

Protective Influence of *Ficus asperifolia* Miq Leaf Extract on Carbon tetrachloride (CCl₄)-Induced Testicular Toxicity in rat's Testes

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ABSTRACT

The aim of this study was to investigate the ability of the polyphenolic rich *Ficus asperifolia* (Miq.) leaf extract in protecting rat testes against carbon tetrachloride-induced testicular damage in male Wistar rats. Thirty rats (weighing 140 - 180 g) were divided into five groups. In each treatment groups, aqueous extract of *F. asperifolia* (100, 200 and 400 mg/kg bw) administered by oral gavage for 21 days before exposure to carbon tetrachloride (CCl₄) 3 mL kg⁻¹.p. were used to test protective influence of the plant extract. Protective influence were observed on antioxidant marker enzymes such as reduced glutathione (GSH) levels, catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), and malonaldehyde (MDA) and histological examination. Animal exposure to the CCl₄ resulted in significant elevation in the MDA with concomitant depletion ($p < 0.05$) in the level of GPx, CAT and SOD activities compared with control. Daily oral administration of *F. asperifolia* showed beneficial and ameliorative effects in all biochemical parameter evaluated. Histopathological alteration in testes was observed in CCl₄ untreated rats and was ameliorated in CCl₄ rats treated with *F. asperifolia*. Result shows that the aqueous leaf extract of *F. Asperifolia* has ameliorative effect against carbontetrachloride-induced testicular toxicity.

INTRODUCTION

Infertility is a major clinical problem, affecting majority of the people medically and psychosocially (Rowe, 2006). In recent years, oxidative stress has been implicated in the progression of male infertility. Evidence has shown that these damaging events are caused by free radicals (Halliwell and Gutteridge, 1993). Oxidative stress results from either a decrease of natural cell antioxidant capacity or an increased amount of reactive oxygen species (ROS) in organisms. Carbon tetrachloride (CCl₄), is a clear, colourless, volatile, heavy and non-flammable industrial liquid, widely used to induce free radical toxicity in various tissues of experimental animals such as

liver, kidneys, heart, lung, testis, brain and blood (Khan *et al.*, 2010). CCl₄ is converted through hepatic microsomal cytochrome P₄₅₀ into tri-chloromethyl-free radical ($\cdot\text{CCl}_3$ or $\cdot\text{CCl}_3\text{OO}$) (Preethi *et al.*, 2009) which in turn, initiate lipid peroxidation process (Adewole *et al.*, 2007; Adewole *et al.*, 2012). The most widely accepted mechanism of CCl₄ is the formation of free radicals which is a rate limiting process in tissue peroxidative damage (Sahreen *et al.*, 2011; Khan *et al.*, 2011).

Maintaining the balance between reactive oxygen species and natural antioxidants could serve as a major mechanism in preventing damage by oxidative stress induced by toxic agents. Antioxidant defense systems that protect the body from free radical damage include the antioxidant nutrients and enzymes (Sreelatha *et al.*, 2009). Antioxidant and radical scavengers have been used to study the mechanism of CCl₄ toxicity as well as to protect tissue cells from CCl₄ induced damage by breaking the chain of lipid peroxidation (Weber *et al.*, 2003).

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Ficus asperifolia Miq belonging to the Moraceae family is a small or average size tree, terrestrial or epiphyte which can reach 20 m in height. It is found in Nigeria, Senegal, Uganda, Tanzania, Natal (South Africa), Madagascar and Cameroon. *Ficus asperifolia* is abundant in the savannah regions, especially along river banks and marshy areas at an altitude of up to 1100 m. The leaves are enormous and displayed spirally, the limb is largely oval or has a form of ellipse and the roots are most often fibrous (Adjanohoun *et al.*, 1996). In many traditional medicines of Africa, the leaf extract of *Ficus asperifolia* is used as an anti-helminthic and a purgative (Soforowa, 1996). *Ficus asperifolia* has been shown to possess many pharmacological and physiological activities such as antioxidants (Ojo and Akintayo, 2014). In view of the fact that CCl₄ has been shown to induce testicular toxicity, the effect being through oxidative stress and the antioxidant properties of phytochemical constituents of *Ficus asperifolia* influence oxidative stress in tissue of rats, we therefore aimed at investigating the protective efficacy of *Ficus asperifolia* on CCl₄ induced testicular derangement in rat.

MATERIALS AND METHODS

Chemicals

Carbon tetrachloride (CCl₄) was bought from a local chemist in Ibadan, Nigeria. Glutathione, hydrogen peroxide, 5, 5'-dithio-bis-2-nitrobenzoic acid (DNBT) and epinephrine were from Sigma Chemical Co., Saint Louis, MO USA. Trichloroacetic acid (TCA) and Thiobarbituric acid (TBA) were purchased from British Drug House (BDH) Chemical Ltd., Poole, UK. Other reagents were of analytical grade and the purest quality available.

Plant material

Fresh sample leaves of *Ficus asperifolia* was procured from local suppliers in Ado-Ekiti (Ekiti State) and authenticated at the Department of Plant Sciences, Ekiti State University, Nigeria. A voucher specimen (U.A.H.E 23) has been deposited in the herbarium of the Plant Science Department, Ekiti State University. The fresh leaves were air dried and finely powdered with an electric grinder.

Extraction

The plant leaves were air dried in the laboratory at ambient temperature (30±2°C) for 10 days, pulverized using a laboratory mechanical grinder (Christy and Norris limited, machine type 8) and the fine powders obtained stored until further use. 50 g of the powdered sample was extracted with distilled water of 500 ml for 48 hours. The mixture was decanted and filtered using sterile Whatman paper No 1. The filtrate measured up to 425 ml and evaporated to dryness using a freeze dryer to obtain 12% yield.

Experimental animals

Thirty Male Wistar albino rats (140-180 g) were maintained in the Laboratory Animal Unit of the College of

Sciences, Afe Babalola University. They were housed in metallic cages and fed with standard chow diet and water *ad libitum*. The animals were exposed to alternate cycle of 12 hours of darkness and light. Male rats were used because of their constant metabolism compared to the variation in the female physiology. Animals were allowed to adapt to the laboratory environment for one week before experimentation. The care and handling of the animals were in accordance with the internationally accepted standard guidelines and were approved by Afe Babalola institutional review board with ethical approval number (ABUAD/ACA/324).

Doses

The dose selection for the aqueous extract of *F. asperifolia* was based on the acute toxicity study, which did not show any adverse effect following oral administration of doses up to 4000 mg/kg. Accordingly, with a slight modification by (Ojo *et al.*, 2014) experimental oral doses of 100, 200 and 400 mg/kg that equal to one-fortieth, one-twentieth and one-tenth of the maximum possible dose of the extract that did not cause mortalities in rats were selected.

Experimental induction of Testes damage

CCl₄ was dissolved in groundnut oil in the ratio 1:1 v/v. Testes damage was induced in rats following subcutaneous (SC) injection of CCl₄ in the lower abdomen at a dose of 3 mL/kg (Theophile *et al.*, 2006).

Biochemical parameters

Thirty male Wistar albino rats were randomly divided into five groups of six animals, each. Rats of the 1st group (normal control) and 2nd group (CCl₄-intoxicated control) groups received 3 mL/kg CCl₄. The 3rd, 4th and 5th groups were treated with the aqueous extract of *F. asperifolia* in doses of 100, 200 and 400 mg/kg, respectively. All medications were administered orally through an oral gavage for 7 consecutive days. Two hours after the last dose, normal control rats were given a single dose of groundnut oil (3 mL/kg, SC), while animals of the 2nd to 5th groups received a single dose of CCl₄ (3 mL/kg, SC). After 24 h of CCl₄ injections, blood sample from each rat (2 mL) was withdrawn by puncturing their retro-orbital plexus of veins and collected in previously labeled centrifuging tubes and allowed to clot for 30 min at room temperature. Serum was separated by centrifugation at 10,000 rpm for 5 min. Testes were dissected out and divided into two parts. One part was kept in liquid nitrogen for determination of antioxidant status and the other part was immediately fixed in buffered formalin 10% and was used for histopathological examination.

Assessment of CCl₄ mediated oxidative stress

Protein contents of the samples were tested by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard. The lipid peroxidation level was tested by the reaction between 2-thiobarbituric acid (TBA) and malondialdehyde

(MDA), a product of lipid peroxides as described by Buege and Aust (1978). The tissue SOD was measured by the nitro blue tetrazolium (NBT) decrease method of McCord and Fridovich (1969) CAT was tested spectrophotometrically by measuring the rate of decomposition of hydrogen peroxide at 240 nm as described by (Aebi, 1974). Reduced GSH level was measured by the method of Beutler *et al.* (1963) This method is on developing a stable (yellow) color when 5',5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent) mix to sulfhydryl compounds. The chromophoric product resulting from Ellman's reagent with the reduced GSH (2-nitro-5-thiobenzoic acid) holds a molar absorption at 412 nm, which is part to the reduced GSH in the test sample. The GPx was tested by the method of Rotruck *et al.* (1973) When this substance is mixed with reduced GSH, its absorption shifts to a longer wavelength (340 nm), and increase at this wavelength provides a direct measurement of the enzymatic reaction.

Histopathology of tissues

The testes from the control and experimental groups were fixed with 10% formalin and embedded in paraffin wax and cut into longitudinal section of 5 μ m thickness. The sections were stained with hemotoxylin and eosin dye for histopathological observation.

Statistical analysis

All the data are expressed as mean \pm SEM. The significance of difference in means between the control and treated animals was determined by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test for analysis of biochemical data using SPSS (20.0). Values were considered statistically significant at $P < 0.05$.

RESULTS

Acute Oral Toxicity Studies

All rats treated with different doses (1000–4000 mg/kg) of *F. asperifolia* extract survived during the 48 h of observation. The animals did not show visible signs of acute toxicity (Table 1).

Table 1: Acute toxicity studies (LD₅₀) of the aqueous extract of *Ficus asperifolia*.

Dose (mg/kg) of administration	No. of Animals before administration	No. of deaths after administration
1000	2	-
1500	2	-
2000	2	-
3000	2	-
3500	2	-
4000	2	-

Key: - = No death

Testicular Oxidative stress

As shown in table 2, the activities of testicular enzyme glutathioneperoxidase (GPx) in CCl₄ treated groups decreased significantly ($p < 0.05$) when compared to the control treated groups. Following pre-administration with *F. asperifolia*, the

activity of GPx increased significantly ($p < 0.05$) in the CCl₄ + *F. asperifolia* treated groups compared to the CCl₄ treated groups but as shown in table 2, the changes in the activities of GPx in the CCl₄ + *F. asperifolia* treated groups was not significant when compared to the control treated groups. The testicular content of Malondialdehyde (MDA) in the CCl₄ treated groups was significantly elevated ($p < 0.05$) when compared to the control treated groups.

There was a significant ($p < 0.05$) decrease in the MDA level of the CCl₄ + *F. asperifolia* treated groups when compared to CCl₄ treated groups. However, the changes were not significant when compared to the control treated groups (Table 2).

Table 2: Changes in the levels of serum testicular (MDA) and testicular antioxidative enzymes (GPx) in CCl₄-induced rats treated with aqueous extract of *Ficus asperifolia* Miq. Leaves.

Treatments	GPx (nmol MDA/mg protein)	MDA (nmol/mg protein)
Control	0.95 \pm 0.02	0.82 \pm 0.12
CCl ₄ untreated	0.39 \pm 0.05*	2.61 \pm 0.01*
CCl ₄ + 100 mg/kg	0.66 \pm 0.04**	0.83 \pm 0.06**
CCl ₄ + 200 mg/kg	0.72 \pm 0.11**	1.42 \pm 0.12**
CCl ₄ + 400 mg/kg	0.90 \pm 0.12**	1.84 \pm 0.17**

Values are means \pm S.E.M. of 6 animals per group, CCl₄ Treated = *Ficus asperifolia* at 100 mg/kg, CCl₄ Treated = *Ficus asperifolia* at 200 mg/kg, CCl₄ Treated = *Ficus asperifolia* at 400 mg/kg, *significantly different from control ($p < 0.05$), ** significantly different from CCl₄ untreated ($p < 0.05$).

The oxidative stress caused by CCl₄ in the testes was assessed by measuring the activity of antioxidant defense enzymes (SOD, GSH and CAT), GSH (Table 3).

Table 3: Changes in the levels of testicular antioxidant enzymes in CCl₄-induced rats treated with aqueous extract of *Ficus asperifolia*.

Treatment	Testes		
	GSH (mg/g tissue)	SOD (U/mg protein)	CAT
Control	0.95 \pm 0.15	0.98 \pm 0.03	0.97 \pm 0.18
CCl ₄ untreated	0.32 \pm 0.18*	0.42 \pm 0.18*	0.35 \pm 0.58*
CCl ₄ + 100 mg/kg	0.62 \pm 0.05**	0.68 \pm 0.30**	0.64 \pm 0.11**
CCl ₄ + 200 mg/kg	0.68 \pm 0.15**	0.71 \pm 0.24**	0.78 \pm 0.25**
CCl ₄ + 400 mg/kg	0.75 \pm 0.27**	0.78 \pm 0.45**	0.82 \pm 0.35**

Values are means \pm S.E.M. of 6 animals per group, CCl₄ Treated = *Ficus asperifolia* at 100 mg/kg, CCl₄ Treated = *Ficus asperifolia* at 200 mg/kg, CCl₄ Treated = *Ficus asperifolia* at 400 mg/kg, *significantly different from control ($p < 0.05$), ** significantly different from CCl₄ untreated ($p < 0.05$).

Results presented in Table 3 showed that subcutaneous injection of CCl₄-induced significant reduction in the activities of SOD and CAT enzymes with a decreased level of GSH content as compared to the normal control group. On the other hand, it increased the MDA level in testes (Table 2). Pre-administration of aqueous extract *F. asperifolia* (100, 200 and 400 mg/kg) reduced the severity of CCl₄ toxicity, as evident from the non-significant differences observed in the oxidative stress indicators and antioxidant enzyme levels in these groups. Histopathological examination revealed severe necrosis, loss of villi and haemorrhagic lesions in CCl₄ treated group (Figure- 1).

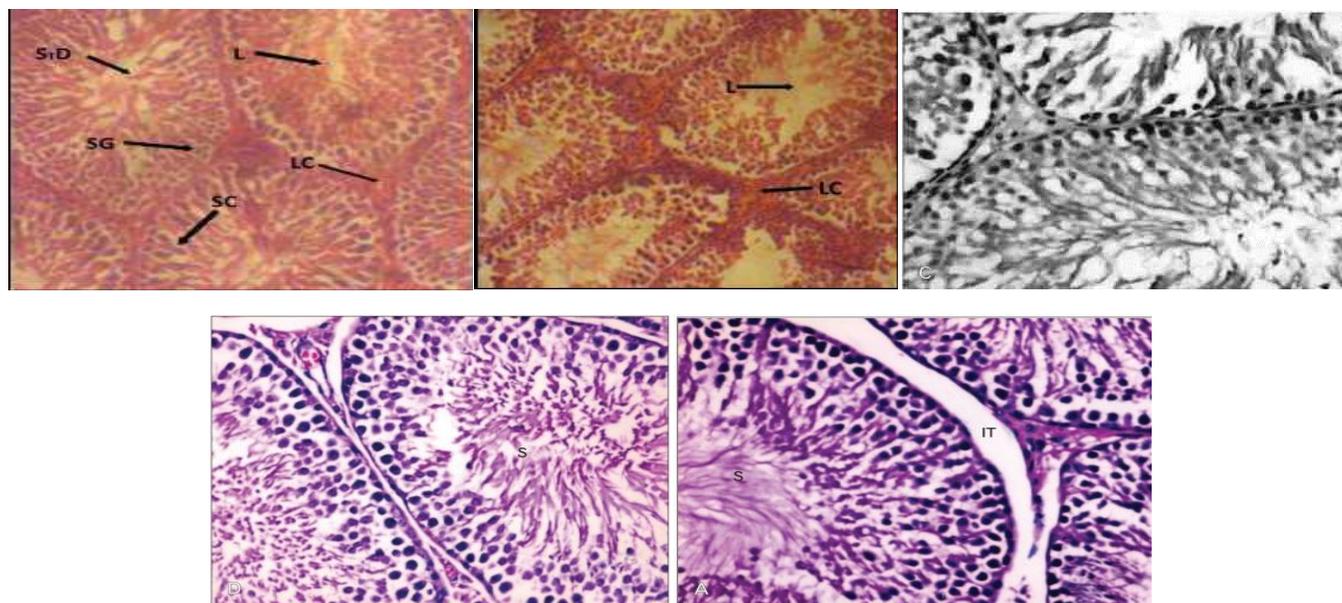


Fig. 1: Histology of Testes tissues. (a) Testes section of normal control rat showing cross section of seminiferous tubules (ST), with stratified epithelium consisting of spermatogenic cells (SG), moderated lumen (L) and Leydig cells (LC), (b) Testes section of CCl₄-treated rats showing closely packed seminiferous tubules, with a wider lumen and the Leydig cells are sparsely separated, degenerated intestinal tissues (c) Testes section of rats treated with CCl₄ and 100 mg/kg of *F. asperifolia* showing seminiferous tubules with closing lumen and spermatogenic cells, (d) Testes section of rats treated with CCl₄ and 200 mg/kg of *F. asperifolia* showing seminiferous tubules with closing lumen and spermatogenic cells, (e) Testes section of rats treated with CCl₄ and 400 mg/kg of *F. asperifolia* showing seminiferous tubules with closing lumen, increase in spermatogenic cells and less sparsely Leydig cells.

DISCUSSION

Reproductive toxicity from heavy and non-flammable industrial liquid in males is one of the areas of concern in toxicology today and our result in this present study showed that exposure to carbon tetrachloride (CCl₄) (3 mL kg⁻¹ CCl₄ orally for 21 days) in rats caused stern testicular toxicity resulting in the obstruction of spermatogenesis and steroidogenesis, evidenced by the increase in the activities of GPx, GSH, CAT and testicular content of MDA of the CCl₄ treated group. Carbon tetrachloride toxicity is very well reported in literature and one of the main mechanisms behind this toxicity has been ascribed to oxidative stress (Sahreen *et al.*, 2011; Khan *et al.*, 2011).

Evaluation of lipid peroxidative activities of antioxidative enzymes such as SOD, CAT, GSH and GPx in tissues has always been used as biomarkers for tissue damage (Stohs and Bagchi, 1995). Hence, increase in the activities of CAT, SOD, GSH and GPx and testicular level of MDA from this study proved that CCl₄ caused oxidative and testicular injury. Pre-treatment with aqueous extracts of *F. asperifolia* leaf extract significantly ($p < 0.05$) ameliorated the activities of tissue parameters and CAT, SOD and GSH with significant ($p < 0.05$) increase levels when compared to CCl₄ treated group (Table 3). Furthermore, the levels of LPO were significantly ($p < 0.05$) increased in CCl₄ treated group when compared with normal group (Table 2). Treatment with *F. asperifolia* significantly ($p < 0.05$) decreased the levels as compared to CCl₄ treated group suggesting that *F. asperifolia* which has been shown to contain flavonoid and other effective antioxidants (Ojo and Akintayo, 2014) resulted in

a notable amelioration of the unbalanced sperm parameters of the testis as seen in the CCl₄ + *F. asperifolia* treated group.

Ojo and Akintayo (2014) reported that *F. asperifolia* contain fundamental antioxidant and phenolic compounds that helps in protecting the testis against spermatogenic and oxidative changes brought about by toxic materials and certain antineoplastic agents.

Knowing that toxic actions of CCl₄ are oxidative in nature, which has been shown by (Sahreen *et al.*, 2011); it is indicative that *F. Asperifolia* was able to attenuate the toxicity of CCl₄ due to its antioxidative potential.

These have shown the antioxidative properties of *F. asperifolia* and its ability to elevate a variety of antioxidant enzymes and testicular biomarkers. This could provide a rationalization for the findings in this study why the experimental group treated with CCl₄ + *F. asperifolia* showed a decrease in activities of GSH, CAT, GPx and testicular MDA. In this reverence, our results showed an increase in the antioxidant enzyme system levels; which implies that *F. asperifolia* treatment can favor reproductive potentials.

Histological examination of the testes revealed that CCl₄ treatment causes the seminiferous tubules to be closely packed with wider lumen and sparsely populated Leydig cells; however treatment with *F. asperifolia* showed noticeable improvement in the histological changes induced by CCl₄. These histological changes seen in the testes of animals exposed to CCl₄ are in agreement with Khan and Ahmed (Khan and Ahmed, 2009) who reported alterations in the seminiferous tubules and reduction in spermatogenic cells in rats following *F. asperifolia* administration.

CONCLUSION

The increased oxidative stress resulting from CCl₄ intoxication in testicular tissue might be accountable, at least in part, for the histopathological changes evidenced in this study. However, *F. asperifolia* leaf extract had a protective effect against the toxicity which evidenced by improvement in testes parameters of rats treated with CCl₄ and *F. asperifolia* leaf extract.

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Author(s)' Statement(s)

Competing Interests

The authors declare no conflict of interest.

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