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Original Article

Barbeya Oleoides Leaves Extract Mitigates Acetaminophen-Induced Nephrotoxicity by Reducing Oxidative Stress and Inflammation in a Rat Model

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ABSTRACT Acetaminophen is widely used as an analgesic and antipyretic, but its potential for nephrotoxicity, particularly in overdose situations, remains a significant concern. Identifying agents that can mitigate this nephrotoxicity is crucial for kidney protection. Barbeya oleoides Schweinfurth (BOL), known for its antioxidant and anti-inflammatory properties, may offer protective effects against acetaminophen-induced kidney injury. This study investigates the nephroprotective potential of an ethanolic extract from BOL leaves against acetaminophen-induced nephrotoxicity. Five groups of rats (n = 6 per group) were treated orally for seven days: Group 1 received a placebo solution (vehicle), Group 2 received a single acetaminophen dose (2 g/kg) on day 5, Groups 3 and 4 received BOL at doses of 100 or 200 mg/kg, respectively, along with acetaminophen on day 5, and Group 5 received only BOL (200 mg/kg). Acetaminophen significantly increased the level of the kidney function biomarkers blood urea nitrogen (BUN), triglycerides (TG), uric acid (UA), and creatinine, indicating renal dysfunction. It also elevated pro-inflammatory cytokines, specifically tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), highlighting an inflammatory response. Furthermore, a notable reduction in antioxidant enzyme levels, namely glutathione (GSH), catalase (CAT), and superoxide dismutase (SOD), was observed, signifying oxidative stress and impaired antioxidant defense. Histopathological examination showed disruption of the glomerular basement membrane, confirming renal tissue damage. BOL co-treatment restored kidney function biomarkers and antioxidant levels, reduced cytokine levels, and preserved renal tissue structure suggesting improved oxidative balance. In conclusion, BOL leaves extract demonstrated significant nephroprotective effects against acetaminophen-induced nephrotoxicity by reducing oxidative stress and inflammation, highlighting its potential as an adjunct therapy to prevent acetaminophen-induced kidney injury.

Keywords: Acetaminophen; Nephrotoxicity; Antioxidants; Antiinflammation; Nephroprotective; Barbeya Oleoides Schweinfurth.

1. INTRODUCTION

The kidneys are crucial in sustaining body homeostasis and regulating multiple physiological processes. They are involved in blood pressure management, acid-base balance, erythropoiesis, and elimination of metabolic waste products [1,2]. In addition to their essential role in drug clearance, recent advances in research have revealed that they are involved in drug metabolism for certain medications such as morphine, and acetaminophen [3–5]. Therefore, maintaining normal kidney functions while consuming medications is essential.

Acetaminophen (APAP) was discovered and introduced to the market in the late nineteenth century. Since then, it has been available as an over-the-counter medication and one of the most commonly utilized analgesics. It is broadly used by the elderly, adults, kids and infants. Also, it is considered a first-line treatment for fever, acute and many chronic pain cases and pregnancy [6]. Nevertheless, the risk of overdosing on APAP persists due to its widespread availability and easy access. Alarmingly, APAP overdose is one primary cause of hospital admission for hepatotoxicity and nephrotoxicity [7]. Although APAP has been used for over a century as an analgesic, its mechanism of action is still poorly understood. An early study by Flower and Vane (1972) showed that APAP analgesic activity is mediated by inhibiting central prostaglandin E2 synthesis [8]. Several in-vivo and in-vitro investigations followed and pointed out that APAP interacts with a central cyclooxygenase enzyme that is responsible for prostaglandin E2 synthesis [6], which was then confirmed in 2002 by Simmons' laboratory [9]. This central cyclooxygenase enzyme was named COX 3.

Research conducted over four decades ago demonstrated that APAP primarily metabolizes through phase II reactions specifically via sulfation and glucuronidation. However, a minor oxidation metabolic pathway of APAP is also involved. Microsomal cytochrome P450 (CYP) enzymes oxidize APAP to form the reactive metabolite N-acetyl-p-benzoquinone imine (NAPQI) [10–13]. Upon interaction with hepatic or extrahepatic tissues, NAPQI causes cellular damage and eventually cell death [14–16]. Numerous investigations have demonstrated that NAPQI substantially contributes to the APAP toxic effects on body organs such as the kidney [10]. Results of these studies showed that NAPQI metabolic pathways such as prostaglandin endoperoxide, N-deacetylase [17–21], renal CYP-450 microsomal enzymes [22–24] and glutathione S-conjugate [17] contribute to APAP's renal toxicity. These pathways are activated by consuming large doses or chronic use of APAP and concurrent alcoholism, causing nephrotoxicity consequent to hepatotoxicity [10]. Nonetheless, a study by Trumper et al., 1992 showed that a highly toxic dose of APAP causes renal toxicity with or without concurrent hepatoxicity [25]. Regardless of the activated metabolic pathway, renal toxicity is one major side effect of APAP. Thus, since APAP is easily accessible and widely used by individuals, it is crucial to develop medications that could serve as therapeutics or prophylactics against APAP-induced renal toxicity.

Given the potential for nephrotoxicity associated with APAP, developing protective agents is essential. Various antioxidant-rich plants, such as Nigella Sative [47], Carica papaya [54] and Cinnamomum zeylanicum L. [55] Curcuma longa [70], and Camellia sinensis [71], have demonstrated nephroprotective effects by reducing oxidative stress and inflammation. Although *Barbeya Oleoides Schweinfurth* (BOL) shares common antioxidant properties with these plants, it remains underexplored for its potential against APAP-induced kidney damage.

BOL is a medicinal plant that is native to North Africa, Ethiopia, Somalia, and the Arabian Peninsula (Saudi Arabia and Yemen) [26]. Leaves, stems and roots of BOL have been empirically used to treat fever, infectious diseases, skin disorders, and inflammatory-related disorders [27,28]. This could be due to BOL wide range of bioactive compounds with several pharmacological properties (Figure 1). For instance, BOL's leaves contain flavonoids and phenols, which are known for their antioxidant effects [29]. Moreover, it has been demonstrated that BOL extracts can mitigate inflammation by decreasing the inflammatory mediators' levels [28]. In his characterization study of BOL, Al-Oqail showed that different parts of BOL, including leaves, exhibit antimicrobial and antispasmodic activities [29]. Recently, Khoja et al., 2021 reported that extracts from BOL leaves may offer therapeutic benefits for type 2 diabetes mellitus by inhibiting the enzymes α-glucosidase and α-amylase. This inhibitory effect was primarily attributed to the presence of polyphenolic compounds within the extract, known for their significant bioactive properties [72]. These properties have sparked interest in investigating the nephroprotective effects of BOL's leaves. This interest stems from the potential of BOL leaves' bioactive compounds to counter the associated alterations in kidney injury such as oxidative stress and inflammation. The availability of diverse pharmacotherapy options to prevent harmful effects on the kidney is highly beneficial for individuals. Nevertheless, to our knowledge, no previous literature has studied the nephroprotective effects of BOL leaves extract against kidney injury caused by APAP. Therefore, this study aims to evaluate the potential nephroprotective effects of extract from BOL's leaves by exploring its anti-inflammatory, antioxidant, and other possible protective mechanisms against APAP-induced kidney injury in rats.

List of abberviations

Full name	Abbreviation
Acetaminophen	APAP
Barbeya oleoides	BOL
Blood Urea Nitrogen	BUN
Catalase	CAT
Glutathione	GSH
Interleukin-1 beta	IL-1β
Lipid Peroxidation	LPO
Malondialdehyde	MDA
Superoxide Dismutase	SOD
Triglycerides	TG
Tumor Necrosis Factor-alpha	TNF-α
Uric Acid	UA
Acetaminophen	APAP
cytochrome P450	CYP-450
N-acetyl-p-benzoquinone imine	NAPQI

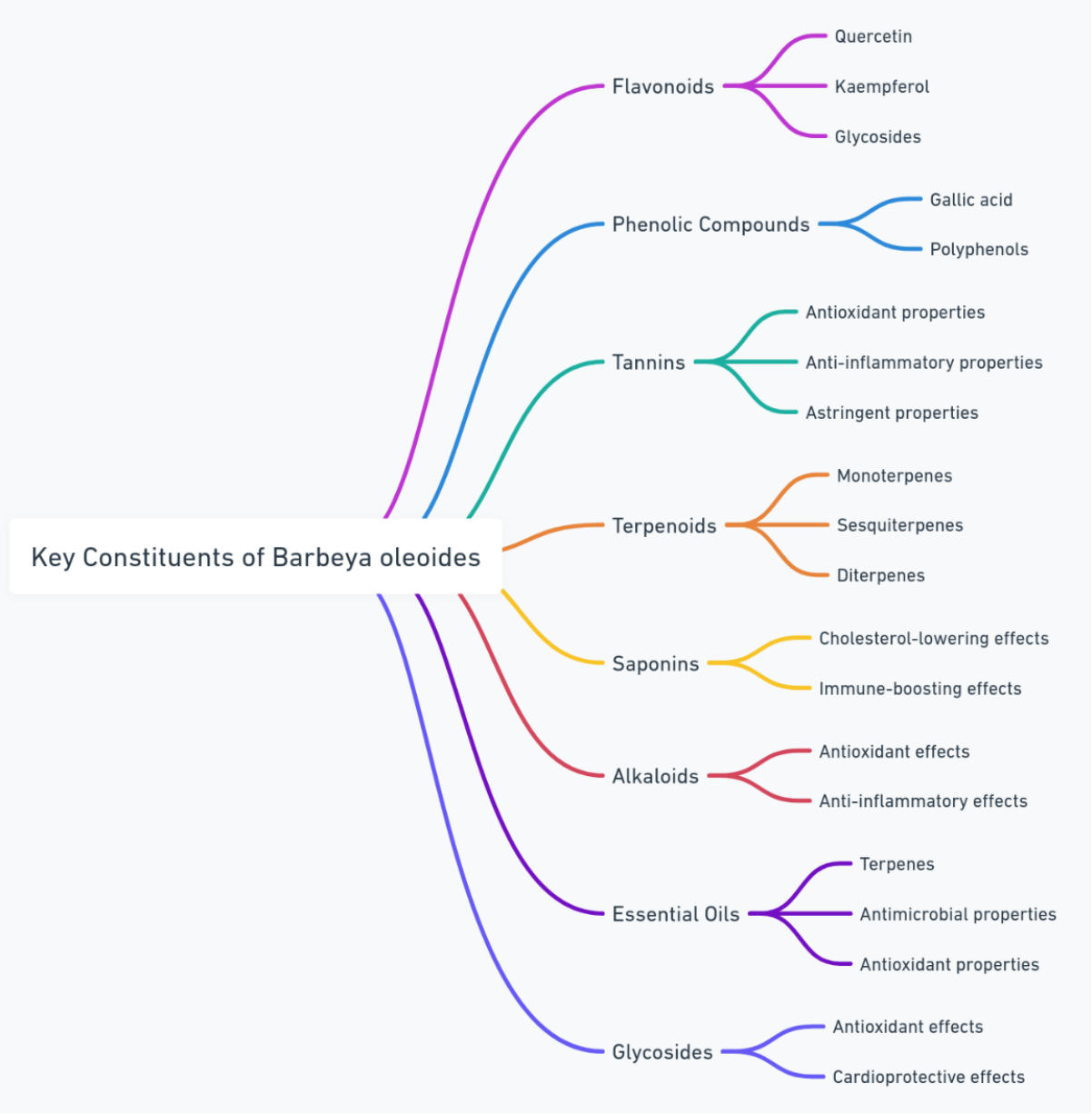

Figure 1. Key constituents of Barbeya oleoides and their associated biological activities. This figure illustrates various phytochemicals found in Barbeya oleoides, including flavonoids [30] (e.g., quercetin, kaempferol, glycosides), phenolic compounds [31] (e.g., gallic acid, polyphenols), tannins [31], terpenoids [32] (e.g., monoterpenes, sesquiterpenes, diterpenes), saponins [33], alkaloids [34], essential oils [32], and glycosides [30]. The content of this figure has been adapted from several previous publications, with specific adaptations to integrate and highlight the therapeutic relevance of each constituent.

2. MATERIALS AND METHODS

2.1. Drugs and Chemicals

D6908 M-13 DNA Isolation Maxi Kit Extraction kits were bought from Solarbio, Tongzhou Dist, Beijing in China. Ethidium bromide, 6X DNA loading dye, TBE buffer and agarose were used for DNA sample preparation, gel formation and electrophoresis applications. Sodium phosphate dibasic and potassium phosphate monobasic solutions were used to prepare phosphate buffer for sample preparation. Dintrobenzoic acid, sulfosalicylic acid and Phosphate buffer were used for Spectrophotometer applications. All the ingredients were sourced from Sigma Aldrich USA.

2.2. Plant Collection and Preparation

Plant Collection: *Barberiya oleiadi* leaves were collected from the Fifa mountains, located in the Jazan territory, southwest of Saudi Arabia. Extraction Process: Collected plants rinsed with water and then placed in a sheltered area to dry. The resulting powder (500g) was extracted using percolation with 95% ethanol for 72 hours. The ethanol was eliminated using a rotary evaporator to extract the pure crude stock. These plant extracts were further used in this experiment.

2.3. Animal Model and Study Design

Male Wistar albino rats weighing 150-180 g were used for this study. The rodents were maintained in an ideal laboratory condition, with unrestricted water access and a daily pellet meal. This project was ethically approved by the ethical committee of Jazan University with reference number REC-45/07/943. The study design and animal numbers were planned using the principles of the 3Rs (Replacement, Reduction, and Refinement) and the Experimental Design Assistant (EDA) tool. The EDA ensured that the minimum number of animals required for robust and reproducible results was used. Five groups, each comprising six rats, were randomly assigned 30 male Wistar albino rats. Group 1 received vehicle orally (p.o.) only for 7 days, serving as the control group; group 2 received a single dose of APAP 2 g/kg p.o. on the fifth day. The toxic APAP dose was previously established in our laboratory [49]. Groups 3 and 4 were given BOL 100 or 200 mg/kg p.o.daily for seven days. A single oral dose of APAP 2 g/kg was given only once on the fifth day one hour after BOL doses in both groups. In group 5 200 mg/kg of BOL was administered p.o. once daily for seven days. On the eighth day, rats were sedated and blood was drawn for biochemical analysis. After that, animals were sacrificed to isolate the kidney for further biochemical and histopathological assay (Figure 2).

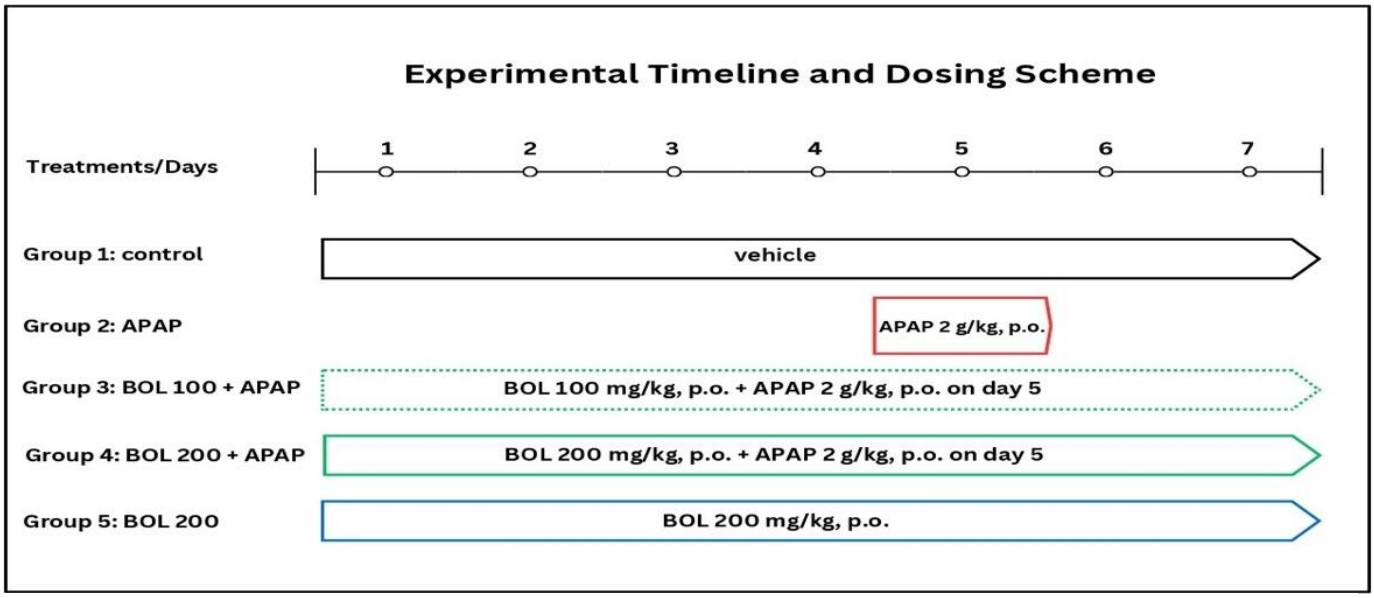

Figure 2. Experimental Timeline and Dosing Scheme: The figure illustrates the experimental timeline and dosing scheme for five groups of rats over seven days. Each group's treatment regimen is represented by color-coded arrows indicating the treatments administered.

2.4. Sample Preparation

The collected blood samples were allowed to coagulate at room temperature without being disturbed for 30 minutes. After that, the sample was centrifuged at 3000 rpm for 10 minutes at 4°C. Finally, the upper layer called Serum was collected for kidney function tests.

Kidney sample preparation was performed as established protocol from our laboratory by Alam et al., 2022 [35]. Briefly, 10% of the kidney homogenate was prepared by homogenizing it in a phosphate-buffered solution (0.1 M and pH 7.4) for oxidative stress markers (GSH, CAT, SOD, and MDA) test. Subsequently, the homogenized material was centrifugated at 800× g for 15 minutes at 4°C to isolate the supernatant. This supernatant is further used for MDA, CAT, and SOD test. The remaining homogenate was subjected to centrifugation at 10,500× g for 15 minutes at 4 °C to get the PMS for the GSH test.

2.5. Blood Serum Test

To evaluate kidney function, Crescent diagnostic testing kits were used to measure uric acid, creatinine, blood urea nitrogen (BUN), and triglycerides. Each marker was analyzed following the manufacturer's standard protocol. Briefly, blood samples were collected and centrifuged at 3,000 × g for 10 minutes to separate the serum. The serum was then processed using the respective kits for each marker, following the detailed step-by-step instructions provided by the manufacturer. The absorbance of each sample was measured at specific wavelengths recommended by the Crescent diagnostic kit using a spectrophotometer, ensuring consistency and accuracy in the readings. All tests were conducted in duplicate to enhance the reliability of the results.

2.6. Protein assay: Lowry et al. (1951) estimated the protein content of each sample [36].

2.7. Antioxidant Assays:

2.7.1. Malondialdehyde (MDA)

The method described by Islam et al. (2002) [37] was employed to measure lipid peroxidation. Specifically, 0.5 mL of supernatant-1 was placed in a metabolic shaker and incubated at 37 °C for 1 hour, while an identical 0.5 mL sample of supernatant-1 was maintained at 0 °C for the same duration as a control. Following the 1-hour incubation period, 0.5 mL of 5% trichloroacetic acid (TCA) and 0.67% thiobarbituric acid (TBA) were added to each sample. The mixture was then centrifuged at 4,000 × g for 10 minutes. The resulting supernatant was collected and heated in a water bath for 10 minutes, during which a pink color developed, indicating the presence of malondialdehyde (MDA). The absorbance of the sample was measured at 535 nm, and the MDA content was expressed as the amount of MDA formed per hour per gram of protein.

2.7.2. Glutathione (GSH)

To test for glutathione (GSH), the sulfhydryl reagent DTNB) is used to react with GSH to form a yellow color TNB. The method outlined by Jollow et al., 1974 [38] were used to analyse the GSH. There was a mix of 0.5 mL PMS and 0.5 mL of 4% SSA and incubated at 4 °C for 1hr. After that, it was spun at 3,000 rpm for 15 minutes at 4 °C. At the end, 0.5 mL of the supernatant, 2 mL of PB, and 0.5 mL of DTNB were mixed to make 3 mL, and further samples were analysed at 412 nm.

2.7.3. Catalase (CAT)

Claiborne methods were employed to ascertain the catalase (CAT) [39]. The variation in absorbance was quantified at 240 nm. The catalase units were expressed in nanomole H2O2 consumed per minute per milligram of protein.

2.7.4. Superoxide Dismutase (SOD)

Marklund methods were employed to determine the activity of superoxide dismutase (SOD) at a 580 nm wavelength [40]. The SOD unit was expressed in units per mg of protein, with 1 unit representing a 50% reduction in pyrogallol autoxidation.

2.8. Inflammatory Cytokine (Elisa Assay for IL-1β)

The cytokine IL-1β was quantified using the protocol of the cytokine assay kit from MyBioSource, Inc. The measurement was performed using an ELISA microplate reader (Bio-Tech ELX800) at a 450 nm wavelength.

2.9. Inflammatory Cytokine (ELISA Assay for TNF-α)

The Rat TNFα -ELISAKit (ab236712) measured serum TNF-α protein following the manufacturer instructions. After adding samples or standards to 96-wells, an antibody mixture was added. The bores were rinsed after incubation to remove unwanted protein. A second confirmatory solution catalyzed the reaction and turned it blue. The blue tint turned yellow when a stop solution stopped the process. The amount of bound analyte determines the signal intensity at 450 nm.

2.10. Renal Histopathology

The isolated tissue samples of the kidney were washed with an ice-cold normal saline solution containing 0.9% and then fixed in 10% formalin. Further dehydration was performed, and kidney tissue was implanted in liquid paraffin before being molded into slabs. The blocks were then split into 3 to 5 µm thick segments using a microtome. After staining with H&E, the samples were viewed under a 40x microscope. The injury score ranged from 0 to 4, with 1 indicating a slight injury, 2 indicating a mild injury, and 3 and 4 indicating a severe injury. The injury score considers observations like tubular necrosis, vacuolization, and glomerulus membrane degradation [41].

2.11. Statistical Evaluation

The results were examined by GraphPad Prism 9.0. One-way ANOVA was employed to compare the means across multiple groups to evaluate the overall treatment effects, as it is suitable for analyzing data from experiments involving more than two groups. Post-hoc analysis was performed using the Tukey-Kramer test, which ensures accurate pairwise comparisons between groups, particularly when group sizes are equal. The significance level was set at $p < 0.05$ to ensure robust and reliable results. All data are presented as Mean ± SEM, with six replicates per group, ensuring precise and consistent reporting of variability within the dataset.

3. RESULTS

3.1. Effect of BOL on Kidney Function Markers

APAP significantly elevated ($p < 0.0001$) the tested kidney function biomarkers levels (BUN, uric acid, TG and creatinine) as opposed to the control (Figure 3). Both doses of BOL (100 and 200 mg/kg) significantly reduced ($p < 0.0001$) the APAP-induced increase in the levels of the tested parameters. Similar to the control group, BOL alone (200 mg/kg) displayed no changes in the tested kidney function marker levels.

3.2. Effect of BOL on Malondialdehyde (MDA)

APAP considerably increased ($p < 0.0001$) the MDA level as opposed to the control (Figure 4). BOL (100 mg/kg) decreased the MDA level in contrast to APAP ($p < 0.001$). Likewise, the higher dose of BOL (200 mg/kg) produced an even greater decrease in the MDA level ($p < 0.0001$). Like the control group, BOL did not affect the MDA level.

3.3. Effect of BOL on Antioxidants (GSH, CAT, and SOD)

As shown in Table 1, APAP noticeably reduced ($p < 0.0001$) the tested antioxidant enzyme levels opposite to the control. BOL (100 mg/kg) partially dampened the APAP-induced reduction in GSH ($p < 0.05$) and SOD ($p < 0.05$) levels and showed a more significant reduction in CAT levels ($p < 0.001$) in comparison to the APAP group. More profoundly, the higher dose of BOL (200 mg/kg) almost diminished the APAP-induced reduction in GSH, CAT ($p < 0.0001$), and SOD ($p < 0.001$) levels as opposed to the APAP group. BOL alone did not cause any changes in the level of the tested antioxidants ($p > 0.05$).

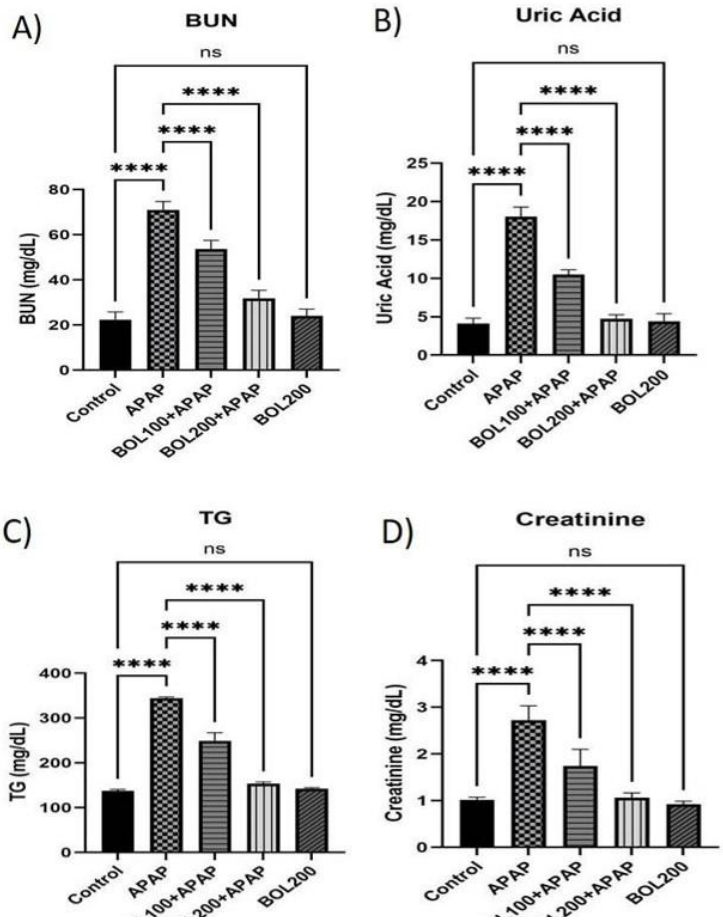

Figure 3. Effect of BOL on kidney function markers; A) Blood Nitrogen Urea (BUN), B) Uric Acid, C) triglycerides (TG) and D) Creatinine. Values are presented as Mean ± SEM (n = 6). **** $p < 0.0001$ (APAP vs. Control, BOL100 +APAP vs. APAP, and BOL200 + APAP vs. APAP) and ^{ns} $p > 0.05$ (BOL200 vs. Control) as assessed by one-way ANOVA followed by Tukey-Kramer post hoc test.

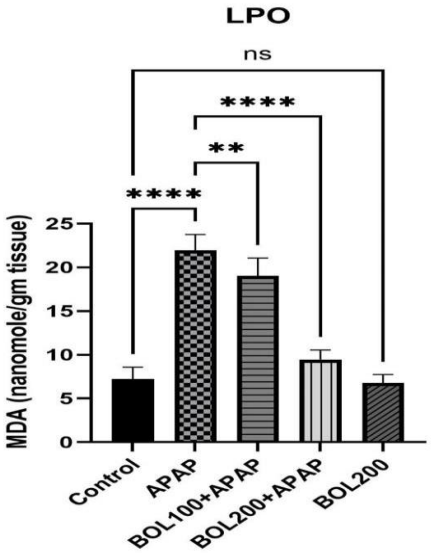

Figure 4. Effect of BOL on lipid peroxidation (LPO). Values are presented as Mean ± SEM (n = 6). **** p < 0.0001 (APAP vs. Control and BOL200 + APAP vs. APAP), ** p < 0.001 (BOL100 + APAP vs. APAP) and ns p > 0.05 (BOL200 vs. Control) as assessed by one-way ANOVA followed by Tukey-Kramer post hoc test.

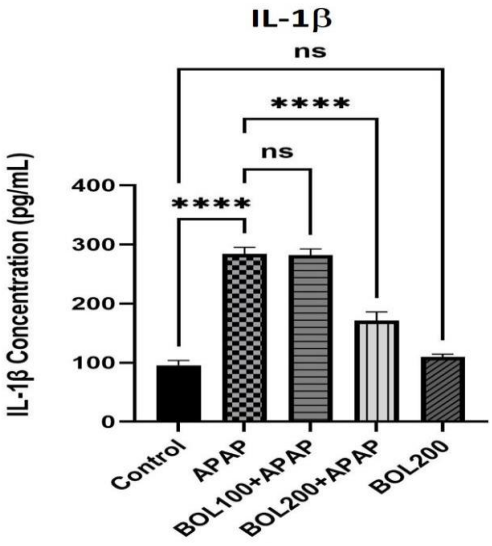

Figure 5. Effect of BOL The of IL-1β Concentration. Values are presented as Mean ± SEM (*n* = 6). **** *p* < 0.0001 (APAP vs. Control and BOL200 + APAP vs. APAP), and ns *p* > 0.05 (BOL100 + APAP vs. APAP and BOL200 vs. Control) as assessed by one-way ANOVA followed by Tukey-Kramer post hoc test.

3.4. Effect of BOL on the IL-1β Concentration

IL-1β concentration was increased by APAP treatment (p < 0.0001) (Figure 5). BOL (100 mg/kg) did not inhibit APAP-induced IL-1β increased concentration (p > 0.05). However, BOL (200 mg/kg) substantially reduced IL-1 β concentration in the kidney following APAP treatment (p < 0.0001) compared to the APAP group. Also, no significant alteration was noticed with BOL alone comparable to the control (p > 0.05).

3.5. Effect of BOL on Tumor Necrosis Factor (TNF-α) Concentration

The levels of TNF-α were elevated by APAP relative to the control group (p < 0.0001). BOL (100 mg/kg) yielded a partial but significant decrease in TNF-α levels (p < 0.001) in contrast to the APAP group (Figure 6). However, BOL (200 mg/kg) produced a remarkable decline in TNF-α levels as opposed to the APAP group (p < 0.0001). No significant difference was observed with BOL alone comparable to the control (p > 0.05).

Table 1. Effect of BOL on The Levels of Antioxidant Enzymes.

Test/Group	Control	APAP	BOL100+APAP	BOL200+APAP	BOL200
GSH (DTNB Conjugate Formed/mg Protein)	16.60±1.35	8.05±0.96***	9.72±1.05*	15.89±2.23***	17.19±1.97ns
CAT nmol of H_2O_2 consumed/min/ mg / protein	13.84±2.20	6.61±0.78***	9.76±0.59**	12.19±1.46***	14.14±1.79ns
SOD (nmol epinephrine protected from oxidation/min/ mg protein)	39.256±2.64	20.18±1.75***	19.075±2.51*	38.40±2.33**	40.72±2.88ns

Table 1. Values are presented as Mean ± SEM (*n* = 6) *** *p* < 0.0001 (APAP vs. Control) (BOL200 + APAP vs. APAP), ** *p* < 0.001 (BOL100 + APAP vs. APAP and BOL200 + APAP vs. APAP), * *p* <0.05 (BOL100 + APAP vs. APAP), ^{ns}p > 0.05 (BOL200 vs. Control) as assessed by one-way ANOVA followed by Tukey-Kramer post hoc test.

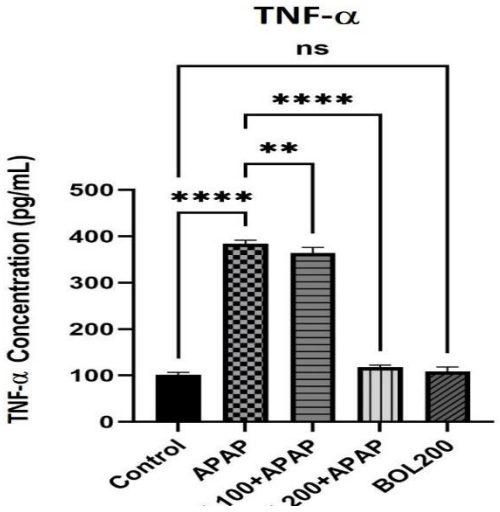

Figure 6. Effect of BOL on tumor necrosis factor (TNF-α) concentration. Values are presented as Mean ± SEM (n = 6). **** $p < 0.0001$ (APAP vs Control and BOL200+ APAP vs APAP), ** $p < 0.001$ (BOL100 + APAP vs. APAP) and ns $p > 0.05$ (BOL200 vs. Control) as assessed by one-way ANOVA followed by Tukey-Kramer post hoc test.

3.6. Effect of BOL on Renal Histology

The control and the BOL-treated groups illustrated a regular kidney architecture with healthy morphology and proper glomerular structure without any pathological lesions; the injury score was 0 (Figure 7 A & 7 E). On the contrary, the APAP group demonstrated a degenerative glomerular basement membrane as indicated by abnormal morphology with damaged glomerular intact and tubular cells; an injury score of 3 was given to the APAP group (Figure 7 B). In contrast, treatment with BOL (100 mg/kg) partially mitigated the degenerative changes in the kidney caused by APAP treatment and improved morphology and glomerular structure of the kidney, and the injury score was 2 (Figure 7 C). BOL (200 mg/kg) remarkably restored the glomerular intact and normal morphology; the injury score was 1 (Figure 7 D). The quantification of injury scores is shown in Figure 7 F.

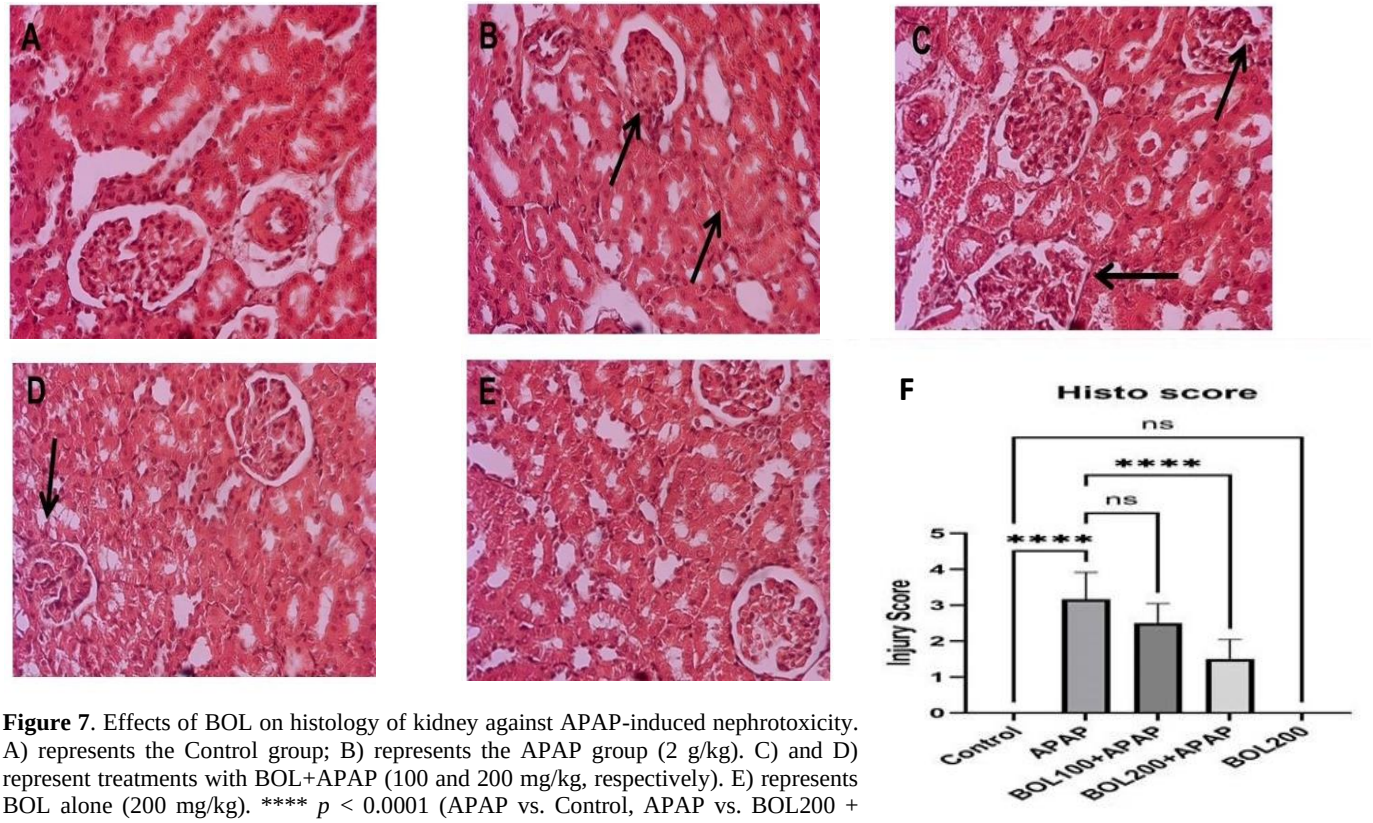

Figure 7. Effects of BOL on histology of kidney against APAP-induced nephrotoxicity. A) represents the Control group; B) represents the APAP group (2 g/kg). C) and D) represent treatments with BOL+APAP (100 and 200 mg/kg, respectively). E) represents BOL alone (200 mg/kg). **** $p < 0.0001$ (APAP vs. Control, APAP vs. BOL200 + APAP), ns $p > 0.05$ (APAP vs. BOL100 + APAP, Control vs. BOL200) as assessed by one-way ANOVA followed by Tukey-Kramer post hoc test. The arrow indicates glomerular membrane degeneration, vacuolization and damaged tubular cells.

DISCUSSION

Despite its broad use as an analgesic and antipyretic, overdosing on APAP or even chronic use presents a substantial risk of nephrotoxicity [16]. One plausible way of causing nephrotoxicity is the generation of toxic metabolite NAPQI [10]. Upon kidney injury, certain indicators are influenced such as kidney function biomarkers, antioxidant enzymes, inflammatory mediators and protein expression, as a response to the damage caused to the renal tissue [42–44]. BOL, originated in North Africa and the Arab Peninsula, is a medicinal plant used for several purposes such as fever, infections and inflammatory-related disorders mainly due to its bioactive constituents [29]. The properties of these bioactive compounds could aid in alleviating APAP's nephrotoxicity by decreasing inflammation, oxidative stress and rebalancing kidney function biomarkers [27–29]. The current study focused on exploring BOL leaves' protection against APAP-developed kidney damage in rats by studying their impact on inflammation, oxidative stress, and DNA fragmentation.

BUN, uric acid, TG and creatinine are critical indicators of kidney health status. Kidney injury resulting from a medication or disease alters the level of these indicators where elevated levels denote impaired kidney function, reflecting the severity of the renal damage [45,46]. In our study, APAP increased the level of the tested indicators suggesting comprised kidney function (Figure 3). This is consistent with previous studies that showed similar [47–49] or lower [50–52] toxic doses of APAP cause kidney damage. On the contrary, both doses of BOL attenuated the APAP-induced elevation in the level of the tested biomarkers. The antioxidant properties of the flavonoids and phenols found in BOL's leaves may be responsible for this beneficial effect. [29]. Consistently, multiple studies have investigated various medicinal plants with similar bioactive compounds that demonstrated their antioxidant effects in reducing elevated kidney function biomarkers induced by APAP. Such plants include curcumin [53], Nigella Sative [47], Carica papaya [54], and Cinnamomum zeylanicum L. [55]. It has been established that APAP oxidative stress dampens kidney performance leading to elevated serum levels of particular biomarkers, mainly as a result of impaired renal glomerular filtration [7]. Thus, counteracting oxidative stress can aid in restoring proper kidney function.

Antioxidant enzymes such as GSH, SOD and CAT are crucial in protecting renal integrity, hence, maintaining proper kidney functions. These antioxidants act like scavengers for reactive oxygen species (ROS) such as superoxide ($O2^{\bullet-}$) or free radicals such as hydrogen peroxide (H2O2). Those could emerge from a variety of sources encompassing endogenous and exogenous factors. Diseases and medications are considered exogenous sources of ROS. Elevated levels of ROS and free radicals cause oxidative stress, which damages cells and contributes to numerous diseases. Antioxidant enzymes collectively work to eventually convert ROS and free radicals into water and oxygen, thereby minimizing oxidative stress and preventing cellular damage [7,56]. As mentioned earlier, upon APAP metabolism via CYP enzymes, NAPQI, a highly reactive metabolite, is generated heavily, particularly when overdosed. This reduces the antioxidant enzymes and saturation of the glutathione system, which then produces ROS and free radicals [7]. Thus, we tested the ability of BOL leaves to restore antioxidant enzyme levels. Consistent with previous studies, APAP reduced GSH, CAT and SOD (Table 1) [25,48,50,51,53,57]. Our study revealed that BOL-treated groups restored GSH, CAT and SOD levels although the higher dose showed a greater magnitude of restoring the antioxidant enzyme levels. Additionally, lipid peroxidation is one hallmark of cellular oxidative stress. Once this stress is initiated, the contents of membrane lipids begin to degrade, and MDA leaks out of the cell membrane as a sign of lipid peroxidation [7]. Hence, we investigated the effect of BOL's leaves on MDA levels. Figure 4 illustrates that APAP raised MDA levels similar to previous studies [48,50,53]. Conversely, BOL dampened APAP-induced MDA levels with the higher dose showing a greater magnitude effect. The antioxidant property of BOL might be the reason for producing such an effect [29]. Natural plants with similar bioactive compounds have shown similar ROS neutralization effects in the kidney, such as resveratrol [58], quercetin [50,59], and thymoquinone [41]. Our findings are consistent with previous studies in which natural antioxidants dampened APAP–induced renal toxicity.

Innate immunity responds to various types of injuries, including renal injury, by initiating an inflammatory response. When oxidative stress and cellular injury occur, inflammatory cytokines, such as TNF-α and IL-1β, are produced by immune cells [7,42]. Within the renal tissue, TNF-α is produced by the renal tubular and IL-1β is produced by the renal epithelial. Both cytokines are key in physiological processes that initiate and extend inflammation and activate proinflammatory pathways. These processes include activation of NF-κB and MAPK signaling pathways, apoptotic induction, and leukocyte infiltration. Several studies have established that an overdose of APAP increased either serum levels or gene expression of both cytokines [7]. In alignment with previous research, our study showed that APAP elevated serum concentration of IL-1β and TNF-α (Figures 5 and 6). Notably, BOL reduced APAP-induced high levels of both mediators, although the low dose did not affect IL-1β levels. The current result suggests that one mechanism for BOL's leaves extract to reduce kidney injury is by modulating the TNF-α and IL-1β inflammatory mediators. Previous studies investigating natural antioxidants have reported similar findings in which they decreased APAP-induced high levels of inflammatory mediators [49,60].

A subsequent consequence of oxidative stress is DNA damage that can be assessed by detecting DNA fragmentation [61]. Our study shows that APAP treatment did not cause DNA smearing and displayed a similar DNA laddering pattern to the respective treatments, denoting no DNA damage occurred (Supplement Figure 1). One possible explanation for this is the APAP treatment period. The main objective of the current study was to establish the nephroprotective effects of BOL against APAP; thus, an acute APAP treatment protocol was adopted in which a single high-toxic dose of APAP (2 g/kg) was used. Previous studies that used a similar APAP dosing regimen protocol in rats did not report DNA damage [48,49].

In contrast to lipid peroxidation and other oxidative stress markers, DNA damage induced by APAP might necessitate a longer exposure duration [7]. Y. Wang et al., 2015 displayed that a 70-day treatment with 400 mg/kg APAP caused DNA damage in mice liver [62]. Moreover, studies that used lower toxic doses of APAP (700-750 mg/kg) for 14 days showed DNA damage in rats' kidneys [63,64]. Another justification is that APAP-induced kidney damage occurs more slowly compared to liver damage. In-vivo and in-vitro analyses reported that tissue necrosis, as a sign of DNA damage, is faster and more evident in the liver than in the kidney [65–6]). Instead, a study by Das et al., 2010 reported that a single dose of 2 g/kg APAP can cause DNA damage in mice [68]. The genetic differences between rats and mice could explain this. Previous studies have shown that rats are more resistant to APAP toxicity in the kidney and liver than mice [66,69]. Furthermore, medications that directly target DNA, such as cisplatin, exhibit faster and more noticeable renal DNA damage [44]. Regardless of the controversies, there is a general agreement that renal DNA damage caused by APAP is affected by factors such as the dose, route of administration, and treatment period of APAP and the genetic differences between rodents [7].

The histopathological alterations induced by APAP and the protective benefits of BOL's leaves are shown in Figure 7. All the impacts caused by APAP, such as disturbance of the glomerular basement membrane, vacuolization, and tubular necrosis, were dampened by BOL's leaves, particularly with the higher dose showing more significant enhancement of renal tissue integrity. This result supports the current biochemical investigations. In accordance, several previous studies have presented similar findings with extracts from medicinal plants that possess similar properties to those of BOL [48–50,53,63].

The nephroprotective effects of BOL against APAP are believed to involve multiple interrelated mechanisms: Antioxidant Defense: BOL enhances the kidney's antioxidant defenses by upregulating key enzymes such as GSH, CAT, and SOD. These enzymes work synergistically to neutralize ROS generated by APAP metabolism, thereby reducing oxidative stress and preventing cellular damage in renal tissues. Anti-inflammatory Action: BOL downregulates pro-inflammatory cytokines, particularly TNF-α and IL-1β. By inhibiting the inflammatory pathways, BOL reduces inflammation and subsequent damage to renal tissues, preserving their structural and functional integrity. Mitochondrial Protection: BOL may protect mitochondrial function by preventing mitochondrial dysfunction induced by APAP. This protection involves maintaining mitochondrial membrane potential, reducing the release of cytochrome c, and inhibiting the activation of the intrinsic apoptotic pathway, thus preventing renal cell death. Enhancement of Detoxification Pathways: BOL may promote the detoxification of APAP by enhancing the conjugation of toxic metabolites with GSH, thus facilitating their safe excretion. This reduces the accumulation of harmful metabolites like NAPQI, minimizing their nephrotoxic effects. Tissue Regeneration: BOL may also support the regeneration and repair of damaged renal tissues by promoting cellular proliferation and inhibiting apoptosis in kidney cells, thereby aiding the recovery of kidney function.

Even though our present study depicts promising protective effects of extract from BOL's leaves against APAP-induced kidney injury, some limitations should be addressed to guide future studies for a better understanding of BOL's nephroprotective mechanism. For example, although the animal model is informative, it may not fully represent human physiological responses. Further studies involving human cell lines or clinical trials would be necessary to confirm the efficacy and safety of BOL in human subjects. Another limitation is that an additional characterization of the ethanolic extract could identify the bioactive components responsible for BOL's effects in the current study. This may include testing different extraction methods or refining purification methods to isolate individual compounds. Moreover, examining BOL's leaves' extract against the long-term treatment of APAP and testing apoptosis induction by measuring the expression of markers such as caspase-3, caspase-9, and NF-κB could provide the extent of the protective mechanism. Further research should also explore varying doses of BOL in both acute and chronic exposure studies to assess potential side effects.

CONCLUSIONS

In conclusion, the ethanolic extract from BOL's leaves shows potential nephroprotective effects against APAP-induced nephrotoxicity. The findings suggest that this protective effect is attained by modulating oxidative stress by restoring normal levels of kidney function biomarkers and antioxidant system status and mitigating inflammation and histopathological kidney injury. BOL leaves extract appears to be a natural antioxidant that could be used as an adjunct agent to reduce the negative impact of APAP on the kidney while preserving APAP analgesic effectiveness.

INFORMED CONSENT STATEMENT
Not applicable

DATA AVAILABILITY STATEMENT
The authors declare that data is available inside the article.

SUPPLEMENTARY MATERIALS
Figure S1: Effect of BOL on DNA fragmentation.

AUTHOR CONTRIBUTIONS:
Conceptualization, writing—original draft preparation, project administration, funding acquisition A.M.J.; methodology, software, writing—review and editing, formal analysis, data curation, M.F.A.; methodology, data curation, S.S.; writing—review and editing, validation, F.K.; writing—review and editing, resources A.Z.; visualization, conceptualization A.H.

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CONFLICTS OF INTEREST
The authors declare no conflict of interest.

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