American Journal of Biomedical Sciences
ISSN: 1937-9080
nwpii.com/ajbms

Hepatoprotective Effect of *Raphiostylis Beninensis* Ethanol Root Extract on Carbon-tetrachloride (CCl_4)-induced Liver Attack and Damage in Rats

Usunobun U.,[1*] Osaigbovo J. Ovbokhan and Okolie N. Paulinaus[2]

[1] Department of Biochemistry, Faculty of Basic Medical Sciences, Edo University Iyamho, Edo State, Nigeria
[2] Department of Biochemistry, Faculty of Life Sciences, University of Benin, Benin City, Nigeria
[*]**Corresponding Author**
Usunobun Usunomena
Department of Biochemistry,
Faculty of Basic Medical Sciences,College of Medical Sciences
Edo University Iyamho,Edo State
Nigeria
Email: usunobun.usunomena@edouniversity.edu.ng
Phone: +2348034174871

Received:24 April 2020; | Revised:10 May 2020; | Accepted:06 September 2020

Abstract

This study was conducted to evaluate hepatoprotective effect of ethanol root extract of *Raphiostylis beninensis*, a plant with several potential therapeutic benefit on Carbon tetrachloride (CCl_4)-induced liver damage in rats. Thirty (30) adult male rats weighing 190-200g divided into five (5) groups of six (6) rats each were used. First group received distilled water and served as normal control. Second group received 1ml/kg CCl_4 for 4days. Third and fourth group received 1ml/kg CCl_4 for 4 days prior to treatment with 150mg/kg and 300mg/kg extract for 8 days respectively. The last group received 1ml/kg CCl_4 for 4 days prior to treatment with 100mg/kg Silymarin. Twenty-four hours after last administration, the rats were sacrificed, blood samples and liver tissues obtained for biochemical assays and histology. Biochemical assays showed liver function enzymes/molecules: asparatate aminotransferase (AST), alanine aminotransferase(ALT), alkaline phosphatase(ALP), lactate dehydrogenase(LDH) and total bilirubin to be significantly increased while liver synthetic molecules (total protein and albumin) where decreased significantly in CCl_4 treated group when compared to control group whereas treatment with *Raphiostylis beninensis* caused a significant decrease in liver function enzymes/molecules (AST, ALT, ALP, LDH and total bilirubin) and an increase in liver synthetic molecules (total protein and albumin) when compared to rats which received CCl_4 alone. Oxidative stress assessment using superoxide dismutase (SOD), catalase (CAT), Glutathione peroxidase (GPx) and Malondialdehyde (MDA) showed significantly decreased SOD, CAT and GPX and a significantly increase MDA in CCl_4-alone group, when compared to control group whereas treatment with *Raphiostylis beninensis* caused significantly increased SOD, CAT and GPX and a decreased MDA when administered with CCl_4 compared to rats which received CCl_4 alone. Histopathologically, *Raphiostylis beninensis* showed reduction in hepatocyte damage, recovery, protection against CCl_4 caused degeneration and necrosis, inflammation and nucleus vacuolation. This study showed

that *Raphiostylis beninensis* reduced oxidative stress by decreasing free radical generation due to its enhancement of antioxidant activity.

Keywords: Carbon tetrachloride, Free radical, Oxidative stress, *Raphiostylis beninensis*, Silymarin

1. Introduction

A decoction of *Raphiostylis beninensis* leaf (and stem) usually taken or drunk by the Yorubas to cure *aiperi* (convulsions) and to treat *afun*, a sickness which makes the skin whitish and transparent in up to 1 year old children [1]. The leaf is normally boiled and the liquid used as a mouthwash in Sierra Leone [2-3], while in Igbo land, the preparation is used to wash sores [2,4]. The root, stem and leaf are decocted in Ghana and Nigeria and the liquor drunk to kill and expel roundworm [1]. Apart from the presence of anthraquinones, cardiac glycosides, flavonoids and triterpenes [5], anti-microbial activity [4,6], cytotoxic activity [7-8], analgesic and anti-inflammatory effects of the root extract [5,9] have been reported of *Raphiostylis beninensis* by various researchers.

Carbon tetrachloride (CCl_4), a highly toxic chemical and one of the most widely used agents for experimental induction of liver injury on animals [10] undergoes transformation to form trichloromethyl free radical which oxidizes cellular macromolecules such as lipids, proteins, and DNA [11] initiating cell damage via covalent binding to membrane proteins and lipid peroxidation [12]. This study was put therefore forward to investigate the protective and anti-oxidative effect of ethanol root extract of *Raphiostylis beninensis* against CCl_4-induced hepatocellular damage and oxidative stress in rats *in vivo*.

2. Materials and Method

2.1 Collection/preparation of plant materials and extraction

Fresh, mature root of *Raphiostylis beninensis* were dug out from under the tree in a garden at Ikpoba Hill, Benin City, Edo state, Nigeria. Thereafter, the plant was authenticated at the Department of Plant Biology and Biotechnology, University of Benin, Nigeria and voucher specimen of the plant $UBHR_b4394$ was deposited at the herbarium of same department. Fresh root of *Raphiosylis beninensis* were sorted, air dried at room temperature and milled into powder and weighed (1700 g). The crushed samples were stored in air-tight sterile containers and used for extraction. Six (6) liters of absolute ethanol was added to the crushed sample (1600) and allowed to stand for 48 hours. It was filtered using filter paper and re-filtered using cotton wool to ensure purity and thereafter extract evaporated to dryness in a rotary evaporator at 40° C to one - tenth volume and then freeze-dried at 37 ° C. The freeze-dried ethanol extract was stored in well-sealed containers and kept in a refrigerator at 4℃ to protect it from light and moisture till it was used.

2.2 Chemicals

Silymarin, Hydrogen peroxide, KMnO4, Epinephrine, thiobabituric acid, Carbon-tetrachloride, Absolute ethanol (99.8%) were purchased from Sigma-Aldrich (USA). Biochemical assays kits were obtained from Randox Diagnostics (Randox, United Kingdom).

2.3 Experimental animals, design and blood collection

Adult male albino rats purchased from Anatomy department, University of Benin were allowed to acclimatize for 7 days, and maintained under standard photoperiodic conditions in the animal house of the Department of Biochemistry, University of Benin. The rats were fed with feeds (grower mash), ethanol root extract of *Raphiostylis beninensis* (orally, using an oral gavage), and food and water (for the control) for the duration of the experiment. Ethical approval was granted and all animals were humanely handled in accordance with ethical guideline for care and use of laboratory animals.

Thirty (30) adult male rats weighing 190-200g divided into five (5) groups of six (6) rats each were used. Group 1 served as normal control and received distilled water. Second group was given Carbon tetrachloride (CCl_4) for 4 days while third and fourth group received CCl_4 for 4 days prior to

treatment with 150mg/kg and 300mg/kg *Raphiostylis beninensis* extract for 8 days respectively. The last group received CCl_4 for 4 days prior to treatment with 100mg/kg Silymarin, standard hepatoprotective drug). With exception of normal control rats, all rats received a mixture of freshly prepared CCl_4 in olive oil (1ml/kg, 1:1 intraperitoneally) for 4 days prior to administration of *Raphiostylis beninensis* ethanol extract or Silymarin for 8 days. Twenty-four (24) hours after last administration, rats from each group were sacrificed by cervical dislocation and blood samples obtained through heart puncture via a syringe into sample bottles containing no anticoagulant. The blood samples were allowed to clot and subsequently centrifuged at 5000rpm for 20mins at room temperature to obtain serum for biochemical assays. The extract dissolved in distilled water was administered by oral gavage for 8 days.

2.4 Tissue collection and preparation of liver homogenate

The liver were excised, rinsed with normal saline, placed in plain containers and stored in ice at -4℃. A 10% liver homogenate was prepared using physiological saline and the homogenates centrifuged at 5000rpm for 10 minutes while the supernatant obtained was used for determination of Malondialdehyde (MDA), Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx). The other part of the liver was place in formalin for histological analysis

2.5 Biochemical assays

Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) were determined in serum using RANDOX Kit according to the manufacturer's instructions. Using RANDOX kit, Serum total protein was determined according to method of Tietz [13] method while serum albumin was determined based on the Bromocresol green (BCG) method as described by Doumas et al [14]. Serum Total bilirubin was determined according to the method of Jendrassik and Grof [15] using the RANDOX Kit. Glutathione peroxidase (GPx) was determined according to method of Nyman [16] based on the oxidation of pyrogallol to purpurogallin by peroxidase activity, resulting to a deep brown color disposition, read at 430nm. MDA was estimated in a colorimetric reaction with thiobarbituric acid according to method of Ohkawa et al., [17]. Superoxide dismutase (SOD) was determined according to the method of Misra and Fridovich [18] based on the ability of the enzyme to inhibit the autooxidation of epinephrine. Catalase (CAT) was determined colorimetrically according to the method of Cohen et al., [19] based on the measurement of the rate of decomposition of H2O2 after the addition of the sample containing the enzyme by reacting it with excess KMnO4 and then measuring the residual KMnO4 spectrophotometrically at 480nm.

2.6 Histology

Liver sections fixed in formol-saline were processed for light microscopy and resultant slides were read and interpreted by a consultant pathologist at the College of Medicine, University of Benin.

2.7 Statistical analysis

Data obtained from this study were expressed as mean value ± standard deviation. Differences between means of control and tested groups were determined by one way ANOVA using Statistical Package for social scientist (SPSS). The mean differences were compared with the Duncan multiple range test. A probability level of less than 5% ($p<0.05$) was considered significant.

3. Results

In this study, as shown in table 1, CCl_4 alone administration caused elevation in liver function enzymes such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) compared to Sylimarin and *Raphiostylis beninensis* treated rats. However at the end of treatment with *Raphiostylis beninensis* (150mg/kg or 300mg/kg), as shown in table 1, the levels of AST, ALT, ALP, and LDH showed towards normal level. The standard drug Silymarin treated group also showed near normal level.

Table 1: Effect of ethanol root extract of *Raphiostylis beninensis* (RB) treatment on liver function enzymes (AST, ALT, ALP and LDH) in CCl_4-administered male Wistar rats

Treatment	AST (U/l)	ALT (U/l)	ALP (U/l)	LDH (U/l)
Control	101.65±3.01[a]	101.53±3.01[a]	91.42±1.99[a]	194.87±10.94[a]
CCl_4 only	209.70±3.73[b]	189.97±3.24[b]	202.47±4.08[b]	976.73±30.05[b]
CCl_4+150mg/kg RB	141.93±2.59[c]	143.45±1.65[c]	138.97±4.75[c]	322.03±13.16[c]
CCl_4+300mg/kg RB	138.92±2.86[c]	141.46±3.73[c]	137.08±3.32[c]	339.07±11.07[c]
CCl_4 + 100mg Silymarin	144.75±5.36[c]	133.77±2.08[c]	135.55±3.58[c]	305.54±10.25[c]

Data are presented as mean± SD; Values with different superscript letters (a, b, c) in each column (between groups) differ significantly, ($p<0.05$). $P<0.05$ was set as significant. [c]: significant compared to CCl_4 alone group; [a]: significant compared to CCl_4 alone group, CCl_4 + 150mg/kg RB group and CCl_4 + 300mg/kg RB group and CCl_4 +Silymarin group

The effect of *Raphiostylis beninensis* (RB) ethanol root extract on liver synthetic molecules in CCl_4-induced liver attack and injury was also evaluated by determining the levels of total protein, albumin and total bilirubin as shown in table 2. Total bilirubin was found to be increased significantly following CCl_4 administration and toxicity whereas *Raphiostylis beninensis* (RB) administration at a dose of 150mg/kg and 300mg/kg significantly decreased the level of total bilirubin that was released into serum as a consequence of CCl_4-induced hepatic injury. Also, total protein and albumin levels were significantly lowered following CCl_4 administration when compared with Sylimarin and *Raphiostylis beninensis* treated rats. However, *Raphiostylis beninensis* (RB) administration at 150mg/kg and 300mg/kg significantly increased ($p<0.05$) total protein and albumin when compared to CCl_4 alone administered rats.

Table 2: Effect of ethanol root extract of *Raphiostylis beninensis* (RB) treatment on liver synthetic molecules (Albumin, Total protein and Total bilirubin) in CCl_4-administered male wistar rats

Treatment	Albumin (g/dl)	Total Protein (g/dl)	Total Bilirubin (mg/dl)
Control	7.51±0.10[a]	31.26±0.45[a]	0.48±0.12[a]
CCl_4 only	2.13±0.04[b]	11.04±1.38[b]	3.19±0.15 [b]
CCl_4+150mg/kg RB	5.34±0.05[c]	20.05±0.41[c]	1.05±0.22[c]
CCl_4+300mg/kg RB	5.34±0.04[c]	20.48±0.56[c]	0.99±0.42[c]
CCl_4 + 100mg/kg Silymarin	5.89±0.12[c]	23.61±1.14[c]	1.03±0.01[c]

Data are presented as mean± SD; Values with different superscript letters (a, b, c) in each column (between groups) differ significantly, *(*$p<0.05$). $P<0.05$ was set as significant. [c]: significant compared to CCl_4 alone group; [a]: significant compared to CCl_4 alone group, CCl_4 + 150mg/kg RB group and CCl_4 + 300mg/kg RB group and CCl_4 +Silymarin group

Antioxidant enzymes activity was measured in all studied groups including control as shown in table 3. The SOD, CAT and GPx activity had a significant decrease in the CCl_4 alone group compared to all other groups ($P<0.05$). However, there was significant increase in SOD, CAT and GPx in CCl_4 group receiving Silymarin (100mg/kg) or *Raphiostylis beninensis* for 8days at 150mg/kg and 300mg/kg compared to the CCl_4 alone group (table 3). On oxidative stress, our results showed markedly increased MDA levels in CCl_4 alone group compared to other studied groups control whereas administration of *Raphiostylis beninensis* for 8days attenuated MDA levels and brought it towards normal.

Table 3: Effect of ethanol root extract of *Raphiostylis beninensis* (RB) treatment on oxidative stress and antioxidant status in CCl_4-administered wistar rats

Treatment	SOD (U/mg wet tissue)	CAT (U/mg wet tissue)	GP_X (U/mg wet tissue)	MDA (U/mg wet tissue)
Control	87.24±5.11[a]	5.62±0.21[a]	7.99±0.17[a]	2.16±0.10[a]
CCl_4 only	16.33±1.11[b]	0.93±0.30[b]	1.01±0.05[b]	9.01±0.20[b]
CCl_4+150mg/kg RB	47.97±4.74[c]	3.21±0.38[c]	5.02±0.51[c]	4.10±0.58[c]
CCl_4+300mg/kg RB	48.67±9.76[c]	3.00±0.40[c]	5.17±0.41[c]	4.05±0.62[c]
CCl_4 + 100mg/kg Silymarin	42.55±4.24[c]	3.51±0.33[c]	5.33±0.37[c]	3.94±0.64[c]

Data are presented as mean± SD; Values with different superscript letters (a, b, c) in each column (between groups) differ significantly, *(*$p<0.05$). $P<0.05$ was set as significant. [c]: significant compared to CCl_4 alone group; [a]: significant compared to CCl_4 alone group, CCl_4 + 150mg/kg RB group and CCl_4 + 300mg/kg RB group and CCl_4 +Silymarin group

On histopathological study, vivid Examination of normal liver section microscopically showed normal tissue architecture, intact parenchymal cells, compactly arranged cells with nucleoli and well fenestrated hepatocytes (Plate A). Liver cell degeneration and necrosis, presence of inflammation, vacuolation of nucleus, fatty changes and loss of cellular boundaries were seen in CCl_4 alone administered rats (Plate B). In rats treated with *Raphiostylis beninensis* ethanol extract at 150mg/kg and 300mg/kg (Plate C and Plate D), there was minimal changes in liver degeneration, minimal swelling, and evidence of reduction in tissue damage compared to Plate B (CCl_4 alone rats). *Raphiostylis beninensis* treatment at 150mg/kg and 300mg/kg showed comparable protection to that offered by silymarin (Plate E).

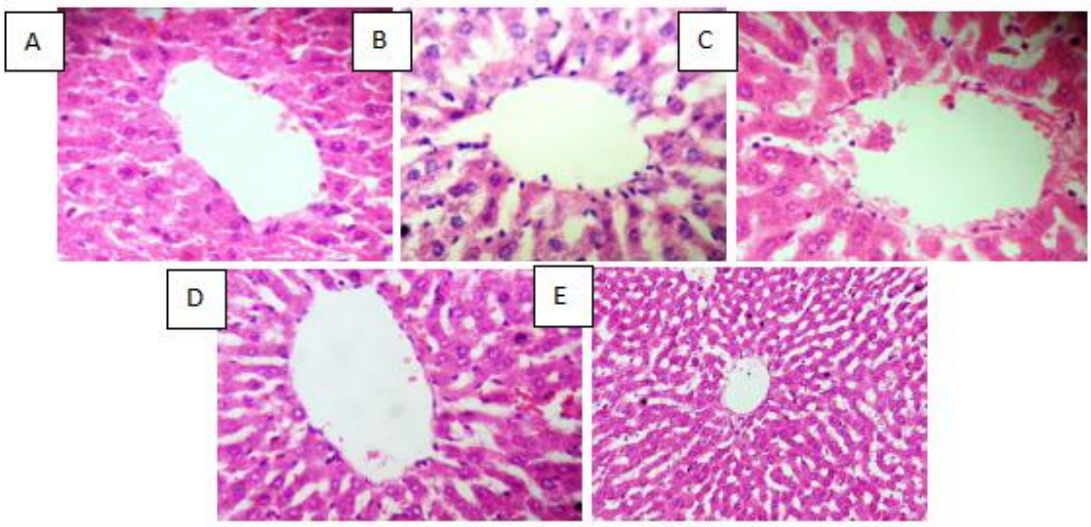

Plate A: Photomicrograph section of the liver of control rat reveals compactly arranged hepatocytes, prominent centriole and intact parenchymal cells.
Plate B: Photomicrograph section of the liver of CCl_4 –treated rat showing necrosis, vacuolation and inflammation
Plate C: Photomicrograph section of the liver of rats given CCl_4 + 150mg/kg *Raphiostylis beninensis* extract showing prominent centriole and hepatocytes with mild swelling and minimal degeneration
Plate D: Photomicrograph section of the liver of rats given CCl_4+300mg/kg *Raphiostylis beninensis* extract showing visible centriole and hepatocytes with minimal inflammation and mild swelling
Plate E: Photomicrograph section of the liver of rats given combination of CCl_4 + Silymarin showing Prominent centriole and hepatocytes with mild and less necrotic damage to the liver

4. Discussion

Liver Fibrosis, Cirrhosis and even liver cancer can occur due to long existence of hepatic injury, thus making liver injury prevention and treatment a key to treating liver diseases [20]. The CCl_4-intoxicated rat model has been widely used for decades to investigate the mechanisms of acute and chronic liver injuries depending on the dose and frequency of injection [21]. The liver is the main target of detoxification and certain drug uptake produce a substantial hepatic shut-off due to prooxidant reactive oxygen species (ROS) production which then activate cellular defect with attack on biomolecules including DNA, Protein and lipids [22]. CCl_4 is characterized by its ability to produce CCl_3 which as a free radical alkylates cellular proteins, leading to liver damage, necrosis and cirrhosis [23]. On free radical induced oxidative stress and antioxidant status, lipid peroxidation (LP), expressed as malondialdehyde (MDA), increased significantly in CCl_4 toxicity whereas, the activity of antoxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) in liver were decreased following CCl_4 administration (Table 3). The high level of MDA and reduced SOD, CAT and GPx activities indicates free radical and reactive oxygen species generation due to CCl_4 induced toxicity. However, Silymarin and *Raphiostylis beninensis* administration for 8 days at 150mg/kg and 300mg/kg markedly increased the activities of these free radical scavenging enzymes, SOD, CAT and GPx when compared to CCl_4 alone administered rats as there were significant reversals towards normal levels thus conferring the anti-lipid peroxidative ability of *Raphiostylis beninensis*. Antioxidant enzymes such as SOD, GPx and CAT play the role of free radical scavengers [24]. Free radicals, including superoxide anion, radical hydroxyl and H_2O_2 [25] are the major substrates for antioxidant enzymes such as SOD, CAT and GPx [26]. After CCl_4 administration and toxicity, SOD, CAT and GPx activity were reduced as a result of increased free radical formation. However *Raphiostylis beninensis* extract administration for 8 days in comparison to CCl_4 alone studied group caused an increase in SOD, CAT and GPx activity by reducing hydroxyl radical, superoxide anion and H_2O_2 formation.

A measure of liver function enzyme levels in serum indicates the extent of liver damage. These liver function enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) mainly concentrated in liver enter circulation upon damage to the liver. In this study, AST, ALT, ALP and LDH were significantly higher in CCl_4 alone treated rats, an indication of hepatic attack and injury, which then caused the cellular enzyme leakage into the blood, thus the seen high AST, ALP, ALP and LDH in blood. It is a known fact that whenever there is damage to liver cell membrane, several enzymes normally located in the cytosol are released into the circulation. However, administration of Silymarin and *Raphiostylis beninensis* (150mg/kg and 300mg/kg) for 8 days to CCl_4 intoxicated rats caused a significant decrease in activities of AST, ALT, ALP and LDH indicating protective effect in improving liver cell functional integrity. The decrease in AST, ALT, ALP, LDH and bilirubin by *Raphiostylis beninensis* indicates plasma membrane stabilization and hepatocyte protection against CCl_4-induced damage. In our previous related study, hepatoprotective activity of Celosia argentea leaf extract was confirmed against CCl_4-induced hepatic injury in rats [10].

It is known that abnormal bilirubin increase in blood indicates hepatobiliary disease and severe hepatocellular function disturbance [27]. In this study, administration of *Raphiostylis beninensis* (150mg/kg and 300mg/kg) for 8 days to CCl_4 intoxicated rats showed significant decrease in level of bilirubin, an indication of protection against hepatic damage caused by CCl_4. Severe liver damage is known to decrease production of various proteins resulting in reduced serum levels of total protein, albumin, and/ or globulin [28-29]. Also decrease in protein production can cause other abnormal test values for instance depletion of coagulation factors may result in prolonged prothrombin or activated partial thromboplastin times [30]. Albumin is one of several proteins made in the liver and needed by the body to fight infections and to perform other functions such as transportation in the blood [31]. Thus, in this study, the decrease in total protein and albumin could be

due to CCl_4-induced hepatic attack and damage which then caused decrease ability of protein production, thus inhibited synthesis of albumin and compromised the rats' immune response. However, *Raphiostylis beninensis* (150mg/kg and 300mg/kg) for 8 days to CCl_4 intoxicated rats was able to offer hepatoprotection, thus the observed significant reversal to near normal in total protein and albumin.

The protective effect of *Raphiostylis beninensis* can be attributed to its ability to inhibit free radicals and ROS generation caused by CCl_4 toxicity. The protection may be due to the presence of phyto-agents and antioxidants in *Raphiostylis beninensis*. Our finding also supports previously published related hepatoprotective studies which elucidated similar liver toxicants and medicinal leaf protection [32-39]. In conclusion, this study showed that *Raphiostylis beninensis* reduced oxidative stress by decreasing free radical generation as well as enhancing SOD, CAT and GPx activity. Thus the hepatoprotective effects of *Raphiostylis beninensis* is because of its antioxidant activity as a free radical scavenger.

Conflicts of Interest

The authors hereby declare no conflicts of interest.

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