Chemical composition, antioxidant properties and hepatoprotective effects of chamomile (*Matricaria recutita* L.) decoction extract against alcohol-induced oxidative stress in rat

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Abstract. The present study assessed the chemical composition, antioxidant properties, and hepatoprotective effects of subacute pre-treatment with chamomile (*Matricaria recutita* L.) decoction extract (CDE) against ethanol (EtOH)-induced oxidative stress in rats. The colorimetric analysis demonstrated that the CDE is rich in total polyphenols, total flavonoids and condensed tannins, and exhibited an important *in vitro* antioxidant activity. The use of LC/MS technique allowed us to identify 10 phenolic compounds in CDE. We found that CDE pretreatment, *in vivo*, protected against EtOH-induced liver injury evident by plasma transaminases activity and preservation of the hepatic tissue structure. The CDE counteracted EtOH-induced liver lipoperoxidation, preserved thiol -SH groups and prevented the depletion of antioxidant enzyme activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). We also showed that acute alcohol administration increased tissue and plasma hydrogen peroxide (H_2O_2), calcium and free iron levels. More importantly, CDE pre-treatment reversed all EtOH-induced disturbances in intracellular mediators.
In conclusion, our data suggest that CDE exerted a potential hepatoprotective effect against EtOH-induced oxidative stress in rat, at least in part, by negatively regulating Fenton reaction components such as H_2O_2 and free iron, which are known to lead to cytotoxicity mediated by intracellular calcium deregulation.

Key words: Chamomile — Ethanol — Liver — Oxidative stress — Rat

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAT, catalase; CDE, chamomile decoction extract; DW, dry weight; EtOH, ethanol; GPx, glutathione peroxidase; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TCA, trichloroacetic acid.

Introduction

Chamomile (*Matricaria recutita* L.) is one of the most widely used and well-documented medicinal plants in the world (Salamon 1992). It has been included for centuries in the pharmacopoeia of several countries including Tunisia. The phytochemical screening of this plant revealed that it is rich in cytoprotective active molecules such as phenolic compounds (McKay and Blumberg 2006). Therefore, owing mainly to their antioxidant (Hernández-Ceruelos et al. 2010) and anti-inflammatory (Bulgari et al. 2012) properties, chamomile extract, or its essential oils are known to exhibit neuro-protective (Ranpariya et al. 2011), anti-allergic (Chan-

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drashekhar et al. 2011), anti-microbial (Silva et al. 2012) and anticancer (Matić et al. 2013) activities.

Alcoholic liver diseases (ALD) remain the principal cause of death in both the advanced and developing countries of the world (Maddrey 2000). They are associated with one-third of all traumatic injury deaths *per* year (Li et al. 1997; Rehm et al. 2003). The administration of ethanol generates reactive oxygen species (ROS), including superoxide anion, hydroxyl radical and hydrogen peroxide (Nordmann 1994; Albano 2008). However, the accumulation of ROS causes lipid peroxidation and the depletion of endogenous antioxidant enzyme activities, such as SOD, CAT, and GPx, which contribute to the pathogenesis of ALD (Samuhasaneeto et al. 2009). Several investigations have been conducted to counteract the liver diseases induced by oxidative challenges during alcohol consumption by reinforcing the endogenous antioxidant defense system (Ozaras et al. 2003; Souli et al. 2013).

Accordingly, the present study was designed to evaluate the putative hepatoprotective role of chamomile (*Matricaria recutita* L.) decoction extract (CDE) (10 days) against oxidative stress induced by acute ethanol exposure and the mechanism involved in such protection.

Materials and Methods

Chemicals

Epinephrine, bovine catalase, 2-thio-barbituric acid (TBA) and butylated hydroxytoluene (BHT) were purchased from Sigma Chemicals Co. (Germany). All other chemicals used were of analytical reagent grade.

Preparation of chamomile decoction extract

Chamomile flowers were collected from the region of Beja (North-West of Tunisia) during March 2013. The chamomile decoction extract was prepared as previously described by Sebai et al. (2014). Briefly, the plant material was later dried in an incubator at 50°C during 72 h and powdered in an electric blender (Moulinex Ovatio 2, FR). The decoction was made with double distilled water (1/5; w/v) at 100°C during five minutes under magnetic agitation and the homogenate was filtered through a colander (0.5 mm mesh size). Finally, the obtained extract (CDE) was stored at –80°C until used.

Animals and treatment

Healthy adult male Wistar rats (200–220 g body weight, 15 weeks old) were purchased from the Pasteur Institute of Tunis and used in accordance with the local ethics committee of Tunis University for the use and care of animals

in accordance with the NIH recommendations. They were provided with standard food (standard pellet diet- Badr Utique-TN) and water *ad libitum* and maintained in animal house at controlled temperature (22 ± 2°C) with a 12 h light-dark cycle. The rats were divided into half a dozen groups of 10 animals each. Groups 1 and 3 served as controls and had a physiological solution (NaCl, 0.9%, p.o.). Group 2 received CDE (50 mg/kg, *b.w., p.o.*). Groups 4, 5, and 6 were pre-treated with various doses of CDE (25, 50, and 100 mg/kg, *b.w. p.o.*). Animals were pre-treated for 10 days. Sixty minutes after the administration of the last dose of CDE, all the animals, except those belonging to the groups 1 and 2, were acutely *per orally* intoxicated by EtOH (6 g/kg, *b.w.*). Two hours later, animals were sacrificed; their livers were rapidly excised and homogenized in phosphate buffer saline. After centrifugation at 10 000 × *g* for 10 min at 4°C, supernatant was used for biochemical determination of protein, free iron, calcium, H_2O_2, -SH groups and MDA levels as well as antioxidant enzyme activities. On the other hand, blood was likewise collected in heparinized tubes. After centrifugation at 3000 × *g* during 15 min, plasma was treated for free iron, H_2O_2, calcium and transaminase determinations.

Total phenolic content

Total phenolic content was determined by the colorimetric Folin-Cieucalteu method (Haseeb et al. 2006). Briefly, 500 µl of the extract was added to 10 ml of water and 0.5 ml of Folin-Cieucalteu reagent. After 5 min, 8 ml of 7.5% sodium carbonate solution was added. The reaction mixture was kept in the dark for 2 h and its optical density was measured at 765 nm using a UV-visible spectrophotometer (Beckman DU 640B). Gallic acid was applied as standard, and results were expressed as mg of gallic acid equivalents (mg GAE/g of dry weight (DW)).

Total flavonoids determination

Total flavonoid content was determined by the AlCl$_3$ colorimetric method (Djeridane et al. 2006). Briefly, 1 ml of the sample was mixed with 1 ml of 2% aluminium chloride solution. After incubation for 15 min at room temperature, the optical density of the reaction mixture was evaluated at 430 nm. Quercetin was used as a citation standard and the total flavonoid content was expressed as mg of quercetin equivalent (mg QtE/g DW).

Condensed tannin determination

The content of condensed tannins in CDE was determined utilizing the modified vanillin assay (Price et al. 1978). Briefly, 250 µl of the sample was added to 750 µl of water,

1.5 ml of vanillin solution (4%) and 750 μl of HCl 12N. The reaction mixture was kept in the dark for 20 min, and its optical density was measured at 500 nm. Catechin was used as standard, and the results were expressed as mg of catechin equivalents (mg CtE/g DW).

HPLC–PDA-MS analysis

The chromatographic separation and mass spectrometric analyses of phenolics contained in the aqueous extracts were carried out on an Agilent 1100 series HPLC systems equipped with a photodiode array detector (PDA) and a triple quadrupole mass spectrometer type Micromass Autospec Ultima Pt (Kelso, UK) interfaced with an ESI ion source. Separation was achieved using a Superspher® 100 (12.5 cm × 2 mm i.d., 4 μm, Agilent Technologies, Rising Sun, MD) at 45°C. The samples (20 μl) were eluted through the column with a gradient mobile phase consisting of A (0.1% acetic acid) and B (acetonitrile) with a flow rate of 0.25 ml/min. The following multi-step linear solvent gradient was employed: 0–5 min, 2% B, 5–75 min, 88% B, 75–90 min, 2% B.

The detection of PDA was performed in the 200–400 nm wavelength range and the mass spectra were recorded in negative ion mode, under the following operating conditions: capillary voltage, 3.2 kV; cone voltage, 115 V; probe temperature, 350°C; ion source temperature, 110°C. The spectra were acquired in the m/z range of 150–750 amu.

The identification of phenolic compounds was based on co-chromatography with authentic standards, when available. PDA spectra and mass spectra were used to affirm the identity of compounds previously reported in the literature (Clifford et al. 2007; Lai et al. 2007; Lin and Harnly 2010; Parveen et al. 2011).

Free radical-scavenging activity on DPPH

The antioxidant capacity of CDE was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity as previously described by Grzegorczyk et al. (2007). Briefly, various concentrations of CDE (20, 50, 100, 150, and 200 μg/ml) were added to 1 ml of 0.1 mm methanol solution of DPPH and incubated at 27°C during 30 min. The optical density of the sample was quantified at 517 nm. DPPH radical-scavenging activity (RSA), expressed as a percentage, was estimated utilizing the following formula:

$$\text{RSA (\%)} = \frac{A_{\text{DPPH}} - (A_{\text{sample}} - A_{\text{control}})}{A_{\text{DPPH}}} \times 100$$

Ascorbic acid was used as a reference molecule in the same concentration as the test extract.

All the analyses were executed in triplicate. The efficacy concentration 50 (EC50) value was determined as the concentration (in μg/ml) of the compound required to scavenge 50% of the DPPH radical.

Lipid peroxidation measurement

Liver lipid peroxidation was determined by MDA measurement according to the double heating method (Draper and Hadley 1990). Briefly, aliquots from liver tissue homogenates were mixed with BHT-trichloroacetic acid (TCA) solution containing 1% BHT (w/v) dissolved in 20% TCA (w/v) and centrifuged at $1000 \times g$ for 5 min at 4°C. Supernatant was blended with solution containing (0.5 N HCl, 120 mM TBA buffered in 26 mM Tris) and then heated at 80°C for 10 min. After cooling, the absorbance of the resulting chromophore was determined at 532 nm. MDA levels were determined by using an extinction coefficient for MDA-TBA complex of $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Thiol group measurement

The total concentration of thiol groups (-SH) was performed according to Ellman's method (Ellman 1959). Briefly, aliquots from liver tissue were mixed with 100 μl of 10% SDS and 800 μl of 10 mM phosphate buffer (pH 8), and the optical density was measured at 412 nm (A_0). After adding 100 μl of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), the reaction mixture was incubated at 37°C during 60 min and a new value (A_1) was determined. The thiol groups concentration was calculated from A_1 to A_0 subtraction using a molar extinction coefficient of $13.6 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. The results were expressed as nmol of thiol groups *per* mg of protein.

Antioxidant enzyme activity assays

The activity of SOD was determined using modified epinephrine assays (Misra and Fridovich 1972). At alkaline pH, superoxide anion O_2^- causes the autoxidation of epinephrine to adenochrome; while competing with this reaction, SOD decreased the adenochrome formation. One unit of SOD is defined as the amount of the extract that inhibits the rate of adenochrome formation by 50%. Enzyme extract was added in 2 ml reaction mixture containing 10 μl of bovine catalase (0.4 U/μl), 20 μl epinephrine (5 mg/ml) and 62.5 mM sodium carbonate/bicarbonate buffer pH 10.2. Changes in absorbance were recorded at 480 nm.

The activity of CAT was assessed by measuring the initial rate of H_2O_2 disappearance at 240 nm (Aebi 1984). The reaction mixture contained 33 mM H_2O_2 in 50 mM phosphate buffer pH 7.0 and the activity of CAT was calculated using the extinction coefficient of 40 mM$^{-1} \cdot \text{cm}^{-1}$ for H_2O_2.

The activity of GPx was quantified by the procedure of Flohé and Günzler (1984). Briefly, 1 ml of reaction mixture containing 0.2 ml of liver supernatant, 0.2 ml of phosphate buffer 0.1 M (pH 7.4), 0.2 ml of GSH (4 mM) and 0.4 ml of H_2O_2 (5 mM) was incubated at 37°C for 1 min and the reaction was stopped by the addition of 0.5 ml TCA (5%, w/v). After centrifugation at $1500 \times g$ for 5 min, aliquot (0.2 ml) of supernatant was combined with 0.5 ml of phosphate buffer 0.1 M pH 7.4 and 0.5 ml DTNB (10 mM) and reading at 412 nm. The activity of GPx was expressed as nmol of GSH consumed/min/mg protein.

Assessment of liver function

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using commercially available diagnostic kits supplied by Randox laboratories (Ardmore, Northern Ireland, UK).

H_2O_2 determination

The tissue and plasma H_2O_2 levels were performed according to Dingeon et al. (1975). Briefly, the hydrogen peroxide reacts with p-hydroxybenzoic acid and 4-aminoantipyrine in the presence of peroxidase leading to the formation of quinoneimine that has a pink color detected at 505 nm.

Iron measurement

Tissue and plasma non haem iron were measured colorimetrically using ferrozine as described by Leardi et al. (1998). Briefly, a solution of guanidine acetate was firstly used to dissociate iron from transferrin-iron complex. After its reduction by adding ascorbic acid, the labile iron reacts with ferrozine leading to the formation of pink complex measured at 562 nm.

Calcium determination

Tissue and plasma calcium were performed using a colorimetric method according to Stern and Lewis (1957). Briefly, at alkaline medium, calcium reacted with cresolphtalein leading to colored complex measurable at 570 nm.

Protein determination

Protein concentration was measured according to Hartree (1972) which is a slight change of the Lowry method. Serum albumin was used as standard.

Statistical analysis

The data were analyzed by one-way analysis of variance (ANOVA) and were expressed as means ± standard error of the mean (S.E.M.). The data are representative of 10 independent experiments. All statistical tests were two-tailed, and a p value of 0.05 or less was considered significant.

Results

Total phenolics, flavonoids, and condensed tannins

As shown in Table 1, the CDE (extraction yield, 5%) exhibited high levels of total phenolics (37.12 mg GAE/g DW), total flavonoids (24.83 mg QtE/g DW) and condensed tannins (1.74 mg CtE/g DW).

Characterisation of phenolic compounds of CDE by LC/MS analysis

The phenolic profile of CDE is shown in Figure 1. The identified compounds are presented in Table 2. Ten phenolic compounds were characterized and 7 (peaks 1–7) of them were unambiguously identified by comparing their retention time, UV and mass spectra with those of reference standards. They were gallic acid (peak 1; Rt = 4.2 min; [M-H]⁻ = 169), protocatechuic acid (peak 2; Rt = 7.7 min; [M-H]⁻ = 153), chlorogenic acid (peak 3; Rt = 16.7 min; [M-H]⁻ = 353), cafeic acid (peak 4; Rt = 17.6 min; [M-H]⁻ = 179), cafeoyl-quinic acid (peak 5; Rt = 20.18 min; [M-H]⁻ = 353), salicylic acid (peak 6; Rt = 21.2 min; [M-H]⁻ = 137) and quercetin (peak 7; Rt = 30.05 min; [M-H]⁻ = 301).

The remaining components (peaks 8–10) were provisionally identified based on the analysis of their UV, mass spectra, and comparison with literature (Clifford et

Table 1. EC50 values of DPPH radical-scavenging activity and contents of total polyphenols, total flavonoids and condensed tannins in chamomile decoction extract (CDE)

	Total polyphenols (mg GAE/g DW)	Total flavonoids (mg QtE/g DW)	Condensed tannins (mg CtE/g DW)	EC50 of DPPH radical-scavenging activity (µg/ml)
CDE	37.12 ± 3.24	24.83 ± 2.78	1.74 ± 0.25	165 ± 2.3
Ascorbic acid	–	–	–	147 ± 0.9

GAE, gallic acid equivalent; QtE, quercetin equivalent; CtE, catechin equivalent; EC50, the effective concentration of sample that can decrease DPPH concentration by 50%; DW, dry weight.
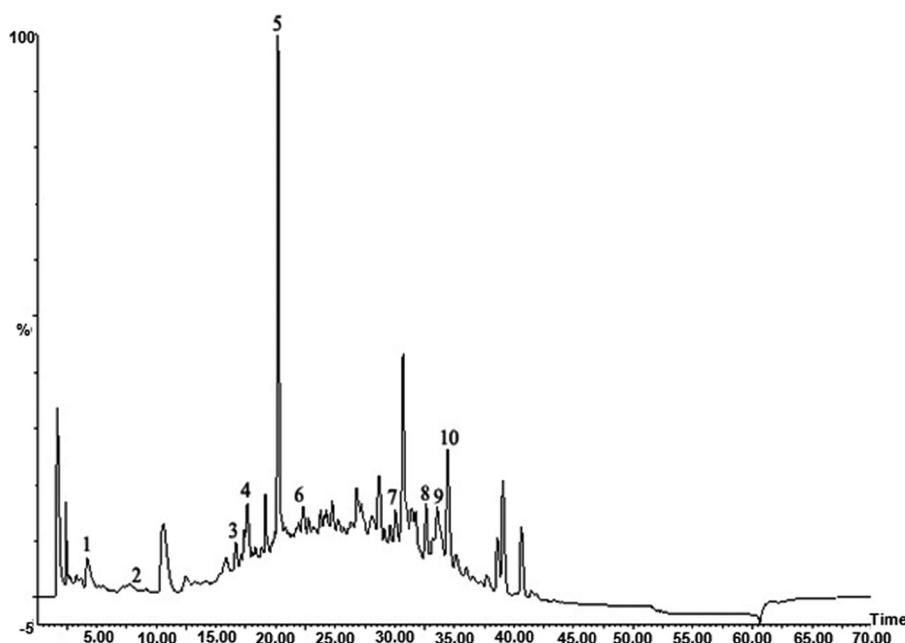
al. 2007; Lai et al. 2007; Lin and Harnly 2010; Parveen et al. 2011). Accordingly, peak 8 (Rt = 32.8 min; [M-H]⁻ = 391), peak 9 (Rt = 33.56 min; [M-H]⁻ = 299) and peak 10 (Rt = 34.43 min; [M-H]⁻ = 329) were tentatively identified as quinic acid derivative, hydroxybenzoic acid-O-hexoside and 5,7,4'-trihydroxy-6,3'-dimethoxyflavone, respectively.

Antioxidant capacity of CDE

Concerning the antioxidant capacity, we have found that the radical-scavenging activity of CDE and ascorbic acid against DPPH radical increased significantly in a dose-dependent manner (Fig. 1). The EC50 values calculated from the graph (Fig. 2) demonstrated that the RSA of CDE

(EC50 = 152.43 µg/ml) appeared significantly higher than ascorbic acid (EC50 = 87.57 µg/ml) as well known reference molecule (Table 1).

Assessment of liver injury

The data from Figure 3 show that acute EtOH (6 g/kg *b.w.*) administration significantly increased the plasma AST and ALT activity used as an index of liver injury while CDE alone at 50 mg/kg; *b.w.* has no effect on both parameters. CDE (25, 50 and 100 mg/kg; *b.w.*) pre-handling significantly and dose-dependently protected against liver injury caused by alcohol administration.

The histological study revealed minor microvesicular steatosis in the liver of rats treated with EtOH alone. CDE

Table 2. Retention time (Rt), wavelength of maximum absorption (λmax), deprotonated molecular mass [M-H]⁻ and tentative identification of the phenolic compounds in chamomile decoction extract

Peak	Rt (min)	λmax (nm)	[M-H]⁻	Tentative identification
1	4.2	255	169	Gallic acid
2	7.7	259, 295	153	Protocatechuic acid
3	16.7	325	353	Chlorogenic acid
4	17.6	323	179	Cafeic acid
5	20.18	328	353	Cafeoylquinic acid
6	21.2	327	137	Salicylic acid
7	30.05	255, 369	301	Quercetin
8	32.8	321	391	Quinic acid derivative
9	33.56	267	299	Hydroxybenzoic acid-O-hexoside
10	34.43	275, 339	329	5,7,4'-Trihydroxy-6,3'-dimethoxyflavone

pre-treatment greatly reduced the histopathological changes induced by acute alcohol intoxication (Fig. 4).

Liver lipoperoxidation

Bearing on the effect of EtOH and CDE on oxidative stress condition, we firstly studied the liver lipoperoxidation (Fig. 5). EtOH *per se* drastically increased the liver MDA level. CDE pre-treatment significantly and dose dependently reversed lipoperoxidation induced by EtOH treatment, while CDE alone at 50 mg/kg; *b.w.* had no effect on hepatic lipid peroxidation.

Changes in tissue -SH groups

We also examined the liver -SH groups level. Treatment with EtOH caused a considerable decrease in the content of -SH groups, which evidenced the induction of oxidative stress and significant thiol depletion during alcoholism. This effect was significantly and dose-dependently corrected by subacute CDE pre-treatment (Fig. 6).

Liver antioxidant enzyme activities

In the present study, the effects of EtOH and CDE treatment on hepatic antioxidant enzyme activities were investigated and the results are presented in Figure 7. As expected, acute EtOH treatment significantly decreased hepatic antioxidant enzyme activities as SOD (Fig. 7A), CAT (Fig. 7B), and GPx (Fig. 7C). CDE pre-treatment significantly reversed all EtOH-induced antioxidant enzymes depletion in a dose-dependent manner. More importantly, CDE alone significantly ameliorated SOD and CAT activities but not GPx.
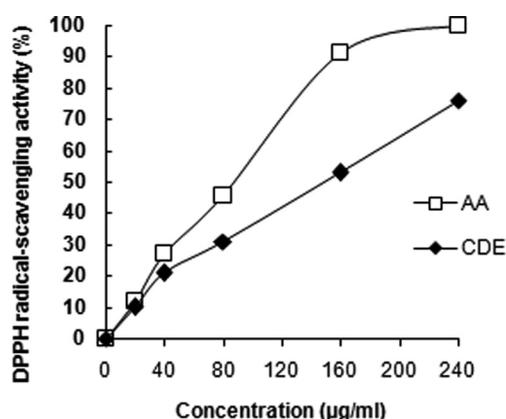
Tissue and plasma iron, H_2O_2 and calcium measurements

We further looked at the effect of ethanol and CDE on intracellular mediators as hydrogen peroxide (Fig. 8), free iron (Fig. 9) and calcium (Fig. 10) levels in plasma and hepatic tissue. As expected, alcohol treatment increased iron, H_2O_2 and calcium levels in plasma and liver. CDE pre-treatment significantly protected against EtOH-induced intracellular mediators disturbances in a dose-dependent manner. CDE alone had no considerable effects on these parameters.

Discussion

In the present work, we investigated the protective effects of CDE on EtOH-induced oxidative stress in rat liver as well
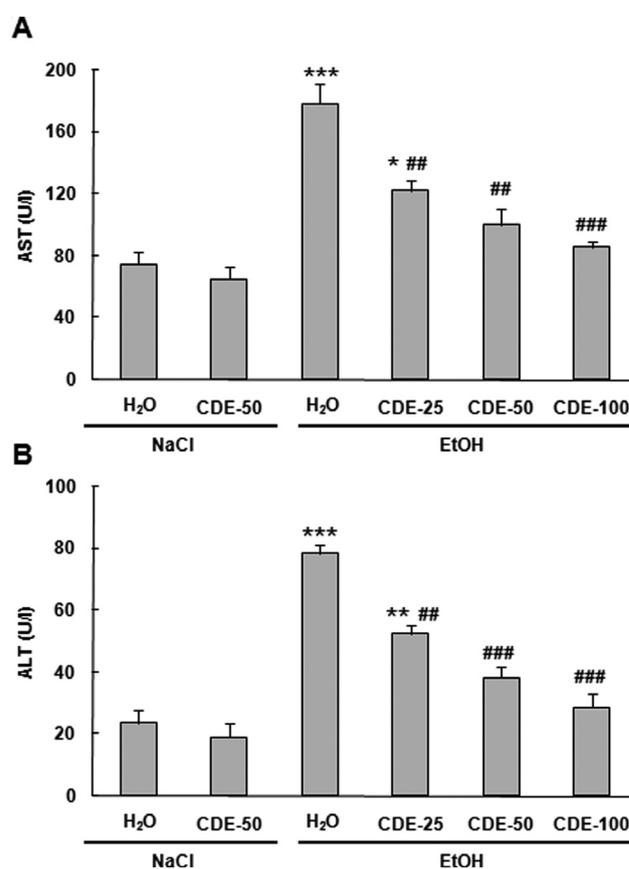

Figure 2. Free radical-scavenging activity of chamomile decoction, extract (CDE) and ascorbic acid (AA) on 2,2-diphenyl-1-picrylhydrazyl (DPPH).

Figure 3. Subacute effect of chamomile decoction extract (CDE) on acute EtOH-induced changes in plasma aspartate aminotransferase (AST, **A**) and alanine aminotransférase (ALT, **B**). Animals were pretreated during 10 days with CDE (25, 50 and 100 mg/kg b.w., *p.o.*) or vehicle (bidistilled H_2O), challenged with a single oral administration of EtOH (6 g/kg b.w.) or NaCl 9‰ for 2 hours. Assays were carried out in triplicate (*n* = 10). * *p* < 0.05, ** p < 0.01, *** *p* < 0.001 compared to control group; ## *p* < 0.01, ### *p* < 0.001 compared to EtOH group.
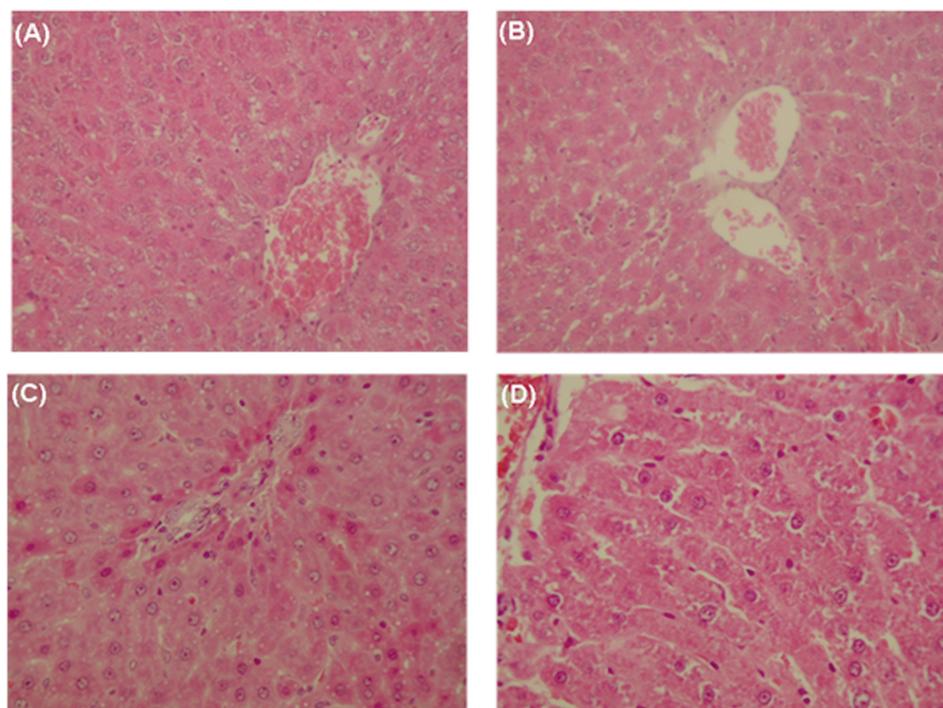
Figure 4. Liver histology showing the protective effect of CDE on EtOH induced hepatic injury in rats. Normal architecture in control (**A**) and CDE-treated animals (**B**). Minor microvesicular steatosis revealed by acute EtOH treatment (**C**) and protected by CDE pretreatment (**D**). CDE, chamomile decoction extract.

as characterized changes in intracellular mediators that may subserve such protection.

Our phytochemical study firstly revealed the presence of total polyphenols, total flavonoids, and condensed tannins in the CDE. The phenolic and flavonoids contents (37.12 ± 3.24 mg EAG/g DW and 24.83 ± 2.78 mg EQt/g DW, respectively) appear higher than those of other medicinal plants such as *Ceratonia siliqua* pods extract known for its anti-
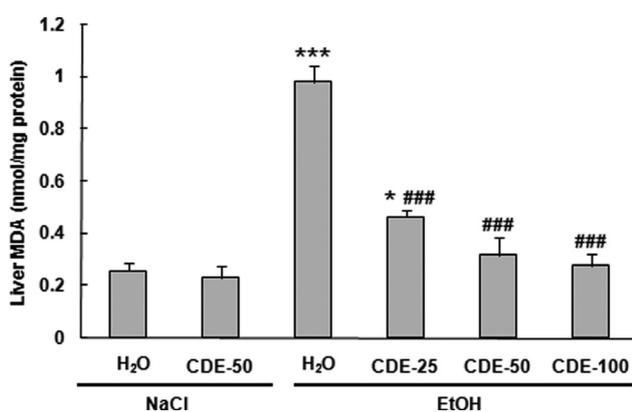

Figure 5. Subacute effect of CDE on acute EtOH-induced changes in liver MDA level. Animals were pre-treated during 10 days with CDE and intoxicated by a single oral administration of EtOH for 2 hours ($n = 10$). * $p < 0.05$, *** $p < 0.001$ compared to control group; ### $p < 0.001$ compared to EtOH group. CDE, chamomile decoction extract; MDA, malondialdehyde .
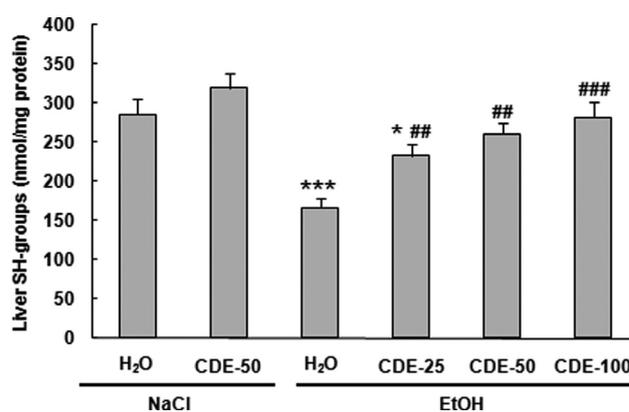
Figure 6. Subacute effect of CDE on acute EtOH-induced changes in liver -SH groups level. Animals were pre-treated during 10 days with CDE and intoxicated by a single oral administration of EtOH for 2 hours ($n = 10$). * $p < 0.05$, *** $p < 0.001$ compared to control group; ## $p < 0.01$, ### $p < 0.001$ compared to EtOH group. CDE, chamomile decoction extract.

oxidant and hepato-protective properties (Sebai et al. 2013; Souli et al. 2013). CDE is richer in polyphenols and flavonoids but not condensed tannins when compared to infusion and maceration (data not shown). The use of HPLC-PDA-MS technique allowed us to identify gallic acid, protocatechuic acid, chlorogenic acid, cafeic acid, cafeoylquinic acid, salicylic acid, quercetin, quinic acid derivative, hydroxybenzoic acid-O-hexoside, 5,7,4'-trihydroxy-6,3'-dimethoxyflavone. Our results generally corroborate previous reports with some discrepancies (Guimarães et al. 2013). However, the variability in chemical composition as well as phenolic compounds content can be attributed to the climatic conditions and the mode of extraction (Papagiannopoulos et al. 2004). Indeed, this variability may also be due to the variety of chamomile as recently described (Sharafzadeh and Alizadeh 2011).

On the other hand, using the DPPH radical-scavenging assay, we found that CDE presents a high scavenging
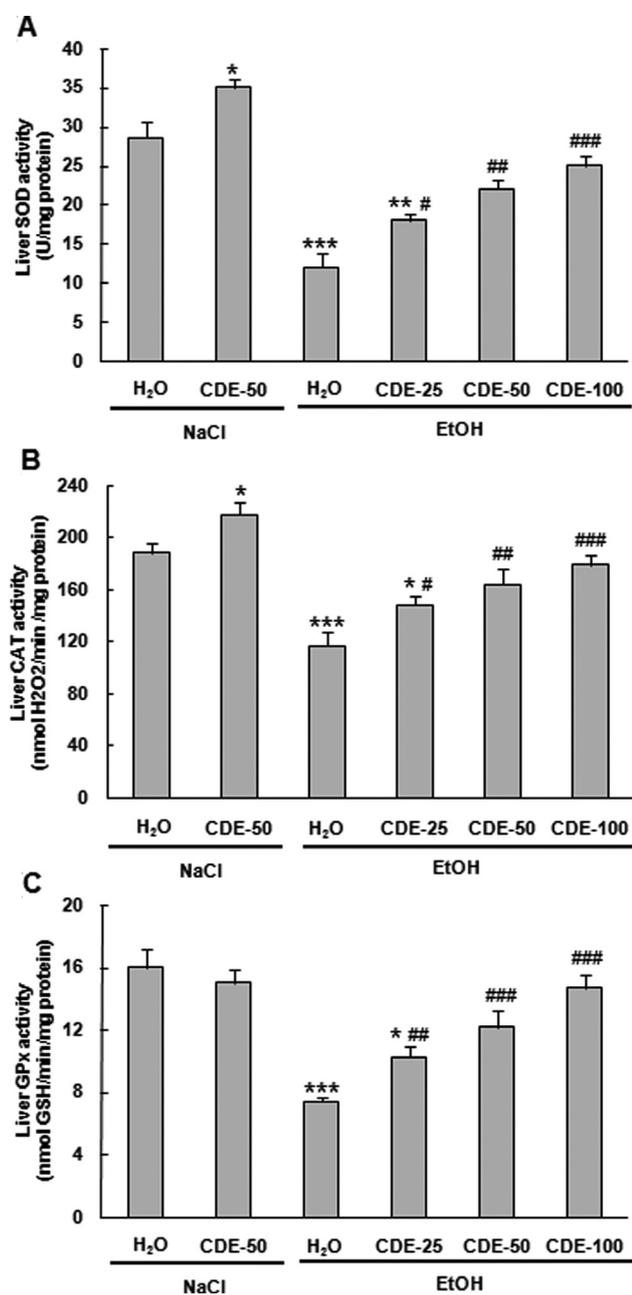

Figure 7. Subacute effect of CDE on acute EtOH-induced changes in liver antioxidant enzymes SOD (**A**), CAT (**B**) and GPx (**C**). Animals were pre-treated during 10 days with CDE and intoxicated by a single oral administration of EtOH for 2 hours ($n = 10$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to EtOH group. CDE, chamomile decoction extract; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.
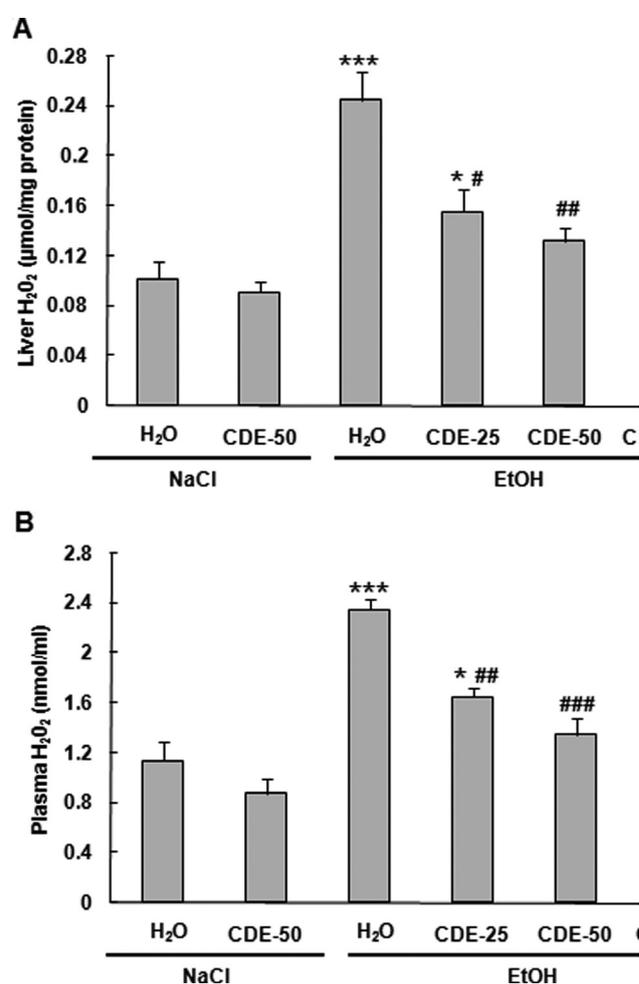

Figure 8. Subacute effect of CDE on acute EtOH-induced changes in liver (**A**) and plasma (**B**) hydrogen peroxide level. Animals were pre-treated during 10 days with CDE and intoxicated by a single oral administration of EtOH for 2 hours ($n = 10$). * $p < 0.05$, *** $p < 0.001$ compared to control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to EtOH group. CDE, chamomile decoction extract.

capacity, albeit lesser than ascorbic acid which was used as a reference molecule. The antioxidant capacity of CDE is mainly related to the higher levels of polyphenols and flavonoids molecules (Guimarães et al. 2013). However, a positive correlation between phenolic compounds and antioxidant capacity is common in the majority of natural extracts (Chon et al. 2009; Hamad et al. 2010).

In vivo, we showed that alcohol administration provoked a clear hepatotoxicity as evidenced by an increase in plasma transaminases (ALT and AST) activity, used as indexes of liver injury. Chamomile pre-treatment significantly reversed EtOH-induced liver toxicity in a dose-dependent manner. The induction of ALT and AST activity following acute alcohol intoxication has been well documented (for review, see Ceccanti et al. 2006). However, it is very important to note that any observed increase in the plasma of these enzymes necessarily results from hepatocyte damage leading

their release into extracellular space (Owumi et al. 2012). Thus, we can suggest that chamomile extract might prevent the activation of phagocytic Kupffer cells by decreasing the formation of inflammatory and fibrogenic mediators, as seen with *Camellia sinenesis* (Zhong et al. 2003). The histological analyses revealed only microvesicular lipid droplets in the livers of ethanol-treated rats. Our results are in line with the previous findings (Zhou et al. 2003; Donohue et al. 2012). However, other reports (Yang et al. 2012; Souli et al. 2013) demonstrated that acute alcohol intoxication induced a more marked structural changes. These discrepancies may result from the variations in experimental species, doses and protocols of ethanol administration.

EtOH-induced hepatotoxicity was also assessed by an increase in lipoperoxidation, decrease in -SH groups as well as depletion of antioxidant enzyme activities such as SOD, CAT and GPx. Alcohol consumption can lead to
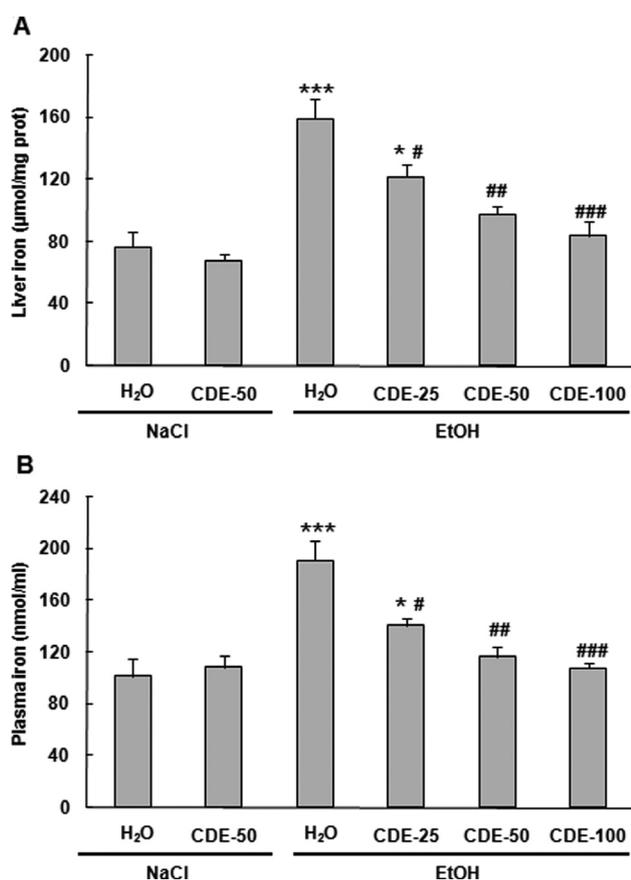

Figure 9. Subacute effect of CDE on acute EtOH-induced changes in liver (**A**) and plasma (**B**) free iron level. Animals were pre-treated during 10 days with CDE and intoxicated by a single oral administration of EtOH for 2 hours ($n = 10$). * $p < 0.05$, *** $p < 0.001$ compared to control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to EtOH group CDE, chamomile decoction extract.
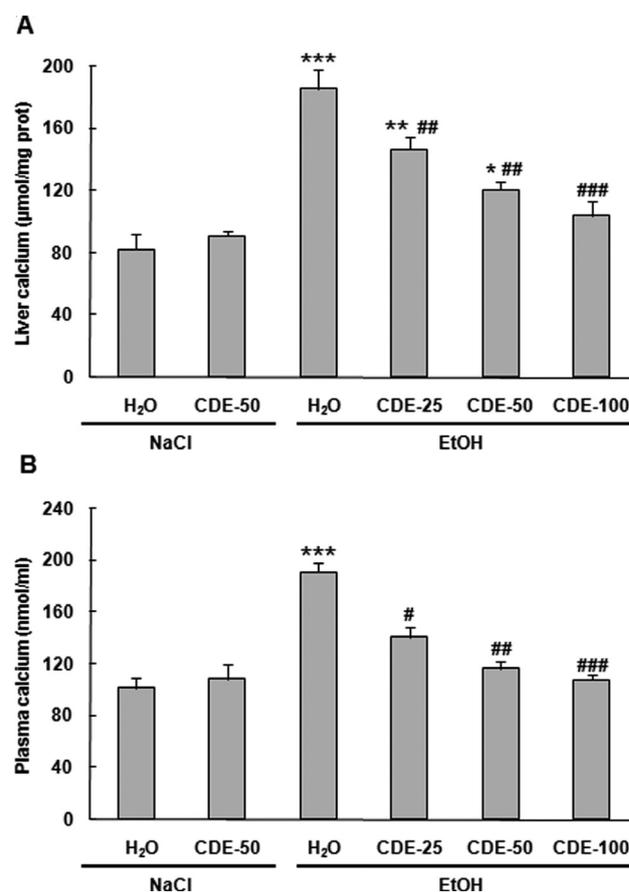
Figure 10. Subacute effect of CDE on acute EtOH-induced changes in liver (**A**) and plasma (**B**) calcium level. Animals were pre-treated during 10 days with CDE and intoxicated by a single oral administration of for 2 hours ($n = 10$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared to EtOH group. CDE, chamomile decoction extract.

oxidative stress through mechanisms associated with EtOH metabolism that generates reactive oxygen species (Owumi et al. 2012). However, ROS produced by cellular metabolic activities, and the alcohol-inducible enzymes can reduce cellular antioxidant defense capacity resulting in oxidative stress (Dey and Cederbaum 2006). Alcohol-induced tissue oxidative stress was widely documented in the liver (Dey and Cederbaum 2006; Nencini et al. 2010; Zhao et al. 2010), kidney, heart and brain (Zloch 1994; Kannan et al. 2004; Ibrahim et al. 2012).

More importantly CDE pre-treatment protected against EtOH-induced hepatic oxidative stress. These data fully corroborated all previously reported *in vivo* (Hernández-Ceruelos et al. 2010; Bulgari et al. 2012) and *in vitro* (Bhaskaran et al. 2012) antioxidant and anti-inflammatory properties of chamomille. EtOH-induced oxidative stress and liver dysfunction have been shown to be attenuated by curcumin (Nanji et al. 2003), resveratrol (Kasdallah-Grissa et al. 2007), N-stearoylethanolamine (Hula et al. 2010), folic acid (Lee et al. 2011), apocynin (Fan et al. 2012) and carob (Souli et al. 2013).

The *in vivo* antioxidant properties of CDE can also be resulting from the presence of phenolic compounds. These molecules are the primal source of the antioxidant ability of this plant, by scavenging free radicals as hydroxyl radical ($OH^\bullet$) which is the major cause of lipid peroxidation (Kogiannou et al. 2013).

We next sought to determine the putative involvement of intracellular mediators in EtOH and CDE mode of action. As expected, alcohol treatment increased H_2O_2, free iron and calcium levels in plasma and liver tissue. However, alcohol-induced liver iron increase was well documented in many previous studies (Houze et al. 1991; Conde-Martel et al. 1992; Shahbazian et al. 1994; Gonzalez-Reimers et al. 1996). Iron plays a central role in many of the ethanol intoxication-associated pathologies such as cardiovascular (Alpert 2004), neurodegenerative (Castellani et al. 2007) and hepatocellular injury (Uchiyama et al. 2008). Furthermore, both iron deficiency and iron excess can lead to cellular dysfunction, maintaining normal iron homeostasis is therefore crucial (Andrews 1999). Powell (1975) demonstrated that iron and H_2O_2 accumulation in ALD catalyzed the highly toxic hydroxyl radical ($OH^\bullet$) production *via* the Fenton reaction leading to hepatocyte membranes lipoperoxidation and enhancement of its permeability to calcium. Indeed, it is well documented that deregulation of calcium homeostasis leading to its overload may have a major role in liver injury after alcohol consumption (Xiao et al. 2005 and González et al. 2008). Importantly, we showed in the present work that subacute pre-treatment with CDE protected against all EtOH-induced intracellular mediators disturbances. Our results also suggest that CDE exerts its beneficial effect by chelating free iron and scavenging H_2O_2 leading to calcium

homeostasis as recently described by Hamlaoui-Gasmi et al. (2011). Further experiments are needed to assess the effect of chamomile extract on hepcidin, an iron shuttling protein, known for its implication in the pathogenesis of iron overload (Papanikolaou et al. 2005).

Conclusion

In conclusion, our data clearly demonstrate that CDE exerts protective effects against acute ethanol-induced injury in the rat liver owing to its antioxidant properties.

Declaration of interest. The authors declare no conflicts of interest.

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