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Research Article

Ricinodendron heudelotii Extract Strongly Protects Mice against Acetaminophen-Induced Liver Injury

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Abstract

Ricinodendron heudelotii (Baill.) Pierre ex Pax. (Euphorbiaceae) is used in Cameroonian traditional medicine to cure liver diseases, malaria, and stomach pain. The present study investigates the effect of *R. heudelotii* stem bark aqueous extract on acetaminophen-induced acute liver injury in BALB/c mice.

R. heudelotii stem bark aqueous extract (100 and 200 mg/kg, p.o.), distilled water and ascorbic acid (50 mg/kg, p.o.) were administered to mice 1 and 12h before liver injury induction by acetaminophen (500 mg/kg, p.o.). Mice were sacrificed 6h later. Biochemical and histological analyses were used to assess liver injury.

Acetaminophen administration resulted in significant ($P < 0.01$) increase of liver relative weight, liver enzymes [aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP)] serum activities, nitrite and malondialdehyde (MDA) liver content. *R. heudelotii* extract significantly ($P < 0.05$) reduced ALT and ALP serum activity, MDA and nitrite's liver content, compared to acetaminophen-treated mice. In addition, *R. heudelotii* extract significantly ($P < 0.05$) prevented depletion of reduced glutathione (GSH) in mice liver. Tumor necrosis factor alpha (TNF-α or TNF) and Interleukin one beta (IL-1β) serum levels were also reduced ($P < 0.05$) by *R. heudelotii* extract. Histological injuries by acetaminophen were remarkably reduced by plant extract administration.

The results revealed that *R. heudelotii* stem bark aqueous extract could provide protection against acetaminophen hepatotoxicity that may be related to its antioxidant and anti-inflammatory properties. The results strongly support the ethnopharmacological uses of *R. heudelotii.*

Keywords: *Ricinodendron heudelotii; Acetaminophen; Liver Injury; GSH; Anti-Inflammatory; Anti-Oxidant*

Abbreviations

R. heudelotii: Ricinodendron heudelotii; APAP: Acetaminophen; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; ALP: Alkaline Phosphatase; MDA: Malondialdehyde; NO: Nitric Oxide; GSH: Reduced Glutathione; TNF-α or TNF: Tumor Necrosis Factor Alpha; IL-1β: Interleukin-1 Beta; IL-10: Interleukin-10; H&E: Hematoxylin/Eosin; NAC: N-Acetyl Cysteine; NAPQI: N-Acetyl-Para-Benzo-Quinone Imine

Introduction

Since 1955 acetaminophen (APAP), also known as paracetamol or N-acetyl-p-aminophenol has been one of the most commonly utilized antipyretic or analgesic drugs worldwide. Intentional and non-intentional overdose of acetaminophen (APAP) is a serious health problem, the leading cause of acute liver failure, accounting for about 50% of cases. It may progress to fulminant hepatic failure and death. It is responsible for approximately 29% of liver transplant cases in the United States, with about 28% mortality rate [1]. Current treatment options for acetaminophen poisoning are limited. N-acetyl cysteine (NAC) is an effective antidote for acetaminophen overdose [1,2]. However, to effectively prevent the formation of toxic metabolite that leads to hepatic injury, NAC should be administered as early treatment, following ingestion of higher doses of acetaminophen [1,3], providing 66% chance of recovery [3].

Therefore, novel therapeutic intervention strategies are needed. Nowadays, increasing efforts are directed towards medicinal plants for the development of new hepatoprotective drugs with long period activity and minimum side effects.

Ricinodendron heudelotii (Baill.) Pierre ex Pax. (Euphorbiaceae) is a fast-growing late secondary forest tree found in the Guinean-Congolean humid forests of West and Central Africa [4]. It can reach 20 - 30m in height. The bark is smooth and grey in color, 1 - 1.5 cm thick, becoming scaly with age. The branchlets are covered with dense brown hairs when young. The leaves digitally alternate and are composed of long elliptic leaflets. The flowers are 4 - 5 mm in size with a yellowish white color. The fruit is 2 - 3 lobed with a thick and hard shell. The fruit contains 2 - 3 red-brown-black rounded, flat seeds, with 1 cm diameter [5]. The seeds, commonly known as "njansang" are ground and used as a flavoring and thickening agent in food [4]. A paste obtained by grinding *R. heudelotii* seeds is applied to treat toothache [6]. Leaves are used to treat fungal infection and reduce fever. Seeds are used against cardiovascular diseases. Roots and stem barks are used to cure diarrhea and sexually transmitted infections. Stem barks used to treat cough are also antidote against poison [4].

Phytochemical analysis revealed the presence of tannins, saponins, flavonoids and alkaloids in *R. heudelotii* barks [7] and seeds, as well as minute amount of phytate, oxalates, carotenoids and anthraquinones in seed extract [8]. In addition, extract from *R heudelotii* barks exhibited free radical scavenging activity against 2,2-diphenyl-1-picrylhydrazyl and inhibited growth of many bacteria species *in vitro*: *Staphylococcus aureus, Klebsiella pneumonia, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Morganella morganii, Citrobacter freundii* and *Enterobacter cloacae* [7].

Toxicological analysis revealed no death of rats after acute administration of *R. heudelotii* barks methanol extract (2 to 10 g/kg) and no significant modification of ALT serum activity in rats treated with 2 to 4 g/kg of *R. heudelotii* extract [7]. However, to the best of our knowledge, no scientific study has been conducted on hepatoprotective effect of *R. heudelotii* extract. The present work evaluates the effect of *R. heudelotii* stem bark aqueous extract on acetaminophen-induced hepatotoxicity in mice.

Materials and Methods

Plant materials

Fresh stem bark of *Ricinodendron heudelotii* was harvested in Buea, South West Region, Cameroon in November, 2015 and identified in the Botanical Garden of Limbe, Cameroon where a voucher specimen was deposited under the serial number SCA3833. The protocol for preparation of plant extract and *R. heudelotii* doses were determined according to the method used in Cameroonian traditional medicine. Briefly, decoction of *R. heudelotii* air dried bark powder (400g) was prepared during 10 minutes into distilled water (2L). After cooling, the mixture was filtered using Whatman paper N°3 and water was evaporated at 40°C in an oven. A paste (20g) representing *R. heudelotii* stem bark aqueous extract was obtained (yield: 5%).

Phytochemical studies

Total phenolic content, sterols, triterpenes and saponins were determined in *R. heudelotii* extract as described by Fotio., *et al* [9]. Alkaloids and tannins were tested in *R. heudelotii* extract as described by Hossain., *et al* [10].

Chemicals and reagents

Acetaminophen, trichloroacetic acid, thiobarbituric acid, sulphanilamide, naphthylethylenediamide, ortho-phosphoric acid and 5,5-Dithiobis (2-nitrobenzoic acid) were purchased from Sigma Aldrich (Germany). TNF-α, IL-1β and IL-10 kits were purchased from Bio-techne (R&D Systems Europe Ltd). ALT, AST and ALP kits were purchased from Chronolab Systems (Spain). All chemicals used were of analytical grade.

Animals

Male and female BALB/c mice (20 - 22g, 8 - 12 weeks old) were used for the investigations. Mice were maintained under conventional laboratory conditions (12:12 h light/dark cycle at 25 ± 2°C) at the Animal House, Department of Zoology and Animal Physiology, University of Buea, Cameroon. They were housed six animals per cage, with free access to standard commercial diet and water. Animals were fasted 12h before liver injury induction. The experiment was carried out in accordance with institutional guidelines and approved by the Cameroon National Ethical Committee (Reg. N° FWAIRD 0001954).

Acetaminophen-induced hepatic injury in mice

Distilled water (neutral control and negative control), ascorbic acid (50 mg/kg, positive control) as previously reported by Okuyucu., *et al*. [11] or *R. heudelotii* stem bark aqueous extract (100 or 200 mg/kg, test groups) were orally administered to mice 1 and 12h before hepatic injury induction [12]. One hour after the second treatment with test products, acetaminophen (500 mg/kg) was orally administered to mice of the negative control, positive control and test groups) [13,14]. Blood obtained from retro-orbital sinuses 6h after acetaminophen (APAP) administration was used for serum separation. Mice were then sacrificed. Liver homogenate (20%) in Tris-HCl (50 mM, pH 7.4) was centrifuged at 3000 rpm for 1h at 4°C and supernatant was used for biochemical assays.

Biochemical analyses

Serum transaminase content

Hepatocyte damage was assessed by measuring aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) serum activity with commercial kits (Chronolab), according to manufacturer's instructions.

Nitrite determination

Nitrite accumulation was measured in liver homogenate as indicator of nitric oxide (NO) production by Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 2.5% ortho-phosphoric acid). Briefly, 500 **µ**L of tissue homogenate (20%) was mixed with 500 **µ**L of Griess reagent and incubated at room temperature for 10 minutes. Absorbance was measured at 570 nm. Nitrite concentration was determined by comparison with reference to the sodium nitrite standard curve [9,12].

Reduced glutathione amounts

Reduced glutathione (GSH) concentration in the liver homogenate was assayed as described by Ellman [15]. Briefly, tissue homogenate supernatant (20 µL) was added to 3 mL of Ellman reagent. The mixture was kept for 1h at room temperature and the absorbance was read at 412 nm.

Malondialdehyde (MDA) determination

Malondialdehyde (MDA) level in liver homogenate was used as indicator of lipid peroxidation. Solutions of trichloroacetic acid (20%, 250 µL), thiobarbituric acid (0.67%, 500 µL) and tissue homogenate supernatant (20%, 500 µL) were mixed together, incubated at 90°C for 1h, then cooled with tap water and centrifuged. Absorbance of supernatant was measured at 530 nm [9,16]. MDA concentration was quantified by the extinction coefficient of 1.56×10^5 M/cm and expressed as µmol of MDA per gram of tissue.

TNF-α, IL-1β, IL-10 determination

Serum level of TNF-α or TNF, IL-1β and IL-10 was assessed by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer instructions.

Histological analyses

Histological analyses of liver were performed 6 h after acetaminophen administration. Livers were fixed in 4% buffered formaldehyde and embedded in paraffin for subsequent hematoxylin/eosin (H&E) staining [12].

Statistical analysis

Values are expressed as mean ± SEM. Statistical differences between groups were determined using one-way Analysis of Variance (ANOVA) followed by Dunnett's test. P values less than 0.05 were considered significant.

Results

Phytochemical screening of *R. heudelotii* extract

Phytochemical analysis revealed the presence of alkaloids, triterpenes, tannins, saponins, flavonoids and polyphenols compounds in *R. heudelotii* stem bark aqueous extract.

Effect of *R. heudelotii* extract on macroscopic aspect of acetaminophen-treated mice liver

Acetaminophen (APAP) administration induced liver swollen, with significant ($P < 0.01$) increase of organ relative weight and massive hepatic toxicity, as revealed by gross morphology of liver of negative control animals (treated with distilled water and APAP), compared to neutral control (vehicle). Pre-treatment of mice with *R. heudelotii* extract or ascorbic acid prevented ($P < 0.01$) acetaminophen to increase liver relative weight (Figure 1). In addition, *R. heudelotii's* extract (100 or 200 mg/kg) and ascorbic acid reduced liver macroscopic toxicity due to APAP treatment (Figure 2).

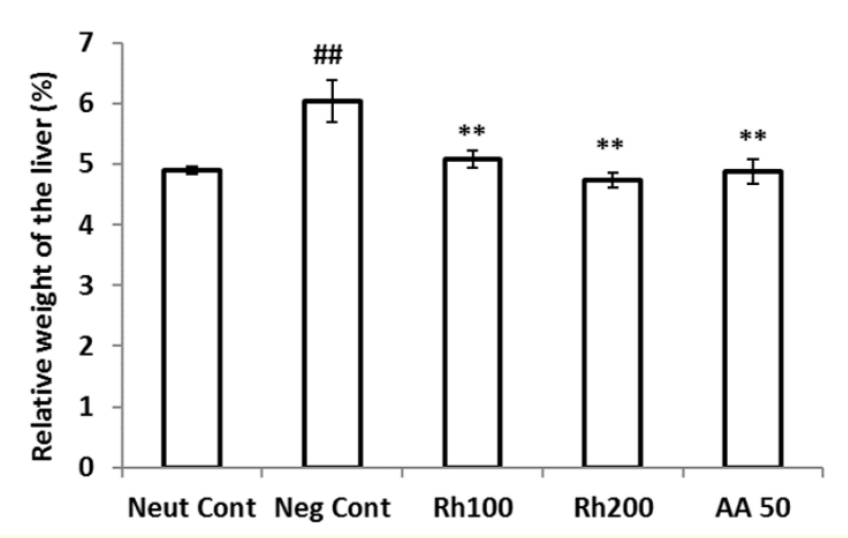

Figure 1: *Effect of stems bark aqueous extract of R. heudelotii on acetaminophen-treated mice liver relative weight. Results are represented as means ± SEM, n = 6. ##P < 0.01 compare to neutral control; **P < 0.01, compared to negative control. Neut Cont: neutral control treated with distilled water; Neg Cont: negative control treated with distilled water and APAP; Rh 100: Ricinodendron heudelotii 100 mg/kg, Rh 200: Ricinodendron heudelotii 200 mg/kg; AA50: ascorbic acid 50 mg/kg.*

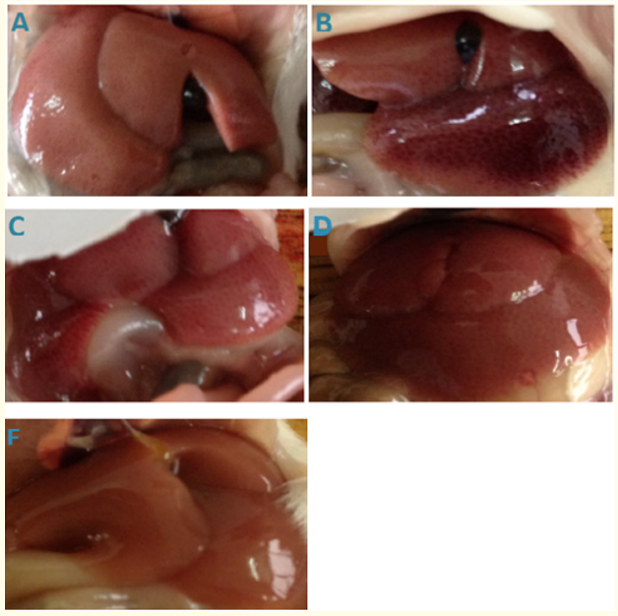

Figure 2: *Stems bark aqueous extract of R. heudelotii protected mice from acetaminophen-induced liver necrosis. A: Liver of mice treated with distilled water; B: Liver of mice treated with distilled water and APAP, C: Liver of mice treated with Ricinodendron heudelotii (100 mg/kg) and APAP; D: Liver of mice treated with Ricinodendron heudelotii (200 mg/kg) and APAP; E: Liver of mice treated with ascorbic acid (50 mg/kg) and APAP. APAP: acetaminophen.*

Effect of *R. heudelotii* extract on malondialdehyde (MDA) level

Six hours after acetaminophen administration, MDA liver level was significantly ($P < 0.01$) increased. *R. heudelotii* extract (100 and 200 mg/kg) significantly ($P < 0.05$, $P < 0.01$) reduced MDA level, compared to negative control animals treated with acetaminophen and distilled water. Plant extract and ascorbic acid treated mice showed similar MDA level to neutral animals treated with vehicle (Figure 3).

Effect of *R. heudelotii* extract on reduced glutathione level

Reduced glutathione (GSH) liver level was significantly depleted ($P < 0.01$), almost threefold, in acetaminophen-treated mice, compared to vehicle-treated animals. Prior administration of *R. heudelotii* extract (100 or 200 mg/kg) significantly ($P < 0.01$) prevented GSH depletion in the liver, compared to APAP-treated mice. Ascorbic acid treatment showed similar results to plant extract (Figure 4).

Effect of *R. heudelotii* extract on liver nitrite level

Liver nitrite level was significantly ($P < 0.01$) increased by acetaminophen treatment, compared to vehicle. *R. heudelotii* aqueous extract (100 and 200 mg/kg) significantly ($P < 0.01$) reduced liver nitrite level, compared to APAP treated mice. Administered at 200 mg/kg, *R. heudelotii* extract exhibited a similar effect when compared to ascorbic acid (Figure 5).

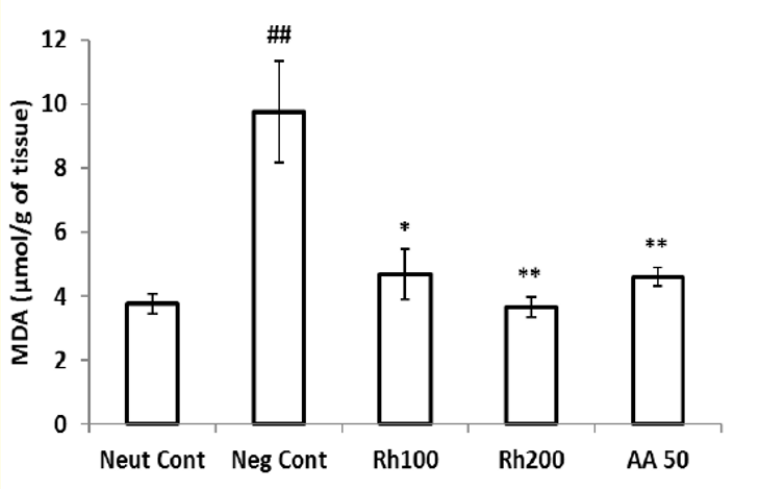

Figure 3: *Inhibition of acetaminophen-induced lipid peroxidation in mice liver by R. heudelotii stem bark aqueous extract. Results are represented as means ± SEM, n = 6. ##P < 0.01, compared to neutral control; *P < 0.05, **P < 0.01, compared to negative control. Neut Cont: Neutral control treated with distilled water; Neg Cont: Negative control treated with distilled water and APAP; Rh 100: Ricinodendron heudelotii 100 mg/kg, Rh 200: Ricinodendron heudelotii 200 mg/kg; AA50: ascorbic acid 50 mg/kg.*

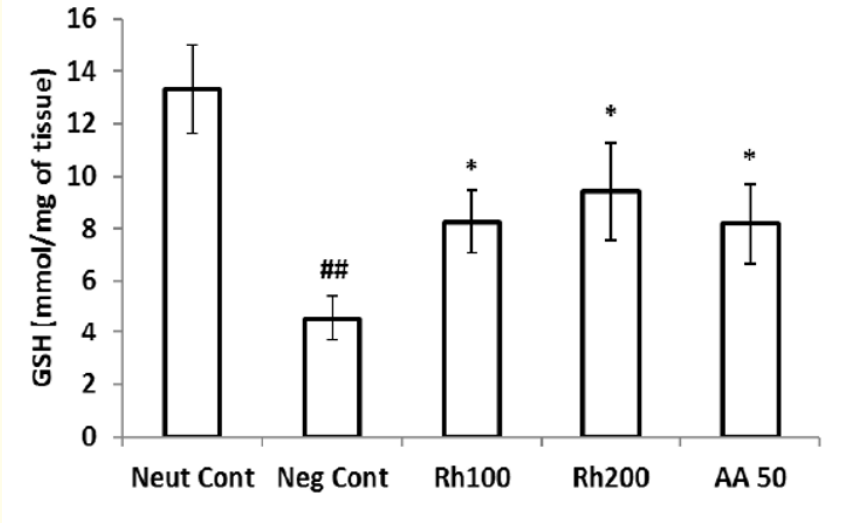

Figure 4: *Effect of R. heudelotii stem bark aqueous extract on GSH liver level of acetaminophen-treated mice. Results are represented as means ± SEM, n = 6. ##P < 0.01, compared to neutral control; *P < 0.05, compared to negative control. Neut Cont: Neutral control treated with distilled water; Neg Cont: Negative control treated with distilled water and APAP; Rh 100: Ricinodendron heudelotii 100 mg/kg, Rh 200: Ricinodendron heudelotii 200 mg/kg; AA50: ascorbic acid 50 mg/kg.*

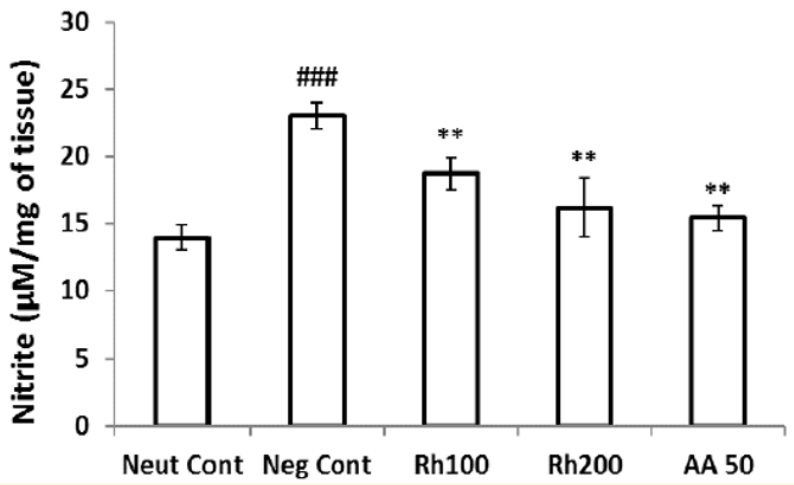

Figure 5: *Reduction of acetaminophen-treated mice nitrite level by R. heudelotii stem bark aqueous extract. Results are represented as means ± SEM, n = 6. ###P < 0.001, compared to neutral control; **P < 0.01, compared to negative control. Neut Cont: Neutral control treated with distilled water; Neg Cont: Negative control treated with distilled water and APAP; Rh 100: Ricinodendron heudelotii 100 mg/kg, Rh 200: Ricinodendron heudelotii 200 mg/kg; AA50: ascorbic acid 50 mg/kg.*

Effect of *R. heudelotii* extract on TNF, IL-1β and IL-10 serum level

Administration of acetaminophen to mice resulted to significant ($P < 0.05$) increase of TNF, IL-1β and IL-10 serum level. Prior administration of *R. heudelotii* extract significantly ($P < 0.05$) reduced serum level of pro-inflammatory cytokines (TNF and IL-1β). These cytokines were also reduced by ascorbic acid. However, IL-10 was reduced, but not to a significant ($P > 0.05$) level, neither by the plant extract nor by ascorbic acid (Figure 6).

Effects of *R. heudelotii* extract on liver enzymes

ALT, ALP and AST serum activities, 3 enzymes that correlate with liver damage were significantly increased in mice treated with APAP. ALT activity was increased eleven-fold ($P < 0.01$) by APAP administration. *R. heudelotii* extract (100 and 200 mg/kg) significantly ($P < 0.05$, $P < 0.01$) inhibited ALT serum activity. Ascorbic acid (50 mg/kg) showed a similar effect, compared to plant extract (200 mg/kg). AST activity that increased almost six-fold ($P < 0.01$) by APAP treatment, was partially and significantly ($P < 0.05$) reduced by *R. heudelotii* extract (100 mg/kg) and ascorbic acid. ALP activity doubled ($P < 0.01$) 6 h after acetaminophen treatment and, was reduced by *R. heudelotii* extract. Ascorbic acid (50 mg/kg) and *R. heudelotii* (200 mg/kg) effects were comparable (Figure 7).

Histopathological examination of liver sections

Figure 8 shows histological micrograph of liver sections excised from acetaminophen-treated mice, submitted to hematoxylin/eosin staining. Liver section of neutral mice (vehicle) showed portal tract and surrounding hepatic sinusoids. Cells and their nuclei are relatively uniform in size and staining characteristics (Figure 8A). Histopathological examination of liver sections confirmed that APAP administration damaged normal liver architecture, with dilated sinusoids, hepatocyte vacuolization and infiltration of inflammatory cells in the liver

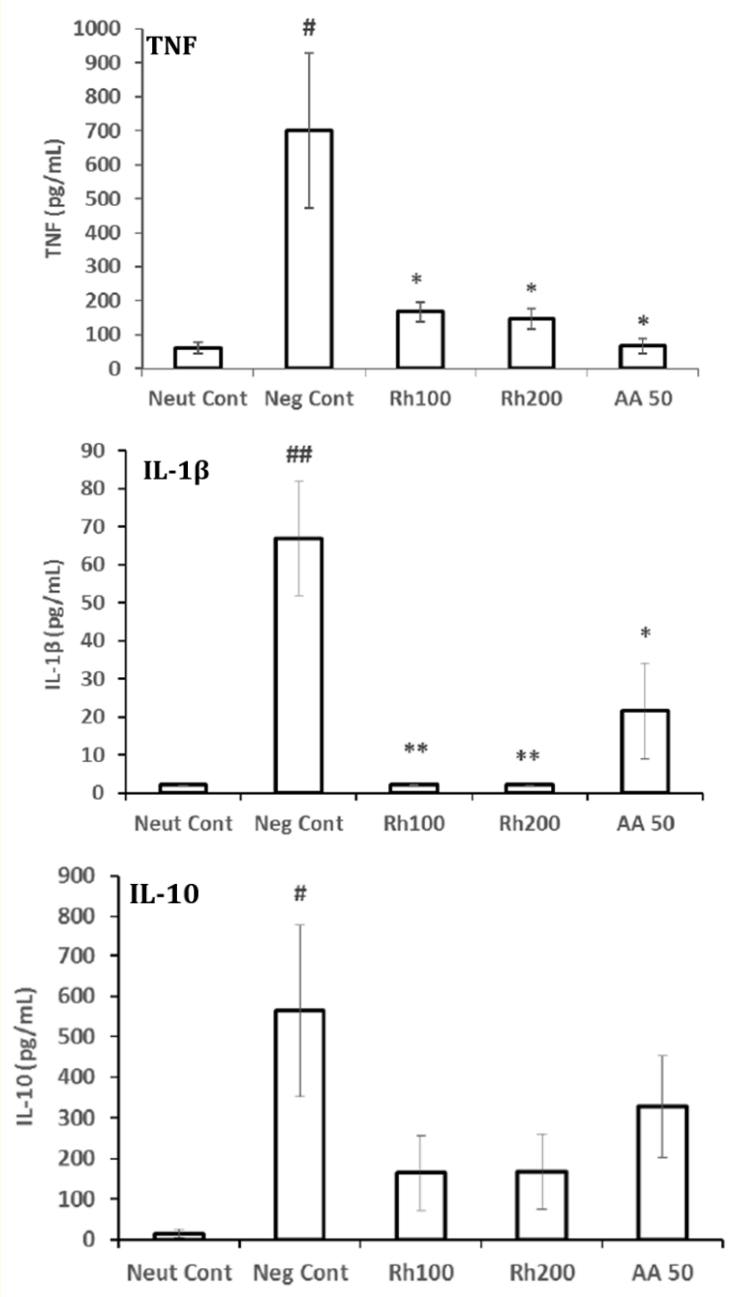

Figure 6: *Effect of R. heudelotii extract on TNF, IL-1β and IL-10 serum level of acetaminophen-treated mice. Results are represented as means ± SEM, n = 6. #P < 0.05, ##P < 0.01, compared to neutral control; *P < 0.05, **P < 0.01, compared to negative control. Neut Cont: Neutral control treated with distilled water; Neg Cont: Negative control treated with distilled water and APAP; Rh100: Ricinodendron heudelotii 100 mg/kg, Rh200: Ricinodendron heudelotii 200 mg/kg; AA50: Ascorbic acid 50 mg/kg.*

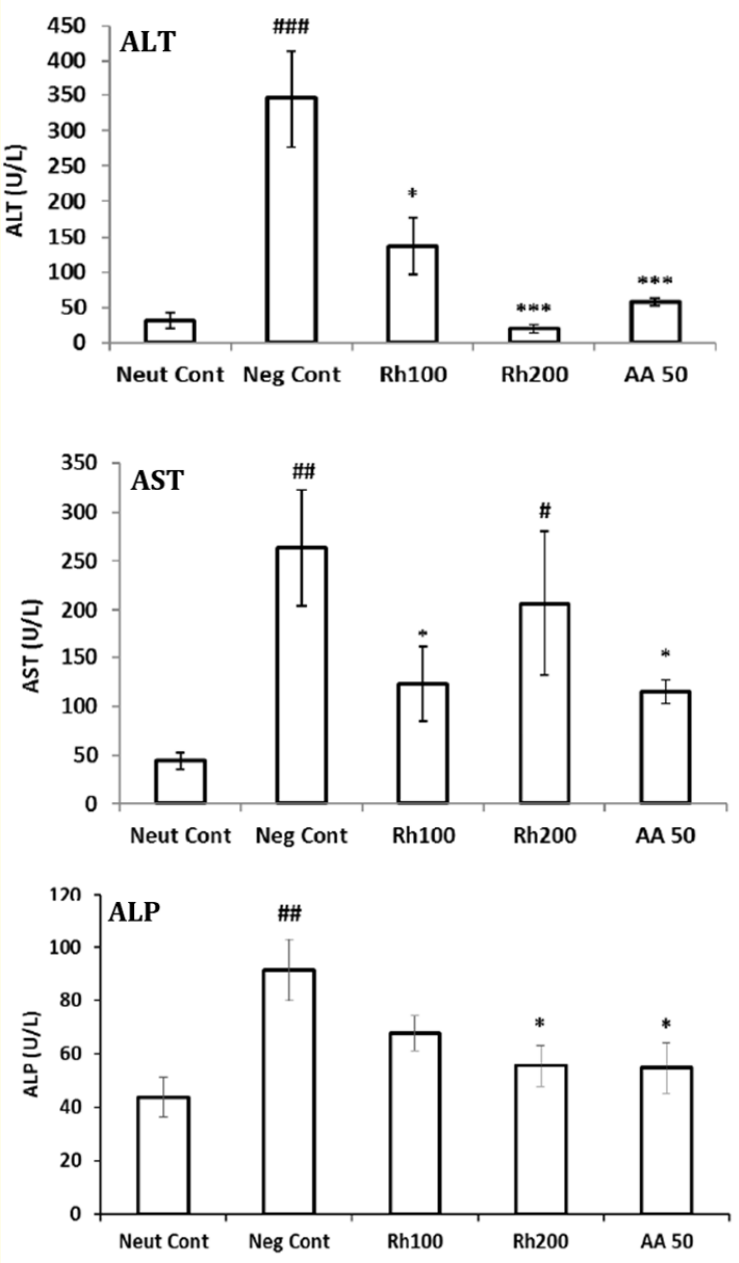

Figure 7: *Reduction of serum transaminases (ALT, ALP and AST) activity by R. heudelotii extract. Results are represented as means ± SEM, n = 6. #P < 0.05, ##P < 0.01, ###P < 0.001 compared to neutral control; *P < 0.05, ***P < 0.001, compared to negative control. Neut Cont: Neutral control treated with distilled water; Neg Cont: Negative control treated with distilled water and APAP; Rh 100: Ricinodendron heudelotii 100 mg/kg, Rh 200: Ricinodendron heudelotii 200 mg/kg; AA50: Ascorbic acid 50 mg/kg.*

parenchyma (Figure 8B). Administration of *R. heudelotii* (100 or 200 mg/kg) extract or ascorbic acid resulted in noticeable improvement in histopathological parameters, and thus, reduction of liver injury (Figure 8C-8E).

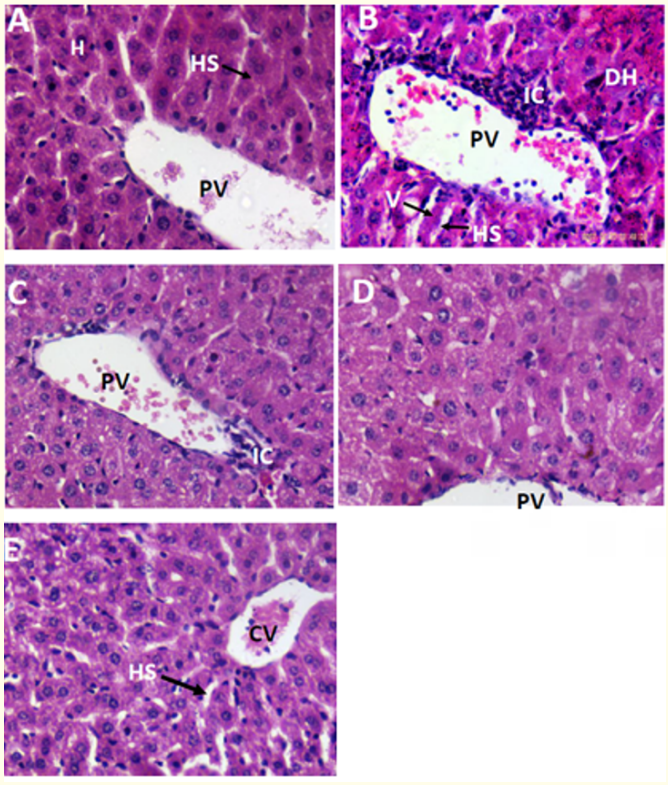

Figure 8: *Effect of R. heudelotii stem bark aqueous extract on Histological changes of acetaminophen-treated mice liver. A: Liver section of control mice treated with distilled water, showing portal vein from which radiate normal hepatocytes in the form of hepatic cords (HC) separated by hepatic sinusoids (HS); B: Histological changes in the liver of acetaminophen treated mice with death of hepatocytes (DH), infiltration of inflammatory cells (IC) into the liver parenchyma and vacuolization (V) of hepatocytes; C: Liver section of mice treated with R. heudelotii (100 mg/kg) and acetaminophen; D: Liver section of mice treated with R. heudelotii (200 mg/kg) and acetaminophen; E: Liver section of mice treated with ascorbic acid (50 mg/kg) and acetaminophen CD&E display reduced liver injury. H: Hepatocyte PV: Portal vein, CV: Central vein (hematoxylin and eosin staining 400×).*

Discussion

The present investigation was undertaken to verify the hypothesis that *R. heudelotii* stem bark aqueous extract can protect mice against acetaminophen-induced liver injury. Acetaminophen treatment resulted in GSH depletion, with increase liver enzymes activity. Prior administration of *R. heudelotii* extract reduced ALT, ALP and AST serum activity, TNF and IL-1β serum level, MDA and nitrite liver level, and prevented GSH depletion in the liver of acetaminophen-treated mice.

Activation of inducible nitric oxide synthase with increase synthesis of NO is involved in APAP-induced hepatotoxicity [17]. In addition to reactive nitrogen species, excessive production of reactive oxygen species leads to imbalance between oxidant and antioxidant agents, inducing oxidative stress in APAP intoxication [1,2,18,19]. Nitrite accumulation was reduced by *R. heudelotii* extract. Lipid peroxidation by acetaminophen was also prevented by *R. heudelotii* extract. Lipids, proteins and DNA are among cellular structures primarily affected by reactive nitrogen and oxygen species [20,21]. APAP hepatotoxicity results from formation of excessive quantities of N-acetyl-para-benzoquinone imine (NAPQI), a noxious metabolite. This subsequently leads to GSH depletion [1,19]. Administration of *R. heudelotii* extract prevented GSH depletion by APAP. GSH is essential for detoxification of APAP metabolites and NAPQI that cause hepatic injury. This suggests that plant extract either reduced APAP metabolism to NAPQI, or stimulated GSH repletion, providing surplus cysteine as substrate for Krebs cycle and playing important role by scavenging free radicals and peroxynitrite [1,22].

Organelle and cellular swelling, increasing liver relative weight was inhibited by plant extract administration in APAP-treated mice. In addition to liver swelling, vacuolization, inflammation and release of alanine aminotransferase are key processes of hepatocyte necrosis [1]. Liver enzymes such as ALT; AST and ALP leak from damaged liver cells into the blood. Determination of serum level of ALT, AST and ALP is used to estimate the extent of hepatic damage [23,24]. ALT, AST and ALP serum activities were reduced by *R. heudelotii* extract. Decrease in liver enzymes activity correlated with reduction of neutrophils accumulation in liver tissue and inhibition of inflammatory injury [12,25], thus prevention of APAP-induced hepatotoxicity by *R. heudelotii* extract. Increase susceptibility to acetaminophen appears to correlate with elevated expression of proinflammatory cytokines (TNF and IL-1β), as well as inducible nitric oxide synthase [17]. Hepatoprotective effect of *R. heudelotii* extract may result from reduction of TNF, IL-1β and nitrite level.

Previous studies revealed the presence of phenolic compounds such as flavonoids in *R. heudelotii* bark. Effects of *R. heudelotii* extract may be attributed to scavenging of free radicals [7] and boosting of antioxidant capacity of the liver by phenolic compounds that have been reported to exhibit strong antioxidant and hepatoprotective effects [26]. *R. heudelotii* extract significantly prevented GSH depletion, lipid peroxidation and nitric oxide synthesis and/or production by hepatocytes. Flavonoids content of *R. heudelotii* correlates with reduced oxidative stress in liver tissue, and thus prevention of hepatic damage by acetaminophen. Hepatoprotective effect of *R. heudelotii* extract was confirmed by significant reduction of ALT, AST and ALP serum activities, prevention of hepatocyte vacuolization and cellular infiltration in the liver parenchyma. Present results corroborated those previously obtained by Gupta., *et al*. [27], while using acetaminophen to induce liver injury in rats, reported that flavonoids rich fractions from three Indian medicinal plants [*Butea monosperma* (Lam.) Taub*.,* (Fabaceae)*, Bauhinia variegata* L.*.*, (Fabaceae). and *Ocimum gratissimum* L.*,* (Lamiaceae)] were able to reduce AST, ALP and ALT serum activities, and thus, protect rats from hepatic necrosis. In addition, triterpenes present in *R. heudelotii* extract may also be involved in hepatoprotective activity of the plant. Medicinal plants have been reported to protect animals against various models of drugs and chemicals-induced liver injury through their antioxidant properties, with inhibition of lipid peroxidation [23,24,28]. Anti-inflammatory effects have also been reported to play a major role in prevention of hepatotoxicity [12].

Conclusion

From these investigations we demonstrate that *R. heudelotii* stem bark aqueous extract Inhibited acetaminophen-induced liver injury. These effects appear to be mediated by GSH repletion, inhibition of lipid peroxidation, nitric oxide, TNF and IL-1β synthesis, and prevention of inflammatory cells accumulation in the liver parenchyma. Considering these results, stem bark aqueous extract of *R. heudelotii* has hepatoprotective activity due to its anti-oxidant and anti-inflammatory properties. The results strongly support the ethnopharmacological uses of *Ricinodendron heudelotii* against liver diseases. However, further investigations are required to purify and identify the active compounds in this extract.

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Declarations of Interest

The author(s) declare that they have no competing interests.

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