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Lucky Legbosi Nwidu*, Yibala I. Oboma, Ekramy Elmorsy and Wayne Grant Carter

Alleviation of carbon tetrachloride-induced hepatocellular damage and oxidative stress with a leaf extract of *Glyphaea brevis* (Tiliaceae)

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Abstract

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Background: *Glyphaea brevis* leaf is reported in ethnomedicine as a treatment for hepatitis and jaundice; however, no studies have hitherto investigated the mechanistic basis of these claims.

Methods: A hepato-protective role of *G. brevis* hydromethanolic (GBH) leaf extract was established against carbon tetrachloride (CCl₄)-induced hepatotoxicity. Twenty-four hours after a CCl₄ challenge, rats were sacrificed and serum hematological indices, lipid profile, and biochemical parameters were determined. The antioxidant enzymes parameters (glutathione, catalase, and superoxide dismutase) and lipid peroxidation product (thiobarbituric reactive substances) levels in liver homogenates were evaluated. Changes in the liver cyto-architecture of different treatment groups were also investigated.

Results: The GBH extract produced no significant impact on weight and hematological indices. Intoxication with CCl₄ significantly ($p < 0.001-0.05$) increased total cholesterol (TC) and low-density lipoproteins (LDL) compared with control rats. Pretreatment with GBH leaf extract significantly reduced triglycerides, TC, and LDL to approaching control levels ($p < 0.001-0.05$). The GBH leaf extract significantly alleviated CCl₄-induced elevation of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, and the CCl₄-induced depression of total protein, and albumin. Liver antioxidant parameters were

significantly increased in plant extract-treated rats, and this antagonized the pro-oxidant effect of CCl₄. Histo-pathological studies also supported a hepato-protective effect of GBH. Collectively, the GBH leaf extract alleviated the CCl₄-induced hepatotoxicity through improvement of innate antioxidant enzyme levels and lipid metabolism and stabilized the hepatocyte cyto-architecture of intoxicated rats.

Conclusions: This study establishes the ethnomedicinal role of *G. brevis* leaf in hepatitis and the mechanistic basis of hepato-protection against CCl₄-induced hepatotoxicity.

Keywords: *Glyphaea brevis* leaf; hepato-protection; hepatotoxicity; lipid profile; oxidative stress.

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Introduction

Enormous scientific advances to treat hepatic diseases exist, yet hepatic diseases persist as a global health challenge. The widespread serious side effects of synthetic drugs have limited their usage, and this favors the use of plants via phytomedicine [1]. The study of plants with hepato-protective activity to establish the scientific basis of their utilization in traditional medicine abounds [2–4]. Herbal drugs have received considerable attention recently because of their safety, efficacy, and cost effectiveness [5]. The need for safer hepato-protective agents has led to extensive screening of an armamentarium of medicinal plants utilized for the management of liver disorders [6–8]. The literature is inundated with hepato-protective agents with antioxidant activities [9, 10], and many of these plants have been reported to influence the hepatic antioxidant system as a mechanistic basis of their hepato-protection [4, 11].

Carbon tetrachloride (CCl₄) is a potent hepatotoxic agent, and injury induced by CCl₄ is extensively used as biomarker for the screening of hepato-protective and antioxidant profiles of plants [12]. The metabolism of CCl₄ produces highly toxic free radicals (CCl₃·) which bind covalently to cell components, abstracting hydrogen from fatty acids, initiating lipid peroxidation, inhibiting lipoprotein secretion, and reacting with free oxygen

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to form $\text{CCl}_3\text{-OO}^\cdot$ radicals that further exacerbate lipid peroxidation, cellular injury [13], and liver damage [14].

Glyphaea brevis (Tiliaceae) is a tree mainly present within forest shrubs in swampy places, rocky savanna, forest galleries, and fallow land [15]. Different parts of the plant, leaves, stem-bark and roots have found use in the traditional treatment of several disorders including hepatitis [16, 17]. It is also reported to be effective in the treatment of impotence [18], possess carminative and anticonvulsant effects [17], and have anti-inflammatory activity [17, 19]. Phytochemical screening has revealed flavonoids, cardiac glycosides, saponins, terpenoid tannins [17, 20], tannins [20], triterpenes, and steroids [21].

A pharmacological investigation of the hepato-protective effects of *G. brevis* leaf extract has yet to be reported. Therefore this study investigated the scientific basis of the utilization of *G. brevis* leaf extract in Ibibio ethnomedicine as an antidote to hepatitis. We evaluated the hepatoprotective and antioxidant effects of a *G. brevis* hydromethanolic (GBH) leaf extract on CCl_4 -intoxicated Wistar albino rats.

Materials and methods

General reagents and chemicals

Carbon tetrachloride, silymarin, diethyl ether, and methanol were purchased from Sigma-Aldrich, St. Louis, MO, USA. Randox Diagnostic kits for serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), conjugated bilirubin (CBIL), and total bilirubin (TBIL) were purchased from Randox Laboratories Ltd., London, UK. Other chemicals and solvents were of highest (analytical) grade commercially available and obtained from either Sigma Aldrich or Merck.

Collection of plant materials

Fresh *G. brevis* leaves were collected in January 2015 from the wild in Itak Ikot village in Akap-Ikono local Government Area of Akwa Ibom State by an herbalist named Mr. Okon Etefia attached to Pharamacognosy Department, University of Uyo, Uyo, Nigeria. The plant was identified by Dr. Margret Basse, Botany Department, University of Uyo, Uyo, Nigeria. A voucher specimen UUPH 20 b was deposited at the University auditorium. The leaves were air dried and powdered with pestle and mortar. The pulverized leaves were stored at room temperature until used.

Methanolic leaf extract preparation

The *G. brevis* leaves were air dried, pulverized, and extracted by cold extraction method (maceration) using 50% methanol as solvent. The

powdered leaves (300 g) were soaked in 500 mL of 50% methanol. After immersing for 72 h the mixture was shaken twice daily to aid extraction. The GBH leaf extract was separated from the marc by filtration through double-layer gauze in a glass funnel. The filtrate was evaporated to dryness in vacuo at 55 °C. The yield obtained was 5.5% and was estimated by weighing to constant dryness. Extracts were stored refrigerated for 3 months before use. The doses employed for the various studies were expressed as milligram of the dried extract per kilogram animal body weight (mg/kg b.w.).

Phytochemical studies

GBH leaf extract was qualitatively screened for the presence of phytochemicals: alkaloids, saponins, tannins, flavonoids, terpenoids, cardiac glycosides, and anthraquinones, adopting standard methods [22].

Experimental animals

Swiss albino mice (28–30 g) of both sexes were obtained from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, Niger Delta University, Bayelsa State, Nigeria. Thirty-six healthy Wistar rats of average weight (210–240 g) of either sex were also purchased from the animal house of the Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Port Harcourt, Rivers State, Nigeria. All the animals were housed in standard cages under laboratory condition in the Department of Pharmacology and Toxicology in Niger Delta University for acclimatization. The animals were fed with standard pellet feeds and clean water ad libitum. All the animals were healthy and kept in wooden laboratory cages with wooden shaving as beddings which were cleaned daily. Experimental techniques and protocols used in this study followed the ‘Guide to the care and use of laboratory animals’ (NIH, 2011) as adopted and approved by the Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.

Experimental procedure

Acute toxicity test (LD_{50} determination): The LD_{50} of the GBH leaf extract in mice was established according to Lorke [23]. The treated mice were monitored for 24 h. The experimental doses chosen were based on 1/20, 1/10, and 1/5 of the LD_{50} calculated dose.

Weight evaluation: The body weight of each rat was measured before and after the experiment by weighing balance (Ohaus, UK). The mean weight of each group was evaluated and recorded.

Experimental design

A total of 36 Wistar rats were utilized for the study. The animals were divided into six groups of six animals of equal sexes per group. Group A served as negative control and was administered distilled water (0.2 mL/kg. p.o.) daily once for 7 days; Group B was given

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distilled water (0.2 mL/kg). Groups C, D, and E were treated with GBH leaf extract dissolved in distilled water (122.5, 245, and 490 mg/kg, p.o., respectively) for 7 days. Group F was treated with the standard drug silymarin (100 mg/kg) dissolved in distilled water p.o. for 7 days. All administration was done with the aid of an orogastric tube. On the 7th day, Groups B to F were treated with a mixture of freshly prepared CCl_4 in liquid paraffin (2 mL/kg b.w., 1:1 intraperitoneally) 2 h after administration of the extract and pure drug. The body weights of all rats were recorded daily throughout the 7 days of treatment.

After 48 h rats were anesthetized using a light dose of diethyl ether prior to sacrifice. Blood was obtained by cardiac puncture into an EDTA vacutainer for determination of hematological parameters using an Automated Hematological Analyzer, SYSMEX-KX21 (supplied by SYSMEX Corporation, Japan). The hemoglobin concentration (Hb), packed cell volume (PCV), red blood cell count (RBC), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), white blood cell count (WBC), and platelet count (PLT) were thus determined.

For biochemical assessment, blood was spun at 3000 rpm for 10 min at 4 °C to separate serum into vacutainer vials and stored at 4 °C until used for analysis. The blood was allowed to stand for 10 min before centrifugation to obtain the serum. Livers were immediately extracted and perfused with ice cooled saline (0.9% sodium chloride) before utilization for further analyses.

$$\text{Hepatoprotective activity (\%)} = 1 - \frac{(\text{GB} - \text{V})}{(\text{C} - \text{W})} * 100$$

GB, C, and W are the measured variables in rats treated with *G. brevis* leaf extract plus CCl_4 , CCl_4 , and distilled water-treated animals, respectively.

Hematological analysis

The effects of the extract on various hematological parameters (PCV, Hb, RBC, MCV, MCH, MCHC, WBC, PLT, etc.) were analyzed using an automated hematology analyzer at Niger Delta University Teaching Hospital (NDUTH), Okolobiri, Bayelsa State, Nigeria, following standard procedures.

Estimation of biochemical parameters

The serum collected was used for analysis to determine the biochemical parameters: ALT, AST, ALP, albumin (ALB), CBIL, TBIL, and total protein (TP) using Randox diagnostic kits. This analysis was performed at the Department of Chemical Pathology, NDUTH, Okolobiri, Bayelsa State, Nigeria.

Measurement of hepatic antioxidant enzymes

Liver tissues taken from experimental animals were quickly perfused in ice-cold saline and stored at -80 °C until required. For assay of antioxidant enzymes, liver pieces (100 mg) were cut with a scapel and then homogenized in 100 mL of 5 mM Tris/HCl buffer (pH 7.4),

1 mM EDTA, and complete mini protease inhibitor cocktail (Roche). Homogenates were centrifuged at 10,000 rpm at 4 °C for 10 min and clear supernatant used for the determination of antioxidant parameters glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), and thiobarbituric reacting substances (TBARS).

Glutathione: GSH levels were determined based upon the method of Ellman [24], with slight modifications. The homogenate (0.2 mL) was mixed with 25% trichloroacetic acid and centrifuged at 3000 rpm for 10 min. The supernatant (0.2 mL) was then mixed with 10 mM of 5,5'-dithio-bis-[2-nitrobenzoic acid] in the presence of phosphate buffer (0.1 M, pH 7.4), and the absorbance read at 420 nm.

CAT activity: The method of Aebi [25] with slight modifications was used to determine the CAT activity. The assay relies upon the ultraviolet absorption of hydrogen peroxide that can be measured at 240 nm. The decomposition of hydrogen peroxide in the presence of CAT on a quartz plate provides a quantitation of CAT activity. Assays were performed in 50 mM phosphate buffer; with hydrogen peroxide decomposition recorded using Optiplex GX 520 microplate reader equipped with Skanlt software for multiskan spectrum version 2.2 (Thermo Electric, Finland). CAT activity was expressed as units/mg protein.

SOD activity: Measurement of liver cytosolic SOD was accomplished following the procedure of Kakkar et al. [26]. Cytosol (0.05 mL) was mixed with sodium pyrophosphate buffer (0.052 M, pH 8.3, 1.2 mL), phenazine methosulphate (0.186 mM, 0.1 mL), nitroblue tetrazolium chloride (0.3 mM, 0.3 mL), and NADH (0.78 mM, 0.2 mL). The reaction was stopped after 90 s by the addition of glacial acetic acid. Color intensity of the chromogen was extracted in butanol solution (2.0 mL) with vigorous shaking. The mixture was then centrifuged at 3000 rpm for 10 min and the supernatant extracted, and the absorbance at 560 nm was determined using Optiplex GX 520 microplate reader equipped with Skanlt software for multiskan spectrum version 2.2 (Thermo Electric, Finland).

TBARS concentration: Lipid peroxidation products were estimated via the level of lipid peroxidation product, malondialdehyde (MDA), as described by Draper and Hadley [27] using spectrophotometric techniques. Normally, MDA reacts with thiobarbituric acid to form a red/pink colored complex which dissolves maximally in acid solution at 532 nm. Spectrophotometric measurements were recorded using Optiplex GX 520 microplate reader equipped with Skanlt software for multiskan spectrum version 2.2 (Thermo Electric, Finland).

Histopathological studies

Liver tissues were cut into small portions of approximately 6 mm³ sizes and immersed in neutral buffered formaldehyde solution (10%) for 24 h. These fixed tissues were processed routinely, embedded in paraffin wax, sectioned (5 μm thickness), de-paraffinized, and rehydrated using standard procedures [28]. The degree of CCl_4 -induced necrosis was assessed by examining the morphological changes in the liver sections stained with hematoxylin-eosin adopting standard techniques. Thereafter the sections were evaluated by high-resolution microscopy after which photomicrographs of the hepatocytes were taken.

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Statistical analysis

All statistical measures were performed using PRISM 5 (GraphPad Software Inc., San Diego, CA, USA). The results are expressed as means \pm standard error of mean (SEM). One-way analysis of variance (ANOVA) was used to compare group data, followed by Tukey's multiple comparisons test. A p-value of <0.05 was considered significant.

Results

Acute toxicity (LD_{50})

The results obtained indicated that the calculated LD_{50} of GBH leaf extract was ~ 2449.5 mg/kg. These results were extrapolated to rats since the LD_{50} assays were conducted in mice.

Phytochemical studies

Preliminary phytochemical screening of GBH leaf extract revealed the presence of alkaloids, saponins, cardiac glycosides, and tannins, with results shown in Table 1.

Weight evaluation

The body weight of rats did not vary significantly between day 0 and day 7 (Figure 1). Two-way ANOVA

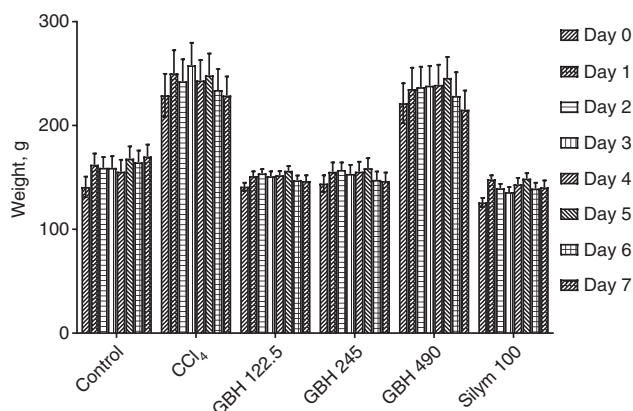


Figure 1: The effect of CCl_4 , GBH leaf extracts, and silymarin on rats' weight throughout the course of the experiment.

results showed that there were no significant changes in the weight of the rats of each group throughout the course of the experiment (0–7 days) (p -value > 0.05). When considering the effect of treatments, a two-way ANOVA showed no significant change among the studied groups when compared with the CCl_4 -treated group. A Bonferroni post-test showed no differences between the weights of the rats of each group in the different days in comparison to day 0 (before the experiments). Data are shown as mean \pm SEM.

Hematological analysis

The effect of the methanolic leaf extract of GBH at different dose levels on carbon tetrachloride-induced

Table 1: Qualitative chemical analysis of GBH leaf extract.

| Constituents | Name of test | Observation | Inference |
|--------------------|--|--|-------------------------------|
| Alkaloids | Mayer's test | Creamy coloration and precipitate formed | Presence of alkaloids |
| | Dragendorff reagent | Deep brownish coloration and precipitate formed | Presence of alkaloids |
| | Wagner's reagent | Brownish coloration and precipitate formed | Presence of alkaloids |
| | Hager's reagent | Yellowish brown coloration and precipitate formed (+) | Presence of alkaloids |
| | Italis Morin test for tropane alkaloid | Presence of violet color (+) | Presence of tropane alkaloids |
| Cardiac glycosides | Salkowski test | Presence of a reddish color at the interface (+) | Cardiac glycosides present |
| | Keller-Kiliani test | No color change | Negative for cardenolides |
| Flavonoids | Ammonium test | Absence of color change | Negative for flavonoids |
| Saponins | Frothing test | Persistent frothing on warming and emulsion with castor oil (++) | Presence of saponins |
| Tannins | Ferric chloride test | Blue-green precipitate formed (++) | Presence of tannins |
| | Bromine test | Dark blue coloration (++) | Presence of tannins |
| Anthraquinone | Borntrager's reagent | No color change observed | Anthraquinones absent |

(+) to (++) = detected in scant to moderate quantities.

Table 2: The effect of GBH leaf extracts on CCl₄-induced hepatotoxicity on hematological indices.

| Parameters | Control | CCl ₄ | GBH 122.5 | GBH 245 | GBH 490 | Silymarin 100 |
|--------------------------------|---------|------------------|-----------|---------|---------|---------------|
| PCV, % | 42±3.7 | 54±4.9 | 42±2.0 | 50±0.5 | 41±6.1 | 40±6.8 |
| Hb, g/dL | 11±2.2 | 15±1.0 | 12±2.0 | 14±0.5 | 15±1.1 | 12±2.1 |
| WBC, ×10 ³ cells/μL | 15±6.6 | 11±6.0 | 7.5±1.2 | 11±9.7 | 12±4.6 | 12±4.3 |
| PLT, ×10 ³ cells/μL | 940±136 | 536±189 | 239±111 | 524±110 | 838±257 | 758±340 |
| RBC, ×10 ⁶ cells/μL | 6.5±2.9 | 8.7±0.86 | 6.7±0.9 | 8.3±0.3 | 8.4±0.9 | 6.8±1.1 |
| MCV, % | 67±2.1 | 62±2.9 | 63±2.3 | 60±2.6 | 59±0.6 | 60±3.4 |
| MCH, pg | 17±2.1 | 17±0.9 | 18±1.1 | 17±0.4 | 18±1.2 | 18±0.7 |
| MCHC, g/dL | 26±4.0 | 28±0.9 | 29±0.4 | 29±1.7 | 31±1.7 | 30±1.7 |
| NEU, % | 22±5.6 | 35±4.8 | 36±8.2 | 39±11 | 53±13 | 43±16 |
| LYM, % | 73±5.1 | 60±3.9 | 55±8.7 | 56±13 | 39±13 | 51±17 |
| MEB, % | 5.8±1.7 | 5.7±1.4 | 4.8±1.9 | 7.3±3.0 | 8.7±1.5 | 6.8±1.6 |

Values are mean ± SEM (n = 6). PCV, packed cell volume; Hb, hemoglobin; WBC, white blood cell; PLT, platelet; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; NEU, neutrophils; LYM, lymphocytes; MEB, monocytes, eosinophils, and basophils.

hepatotoxicity is shown in Table 2. There were moderate increases of Hb and RBC in the CCl₄-intoxicated group compared with the control group, but these did not reach significance (p > 0.05). Treatment with GBH (122.5 mg/kg) leaf extract and silymarin (100 mg/kg) normalized these CCl₄-mediated increases of Hb and RBC. Other doses did not produce any significant effects on Hb or RBC. The effects on platelets show no significant reduction of PLT in the CCl₄-treated group compared with the control group. Treatment with GBH leaf extract and silymarin showed no significant effects on PLT reduction following intoxication with CCl₄. Similarly, the treatment with GBH leaf extract and silymarin did not have statistically significant effects on the other hematologic indices following intoxication with CCl₄.

Effects of GBH leaf extracts on lipid profile

The effect of the GBH leaf extracts at different dose levels on CCl₄-induced lipid profiles are shown in Figure 2. Intoxication with CCl₄ produced a statistically significant (p < 0.001) increase in the concentration of total cholesterol (TC) and low-density lipoproteins (LDL). Pretreatment with GBH leaf extract at 122.5, 245, and 490 mg/kg produced a significant (p < 0.01–0.05) reduction of triglycerides, significant (p < 0.001–0.01) decrease in TC, and significant (p < 0.001–0.01) decrease in LDL compared with the CCl₄-treated group. Silymarin also significantly (p < 0.001) reduced triglycerides, TC (p < 0.001) and LDL (p < 0.01) relative to the CCl₄ group. No significant effects of the GBH extracts at all

doses or from silymarin (100 mg/kg) were observed for HDL.

Effect of GBH leaf extract on serum biochemical markers

The results of the effect of GBH extract at 122.5, 245, and 490 mg/kg body weight on CCl₄-induced hepatotoxicity is shown in Table 3. ALT, AST, and ALP were significantly (p < 0.001) elevated by 49%, 65%, and 48%, respectively; TBIL significantly (p < 0.01) increased by 90%, and CBIL (p > 0.05) increased by 22% following intoxication with CCl₄ compared with controls. Pretreatment with GBH leaf extracts at 122.5 mg/kg significantly (p < 0.001) decreased ALT, AST, and ALP by 28%, 35%, and 25%, respectively; TBIL was significantly (p < 0.01) decreased by 58%, and TP significantly (p < 0.001) increased by 43% compared with the CCl₄-intoxicated group. At 245 mg/kg of GBH extract administration, ALT, AST, ALP, and TBIL were significantly (p < 0.001) decreased by 32%, 39%, 28%, and 53%, respectively; TP and ALB significantly (p < 0.001) increased by 74% and 43%, respectively. While at 490 mg/kg of GBH extract, ALT, AST, and ALP were significantly (p < 0.001) decreased by 48%, 56%, and 44%, respectively, CBIL and TBIL significantly (p < 0.01) decreased by 71% and 69%, respectively; TP and ALB significantly (p < 0.001–0.01) increased by 129% and 53%, respectively, compared with the CCl₄-intoxicated group. With the pure drug, silymarin, ALT, AST, ALP, CBIL, and TBIL were significantly (p < 0.001) decreased by 48%, 64%, 44%, 71%, and 69%, respectively, while TP and ALB

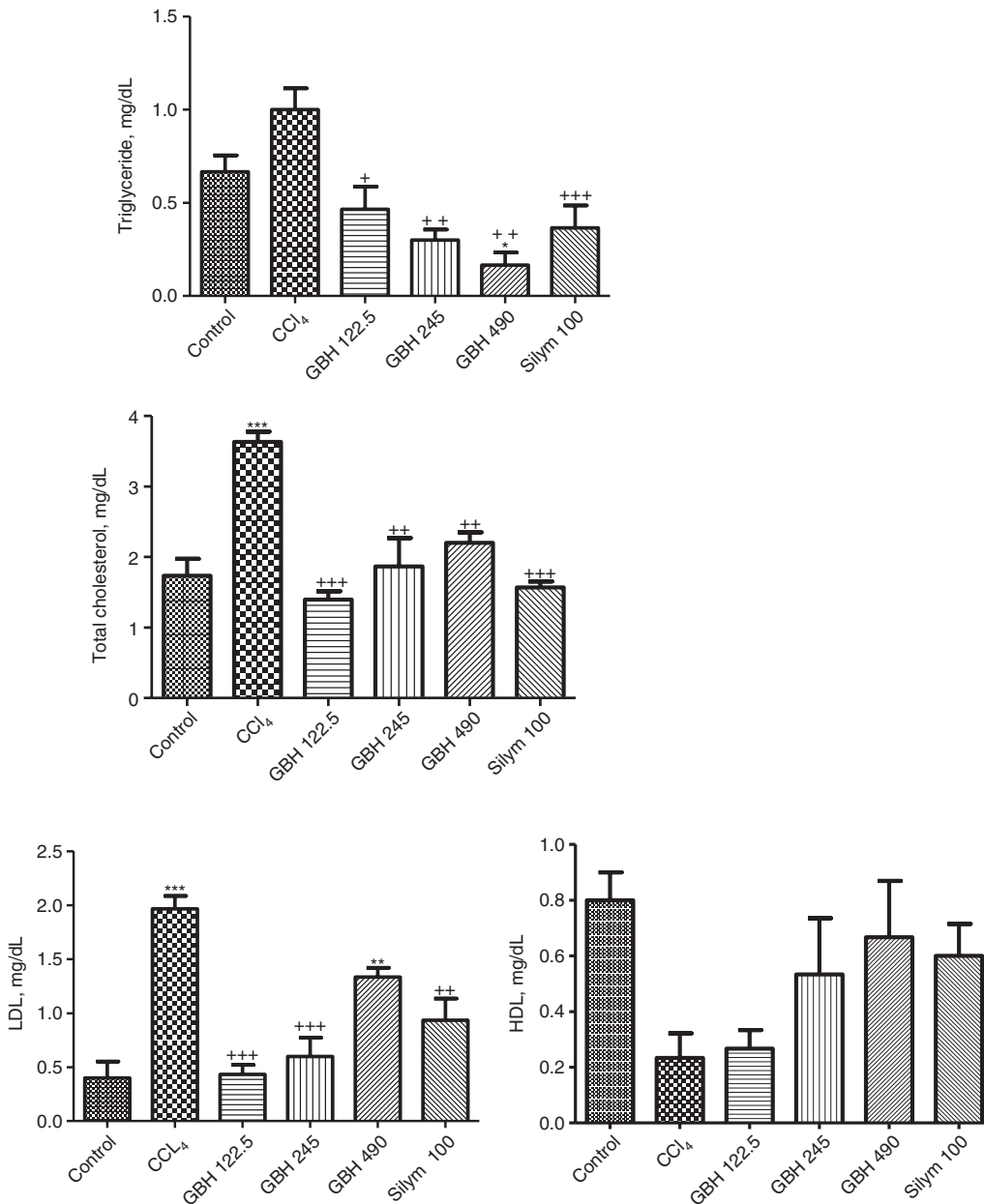


Figure 2: Effect of GBH leaf extract and silymarin on lipid profile.

Values are mean \pm SEM (n = 6). *p < 0.05; **p < 0.01; ***p < 0.001 compared with control group; +p < 0.05; ++p < 0.01; +++p < 0.001 compared with CCl₄ group. TC, total cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

were significantly ($p < 0.001$) increased by 113% and 56%, respectively, compared with the CCl₄-intoxicated group.

The percentage of hepato-protection of GBH leaf extract demonstrated dose-dependent protection at 122.5, 245, and 490 mg/kg of GBH extract. For ALT this was 57%, 65%, and 96%, respectively; AST, 54%, 61%, and 87%, respectively; ALP, 52%, 59%, and 92%, respectively; CBIL, 11%, 148%, and 396%, respectively; TBIL, 124%, 111%, and 146%, respectively; ALB, 55%, 85%, and 104%, respectively; and TP, 38%, 66%, and 114%, respectively.

Silymarin at 100 mg/kg produced comparative hepato-protection of ALT (97%), AST (98%), ALP (92%), CBIL (396%), TBIL (146%), TP (100%), and ALB (111%).

Histopathological analysis

The effects of GBH extract at 122.5, 245, and 490 mg/kg and silymarin at 100 mg/kg on liver histology of CCl₄-intoxicated rats' hepatocytes are presented in Figure 3.

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Table 3: Evaluations of GBH leaf extract on serum biochemical markers.

| Treatment | Dosing regimen | ALT, U/L | AST, U/L | ALP, U/L | ALB, g/L | TP, g/dL | CBIL, $\mu\text{mol/L}$ | TBIL, $\mu\text{mol/L}$ |
|---------------------------|--|-------------------------------|------------------------------|------------------------------|-------------------------------|-------------------------------|------------------------------|------------------------------|
| Control (distilled water) | 0.0 | 45.0 \pm 0.58 | 30.3 \pm 0.3 | 39.8 \pm 0.5 | 46.67 \pm 2.4 | 81.67 \pm 3.2 | 1.23 \pm 0.1 | 6.33 \pm 0.9 |
| | 1 mL/kg CCl ₄ | 88.3 \pm 0.75 ^{cd} | 85.8 \pm 1.4 ^{cd} | 76.3 \pm 0.8 ^{cd} | 31.00 \pm 3.6 ^{bd} | 38.33 \pm 0.3 ^{cd} | 1.50 \pm 0.2 | 12.0 \pm 0.5 ^{bd} |
| GBH extract | 1 mL/kg CCl ₄ + 122.5 mg/kg | 63.7 \pm 0.33 ^{ce} | 56.0 \pm 2.1 ^{ce} | 57.3 \pm 1.7 ^{ce} | 39.67 \pm 0.9 | 55.00 \pm 3.5 ^{ce} | 1.47 \pm 0.1 | 5.0 \pm 1.5 ^{ce} |
| | 1 mL/kg CCl ₄ + 245 mg/kg | 60.3 \pm 0.33 ^{ce} | 52.0 \pm 1.0 ^{ce} | 54.6 \pm 0.7 ^{ce} | 44.33 \pm 0.3 ^{be} | 66.67 \pm 0.3 ^{ce} | 1.10 \pm 0.1 | 5.7 \pm 1.2 ^{ce} |
| Silymarin | 1 mL/kg CCl ₄ + 490 mg/kg | 46.7 \pm 0.3 ^{ce} | 37.8 \pm 0.7 ^{ce} | 42.7 \pm 0.9 ^{ce} | 47.33 \pm 0.9 ^{be} | 87.67 \pm 0.9 ^{ce} | 0.43 \pm 0.0 ^{ce} | 3.7 \pm 0.3 ^{ce} |
| | 1 mL/kg CCl ₄ + 100 mg/kg | 46.3 \pm 0.3 ^{ce} | 31.3 \pm 1.2 ^{ce} | 42.7 \pm 1.5 ^{ce} | 48.33 \pm 0.7 ^{ce} | 81.67 \pm 0.9 ^{ce} | 0.43 \pm 0.1 ^{ce} | 3.7 \pm 0.3 ^{ce} |

Values represent mean \pm SEM (n = 6). ^ap < 0.05, ^bp < 0.01, ^cp < 0.001. ^dValues are compared with the distilled water control group. ^eValues are compared with the CCl₄ group. Statistical analysis was performed using one-way ANOVA. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; ALB, albumin; TP, total protein; CBIL, conjugated bilirubin; TBIL, total bilirubin.

The section of the control rat's liver shows normal liver tissue displaying a central vein with portal tract radiating numerous normal sinusoids and hepatocytes and differentiated blood vessels and viable hepatocytes (Figure 3A). Liver sections of rats intoxicated with CCl₄ reflect lipid necrosis at centrilobular (zone 3) and mid-portions of the hepatic lobule that were completely necrotic, with extensive fatty accumulation and polymorphonuclear cell infiltration, and with loss of cellular boundaries of the hepatocytes (Figure 3B). However, pretreatment with GBH extract at 122.5 mg/kg showed only mild centrilobular necrosis with little infiltration of inflammatory cells (Figure 3C). Following pretreatment with GBH extract at 245 mg/kg after intoxication with CCl₄, liver tissue revealed congestion of the central vein and periportal necrosis (Figure 3D). Hepatocytes of rats pretreated with 490 mg/kg GBH extract following CCl₄ intoxication showed liver tissue with numerous infiltration of inflammatory cells within the portal tract and some also at the stroma. The presence of mitotic bodies was detected at various zones of the liver indicating a regeneration process (Figure 3E). Silymarin at 100 mg/kg showed liver tissue with areas of severe necrosis but with numerous fibroblasts and mitotic bodies indicative of repair processes (Figure 3F).

Effect of GBH on hepatic antioxidant markers

The results of GBH leaf extracts on hepatic antioxidant markers are summarized in Figure 4. The levels of the liver antioxidant GSH and the activities of CAT and SOD were decreased significantly (p < 0.001) by 59%, 71%, and 70%, respectively, and TBARS were increased by 183% following intoxication with CCl₄. However, oxidative stress markers were increased significantly, GSH (p < 0.05) by 66%, CAT (p < 0.05) by 93%, and SOD (p < 0.05) by 83% with pretreatment with GBH (245 mg/kg). Silymarin (100 mg/kg) significantly (p < 0.001) increased GSH, CAT, and SOD concentrations by 85%, 86%, and 96%, respectively, compared with the CCl₄-intoxicated group. GBH (245 mg/kg) significantly (p < 0.001) decreased lipid peroxidation products, as measured by the lowering of TBARS in treated rats, compared with the CCl₄-intoxicated group. The reduction of TBARS was significant (p < 0.01) from 183% to 156% for GBH (245 mg/kg), and standard drug, silymarin, significantly (p < 0.001) reduced TBARS from 183% to 129%. GBH extracts at 122.5 mg/kg and 490 mg/kg partially restored antioxidant markers to limit the CCl₄ induction of TBARS, but these improvements did not reach significance.

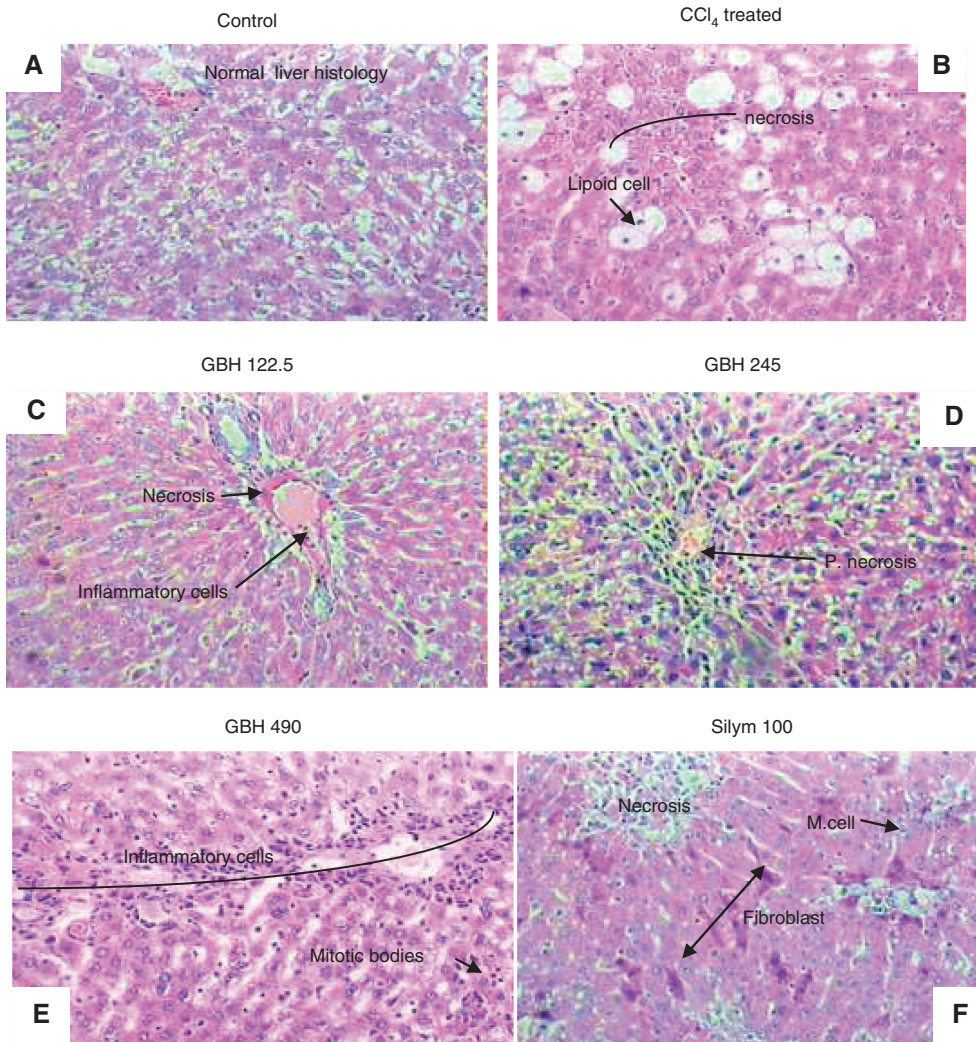


Figure 3: Effect of GBH extract on liver histopathology.

(A) Normal liver tissue displaying a central vein with portal tract radiating numerous normal sinusoids and hepatocytes (control); (B) lipid necrosis at centrolobular (zone 3) region, and mid-portions of the hepatic lobules are completely necrotic (CCl_4 treated); (C) mild centrolobular necrosis with few infiltration of inflammatory cells (GBH 122.5 mg/kg); (D) congestion of central vein with periportal necrosis (GBH 245 mg/kg); (E) numerous infiltration of inflammatory cells within the portal tract and some at the stroma. There exists the presence of mitotic bodies at various zones of the liver indicating regeneration process (GBH 490 mg/kg); (F) area of severe necrosis with numerous fibroblast and mitotic bodies showing repair process (silymarin 100 mg/kg).

Discussion

The liver is involved in numerous metabolic and detoxification functions as well as having a role in the performance, maintenance, and regulation of body homeostasis. The liver is predisposed to various insults arising from metabolic disorders, viral infections, inflammation (hepatitis), alcohol, and drug-induced liver injury [29]. Our initial toxicity analyses established that the GBH leaf extract displayed a wide safety margin above 1000 mg/kg. Weight and hematological indices from pretreatment of rats with GBH leaf extract did not

produce any concentration-dependent effects. CCl_4 is a potent hepatotoxic agent, and injury induced by CCl_4 is extensively used as biomarker for the screening of the hepato-protective and antioxidant profile of drugs and plant extracts [30]. Metabolism of CCl_4 produces highly toxic free radicals ($\text{CCl}_3\cdot$) that covalently bind to cellular components, abstracting hydrogen from fatty acids and initiating lipid peroxidation. These radicals also lead to an inhibition of lipoprotein secretion and also react with free oxygen to form $\text{CCl}_3\text{-OO}\cdot$ radicals which further exacerbate lipid peroxidation, promoting cellular injury and liver damage [31].

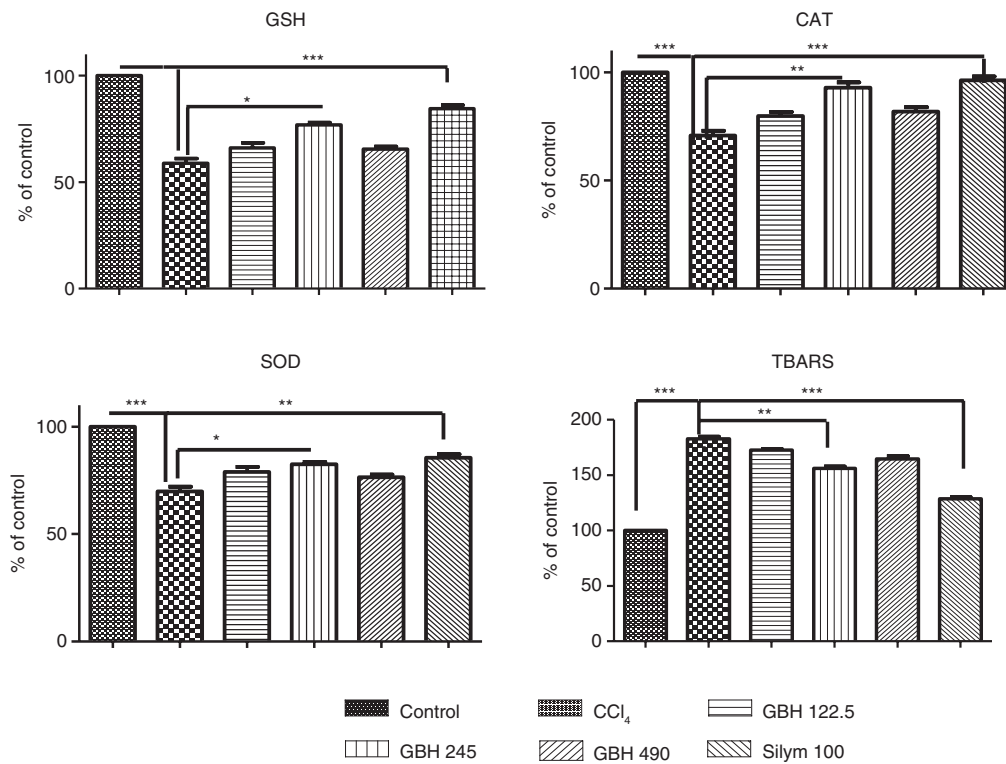


Figure 4: Effects of GBH leaf extract on markers of oxidative stress.

Oxidative stress markers (GSH, CAT, SOD, and TBARS) were measured in homogenized liver samples. Histograms are results displayed relative to CCl₄-treated values, with significant changes from controls marked with asterisks. For significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. GSH, glutathione; CAT, catalase; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances.

CCl₄ treatment exhibited marked effects on lipid metabolism, with serum triglyceride levels increased by ~50% (although not significantly), serum cholesterol levels significantly increased by ~2.1-fold, and serum LDL levels significantly increased by ~5-fold. The rise in the levels of serum TBIL and CBIL observed in the CCl₄ group usually reflects liver damage [29, 32]. Likewise, liver enzymes (AST, ALT, and ALP) are useful biomarkers of liver health, with circulatory levels elevated as a consequence of liver damage such as via CCl₄-induced hepatotoxicity [33–36]. Serum ALB and TP levels were also reduced after CCl₄ intoxication, indicative of severe liver injury or cirrhosis and biliary damage [37, 38].

Pretreatment of rats with the GBH extracts lowered the CCl₄-induced elevations of ALT, AST, and ALP, with the extent of response reflective of GBH dose. Similarly, GBH extracts restored circulatory levels of ALB and TP to approaching control levels and lowered the levels of circulatory bilirubin, indicative of hepato-protection. This hepato-protection was also evidenced by the improvement to the CCl₄-induced histo-pathological damage to the liver cyto-architecture with pretreatment of rats with GBH extracts. In accordance with our results, livers from

CCl₄-intoxicated rats display necrosis, fatty accumulation, disappearance of nuclei, and extensive vacuolization and infiltration of inflammatory cells [33–42]. By contrast, after pre-incubation with GBH extracts or silymarin, the hepatocytes normalized with the presence of mitotic bodies, and numerous fibroblasts were present at various zones of the liver indicating a regeneration process. An improvement of liver function would be expected after restoration of functional integrity and stability of the cellular membranes [43]. Serum levels of transaminases return to normal with healing of hepatic parenchyma and the regeneration of hepatocytes [37, 38].

Oxidative stress is generally an undesired product of hepatic toxins such as CCl₄. Oxidative damage can be efficiently alleviated by boosting the innate antioxidant system that is otherwise diminished following CCl₄ intoxication with associated free radical damage and lipid peroxidation [44]. Our results show that CCl₄ challenge significantly decreased the levels of GSH and activities of CAT and SOD, with an associated elevation of TBARS. However, treatment with GBH leaf extracts significantly elevated GSH, CAT, and SOD activities and hence ameliorated the elevation of TBARS.

Phytochemical analyses revealed the presence of alkaloids and cardiac glycosides and moderate quantities of saponins and tannins but an absence of flavonoids and anthraquinones. The phytochemicals present can display free-radical scavenging actions and alter gene expression to combat hepatotoxicity [45–47]. The phytochemicals present within the GBH leaf extract could act singly, additively, or even synergistically to produce the hepato-protective effects we have observed.

Conclusions

Our results suggest that the mechanism of hepato-protection demonstrated with the GBH leaf extracts against CCl_4 -induced liver damage is in part due to a reduction of lipid peroxidation and improvement of the cellular antioxidant system (GSH levels and CAT and SOD activities), thereby resisting cellular oxidative injury. Our histopathological analyses corroborate this posit, with less CCl_4 -induced centrilobular damage and the detection of restorative and regenerative processes. *Glyphaea brevis* may therefore provide a useful and viable source of bioactive compounds that could be exploited for the treatment and management of liver damage. Further studies regarding the isolation and characterization of the active agent(s) responsible for hepato-protective properties are in progress in our laboratory.

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