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Protective activity of root extract of *Rhaphiostylis beninensis* against carbon tetrachloride-induced hepatotoxicity in Wistar rats

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ABSTRACT: Liver diseases pose an enormous problem worldwide irrespective of the many strides in modern medicine. This has necessitated the need to depend on complementary and alternative systems of medicine for liver ailments. *Rhaphiostylis beninensis* is a culinary spice with various medicinal applications. The current study examined the hepatoprotective effects of methanol root extract of *Rhaphiostylis beninensis* in Wistar rats for a 14-day period. Twenty experimental rats were randomly divided into four groups labelled A-D comprising five rats each. Rats in group A were fed with pellets and water *ad libitum* for 14 days as control. Group B rats received a single intraperitoneal injection of carbon tetrachloride in a dose of 3 mL/kg of a 50 % (v/v) solution in liquid paraffin only on day 1. Group C rats were administered the same dose of carbon tetrachloride on day 1 prior to thirteen days administration of the root extract (500ml/kg body weight). A similar dose of the root extract was given to rats in group D for thirteen days sequel to Carbon tetrachloride administration on day 14.The substantive elevated (p<0.05) serum levels of liver biochemical parameters, liver malondialdehyde levels and decreased liver antioxidant enzyme levels (p<0.05) in group D rats, were restored towards normalcy in rats pre-treated and treated with the spice extract (D and C) as compared with the control group. The results of this study strongly indicate that the root extract of *Rhaphiostylis beninensis* possess hepatoprotective activity against carbon tetrachloride induced hepatotoxicity in Wistar albino rats.

Keywords: *Rhaphiostylis beninensis,* hepatotoxicity, carbon tetrachloride, malondialdehyde

Introduction

The liver is the chemical plant of the body that is often exposed to a variety of xenobiotics and therapeutic agents. The body depends on the liver to regulate, synthesize, store and secrete many important proteins, nutrients, chemicals and to purify and clear toxins or unnecessary substances from the body (Garba *et al,* 2009).

Carbon tetrachloride (CCl_4) is a well-known liver toxicant and has been frequently used for generating free radical induced liver injury in rat models (Minami *et al,* 2005; Junnila *et al,* 2000; Cui *et al,* 2009; Kim *et al,* 2010). The hepatotoxicity of CCl_4 has been reported to be due to its biotransformation by cytochrome

P_{450} system to produce trichloromethyl free radical (CCl_3) which readily reacts with molecular oxygen to form trichloromethyl peroxy radical (Raucy *et al*, 1993).

Liver disease remains a worldwide health problem in spite of great advancements in modern medicine (Sowjanya *et al.,* 2013). Different kinds of herbal medicines derived from plant extracts with relatively little knowledge regarding their modes of action, are being utilized increasingly to treat a wide variety of clinical diseases (Mathews *et al.,* 1999*)*. Besides, studies have demonstrated that dietary intake of a variety of plant phytochemicals confer inhibitory effects against oxidative damage (Hollman and Katan, 1999; Yemitan and Izegbu, 2006). For this reason, a growing interest has been paid to the research of natural antioxidants, among which spices occupy an important position (Pokorny *et al.,* 2001). Antioxidants retard or inhibit oxidation of other substances by inhibiting the initiation or propagation of oxidizing chain reactions (Velioglu *et al*., 1998). Hence, natural antioxidants can protect the biologically important cellular components from oxidative processes caused by reactive oxygen species (Su *et al*., 2007). Included in the purview of spices with antioxidant potential is *Rhaphiostylis beninensis*.

Rhaphiostylis beninensis Planch ex Benth (Icacinaceae) is a woody climber with various medicinal and culinary applications. It is found growing in South-western Nigeria and the West African sub region. In Nigeria, it has many vernacular names, depending on the location and usage. The plant is called *atapata* (Yoruba), *osumadin* (Benin), *kpolokoto* (Ibos), *umeni* (Urhobos) and *kumeni* (Itsekiris).

The family to which *R. beninensis* belongs (Icacinaceae) has been reported to contain numerous bioactive phytochemicals (Nkafaniya *et* al., 2007; Gandiza *et al*., 1993). Studies have also revealed various pharmacological and biological properties of the root bark extracts of the plant. Pharmacological activities reported for the plant include anti-bacterial, analgesic and anti-inflammatory (Edema *et al,* 2006; Lasisi *et al.,* 2010; Ofeimun and Onwukaeme 2006).

In folkloric medicine, the leaf and root are used in the management of arthritis and rheumatism, skin diseases, mental disorder, convulsion and eye problem (Burkill, 1994; Odugbemi, 2008) while the leaf decoction is used as a mouth wash and a wash for sores. Oil obtained from the root and various extracts of the bark and fruit exhibited anti-microbial activity against gram positive and gram-negative bacteria as well as fungi (Edema *et al.,* 2009; Adebayo-Tayo *et al.,* 2010).

The plant was reported to contain anthraquinones, flavonoids and triterpenes (Ofeimum and Onwukaeme, 2006). In addition, a thiourea derivative namely, N,N-di (4-methyoxybenzyl) thiourea with anti-inflammatory activity has also been isolated from the root of the plant (Ofeimum *et al.*, 2014).

Till date, mankind has not been able to find an ideal curative and prophylactic agent for liver disorders. Thus, the need for an alternative/complementary medicine for liver ailments cannot be overemphasized. Although several reports abound on some biological and pharmacological properties of *Rhaphiostylis beninensis* spice extracts, there is no documentation on the antioxidant ability of the spice in relation to its hepatoprotective effect on carbon tetrachloride-induced changes in rats. Hence, the present investigation of the plant.

Materials and Methods

Chemicals

CCl_4 was obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and kits (from Randox laboratories, Crumlin, United Kingdom) used throughout this investigation were of the highest analytical grade commercially available while the water was glass distilled.

Collection and Authentication of the Plant Material

The roots of *Raphiostylis beneninsis* were bought from a local market, in Oghara, Delta state. The roots were identified and validated by Dr. H. A Akinnibosun of the Department of Plant Biology and Biotechnology, University of Benin. The plant specimen with serial number, UBHp0262 was deposited in his herbarium.

Preparation and Extraction of the Plant Material

The plant was washed free of earthy material and the root bark was scrapped off and dried in the laboratory at room temperature for 1 week and thereafter in an oven maintained at 40 ºC for 30 min. The dried plant material (1500g) was milled and subjected to extraction with 70 % methanol by cold maceration technique at 20 mg/ml for 4 days. The extract was then concentrated to dryness by a rotary evaporator (Buchi Rotavator-R, China) to a brownish slurry at 40ºC. This was then dried in an open water bath at 40ºC and weighed to determine the yield. The concentrated extract was stored in an air-tight container at 4ºC until required.

Phytochemical Screening of the Plant Material

The phytochemical components of *Raphiostylis beninensis* roots were determined using the methods of (Sofowora, 1993; Harbone, 1973) with some minor modifications.

Experimental Animals

Twenty (20) male Wistar rats (110-170 g) were selected for the study. The animals were procured from the animal unit of the Anatomy Department, University of Benin, Benin City and kept in cages for four (4) weeks.

The animals were allowed to acclimatize to the new environment for two (2) weeks and thereafter, subjected to two (2) weeks of treatment with free access to pelleted livestock feed, and water *ad libitum* with a 12-12 h light/dark cycle respectively.

The handling and treatment of the rats were done based on the approved ethics for the use and care of experimental animals.

Experimental Design

The animals were randomly divided into four groups labelled A-D comprising five rats each. Rats in group A were fed with pellets and water *ad libitum* for 14 days as control. Group B rats received a single intraperitoneal injection of CCl_4 in a dose of 3ml/kg of a 50 % (v/v) solution in liquid paraffin only on day 1.

Group C rats were administered CCl_4 (3ml/kg body weight of a 50% (v/v) solution in liquid paraffin on day 1 prior to thirteen days administration of Methanol root extract of *Rhaphiostylis beninensis* (MERB) at a dose of 500ml/kg body weight.

Group D rats were administered 500 mg/kg body weight of MERB for thirteen days sequel to CCl_4 (3ml/kg body weight of a 50% (v/v) solution in liquid paraffin) administration on day 14. On the 15^{th} day, animals were fasted overnight, sacrificed by cervical dislocation and then dissected.

Preparation of Serum and Liver homogenate

Blood samples were collected by heart puncture using a hypodermal syringe and needle. Blood sample was collected from the heart vessels into the non-heparinized bottles for serum biochemical analyses, using standard assay kits (Randox Lab Ltd. UK) and the liver was quickly excised from each rat immediately after sacrifice. It was rinsed in ice-cold 1.15% potassium chloride, blotted with filter paper and weighed.

Weighed portions were minced with scissors in 4ml of ice-cold 0.1M phosphate buffer, pH 7.4 and homogenized in a Potter–Elvehjem homogenizer. The homogenates were later centrifuged using refrigerated centrifuge at 3,000 x *g* for 15 min at 4ºC to obtain clear supernatants, which were used for subsequent oxidative stress indicators.

Biochemical Analyses

Biochemical analyses carried out include measurement of the activities of plasma alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (γ-GT), and alkaline phosphatase (ALP); plasma total protein and albumin concentrations. The determination of the

concentrations of these biochemical parameters was done using commercially available test kits, products of Randox Laboratories (Crumlin, United Kingdom).

Lipid peroxidation and Antioxidant Biomarkers

Lipid peroxidation was assessed by measuring the formation of malondialdehyde according to the method described by Iqbal *et al.* (1996). Catalase (CAT) activity was determined according to the method of Aebi (1984) and Luck (1974). The level of superoxide dismutase (SOD) activity was measured as described by Misra and Fridovich (1972).

Statistical Analysis

Data generated in the present study were expressed as Mean ± SEM for the five determinations of each experimental group. They were statistically analyzed using one-way analysis of variance (ANOVA) and tukey multiple comparison (TMC) test. The mean results with probability less than 0.05 ($p < 0.05$) were considered significant.

Results

Results of the quantitative phytochemical constituents of methanol root extract of *R. beninensis* spice (**Table 1**) indicated that flavonoids and phenols were present in higher concentration in the root extracts than alkaloids, tannins and saponins.

Table 1: Phytochemical constituents of methanol root extracts of *R. beninensis* spice

Phytochemical	Quantity (%)
Flavonoids	3.72 ± 0.13^{a}
Tannins	0.78 ± 0.04^{b}
Alkaloids	1.74 ± 0.07^{c}
Phenols	2.03 ± 0.07^{d}
Saponins	0.23 ± 0.01^{e}

Values are expressed as mean ± SEM (n=3). Values with different letters are significantly different ($p<0.05$).

Table 2 reveals relative weight of liver in the control and experimental groups. The relative weight of Liver for group B animals (carbon tetrachloride administered) were significantly higher ($p<0.05$) than that of group A (control) and the other experimental groups (B and C). However, values for groups A, C and D were significantly similar ($p<0.05$).

Table 2: Comparison of mean relative weight of the liver

PARAMETER	GROUP A (CONTROL)	GROUP B (CCl_4)	GROUP C (MERB + CCl_4)	GROUP D (CCl_4 + MERB)
Liver weight(g)	4.28 ± 0.29^{a}	6.02 ± 0.21^{b}	4.32 ± 0.13^{a}	4.14 ± 0.15^{a}

Values are expressed as mean ± SEM (n=5). Values with different letter along the same row are significantly different ($p<0.05$).

Table 3 shows the effect of methanol root extract of *R. beninensis* on CCl4 - induced changes in serum levels of some Liver biochemical parameters. There was significant increase ($p<0.05$) in the concentrations of gamma-glutamyl transpeptidase (GGT), aspartate aminotransaminase (AST), alanine aminotransaminase

(ALT), alkaline phosphatase (ALP) and decreased total protein (TP) and albumin(ALB) levels were observed following an intraperitoneal administration of CCl_4 when compared with normal control. However, treatment with MERB before and after administration of CCl_4 in groups C and D respectively, resulted in significant increased level (p<0.05) of total protein and albumin and a concomitant decreased levels (p<0.05) of other serum biochemical parameters in comparison with the CCl4 treated group. In general, treatment with MERB extract produced a non-significant difference in the aforementioned biochemical parameters when compared with the normal control group.

Table 3: Effect of methanol root extract of *R. beninensis* on CCl_4-induced changes in serum levels of some liver biochemical parameters

Groups	GGT(U/L)	ALB (g/L)	TP(g/L)	ALP (U/L)	AST (U/L)	ALT(U/L)
A	110.10±26.80^{a}	13.80±0.30^{c}	23.69±0.40^{d}	7.10±0.3.10^{b}	12.03±4.30^{e}	60.75±4.01^{f}
B	126.22±0.00^{b}	6.28±0.00^{d}	16.16±0.00^{e}	12.65±0.00^{c}	15.00±1.41^{f}	94.00±0.00^{h}
C	112.67±0.18^{a}	12.81±0.76^{c}	22.98±1.85^{d}	7.38±1.65^{b}	11.90±0.03^{e}	59.71±5.04^{f}
D	113.99±0.15^{a}	13.23±0.14^{c}	23.38±0.12^{d}	7.46±3.03^{b}	14.84±3.86^{e}	64.08±0.62^{f}

Values are expressed as mean ± SEM (n=5). Values with different letter along the same column are significantly different (p<0.05).

Table 4 shows the effect of methanol extract of the roots of *R. beninensis* on CCl_4-induced changes in liver lipid peroxidation product (MDA), Superoxide dismutase (SOD) and Catalase (CAT).

In group B animals, Malondialdehyde (MDA), an index of lipid peroxidation was significantly increased (p<0.05) by the administration of CCl_4 when compared with normal control (A) and the other experimental groups (B and C).The induced peroxidation due to this increase was attenuated by treatment with MERB before and after CCl_4 administration (groups C and D).

Liver antioxidant marker enzymes (SOD and CAT) activities on administration of CCl_4 on rats were significantly reduced in group B animals when compared with normal control group (A) and the other experimental groups (C and D). However, administration of MERB gave a significant increase (p<0.05) in their activities in groups C and D when compared with the normal control.

Table 4: Effect of methanol root extract of *R. beninensis* on CCl_4-induced changes in levels of catalase, superoxide dismutase and malondialdehyde in liver of rats

Groups	CAT(µmol/g Tissue)	MDA(µmol/g Tissue)	SOD(µmol/g Tissue)
A	38.73±2.99^{d}	45.40±4.67^{b}	22.55±5.94^{e}
B	32.40±3.24^{e}	73.91±0.00^{c}	16.12±0.00^{f}
C	40.10±0.00^{d}	47.01±2.89^{b}	24.31±1.22^{e}
D	38.92±0.91^{d}	45.10±2.75^{b}	23.20±2.61^{e}

Values are expressed as mean ± SEM (n=5). Values with different letter along the same column are significantly different (p<0.05).

Discussion

Liver disorders are one of the world's problems. The conservative treatments of liver problems, such as acute and chronic liver hepatitis, liver cirrhosis, and fatty liver are often inadequate due to hazardous effects initiated by hepatotoxic drugs of chemical origin (Weber *et al,* 2003). The occupational exposure to chemical compounds such as aliphatic hydrocarbons alters the liver structure and functions (Jaeschke,

2008). Moreso, oxidative stress-induced peroxidation is a prominent feature of CCl_4- induced liver injury (Adewale and Orhue, *2015)*.

Phytochemical analysis of the root extract of *R. beninensis* reveal that flavonoids and phenols were present in higher concentration in the root extracts than alkaloids, tannins and saponins. The relative composition of each phytochemical in the plant is contrary to the analysis of phytochemicals in the stem extract of *R. beninensis* from an earlier study (Lasisi *et al*, 2011).The reason may be due to the differential quantities of phytochemicals in different parts of a plant (Kouki and Manetas, 2002; Monteiro *et al*., 2006). However, the presence of these chemical constituents in the methanol root bark extract of *R. beninensis* is an indication that this plant if properly screened would yield drugs of plant origin with pharmacological significance. This is better supported by the fact that various parts of the plant are known to be involved in ethnomedicine for the management of numerous ailments.

Alkaloids are made up of heterocyclic nitrogen that has been shown to exhibit antimalarial, antihypertensive, antiarrhythmic, and anticancer properties (Heikens *et al.*, 1995). Alkaloids have also been reported to act as CNS stimulant and powerful analgesics (Ashok and Upadhyaya, 2012). Saponins have been reported to have antimalarial effect (Besong *et al.*, 2016). Flavonoids have been reported to possess antioxidant, anti-inflammatory, antitumor, antiallergic, and antiplatelet activity (Pal and Verma, 2013).

The presence of these phytochemicals in *R. beninensis* could be responsible for the various reported pharmacological activities of the plant (Edema *et al*, 2006; Lasisi *et al.*, 2010; Ofeimun and Onwukaeme 2006).

In accordance with previous studies (Adewale and Orhue, 2015; Hai *et al*, 2011; Krishnan and Muthukrishnan, 2012), administration of CCl_4 brought about increased levels of AST, ALT, and ALP, and GGT in serum of the CCl_4 treated group.

This outcome can be attributed to hepatic damage, resulting in an increased rate of synthesis or release of functional enzymes from bio membranes (Pari and Prasath, 2008). In particular, the relative increase in plasma levels of ALT is an indicator of hepatocyte damage (Brent and Rumack 1993).

Elevated plasma level of ALP is suggestive of biliary obstruction, such as occur in cholestatic disease of the liver. Differential diagnosis to ascertain the source of plasma ALP is provided by assay of gamma glutamyl transferase (GGT).

GGT is a membrane bound enzyme whose activity in plasma increases significantly alongside that of plasma ALP following biliary obstruction (Zimmerman *et al*, 1965; Ratanasavanh *et al*, 1982). Biliary obstruction, as suggested by the data obtained for both ALP and GGT for the CCl_4- treated group in this study, implies a failure of biliary secretion.

The significant decrease ($p<0.05$) in albumin levels in CCl_4 treated groups when compared with the control, suggest liver injury, since these are reliable indices of liver toxicity (Omoniyi and Mathew, 2006). Lipid peroxidation has been categorized as one of the most important causes of CCl_4-induced hepatic injury (Basu, 2003). Malondialdehyde (MDA) is a cytotoxic reactive aldehyde formed as a byproduct of lipid peroxidation (Manibusan *et al*, 2007).

An increase in the hepatic MDA level suggested the enhancement of lipid peroxidation, consequently leading to hepatic damage as well as the inactivation of the antioxidant defense system in the CCl_4- treated group.

Administration of methanol root extracts of *R. beninensis* (MERB) significantly ($p<0.05$) protected the rats against CCl_4-induced hepatotoxicity as demonstrated by its inhibition of the elevation of serum AST ALT, ALP, MDA, GGT and Total protein activities in the pre-treated and treated groups. The ability of MERB to effectively protect the liver against carbon tetrachloride toxicity may be related to the remarkable antioxidant and anti-inflammatory properties of its chemical constituent, thiourea (Ofeimum *et al*, 2014; Kajimoto and Murakami, 1998; Kulkarni *et al*, 2008).

This reduction in the elevated enzyme levels in the pre-treated and treated groups, indicate that MERB interferes with the action of CCl_3 free radicals produced.

Animals pretreated and treated with MERB showed a significant reduction in the levels of hepatic peroxidative markers with concomitant improvement in the hepatic antioxidative defense system.

The suppression of MDA production plausibly promote the activities of SOD and CAT. Therefore, an increase in antioxidant activity and the inhibition of free radical generation is positively correlated with hepatic protection.

In conclusion, the results of this study have demonstrated for the first time that, *R. beninensis root extract* possess hepatoprotective potential seemingly through enhancing hepatic antioxidant enzyme activities and inhibition of lipid peroxidation in rats. The hepatoprotective effects of the extract may be traceable to the presence of potent phytoconstituents of the roots. However, further studies are in progress to identify the antioxidant ability and characterize the active principles of *R. beninensis* root extract so as to propose the fingerprint of the mechanism of action of the plant.

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