HEPATOPROTECTIVE AND ANTIOXIDANT EFFECT OF *RHAPHIOSTYLIS BENINENSIS* ETHANOL ROOT EXTRACT ON CARBON TETRACHLORIDE (CCL_4)-INDUCED HEPATOTOXICITY AND OXIDATIVE STRESS

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ABSTRACT

This study was conducted to evaluate the hepatoprotective and antioxidative effects of ethanolic root extract of Rhaphiostylis beninensis on liver function, cell integrity and survival of male albino rats exposed to carbon tetrachloride (CCl_4). Administration of CCl_4 at 1ml/kg body weight of rat for four (4) days induced hepatotoxicity, cellular attack and damage, while R. beninensis ethanolic root extract was administered (150 and 300 mg/kg) for 4 days. CCl_4-induced hepatotoxicity significantly increased ($p<0.05$) alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total bilirubin (TB) and malondialdehyde (MDA) and significantly decreased albumin (ALB), total protein (TP), glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) levels. R. beninensis-treated groups had significantly decreased ($p<0.05$) ALT, AST, ALP, LDH, AST, TB and MDA levels, and significantly increased ($p<0.05$) ALB, TP, GPx, SOD and CAT levels. Histologically, CCl_4 caused necrosis, inflammation, severe dilatation, congestion of blood vessels, vacuolation and hepatocellular degeneration, whereas R. beninensis administration on CCl_4 exposed rats showed regions of recovery and significant hepatoprotection against CCl_4-induced hepatic attack and damage as well as absence of vacuolation. In conclusion, this study demonstrated that R. beninensis root extract offered hepatoprotection against carbon tetrachloride (CCl_4)-induced hepatotoxicity by improving/enhancing liver function and cellular integrity as well as the antioxidative status of the liver.

Keywords: Carbon tetrachloride, Ethanol, Antioxidant, Oxidative stress, *Rhaphiostylis beninensis* root

INTRODUCTION

Toxicity of carbon tetrachloride (CCl_4), a well-known industrial solvent results from CCl_4 bioactivation into trichloromethyl (CCl_3) free radical by cytochrome P450 system in liver microsomes causing lipid peroxidation of membranes thereby leading to liver damage. Liver is not the only target organ of CCl_4 but it also affects several other organs of the body including lungs, hearts, testes, kidneys and brain (Ogeturk *et al.,* 2004).

Modern medicines have very little to offer in treatment and alleviation of liver diseases and attention is now shifted to plant based preparations for their treatment (Muthulingam, 2008), because the available synthetic drugs for treating liver disorders further damage the hepatic cells. Antioxidants play a significant role protecting the liver from toxic effect of various chemicals by preventing free radical formation (Sheweita *et al.,* 2001). Series of research are now been done on plants sources and a large number of medicinal plants

have so far affirmed the importance of herbs for medicinal remedies (Alrheam, 2015). Hence, standardized experimental procedures should be employed to test the efficacy of medicinal plants.

Rhaphiostylis beninensis (Hook.f. ex Planch) has many Nigerian vernacular names such as *Atapata* (Yoruba), *Usuende* (Binis), *Kpolokoto* (Igbo), *Umeni* (Urhobos) and *kumeni* (Itsekiris) and possess mosquito-repellant property according to the natives of Cote d'Ivoire (Adjanohoun and Ake, 1979; Lasisi *et al.,* 2013). The root, stem and leaves are boiled and drunk to kill and expel round worm (Adjanohoun and Ake, 1979). In South Western Nigeria, *R. beninensis* is reportedly used as a tonic for children between the ages of two to three years, and for treating a disease called *afun*, a diseased condition where the entire skin turns white (Lasisi *et al.,* 2013). Apart from the presence of phytochemicals such as anthraquinones, cardiac glycosides, flavonoids and triterpenes (Ofeimun and Onwukeame, 2006), anti-microbial activity against Gram-positive and Gram-negative bacteria as well as fungi have been demonstrated (Edema *et al.,* 2009; Adebayo-Tayo *et al.,* 2010), cytotoxic activity against brine shrimp (Lasisi *et al.,* 2013) as well as analgesic and anti-inflammatory effects of the root extract (Ofeimun and Onwukeame, 2006; Ofeimun *et al.,* 2014) have been reported of *R. beninensis* by various researchers. This study is aimed at evaluating hepatoprotective and antioxidative effect of ethanol root extract of *R. beninensis* on liver function, integrity and survival in rats exposed to CCl_4.

MATERIALS AND METHODS

Ethanolic Root Extract of *R. beninensis:* Fresh mature root of *R. beninensis* were dug out from under the tree in a garden at Ikpoba Hill, Benin City, Edo state, Nigeria. Thereafter, the plant was identified (TPL, 2010) and authenticated by a plant taxonomist 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 *R. 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 litres of absolute ethanol was added to the crushed sample (1600 g) and allowed to stand for 48 hours. It was filtered using filter paper and re-filtered using cotton wool to ensure purity and thereafter the extract evaporated to dryness in a rotary evaporator at 40ºC to one – tenth volume and then freeze-dried. The freeze-dried extract was stored in well-sealed containers and kept in a refrigerator at 4ºC to protect it from light and moisture till it was used. The phytochemical properties of *R. beninensis* to include: anthraquinones, cardiac glycosides, flavonoids and triterpenes have previously been reported by Ofeimun and Onwukeame, (2006) as well as the non-toxic nature of *R. beninensis* have been reported by Iwueke *et al.* (2011) and Ofeimun and Ayinde (2017) even at 3808 mg/kg.

Animals and Experimental Design: Thirty adult male albino rats weighing 190 – 200 g purchased from Anatomy Department, University of Benin were allowed to acclimatize for 7 days in the Animal House of the Department of Biochemistry, University of Benin and maintained under standard conditions, provided pelleted grower's mash (containing 18 % crude protein and 2600 Kcal/kg metabolizable energy, Guinea Feed, Nigeria PLC) and drinking water *ad libitum*. A daily cycle of 12 hours of light and 12 hours of darkness was maintained throughout the experimental period. The rats were randomly assigned to five treatment groups, replicated twice with each replicate having three albino rats. This study was carried out in accordance with the guidelines for ethical conduct in the care and use of nonhuman animals in research (APA, 2012).

One group received distilled water and served as normal control. Second group received carbon tetrachloride (CCl_4) for 4 days. Third and fourth group received CCl_4 for 4 days prior to treatment with 150 and 300 mg/kg extract for 4 days respectively. The last group

received CCl_4 for 4 days prior to treatment with Silymarin (100 mg/kg). With exception of normal control rats, all rats received a mixture of freshly prepared CCl_4 in olive oil (1 ml/kg, 1:1 intraperitoneally) for 4 days prior to administration of *R. beninensis* ethanol extract or Silymarin. The dosage of 150 and 300 mg/kg were used based on previous non-toxic nature of *R. beninensis* reported by Iwueke *et al.* (2011) and Ofeimun and Ayinde (2017) even at 3808 mg/kg.

Twenty four hours after last administration, rats from each group were placed under chloroform aesthesia and blood collected through heart puncture via a syringe into sample bottles containing no anticoagulant. The blood samples were allowed to clot and subsequently centrifuged at 5000 rpm for 20 minutes at room temperature to obtain serum for biochemical assays.

Preparation of Liver Homogenate: The liver were excised, rinsed with normal saline, placed in plain containers and stored in ice at -4^{o}C. A 10 % liver homogenate was prepared by crushing 1 g liver tissue in 8 ml physiological saline with a mortar and pistle and adding more 2ml physiological saline to make it 10 ml. The homogenates was centrifuged at 5000 rpm for 20 minutes. The supernatant obtained was used for determination of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). The left over part of the liver was fixed in 10 % buffered formalin for histological analysis.

Hepatoprotective Activity Assay: Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) was determined according to the method of Reitman and Frankel (1957). Alkaline phosphatase (ALP) assay was carried out according to method of Kind and King (1954), while lactate dehydrogenase was determined according to the method of Spiegel *et al.* (1972). Lowry *et al.* (1951) method was used for Total protein determination, while Doumas *et al.* (1971) method was used for albumin determination. Total bilirubin was determined according to the method of Jendrassik and Grof (1938). Test kits from Randox Laboratories, United Kingdom were used in the assays determination.

***In Vivo* Antioxidant Assay**: Superoxide dismutase (SOD) was determined according to the method of Misra and Fridovich (1972) based on the ability of the enzyme to inhibit the autoxidation of epinephrine. Catalase (CAT) was determined colorimetrically according to the method of Cohen *et al.*(1970) based on the measurement of the rate of decomposition of H_2O_2 after the addition of the sample containing the enzyme by reacting it with excess $KMnO_4$ and then measuring the residual $KMnO_4$ spectrophotometrically at 480 nm. Glutathione peroxidase (GPx) was determined according to method of Nyman (1959) based on the oxidation of pyrogallol to purpurogallin by peroxidase activity, resulting to a deep brown color disposition, read at 430 nm. Malondialdehyde (MDA) was estimated in a colorimetric reaction with thiobarbituric acid according to method of Ohkawa *et al.* (1979).

Histopathological Examination: Based on Bancroft and Gamble (2002) method, the livers removed from the animals and the liver tissues were fixed in buffered formol saline (10 %) and dehydrated in grades of ethanol in ascending order, followed by overnight clearance of the liver tissues in chloroform, infiltration and embedment in molten paraffin wax. Trimmed blocks were thereafter sectioned at 5 microns and the sections deparaffinized in xylene before being mounted on clean slides and thereafter stained with Haematoxylin and Eosin (H and E). Olympus/3H light microscope was then used for examination and the liver photomicrographs captured using a Moticam Images Plus 2.0 digital fitted to the light microscope.

Statistical analysis: The data obtained from this study were analysed using analysis of variance (ANOVA) and the results expressed as Mean ± SE. Statistical significance between means were separated using Duncan multiple range test. The level of significance was $p<0.05$. All statistical analyses were done using GraphPad Prism 6.05.

RESULTS

Liver function assays shown in Table 1 revealed that serum levels of AST, ALP, ALT and LDH were significantly increased in CCl_4 alone group, when compared to *R. beninensis* and control treated groups. However, animals given CCl_4 and treated with ethanolic root extract of *R. beninensis* for 4 days at 150 and 300 mg/kg caused a significant decrease in AST, ALT, ALP and LDH activities, when compared to rats which received CCl_4 alone.

Liver synthetic molecules assays shown in Table 2 indicated that the total protein (TP) and albumin (ALB) were significantly decreased in CCl_4 alone group, when compared to *R. beninensis* and control group, while total bilirubin (TB) showed a significant increase in CCl_4 alone compared to control. However, animals given CCl_4 and treated with *R. beninensis* for 4 days at 150 and 300 mg/kg had significant increase ($p<0.05$) in ALB and TP, and a decrease in TB, when compared to rats which received CCl_4 alone.

The result on oxidative stress indicators is shown in Table 3 on the effect of *R. beninensis* treatment on oxidative stress and antioxidant status in CCl_4-treated rats. As observed, catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were significantly decreased ($p>0.05$), while malondialdehyde (MDA) was significantly increased ($p<0.05$) in the CCl_4-treated rats. However, animals given CCl_4 and treated with ethanolic root extract of *R. beninensis* for 4 days at 150 and 300 mg/kg showed significant decrease in MDA and also a significant increase in CAT, SOD and GPx activities, when compared to rats which received CCl_4 alone.

On the histology of the liver, control rats (Figure 1) showed normal hepatic architecture with central canal having radiating hepatocytes. Rats treated with CCl_4 alone had marked liver damage with loss of liver tissue architecture, necrosis, severe dilatation, congestion of blood vessels, vacuolation and hepatocellular degeneration (Figure 2). The liver of the rats treated with CCl_4 and *R. beninensis*at 150 and 300 mg/kg (Figures 3 and 4) showed significant hepatoprotection against CCl_4-induced hepatic attack and damage with observed mild congestion, mild stenosis, mild mononuclear cells in sinusoids and absence of vacuolation. The liver of the rats given *R. beninensis* treatment showed regions of recovery at 150 and 300 mg/kg and comparable protection to that offered by Silymarin.

DISCUSSION

Carbon tetrachloride is a well-known animal model for hepatotoxicity and can be justified by increased level of liver function marker enzymes (Reddy *et al.,* 1993; Alrheam, 2015). It is well known that aminotransferases are sensitive indicators of liver cell injury and are released into the blood in increasing amounts whenever the liver cell membrane is damaged (Pratt and Kaplan, 1999). AST, ALT, ALP and LDH all indicators of liver inflammation and necrosis, showed significant increases in activities in animals that received CCl_4 alone compared to other groups. This is an indication that these liver enzymes crossed the liver membrane as a result of damage of the liver cell membrane. Bilirubin is a useful index of the excretory function of the liver. It is known that abnormal increase in serum bilirubin indicates hepatobiliary disease and severe disturbance of hepatocellular architecture (Martin and Friedman, 1992). Bilirubin usually conjugates with glucoronic acid in the liver in a reaction catalysed by bilirubin-UDP-glucuronyltransferase which renders it soluble and subsequently excreted into the bile (Saidu *et al.,* 2010). High level of serum bilirubin as observed in animals treated with CCl_4 alone compared to other groups is an indication of liver cell damage or impairment and thus impairment of liver function. The high bilirubin level in rats treated with CCl_4 alone can be said to be due to the liver inability to conjugate bilirubin with glucoronide resulting in unconjugated bilirubin accumulation in the blood. Fortunately, Silymarin and *R. beninensis* administration for 4 days at 150 and 300 mg/kg modulated bilirubin level in the liver thus mitigating liver damage. The reduction in bilirubin by Silymarin and *R. beninensis* also indicates an improvement in the liver secretory function.

Table 1: Effect of ethanol root extract of *Rhaphiostylis beninensis (RB)* treatment on liver function enzymes in CCl_4-administered Wister rats

Treatment	AST (U/l)	ALT (U/l)	ALP (U/l)	LDH (U/l)
Control	109.65 ± 2.01^a	111.53 ± 3.61^a	112.42 ± 3.70^a	201.87 ± 17.94^a
CCl_4 only	213.70 ± 5.71^c	196.97 ± 6.54^c	214.47 ± 5.48^c	943.73 ± 50.15^c
CCl_4 + 150 mg/kg RB	160.54 ± 1.41^b	152.25 ± 1.56^b	154.07 ± 4.42^b	326.08 ± 32.61^b
CCl_4 + 300 mg/kg RB	166.65 ± 5.53^b	154.79 ± 3.44^b	152.23 ± 1.15^b	348.28 ± 56.18^b
CCl_4 + 100 mg/kg Silymarin	164.75 ± 5.36^b	153.77 ± 2.08^b	155.55 ± 2.58^b	315.54 ± 51.25^b

Values are mean ± SE. Mean values in each column (between groups) having different superscripts are significantly different ($p<0.05$)

Table 2: Effect of ethanol root extract of *Rhaphiostylis beninensis* (RB) treatment on liver synthetic molecules in CCl_4-administered Wister rats

Treatment	ALB (g/dl)	TP (g/dl)	TB(mg/dl)
Control	6.58 ± 0.10^d	27.96 ± 0.38^d	0.57 ± 0.02^a
CCl_4 only	2.20 ± 0.07^a	10.04 ± 1.47^a	5.05 ± 0.05^c
CCl_4 + 150 mg/kg RB	4.43 ± 0.01^b	19.24 ± 0.32^b	1.18 ± 0.04^b
CCl_4 + 300 mg/kg RB	4.55 ± 0.14^b	19.42 ± 0.51^b	1.11 ± 0.05^b
CCl_4 + 100 mg/kg Silymarin	5.69 ± 0.14^c	22.54 ± 1.04^c	1.08 ± 0.08^b

Values are mean ± SE. Mean values in each column (between groups) having different superscript are significantly different ($p<0.05$)

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

Treatment	SOD (u/mg wet tissue)	CAT (u/mg wet tissue)	GP_X (u /mg wet tissue)	MDA (u/mg wet tissue)
Control	74.27 ± 3.81^c	4.53 ± 0.14^c	7.21 ± 0.19^c	2.01 ± 0.14^a
CCl_4 only	17.29 ± 3.00^a	0.44 ± 0.21^a	0.71 ± 0.02^a	10.00 ± 0.19^c
CCl_4 + 150 mg/kg RB	49.41 ± 5.44^b	3.18 ± 0.12^b	5.23 ± 0.70^b	3.39 ± 0.34^b
CCl_4 + 300 mg/kg RB	52.80 ± 8.41^b	3.42 ± 0.20^b	6.55 ± 0.78^b	3.27 ± 0.27^b
CCl_4 + 100 mg/kg Silymarin	58.98 ± 6.54^b	3.44 ± 0.58^b	5.98 ± 0.52^b	4.59 ± 0.85^b

Values are mean ± SE. Mean values in each column (between groups) having different superscript are significantly different ($p<0.05$)

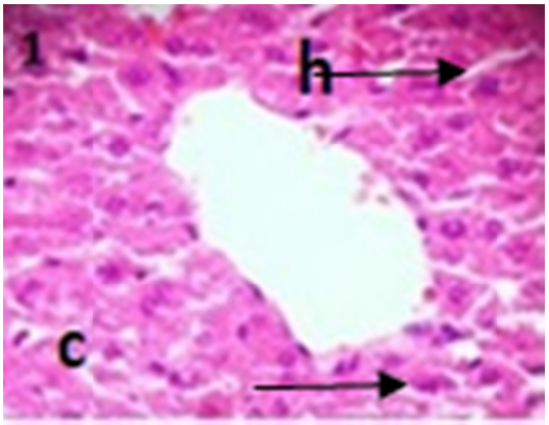

Figure 1: Photomicrograph section of the liver of control rat reveals prominent centriole (c) and normal hepatic architecture with central canal having radiating hepatocytes (h), H&E, Mag. x400

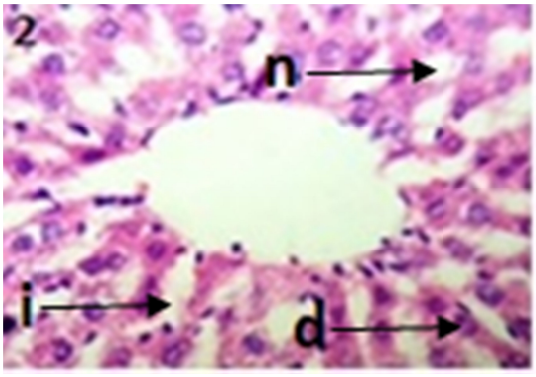

Figure 2: Photomicrograph section of liver of CCl_4 alone treated rat reveals necrosis (n), inflammation (i), severe dilatation (ds), vacuolation and fatty changes, H&E, Mag. x400

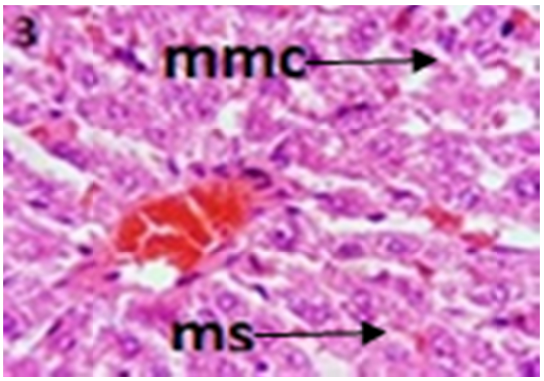

Figure 3: Photomicrograph section of liver of rats given CCl_4 and 150 mg/kg *Rhaphiostylis beninensis* showing mild stenosis (ms), mild mononuclear cells (mmc) in sinusoids and absence of vacuolation, H&E, Mag. x400

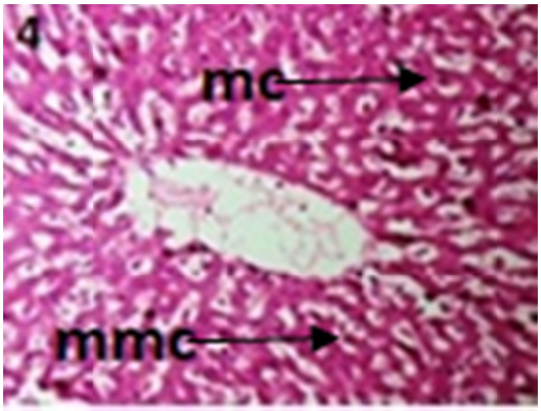

Figure 4: Photomicrograph section of liver of rats given CCl_4 and 300 mg/kg *Rhaphiostylis beninensis* showing mild Congestion (mc), mild mononuclear cells (mmc) in sinusoids but no vacuolation, H&E, Mag. x400

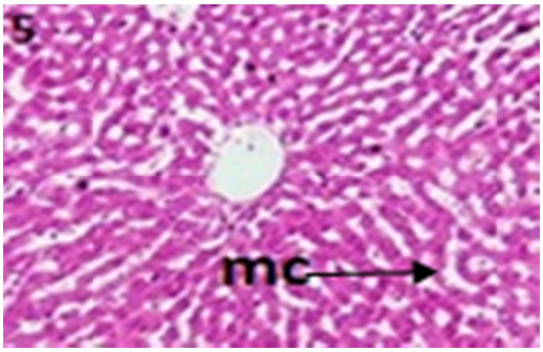

Figure 5: Photomicrograph section of the liver of rats given CCl4 and 100 mg/kg Silymarin, showing mild congestion (mc) and absence of vacuolation, H&E, Mag. x400

Although CCl_4-induced hepatotoxicity was shown in the significantly increased levels of AST, ALT, ALP, LDH, Silymarin and *R. beninensis* administration for 4 days at 150 and 300 mg/kg significantly mediated effective reduction of AST, ALP, ALP, LDH, Bilirubin compared to rats which received CCl_4 alone. This is an indication that the *R. beninensis* extract and Sylimarin offers hepatic protection and enhances liver integrity.The reduction in liver function enzymes and bilirubin observed after *R. beninensis* administration, indicates plasma membrane stabilization as well as hepatic tissue damage reversal. In our previous study in 2019, *Celosia argentea* leaf demonstrated hepatoprotection against CCl_4 induced hepatotoxicity (Usunobun *et al.,* 2019) is similar to findings in this study. The histology of the liver in this study showed necrosis, inflammation and vacuolation in animals administered CCl_4 alone was similar to study by Wagh and Shinde (2010) and corroborating our observed leakage of AST, ALT, ALP, LDH and bilirubin across the liver cell membrane into the blood.

Hepatotoxins are known to impair liver capacity to synthesize albumin (Dubey *et al.,* 1994). In this study total protein and albumin levels were significantly reduced in group that received only CCl_4, whereas groups that received Sylimarin and *R. beninensis* had significantly normalized total protein and albumin levels. Thus Sylimarin and *R. beninensis* mitigated hepatic damage compared to the only CCl_4 intoxicated rats.The reduction in serum total protein and albumin in CCl_4 treated rats can be said to be due to impaired protein synthesis due to the damaged of the liver tissue (Orhue *et al*., 2005; Akpan *et al.,* 2012).

Carbon tetrachloride undergoes metabolism in liver to form trichloromethyl peroxyl (CCl_3O_2) radical (Tapel, 1975). Several lines of evidences suggested that the free radicals oxidize essential macromolecules such as DNA, proteins and lipids, and eventually produce cytotoxicity (Ames *et al.,* 1993; McCord, 1993).

Antioxidants such as SOD, CAT and GPx are known to neutralize excess free radicals and hence protect cells against their toxic effects (Wahid *et al.,* 2016). In this study, the level of antioxidant enzymes (SOD, CAT and GPx) were significantly decreased in rats administered CCl_4 only compared to rats in other groups. This can be attributed to free radical and oxidative stress formation as a result of elevated MDA level in only CCl_4 intoxicated rats. Enhancement of lipid peroxidation and reduced activities of GPx, SOD and CAT is an indication of generation of free radical and oxidative stress, a mark of hepatic

damage (Wagh and Shinde, 2010). Increased reactive oxygen species (ROS) and free radical generation from CCl_4 toxicity produced oxidative stress and increased lipid peroxidation as shown in rats intoxicated with CCl_4 alone. It is a known fact that processes that elevates lipid peroxidation, free radical formation and oxidative stress depletes hepatic antioxidants leading to the development of hepatic diseases (Wagh and Shinde, 2010). Although CCl_4 induced hepatotoxicity was evidenced in decreased levels of SOD, CAT and GPx. Silymarin and *R. beninensis* administration for 4 days at 150 and 300 mg/kg significantly prevented the depletion of the antioxidant status of rats compared to rats that received CCl_4 only. The increased antioxidant levels in Silymarin and *R. beninensis* treated rats when compared to rats treated with CCl_4 alone could be the result of stimulation of SOD, CAT and GPx synthesis and increased levels of other antioxidants as well as regeneration of hepatocytes that restore the structural and functional integrity of liver (Wagh and Shinde, 2010).The decreased MDA level following *R. beninensis* administration can be attributed to the *in vivo* elevated levels of antioxidants and decreased formation of free radicals. Our findings in this study are similar to previous results which elucidated similar liver toxicants and medicinal leaf protection (Usunobun *et al.,* 2015; Usunobun *et al.,* 2019). Silymarin has been used for many years as a hepatoprotective agent (Flora *et al.,* 1998; Wellington and Jarvis, 2001). In this study compared to rats that received only CCl_4, Silymarin increased SOD, CAT and GPx activities and decreased lipid peroxidation, total bilirubin, AST, ALT, ALP and LDH leakages, while it also significantly increased levels of total protein and albumin similar to previous work of Wagh and Shinde (2010).

Conclusion: Based on findings of this study, it can be concluded that *R. beninensis*leaf had protective effect on CCl_4-induced free radical mediated hepatotoxicity due to its antioxidant and anti-inflammatory properties.

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