Yüksek İhtisas Üniversitesi
Sağlık Bilimleri Dergisi
Yüksek İhtisas University Journal of Health Sciences
ISSN 2717-8439
E-ISSN 2717-9257

YIU Saglik Bil Derg 2026;7(2):47–56
https://doi.org/10.51261/yiu.2026.1908860

ARAŞTIRMA MAKALESİ / RESEARCH ARTICLE

Asiatic Acid Ameliorates Chlorpyrifos-Induced Toxicity in Rats: Biochemical and Histopathological Insights

Sıçanlarda Klorpirifos Kaynaklı Toksisite Üzerine Asiatik Asidin Etkileri: Biyokimyasal ve Histopatolojik Bulgular

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ABSTRACT

Introduction: Chlorpyrifos (CPF), one of the most widely used organophosphorus (OP) insecticides, exerts its toxic effects in part by inducing oxidative stress. Despite increasing recognition of natural antioxidants as potential countermeasures, the efficacy of asiatic acid (AA), a bioactive triterpenoid from Centella asiatica, against CPF-induced toxicity remains unexplored. This study aimed to investigate the protective effects of AA against CPF-incuded toxicity model in rats by analyzing biochemical and histological parameters in serum, liver, and brain tissues.

Material and Methods: Adult male Sprague Dawley rats (n=30) were randomly divided into five groups: control, CPF (279 mg/kg, subcutaneously), and three AA-pretreated groups (35, 70, and 140 mg/kg, per os) for 14 days prior to CPF exposure. Oxidative stress markers [malondialdehyde (MDA), advanced oxidation protein products (AOPP)] and antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)] were measured in serum, liver, and brain. Histopathological and immunohistochemical analyses assessed tissue injury and apoptotic responses.

Results: AA administration significantly attenuated CPF-induced oxidative damage and restored antioxidant defenses in a dose-dependent manner. Histopathological analyses confirmed that AA mitigated hepatic and neuronal damage and preserved tissue architecture. Immunohistochemistry revealed that AA suppressed CPF-induced upregulation of the pro-apoptotic protein Bcl-2-associated X protein (Bax), indicating reduced apoptosis.

Conclusion: This study provides the first demonstration that AA exerts robust systemic antioxidative, hepatoprotective, and neuroprotective effects against CPF-induced toxicity. These findings highlight AA's therapeutic potential for mitigating organophosphate pesticide poisoning, warranting further translational research.

Keywords: Asiatic acid, chlorpyrifos, hepatoprotection, neuroprotection, organophosphate toxicity, oxidative stress

ÖZ

Giriş: Klorpirifos (CPF), yaygın olarak kullanılan organofosforlu (OP) insektisitlerden biridir ve toksik etkilerini kısmen oksidatif stres mekanizmaları yoluyla göstermektedir. Doğal antioksidanların pestisit kaynaklı toksisiteye karşı potansiyel koruyucu etkileri giderek daha fazla araştırılmaktadır. Centella asiatica bitkisinden elde edilen biyolojik olarak aktif bir triterpenoid olan asiatic asidin (AA) antioksidan ve sitoprotektif özellikleri bilinmesine rağmen, CPF kaynaklı toksisite üzerindeki koruyucu etkileri henüz yeterince araştırılmamıştır. Bu çalışmanın amacı, sıçanlarda oluşturulan CPF kaynaklı toksisite modelinde AA'nın koruyucu etkilerini serum, karaciğer ve beyin dokularındaki biyokimyasal ve histolojik parametreler üzerinden değerlendirmektir.

Materyal ve Metodlar: Erişkin erkek Sprague Dawley sıçanlar (n=30) rastgele beş gruba ayrılmıştır: kontrol, CPF (279 mg/kg, subkutan) ve CPF uygulamasından önce 14 gün boyunca farklı dozlarda AA verilen üç ön tedavi grubu (35, 70 ve 140 mg/kg, oral). Serum, karaciğer ve beyin dokularında oksidatif stres belirteçleri [malondialdehit (MDA), ileri oksidasyon protein ürünleri (AOPP)] ile antioksidan enzim aktiviteleri [süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GPx)] ölçülmüştür. Ayrıca doku hasarı ve apoptotik yanıtları değerlendirmek amacıyla histopatolojik ve immünohistokimyasal analizler gerçekleştirilmiştir.

Bulgular: AA uygulaması, CPF'nin neden olduğu oksidatif hasarı anlamlı düzeyde azaltmış ve antioksidan savunma sistemini doza bağımlı olarak iyileştirmiştir. Histopatolojik incelemeler AA'nın hepatik ve nöronal hasarı azalttığını ve doku mimarisini koruduğunu göstermiştir. İmmünohistokimyasal analizler ise AA'nın pro-apoptotik protein Bax ekspresyonundaki CPF kaynaklı artışı baskıladığını ortaya koymuştur.

Sonuç: Bu çalışma, AA'nın CPF kaynaklı toksisiteye karşı güçlü antioksidan, hepatoprotektif ve nöroprotektif etkilerini ilk kez ortaya koymaktadır. Bu bulgular, AA'nın organofosfat pestisit toksisitesinin azaltılmasında potansiyel bir terapötik ajan olabileceğini ve bu konuda ileri translasyonel çalışmaların yapılması gerektiğini göstermektedir.

Anahtar Sözcükler: Asiatik asit, klorpirifos, hepatoprotektif etki, nöroprotektif etki, organofosfat toksisitesi, oksidatif stres

Cite this article as: Darakcı Saltık Ö, Balcı H, Güzelgül F, Öksel N, Hatipoğlu Aktemur B, Abdullahoğlu R, Bozkurt A. Asiatic Acid Ameliorates Chlorpyrifos-Induced Toxicity in Rats: Biochemical and Histopathological Insights. YIU Saglik Bil Derg 2026;7(2):47–56. https://doi.org/10.51261/yiu.2026.1908860

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Geliş Tarihi/Received: 13.03.2026, **Kabul Tarihi/Accepted:** 09.04.2026, **Çevrimiçi Yayın Tarihi/ Available Online Date:** 27.08.2026

Introduction

Organophosphorus (OP) insecticides, a class comprising over 200 chemical compounds, are among the most widely used pesticides globally, playing a crucial role in pest control and agricultural productivity. However, their excessive and indiscriminate use has raised serious public health and environmental concerns. Acute or chronic OP exposure occurs via multiple routes, including ingestion, inhalation, and dermal absorption, leading to a broad spectrum of toxic effects in multiple organs. Growing evidence suggests that these toxic effects are associated with oxidative stress (1).

Chlorpyrifos (CPF), a widely used broad-spectrum OP insecticide, poses significant toxic effects on human and animal health due to its extensive application in agriculture. Given its detrimental impact, effective protective strategies are needed to mitigate CPF toxicity. Oxidative stress contributes to CPF toxicity by inducing the generation of reactive oxygen species (ROS) and reducing the activity of antioxidant enzymes, such as CAT, SOD, and GPx. Thus, the excessive accumulation of ROS triggers lipid peroxidation and protein oxidation and induces apoptosis, processes that play a critical role in the hepatotoxic and neurotoxic effects of CPF (2,3).

Antioxidants have been introduced as potential therapeutic agents to counteract CPF-induced tissue damage. Among these, natural antioxidants derived from medicinal plants have attracted significant attention due to their potent free radical scavenging properties (4,5). *Centella asiatica (C. asiatica)*, a medicinal plant extensively utilized in Ayurvedic and traditional Chinese medicine, is recognized for its potent antioxidant, neuroprotective, antiapoptotic, and mitoprotective properties. Asiatic acid (AA), a bioactive pentacyclic triterpenoid derived from C. asiatica, is primarily responsible for its therapeutic effects (6,7). Over the past two decades, AA has demonstrated significant protective effects in various preclinical models of organ toxicity (7). For instance, in a rat model of doxorubicin-induced organ toxicity, AA administration ameliorated cardiac and hepatorenal toxicity by reducing oxidative stress (8). Although growing evidence suggests that various pentacyclic triterpenoids exert protective effects against CPF-induced hepatic and neurotoxicity, no study has assessed the potential role of AA in mitigating CPF or any other organophosphate-induced toxicity, a pervasive environmental health risk (9).

In this study, we employed an experimental rat model of CPF toxicity to investigate whether subacute AA administration could attenuate CPF-induced tissue damage and, if so, to determine the role of the oxidative system in this effect. Through biochemical and histopathological analyses, this study provides the first comprehensive evaluation of AA as a potential protective agent against organophosphate toxicity, contributing to therapeutic intervention strategies.

Material and Methods

Reagents

Chlorpyrifos (CPF; Riedel-de Haen, Germany) was dissolved in pure olive oil (Sigma-Aldrich, USA), while asiatic acid (AA; Sigma Chemical Co., St. Louis, MO, USA) was dissolved in propylene glycol (Sigma Chemical Co., St. Louis, MO, USA). CPF was administered subcutaneously (s.c.) at a volume of 1 mL/kg body weight, whereas AA was administered per os (p.o.) via an intragastric feeding tube at a volume of 1 mL/kg (10,11). Because CPF is a highly lipophilic compound with limited aqueous solubility, an oil-based vehicle was selected to ensure homogeneous dissolution, dose stability, and controlled systemic exposure.

Experimental Design

The study, approved by OMU-HADYEK (Protocol number 2023/08), was conducted at Ondokuz Mayis University Faculty of Medicine and adhered to both OMU-HADYEK guidelines and the European Directive 2010/63/EU on laboratory animal welfare.

Thirty adult male Sprague Dawley rats (250–275 g, 10–12 weeks of age) were obtained from the Experimental Animal Research Center of Ondokuz Mayis University (OMÜ-DEHAM). Animals were housed in polycarbonate cages with wood shavings bedding. The rats were maintained under controlled environmental conditions (25 ± 1°C, 12 h light/dark cycle, 50% ± 5% humidity) with ad libitum access to standard pelleted chow (Nükleon, Türkiye) and tap water.

Following a one-week acclimatization period, the rats were randomly assigned to five groups of six animals each. Asiatic acid (35, 70, or 140 mg/kg) or its vehicle (propylene glycol) was administered orally for 14 consecutive days. On day 15, a single dose of CPF (279 mg/kg, s.c.) was administered to all rats except those in the control group, which received propylene glycol instead. Figure 1 illustrates the experimental timeline, treatment groups, and evaluated parameters.

The timing and dose of CPF were determined based on studies defining this exposure as an acute toxicity model in adult rats (10,12). The dose and administration of AA were determined based on previously reported experimental protocols. The selected doses (35, 70, and 140 mg/kg) were chosen to represent a graded dose range (low, moderate, and high), enabling the assessment of dose–response relationships. Previous studies have demonstrated that these doses are pharmacologically effective in reducing oxidative stress and tissue injury, while remaining within a safe and non-toxic range in experimental animal models (13,14). The animals were weighed daily, and doses were adjusted accordingly. The rats' body weight and temperature were measured and recorded just before (day 15) and 24 hours after (day 16) CPF administration. All animals were

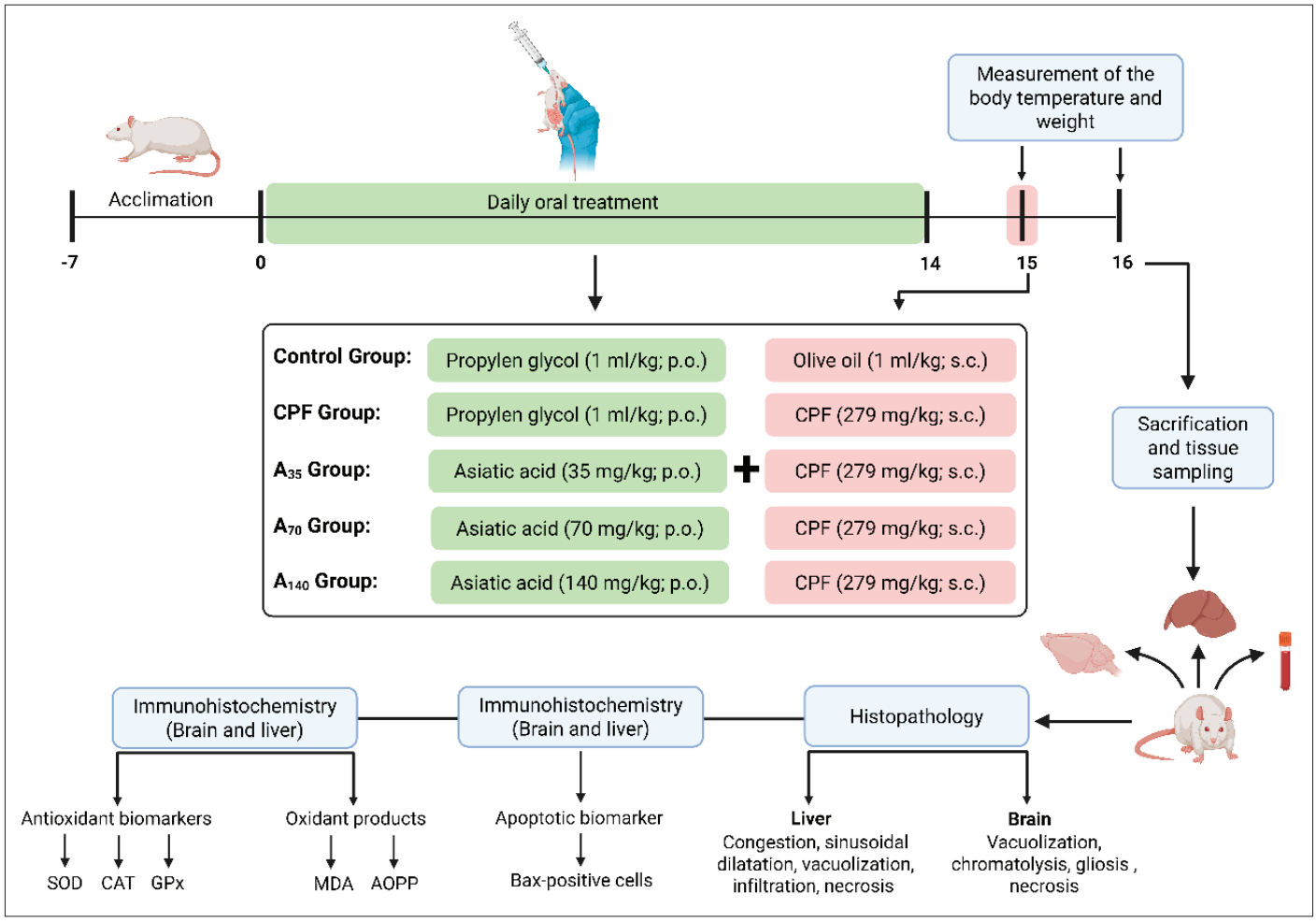

Figure 1. The Schematic Illustration of the Experimental Design. The image was created with BioRender.

then anesthetized with ketamine (100 mg/kg) and chlorpromazine (0.75 mg/kg) and sacrificed. Blood samples, liver, and brain tissues were collected for biochemical, histopathological, and immunohistochemical analyses.

Sample Preparation

After anesthesia, 5 mL of blood was collected directly from the heart. Serum was then obtained by centrifugation (Ohaus Frontier 5707, USA) at 3000 x g for 10 minutes at room temperature then stored at -80°C for biochemical analysis. Liver and brain tissues were extracted and washed with physiological saline (0.9% NaCl). The left hepatic lobe and left hemisphere of the brain were promptly frozen at -80°C until biochemical analysis was performed to determine oxidant and antioxidant levels. The right hepatic lobe and right hemisphere of the brain were fixed in 10% neutral buffer formalin for histological analysis.

Biochemical Measurements

Brain and liver tissues were homogenized for 1 minute in Eppendorf tubes containing 900 µL of ice-cold phosphate-buffered saline (PBS, pH 7.4, 1 mM) per 100 mg of tissue using an Ultra Turrax homogenizer (Ika T18 Basic, Germany), followed by sonication for 10 minutes at 4 °C using a sonicator (METU Electromechanical, Germany). Homogenates were centrifuged (3000 × g for 15 min at 4°C), and supernatants were analyzed for malondialdehyde (MDA), advanced oxidation protein products (AOPP), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) concentrations using enzyme-linked immunosorbent assay (ELISA) kits. Standard curves were generated for each ELISA analysis to ensure accuracy and reproducibility. The protein concentration was determined using the Bradford protein assay (15).

Lipid Peroxidation and Protein Oxidation Measurements

MDA levels, which indicate lipid peroxidation, and AOPP levels, which indicate protein oxidation, were measured in serum and supernatants from rats' brain and liver tissues using commercial kits [ELK7832 (LOT:55777764), ELK0784 (LOT: 55760540), respectively] via an ELISA reader at 450 nm (Organon Teknika Microwell System Reader 230S, Germany). Serum results were expressed as ng/mL (for MDA) and pg/mL (for AOPP), whereas liver and brain tissue results were normalized to total protein content and expressed as ng/mg protein (for MDA) and pg/mg protein (for AOPP).

Superoxide Dismutase, Catalase and Glutathione Peroxidase Antioxidant Biomarkers Measurements

SOD, CAT, and GPx levels were measured by using commercial measurement kits [ELK8178 (LOT: 55777102), ELK5986 (LOT:55772662) and ELK2222 (Lot:55763376), respectively] via an ELISA reader at 450 nm (Organon Teknika Microwell System Reader 230S, Germany). Serum results were expressed as ng/mL (for SOD and CAT) and pg/mL (for GPx), whereas liver and brain tissue results were normalized to total protein content and expressed as ng/mg protein (for SOD and CAT) and pg/mg protein (for GPx).

Histopathology

Liver and brain tissues were processed using standard paraffin embedding techniques. Sections (5 µm) were stained with hematoxylin and eosin (H&E) and analyzed under a light microscope. For each animal, three non-overlapping randomly selected microscopic fields were evaluated per tissue section (liver and brain). Liver damage was assessed based on vascular congestion, sinusoidal dilatation, vacuolation of hepatocytes, mononuclear cell infiltration, and necrosis. Cerebral cortex analysis included vacuolization, chromatolysis, gliosis, and necrosis. A semiquantitative scoring system (0: no damage, 1: minimal damage, 2: moderate damage, 3: severe damage) was used for histopathological grading (16,17). For each parameter, the scores obtained from the three evaluated fields were averaged to obtain a single histopathological score for each animal. Subsequently, the mean of these individual animal scores was calculated for each group, and the results were expressed as mean ± SEM (n=6 animals per group).

Immunohistochemical Evaluation

Immunohistochemical analysis was performed to evaluate Bax protein expression in liver and brain tissues. For each animal, a total of three tissue sections were obtained from both the liver and brain. Each section was deparaffinized and rehydrated using a graded alcohol series. Antigen retrieval was conducted in citrate buffer (pH: 6) at 95°C for 30 minutes. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide, and a blocking solution (5% goat serum + 2% BSA + 0.1% Triton X-100 in PBS) was applied to prevent nonspecific binding.

Sections were incubated overnight at 4°C with Bax primary antibody (E-AB 81433, 1:100 dilution) and then treated with secondary antibody (E-AB 1003, 1:1000 dilution) for 1 hour at room temperature. The reaction was visualized with 3,3'-diaminobenzidine (DAB) for 5 minutes, and counterstained with Mayer's hematoxylin. After dehydration through a graded alcohol series, the sections were mounted for microscopic evaluation (10,17). For each tissue section, three randomly selected regions of interest (ROIs) were analyzed under ×200 magnification using a Nikon Eclipse E2000 microscope. Bax-positive cells were manually counted using ImageJ software (version 1.54f, NIH, USA) and expressed as a percentage of total cells in each ROI. Each immunohistochemical image includes a 100 µm scale bar. Negative control staining (omission of primary antibody) was also performed to confirm antibody specificity.

Statistical Analysis

Sample size was determined by an a priori power analysis using G*Power (v3.1) with an effect size of 0.7, α=0.05, and power=80%, resulting in six animals per group. Statistical analyses were performed using IBM SPSS Statistics 20 (IBM Corp., Armonk, NY, USA). Data normality was assessed using the Shapiro–Wilk test. Normally distributed data were analyzed by Student's t-test or one-way ANOVA followed by Tukey's post-hoc test, whereas non-normally distributed data were analyzed using Mann–Whitney or Kruskal–Wallis tests with Dunn's post-hoc test. Results were expressed as mean ± SEM or median (min–max), and $p<0.05$ was considered statistically significant. Graphs were generated using GraphPad Prism 8.0.2.

Results

Effect of Asiatic Acid on Oxidative Stress Markers in CPF-Induced Toxicity

MDA concentrations were significantly elevated in the CPF group in serum, brain, and liver ($p<0.01$). AA suppressed MDA levels in serum at all doses, while 70 and 140 mg/kg reduced MDA levels in brain and liver ($p<0.05$–0.01) (Figure 2A).

AOPP concentrations were also increased in the CPF group in serum, brain, and liver ($p<0.05$–0.001). AA (140 mg/kg) reduced AOPP levels in serum and liver ($p<0.01$), whereas 35 and 70 mg/kg reduced AOPP levels in the brain. The highest dose fully prevented CPF-induced AOPP elevation in the brain ($p<0.001$) (Figure 2B).

Effect of Asiatic Acid on Antioxidant Enzymes in CPF-Induced Toxicity

SOD levels were substantially lower in the CPF group in serum ($p<0.01$), brain ($p<0.001$), and liver ($p<0.05$). AA at 70 and 140 mg/kg restored serum SOD levels ($p<0.05$–0.01), while 140 mg/kg restored SOD concentration in the brain ($p<0.01$) and liver ($p<0.05$) (Figure 3A).

CAT levels were reduced in the CPF group in serum, brain, and liver ($p<0.05$). AA (140 mg/kg) restored serum CAT levels ($p<0.05$), while 70 and 140 mg/kg reversed CPF-induced reductions in liver CAT levels ($p<0.05$). No AA dose affected CAT levels in the brain (Figure 3B).

GPx levels were lower in serum ($p<0.01$) and brain ($p<0.001$) in the CPF group, while liver GPx levels remained unaffected. AA (140 mg/kg) restored serum GPx levels ($p<0.05$), and 70 and 140 mg/kg increased GPx levels in the brain ($p<0.05$) (Figure 3C).

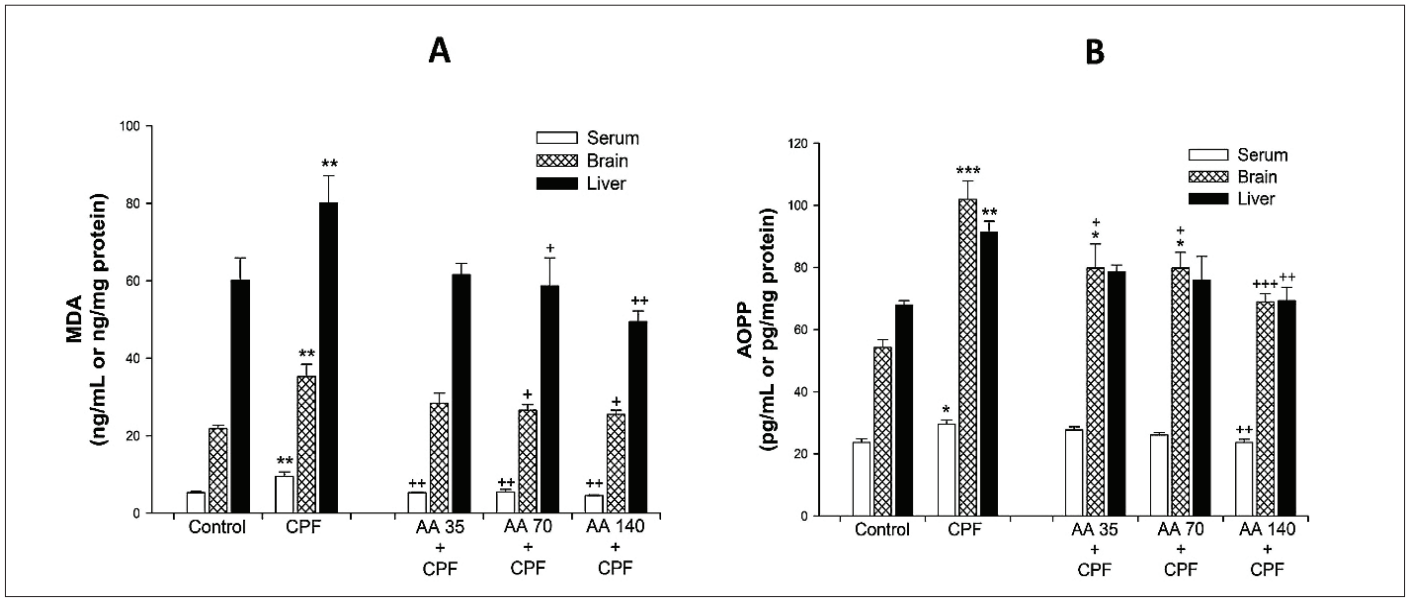

Figure 2. Effect of AA on Oxidative Stress Markers (MDA and AOPP) in Serum, Brain, and Liver of CPF-intoxicated Rats. (A) MDA and (B) AOPP concentrations. The levels of lipid peroxidation and protein oxidation were measured in terms of MDA and AOPP concentrations, respectively. Data are expressed as mean ± SEM (n=6). *p < 0.05, **p<0.01, and ***p<0.001 indicate significant differences compared with the control group, while +p<0.05, ++p<0.01, and +++p<0.001 indicate significant differences compared with the CPF group.

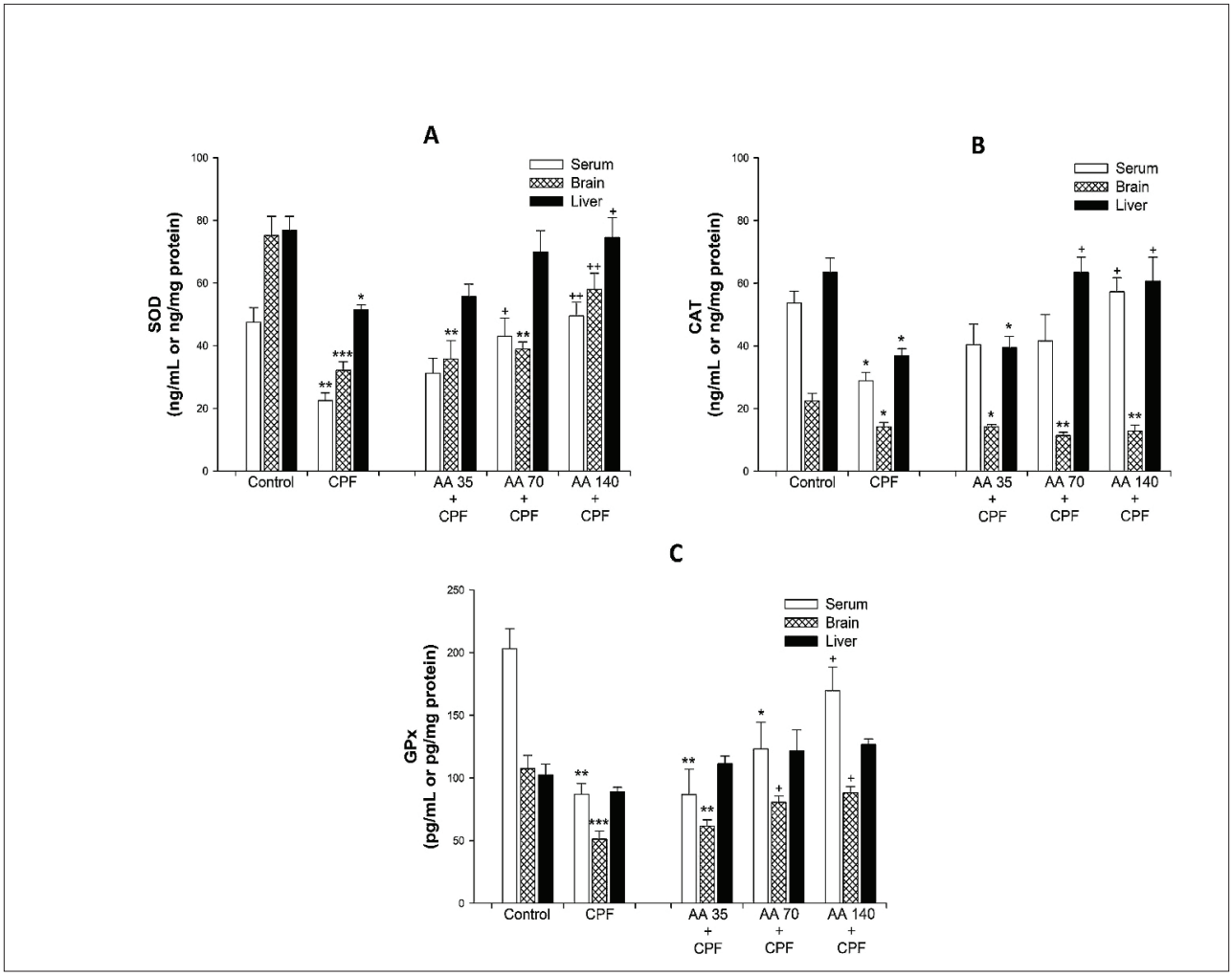

Figure 3. Effect of AA on Antioxidant Enzyme Concentrations (SOD, CAT, GPx) in Serum, Brain, and Liver of CPF-intoxicated Rats. (A) SOD, (B) CAT, and (C) GPx concentrations. Data are expressed as mean ± SEM (n=6). *p<0.05, **p<0.01, and ***p<0.001 indicate significant differences compared with the control group, while +p<0.05 and ++p<0.01 indicate significant differences compared with the CPF group.

Effect of Asiatic Acid on Histopathological Parameters in CPF-Induced Toxicity

Histopathological evaluation of liver tissues showed that CPF increased mononuclear cell infiltration, sinusoidal dilatation, vascular congestion, vacuolation, and necrosis ($p<0.05$). AA alleviated these histopathological changes ($p<0.05$). Liver architecture in the AA70 and AA140 groups was largely comparable to that of the control group, except for persistent sinusoidal dilatation in the AA140 group (Figure 4, Table 1).

In the cerebral cortex, CPF toxicity increased vacuolation, chromatolysis, gliosis, and necrosis ($p<0.05$). AA treatment mitigated these alterations ($p<0.05$), with the AA70 and AA140 groups showing histological profiles resembling the control group (Figure 5, Table 2).

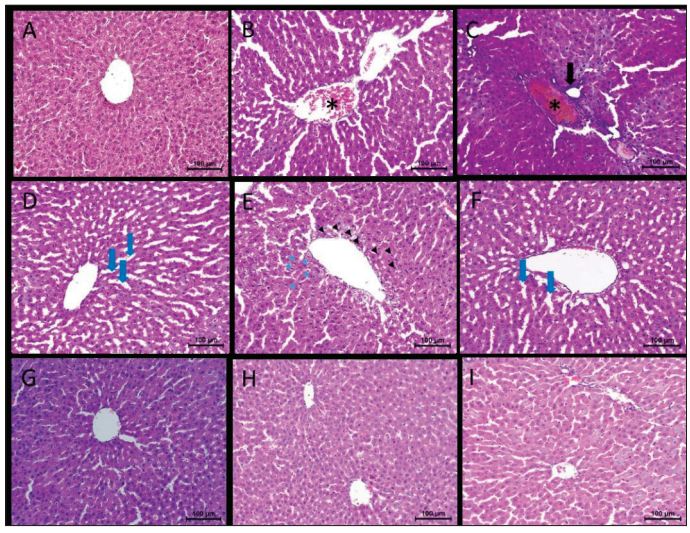

Figure 4. Histopathological Changes in Liver Tissues of CPF-intoxicated Rats Treated with AA. AA exhibits a marked restoration of normal architecture in liver tissues. Histological changes were observed using H&E staining (×20). "A" represented the control group. "B" to "F" represented the CPF group. "G" to "I" represented AA35, AA70, AA140 groups, respectively. *: Central vein congestion, Black arrow: Mononuclear cell infiltration, Blue arrow: Sinusoidal dilatation, Blue arrowhead: Vacuolization, Black arrowhead: Hepatocytes with pyknotic nuclei.

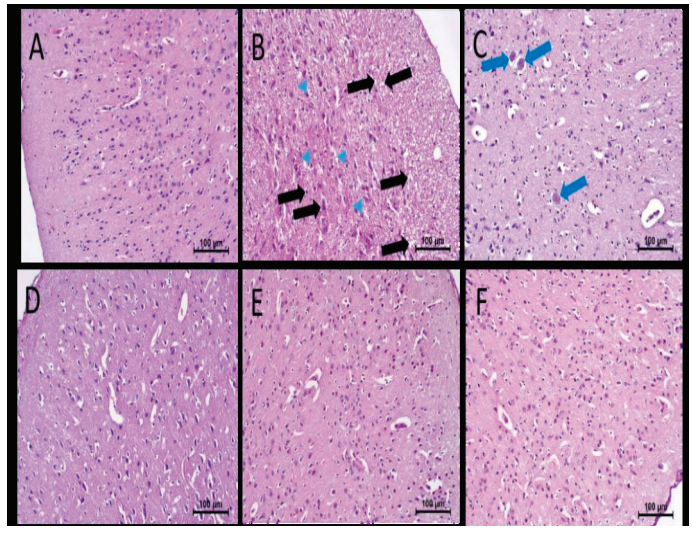

Figure 5. Histopathological Changes in the Cerebral Cortex of CPF-intoxicated Rats Treated with AA. AA exhibits a marked restoration of normal architecture in brain tissues. Histological changes were observed using H&E staining (×20). "A" represented the control group. "B" to "C" represented the CPF group. "D" to "F" represented AA35, AA70, AA140 groups, respectively. Black arrow: Vacuolization, Blue arrow: Chromatolysis, Blue arrowhead: Pyknotic nucleus.

Table 1. Histopathological Damage Scoring in Liver Tissues

Parameter Group	Mononuclear cell infiltration	Sinusoidal dilatation	Vascular congestion	Vacuolation	Necrosis
Control	0.33±0.114	0.44±0.121	0.61±0.118	0.28±0.109	0.56±0.121
CPF	2.78±0.101 [a]	2.89±0.176 [a]	2.44±0.121 [a]	2.33±0.114 [a]	2.72±0.109 [a]
AA35	1.44±0.166 [a,b]	1.78±0.152 [a,b]	1.28±0.135 [a,b]	1.44±0.145 [a,b]	1.56±0.185 [a,b]
AA70	0.72±0.158 [b]	0.67±0.140 [b]	0.72±0.177 [b]	0.56±0.121 [b]	0.83±0.167 [b]
AA140	0.56±0.166 [b]	1.50±0.167 [a,b]	0.89±0.137 [b]	0.50±0.146 [b]	1.00±0.181 [b]

Note: Data are expressed as mean ± SEM (n=6/group). a: $p<0.05$ as compared to control group [b]: $p<0.05$ as compared to CPF group.

Table 2. Histopathological Damage Scoring in Cerebral Cortex

Parameter Group	Vacuolization	Chromatolysis	Gliosis	Necrosis
Control	0.61±0.118	0.39±0.118	0.22±0.101	0.67±0.114
CPF	2.83±0.090 [a]	2.00±0.114 [a]	2.50±0.121 [a]	2.72±0.109 [a]
AA35	1.72±0.158 [a]	1.22±0.152 [a,b]	1.44±0.145 [a,b]	1.61±0.143 [a,b]
AA70	0.94±0.171 [b]	0.67±0.140 [b]	0.78±0.152 [a,b]	1.06±0.171 [a,b]
AA140	1.33±0.162 [a,b]	1.11±0.159 [a,b]	0.94±0.151 [a,b]	1.28±0.158 [a,b]

Note: Data are expressed as mean ± SEM (n=6/group). a: $p<0.05$ as compared to control group b: $p<0.05$ as compared to CPF group.

Effect of Asiatic Acid on Bax Immunoexpression in CPF-Induced Toxicity

CPF exposure significantly increased Bax (+) hepatocytes ($p<0.001$). AA treatment reduced Bax immunoexpression ($p<0.01–0.001$), with the greatest suppression in the AA70 and AA140 groups (Figure 6 and 7). Similarly, CPF increased Bax (+) cells in the cerebral cortex ($p<0.001$), which was reduced in all AA treatment groups ($p<0.01–0.001$) (Figure 6 and 8).

Effect of Asiatic Acid on Body Weight and Temperature in CPF-Induced Toxicity

CPF toxicity decreased body weight and temperature, resulting in a negative percentage change ($p<0.05$). AA at 70 and 140 mg/kg ameliorated body temperature changes ($p<0.05–0.01$), whereas AA reduced body weight changes without reaching statistical significance (Figure 9).

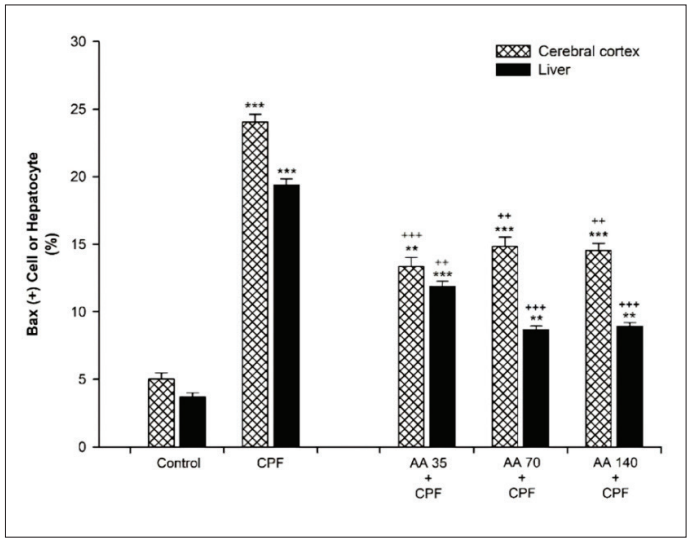

Figure 6. Effect of AA on the percentage of Bax-positive Cells in the Cerebral Cortex and Liver Tissues of CPF-intoxicated Rats Treated with AA. Data are expressed as mean ± SEM (n=6/group). **p<0.01 and ***p<0.001 significantly are different from the control group, while ++p<0.01 and +++p<0.001 are significantly different from CPF group.

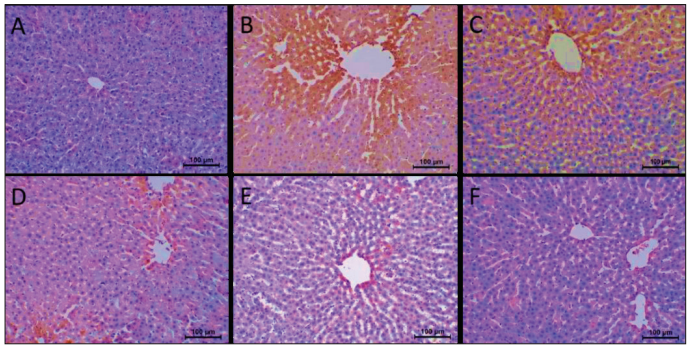

Figure 7. Bax Immunoexpression in Liver Tissues of CPF-intoxicated Rats Treated with AA (DAB, ×20). "A" represented the control group. "B" to "C" represented CPF group. "D" to "F" represented AA35, AA70, AA140 groups, respectively.

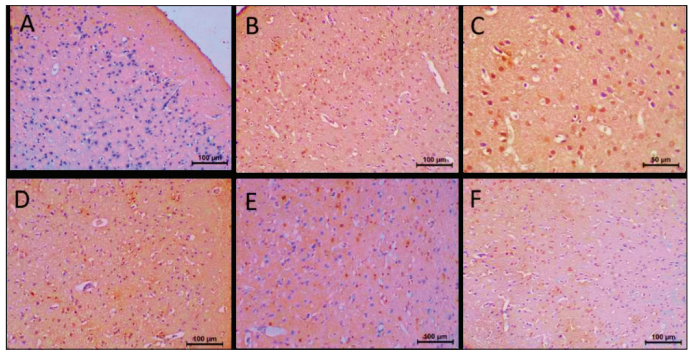

Figure 8. Bax Immunoexpression in Brain Tissues of CPF-intoxicated Rats Treated with AA (DAB Staining, ×20, ×40). "A" represented the control group. "B" represented the CPF group (x20). "C" represented CPF group (×40). "D" to "F" represented AA35, AA70, AA140 groups (×20), respectively.

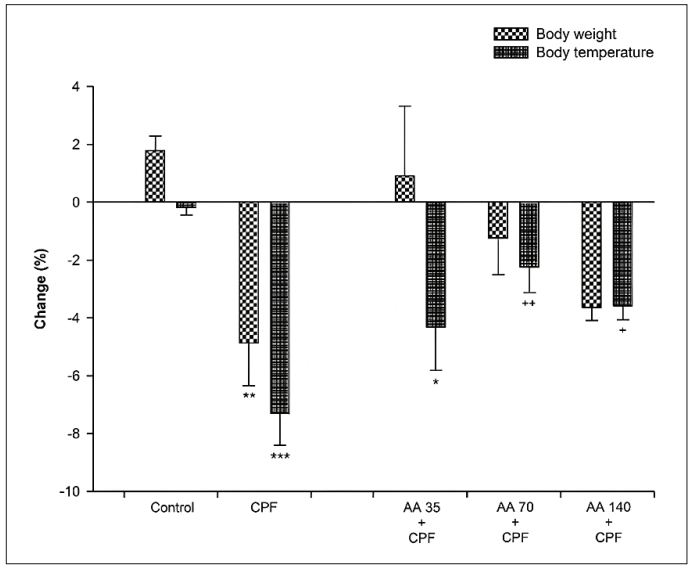

Figure 9. Effect of AA on the Percentage Change in Body Weight and Temperature of CPF-intoxicated Rats. Data are expressed as mean±SEM of six rats. *p<0.05, **p<0.01 and ***p<0.001 significantly different from control group and +p<0.05 and ++p<0.01 significantly different from CPF group.

Discussion

Organophosphorus (OP) insecticide exposure causes diverse toxic effects in multiple organs. Due to the widespread and harmful effects of CPF, one of the most commonly implicated OP insecticides in toxicity cases, there is a need for effective protective strategies against its toxicity. This study provides the first experimental evidence demonstrating the protective effects of AA against CPF-induced toxicity in adult rats, integrating biochemical, histological, and immunohistochemical analyses. The CPF dose used in this study (279 mg/kg; s.c.) represents the maximum tolerated dose in adult rats and has previously been reported to cause comprehensive inhibition of AChE activity (10,12,18). Our results confirm that acutely administered CPF induces increases in oxidative stress-related toxicity markers in the serum, brain, and liver. Furthermore, histopathological analyses also revealed extensive CPF-induced damage in hepatic

and brain tissues. Of particular importance, our study provides novel indications that AA may exert potent neuroprotective and hepatoprotective effects by enhancing antioxidant defense mechanisms and mitigating CPF-induced histological damage.

In the present study, the oxidant and antioxidant enzyme profiles of brain and liver tissues — the primary target organs of OP insecticides — as well as blood samples providing critical insights into the physiological and pathological status of these organs, were evaluated through biochemical analyses. Previous studies have shown that CPF induces excessive ROS production (3,19). The oxidation of cellular macromolecules caused by excess ROS can result in lipid peroxidation (LPO) and protein oxidation, leading to the inactivation of several proteins, including antioxidant enzymes (SOD, CAT, and GPx). Thus, CPF induces ROS accumulation, exacerbating oxidative stress-related damage in the liver, brain, and systemic tissues (19,20). In addition, excessive ROS accumulation is known to trigger mitochondrial apoptotic pathways by upregulating pro-apoptotic proteins such as Bax, ultimately leading to apoptotic cell death.

Findings from this research indicate that the level of MDA, an indicator of LPO, was significantly increased in the serum, liver, and brain tissues of rats in the CPF group, in agreement with the findings of Avci et al. (2018) (10). In addition, AOPP levels were elevated following CPF exposure, consistent with the study by Berkoz et al. (2019), who suggested that CPF increases AOPP formation by enhancing ROS flux into the liver and brain, leading to functional impairment of ROS-targeted proteins (21). CPF exposure has also been reported to disrupt the balance between pro-oxidants and antioxidants, resulting in excessive ROS production that promotes lipid and protein oxidation, ultimately causing damage to cellular macromolecules and potential cell death (4). Notably, CPF intoxication significantly reduced antioxidant enzyme levels (SOD, CAT, and GPx) in the serum, brain, and liver tissues of rats. These findings are supported by Ibrahim and Khwanes (2018), who demonstrated that CPF-induced ROS overproduction leads to a marked reduction in antioxidant enzyme activity, suggesting impaired detoxification capacity in exposed tissues (22). These observations are further corroborated by Avci et al., (2018), who showed that CPF-induced ROS accumulation inhibits CAT, SOD, and GPx activities in serum, liver, and brain tissues, thereby disrupting oxidant/antioxidant balance; inhibition of CAT and GPx may promote hydrogen peroxide accumulation, which in turn contributes to SOD suppression and further oxidative damage (10).

Pentacyclic triterpenoids such as AA exhibit antioxidant and hydroxyl radical scavenging activities due to their ability to donate hydrogen in different toxicity models, as proposed by Ramachandran et al., (2008) (23). Previous studies demonstrated that AA increases antioxidant enzyme activities and reduces oxidative stress in various toxicity models (24-26). Similar to the findings of the aforementioned studies, our results indicate that AA reduces CPF-induced protein oxidation and LPO in a dose-dependent manner by restoring antioxidant enzyme levels in the serum, liver, and brain tissues of rats. Thus, our study demonstrates and strengthens the suggestion that AA supports the antioxidant defense system, thereby reducing oxidative damage and preserving cellular integrity in CPF toxicity.

According to biochemical and histological studies, the liver and brain are the organs most likely to accumulate CPF (27,28). In the present study, microscopic analysis of the liver in the CPF group revealed significant histological modifications, including notable alterations in hepatic architecture and an increased rate of hepatocellular degeneration. These findings support previous research demonstrating that the lipophilic properties of CPF facilitate its passage through cell membranes, leading to liver injury (29). In the brain tissues, the CPF group exhibited significant histopathological alterations, aligning with both our biochemical data and previous findings that acute CPF intoxication damages rat brain tissue (12, 28). Additionally, Latuszynska et al., (2003) reported that CPF induces extensive histopathological changes in the cerebellum and cerebral cortex of rats (30). In addition to the biochemical data obtained in our study, the histopathological changes observed in the CPF group may be associated with oxidative stress, which induces severe hepatocyte and brain damage. This damage is likely exacerbated by a reduction in antioxidant defense mechanisms in both tissues, leading to excessive ROS production, which in turn results in cellular damage and apoptosis (28). Notably, AA treatment dose-dependently reduced histopathological damage scores in liver and brain tissues and promoted the restoration of histological structure. This observation is consistent with previous reports indicating that AA significantly ameliorates histopathological changes in various pathological conditions affecting the liver and brain (6-8).

The protective effect of AA against CPF-induced toxicity was further supported by immunohistochemical analysis of apoptosis-related markers, particularly Bax, a key pro-apoptotic protein. The number of Bax (+) cells significantly increased in the liver tissues of CPF-exposed rats, suggesting that Bax activation plays a key role in hepatocellular apoptosis. This finding corroborates previous studies reporting Bax involvement in CPF-induced hepatic cell death (10,28). Similarly, an increase in Bax (+) cells was observed in the brain tissues of the CPF group, likely due to the transcriptional upregulation of key apoptosis-related genes. In addition to the pro-apoptotic effects of excessive ROS in CPF-exposed tissues, CPF may contribute to the upregulation of apoptotic mediators in the brain (31). Importantly, AA treatment significantly and dose-dependently reduced the number of Bax (+) cells in the brain and liver tissues of CPF-exposed rats. This finding is in agreement with studies suggesting that the downregulation of pro-apoptotic proteins

may be attributed to the anti-apoptotic properties of triterpenoids such as AA, which exerts potent antioxidant effects (6,32). It is known that ROS generated by CPF exposure increases Bax protein expression (32). The observed reduction in Bax (+) cells in AA-treated groups may be attributed to AA's ability to suppress ROS formation by increasing the levels of antioxidant enzymes. These findings collectively suggest that AA may exert protective effects by preserving histological structure and inhibiting apoptosis in CPF-induced toxicity.

Body weight and temperature are critical indicators for assessing the toxic effects of substances on human health. In the present toxicity model, a significant reduction in body weight and temperature was observed in rats following a single dose of 279 mg/kg CPF, which aligns with previous studies reporting similar CPF-induced effects (10,12). Interestingly, no significant change in dietary intake was observed despite the weight loss, suggesting that the weight reduction caused by CPF may be attributed to increased lipid and protein catabolism, as previously documented (29,33). The decrease in body temperature, on the other hand, may result from CPF-induced AChE inhibition, which elevates acetylcholine levels and triggers hypothermia through enhanced heat loss mechanisms within central thermoregulatory pathways (34). Notably, AA administration in CPF-treated rats exhibited an ameliorative effect on body temperature but had a limited impact on body weight. Specifically, while AA at 70 and 140 mg/kg significantly mitigated CPF-induced hypothermia, the 35 mg/kg dose was insufficient to prevent the drop in body temperature. On the other hand, AA administration did not produce a statistically significant improvement in CPF-induced body weight loss, despite a slight trend toward recovery.

These findings indicate that CPF-induced metabolic and thermoregulatory disturbances may be partially alleviated by AA, possibly through its antioxidant and neuroprotective properties. The observed improvement in body temperature following AA administration may be attributed to its ability to reduce oxidative stress, which is known to modulate thermoregulatory pathways. Previous studies have demonstrated that oxidative stress can disrupt redox homeostasis in the brain, leading to impaired temperature regulation (34,35). By mitigating oxidative damage, AA might enhance redox stability and restore thermoregulatory control mechanisms, thereby preventing excessive CPF-induced hypothermia. However, the persistent weight loss suggests that CPF-related metabolic dysregulation might involve mechanisms beyond oxidative stress, such as disrupted energy homeostasis or cholinergic overactivation, which AA alone may not effectively counteract. Further studies are warranted to explore alternative therapeutic interventions targeting CPF-induced metabolic imbalances.

The findings of the present study suggest that the protective effects of AA against chlorpyrifos-induced toxicity are primarily mediated through the reinforcement of endogenous antioxidant defense systems. By restoring the activities of key antioxidant enzymes, AA limits excessive reactive oxygen species generation and suppresses lipid and protein oxidation, thereby preserving tissue integrity in the liver and brain. The attenuation of oxidative stress may also inhibit the Bax-dependent mitochondrial pathway, as reflected by the reduced Bax expression observed in AA-treated groups. Although AA partially ameliorated CPF-induced hypothermia, its limited effect on body weight indicates that some metabolic disturbances may involve mechanisms beyond oxidative stress.

A limitation of the present study is the absence of an AA-alone treatment group. However, previous studies have demonstrated that AA, when administered alone at similar doses, does not significantly alter oxidative stress markers or apoptotic parameters in healthy animals (22,36). Therefore, the present study focused primarily on evaluating the protective effects of AA under CPF-induced toxic conditions.

Conclusions

This study demonstrates that AA protects against acute CPF-induced liver and brain injury. AA dose-dependently attenuated CPF-induced oxidative stress, histopathological alterations, and Bax-mediated apoptosis through reinforcement of endogenous antioxidant defenses. These findings suggest that asiatic acid is a promising neuroprotective and hepatoprotective agent against organophosphate toxicity. Further studies using chronic exposure models are needed to confirm its long-term protective efficacy.

Ethical Considerations: This study was approved by the Ondokuz Mayis University Faculty of Medicine Ethics Committee (2023-08/23.02.2023).

Peer-review: Externally peer-reviewed.

Conflict of Interest: No conflict of interest was declared by the authors.

Funding: This research was funded by Ondokuz Mayis University Scientific Research Projects Unit, grant number PYO.TIP.1901.23.006

Consent of Patients: The participants were informed in detail, and informed consent was obtained.

Data Availability Statement: All relevant data are within the paper and they are available from the corresponding author on reasonable request.

Author Contributions: Concept - ÖDS, HB, AB; Design - ÖDS, HB; Supervision - ÖDS, FG, HB, AB; Data Collection and/Or Processing - NÖ, FG, ÖDS; Providing Resources and Funding - AB, ÖDS, HB, RA, BH; Literature Search - ÖDS, AB; Analysis or Interpretation - NÖ, FG, ÖDS, HB, AB; Writing - ÖDS, HB, AB; Critical Review - NÖ, AB

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