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Research Article

Synergistic Hepatoprotective Effect of Chrysin and *Ginkgo biloba* Against Lead Toxicity in Adult Wistar Rats

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Abstract | Lead (Pb) poisoning is primarily caused by the accumulation of lead in multiple tissues and its interactions with bio-elements, essential for various biological functions. This study aimed to investigate the combined protective effects of Chrysin (Chy) and *Ginkgo biloba* (Gnk) against the bioaccumulative toxicity of Pb+2 on histological liver profiles and antioxidant enzyme activities in male adult rats. Adult male Wistar rats (250±10 g) were divided into four groups: group I (n = 6) served as the control and received 2.5 mg NaCl/kg/day as a sodium chloride solution, group II (n = 6) received 2.5 mg/kg/day as lead chloride solution orally, group III (n = 6) received 50 mg Chy and 50 mg Gnk/kg/day, and group IV (n = 6) received 50 mg Chy, 50 mg Gnk, and 2.5 mg Pb/kg/day. All animals were sacrificed 24 hours after the last oral dose administration upon completion of the 30-day trial. The liver was promptly removed for an assessment of the changes in malondialdehyde (MDA), glutathione (GSH), and myeloperoxidase activity (MOP). Proinflammatory cytokines (TNF-α and IL-1β) were measured in the plasma samples, and an evaluation of the liver histological profile was conducted on liver samples. The results indicated that lead toxicity significantly ($p \leq 0.05$) increased MDA and MOP levels while decreasing GSH activity in the liver compared to the control group. However, this effect was significantly ($p \leq 0.05$) altered by the supplementation of Chy and Gnk. Similarly, the levels of TNF-α and IL-1β were significantly decreased ($p \leq 0.05$) by the treatment with Chy and Gnk, which were markedly ($p \leq 0.05$) elevated by the Pb. The ameliorative effects of Chy and Gnk were also observed on the histological structures of the liver that had been damaged by lead. Generally, these results demonstrated that combined treatment of Chy and Gnk is effective against Pb-induced hepatic toxicity.

Keywords | Chrysin, *Ginkgo biloba*, Lead toxicity, Liver, Histology, Antioxidant enzymes

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INTRODUCTION

Due to its substantial contribution to modern industry, lead is now a common environmental pollutant (Abdul Kareem, 2014). Exposures at work and to the environment, however, continue to be a significant issue in many emerging and industrializing nations (Sawsan et al., 2018). Because of its dispersal in the surrounding air, drinking water, numerous foods, and dust, lead is effectively the most abundant non-essential element in the human organism. Pb^{+2} is one among the top ten chemicals of public health concern, according to the World Health Organization (Shojaeepour et al., 2018). Its toxicity is linked to its build-up in specific tissues and interference with bio elements, which play key roles in various bio- functional processes. It has numerous negative consequences such as neurological (Omotoso et al., 2015). behavioral (Al-Megrin et al., 2019), renal (Abd AL-Zahra et al., 2023), hepatic (Patra et al., 2001), and hematological (Omotayo et al., 2022). Pb^{+2} biological excretion is exceedingly challenging and can persist for a very long period in soft tissues, bones, and other vital organs (Abd El-Hack et al., 2019). Studies in-

dicate that oxidative stress may play a significant role in activating Pb^{+2} toxicity, even if the precise mechanism of Pb^{+2} toxicity in the tissue is not yet fully understood (Farmand et al., 2005). When heavy metal toxicity, such as Pb^{+2} poisoning, is being treated, chelation are employed in the removal of heavy metals from the body by a type of bonding of ions and molecules to metal ions, these ligands are called chelating agents, or sequestering agents (Caglayan et al., 2019). It has been observed that some unfavorable side effects are caused by the chelators used in lead treatment (Alcaraz-Contreras et al., 2016), as well as it did not have effective effect on subtraction and reduction of lead content accumulated in the tissue (Andersen and Aaseth, 2016). Consequently, a considerable amount of recent research has focused on various methods for treating lead toxicity, with a particular emphasis on plant-based medications (Abd El-Hack et al., 2019). The active ingredients in herbal products protect the tissues and inhibit the stages of oxidative stress (Caglayan et al., 2019). Generally, herbal materials have been included in recent scientific concerns as natural alternatives in detoxification of heavy metals (Caglayan et al., 2019). Flavonoids are phenolic chemicals, that represent plant secondary metabolites. They are commonly found in foods and exhibit antioxidant, antibacterial, anti-cancer, anti- mutagenic, and anti-inflammatory properties. (Celik et al., 2019). Flavonoid and glycosides have an inhibitory effect on the expression of inducible nitric oxide and nitric oxide synthesis, according to (Zaheer et al., 2020). Because there is conflicting information in the literature regarding the potential protective effects of Chrysin (Chy) and *Ginkgo biloba* (Gnk) against Pb^{+2} induced oxidative damage to the liver (Abd El-Hack et al., 2019). The aim of this current investigation was to assess potential protective effects of Chy and Gnk against lead-induced oxidative liver damage. Biological methods were employed as biomarkers of lead toxicity, including measurements of malondialdehyde (MDA), glutathione (GSH) and myeloperoxidase (MPO) activities, plasma tumor necrosis factor-alpha (TNF-α) and interleukin one beta (IL-1β) level along with an evaluation of liver histological profile.

MATERIALS AND METHODS

Chemical and drugs

The chemicals used in the experiment were included, Lead (II) chloride ($PbCl_2$), and chrysin (5,7-dihydroxyflavone [$C_{15}H_{10}O_4$] with 97% purity, were supplied by the Sigma-Aldrich Chemical Company (St Louis, MO, USA). *Ginkgo biloba* (contain 24% flavanol glycosides), and terpene trilactones (TTLs) (6%, including four kinds of ginkgo ides and bilobalide), purchased from Hisham Abdullah Pharmacy and the drug originates from China (Zhejiang Comba Pharmaceutical Co.).

Animals

All experiments were carried out on male Wistar rats (n=24) weighing 250 ± 10 g, brought from the animal house of the College of Veterinary Medicine, University of Basra. The animals were housed in clean plastic cages and given a week to acclimate in the laboratory setting (temperature = 22.5 C^0 with 12-hour dark/light cycle and ventilation system). During the trial, animals had unlimited access to food and water. Before conducting the study, official approval was obtained from the Professional Ethics Committee of the College of Veterinary Medicine, University of Basra. All ethical standards were strictly adhered to during this study.

Design of an experiment

The animals were divided into four groups of 6 rats each at random, and they received treatment for 30 days. The Group I served as the negative control and received daily oral administration of sodium chloride solution at a dose of 2.5 mg/kg/day. Group II rats served as the positive control and received by orogastric intubation 2.5 mg/kg/day lead chloride solution. Group III received 50 mg Chy + 50 mg Gnk /kg/day. Group IV received 50 mg Chy + 50 mg Gnk + 2.5mg Pb/kg/day. After the treatment, 24 hours following the last dose, animals were decapitated without anesthetic, and arteriovenous blood was promptly collected. A transverse abdominal incision was used to remove the liver, which was then frozen for use in later transactions.

Tissue extract preparation

Livers were promptly removed, rinsed with 0.9% ice-cold physiologic saline solution, blotted dry, and weighed. All tissues were homogenized in ten volumes of ice-cold trichloracetic acid (1 g tissue plus 10 mL 10% TCA) for 30 seconds. To remove cell debris, nuclei, and mitochondria from homogenates, they were centrifuged at a speed of 10,000*g* for 10 min at 4°C. The MDA levels were measured for products of lipid peroxidation through monitoring thiobarbituric acid responsive substance formation as described earlier (Beuge and Aust, 1978). Lipid peroxidation was calculated as nmol MDA/g tissue and represented as MDA equivalents using an extinction coefficient of 1.56 x 10^5 M^{-1} cm^1.

Glutathione (GSH) assay

A modified version of the Ellman procedure was used to measure glutathione (Beutler, 1978). Briefly, 2 mL of 0.3 mol/L $Na_2HPO_4.2H_2O$ solution was added to 0.5 mL of supernatant following centrifugation at 2,000*g* for 10 min. Immediately after adding a 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate), the absorbance at 412 nm was measured by spectrophotometer (model UV-1700, Shimadzu, Japan). The extinction coefficient used to compute glutathione levels was 1.36 x 10^5

M^{-1} cm1. The outcomes are displayed in mol GSH/g tissue.

Myeloperoxidase activity (MPO) assay

MPO activity of tissue was evaluated by determining the H_2O_2- dependent oxidation of dianizidine -o- 2HCl. Briefly, hexa- decyltri- methyl-ammonium bromide (HETAB; 0.5%, w/v) was added to 10 ml of ice-cold potassium phosphate buffer (50 mmol /L K2HPO4, pH 6.0) to 0.2–0.3 g of homogenize tissue samples. By detecting the H_2O_2-dependent oxidation of dianizidine- o- 2HCl, MPO activity was determined. The amount of MPO present per gram of tissue that resulted in a 1.0/min change in absorbance at 460 nm was defined as one unit of enzyme activity by spectrophotometer (model UV-1700, Shimadzu, Japan) (Hillegass et al., 1990).

Pro-inflammatory cytokines detection (TNF-α and IL-1β)

Enzyme-linked immunosorbent assay (ELISA) kits were used that were designed specifically for measuring TNF-α and IL-1β of blood plasma according to the manufacturer's instructions and guidelines. ELISA microplate reader (Spectra Max 190, Molecular Devices Corporation, Sunnyvale, CA) was utilized to record the absorbance at 450 nm. The careful examination of five independent standard series replications, the assessment of the deviation of TNF- and IL-1β concentrations in experimental animal groups, and the evaluation of visual analysis of whole UV-Vis spectra were all meticulously carried out.

Histopathological assay

Each sacrificed rat's liver were removed at the end of the trial. They were then prepared for paraffin sectioning by dehydrating in various concentrations of alcohol, employing xylol to clean, and embedding in blocks of paraffin after being fixed in 10% buffered formalin. For histopathological investigation, sections of tissue with a thickness of around 5 µm were stained with Harris hematoxylin and eosin staining (H&E) (Ada and Delia 2014), and examined under a microscope at 400X magnification.

Statistical analysis

The data were analyzed statistically using one-way ANOVA with the SPSS 18th version. The mean± SD was used to express all data. All significant differences were represented at p≤0.05.

RESULTS

MDA as expression to Lipid peroxidation assay

The lipid peroxide level of liver tissue was expressed through MDA and results were presented in Fig.1-A. Rats of group II exhibited a statistically significant (p≤0.05) increase (93±8.99) when compared with other groups. While group IV showed a statistically significant (p≤0.05) decrease (63±3.4) compared to the group II, and an increase compared to the control group (p≤0.05) and the group III (p≥0.05).

Glutathione (GSH) activity

We noted a significant (p≤ 0.05) decrease in the GSH level of liver tissue in group II rats treated with 2.5mg Pb^{+2}/kg BW, compared with other experimental treatments. GSH level in group IV rats remained parallel (p≥0.05) with that of control group (Fig.1-B).

MPO level

A significant (p≤0.05) increase in MOP content was noticed in group II compared with all other groups. On the other hand, the group IV reflected a moderate increase in the MOP of rats, which was significantly different (p≤0.05) as compared to group III and control (Fig.1-C).

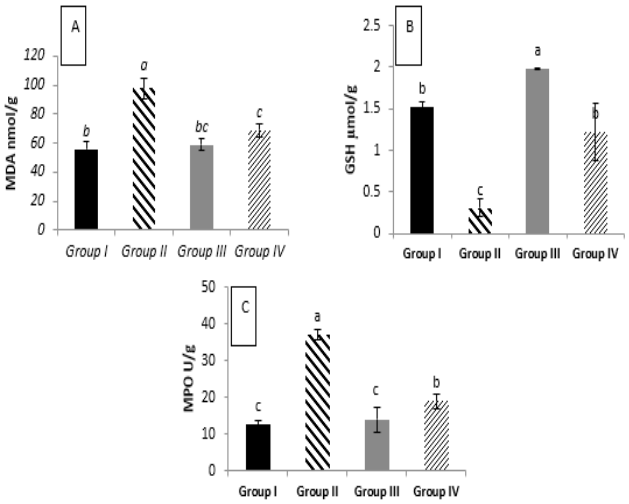

Figure 1: Measure of antioxidant enzymes concentration in Wistar rats. (A) MDA level (nmol/g); (B) GSH level (µmol/g); and (C) MPO activity (U/g). Values are presented as means ± S.D of 6 individuals. Different letters indicate significant (p≤0.05) differences, and similar letters indicate no significant differences among experiment groups

Effect of Chrysin and *Ginkgo biloba* on plasma pro-inflammatory cytokines

TNF-alpha and IL-1β levels of blood plasma were noticeably higher (p≤0.05) in group II, confirming that lead poisoning is tightly linked to inflammatory and oxidative processes. Group IV exhibited a significant (p≤0.05) decrease in these cytokines level and reached almost similar level as that of control and group III rats (Fig.2- A and B).

Histological findings

The histological alterations in the liver tissues were evaluated as outlined in Figure 3. The hepatic parenchyma of rats in group I exhbited multiple hepatic lobules separated

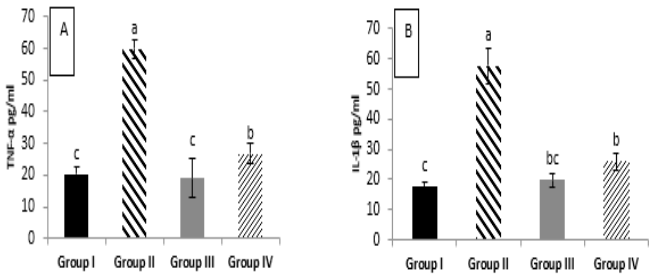

Figure 2: Measure of plasma pro-inflammatory cytokines concentration in Wistar rats. (A) TNF-alpha, and (B) interleukin 1-beta (IL-1β) levels. Values are presented as means ± S.D of 6 individuals. Different letters indicate significant ($p \leq 0.05$) differences, and similar letters indicate no significant differences among experiment groups.

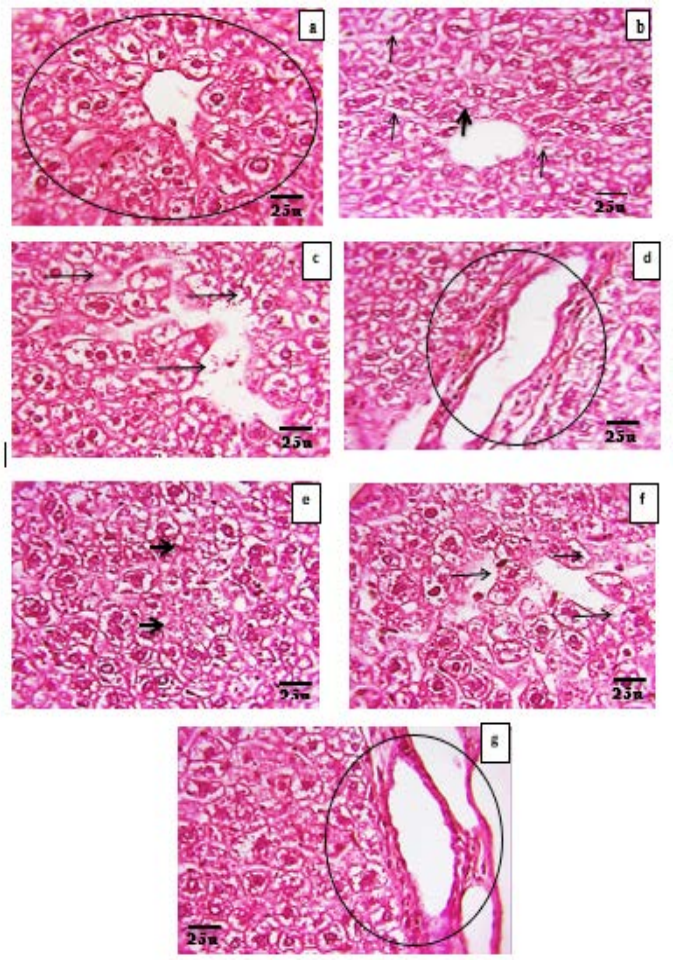

Figure 3: (a): Photomicrograph of a liver from the control group represents normal architecture (circle). (e, f): A section of the liver from group III on day 30 showing semi-normal architecture, except for slight expansion of the hepatocytes sinusoids (arrow) and cytoplasmic degeneration (thick arrow). (c, d): section of liver from group II at day 30 showing acute hepatocyte necrosis (arrow), cytoplasmic degeneration (thick arrow), expansion, and necrosis of blood vessels (circle). (g): A section of the liver from group IV on day 30 showing a limited extent of hepatocyte necrosis (arrow), and cytoplasmic degeneration (thick arrow), which also showed limited expansion and degeneration of blood vessel walls (circle). H and E. X400, (Scale bar 25u).

by delicate connective tissue septa. Each lobule contained a central vein with thin walls, surrounded by hepatic cords extending to the periphery (Fig. 3 a). While no prominent alteration were noticed in the hepatocytes tissue of rats treated with Chy + Gnk, the hepatocytes showed with a normal pattern, while dilatation of the sinusoidal of the portal vein was still identified (Fig. 3 b). Lead poisoning rats (group II) showed severe damage to liver tissue, including fatty alterations degeneration of cytoplasmic, focal necrosis, karyolysis, pyknotic nuclei, the proliferation of Kupffer cells, inflammatory cells, as well as of bile ductless (Fig. 3. c, d, e, and f). Group IV reflected the susceptibility of the two treatments, Chy and Gnk, to inhibition of liver tissue damage due to lead's harmful impact. Generally, the noted improvement partly in liver tissue, represented by a few lipid droplets, a small localized necrotic region, and cytoplasmic degeneration of hepatocytes accompanied by some intravenous congestion (Fig. 3. g).

DISCUSSION

The fifth most often used metal in the world, lead (Pb^{+2}), is harmful to people, and its poisonousness is linked to the production of oxidative stress factors (Kao and Rusyniak, 2016). The route of exposure, the subject's age, health state, the number of exposures, the duration of exposure, and the person's genetic makeup all play a role in the physiological damage caused by lead (Chowdhury et al., 2014). Lead is absorbed in its organic and inorganic state with the help of the respiratory chain and the gastrointestinal tract. Generally lead has high lipid-solubility characteristic, this makes its transport throughout organs and tissues easier (Rosin, 2009). Our results confirmed that increases in lipid peroxidation and myeloperoxidase activity caused by lead toxicity were followed by significant decreases in hepatic GSH levels in the current investigation. Additionally, higher plasma levels of the cytokines TNF-alpha and IL-1β, as well as histological studies, revealed the severity of the lead-induced systemic inflammatory response. Generally, in the current investigation, it was discovered that lead changes liver tissue's oxidative stress-related metrics. Lead has an induced property that throws off the body's antioxidant system's delicate equilibrium, leading to the development of reactive oxygen species and oxidative stress (Taslimi et al., 2019). Lipid peroxidation makes significant contributions to the assessment of liver cell injury, lipid peroxidation produces MDA as a breakdown byproduct (Zheng et al., 2020). According to studies, Pb^{+2} reduced the activity of antioxidant enzymes by attaching to their SH- groups, which also led to GSH depletion, a non-enzymatic antiox-

idant, and lipid peroxidation are two examples. As a result, the goal of treating Pb^{+2} poisoning is to remove Pb^{+2} from the body while also removing reactive oxygen species to avoid the development of oxidative stress (Alcaraz-Contreras et al., 2016). As demonstrated in this investigation, the antioxidant Chy and Gnk combined treatment dramatically suppressed MDA formation while simultaneously replenishing tissue GSH concentration, signifying a reduction in lipid peroxidation and cellular injury, which protects liver tissues from lead-induced oxidative damage. Moreover, our findings indicate a noteworthy elevation in plasma levels of TNF-alpha and IL-1β due to lead toxicity. Conversely, the combined treatments mentioned earlier resulted in the inhibition of tumor necrosis factors levels. Chy is one of the flavonoids present in many plant extracts that is frequently used as a traditional medication (Hanedan et al., 2018), while numerous distinct terpenoids and flavone glycosides are present in *Ginkgo biloba* extract. The antioxidant activity of flavonoids is thought to be one of the primary processes underlying the pharmacological actions of the extract, which is one of its primary impacts. Generally, in the study by Zhang et al. (2004), the suppression of lipid peroxidation was credited with the protective effect of Gnk on hepatic endothelial cells in rats with chronic liver injury. Additionally, it was discovered that Gnk increased the activity of glutathione peroxidase and superoxide dismutase, two antioxidant enzymes that protect brain regions from ischemia/reperfusion injury (Janssens et al., 2000). On the other hand, Akdere et al. (2014) established that terpenic components suppress free radical production. Flavonoids play an important role in the regulation of cellular functions such as cell cycle signals and inflammatory pathways (Gargouri et al., 2013). Generally, the relationship between oxidative stress and inflammatory response is becoming clearer, as oxidative stress contributes significantly to the inflammatory process (Lugrin et al., 2014).

According to reports, oxidant molecules have an impact on all stages of the inflammatory process, including the release of endogenous danger signal molecules, recognition of these molecules by natural immune cells via the toll-like receptor (TLR) and NOD-like receptor (NLR) families, and activation of signal pathways that initiate an adaptive cellular response to these signals are all examples of how these signals are processed (Khalil et al., 2019), Hence, oxidative stress initiates NF-κB, instigating the inflammatory process (Turillazzi et al., 2016). Subsequently, NF-κB promotes the release of TNF-α and IL-1β, which are pro-inflammatory cytokines. Furthermore, NF-κB regulates the expression of COX-2 and iNOS proteins. Therefore, inhibiting NF-κB becomes essential for therapeutic purposes (Caglayan et al., 2019). According to Liu et al. (2017), Pb^{+2} damages tissue and triggers NF-κB activation and inflammation. Flavonoids are crucial for controlling cellular processes such as modulating inflammatory pathways and cell cycle signals (Gargouri et al., 2013).

The histology findings observed in current study supported biological measurements that showed lead caused oxidative damage, when compared to the usual control group. Generally, exposure to lead causes a noticeable liver tissue injury represented by dilation in the central vein and blood vessels, severe necrosis and degradation of hepatocytes, high accumulation of Kuppfer cells, and dilated sinusoids. The Chy and Gnk mixed treatment clearly reduced the overall necrosis and degeneration of hepatocytes. Chy and Gnk, as antioxidants, reduced oxidative stress of MDA and MPO in hepatic tissues and maintained the shape, parameters, and function of hepatocytes, increased plasma antioxidant capacity and cytokine suppression.

CONCLUSION

Our data demonstrated that oxidative stress caused lead poisoning in the liver. The antioxidant and anti-inflammatory activities of Chy and Gnk were found to be applicable to Pb-induced hepatic toxicity, and the combined treatment of Chy and Gnk is an encouraging blend in reducing liver damage caused by lead exposure. However, more research is needed to support the mechanism of this effect of combined Chy and Gnk treatment.

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ETHICAL STANDARDS COMPLIANCE

All ethical standards were strictly adhered to during this study. Official approval was obtained from the Professional Ethics Committee of the College of Veterinary Medicine, University of Basra

CONFLICT OF INTEREST

The authors state that they do not have any conflicts of interest.

AUTHORS' CONTRIBUTION

Afrah A. Hassan (First Author), Introduction Writer and Statistical Analyst (40%), Hawraa H. Alowaid (Second Author), Methodologist/ (30%), Majdy F. Majeed (Third Author), Discussion Writer (30%).

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