0.015	2.12±0.0134	100.6±0.346

ME; methanolic extract, CHE; chloroform extract, EAE; ethyl acetate extract, AqE; aqueous extract. Results are expressed as means ± SD (n = 3)

Flavonoids were quantified using $AlCl_3$ method. The results showed that total flavonoids content of various plant fractions decreased in the following order: EAE > CHE > ME >AqE. EAE extract contain high amounts of phenolics (396.04±1.204 GAE/g DW) and flavonoids compounds (32.62±0.033 QE/g DW), indicating that ethyl acetate was to be the best solvent to concentrate phenolic substances of intermediate polarity. This is in accordance with the findings of other authors [44, 45, 46].

Nevertheless, EAE from *C .azarolus* presents higher contents in total polyphenols and flavonoids in regard to the same extract prepared from flower buds and open flowers of *C.*

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azarolus. var. *aronia* grown in Tunisia (16.4 mg gallic acid equivalents/ g DW; 7.3 rutin equivalent /g DW) [37].

Tannins are complex polyphenols found in plant extracts and could be good candidates, in a perspective of reducing the cost of prevention of lipid oxidation. In fact, it has been demonstrated that the most efficient inhibitors of oxidation are the polyhydroxylated phenyl compounds, which are also present in tannins [47]. As shown in Table 1, *C. azarolus* extracts contained similar levels of tannins contents. It was found that CHE contained the highest tannins concentration of 126.53±1.045 mg TAE /g DW followed by ME (115.33±1.191 TAE/ g DW) , and AqE(100.6±0.346 TAE/ g DW), while the low tannins content was noticed for EAE (90.26±0 .834 TAE/ g DW). The results strongly suggest that the phenolics are important components of this plant and some of the pharmacological effect could be attributed to the presence of these invaluable components.

Antioxidant activity evaluation

DPPH radical scavenging activity

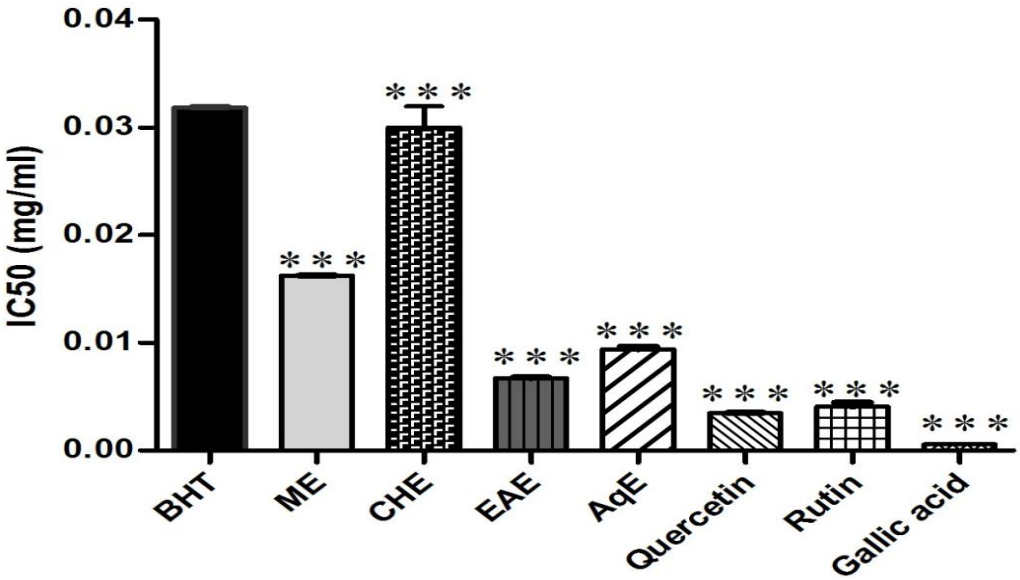

Fig. 2. DPPH free radical scavenging activity of different *C.azarolus* extracts. ME: methanolic extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as IC_{50} means ± SD (n = 3). *(p < 0.001) compared to BHT as standard.**

The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples [28]. DPPH is a stable nitrogen-centered free radical whose color changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers [48]. The antioxidant activities obtained by the DPPH method for the *C. azarolus* extracts are displayed in Fig. 2. This activity was compared with BHT as a synthetic antioxidant. The results show that all fractions can reduce the signal intensity of DPPH and their radical-scavenging activities increased with increasing concentration. IC_{50} for DPPH radical-scavenging activity was in the order: EAE (0.006±0.0001mg /ml) > ME(0.016 ±0.0004mg /ml) > CHE (0.049±0.0016mg /ml) >AqE (0.066±0.0004mg /ml) respectively. Hence, EAE, ME

and CHE fractions were significantly more active than our positive control, BHT ($p < 0.001$). From our finding, it may be postulated that *C. azarolus* contained substrates mainly were polyphenols , which were electron donors and could react with free radicals to convert them to more stable products and terminate the radical chain reaction. Also, it is reported that polyphenolic compounds in plants possess strong antioxidant activity and help in protecting cells against oxidative damage caused by free radicals [49]. In previous studies, it was found that ethyl acetate extract prepared from the floral buds and open flowers of *C. azarolus*[37] and fruit extracts of *C. monogyna* prepared with ethanol and water were effective in scavenging DPPH. Clear synergistic effects were observed among different phenolic compounds in scavenging free radicals [42].

ABTS radical scavenging activity

ABTS radical is relatively stable but readily reduced by antioxidants. The scavenging activity against cationic ABTS radical indicates the ability of fractions to act as electron donors or hydrogen donors in free radical reactions [50]. To assay the ABTS radical scavenging of *C. azarolus* fractions, cationic ABTS radical decolorization was carried out. Similar to DPPH assay, the results shown in Fig.3 revealed that EAE exhibits a strongest antioxidant activity in ABTS method (IC_{50} = 0.0035±0.0007mg/ml), which is comparable to the standard used. The other fractions also showed good ABTS radical-scavenging activities in the order of EAE > ME > CHE >AqE. It was found that no significant difference ($p > 0.05$) in antioxidant activities between EAE, ME and trolox as standard, while CHE and AqE showed a significant difference ($P>0.001$). The effect of EAE and ME is very probably attributed to their high phenolic compounds and flavonoids.

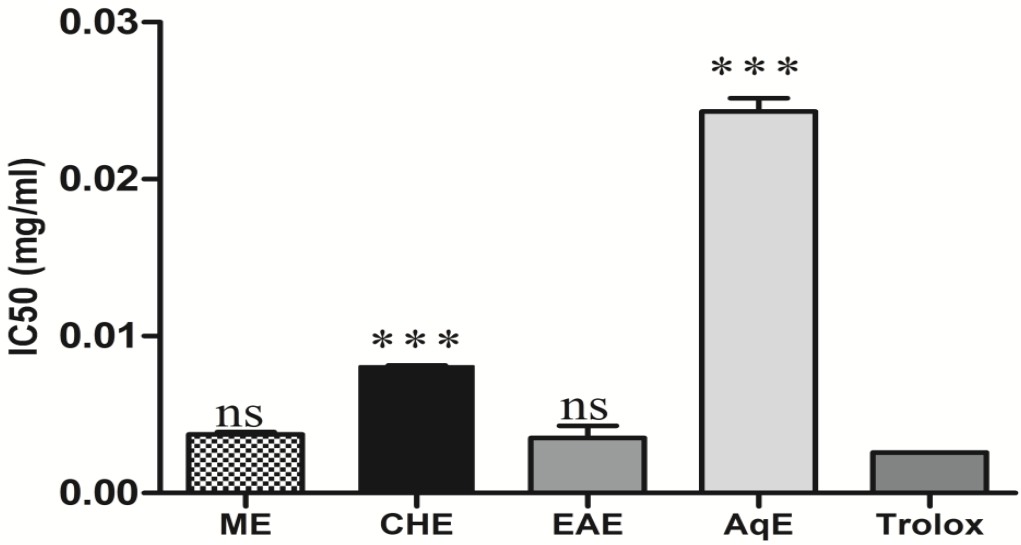

Fig.3. ABTS radical scavenging activity of different *C.azarolus* extracts. ME: methanolic extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as IC50 means ± SD (n = 3).(ns: no significant difference , *** p<0.001) compared to trolox as standard.

Metal chelating activity

The metal chelating capacity is important, since it reduces concentration of the catalyzing transition metal in lipid peroxidation [51]. In the presence of chelating agents, the

ferrozine-Fe^{2+} complexes are disrupted, resulting in a decrease in the red color of the complex. Iron chelating activity of *C. azarolus* fractions was measured as antioxidant activity (Fig. 4). In this study, EDTA was used as a positive control to compare the results and it exhibited the highest chelating activity (IC_{50} = 0.018 ± 0.0002 mg/ml), which is significantly higher than all fractions ($p<0.001$). Among the extracts, AqE showed better metal chelating activity (IC_{50} =0.35 ±0.05 mg/ml), while the lowest chelating effect on ferrous ions was showed for CHE (IC_{50} =3.19 ±0.11 mg/ml).

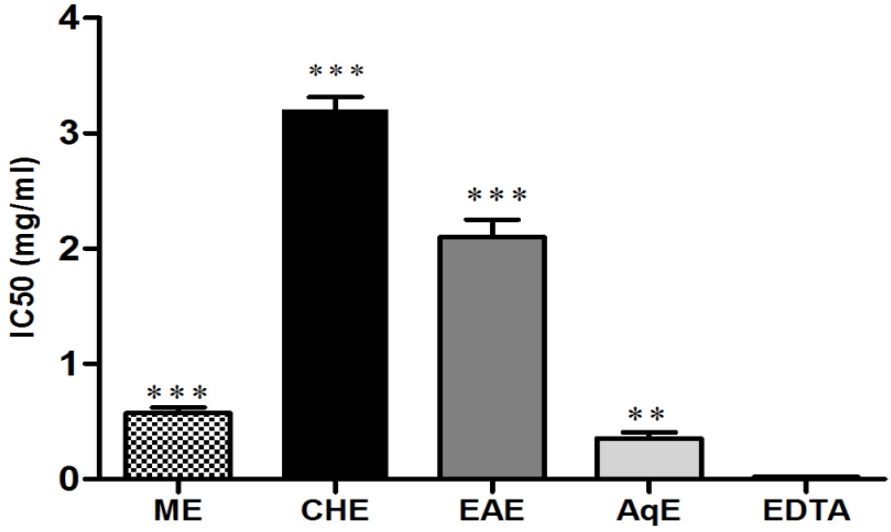

Fig. 4. Metal chelating activity of different *C.azarolus*extracts. ME: methanolic extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as IC_{50} means ± SD (n = 3).(p ≤0.01; *** p<0.001) compared to EDTA as standard.**

Phenolic compounds have been reported to be chelators of free metal ions [52]. But in this study a poor correlation (R^2 = 0.15) was observed between the chelating extracts of *C. azarolus* leaves and their contents in phenolic compounds ($p<0.05$), indicating that polyphenols might not be the main ion chelators. Indeed , our results are in good agreement with those obtained by Shahidi and Maryan (2003) [53], who reported that differences in the antioxidant activities of plant extracts could be due to different qualitative and quantitative compositions of their phenolic constituents. It is very important to evaluate the molecular mechanisms of the radical-scavenging activities of polar extracts for better understanding of the mode of action.

Hydroxyl radical scavenging activity

Hydroxyl radical is one of the ROS that easily reacts with biomolecules, such as amino acids, proteins and DNA. Therefore, removal of hydroxyl radicals can protect humans against some diseases [54]. In this study, hydroxyl radical was generated by 1, 10-phenanthroline/ (EDTA)/H_2O_2 system to determine the hydroxyl radical scavenging capacity of *C. azarolus* fractions. As shown in Fig.5, *C. azarolus* possess good in vitro scavenging activity against hydroxyl radicals generated in a Fenton reaction system , and hydroxyl radical scavenging activity of all extracts were significantly ($p< 0.05$) lower than vitamin C (IC_{50} was 0,084 ±0.007 mg/ml). The IC_{50} of EAE, CHE, ME and AqE were (0.283± 0.01 mg /ml), (0.294±0.007 mg/ml), (0.330 ±0.02mg/ml) and (1.869± 0.03 mg/ml) respectively. This

finding reveals that *C. azarolus* extracts displayed different potential in scavenging hydroxyl radical which is positively related to their amount of total phenolic, flavonoids contents, andhave active hydrogen donor ability of hydroxyl substitution. Similarly, the hydroxyl radical was inhibited by *C. oxycantha* extract [55] and *C. pinnatifida* var. *major* fruit extract [56].

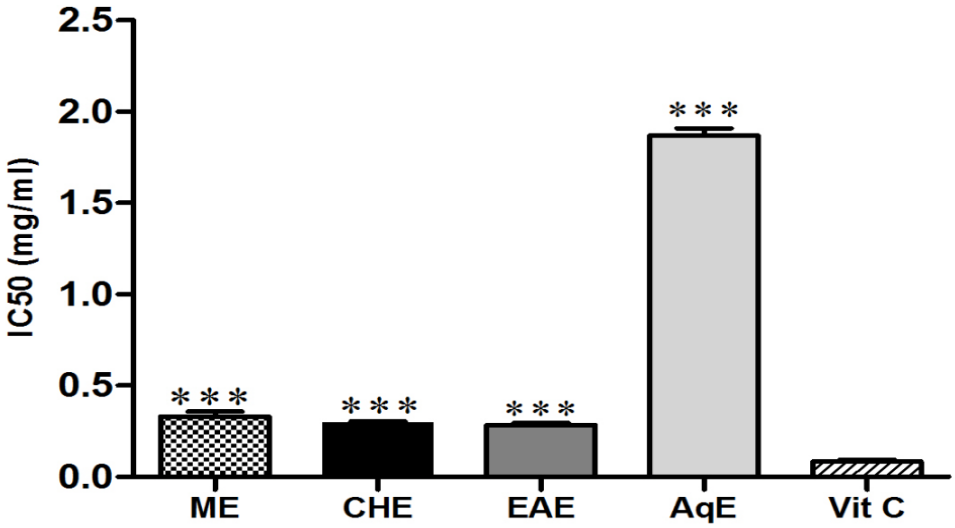

Fig. 5. Hydroxyl radical scavenging activity of different *C.azarolus* extracts. ME: methanolic extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. Data were presented as IC_{50} means ± SD (n = 3).(* p<0.001) compared to vitamin C as standard.**

Reducing Power

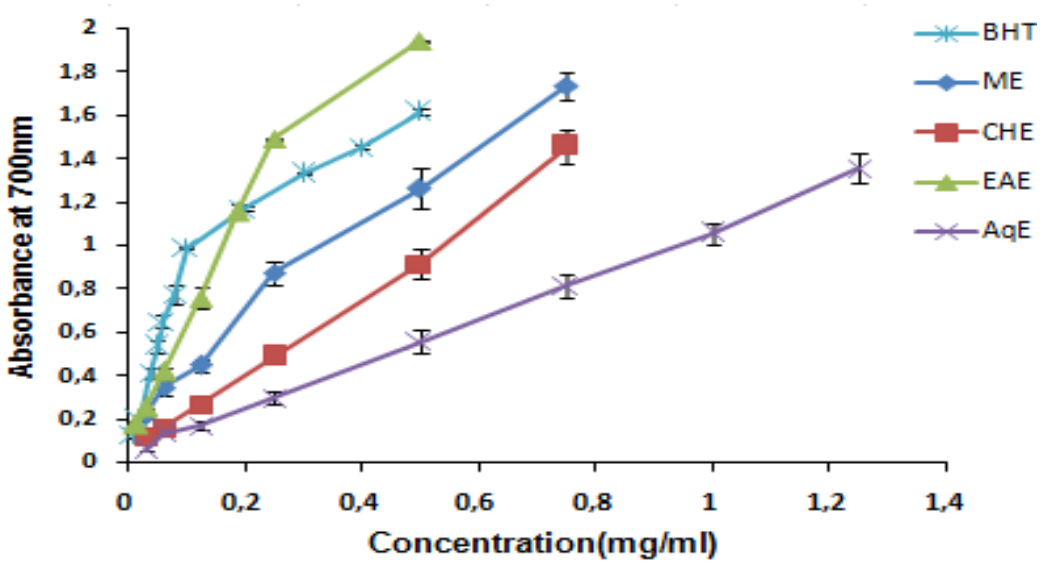

Fig.6. Reducing power of different *C. azarolus* extracts. ME: methanolic extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. BHT: Positive standard. Values are means ± SD (n = 3).

Different studies have indicated that antioxidant activity and reducing power are related [57]. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of

reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration [58]. Fig. 6 shows the dose-response curves for the reducing powers of various extracts from *C. azarolus.* It was found that all extracts exhibited ferric reducing power greatly in concentration dependent manner, whereas the highest ferric reducing power activity was noticed by EAE. However, the other extracts exhibited less potent reducing power when compared to BHT at all tested concentrations. This finding reveals that the favorable antioxidant activity observed in the EAE is most possibly due to its high total contents of phenolic compounds, flavonoid among these four extracts. At 0.5mg/ml, reducing power of plant fractions and standard compound (BHT) exhibited the following order: EAE (1.943±0.005)> BHT (1.615±0.01)>ME (1.262 ± 0.09) > CHE (0.915±0.06) >EqE (0.558±0.05).Because the reductive abilities of these extracts were excellent, it was evident that these extracts did show reductive potential and could serve as electron donors, terminating the radical chain reaction.

β -Carotene/linoleic acid bleaching assay

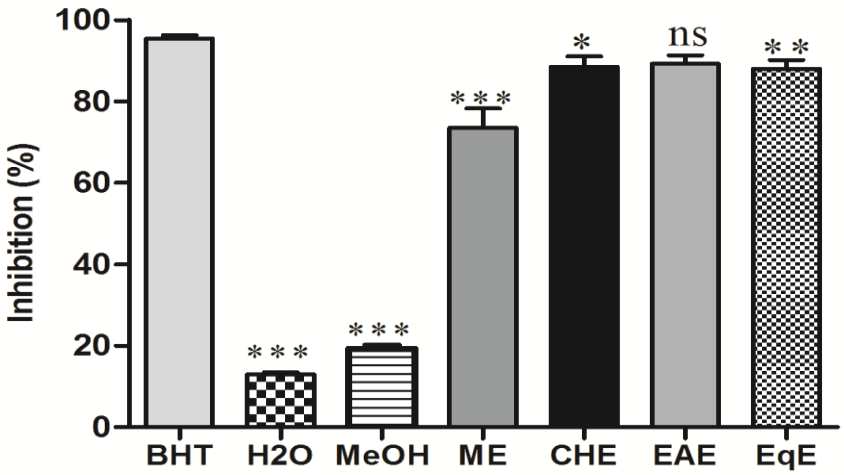

Fig.7. Antioxidant activities of *C. azarolus* extracts (2 mg/ml at 24 hours of incubation)measured by β-carotene bleaching method. ME: methanolic extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract. BHT was used as reference antioxidant. Values are means ± SD (n = 3). (ns: no significant difference ,*p < 0.05, ** p < 0.01, *** p < 0.001) compared to BHT as standard.

In this method, the linoleic acid free radical formed attacks the highly unsaturated β -carotene molecules and in the absence of an antioxidant rapidly bleached the orange color of β-carotene. The extent of discoloration is monitored spectrophotometrcially at 490 nm [59]. The lowest β -carotene discoloration rate exhibited the highest antioxidant activity. As shown in Fig.7, all *C. azarolus* extracts inhibit the oxidation of β-carotene in a very important level compared to BHT(95.4 ± 0.51% inhibition). This effect is due to either; the inhibition of linoleic acid peroxidation or the radical scavenging hydroperoxides formed during the peroxidation of linoleic acid (scavenger effect). EAE is the best inhibitor of the oxidation of β-carotene in 24h (89.21 ± 2.087%), which was as strong as the positive reference, BHT (no significant difference, *P* >0.05), followed by CHE (88.52±2.48%), AqE (87.96±1.76%) and ME (73.51±4.72%) respectively. A similarly, Ljubuncic et al [17] demonstrated that aqueous

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extract from a decoction of leaves and unripe fruits of *C. aronia,* a hawthorn indigenous to Jordan, and Palestine, inhibited the oxidation of β -carotene in the coupled oxidation of β -carotene and linoleic acid and scavenged peroxide radical. The potential antioxidant abilities of these plant extracts might be due to their phenolics contents, which can significantly inhibit oxidation of linoleic acid. Several studies have exhibited that the antioxidant activity is essentially correlated to phenolic and flavonoid contents [11].

Ferric thiocyanate method

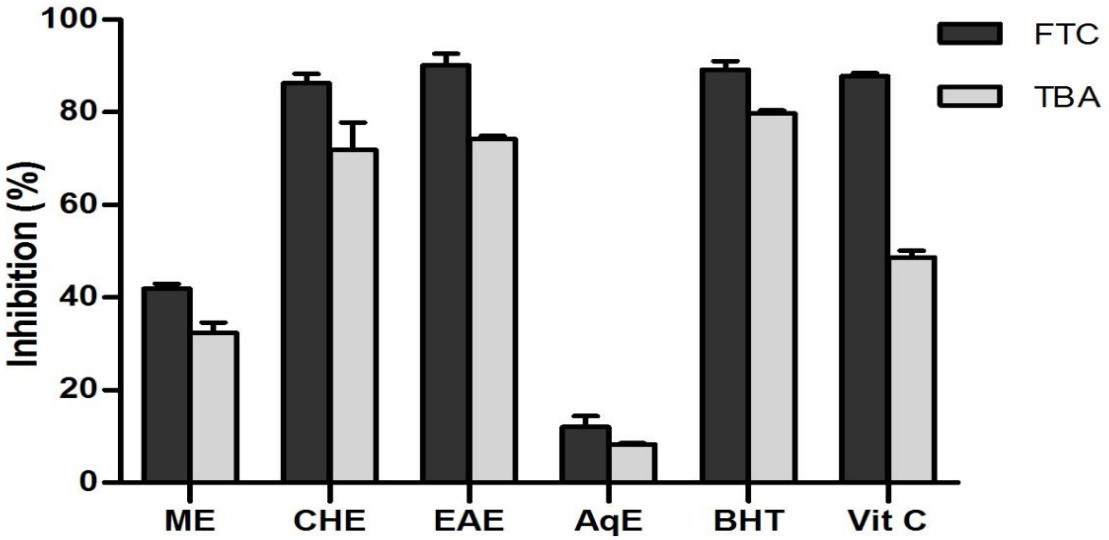

Fig.8.A comparison between total antioxidant activity using the FTC and TBA methods of *C. azarolus* extracts. ME: methanolic extract, CHE: chloroform extract, EAE: ethyl acetate extract, AqE: aqueous extract, BHT and Vit C: Positive standards. Values are means ± SD (n=3).

The ferric thiocyanate method measures the amount of peroxide, which is the primary product of oxidation produced during the initial stages of oxidation. This method was used to measure the ability of antioxidants to scavenge peroxyl radicals through hydrogen donation during polyunsaturated fatty acid (PUFA) oxidation. Peroxides are generated during linoleic acid oxidation, and react with Fe^{2+} to form Fe^{3+}. Ferric ions then react with thiocyanate to produce a red-colored complex, and this complex has a maximum absorbance at 500 nm [60]. In this assay, all extracts exhibited strong antioxidant potential with percent inhibition in the range of 12.5% – 90.13% as compared with Vitamin C and BHT (Fig.8).The results indicate that both EAE and CHE exerted marked effects on inhibition of linoleic acid oxidation with 90,13 ± 2.51 % and ± 86.27 ± 2.05% inhibition value respectively , and were as strong as the positive references, BHT and vitamin C (no significant difference, *P* >0.05),while AqE showed significantly(p>0.001) lower percent inhibition (12.5%) compared with BHT (89.19±1.88%). The auto-oxidation of linoleic acid emulsion without *C. azarolus* extract or standard compounds was accompanied by a rapid increase of peroxides during the 5 days of incubation. Consequently, these results clearly indicated that *C. azarolus* fractions had effective and powerful antioxidant activity which is related to their polyphenolic content. Phenolic compounds in plants are powerful free radical-scavengers which can inhibit lipid peroxidation by neutralizing peroxyl radicals generated during the oxidation of lipids [61]. Therefore, inhibition of lipid peroxidation by the C*. azarolus* leaf extracts can be a fundamental property through which they can mitigate the initiation

and/or propagation of oxidative stress related diseases. A similar observation has been reported by Ljubuncic et al [17]with *C. aronia* decoction on iron-induced lipid peroxidation in rat liver homogenates.

Thiobarbituric acid method

Different from the FTC test, which is related to the peroxide formation in the initial stage of lipid oxidation, the thiobarbituric acid (TBA) test measures the amount of malondialdehyde (MDA) produced after the decomposition of the lipid peroxide during the oxidation process. MDA is a very unstable compound causing mutagenic and cytotoxic events [62, 63, 64]. At a low pH and high temperature (100 °C), MDA binds TBA to form a red complex that can be measured at 532 nm in the final day of FTC (5th day). Fig.8 showed the total antioxidant activity of the FTC method compared to the TBA method. In this study, the results of TBA test were confirmed with the FTC data. Based on these results, all fractions, except for AqE, showed a significantly (P <0.05) high antioxidant activity within the range of 8.27% – 74.23 % when compared with BHT (79. 75 ± 0.61%). The inhibition of lipid peroxidation decreased in the following order: EAE (74.23±0.61 %) > CHE (71.91 ± 5.94 %)> ME (32.33 ± 2.03%)>AqE (8.27 ±0.24 %). This result may be related to the difference in the amount of flavonoid and phenolic compounds in these extracts. The antioxidative effectiveness in natural sources has been reported to be mostly due to phenolic compounds. According to previous studies, the therapeutic effects of *Crataegus* leaves were closely related with its polyphenol compounds [65, 22] which possessed favourable antioxidant activities due to their ideal chemical structures for free radicals scavenging and anti- lipid peroxidation [66].

Hypotensive effect in anesthetized rats

This study demonstrated for the first time that the ME and EAE of *C.azarolus* possessed antihypertensive effect. The intravenous administration of ME and EAE of *C. azarolus* decreased SBP , DBP and MABP in anesthetized rats in dose dependant manner at the range doses of 0.04 to 12 mg/kg body weight (Fig. 9 and 10). The effect of both extracts was transient, since blood pressure returned to the original baseline after the maximal response was obtained in about 137 ± 3.1 sec and 108 ± 5.2 sec for EAE and ME, respectively. At the maximal dose of the ME (12 mg/kg) injected, average SBP and DBP were decreased significantly by 28.12±2.34% and 26.50± 2.03%, respectively, whereas a percent decrease in SBP and DBP for EAE was about by 38.29±5.69 %and 41.54±5.13%respectively (Fig.9). The extracts decreased both SBP and DBP of the anesthetized rats but the fall in DBP was greater than that in SBP for EAE. This kind of blood pressure lowering effect has been observed with β-adrenergic receptor agonistic drugs, which cause vasodilation with increased cardiac output, resulting in greater fall of DBP [67]. However, the involvement of other receptors and ion channels cannot be ignored. The hypotensive response was found to be significant (p < 0.001) at doses 0.4 , 1.2 , 4 and 12 mg/kg and it is more potent with the EAE for which the maximal fall in MABP is about 39.37 ± 3.41% at a dose of 12 mg/Kg in comparison with the maximal fall observed with the ME (27.58±1.3%) (Fig.10).It seems that the strong hypotensive activity of the EAE, compared with the ME, would be due to its richness by polyphenols.

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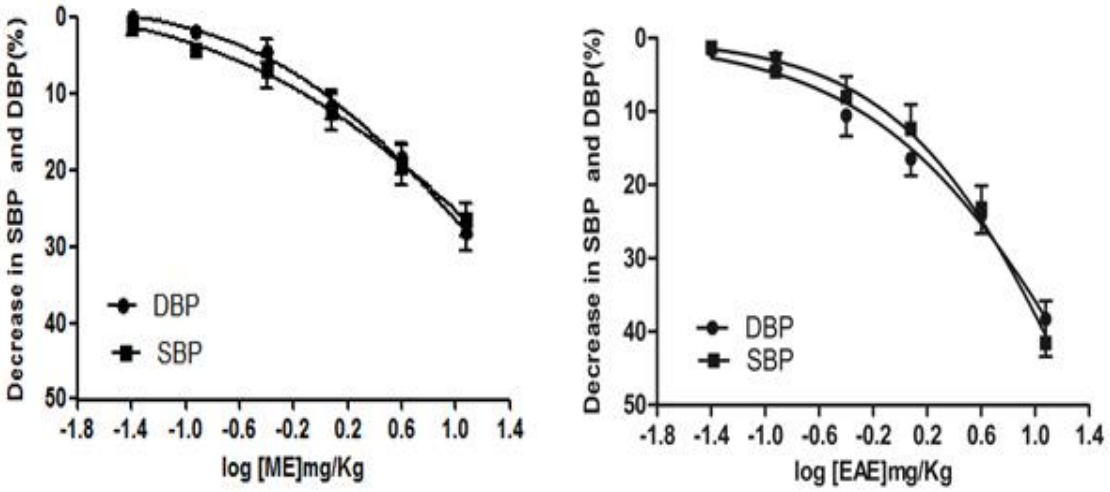

Fig. 9. Concentration-response curves of ME and EAE of *C. azarolus* on systolic (SBP) and diastolic (DBP) blood pressure of anesthetized rats. Values are means ± SEM(*n*=6).

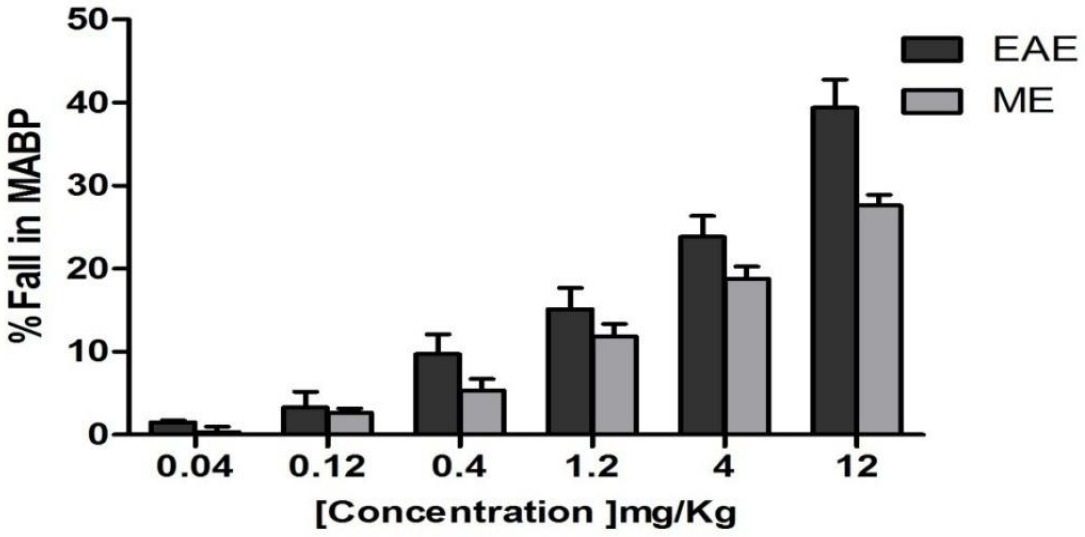

Fig.10 . Dose-dependent hypotensive effect of ME and EAE of *C. azarolus* on mean arterial blood pressure (MABP) in anaesthetized rats. Values shown are mean±SEM(*n*=6).

These results are consistent with the effect of related *crataegus* species in various hypertensive rat models and in clinical studies [68, 69, 70, 71]. The Hawthorn leaves extract is well known rich source of flavonoids and polyphenolic compounds [16]. In the present study, the phytochemical screening confirmed the presence of tow flavonoids which are (+) - catechin and hyperoside in the EAE. Thus, The hypotensive activity showed by ME and EAE could probably be attributed to polyphenols, which have widely been reported as antioxidant and vasodilatory agents positively correlated in the treatment of cardiovascular diseases due to their antihypertension properties [72].

Hawthorn extracts have been reported to cause peripheral vasodilatation [15], induce endothelium-dependent NO-mediated vasorelaxation and inhibition of Ca^{2+}influx to the smooth muscle [71]. Indeed, Hyperoside has been shown to inhibit calcium influx

induced by the activation of G-protein-coupled receptors leading to vasorelaxant effect [73]. The results of Schroeter et al [74]suggest that oral administration of pure (-)-epicatechin showed similar acute vascular effects as catechin - rich cocoa. These effects were likely due to augmentation of NO synthesis by flavanols. Thus, the bioactive principles identified in this work (hyperoside and (+) – catechin), at least in-part, may be responsible for the observed hypotensive effect of *C. Azarolus* extract.

CONCLUSION

This work revealed that extracts of the leaves of *C. azarolus* contain high levels of phenolics and flavonoids, and possess significant antioxidant activities. The EAE extract exhibited remarkable free radical scavenging and antioxidant activity, which may due to the presence of high polyphenolic content. At least to our known, this is the first study showing that ME and EAE obtained from *C. azarolus* leaves, are able to cause blood pressure reduction in rats. This findings provide scientific support for the traditional uses of *C. azarolus* leaves in the treatment of hypertension. Knowing the cellular and molecular mechanisms through which the *C. azarolus* extracts act in arterial hypertension requires further studies. It is also suggested that *C. azarolus* be viewed as a potential source of natural antioxidants which can provide precious functional ingredients useful for the prevention of diseases related to oxidative stress such as cardiovascular diseases.

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