Chamomile decoction extract inhibits human neutrophils ROS production and attenuates alcohol-induced haematological parameters changes and erythrocytes oxidative stress in rat

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Abstract

Background: The aim of this study was to evaluate the protective effects of subacute pre-treatment with chamomile (Matricaria recutita L.) decoction extract (CDE) against stimulated neutrophils ROS production as well as ethanol (EtOH)-induced haematological changes and erythrocytes oxidative stress in rat.

Methods: Neutrophils were isolated and ROS generation was measured by luminol-amplified chemiluminescence. Superoxide anion generation was detected by the cytochrome c reduction assay. Adult male wistar rats were used and divided into six groups of ten each: control, EtOH, EtOH + various doses of CDE (25, 50, and 100 mg/kg, b.w.), and EtOH+ ascorbic acid (AA). Animals were pre-treated with CDE extract during 10 days.

Results: We found that CDE inhibited (P ≤ 0.0003) luminol-amplified chemiluminescence of resting neutrophils and N-formyl methionylleucyl-phenylalanine (fMLF) or phorbolmyristate acetate (PMA) stimulated neutrophils, in a dose-dependent manner. CDE had no effect on superoxide anion, but it inhibited (P ≤ 0.0004) H₂O₂ production in cell free system. In vivo, CDE counteracted (P ≤ 0.0034) the effect of single EtOH administration which induced (P < 0.0001) an increase of white blood cells (WBC) and platelets (PLT) counts. Our results also demonstrated that alcohol administration significantly (P < 0.0001) induced erythrocytes lipoperoxidation increase and depletion of sulphydryl groups (−SH) content as well as antioxidant enzyme activities as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). More importantly, we found that acute alcohol administration increased (P < 0.0001) erythrocytes and plasma hydrogen peroxide (H₂O₂), free iron, and calcium levels while the CDE pre-treatment reversed increased (P ≤ 0.0051) all these intracellular disturbances.

Conclusions: These findings suggest that CDE inhibits neutrophil ROS production and protects against EtOH-induced haematological parameters changes and erythrocytes oxidative stress. The haematoprotection offered by chamomile might involve in part its antioxidant properties as well as its opposite effect on some intracellular mediators such as H₂O₂, free iron, and calcium.

Keywords: Chamomile, Ethanol, Haematological parameters, Oxidative stress, Rat
Background
Reactive oxygen species (ROS) are involved in a wide
range of processes, such as aging and diseases [1].
Nevertheless, ROS are not only an aspect of normal me-
tabolism but are also implicated in several physiological
phenomena such as the substantial protection against
severe infections [2] and the redox regulation of protein
phosphorylation, ion channels, and transcription factors
[3]. However, the enhanced ROS production may lead to
oxidative stress and oxidation of vital cellular compo-
ments, which induce cellular damage and cell death [4].
Therefore, the cytotoxicity of ethanol was attributed to
increased ROS generation [5, 6], which in turn conse-
quently led to injuries and oxidative stress in many
organ systems [7]. To protect cells against these harmful
species we use a synthetic or natural antioxidants mole-
cules [8, 9]. These latter are able to scavenge ROS and
to up-regulate endogenous antioxidant defense systems
[10]. Mammalian erythrocytes are endowed with extra-
ordinarily efficient enzymatic and non-enzymatic anti-
oxidant defense systems that act as ROS scavengers to
limit their imposed damage [11]. The importance of the
protective mechanisms of erythrocytes is evident from a
consideration of human haemolytic disorders due to a
variety of enzyme deficiencies involving pathways that
maintain intracellular reductive molecules [12]. Deficiencies
compromising the capacity to detoxify oxidant molecules
such H$_2$O$_2$ and O$_2^-$ radicals result in oxidant-induced
denaturation of intracellular molecules and premature
destruction of erythrocytes. Nevertheless, despite the lim-
ited biosynthetic repertoire available to mature erythrocytes,
they are resilient to oxidant-induced damage. Clearly, anti-
oxidants in the form of scavengers and detoxifying enzymes
provide an important protective system in erythrocytes [12].
However, erythrocytes are considered as passive ‘reporter
cells’ for the oxidative status of the whole organism and an
increasing amount of attention is being paid to the use of
plant molecules such as polyphenolic and carotenoid com-
ponents [13] in the prevention and cure of various [13].

Chamomile (Matricaria recutita L.) is a medicinal
plant belonging to Compositae family. It is one of the
ancient and most popularly consumed beverages world-
wide, including Tunisia [14]. This species is traditionally
known for its beneficial effects for the treatment of
hepatic and gastrointestinal disorders such as diarrhea
[15–17]. From the experimental and clinical studies
performed on Matricaria recutita, it seems that the
majority of its pharmacological actions are related to
its antioxidant activity which is mainly due to its ability to
scavenge free radicals and/or inhibit lipid peroxidation
[17, 18]. For this reason, chamomile extracts are known to
exhibit many beneficial health effects as neuro-protective
[19], anti-allergic [20], anti-microbial [21], anti-cancer
[22], and anti-inflammatory [23].

The present study was undertaken to investigate the
protective effect of chamomile decoction extract on
haematological parameter disorders and erythrocytes-
induced oxidative stress after the acute alcohol ad-
ministration. We also studied the implication of some
intracellular mediators as H$_2$O$_2$, free Fe, and Ca in
such protection.

Methods
Chemicals
PMA, fMLF, protease inhibitors, and cytochrome c were
from Sigma–Aldrich (St Quentin Fallavier, France).
Epinephrine, bovine catalase, 2-Thio-barbituric acid
(TBA), and butylated hydroxytoluene (BHT) were 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
plant material was later dried in an incubator at 40 °C
during 72 h and powdered in an electric blender. 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.

Isolation and preparation of human neutrophils
Venous blood was collected from healthy adult volun-
teers and neutrophils were isolated by Dextran sedimen-
tation and density gradient centrifugation as previously
described by El-Benna and Dang [24]. Erythrocytes were
removed by hypotonic lysis. Following isolation, the cells
were resuspended in Hank’s balanced salt solution
(HBSS). The cells were counted and their viability was
determined with the trypan blue exclusion method.

Ethics
Neutrophils were isolated from venous blood of healthy
volunteers managed in the hematology and immunology
department of Bichat Hospital, Paris, France. The investi-
gations were approved by the local ethics committee and
samples were obtained with the volunteers’ and patients’
written informed consent. All experiments were approved
by the ‘Institut National de la Santé et de Recherche
Médicale (INSERM)’ institutional review board and
ethics committee. Data collection and analyses were
performed anonymously.

Measurement of ROS production by chemiluminescence
Isolated cells were resuspended in HBSS at a concentra-
tion of 1 million per mL. Cell suspensions ($5 \times 10^5$) in
0.5 mL of HBSS containing 10 μM luminol in the
presence or absence of CDE were preheated to 37 °C in the thermostatted chamber of a luminometer (Berthold-Biolumat LB937) and allowed to stabilize. After a baseline reading, cells were stimulated with 0.1 μM fMLF or 100 ng/mL PMA. Changes in chemiluminescence were measured over a 30-min period.

**Measurement of superoxide anion production**
Isolated cells were also resuspended in HBSS at a concentration of 1 million per mL. Cell suspensions in 1 mL of HBSS containing 1 mg/mL cytochrome c in the presence or absence of CDE were preheated to 37 °C in the thermostatted chamber of a spectrophotometer (Uvikon) and allowed to stabilize. After a baseline reading, cells were stimulated with 0.1 μM fMLF or 100 ng/mL PMA. Changes in absorbance were measured at 550 nm over a 15-min period.

**Measurement of H$_2$O$_2$ inhibition by chemiluminescence**
The effect of CDE on H$_2$O$_2$ was tested in a cell free system using horseradish peroxidase (HRPO). The reaction mixture contained 10 μM luminol in the presence or absence of MBSAE. The reaction was started by addition of 2.5 U/mL horseradish peroxidase (HRPO), and lucigenin chemiluminescence was measured at 37 °C for 30 min in a luminometer (Berthold-Biolumat LB937).

**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 2 served as controls and received bidistilled water. Groups 3, 4, and 5 were pre-treated with various doses of CDE (25, 50, and 100 mg/kg, b.w. p.o.) while group 6 received ascorbic acid (250 mg/kg, b.w. p.o.). Animals were pre-treated during 10 days. After 60 min of the last administration, each animal, except those of group 1, was intoxicated by acute oral administration of EtOH (4 g/kg, B.w.).

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**Fig. 1** Effect of CDE on luminol-amplified chemiluminescence in human neutrophils. Human neutrophils ($5 \times 10^5$) were incubated in the presence or absence of different CDE concentrations and stimulated with PMA(a) or fMLF(b). Luminol-amplified chemiluminescence was measured for 30 min (Data are presented as means ± S.E.M. of five independent experiments, *p < 0.05)
Blood cells count and erythrocytes preparation
Two hours after the EtOH intoxication, 0.5 mL of blood was firstly collected by ocular ponction in EDTA tubes for blood cells count using a haematology analyzer Coulter MAXM (Beckman Coulter, Inc., Fullerton, USA). Then, animals were immediately sacrificed and blood was collected in heparinized tubes. Erythrocytes were isolated by gentle centrifugation (2 000 g, 15 min at 4 °C), resuspended in isotonic phosphate buffer pH 7.4, and lysed with a hypotonic solution consisting of 20 mM Tris–HCl pH 7.2. Obtained homogenates were after used for biochemical determination of protein, free iron, calcium, H$_2$O$_2$, SH-groups, and MDA levels as well as antioxidant enzyme activities.

Biochemical estimations
SOD activity was estimated according to the method described by Misra and Fridovich [25]. CAT activity was measured using Aebi’s method [26]. GPx activity was determined according to the method described by Flohé and Günzler [27]. Thiol groups (–SH) was performed according to Ellman’s method [28]. MDA was estimated using the thiobarbituric acid test [29]. H$_2$O$_2$ was estimated using the method of Dingeon et al.[30]. Erythrocytes non haem iron was measured by colorimetrically using ferrozine as described by Leardi et al.[31]. Calcium was performed according to Stern and Lewis method [32]. The protein content was determined according to Hartree [33] which is a slight change of the Lowry method.

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
Effect of CDE on luminol-amplified chemiluminescence in human neutrophils
To investigate the antioxidant effect of CDE on human neutrophils, we first looked at the luminol-amplified chemiluminescence stimulated with PMA (Fig. 1a) and fMLF (Fig. 1b) in these cells. Compared with cells not stimulated with any chemical or resuspended in HBSS alone, CDE significantly (P ≤ 0.0003) and dose-dependently inhibited luminol-amplified chemiluminescence.

Effect of CDE on fMLF and PMA-induced neutrophils superoxide anion production
Next we focused on the experimental production of superoxide anion in human neutrophils treated with fMLF and PMA (Fig. 2). First, we pretreated neutrophils with various concentrations of CDE from 0 to 20 μg/mL. After neutrophils suspension in HBSS solution and their stabilization, they were stimulated with fMLF and PMA. Then, we investigated the change in superoxide anion production induced by fMLF and PMA over time. Although the exposure of neutrophils to various doses of CDE, it does not affect the production of superoxide anion (Fig. 2).

Effect of CDE on H$_2$O$_2$ production in a cell free system
To verify the role of CDE on H$_2$O$_2$ production in a cell free system, we evaluated the luminol-amplified chemiluminescence stimulated with horseradish peroxidase
(HRPO) (Fig. 3). CDE dose-dependently showed dramatic and significant \((P \leq 0.0004)\) inhibition of \(\text{H}_2\text{O}_2\) production.

**Effect of EtOH and CDE on haematological parameters**

The main haematological parameters analysed were WBC and PLT. Both were significantly \((P < 0.0001)\) increased after the acute administration (6 g/kg, b.w., p.o.) of EtOH (Fig. 4).

CDE pre-treatment significantly \((P \leq 0.0034)\) and dose-dependently abrogated these haematological deregulations induced by EtOH intoxication, with the same efficiency than ascorbic acid.

**Effect of EtOH and CDE on Erythrocytes lipoperoxidation**

Concerning the effect of EtOH and CDE on oxidative stress condition, we firstly studied the erythrocytes lipoperoxidation (Fig. 5). Acute EtOH administration drastically \((P < 0.0001)\) increased the erythrocyte MDA levels. However, CDE and AA pre-treatment significantly \((P < 0.0001)\) and dose-dependently reversed lipoperoxidation induced by alcohol intoxication. With the dose of 100 mg/kg the protective effect was similar to that of ascorbic acid.

**Effect of EtOH and CDE on Erythrocytes- SH groups content**

In the present study, the effects of EtOH and CDE treatment on SH-group levels were also examined. The data from Fig. 6 showed that acute EtOH administration significantly \((P < 0.0001)\) decreased the content of -SH groups. CDE (25, 50, and 100 mg/kg; b.w.) pre-handling significantly \((P \leq 0.0024)\) and dose-dependently protected erythrocytes sylphhydryls against depletion caused by alcohol administration. Ascorbic acid pre-treatment, used as
reference molecule, also abrogated sulphydryl groups’ decrease.

**Effect of EtOH and CDE on Erythrocytes antioxidant enzyme activities**

We further examined the effect of EtOH and CDE on erythrocytes antioxidant enzyme activities (Fig. 7). We showed that alcohol administration significantly ($P < 0.0001$) decreased erythrocytes antioxidant enzyme activities as SOD (A), CAT (B), and GPx (C). CDE pre-treatment significantly ($P \leq 0.0051$) reversed all EtOH-induced antioxidant enzymes depletion in a dose-dependent manner. Ascorbic acid, an antioxidant reference molecule, also exhibited the same protection.

**Effect of EtOH and CDE on Erythrocytes $H_2O_2$, free iron, and calcium levels**

We reported in Table 1 the effect of Ethanol and CDE on intracellular mediators as hydrogen peroxide, free iron, and calcium levels. EtOH *per se* drastically ($P < 0.0001$) increased iron, $H_2O_2$, and calcium levels. CDE
Table 1 Subacute effect of chamomile decoction extract (CDE) on acute EtOH-induced changes in erythrocytes hydrogen peroxide, free iron, and calcium levels

| Groups         | H$_2$O$_2$ (μmol/mg protein) | Free iron (μmol/mg protein) | Calcium (μmol/mg protein) |
|----------------|-------------------------------|-----------------------------|---------------------------|
| Control        | 0.99 ± 0.062                  | 9.04 ± 0.55                 | 220.48 ± 14.25            |
| EtOH           | 1.85 ± 0.068*                 | 22.72 ± 1.04*               | 370.15 ± 10.13*           |
| EtOH + CDE-25  | 1.60 ± 0.054*                 | 17.11 ± 1.15*               | 321.22 ± 12.47*           |
| EtOH + CDE-50  | 1.23 ± 0.056*                 | 14.37 ± 0.61*               | 293.03 ± 9.38*            |
| EtOH + CDE-100 | 1.09 ± 0.077*                 | 11.23 ± 0.54*               | 237.23 ± 17.65*           |
| EtOH + AA      | 1.03 ± 0.041*                 | 10.71 ± 0.69*               | 235.18 ± 13.96*           |

Animals were pre-treated during 10 days with CDE (25, 50 and 100 mg/kg b.w., p.o.) or vehicle (bidistilled H$_2$O), challenged with a single oral administration of EtOH (4 g/kg b.w.) or NaCl 9% for 2 h. Assays were carried out in triplicate. *: $p < 0.05$ compared to control group and #: $p < 0.05$ compared to EtOH group.

Discussion

The current study was designed to investigate the effect of CDE on human neutrophil reactive oxygen species (ROS) production in vitro as well as to determine its protective effects on EtOH-induced haematological alterations and erythrocytes oxidative stress in rat.

We firstly tested the CDE on human neutrophils total ROS production, in response to chemotactic peptide (fMLF) and phorbolmyristate acetate (PMA) stimulation, as well as on H$_2$O$_2$ accumulation in a cell free system. Our data showed that CDE (5, 10 and 20 μg/mL) treatment significantly inhibited luminol-amplified chemiluminescence in neutrophils and H$_2$O$_2$ production in a cell free system, in a dose-dependent manner. However, CDE had no effect on cytochrome c reduction in human neutrophils stimulated with fMLF or PMA, suggesting that it does not affect NADPH oxidase activity or does not scavenge superoxide anions (O$_2^-$) as previously described for other medicinal plant extracts such as Punica granatum [34] and Myrtus communis [35].

The oxygen consumed by neutrophils is enzymatically converted to O$_2^-$ by univalent transfer of 2 electrons from the cell NADPH [36]. This reaction is catalyzed by NADPH oxidase. The O$_2^-$ is the source of other ROS such as H$_2$O$_2$ and the highly toxic hydroxyl radical OH$^-$ [36]. ROS may induce several biochemical lesions, including lipid peroxidation, cell membrane disruption, oxidation of sulfhydryl groups, and DNA mutation [37]. More importantly, the present data clearly demonstrated that CDE protects against ROS attacks by scavenging the H$_2$O$_2$ molecules. Furthermore, other authors reported that this protection might also be provided by inhibition of the myeloperoxidase activity in myrtle berries seeds extract [35].

In vivo, we showed that acute EtOH administration significantly decreased the number of WBC and PLT. Our results partially corroborated those of Kawashima et al. [38] who demonstrated that the administration of 2.0 g/kg of b.w. of ethanol significantly increased the number of neutrophils, basophils, monocytes, and total WBCs without changing the number of PLTs. EtOH had no effect on erythrocytes number or haemoglobin (Hb) and hematocrit (Ht) levels (data not shown). These results are in agreement with previous findings, suggesting that a single administration of ethanol (4 g/kg b.w.) to rats markedly increased the number of natural immunity cells without changing the number of acquired cells [38]. In contrast, chronic administration of alcohol significantly reduced the erythrocytes, WBC, and PLT numbers, Hb concentration, Ht value, mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC) when compared to control group [39]. However, these discrepancies may be due to the ethanol dosages, the route of administration as well as the period of treatment [40]. The increase in WBC might be due to the marker activation of defence and immune systems and showed that there were inflammations in the tissues [41]. More importantly, we demonstrated in the present work that subacute CDE pre-treatment abrogated all ethanol induced haematological parameters disturbances. The protection offered by CDE against WBC or PLT decrease might be due to its antioxidant [42] and anti-inflammatory [23] properties.

These findings also showed that alcohol administration clearly induced erythrocytes lipoperoxidation increase, sulfhydryl groups decrease, and depletion of antioxidant enzyme activities such as SOD, CAT, and GPx. Ethanol-induced tissue oxidative stress was widely documented in many organ systems such as liver, kidney, heart, brain, and erythrocytes [42–46]. Alcohol consumption can lead to oxidative stress through mechanisms associated to EtOH metabolism that generates ROS [47]. However, ROS production associated to the alcohol-induced depletion of antioxidant enzymes can reduce cellular antioxidant defence capacity, leading to oxidative stress.
status [48]. More importantly, EtOH-induced erythrocytes oxidative stress has been shown to be attenuated by subacute chamomile pretreatment. EtOH-induced erythrocytes oxidative stress has been previously shown to be attenuated by caffeic acid [49], Gymnema montanum [48], beta-carotene [50], olive oil [51], and Opuntia ficus indica [46]. The erythroprotective effects of CDE against oxidative stress induced by acute ethanol administration may be due to its richness in biomolecules with significant antioxidant capacity such as phenolic compounds. According to the study done in our laboratory, phytochemical studies of CDE revealed the presence of high concentrations of total polyphenols, total flavonoids, and condensed tannins. The use of HPLC-PDA-MS allowed to the identification of gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, caffeoylquinic acid, salicylic acid, quercetin, quinic acid derivative, hydroxybenzoic acid-O-hexoside, 5,7,4’-Trihydroxy-6,3’-dimethoxyflavone [52]. These molecules are the primal source of antioxidant ability of this plant that act as scavengers of free radicals [53].

We next sought to determine the putative involvement of some intracellular mediators in EtOH and CDE modes of action. We firstly showed that alcohol administration significantly increased the plasma (data not shown) and erythrocytes H$_2$O$_2$, free iron, and calcium levels. The implication of these intracellular mediators in the EtOH mode of action has been previously well documented in the hepatic tissue [54–57]. Furthermore, both iron and H$_2$O$_2$ accumulation catalyzed the highly toxic OH$^-$ production via the Fenton reaction leading to membranes lipoperoxidation and enhancement of its permeability to calcium [58]. Interestingly, our data showed that subacute CDE pre-treatment significantly attenuated all EtOH-induced intracellular mediators’ disturbances.

The possible mechanism by which CDE exerts its beneficial effect on erythrocytes could be its ability of chelating free iron and scavenging H$_2$O$_2$, leading to calcium homeostasis as previously proposed for other extracts rich in phenolic compounds as grape seeds and skin extracts [59, 60], myrtle berries seeds extract [35] and Myrtus communis leaves essential oils [61]. CDE could also act on calcium channels known for their implication in iron-overload disorders [62]. Further works 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 [63].

Conclusions

In the present work, we clearly demonstrated that subacute CDE pre-treatment exerts protective effects against ethanol-induced haematological parameters disturbances and erythrocytes oxidative stress. The beneficial effect of CDE may be explained owing to its ROS scavenging properties and opposite effects on some intracellular mediators such as H$_2$O$_2$, free iron, and calcium.

Abbreviations

CAT: Catalase; CDE: Chamomile decoction extract; EtOH: Ethanol; GPx: Glutathione peroxidase; H$_2$O$_2$: Hydrogen peroxide; MDA: Malondialdehyde; PLT: Platelets; ROS: Reactive oxygen species; SOD: Superoxide dismutase; WBC: White blood cells.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

MA-I and KR designed the study and carried out the biochemical analyses. MS and HS performed the statistical analysis and drafted the manuscript with contributions from JE-B, LM, and MS. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank all members of U1149, “Center for Research on Inflammation” Paris France for assistance and helpful discussion. Financial support of INSERM and Tunisian Ministry of Higher Education and Scientific Research is gratefully acknowledged. Financial disclosures: none declared.

Declaration of interest

The authors alone are responsible for the content of this paper.

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Received: 6 November 2015 Accepted: 22 March 2016

Published online: 31 March 2016

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