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Original Article

Acute paracetamol toxicity-induced inflammatory and oxidative effects are relieved by Aleppo galls: a novel experimental study

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Abstract: Background: Paracetamol (acetaminophen) is an over-the-counter non-steroidal anti-inflammatory drug that may cause acute toxic overdosage particularly in neuropsychiatric patients. Paracetamol is also very commonly prescribed as an analgesic and antipyretic agent. Paracetamol toxicity causes decreased reduced glutathione and oxidative tissue damage. Aleppo galls is a promising natural remedy exerting antioxidant and tissue-protective effects that may combat acetaminophen-induced oxidative tissue damage. Methodology: Biochemical and toxicological effects of a toxic dose of paracetamol (250 mg/kg) were investigated for three consecutive days versus the tissue-protective effects of Aleppo galls. Eighteen white albino mice were randomly allocated in this study and divided into three experimental groups (six mice per group): negative control (received intraperitoneal sterile water injection), paracetamol toxicity group (received intraperitoneal paracetamol injection) and the third group (received paracetamol injection at 250 mg/kg/day together with oral Aleppo galls treatment at 250 mg/kg/day for 3 consecutive days). All mice were sacrificed by the end of the study. Results: Our data revealed that paracetamol toxicity exerted significant oxidative stress damaging effects (high serum malondialdehyde, decreased serum catalase and decreased total antioxidant capacity), and significant inflammatory effects (high serum IL-6) and significant tissue-damaging effects (high serum LDH). Aleppo galls treatment significantly protected against acetaminophen toxicity-induced oxidative stress effects ($P<0.001$), inflammatory effects ($P<0.001$) and tissue-damaging effects ($P<0.001$). Conclusion: Aleppo galls are promising for future drug therapeutics and for the synthesis of natural remedies for treating paracetamol toxicity. We recommend formulating Aleppo galls extract as a pharmaceutical nutrition and to be given to those who need to take large doses of paracetamol.

Keywords: Aleppo galls, paracetamol, malonedialdehyde, total antioxidant capacity, LDH

Introduction

Paracetamol (acetaminophen) is a non-steroidal anti-inflammatory drug that is commonly used to treat pain and fever and is typically used for mild to moderate pain relief. Paracetamol may undergo toxic overdosage particularly in neuropsychiatric patients. Paracetamol

is both a prescription (prescribed by many physicians in different specialties) and an over-the-counter drug [1]. Paracetamol is also used for treating severe pain e.g. cancer pain and post-operative pain in combination with opioid pain medications and is given orally, rectally and intravenously [2]. Currently, paracetamol is on the World Health Organization's list of essential medicines, which lists the most effective and safe medications needed in human health system [3]. In paediatric cardiology, paracetamol is used to treat patent ductus arteriosus [4]. Paracetamol does not inhibit cyclooxygenase enzyme outside the central nervous system, so that paracetamol is not effective as a general anti-inflammatory agent. However, inside the CNS, paracetamol selectively inhibits cyclooxygenase enzyme. This also explains the effectiveness of paracetamol in treating fever and pain [5]. Interestingly, paracetamol is lethal to snakes, and has been suggested as a chemical control program for the invasive brown tree snakes where doses of 80 mg inserted into dead mice may be scattered by helicopter aeroplanes to kill snakes [6].

Paracetamol is metabolised basically in the liver into toxic and nontoxic metabolites where three metabolic pathways are described: Glucuronidation (50%), sulfation (30%) [7] and glutathione conjugation (15%) [8]. The liver enzyme cytochrome P450 enzyme system metabolizes paracetamol into N-acetyl-p-benzoquinone imine (NAPQI) that undergoes glutathione conjugation [8].

Despite its well-known safety, paracetamol may cause potential toxicity. Healthy adults may not experience symptoms of paracetamol toxicity when taking regular doses up to 4,000 mg per day apart from a mild to moderate elevation of serum liver enzymes (ALT and AST) i.e. abnormal liver function tests [9]. Acute acetaminophen overdosage and toxicity may induce seriously fatal liver damage. Paracetamol poisoning is the most common cause of acute liver failure in Europe, USA, Australia, and New Zealand. FDA reported that in USA, "56,000 emergency room visits, 26,000 hospitalizations, and 458 deaths are met every year due to paracetamol-associated overdoses during the 1990s. Accidental paracetamol overdosage constitutes about 25% of the emergency department visits, 10% of the hospitalizations, and 25% of the deaths" [9]. Side effects and toxicity of paracetamol are exaggerated when accompanied by alcoholic drinks particularly in chronic alcoholics causing alcohol-acetaminophen syndrome. Even moderate alcoholics may be at risk of that [10]. A small fraction of paracetamol is metabolised by the CYP450 system to NAPQI that is toxic to the liver. NAPQI is conjugated with glutathione causing depletion of reduced glutathione. Alcohol and other drugs induce CYP450 causing increased NAPQI production and further depletion of reduced glutathione [11].

Galls may occur at the leaves of the Aleppo oak (*Quercus infectoria)* in response to the larvae of the gall wasp (*Cynips quercusfolii)* [12] that are seen on Quercus Infectoria tree branches (**Figure 1A-D**) after the gall wasps sting the oak and start depositing their larvae. A chemical reaction soon arises causing the oak tree to start the formation of hard galls [13]. The galls from *Quercus infectoria* contain the highest naturally occurring levels of tannins (50-70%) [14], syringic acid, β-sitosterol, amentoflavone, hexamethyl ether, isocryptomerin, methyl betulate, methyl oleanate and hexagalloyl glucose [15, 16]. Galls are also rich in gallic acid (2-4%) and ellagic acid that are polymerized to make tannins [17, 18]. Tannins (of galls) are vital medicinal ingredients used nowadays in many medical specialties e.g. dermatology [19] and have been used for tanning of leather. For all these active ingredients, medicinal properties of the galls are wide and diverse. Quercus infectoria exert astringent, antidiabetic, antiheminths, local anaesthetic, antiviral, antibacterial, antifungal, larvicidal and anti-inflammatory effects. As mentioned above, the main constituents found in Quercus infectoria galls are tannin (50-70%) and small amount of free gallic acid and ellagic acid. Recently, new pharmacological applications were added to galls e.g. preventing cataract formation induced by glucose in experimental animals [20], exerting effects against pathogenic bacteria, enhancing prebiotic bacteria (lactobacilli) [21] and increasing the contractile response of uterine smooth muscle i.e. galls may help treating postpartum haemorrhage [22].

The wide range of pharmacological activities of *Quercus infectoria* might support its efficacy as a natural extract preparation. Quercus infecto-

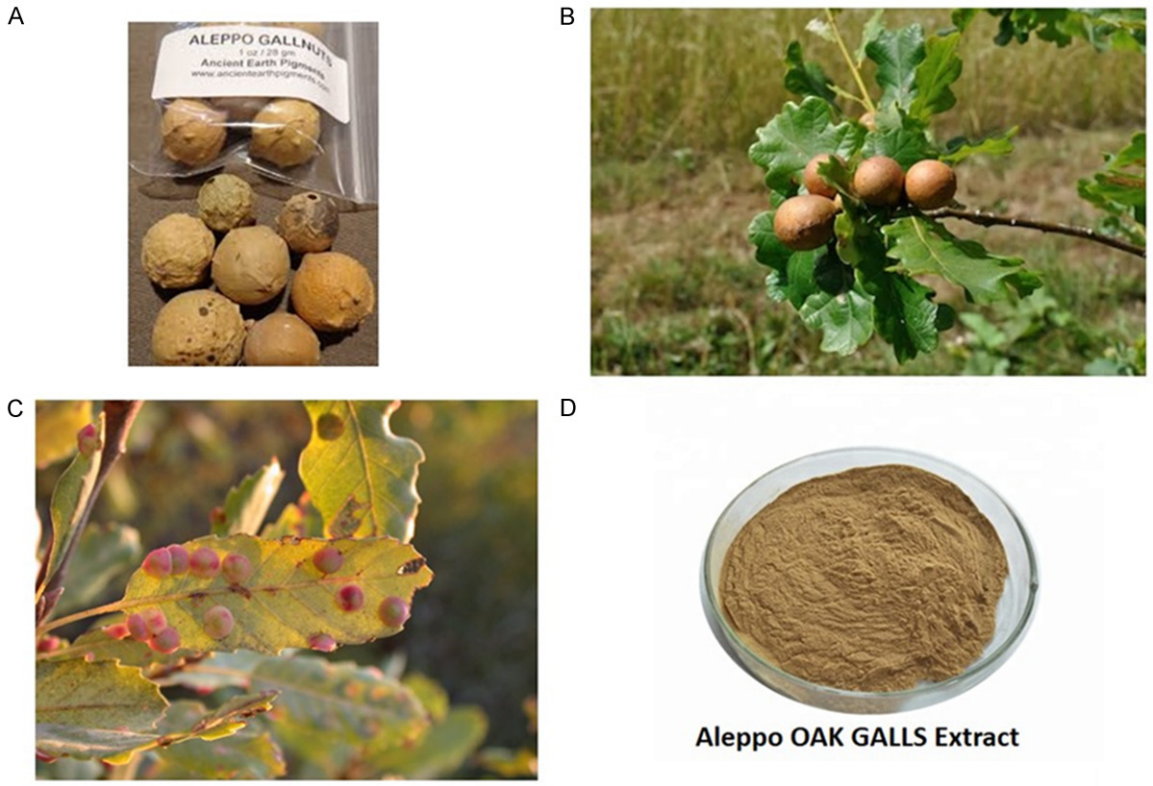

Figure 1. Aleppo galls are natural, cheap and therapeutic remedies. A, B. Aleppo galls. C. Aleppo galls associated with plants leaves. D. Extract of Aleppo oak galls (Quercus Infectoria), a natural pure bulk extract.

ria is traditionally used in Malaysia for treating many kinds of health problems for decades. The nutgalls have been pharmacologically documented for their antiamoebic, anticariogenic and anti-inflammatory activities, and to treat skin infections and gastrointestinal disorders [23-25]. In this study, we investigated the potential of Aleppo galls extract to treat acetaminophen toxicity via investigating the antioxidant, anti-inflammatory and tissue-protective effects of Aleppo galls.

Materials and methods

Relevant research indicators of the study

Aleppo galls are well-known for their rich antioxidant and anti-inflammatory ingredients that may help treating paracetamol toxicity-induced oxidative stress, inflammation and tissue damage. The relevant indicators of this study include assaying serum oxidants e.g. malondialdehyde and serum antioxidants e.g. serum catalase and total antioxidant capacity after exposure to paracetamol toxicity in both presence and absence of Aleppo galls extract. In addition, serum markers of inflammation e.g. IL-6 and markers of tissue damage e.g. serum LDH were also assayed after exposure to paracetamol toxicity in both presence and absence of Aleppo galls extract.

Plant material and extraction procedure

The Aleppo galls were purchased from a local herb store in Al-Madinah Al-Munawwarah, Saudi Arabia then Aleppo galls were identified by an expert pharmacognosist. The specimen has been kept at the department of Pharmacognosy and Pharmaceutical Chemistry, College of Pharmacy, Taibah University, Saudi Arabia. Aleppo galls were powdered using a grinder. The extraction experiments were performed using one litre of hydro-alchohol (80%) that was added to the galls powder and that was followed by a twice incubation at room temperature for 48 hours. The combined extracts were filtered using whatman filter paper and the ethanol was eliminated by a rotary vacuum evaporator at 50°C. The plant extracts

were dissolved in 50 ml distilled water and kept in a refrigerator at 4°C for performing further tests and applications.

Animal preparation and experimentation

White albino mice (150-200 grams) were brought from the central animal facility of Assuit University (Assuit, Egypt). Animals were maintained in pathogen-free conditions at the central animal house of Minia University in Egypt. Animal maintenance took place in their polypropylene cages at a temperature of 22 ± 3°C. Animals were fed per mouth using a standard laboratory pellet chow diet with pathogen-free open access to water source during the whole period of the study. The whole experimental study was conducted at Minia University. A prior ethical committee approval (no. COPTU-REC-15-20210401) was gained from The Faculty of Pharmacy, Taibah University before starting the experimental work. Eighteen mice were randomly allocated into three experimental groups (6 mice per group).

Experimental work

The aim of the current study was to investigate the antioxidant, anti-inflammatory and the tissue-protective effects of Aleppo galls extract against paracetamol toxicity-induced oxidative tissue damage. This study also aimed at assessing the possible anti-inflammatory and tissue-protective effects of Aleppo galls. Animals were acclimatized for 2 weeks in the animal house prior to experimentation. All animals were maintained at a relative humidity of 75%, and a temperature of 20°C. The use of animals was approved by the Ethical Committee of the Animals Research Facility (ECARF). Eighteen healthy white albino mice of both sexes (10 males and 8 females) were randomly divided in this study into three different experimental groups, each group included 6 mice:

- 1st group (untreated control = negative control).
- 2nd group (paracetamol toxicity group): received paracetamol 250 mg/kg/day via intraperitoneal injection for 3 consecutive days.
- 3rd group (treatment group): received paracetamol 250 mg/kg/day via intraperitoneal injection for 3 consecutive days and oral Aleppo galls extract (250 mg/kg/day for 3 consecutive days).

N.B. The basis for choosing this high dose of paracetamol was the report by Rostami et al. who used 300 mg/kg paracetamol in mice and reported that paracetamol lethality occurs when paracetamol is taken in a single dose of 300 mg/kg [26]. We used a lower dose than this reported lethal dose. Proving acute paracetamol toxicity in mice occurs upon estimating oxidative stress markers e.g. increased serum malondialdehyde, LDH, IL-6 and serum hydrogen peroxide and decreased serum total antioxidant capacity as was previously reported [27].

Biochemical evaluation

All animals were kept in the previously mentioned experimental conditions until sacrifice. Under ether anaesthesia, the animals were sacrificed by decapitation and blood samples were collected. Decapitation was done to collect as much blood as possible for the needed biochemical investigations. Lack of decapitation does not allow us to collect enough blood for subsequent serum assays. Blood samples were drawn (after decapitation) and cardiac puncture. Blood was collected in sterile plain tubes and centrifuged (4000 rpm/minute) and the serum was kept in -30°C for future biochemical assays. Serum malondialdehyde (Bio-Systems kits, Barcelona, Spain), serum catalase (elabscience, TX, USA) and serum total antioxidant capacity (elabscience, TX, USA) were assessed. Moreover, serum IL-6 (elabscience, TX, USA) and serum LDH (Sigma, MD, USA) were also assayed according to the manufacturer's instructions.

Assay of serum malondialdehyde (MDA)

Serum MDA (μM) levels were estimated in the sera of all mice using MDA ELISA Kit (E-EL-0060, Elabscience, TX, USA) having a detection range between 31.25 and 2000 ng/mL. Both standards and test samples were prepared inside the microplate wells that are coated with a monoclonal antibody and incubated. The test also included both positive and negative controls. Biotin was added to all the wells followed by adding streptavidin-HRP and then incubation was done. Washing was performed

many times to remove the unbound reagents. Chromogen solutions were added followed by adding the stop solutions. Biotek Synergy multimode microplate reader (VT, USA) was utilized to estimate the optical density at 450 nm. The concentrations of MDA were calculated from the standard curve for each assay.

Total antioxidant capacity assay

Using total antioxidant capacity assay kits (elabscience, TX, USA), we assayed total antioxidant capacity. Briefly, collected fresh blood was centrifuged at 2000 g for 15 min at 4°C to obtain the serum that was later frozen at -30°C. Then, 100 µL of buffer solution was added to the test tubes, to 1.5 mL eppendorff tubes followed by adding 10 µL of serum. That was followed by adding 200 µL of the chromogenic agent working solution and 50 µL of ferric salt stock solution to the sample tubes and the control tubes. Full mixing was done and the reaction was allowed at 37°C for 30 min. That was followed by the sequential addition of ferric salt diluent, stop solution and finally the clarificant. Then, full mixing was done followed by standing at 10 min. at room temperature. We then added 300 µL of the reaction liquid to the 96 well microplates. Biotek Synergy multimode microplate reader was used to measure the OD value of each well at 520 nm.

Serum catalase assay

Catalase assay is based on the principle that catalase decomposes H_2O_2 and that can be quickly stopped by ammonium molybdate. Using catalase assay kits (Elabscience, TX, USA), serum catalase activity (antioxidant) was assayed. Kit components include buffer solution, reagent and chromogenic agents supplied in the kits. Serum catalase assay was estimated based on the manufacturer's instructions. Remaining H_2O_2 reacts with ammonium molybdate to form a yellowish complex. Blank tubes contained distilled water while test tubes contained mice serum. Both were also prepared. All tubes remained at room temperature for 10 minutes. That was followed by taking 200 µL of reaction solution to the microplate with a micropipette. Using Biotek Synergy multimode microplate reader (VT, USA), absorbance readings were taken.

LDH assay

Serum LDH samples were assayed using LDH assay kits (sigma, MD, USA) according to the manufacturer's instructions. Briefly, that was done via adding 20 µL of the samples into the triplicate wells of the 96 well plate. Samples were brought to a final volume of 50 µL via adding LDH assay buffer. Several sample dilutions were tested to ensure that the readings are within the linear range of the standard curve. The Master Reaction Mix was prepared by mixing LDH assay buffer and LDH substrate mix in a ratio of 24:1. Then, 50 µL of the master reaction mix wad added to each of the wells, followed by a gentle mixing using a horizontal shaker or by pipetting. The plate was incubated at 37°C. After 3 minutes, the initial measurement was taken and the absorbance was measured at 450 nm using Biotek Synergy multimode microplate reader. The background was corrected via subtracting the final measurement obtained for the blank from all the readings, and NADH standard from the final measurement of the standards. Then, the NADH standard curve was plotted.

IL-6 assay

IL-6 was assayed using IL-6 assay kits (elabscience, TX, USA) via adding 100 µL of the standard solution or sample to the wells. That was followed by incubation for 90 min at 37°C and discarding the extra liquids immediately. Then, 100 µL of biotinylated antibody detection working solution was added to each well. Incubation was done for 60 min at 37°C followed by aspiration and washing of the plates for 3 times. Then, 100 µL HRP conjugate working solution was added followed by incubation for 30 min at 37°C. Aspiration of the wash solution of the plate was done 5 times followed by adding 90 µL of the substrate reagent. Incubation was done for 15 min at 37°C and then followed by the addition of 50 µL stop solution. Using Biotek synergy multimode microplate reader, the wells of the plate were read at 450 nm immediately and calculation of the results was done.

Statistical analysis

Data was collected, analyzed using IBM SPSS software (version 20) and presented as mean ±

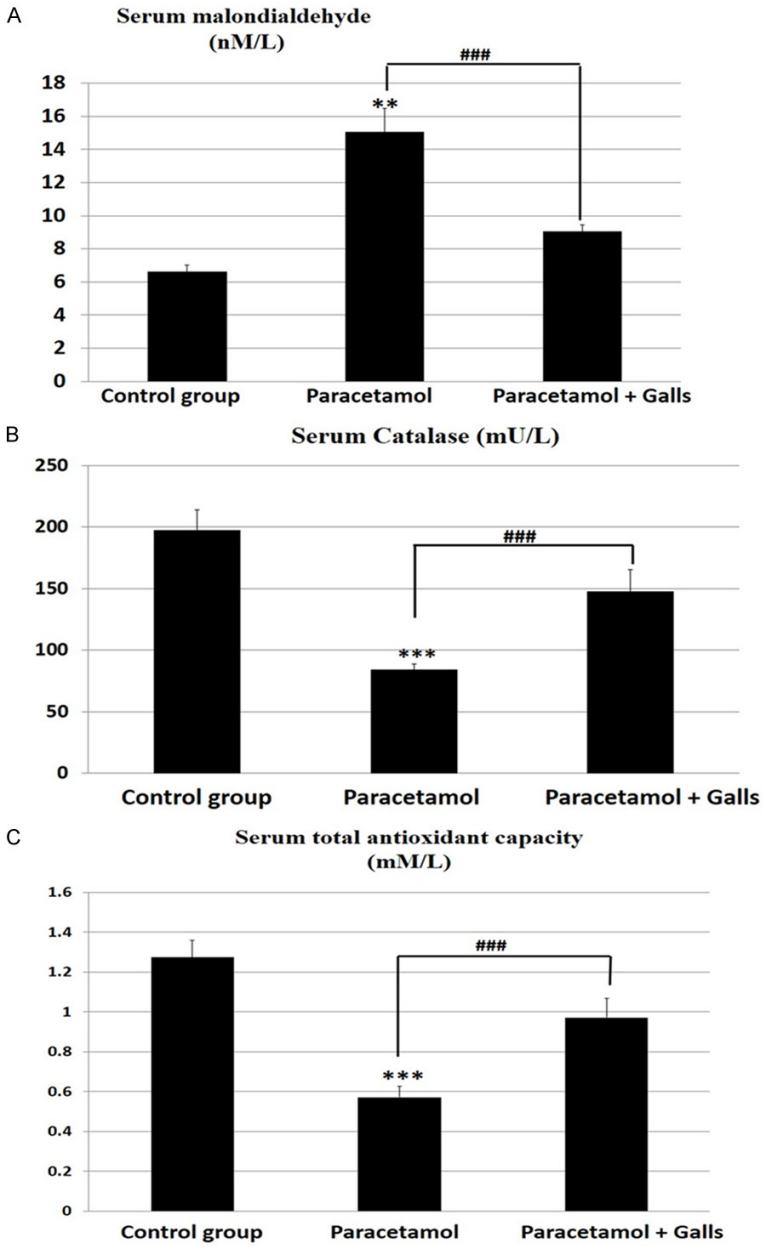

Figure 2. Paracetamol toxicity induces oxidative stress effects that are relieved by Aleppo galls. A. Effects of Paracetamol toxicity on serum malondialdehyde (a byproduct free radical due to cellular lipids peroxidation measured in nM/L) and the relieving effects induced by Aleppo galls. B. Effects of paracetamol toxicity on serum catalase vs the relieving effects induced by Aleppo galls. C. Effects of paracetamol toxicity on total antioxidant capacity vs the relieving effects induced by Aleppo galls. ** means P<0.01 and *** means P<0.001 to indicate significant differences between treatment groups versus the negative control. ### means P<0.001 to indicate significant differences among the different treatment groups.

standard error of mean. Paired samples t test was used to compare the results between the experimental groups versus the negative control and among the different treatment conditions. *P* value <0.05 was considered as statistically significant.

Results

Paracetamol toxicity caused oxidative tissue damage that was relieved by Aleppo galls extract

Toxic dose administration of paracetamol (250 mg paracetamol/kg/day) caused a significant oxidative tissue damage that was confirmed by a significant increase (P<0.01) in serum malondialdehyde (**Figure 2A**). MDA increased significantly (P<0.01) from baseline (6.65 ± 0.36 nM/L) to (15.7 ± 3.49 nM/L). After treatment with Aleppo galls, MDA significantly decreased (P<0.001) to near baseline levels (9.1 ± 0.38 nM/L). Moreover, paracetamol toxicity caused a significant decrease (P<0.001) in serum catalase from baseline (197.66 ± 16.33 mU/L) to (84.33 ± 9.72 mU/L) (**Figure 2B**). Treatment with Aleppo galls significantly (P<0.001) raised serum catalase to near baseline values (148 ± 17.33 mU/L). Consequently, paracetamol toxicity significantly (P<0.001) decreased serum total antioxidant capacity from baseline (1.27 ± 0.09 mM/L) to (0.57 ± 0.05 mM/L) (**Figure 2C**). Treatment with Aleppo galls caused significantly (P<0.001) elevated serum total antioxidant capacity to near normal values (0.97 ± 0.09 mM/L).

Paracetamol toxicity induced an inflammatory process that was relieved by Aleppo galls

A toxic dose of paracetamol (250 mg/kg) caused a significant increase (P<0.01) in serum IL-6 (a pro-inflammatory cytokine) from baseline (4.63 ± 0.68 ng/L) to (16.23 ± 1.01

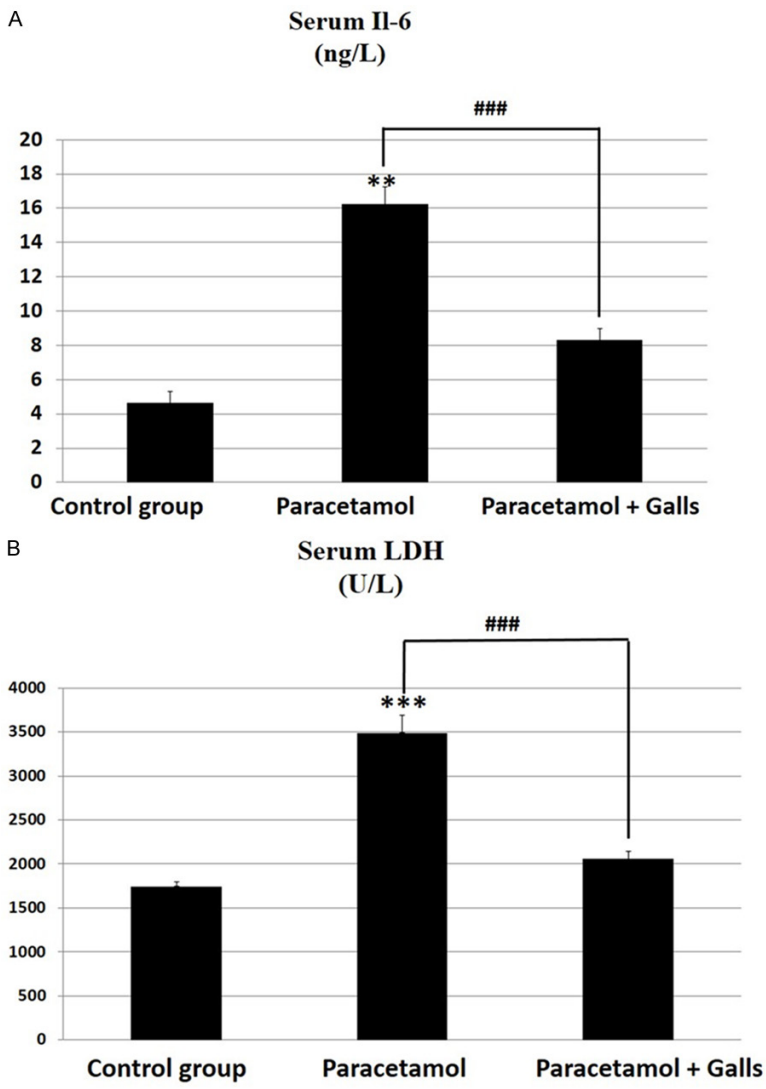

Figure 3. Paracetamol toxicity induces tissue damage that's significantly alleviated by Aleppo galls. A. Effects of paracetamol toxicity on serum IL-6 levels (a proinflammatory cytokine) and the relieving effects induced by Aleppo galls. B. Effects of paracetamol toxicity on serum LDH levels (a marker of tissue damage) and the relieving effects induced by Aleppo galls. ** means P<0.01 and *** means P<0.001 to indicate significant differences between treatment groups versus the negative control. ### means P<0.001 to indicate significant differences among the different treatment groups.

ng/L). Treatment with Aleppo galls significantly (P<0.001) decreased serum IL-6 to near normal values (8.3 ± 0.68 ng/L) (**Figure 3A**).

Paracetamol toxicity caused tissue damage that was relieved by Aleppo galls

In the same context, a toxic dose of paracetamol significantly increased (P<0.001) serum lactate dehydrogenase (LDH, a marker of tissue damage) from baseline (1745.33 ± 48.88 U/L) to (3488.48 ± U/L). Aleppo galls treatment significantly decreased (P<0.001) serum LDH to near baseline values (2059.16 ± 85.44 U/L) (**Figure 3B**).

Discussion

Nonsteroidal anti-inflammatory drugs are among the most commonly used medications to relieve pain, headache, fever and others. Many of them e.g. paracetamol are categorized as over-the-counter drugs that can be purchased without a prior medical recipe, hence the possibility of overdosage and toxicity is great. Paracetamol is among the most widely prescribed antipyretic drugs. Overdosage and toxicity due to paracetamol administration is expected in so many clinical scenarios. The main side effects due to paracetamol toxicity are closely related to paracetamol-induced depletion of glutathione (a vital intracellular and extracellular tripeptide antioxidant). Based on that, paracetamol-induced toxicity exerts oxidative stress-induced tissue damaging effects [28].

Natural products e.g. Aleppo galls (**Figure 1A-D**) are well-known for their antioxidant power that confers a tissue-protective effect that may counteract many of the oxidative damaging effects induced by many currently used drugs and chemotherapeutics particularly at large doses. In this study, a toxic dosage of paracetamol significantly induced tissue damage through an oxidative stress mechanism (**Figure 2A-C**) and that was evident due to high serum MDA, decreased serum catalase and decreased serum total antioxidant capacity. That was relieved to near normal baseline conditions upon treatment with Aleppo galls. Toxicity with paracetamol significantly caused

increased serum levels of MDA (a byproduct free radical formed due to cellular lipids peroxidation) (**Figure 2A**). This denotes that paracetamol toxicity causes tissue damage through generation of oxidants and free radicals that impair the integrity of the cell membranes lipid bilayer causing the generation of MDA. Also, our data confirmed that Aleppo galls significantly decreased paracetamol-induced high serum MDA (**Figure 2A**). This confirms the antioxidant and tissue-protective effects induced by Aleppo galls. For the authors, this is explained by the high contents of the anti-inflammatory and antioxidant ingredients in Aleppo galls.

Catalase is an antioxidant enzyme found in nearly all living organisms and catalyses the decomposition of hydrogen peroxide to water and oxygen [29]. It is a very important enzyme in protecting the cells from oxidative damage by reactive oxygen species (ROS). Likewise, catalase has one of the highest turnover numbers among all the enzymes; one catalase molecule can convert millions of hydrogen peroxide molecules to water and oxygen each second.

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

In line with the previous findings, paracetamol toxicity significantly induced a decrease in serum catalase enzyme (reflects a decrease in antioxidant power) (**Figure 2B**). That was significantly relieved by Aleppo galls (**Figure 2B**) to near baseline values. Consequently, serum total antioxidant capacity that reflects the total antioxidant reserve in serum underwent a significant decrease upon treatment with a toxic dose of paracetamol (**Figure 2C**). That was significantly corrected to near baseline values upon treatment with an extract of Aleppo galls. For the authors, this may be explained by the high contents of anti-inflammatory and antioxidant ingredients in Aleppo galls.

In the same context, this study investigated the effects of a toxic dose of paracetamol on serum level of IL-6. IL-6 is a proinflammatory cytokine [30] that enhances the inflammatory and autoimmune processes in many disease conditions e.g. diabetes mellitus [31], atherosclerosis [32], depression [33], Alzheimer's Disease [33], systemic lupus erythematosus [34], multiple myeloma [35], prostate cancer [36], Behçet's disease [37], and rheumatoid arthritis [38]. Hence, there is an interest in developing anti-IL-6 agents as a promising treatment against many of these diseases [39, 40] e.g. tocilizumab was approved as an anti-IL-6 agent for treating rheumatoid arthritis [41].

This study confirmed also that toxicity with paracetamol significantly caused increased serum levels of Il-6 (**Figure 3A**) i.e. paracetamol toxicity causes inflammation-induced tissue damage through generation of oxidants and free radicals that impair the integrity of the cell membranes. Our data also confirmed that Aleppo galls significantly decreased the paracetamol-induced high serum IL-6 levels (**Figure 3A**). This confirms the antioxidant and tissue-protective effects induced by Aleppo galls. For the authors, the rich content of the anti-inflammatory and antioxidant ingredients in Aleppo galls suppressed the paracetamol toxicity-induced inflammation and restored an almost normal condition.

Our data confirmed also that toxicity of paracetamol significantly caused increased serum levels of lactate dehydrogenase (LDH, a marker of cellular damage) (**Figure 3B**). This denotes that paracetamol toxicity causes an evident tissue damage with impairment of the integrity of cell membranes. Our data confirmed that Aleppo galls significantly decreased the paracetamol-induced high serum LDH (**Figure 3B**). This confirms the antioxidant and tissue-protective effects induced by Aleppo galls.

$$\text{Pyruvate} + \text{NADH.H} \xleftrightarrow{\text{LDH}} \text{Lactate} + \text{NAD+}$$

High serum level of LDH usually reflects occurrence of tissue damage that may result from so many causes e.g. acute kidney disease [42], acute liver disease [42], rhabdomyolysis [43], and pancreatitis [42]. For the authors, Aleppo galls ingredients conferred an antioxidant and an anti-inflammatory status that minimized paracetamol toxicity-induced tissue damage. We strongly recommend using Aleppo galls for future drugs industry and also for better synthesis of new active antioxidant and anti-inflammatory compounds. Aleppo galls are promising medicinal plants for the production of potential active ingredients and future therapeutics for the treatment of paracetamol-induced toxicity. Our clinical impression is that Aleppo galls should be included as a medicinal nutrition and should be given to patients suffering from

paracetamol toxicity and admitted to clinical toxicology departments.

The shortcomings of this study is the recommendation to formulate Aleppo galls extract as a pharmaceutical nutrition to be given as an adjuvant treatment to those who need to take large doses of paracetamol.

Conclusion

In conclusion, Aleppo galls are promising sources of future drugs and therapeutic remedies for treating paracetamol overdosage and toxicity.

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Disclosure of conflict of interest

None.

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