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Antioxydant and protective effect of *Cynara cardunculus* against paracetamol induced liver mitochondria oxidative stress.

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Abstract

Cynara cardunculus L. (Asteraceae), commonly known as cardoon, is a Mediterranean species that grows naturally in harsh habitat conditions. It is used as a food for its nutritional value and ethnomedicinal properties linked to liver cleansing. The aim of the present study was to evaluate the hepatoprotective action of *Cynara cardunculus* butanolic extract (CCBE) against paracetamol (APAP) induced acute liver injury in rats. Results showed that APAP intoxication caused increase levels of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). This phenomenon was paralleled by an impaired liver mitochondria redox status (reduced glutathione (GSH)), glutathione peroxidase (GPx), glutathione S-transferase (GST), and superoxide dismutase (SOD) and increased lipid peroxydation (LPO) in APAP treated rats. The pretreatment with CCBE blocked the increases of serum enzymes, reversed the mitochondrial LPO levels, and restored the mitochondrial antioxidant defense system (GSH, GST, GPX and SOD). The effect of CCBE was comparable to that of standard antioxidant *N*-acetylcysteine. Moreover, the antioxidant property of CCBE was also proved by *in vitro* assays as established by DPPH and hydroxyl radical scavenging activity and iron chelating ability. Our results indicate that the protective mechanism of CCBE may underlie the radical scavenging activity and the enhancement of mitochondrial antioxidant system.

Keywords: Cynara cardunculus, antioxydant, paracetamol, liver, mitochondria.

Introduction

The liver is the chief organ involved in the metabolism of xenobiotics. Among these compounds is paracetamol (APAP). APAP is a widely used analgesic and antipyretic drug that is safe at therapeutic doses but which can precipitate liver injury at high doses [1]. At therapeutic doses, APAP is rapidly metabolized in the liver, mainly by glucuronidation and sulfation. A small amount of APAP is oxidized by the cytochrome P450 system to the highly reactive and cytotoxic-intermediate metabolites N-acetyl-p-benzoquinone imine; (NAPQI) [2]. Under normal conditions, NAPQI is detoxified by the presence of reduced GSH; however, following large doses of APAP, the glucuronidation and sulfation pathways are overwhelmed and hepatic GSH is depleted [1]. Once cellular GSH is consumed, NAPQI covalently binds to cellular proteins including a number of mitochondrial proteins [3]. The well-established effects of an APAP overdose on mitochondria include inhibition of mitochondrial respiration, enhancement of reactive oxygen species ROS and peroxynitrite formation, and ultimately opening of the mitochondrial membrane permeability transition pore (MPTP) [4-5].

Many dietary polyphenols are antioxidants, and the possibility exists that they protect against oxidative damage by directly neutralizing reactive oxidants and to increase the capacity of endogenous antioxidant defenses and modulate the cellular redox state [6]. *Cynara cardunculus* is a member of the Asteraceae (Compositae) family, and originates from the Mediterranean basin. Several *in vitro* and *in vivo* studies have investigated the antoxidative and hepatoprotective properties of globe artichoke leaf extracts, and their constituents, against liver cell damage induced by different hepatotoxins [7-9]. In addition, the plant has been reported to prevent LDL oxidation [10]. Furthermore, antigenotoxic effect of *Cynara cardunculus* were reported [11]. These variable therapeutic functions cannot be attributed to a single active compound; however it could be due to the presence of several bioactive components which generate synergistic pharmacologic effects. Extensive studies on the chemical components of the Cynara species have revealed it to be a rich source of the polyphenol compounds with antioxidant properties [8, 10, 12]. In order to supply more scientific evidences on the globe artichoke leaf extract and because there is no scientific report about *Cynara cardunculus*, Algerian wild artichoke, the purpose of the present study is to evaluate the antioxidant activity of *Cynara cardunculus* butanolic extract (CCBE) using various in vitro assay methods and

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to evaluate its hepatoprotective effect against APAP mediated mitochondria oxidative stress.

Material and Methods

Chemicals and Reagents

1,1-diphenyl-2-picryl-hydrazyl (DPPH.), Folin- Ciocalteus's phenol reagent, Thiobarbituric acid (TBA), 5,5-dithiobis-2-nitrobenzoic (DTNB), 1-Chloro-2, 4-dinitrobenzene (CDNB), deoxyribose,ferrozine ,nitrobluetetrazolium (NBT), Trichloracetic acid(TCA), Ethylenediaminetetraacetic acid(EDTA), reduced nicotinamide adenine dinucleotide phosphate (NADPH), were procured from Sigma Chemical Co.(St. Louis, Mo). The solvents and/or reagents were of analytical grade.

Plant material and extract preparation

The plant material was collected, from Ben Badis region - Constantine –East of Algeria. The dried aerial parts of *Cynara cardunculus* (1890g) were macerated with EtOH-H_2O (70:30 v/v) three times for 24, 36 and 48 hours successively. The crude extract was concentrated at room temperature. The hydroethanolic extract was diluted with 756 ml H_2O. After filtration, the remaining aqueous solution was extracted successively with petroleum ether, $CHCl_3$, EtOAc, and n-ButOH. The organic layers were dried with Na2SO4 giving, after removal of solvents under reduced pressure, $CHCl_3$ (1.55g), EtOAc (11.97g), and n-ButOH (28.04g) extracts. The obtained *Cynara cardunculus*butanolic extract (CCBE) fraction was subsequently subjected for *in vitro* and *in vivo* assays.

Total polyphenols quantification

The concentrations of phenolic compounds in CCBE, expressed as gallic acid equivalents, were measured using a modified method of Singleton and Rossi [13]. Basically, 100 µl of extract was mixed with 250 µl of Folin-Ciocalteu reagent (1N) and allowed to stand at room temperature for 2 min. Then, 1250 µl of 20% sodium carbonate solution was added, mixed and allowed to stand at room temperature in the dark for 2 h. The absorbance was read at 765nm, and the total polyphenols concentration was calculated from a calibration curve, using gallic acid as standard. The results were expressed as mg gallic acid equivalents (GAEq)/g extract.

In vitro antioxidant assays

DPPH radical scavenging activity

The determination was based on the method proposed by Blois *et al.*, [14] but with slight modification. Briefly, 1ml of a 0.2mM methanol solution of DPPH was incubated with varying concentrations of CCBE (5-150µg/ml).The mixture was shaken an allowed to stand of room temperature for 30min and the absorbance was measured at 517nm. Ascorbic acid was used as standard. The percent inhibition of DPPH was calculated as follows:

% Inhibition = [(Abs C – Abs S) /Abs C] x100 (1)

Where Abs C was the absorbance of the control, and Abs S was the absorbance of the extract or standard.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of the CCBE was evaluated by the deoxyribose method described by Halliwell *et al.*, [15]. Hydroxyl radical were generated by incubating the following reagents in a final volume of 1.2 ml 10 mM phosphate buffer (pH 7.4) at 37 C for 60 min: 1.4 mM H_2O_2, 100 µM $FeCl_3$ and 2.8 mM deoxyribose, 100 µM EDTA and 100 µM ascorbic acid in presence or absence (control) of the extract. Degradations of deoxyribose sugar induced by hydroxyl radical was determined by addition of 1 ml TBA (1%) and 1 ml TCA (5%); the contents were mixed well and boiled at 100 C for 20 min. The pink chromogen formed was determined by measuring its absorbance at 535 nm. Ascorbic acid was used as standard. The percentage inhibition effect on hydroxyl radicals was calculated according to equation (1).

Iron chelating activity

The chelating effect of ferrous ions Fe^{2+} was estimated by the method of Dinis *et al.*, [16]. Various concentrations of CCBE were mixed with 2mM $FeCl_2$After 1min, 5mM ferrozine was added. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. EDTA was used as standard. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated using equation (1).

In vivo hepatoprotective activity

Animals and experimental design

Male albino rats weighting about 195-200g were obtained from the Pasteur institute in Algiers, Algeria. The animals were maintained in the controlled temperature with 12hours period of light and dark and fed with standard feed and water. Animals were randomly divided into 4 groups (n=5). Animals in group 1 (control) were treated only with 5ml/kg NaCl (0.9%) throughout the duration of the experiment. Those in group 2 (APAP) were treated with 5ml/kg NaCl (0.9%) for 10 days. Animals in group 3 (NAC+APAP) were fed orally standard drug N-acetylcysteine (200 mg/kg) once daily for 10 consecutive days [17]. Animals in groups 4 (CCBE+APAP) were fed orally CCBE (300 mg/kg) once daily for 10 consecutive days. On10th day, group 2, 3 and 4 received a single dose of APAP (750 mg/kg) to induce hepatotoxicity [18].

Estimation of serum enzymatic markers of liver function

After 18 hours of APAP challenge, the blood samples were collected and were centrifuged at 1000 g for 10 minutes at 40C.

The obtained serum was stored at 4 C for the estimation of biochemical markers of hepatic injury, namely ALT, AST andALP. These estimations were done according to the standard procedures given along with the kits purchased.

Estimation of liver mitochondria oxidative stress markers

After collection of blood samples, rats were decapitated, and the liver was rapidly excised, weighed and processed for mitochondria oxidative stress markers. Liver mitochondria were isolated by the method of Johnson and Lardy [19]. The weighed amount of liver tissue was homogenized in ice-cold sucrose (0.25 M) buffer. This homogenate was centrifuged at 600 g for 10 min. The supernatant fraction was decanted and centrifuged at 15000 g for 5 minutes. The resultant mitochondrial pellet was then washed and resuspended in the same buffer. Mitochondrial LPO was determined by the procedure of Ohkawa *et al.*,[20]. Malondialdehyde (MDA), formed as an end product of the peroxidation of lipids, served as an index of oxidative stress. LPO results were expressed as µmoles of MDA/ mg protein. Total reduced glutathione (GSH) was determined by the method of Ellman *et al.*, [21]. GSH levels were expressed as µmole of GSH /mg protein. Superoxide dismutase (SOD) was assayed according to the method of Winterbourn *et al.*, [22]. SOD unit was defined as the amount of enzyme required to inhibit the reduction of NBT by 50%. Glutathione peroxidase (GPx) was assayed by the method of Paglia and Valentine [23] by monitoring the oxidation of reduced NADPH at 340 nm. GPX unit was defined as the number of µmoles of NADPH oxidized/ minute. Glutathione S-transferase (GST) was assayed by the method of Habig *et al.*, [24]. GST unit was expressed as nmol of CDNB–GSH conjugate formed/ min. The specific activity of SOD, GPX and GST was expressed as a unit/ mg protein. The protein content was determined by the method of Lowry *et al.*, [25].

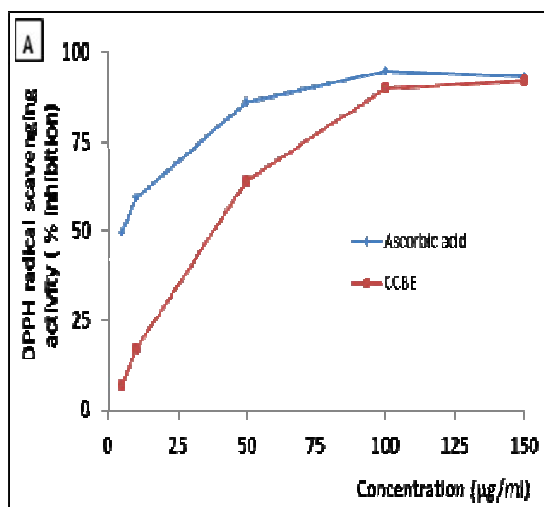
Statistical analysis

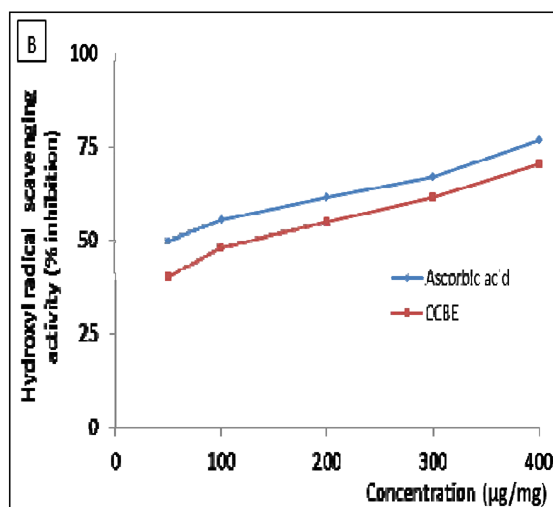
In vivo experimental results were statistically analyzed using the Student's *t*-test for unpaired data, with P 0.05 considered significant. Values reported are means ± S.E.M (n=5 rats). All *in vitro* measurements were replicated three times and the IC50 values were calculated from linear regression analysis.

Results

Polyphenols amount and *in vitro* antioxidant activity

The amount of total phenols in the CCBE was investigated by the Folin-Ciocalteu method, which is an oxidation/reduction reaction based on the redox properties of antioxidant compounds that can react with the Folin-Ciocalteau reagent enhancing the measurement of phenolic concentration. In this study, the total phenolic content in CCBE was recorded as 303.66±15.91mg GAEq /g extract. Data regarding *in vitro* antioxydant activities of CCBE are depicted in Figure 1.The extract was capable of scavenging DPPH radical in concentration dependent manner (Figure 1A).The IC50 value of DPPH scavenging activity of the CCBE was found to be 29.32µg /ml while for standard it was 3.39 µg/ml. In hydroxyl scavenging assay, the activity was increased on increasing CCBE concentration from 50 to 400 µg/ml (Figure 1B). The IC50 value for inhibition of hydroxyl radical was found to be113.89±1.91µg/ml and 59.78±4.69µg/ml for CCBE and standard respectively. As observed in DPPH and hydroxyl radical scavenging assays, the percentage of metal chelating activity was determined to be increased with the increase in the CCBE concentration (Figure1C). The IC50 value for metal chelating activity of CCBE was found as 118.91±10.54 µg/ml, whereas the IC50 value of standard was found as 17.40± 2.94 µg/ml.





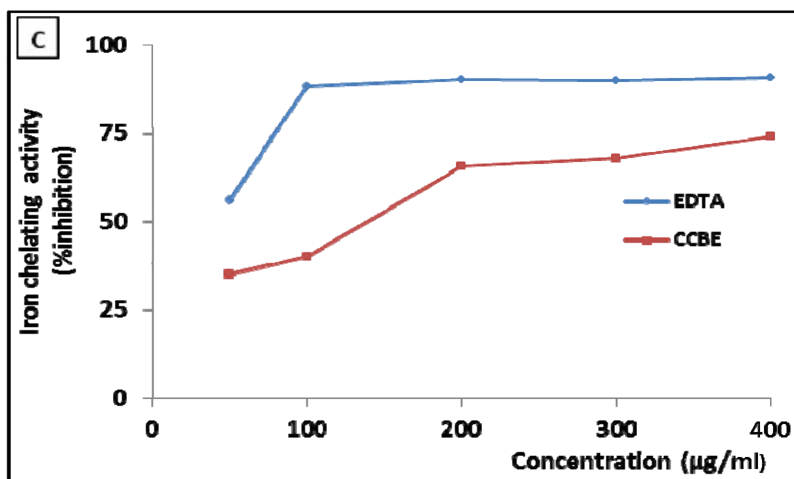


Figure 1. *In vitro* antioxydant activities of *C.cardunculus* extract (CCBE).
(A) DPPH radical scavenging activity.(B) Hydroxyl radical scavenging activity.
(C) Iron chelating activity. Values expressed as Mean± S.E.M; (n=3).

In vivo hepatoprotective activity

Serum enzymatic markers of liver function

A statistically significant increase was detected in the serum AST, ALT and ALP levels of the APAP treated rats in comparison with the control (Figure 4). Pretreatment with CCBE (300mg/kg) for 10 days significantly prevented (P 0.005) the elevation of serum enzymes as compared with those of APAP treated group. NAC (200mg/kg) pretreated rats also showed significant decrease in AST, ALT and ALP activities when compared to APAP treated rats. The results are compiles in (Figure 2).

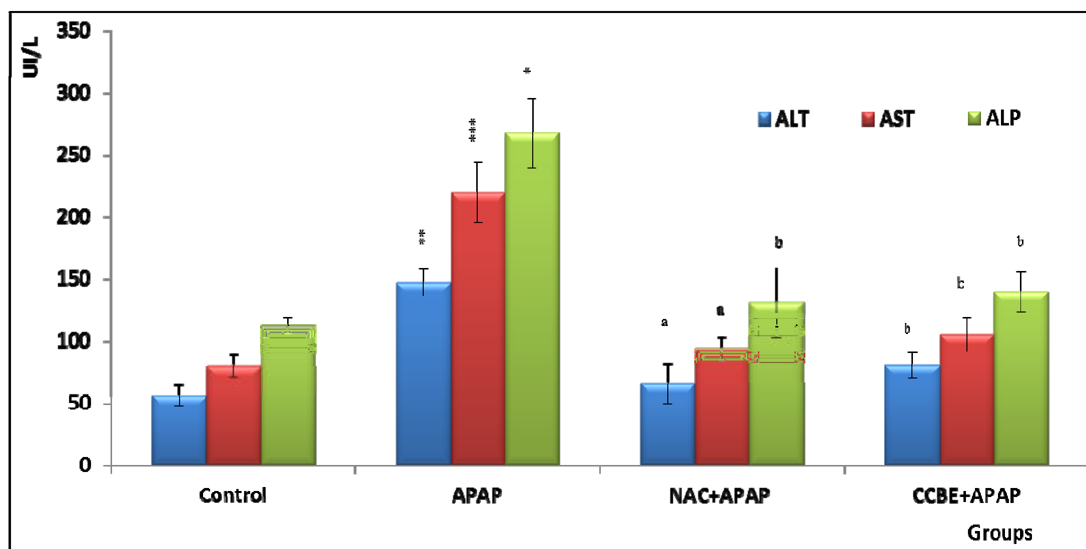


Figure 2. Effect of *C. cardunculus* extract (CCBE) on serum enzymes of liver function in paracetamol intoxicated rats.
*P 0.05, **P 0.01, ***P 0.001 compared with control group; ^{b}P 0.05, ^{a}P 0.001 compared with APAP alone group. Values are Mean± S.E.M (n=5).

Oxidative stress markers in liver mitochondria

As shown in Table 1, the mitochondrial LPO level in APAP treated rats was increased significantly (P 0.001) as compared with controls, while pretreatment with CCBE significantly (P 0.001)

prevented the increase in mitochondrial LPO level. The effect of CCBE was comparable with that of standard drug (NAC). APAP administration alone significantly altered the mitochondrial GSH level and antioxidant enzymes (GST, GPx and SOD) activities, whereas pretreatment with CCBE resulted in a significant (P 0.001) enhancement in mitochondrial GSH level and antioxidant enzymes activities when compared to APAP treated rats. Liver mitochondria isolated from NAC treated rats also showed a significant increase in estimated antioxidant markers when compared with APAP treated rats.

Table 1. Effect of *C.cardunculus* extract(CCBE) on liver mitochondrial oxidative stress markers in paracetamol intoxicated rats.

Groups	LPO nmol/mg protein	GSH µmole/mg protein	SOD U/mg protein	GPx U/mg protein	GST U/mg protein
Control	0.98±0.17	4.69±0.43	47.75±3.93	65.00±5.97	6.54±0.34
APAP	2.04±0.18*	2.18±0.35*	28.49±5.83*	33.54±3.52*	3.04±0.32*
NAC+APAP	1.49±0.31[a]	4.01±0.31[a]	44.45±3.70[a]	56.59±7.36[a]	5.24±0.96[a]
CCBE+APAP	1.33±0.23[a]	4.09±0.60[a]	47.02±5.62[a]	61.82±1.67[a]	5.06±0.16[a]

*P 0.001 compared with control group; [a]P 0.001 compared with APAP alone group.Values are expressed as mean ± S.E.M (n=5).

Discussion

Modern screenings of the plant Kingdom for the discovery of new medications are not performed randomly, but rely on ethnobotanical knowledge and previous phytochemical and bioactivity investigations. Metal chelation propriety, DPPH radical and hydroxyl radical scavenging activities are most commonly used for the *in vitro* evaluation of the total antioxidant behavior of extracts. In this connection, our results indicated that CCBE show a noticeable effect on scavenging DPPH radical and act as primary antioxidant. This can be explained in terms of the presence of active compounds such as polyphenols. Phenolic compounds generally exhibited significant scavenging effect against the DPPH radical [12]. In another set of experiment, CCBE shows a good scavenging activity against hydroxyl radical generated in a Fenton reaction system and thereby preventing deoxyribose degradation, which is in agreement with findings of Valentao *et al.*, [26]. Hydroxyl radical has the capacity to join nucleotides in DNA and cause strand breakage [27] and is known to be capable of abstracting hydrogen atoms from membrane lipids and brings about peroxidic reaction of lipids [10, 27]. The chelating property of the phytochemicals can contribute significantly towards antioxidant behavior [28]. The high amount of phenolic compounds found in CCBE was likely to be the main contributors of antioxidant activity of the extract. This is in accordance to results obtained by previous reports which demonstrated that polyphenols are able to act as antioxidants by virtue of their hydrogen donating and metal chelating capacities [10, 12, 26]. The observed *in vitro* activities suggest that the investigated plant extractcould exert protective effects also *in vivo* against oxidative and free radical injuries occurring in different pathological conditions.

Liver cells participate in variety of metabolic activities and thus contain a host of enzymes. An obvious sign of hepatic injury is leakage of cellular enzymes into serum. The extent of APAP induced hepatotoxic effect was assessed by the levels of released cytoplasmic enzymes such as ALT, AST and ALP in circulation, indicating liver necrosis. The normalization of serum markers by CCBE suggests that they are being able to condition the hepatocytes so as to protect the membrane integrity against APAP induced leakage of liver specific enzymes in blood stream. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma in the presence of protective compounds [8]. Polyphenols are thought to be involved in the prevention of APAP induce hepatotoxicity by reduction of NAPQI formation, with the inhibition of phase I enzymes probably playing the most important role [29].

It is widely accepted that APAP can produce potentially fatal liver necrosis, via an intermediate toxic metabolite NAPQI [2]. Thereafter, NAPQI binds to intracellular proteins including a number of mitochondrial proteins and causes mitochondrial dysfunction and superoxide formation. Superoxide reacts with nitric oxide to form peroxynitrite, which is responsible for intracellular protein nitration upon APAP overdose [3- 4]. SOD is the first enzyme involved in the mitochondria antioxidant defense by catalysis the dismutation of superoxide into hydrogen peroxide, which is less harmful. GPx detoxifies this hydrogen peroxide by reducing it to water, thus completely decreases the risk of hydroxyl radicals formation via the metal catalyzed Haber-Weiss reaction. Also, GPx is of major importance in detoxification of lipid peroxides [30]. GR is a crucial enzyme in glutathione metabolism because it reduces glutathione disulphide (GSSG) back to the reduced form [31]. Besides, mitochondrial GST may play an important role in defense against chemical and oxidative stress. GST achieves detoxication by catalyzing the conjugation of GSH to various electrophilic substrates such as NAPQI [2]. Mitochondrial GSH is not only an antioxidant but also is an essential factor for maintenance of thiol groups of mitochondrial proteins in the reduced state [32]. In fact, oxidation of mitochondrial GSH has greater toxic effect on cell viability compared to cytosolic GSH oxidation [33]. The balance between the production and scavenging of ROS within mitochondria leads to homeostasis in general; however, the balance is somehow shifted towards the formation of free radicals, which through a series of events deregulates mitochondrial functions [3, 4, 32- 33]. LPO not only leads to increase of ROS production but also could damage mitochondrial membrane integrity and open the membrane permeability transition pore (MPTP) [33]. The opening of the MPTP

is an important step in both necrosis and apoptosis mechanisms in APAP- hepatotoxicity [4-5].

In the present study, APAP administration caused a significant elevation in the level of LPO with concomitant decline in the level of GSH and in the activities of GPX, GST and SOD in liver mitochondria. Thus suggests mitochondria oxidative stress. However, pretreatment with CCBE prior to APAP intoxication could not only prevent the APAP increased mitochondria LPO, but also protect the antioxidant machineries of the mitochondria as revealed from the enhanced levels of GPx, GST, and SOD activities, increased level of GSH content. This suggests that in order to overcome the oxidative damage in liver mitochondria, some other compensatory mechanisms exist in addition to antioxidant enzymes. Any compound, natural or synthetic, with antioxidant properties might contribute towards the partial or total alleviation of this type of damage. NAC is the primary antidote for an APAP overdose; it acts as an ROS scavenger and hepatic GSH precursor [1,4]. Reversal of decreased enzymes and inhibition of LPO appears to be due to direct free radical scavenger activity and/or activation of antioxidant system in the presence of CCBE polyphenolic compounds. Our present data are in agreement with other reports in the literature which indicate that artichoke leaf extract, and especially its luteolin-7-*O*-glucoside compound exhibited protective effects against DMSO-induced HepG2 cytotoxicity, such effect indicating a direct interaction with the detoxifying agent and an induction of repair mechanisms [34]. Recently, Juzyszyn *et al.*, [35] have shown that the salubrious effects of artichoke extracts may rely in part on the effects of their active compounds on the activity of the mitochondrial respiratory chain system. Furthermore, Reid and his collaborators [5] have suggested that iron chelators offered protection against APAP-induced liver mitochondria dysfunctions. From our *in vitro* assays, CCBE was demonstrate an effective capacity for iron binding, suggesting that its action as antioxidant may be related to its phenolic compounds that can chelate metal ions, thereby inhibiting mitochondrial LPO process. Polyphenols had the ability to reduce the concentration of the catalysing transition metal involved in the peroxidation of lipids [10, 28]. According to obvious views, it is plausible that observed hepatoprotective effect of CCBE against APAP toxicity may be related in part to its polyphenols iron chelating activity, free radical scavenger activity and enhancement of mitochondria anti-oxidative stress mechanisms.

Conclusion

In summary, increasing the antioxidant capacity of the mitochondrial compartment represents a potential protective effect of CCBE against APAP induced liver injury. The gained antioxidant activities could be correlated with the polyphenolic compounds present in the extract. So, whether the results of *in vitro* experiments are meaningful in the context of *in vivo* situation needs to be investigated further.

Author's contribution

NB: performed the experiments, data analysis and wrote the paper.
ZB: collected the plant and prepared the plant extracts.
AM: calculated IC50 values for *in vitro* assays.
SA: supervisedand designed this study and approved final paper.
FB and SB: have general supervision of VARENBIOMOL research unit.

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