

Effects of Methanolic Extract of *Crataegus azarolus* L. on Plasma Antioxidant Capacity and Biomarkers of Oxidative Stress in Liver and Kidney of Rats

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Available Online: 9th June, 2016

ABSTRACT

The aim of the present study was to investigate the effect of methanolic extract of *Crataegus azarolus* (MECA) on the plasma antioxidant capacity and oxidative stress markers in liver and kidney of rats. Albino rats were categorized into four groups. The first group served as the normal control, the second and the third groups were treated with MECA at dose of 100 and 200 mg/kg, respectively. Fourth group (positive group) was treated with 100 mg/kg of vitamin C for 14 consecutive days. The levels of protein, malondialdehyde (MDA) and reduced glutathione (GSH) and catalase (CAT) activity were assayed in liver and kidney homogenates using standard procedures. The results showed that MECA (100 and 200 mg/kg) caused significant elevation of plasma antioxidant capacity using DPPH radical scavenging activity, whereas only the dose of 200 mg/kg was found to increase significantly the plasma reducing power. Also, the treatment with MECA increased protein level in liver and enhanced the antioxidant status in liver and kidney by reducing MDA level and increasing CAT activity and GSH level. These findings suggest that MECA has significant potential as a natural antioxidant to promote health and to reduce the occurrence or progress of diseases related to oxidative stress.

Keywords: *Crataegus azarolus*, Catalase, Lipid peroxidation, *In vivo* antioxidant, Rat.

INTRODUCTION

Oxidative stress is considered as the imbalance between reactive oxygen species production and antioxidant protective system in the body^{1,2}, resulting in potential damage in the living cells through the generation of free radicals and peroxides that can destroy the lipids, proteins and DNA of the cells and all these events may lead to number of diseases such as nephrotoxicity, liver cirrhosis, cancers, atherosclerosis, diabetes^{3,4}, cardiovascular diseases, neurodegenerative diseases, and even aging¹. Therefore, the living system can defense to protect against harmful effect of oxygen and nitrogen reactive species using endogenous antioxidant enzymes such as catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD); and nonenzymatic antioxidants as reduced glutathione (GSH), vitamins, micronutrients, carotenoids and polyphenols^{4,5}. Antioxidants help to improve natural defense mechanisms of the body and the main source of them are medicinal plants³. Flavonoids and phenolic compounds of plant origin have been reported as good scavengers of free radicals. Hence, nowadays search for natural antioxidant source is gaining much importance⁶ due to lesser side effects and multiple therapeutic advantages. Subsequently, plant products are considered as

helpful dietary supplements in numerous diseases⁷. *Crataegus* species (Rosaceae), known as "Hawthorn" are native to Mediterranean region, North Africa, Europe and Central Asia⁸. Most species of Hawthorn have prominent, long, straight and sharp thorn. In Mediterranean region, the predominant species of the genus *Crataegus* is *Crataegus azarolus* L. (*C. azarolus*) that populates the mountains of these areas⁹. In Algeria, it is especially localized in the tell of Constantine region, and known under the name of "zaaroura"¹⁰. *Crataegus* species have been extensively used in traditional medicine for treating cardiovascular diseases as congestive heart failure, angina, hypertension, hyperlipidaemia and diabetes mellitus^{11,12}. However, *C. azarolus* was used to treat cardiovascular diseases, as well as cancer, diabetes, sexual weakness¹³ and hypertension^{14,15}. *C. azarolus* reveals the presence of bioactive constituents such as polyphenols, flavonoids as hyperoside¹⁵, rutin, spiraeoside, isoquercitrin and quercetin¹⁶, glycosides^{16,17}; and tannins¹⁵. Furthermore, pharmacological studies reported that *C. azarolus* has anti-lipid peroxidation¹³, anticoagulant¹² and hypotensive effects¹⁵. The anti-oxidant properties of *C. azarolus* have been studied in various *in vitro* models which showed that azarole extracts have free radical scavenging, chelating,

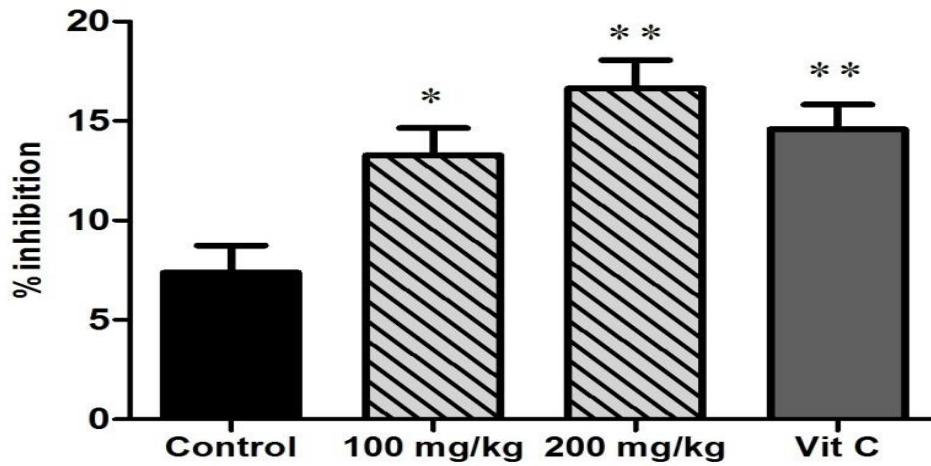


Figure 1: Effect of methanolic extract of *Crataegus azarolus* and vitamin C on plasma antioxidant capacity using DPPH radical scavenging activity. Data were presented as % means \pm SEM (n = 6). (** p < 0.01; * p < 0.05) compared to control group.

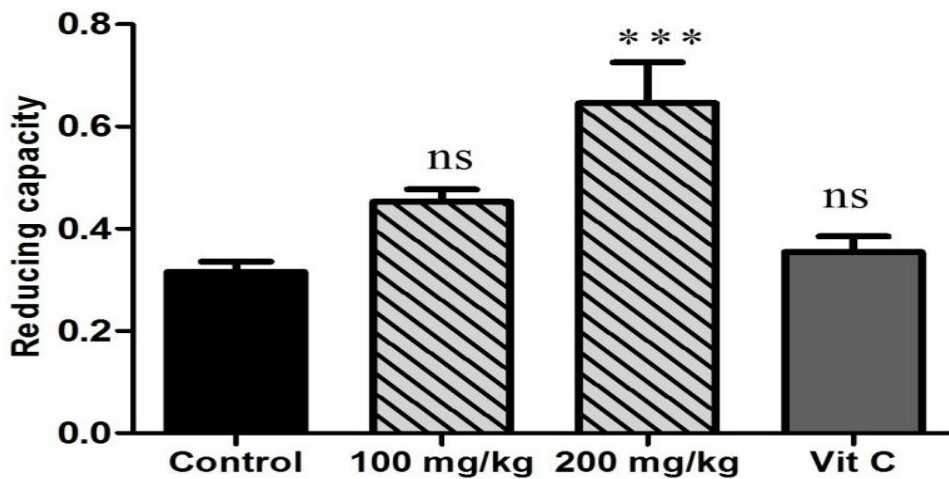


Figure 2: Effect of methanolic extract of *Crataegus azarolus* and vitamin C on antioxidant capacity using reducing power assay. Data were presented as means \pm SEM (n = 6). (ns: no significant difference; *** p < 0.001) compared to control group.

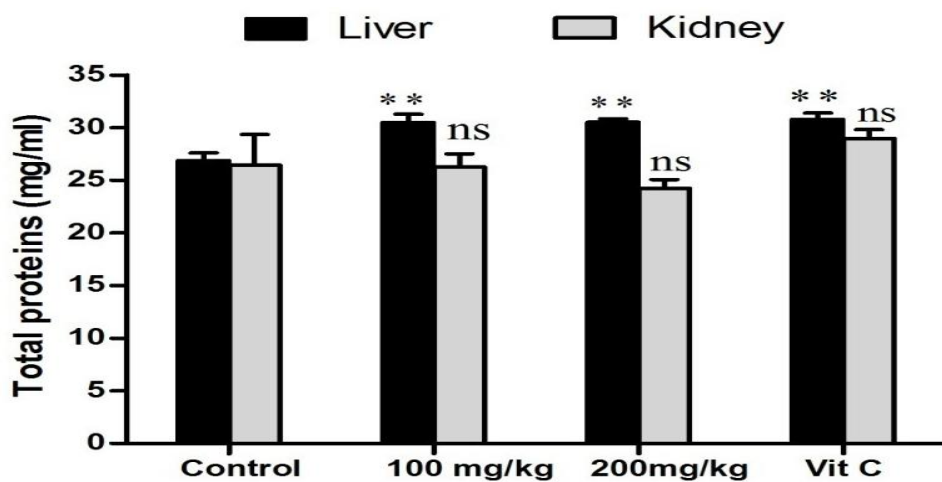


Figure 3: Effect of methanolic extract of *C. azarolus* and vitamin C on total proteins level in liver and kidney of rats. Values are given as means \pm SEM (n=6). (ns: no significant difference; ** p < 0.01) compared to control group.

reducing, and anti-lipid peroxidant activities by various *in vitro* antioxidant assays¹⁵. However, no scientific report of

this plant *in vivo* has ever been recorded in the literature showing the antioxidant efficacy in living tissues.

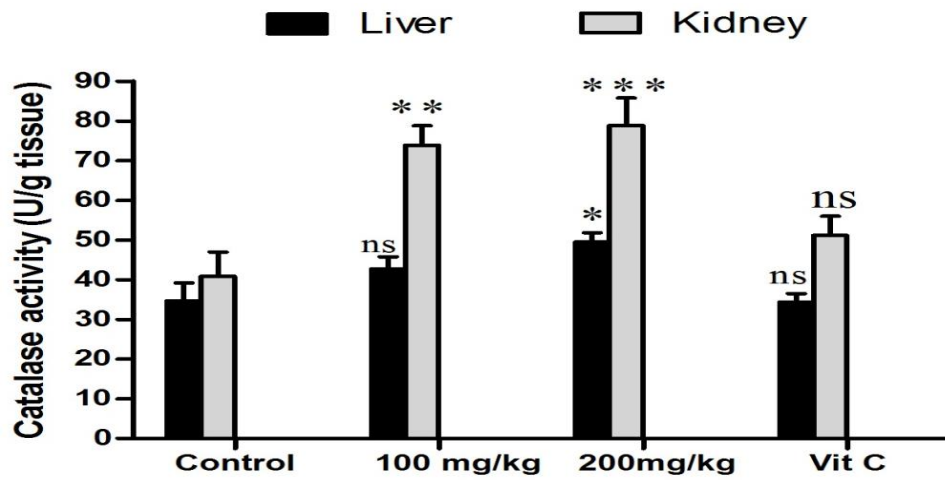


Figure 4: Effect of methanolic extract of *C.azarolus* and vitamin C on CAT activity in liver and kidney of rats. Values are given as means \pm SEM (n=6). (ns: no significant difference; * p < 0.05; ** p < 0.01; *** p < 0.001) compared to control group.

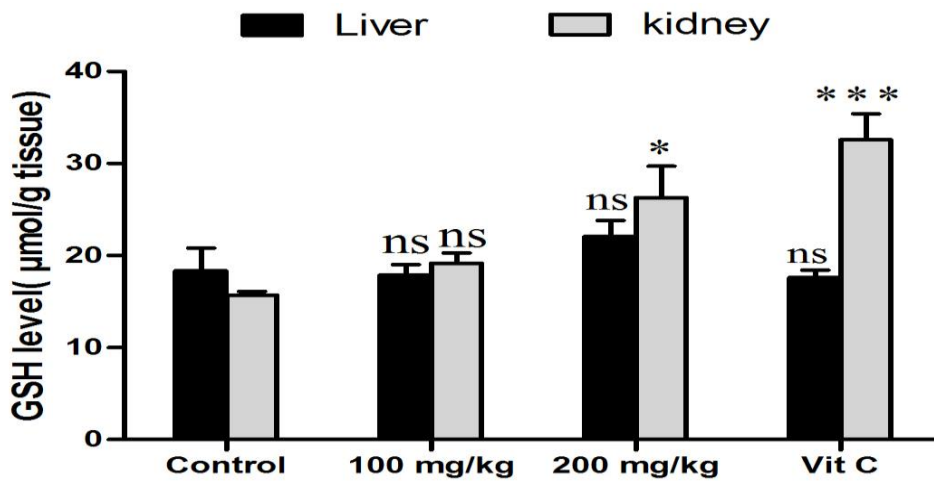


Figure 5: Effect of methanolic extract of *C.azarolus* vitamin C on reduced glutathione level in liver and kidney of rats. Values are given as means \pm SEM (n=6). (ns: no significance difference; * p < 0.05; *** p < 0.001) compared to control group.

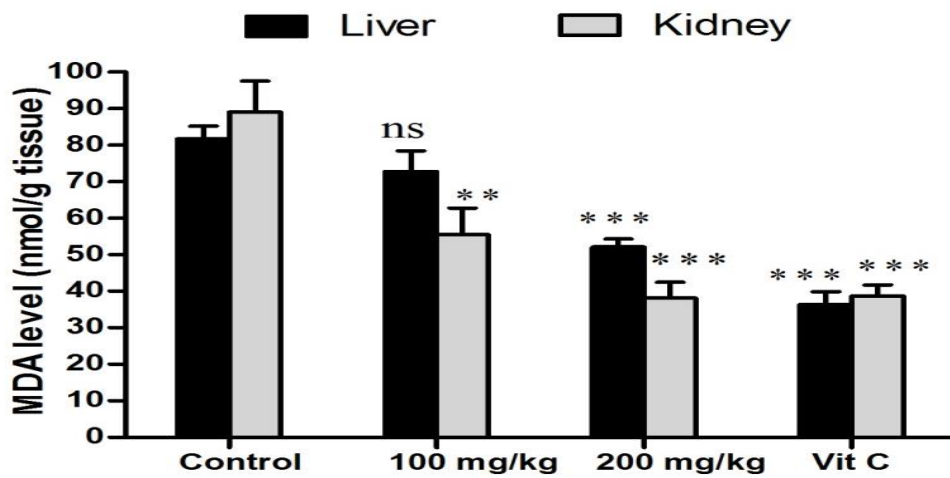


Figure 6: Effect of methanolic extract of *C. azarolus* and vitamin C on MDA level in liver and kidney of rats. Values are given as means \pm SEM (n=6). (ns: no significant difference; * p < 0.05; ** p < 0.01; *** p < 0.001) compared to control group.

Therefore, the aim of the present study was to evaluate the effect of methanolic extract from *C.azarolus* leaves on plasma antioxidant capacity and antioxidative status in liver, kidney tissues of rats by assaying various biomarkers.

MATERIALS AND METHODS

Plant Material

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 Nature and Life Sciences, University Ferhat Abbas, Setif, Algeria. The leaves were separated, dried under shadow and powdered.

Animals

Healthy albino rats, weighing 170-200 g were purchased from Pasteur Institute in Algiers, Algeria, and were acclimatized for one week, prior to experiments. The animals were housed in an air-conditioned animal room, and maintained with free access to water and standard diet. The experimental protocol in rats was conducted after the experimental procedures were revised and approved by the Animal Ethics Committee of Institute of Nature and Life Sciences, University Ferhat Abbas, Setif 1, Algeria.

Preparation of methanolic extract

For preparation of dry leaves methanolic extract, the powdered plant material (100 g) was extracted with methanol (85% and 50%), at room temperature for 5 days, according to the method of Markham¹⁸. Then, the suspension was filtered through a Buchner funnel and concentrated under reduced pressure on a rotary evaporator to give crude methanolic extract (ME), and was then stored at -20 °C until use.

Experimental design

Rats were randomly divided into four groups of six animals each, and the treatment was given every day via orogastric tube for 14 days as the following:

Group 1: received normal saline (0.9%) and served as control group.

Group 2: received ME of *C. azarolus* at the dose of 100 mg/kg.

Group 3: was given ME of *C.azarolus* at the dose of 200 mg/kg.

Group 4: received vitamin C (100 mg/kg) and served as reference control.

Drugs were suspended in saline and administered at dose of 5 ml/kg.

On day 15, all animals were sacrificed. Blood was collected and centrifuged at 3000 rpm for 15 min for determination of plasma antioxidant capacity. The liver and kidney were removed and washed with ice-cold saline, blotted with filter paper and kept in plastic vials at -20 °C until use.

Preparation of tissue homogenates

Liver or kidney tissues were cut down into small pieces, placed in 1.15M KCl buffer, and homogenized using dounce homogenizer in ice-cold condition to obtain 10 % homogenate. The homogenate thus obtained was centrifuged at 4000 rpm for 15 min and the supernatant collected was used for the determination of MDA as a lipid

peroxidation marker. CAT, reduced glutathione and total protein were also assayed.

Estimation of plasma antioxidant capacity

DPPH radical scavenging assay

In this test, the capacity of plasma to trap the DPPH radical (2, 2-diphenyl- 1-picrylhydrazyl) was evaluated according to the method of Burits and Bucar¹⁹ with some modifications. Briefly, a volume of plasma was added to DPPH methanolic solution (0.004%). After 30 min of incubation in the darkness followed by a centrifugation, the absorbance at 517 nm is measured and the plasmatic antioxidant capacity was then calculated according to the equation below:

$$\% \text{ scavenging activity} = [(\text{Abs control} - \text{Abs sample}) / \text{Abs controls}] \times 100$$

A control: is the absorbance of the blank solution

A sample: is the absorbance in the presence of the test compound.

Plasma reducing power

The plasma reducing power was determined according to the method of Chung *et al.*²⁰. In brief, plasma (0.1 ml) was mixed with 0.1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.1 ml of 1 % potassium ferricyanide. The mixture was then incubated for 20 min at 50°C. After that, 0.250 ml of 1% trichloroacetic were added, the mixture was then centrifuged for 10 min at 3000 rpm. The aliquot (0.250 ml) of the upper layer was mixed with 0.250 ml of distilled water and 0.5 ml of ferric chloride (0.1%), and the absorbance at 700 nm was measured. A higher absorbance indicated a higher reducing power.

Quantification of oxidative stress biomarkers in tissue homogenates

Assessment of total protein level

Tissue protein concentration was assayed according to Gornall *et al.*²¹ using the Biuret reagent and bovine serum albumin as a standard. In brief, 1 ml biuret reagent was mixed with 25 µl sample or standard (albumin), and the absorbance (540 nm) was measured after 10 min of incubation at 37 °C.

Estimation of CAT activity

CAT activity was measured according to the method of Clairborne²². The breakdown of hydrogen peroxide in the presence of CAT was followed by observing the decrease in absorption of peroxide solution in the ultraviolet (UV) region. In brief, an aliquot (50 µl) of each tissue supernatant was added to cuvette containing 2950 µl of 19 mM H₂O₂ prepared in 0.1M phosphate buffer (pH 7.4). The rate of decomposition of H₂O₂ was measured at 240 nm at 1 min interval for 2 min using UV-visible spectrophotometer. Units of CAT were expressed as the amount of enzyme that decomposes 1 µM H₂O₂ per minute at 25°C. Thus, the specific activity was expressed in terms of units per gram of tissue.

Estimation of reduced glutathione

Total reduced glutathione (GSH) content was measured following the method of Ellman²³, which measures the reduction of 5,50-dithio-bis (2-nitrobenzoic acid) (DTNB) (Ellman's reagent) by sulfhydryl groups to 2-nitro-5-mercaptobenzoic acid, which has an intense yellow color and maximum absorption at 412 nm. Briefly, 50 µl of

supernatant were diluted in 10 ml phosphate buffer (0.1 M, pH 8). Then, to 3 ml of the mixture of dilution, 20 μ l of DTNB (0.01 M) were added. The yellow color developed was read at 412 nm after 5 min. GSH concentrations were calculated using the standard curve of GSH, and results are expressed as μ mol/ g tissue.

Estimation of lipid peroxidation

MDA levels were measured by the method of Ohkawa *et al.*²⁴, which is based on spectrophotometric measurement of the pink color generated by the reaction of thiobarbituric acid (TBA) with MDA. Briefly, 0.5 ml of tissue homogenate was mixed with 0.5 ml of trichloroacetic acid (TCA) (20 % w/v) and 1 ml of TBA (0.67 % w/v). Then, the mixture was boiled in a water bath for 15 min. After cooling to room temperature, 4 ml of n-butanol were added to each sample and centrifuged at 3000 rpm for 15 min. The absorbance of the supernatant was measured at 532 nm with respect to the blank solution. The concentration of MDA was determined from a standard curve of 1, 1, 3,3 tetraethoxypropane in the same conditions and it was expressed as n mol/ g tissue.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism (version 5.01 for Windows). All experiments were done in triplicate and results were reported as mean \pm S.E.M. (n = 6). The data were analyzed by one-way ANOVA, and statistically significant effects were further analyzed by means comparison using Tukey's multiple comparison analysis using GraphPad program. The $p < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Plasma antioxidant capacity

Several methods have been used to evaluate the antioxidant capacity of plant extracts and that of human plasma after dietary supplementation. These methods include the ferric-reducing antioxidant potential (FRAP); oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC); and total radical-trapping antioxidant parameter (TRAP). All these assays assess either the radical-scavenging (hydrogen atom transfer) or reducing (electron transfer) capacity of the compound or (biological) fluid under investigation²⁵.

Plasma antioxidant capacity using DPPH radical scavenging activity

To evaluate the effectiveness of an antioxidant treatment, the scavenger effect against DPPH radical was assessed. This method is based on the ability of the antioxidants to reduce the purple DPPH radical to a yellow-colored compound, diphenylpicrylhydrazine upon reduction by either the process of hydrogen or electron donation. In this study, the obtained results (Figure. 1) showed that the administration *per os* of ME of *C. azarolus* at doses of 100 and 200 mg/kg in rats increased significantly the plasma antioxidant capacity with values of 13.28 ± 1.36 % and 16.65 ± 1.40 %, respectively compared to control group (7.37 ± 1.36 %), and were as stronger (no significant difference; $p > 0.05$) as vitamin C (14.90 ± 1.24 %) to increasing the total plasma antioxidant capacity.

Plasma reducing power

Plasma antioxidant capacity of this plant was reinforced by the reducing power assay. Data demonstrated that administration of ME of *C. azarolus* at the two doses (100 and 200 mg/kg) possess similar DPPH-free radical scavenging pattern (Figure. 2), and showed electron donation capacity dose dependently, but only the plasma reducing power of the dose 200 mg/kg (0.64 ± 0.08) were significantly higher than that of control and positive groups which had values of 0.31 ± 0.02 and 0.35 ± 0.03 , respectively. These obtained results showed a positive association between ME administration and plasma antioxidant capacity using DPPH scavenging assay and reducing power and these effects may be primarily attributed to its high phenolic and flavonoids contents which were estimated previously with values of 188.91 mg gallic acid equivalent/g of extract and 21.03 mg quercetin equivalent /g of extract, respectively¹⁵. Many studies have found highly significant correlation between the measured plasma antioxidant capacity and the total phenol content of plant-derived extracts and beverages²⁶⁻²⁹. It was reported that the consumption of extra virgin olive oil rich in phenolic compounds (30 days, 50 ml/day) increases the antioxidant activity by 40%³⁰ and the consumption of 482 ml/day coffee rich in phenolic acids such as caffeic, ferulic, chlorogenic, and p-coumaric acids for 4 weeks increases also the plasma total antioxidant status subjects by 26 %³¹. Addition of catechins or tea extract to human plasma and tea consumption by humans were also reported to increase the total antioxidant capacity of plasma³².

Oxidative stress biomarkers in liver and kidney homogenates

It is well known that free radicals can cause cell damage through covalent binding and lipid peroxidation with subsequent tissue injury. Liver and kidney are most vulnerable to injury by free radicals resulting in serious health problems³³. Thus, in this study, the ME of *C. azarolus* was screened for its possible *in vivo* antioxidant activity, which served as a significant indicator of its potential antioxidant activity to maintain redox status.

Total protein level

Liver is one of the largest glands in the human body and the chief site for intense metabolism and excretion. So, it has the surprising role in the maintenance, performance and regulating the homeostasis of body and their major functions are carbohydrate, protein and fat metabolism, detoxification, storage of vitamin and formation of bile^{34,35}. Thus, we tried in this study to evaluate the effect of extracts on total proteins level in liver and kidney. As seen in Figure 3, the treatment with ME at the doses of 100 and 200 mg/kg and vitamin C (100 mg/kg) resulted in significant ($p < 0.05$) increase of total protein level in liver with values of 30.50 ± 0.77 mg/ml, 30.51 ± 0.32 mg/ml, respectively when compared to control group (26.87 ± 0.74 mg/ml). However, the administration of the same doses of ME and vitamin C had no effect (no significant difference, $p > 0.05$) on proteins level in kidneys compared to control (26.43 ± 2.91 mg/ml) and vitamin C groups (28.99 ± 0.82 mg/ml). Stimulation of protein synthesis has been advanced as a contributing protective mechanism

which accelerates regeneration of cells³⁶. Thus, an increase in total protein level evaluated in this study suggests the stabilisation of endoplasmic reticulum by *C. azarolus* ME treatment, leading to protein synthesis in liver and this supports other reports³⁷.

Estimation of CAT activity

Endogenous antioxidant, SOD detoxifies the super anion radicals into H₂O₂ and CAT converts this by-product into water and molecular oxygen, which contribute to the protective

function by scavenging for any excess lipid peroxidation products generated^{38,39}. Thus, *in vivo* effects of ME of *C. azarolus* and vitamin C on CAT activity in liver and kidney of rats were evaluated and shown in Figure. 4. It was observed that the treatment of rats with the ME of *C. azarolus* at doses of 100 and 200 mg/kg exhibited increased activity of CAT in liver, but this increase didn't reach statistically significant difference except for the dose 200 mg/kg (49.53 ± 2.31 U/ g tissue) when compared to control group (34.67 ± 4.53 U/g tissue). However, it was observed a significant increase ($p < 0.01$) in the activity of renal CAT in the groups treated with doses of 100 and 200 mg/kg of *C. azarolus* extract (73.93 ± 4.89 U/ g tissue and 78.88 ± 6.84 U/ g tissue, respectively) when compared with control group (40.84 ± 6.11 U/g tissue). In the present study, the increase in CAT activity observed after the treatment with ME of *C. azarolus* may be due to the high levels of exogenous antioxidants as polyphenols in this extract. Similar studies have shown a positive effect of different classes of polyphenols on antioxidant enzyme activities (SOD, CAT and GPx) in animal model and human studies^{29,40-44}. The mechanisms underlying the activation of antioxidant enzymes by polyphenols are not fully understood but a number of studies indicated also an association between polyphenols and antioxidant enzyme expression via nuclear factor-erythroid-2-related factor 2 (Nrf 2) activation *in vivo* and in various cultured cell lines^{45,46}. Oliveras-López *et al.*³⁰ demonstrated that oral extra virgin oil intake increase CAT and GPx activities and their mRNA expressions in healthy adults. Hence, these findings that may support our hypothesis in that the ME of this plant might exert antioxidant activities based on its ability to stimulate activity of antioxidant enzymes and an induction of gene expression.

Estimation of GSH level

ME of *C. azarolus per os* treatment to rats at doses of 100 and 200 mg/kg increased the level of GSH in liver without reaching the statistical significance when compared with control group (18.34 ± 2.47 μ mol/g tissue) and vitamin C groups (17.61 ± 0.80 μ mol/g tissue) (Figure.5). Also, administration of same doses in rats didn't produce significant increase in renal GSH level except in the case of dose of 200 mg/kg which had the value of 26.29 ± 3.41 μ mol/g tissue. However, the treatment with the vitamin C showed a considerable effect in increasing renal GSH level (32.60 ± 2.822 μ mol/g tissue) compared to control group and *C. azarolus* treated group. GSH plays a primary role in the maintenance of a balanced redox status, where GPx and GST work together with GSH in the decomposition of hydrogen peroxide, superoxide anions. Hence, it can serve

as a potential marker of susceptibility, early and reversible tissue damage, and of decrease in antioxidant defense^{47,48}. Reportedly, it was found that the increase in lipid peroxidation was associated with GSH depletion^{37,49}. In this study, ME administered at dose of 100 and 200 mg/kg increased slightly the hepatic and renal reduced glutathione except for the *C. azarolus* (200 mg /kg), where the increase in GSH was found significant. This finding is consistent with that of Akila and Devaraj⁵⁰ whose demonstrated that oral administration of tincture of *Crataegus* (TCR) made from alcoholic extract berries of *Crataegus oxyacantha* (0.5 ml/100 g body weight) to rats fed with an atherogenic (4% cholesterol, 1% cholic acid, 0.5% thiouracil) diet caused a significant decrease in lipid accumulation in the liver and aorta and significantly restored the activity of antioxidant enzymes such as SOD, CAT, GPx, and glutathione. This effect could be attributed to the synergistic activity of flavonoids in TCR Taking into account the fact that ME of *C. azarolus* contained high concentration of phenolics and flavonoids, such constituents have demonstrated previously significant increase of GSH level^{48,51}, thus, their presence could explain the elevated content of GSH by ME of *C. azarolus* treatment in rats or maintaining the normal ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) and a normal redox state in liver and kidney. Furthermore, the increase in the levels of GSH may possibly be due to *de novo* synthesis and/or GSH regeneration³⁷ via glutathione reductase activation and inhibiting free radical formation.

Estimation of lipid peroxidation

The concentration of MDA was measured as an index of the extent of lipid peroxidation in different extracts treated groups. It was observed that treatment (Figure.6) with ME of *C. azarolus* at dose of 200 mg/kg significantly reduced ($p < 0.001$) the level of MDA in liver (52.1 ± 2.16 nmol/ g tissue) compared to control group (81.69 ± 3.49 nmol/ g tissue). The same results were obtained in kidney homogenates of rats treated with ME of *C. azarolus*. There was significant decrease in the levels of MDA in rat groups treated with 100 and 200 mg/kg of ME of *C. azarolus* with values of 55.56 ± 7.23 and 38.13 ± 4.29 nmol/ g tissue, respectively when compared with control group (89.04 ± 8.52 nmol/ g tissue). The same doses exhibited similar effect to that of rats receiving the standard drug; vitamin C (38.74 ± 2.98 nmol/ g tissue). Propagative lipid peroxidation is a degenerative process that affects cell membranes and other lipid containing structures under conditions of oxidative stress⁵².

As mentioned above, the observed results may be due to the high polyphenolic content of the plant extract. Accordingly, a lower level of oxidative stress and improvement in antioxidant status was found in many researches after polyphenol-rich food administration mainly based on the reduction of thiobarbituric acid reactive substance levels (TBARS)^{43,53,54}. Moreover, the reduction of the lipid peroxidation level in groups treated with ME of *C. azarolus* could be due to the increase in the antioxidant status, resulting in an increase in the activity of CAT and concentration of the GSH compared to the control group (Figure. 4 and 5). It was reported that if the

CAT and GSH activities are not sufficiently enhanced to metabolize hydrogen peroxide, this can lead to increased hydrogen peroxide and TBARS levels. It is noteworthy that, in mice supplemented with ferulic acid and clofibrate, the increase of antioxidant enzyme activities resulted in significant reduction of hepatic TBARS levels compared with the control group⁵⁵. Finally on the basis of these investigations, we could conclude that the administration of *C. azarolus* ME caused an increase in the total plasma antioxidant capacity and an improvement of antioxidant status and decreasing lipid peroxidation by lowering MDA level in liver and kidney tissues. This *in vivo* antioxidant activities might be due to the synergistic action of bioactive compounds present in the extract which are probably polyphenols, emphasizing the influence of these antioxidants in the diet for human health. As the present findings are obtained in healthy rats with no oxidative stress induction, this indicates that *C. azarolus* extract can improve the base line of the defense mechanism against possible oxidative stress, thus decreasing susceptibility to diseases related to oxidative stress as cardiovascular and liver diseases. Further studies are required to gain more insight in to the possible mechanisms of action of this plant extract.

ACKNOWLEDGEMENTS

This work was supported by the Algerian Ministry of Higher Education and Scientific Research (MESRS) and by Thematic Agency for Research in Health Sciences (Grant No. 361/25/09/2011). We express our gratitude to these organizations.

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