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Article *in* WORLD JOURNAL OF PHARMACY AND PHARMACEUTICAL SCIENCES · June 2017

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SJIF Impact Factor 6.647
Volume 6, Issue 6, 338-362 Research Article ISSN 2278 – 4357

EFFECTS OF *PIPER UMBELLATUM* LINN. (PIPERACEAE) LEAVES EXTRACT ON ALUMINIUM CHLORIDE REPRODUCTIVE TOXICITY IN MALE RATS

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Article Received on
14 April 2017,
Revised on 04 May 2017,
Accepted on 24 May 2017,
DOI: 10.20959/wjpps20176-9379

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ABSTRACT

Numerous studies found that aluminium has detrimental effects on body systems, including the reproductive one. *Piper umbellatum*, a plant used as spice and vegetable in the southern part of Cameroon, has been reported having many therapeutic properties such as anti-inflammatory, analgesic, antioxidant and aphrodisiac. So, this study was undertaken to assess the effects of *Piper umbellatum* leaves extract on aluminium chloride reproductive toxicity in male rats and to perform the phytochemical screening of the extract. Rats first received aluminium chloride for 35 days. Then, for 60 days, rats were treated with *Piper umbellatum* aqueous leaves extract at the doses of 75 and 150 mg/kg. A libido test was performed the 55th day. The 61st day, rats were sacrificed and blood taken for biochemical analysis. Sexual organs were weighted. The epididymal sperm was collected for evaluation of sperm count and motility. The testis and the epididymis were homogenised for biochemical analysis. The testis and the epididymis were kept in the Bouin's solution for histological analysis. The phytochemical screening revealed the presence of alkaloids, flavonoids and saponins in the extract. Aluminium caused a significant decrease in organ weight ($p < 0.05$), sperm count and motility ($p < 0.05$) and oxidative stress ($p < 0.05$). In addition, histological damages were observed in the testes and epididymis. Our results revealed that aluminium reproductive toxicity was reversed by *Piper umbellatum* extract

which exhibited pro-fertility properties. This may justify the use of the plant for the management of male fertility problems.

KEYWORDS: *Piper umbellatum*, reproductive toxicity, antioxidant, male fertility, libido.

INTRODUCTION

Aluminium is the third most abundant element on the earth crust and its compounds are widely distributed in nature. It is used in the manufacturing of many everyday products like toothpastes, antiperspirants, cosmetics, processed foods, adjuvants in various parenteral preparations and pharmaceutical agents.[1,2] This metal is incorporated in some medications such as antacids, buffered aspirins and anti-diarrheal products. Aluminium sulfate is extensively added as a coagulant agent during the purification process of drinking water in order to folliculate the organic matter, to clarify the water.[3] Aluminium is a metal of choice in making various kinds of household cookware and storage utensils.

Aluminium can enter the body through inhalation of air contaminated with aluminium compounds, through ingestion of aluminium dusts or with food and drinking water and through dermal contact. In the body, aluminium accumulates mainly in bones, liver, testes, kidney and brain.[4] Different forms of aluminium are environmental xenobiotics that induce free radical-mediated cytotoxicity.[5] Aluminium accelerates oxidative damage to lipids, proteins and nucleic acids. Different studies demonstrated that the main toxic effects of aluminium in a chronic exposure were neurological with encephalopathy and psychomotor functions disturbances[6], in bone with osteomalacia[7] and haematological with microcytic anaemia.[8] An experiment reported that aluminium induced toxicity in epididymis, vas deferens, seminal vesicle and ventral prostate in mice.[9] According to another study, it has been shown that aluminium chloride induced reproductive toxicity and exerted a significant adverse effect on the steroidogenesis.[10] It has been shown in a study that, *in vitro*, aluminium chloride provoked deterioration in sperm motility and viability and enhancement of free radicals and alterations in enzyme activities on rabbit sperm.[11] Alterations in the metabolism of testis and epididymis, leading to a reduction in fertility rate in mice treated with aluminium chloride were also observed in an individual study.[5]

From an epidemiological evaluation, infertility due to male factor was ranged from 20% to 70% and infertility rates were highest in Africa and Eastern Europe.[12] There are evidences to

show that sperm counts have been declining over the last 50 years, with a consequent increase in male infertility.[13]

The treatment of male infertility includes administration of androgens or gonadotropins, aphrodisiacs like sildenafil citrate or surgery and assisted reproductive technology. Chemical drugs like aphrodisiacs also cause side effects like flushing, congestion, headache and dyspepsia. In developing countries, particularly in Sub-Saharan Africa these various treatments are not affordable for about 50% of the population.[14]

The use of plants materials for medical purposes dates back to the history of mankind.[15] Traditional medicine is still being used nowadays in all parts of the world and has been growing in economic importance particularly by the use of medicinal plants that have a respectable position today, especially in developing countries, where modern health services are limited and represent the only accessible treatment. It is also well-known that most of the plants used in the folk medicine exert antioxidant activity. Members of the *Piper* genus are of commercial, economical and medicinal importance, known to contain molecules of therapeutic importance.[16] *Piper umbellatum* (*P. umbellatum*) is widely used in many countries as vegetable or condiment, besides its medicinal uses. Its leaves have been shown effective in the treatment of several ailments such as ascite, anaemia, urinary and kidney problems, venereal infections, menstrual and stomach problems. The plants' roots are used for their aphrodisiac properties and to treat infertility.[17] An ethnobotanical survey revealed that leaves can be used in the treatment of dysmenorrhoea in the upper Nyong valley in Cameroon.[18] According to another study a methanolic extract of *Piper umbellatum* leaves exhibited contraceptive activity in female rodents and inhibited ovulation and regular oestrus cycle.[19] In some part of the Centre region of Cameroon, *Piper umbellatum* leaves are used for the treatment of male infertility. However, the scientific evidence of this effect has not yet been investigated.

The present study was therefore undertaken to evaluate the effects a 60-days treatment with *Piper umbellatum* leaves extract on some parameters of male rat reproductive function impaired aluminium chloride injection for 35 days.

MATERIAL AND METHODS

Collection of plant material and extraction

Piper umbellatum leaves were collected in a rural area, Mbele village, near Obala town in the Centre Region of Cameroon. The plant was authenticated at the national herbarium under the voucher number 10391SRF/Cam in comparison with a sample of the collector J.F. Breteler 429. Fresh leaves were shade dried and crushed in a mortar. Thereafter, 220g of leaves powder were soaked in a volume of 6 litres of tap water for 12 hours. After this time a first filtration was performed with a sieve of 0.5 mm meshing and a second filtration with Whatman N°3 paper. The solution was then lyophilised and a powder of 34.29 g was obtained for an extraction yield of 15.58%.

Phytochemical analysis

Phytochemical screening of the aqueous extract of the leaves of *Piper umbellatum* was carried out according to methods described by Ayoola *et al.*[20]

Experimental animals

The study was performed on healthy male rats, weighing 90-120 g and 6-7 weeks old of age at the beginning of the experiment. Animals were obtained from the animal house of the Laboratory of Animal Physiology of University of Yaoundé I. The animals were housed in clean cages placed in well-ventilated housed conditions. Rats were given free access to food and tap water and received a multivitamin complex (Kelavitasol®) every fortnight. Adult female were used for the libido test. The study was conducted according to the guidelines of the Cameroon National Ethical Committee on the use of laboratory animals for scientific research (Ref No.FW-IRB00001954).

Chemicals

Aluminium trichloride anhydrous was provided by *Prolabo VWR International*. Assay kits were used to determine total cholesterol level, gamma glutamyltransferase (GGT) activity and proteins levels. These kits were supplied by *Fortress diagnostics*, United Kingdom. Drugs used during the test were vitamin C (Vitascorbol®, *Cooper* France), oestradiol (Sigma Chemicals, USA), ketamine (Trittau, Germany), diazepam (Valium Roche) and progesterone (Progesterone Retard® *Bayer Schering Pharma* Laboratories, Germany). Different buffers and solutions used were prepared in laboratory in flasks with distilled water.

Experimental design

The study was performed in two steps. During the first step, made for induction of testicular damage and which lasted 35 days, 25 rats were divided into two unequal groups, one of 5 rats (group I) and the other of 20 rats (group II). Group I received an intraperitoneal injection of a normal saline solution at the dose of 0.1 mL/kg body weight (bw). Group II received intraperitoneal injection of aluminium chloride dissolved in a normal saline solution at the dose of 30 mg/kg bw according to the procedure of Khattab.[21]

The second stage of the study which was the treatment period with plant extract and reference drug lasted 60 days. During the treatment phase, group I constituted the normal control group. Group II was randomly divided into 4 groups of 5 rats each IIA, IIB, IIC and IID and the different groups of rats were treated as follows:

-Group I: (Normal control): distilled water orally at the dose of 10 mL/kg for 60 days;

- Group IIA ($AlCl_3$ group: negative control): distilled water at the dose of 10 mL/kg for 60 days orally;

- Group IIB (Vitamin C group: positive control): vitamin C at the dose of 30 mg/kg *per os* for 60 days;

- Group IIC: aqueous extract of *Piper umbellatum* at the dose of 75 mg/kg for 60 days through oral pathway;

- Group IID: aqueous extract of *Piper umbellatum* at the dose of 150 mg/kg for 60 days orally.

Libido test

The libido test necessitated, male and female rats. The female rats used were ovariectomised according to the method used by Cariton.[22] For the test, these female rats were made receptive by sequential injection of oestradiol benzoate (100 µg/kg body weight) and caproate of hydroxyprogesterone (5 mg/kg body weight), through respectively subcutaneous and intramuscular pathways, 48 h and 4 h prior to pairing.[23] The libido test was carried out according to the modified procedure of Oyeyipo *et al.*,[24], the day 55 from 6 PM local time, under dim light, in a quiet atmosphere. One hour after administration of different substances, male rats were introduced in observation cages (one rat per cage). After 5 minutes of acclimation, a female rat made artificially receptive was also introduced in the observation cage. For 10 minutes, the number of mounts and the number of intromissions were recorded.

Sacrifice and sample collection

Initial and final body weights of the animals were recorded. Sacrifice of rats was carried on the 61st day. At the time of the sacrifice, rats were anesthetized by intraperitoneal injection of diazepam. Under anaesthesia, the neck was cleared to expose jugular veins. Then a sharp blade was used to cut the jugular vessels and the rats were made to bleed into clean and dry centrifugation tubes. The blood was centrifuged at 3000 rpm for 15 minutes and different sera were aspirated with pipettes into clean and dry Eppendorf tubes and stored at -20°C for further biochemical analysis.

Thereafter, the rats were quickly dissected, the testes and the epididymis removed. These organs were cleaned of superficial fatty layer and weighed for the determination of the testes/body and epididymis/body weight ratio.

Collection of epididymal sperm

Shortly after, the cauda of the right epididymis measuring 6 mm length was dissected out and used for the estimation of the number of spermatozoa. The cauda was cut into small pieces in a stemmed glass containing 10 mL of NaCl 0.9% solution and incubated in a water bath at 34°C temperature. This sperm was further used for determination of sperm count, sperm motility and viability.

Evaluation of sperm characteristics

Sperm count

A volume of 20 µl of the sperm suspension was aspirated with a micropipette and deposited on a Malassez cell. It was observed in a photonic microscope (Olympus Japan), X400 and the number of spermatozoa was rapidly counted in four areas. The number of spermatozoa per mL of sperm (N) was estimated.[25]

Sperm viability

Sperm viability assessed from eosin staining that discriminate life sperm from dead sperm by staining cytoplasm of cell. A volume of 10 µL of the sperm sample previously obtained was placed on a microscopic slide and 10 µL of eosin 0.5% added to it and then covered with a slide and observed with light microscope x 400. A total of 100 sperms were counted within 2 minutes after the addition of the stain. Evaluation of live (unstained) and necrospermic (stained) spermatozoa were done with light microscopy[26] (Fig. 1).

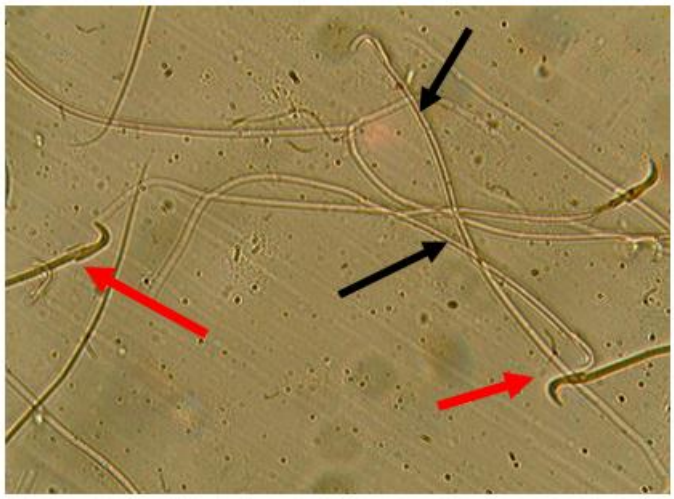

Fig. 1: Light microphotography showing live sperm and necrospermic sperm stained with eosin. Unstained sperm are pointed with black arrows and stained with red arrows; X400.

Preparation of homogenates

The testes were then ground in a mortar and homogenised at 20% in the phosphate sodium buffer (S buffer) pH =7.4 on an ice tray. The epididymis were also ground in a mortar and homogenised in a phosphate potassium buffer, at 20% on an ice tray. Then, the homogenates were centrifuged at 3000 rpm for 45 minutes at 4°C. The supernatants were removed with pipettes into dry and clean Eppendorf tubes and stored at -20°C. These homogenates were further used for biochemical assays.

Biochemical analysis

Total cholesterol levels in the blood and the testes, total proteins concentrations in the blood, the testes and the epididymis and the gamma glutamyltransferase activity in the testes and epididymis, were determined with the help of colorimetric and the kinetic methods as prescribed in commercial kits. Superoxide dismutase (SOD) was determined using Misra and Fridovish procedure.[27] Catalase activity was evaluated respectively with the help of Sinha methods.[28] The level of malondialdehyde (MDA) was determined following the method used by Wilbur *et al.*[29]

Histology

The left testis and the cauda of the left epididymis of each animal were kept into the Bouin solution for fixation for two weeks. After fixation, transversal sections of the testis and the cauda epididymis measuring about 3 mm were made with a scalpel. Fixed tissues were then

transferred to graded series of alcohol (50°, 70°, 95° and 100°) and cleared in the xylene. Cleared tissues were infiltrated in molten paraffin wax at 60°C. Sections of 5 µm thickness were obtained using a microtome from solid paraffin blocks of tissue, cleared, fixed on clean slides and stained with haematoxylin-eosin (HE) stains. The observations were made under a light microscope. Photography of slides was made with the help of *Minisee* program.

Statistical analysis

Results were expressed as the mean ± Standard Error of the Mean (S.E.M.). The data were analysed using ANOVA followed by Tukey post-test to compare control and test groups with GraphPad Prism software version 5.03. Values of $p < 0.05$ were considered statistically significant against negative control.

RESULTS

The phytochemical screening carried out on *Piper umbellatum* leaves extract revealed that it contained alkaloids, saponins, flavonoids, cardiac glycosides, reduced sugars and tannins.

Effects of *Piper umbellatum* extract on relative weight of the testes, the epididymis, the seminal vesicles and the ventral prostate

The results of the effects of *Piper umbellatum* leaves extract on the relative weight of the testes, the epididymis, the seminal vesicles and the ventral prostate are summarised in Table 1. Intraperitoneal injection of $AlCl_3$ for 35 days induced a decrease of testis and epididymis relative weight, in comparison with the normal group. This effect was reversed by *Piper umbellatum* leaves extract treatment which induced a significant ($p < 0.05$) rise in relative weight of testis at the two doses, compared negative control group. A similar effect was observed on the epididymis and the ventral prostate, where *Piper umbellatum* leaves extract induced a remarkable increase ($p < 0.001$) of the relative weight of these organs at the two doses, compared to aluminium chloride treated group. For the seminal vesicles the treatment with the extract exhibited a significant increase of the relative weight of these glands at the doses of 75 and 150 mg/kg ($p < 0.05$ and $p < 0.01$ respectively). The administration of vitamin C for 60 days induced a significant increase of the relative weight of the testes, the epididymis, the seminal vesicles and the prostate gland, in a similar manner to *Piper umbellatum* extract.

Table 1: Effects of *Piper umbellatum* extract on the relative weight of some sexual organs (g/100 g body weight)

Groups	Normal	$AlCl_3$	Vitamin C (30 mg/kg)	*P. umbellatum* 75 mg/kg	*P. umbellatum* 150 mg/kg
Testis	1.41 ± 0.04	1.03 ± 0.08	1.37 ± 0.07	1.58 ± 0.11*	1.53 ± 0.01*
Epididymis	0.50 ± 0.01	0.27 ± 0.02	0.44 ± 0.02***	0.47 ± 0.02***	0.42 ± 0.007***
Seminal vesicles	0.38 ± 0.04	0.009 ± 0.02	0.32 ± 0.03*	0.30 ± 0.01*	0.43 ± 0.05**
Ventral prostate	0.25 ± 0.04	0.06 ± 0.008	0.30 ± 0.03***	0.21 ± 0.02***	0.33 ± 0.02***

Data values represent mean ± SEM ; n=5. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$: significant difference when compared with $AlCl_3$.

Effects of *Piper umbellatum* leaves extract on total cholesterol

The effects of *Piper umbellatum* extract are shown on Fig.2. $AlCl_3$ treated-group showed a decrease of total cholesterol in the serum when compared to normal rats. *P. umbellatum* leaves extract induced a significant rise ($p < 0.001$) of total cholesterol in the serum at the dose of 75 mg/kg, while that increase was not significant at the dose of 150 mg/kg, compared to aluminium chloride treated rats (Fig. 2A). Vitamin C provoked a significant rise ($p < 0.01$) in total serum level compared to negative control group. In the testis, there was an enhancement of total cholesterol concentration in the group which received $AlCl_3$ in comparison with those who received plant extract or vitamin C (Fig. 2B). *Piper umbellatum* extract and vitamin C both induced the decrease of this parameter. The decrease was significant in the group treated with vitamin C ($p < 0.01$) and the plant extract at the dose of 150 mg/kg plant extract ($p < 0.001$) in comparison with aluminium chloride group.

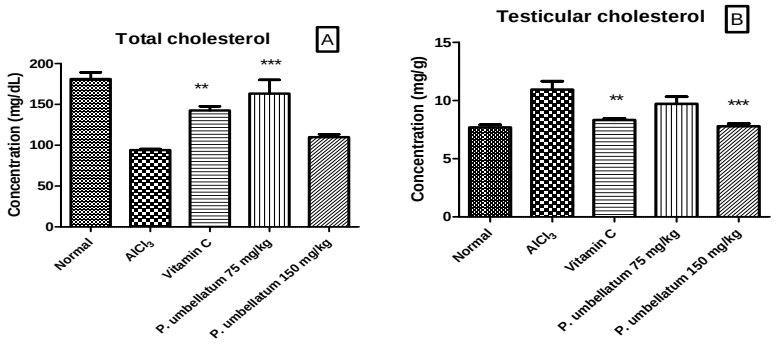

Fig. 2: Effects of *Piper umbellatum* leaves extract on total cholesterol in serum (A) and the testis (B).

Data bars represent mean ± SEM ; n=5. $^{**}p < 0.01$; $^{***}p < 0.001$: significant difference when compared with $AlCl_3$.

3-Effects of *Piper umbellatum* extract on total proteins

The intraperitoneal injection of $AlCl_3$ for 35 days provoked a reduction of total proteins levels in the serum, testis and epididymis in comparison with distilled water treated group (Fig. 3). Treatment with the plant extract induced a significant rise of proteins in the serum, in a dose-dependent manner ($p < 0.01$ and $p < 0.001$ respectively at the doses of 75 and 150 mg/kg) (Fig. 3A). In the testis, a significant increase of total proteins ($p < 0.001$) was observed only at the dose of 75 mg/kg (Fig. 3B). In the epididymis, total proteins were notably increased ($p < 0.001$) at the two doses (Fig. 3C).

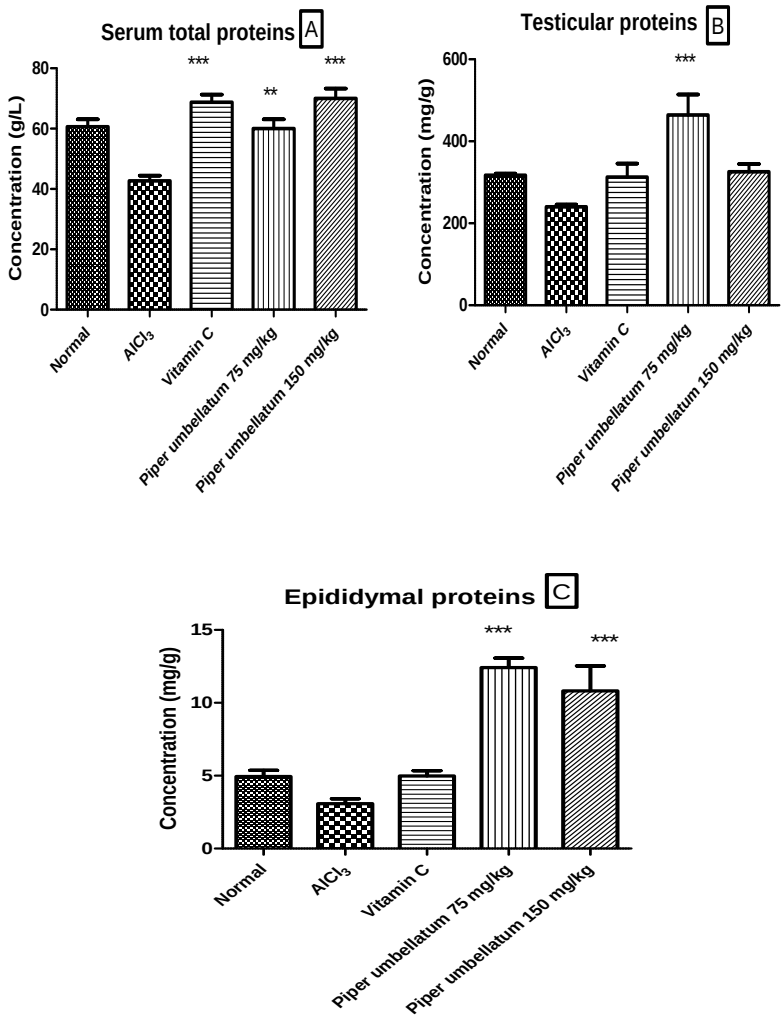

Fig. 3: Effects of *Piper umbellatum* extract on total proteins levels in the serum (A), the testis (B) and the epididymis (C).

Each bar represents mean ± SEM ; n=5. $^{**}p < 0.01$; $^{***}p < 0.001$: significant difference when compared with $AlCl_3$.

Effects of *Piper umbellatum* extract on testicular and epididymal GGT activity

The administration of $AlCl_3$ induced a reduction of GGT activity in the testis and the epididymis when compared to normal group. The treatment with the plant extract exhibited a rise of GGT activity in both the testis and the epididymis. This elevation of GGT activity was not significant in the testis (Figure 4A). However, *Piper umbellatum* leaves extract provoked a significant rise of GGT in the epididymis, $p < 0.001$ and $p < 0.01$ respectively at the doses of 75 and 150 mg/kg (Figure 4B) as compared to $AlCl_3$ treated group.

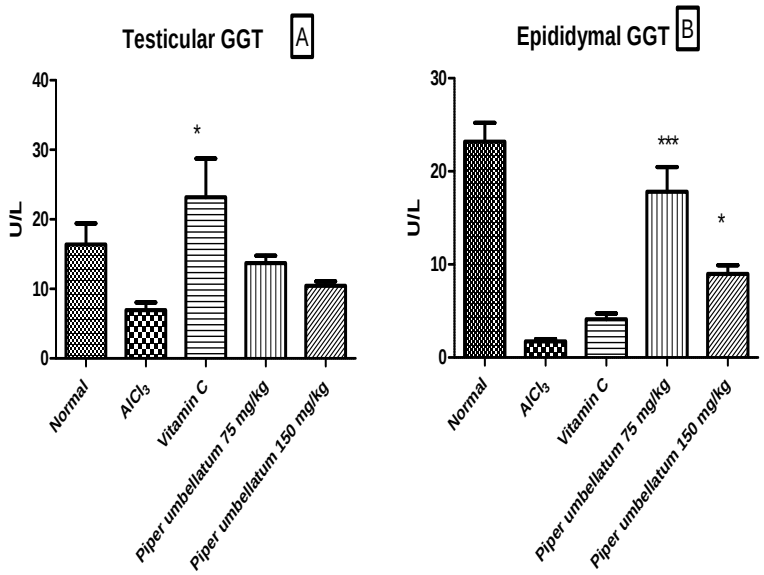

Fig. 4: Effects of *Piper umbellatum* leaves extract on GGT activity in the testis (A) and the epididymis (B).

Bars represent mean ± SEM ; n=5. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$: significant difference when compared with $AlCl_3$.

Effects of *Piper umbellatum* leaves extract on some oxidative stress parameters

Table 2 summarises the results of *Piper umbellatum* leaves extract on some oxidative stress parameters in the testes and the epididymis, notably malondialdehyde (MDA) levels, superoxide dismutase (SOD) and catalase activities. In aluminium chloride group, a marked increase of MDA was noted, in the testes and the epididymis, when compared to the normal group (Table 2). Treatment with the plant extract for 60 days induced a significant decrease ($p < 0.001$) of MDA levels in both the testes and the epididymis, at the two doses, as compared with $AlCl_3$-group. Vitamin C had similar effects to *P. umbellatum* extract in comparison with aluminium chloride.

$AlCl_3$-group exhibited a decrease of SOD and catalase levels in the testes and epididymis when compared to normal group. Testicular SOD was significantly increased in a dose-dependent manner, $p < 0.01$ and $p < 0.001$, respectively at the doses of 75 and 150 mg/kg, in comparison with aluminium chloride group. In the epididymis, a non-significant increase of SOD was observed (Table 2). As for testicular catalase, a notable rise ($p < 0.001$) has been observed only at the dose of 150 mg/kg of the extract compared to $AlCl_3$ group (Table 2). Epididymal catalase was also significantly ($p < 0.05$) increased only at the dose of 150 mg/kg of *Piper umbellatum* leaves extract (table 2). For the two parameters, vitamin C induced a significant increase in both the testis and the epididymis when compared to aluminium chloride treated group.

Table 2: Effects of $AlCl_3$ and *Piper umbellatum* extract on MDA, reduced glutathione, SOD and catalase in the testes and the epididymis

Groups	Normal	$AlCl_3$	Vitamin C (30 mg/kg)	*P. umbellatum* 75 mg/kg	*P. umbellatum* 150 mg/kg
MDA (mmol/gx10^{-6})	2.45 ± 0.08	4.34 ± 0.21	2.12 ± 0.06***	2.64 ± 0.12***	2.68 ± 0.18***
	4.67 ± 0.14	6.94 ± 0.33	4.17 ± 0.10***	4.37 ± 0.34***	4.12 ± 0.15***
SOD (µmole/mg protein)	12.69 ± 0.12	1.35 ± 0.25	14.96 ± 0.61***	8.40 ± 0.46**	0.90 ± 0.25
	0.58 ± 0.19	0.08± 0.01	1.40 ± 0.36*	12.90 ± 2.73**	1.03 ± 0.32
Catalase (µMH_2O_2/min/mg of proteins)	0.04 ± 0.003	0.02 ± 0.002	0.06 ± 0.00***	0.04 ± 0.002	0.06 ± 0.001***
	2.52 ± 0.23	0.75 ± 0.07	2.35 ± 0.31**	1.09 ± 0.18	1.81 ± 0.29*

Values represent mean ± SEM ; n=5. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$: significant difference when compared with $AlCl_3$.

Effects of *Piper umbellatum* leaves extract on some sperm characteristics

The effects of *P. umbellatum* leaves extract on some sperm characteristics are found in Table 3. $AlCl_3$ group had lower sperm count, motility and viability than the normal group (Table 3). The plant extract at the dose of 75 mg/kg induced a significant rise ($p < 0.001$) of sperm motility, sperm viability when compared to the negative control group. Rats treated with the dose of 150 mg/kg of *P. umbellatum* leaves extract exhibited a remarkable increase of the sperm count, sperm motility and sperm viability in comparison with $AlCl_3$-group. Similar results to those of the extract were observed in vitamin C treated-group (Table 3).

Table 3: Effects of *P. umbellatum* leaves extract on epididymal sperm concentration, sperm motility and viability

Groups	Normal	$AlCl_3$	Vitamin C	*P. umbellatum* 75 mg/kg	*P. umbellatum* 150 mg/kg
Sperm count ($x10^6$/epididymis)	547.00 ± 48.41	168.00 ± 60.84	421.00 ± 95.90*	383.20 ± 23.88	462.40 ± 16.23*
Motility (%)	92.09 ± 6.13	37.00 ± 12.68	97.37 ± 0.90***	84.34 ± 6.01***	95.15 ± 0.77***
Viability (%)	89.40 ± 1.36	20.00 ± 12.45	87.80 ± 1.98***	84.60 ± 2.63***	87.00 ± 1.64***

Values represent mean ± SEM ; n=5. *p < 0.05; **p < 0.01; ***p < 0.001: significant difference when compared with $AlCl_3$.

Effects of *Piper umbellatum* leaves extract on the libido of the rats

Aluminium chloride treatment induced a decrease of the number of mounts intromissions in comparison with the normal group. *P. umbellatum* extract treatment for 60 days provoked a notable rise of the number of mounts, p <0.05 and p < 0.001, respectively at the doses of 75 and 150 mg/kg (Fig. 5). The plant extract also induced a significant increase of the number of intromissions at the doses of 75 mg/kg (p < 0.05) and 150 mg/kg (p < 0.01), when compared with negative control group. Vitamin C had plant extract like-effects on the testis and epididymis when compared to $AlCl_3$ treated group.

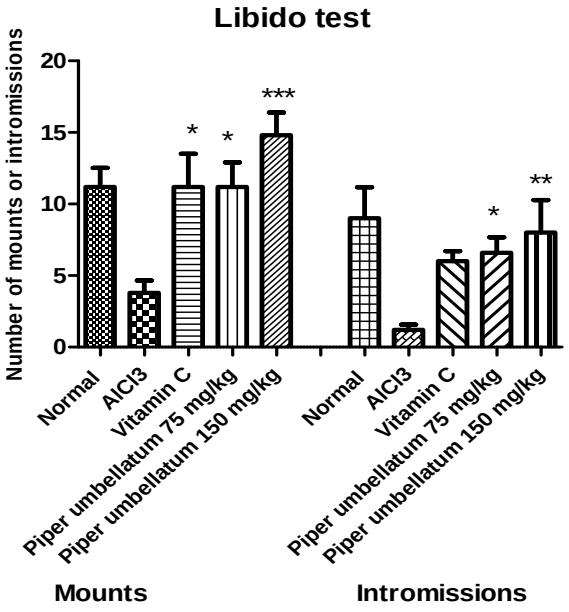

Fig. 5: Effect of *Piper umbellatum* leaves extract on the libido of male rats after 55 days of treatment.

Each bar represents mean ± SEM; n=5. *p < 0.05; **p < 0.01; ***p < 0.001: significant difference when compared with $AlCl_3$.

Effects of *Piper umbellatum* leaves extract on the histology of the testis and the epididymis

Microphotography of the testis of rats which received distilled water showed normal tissue architecture, with a normal ongoing spermatogenesis process in the seminiferous tubules. Lumen is full of spermatozoa and interstitial tissue is well organised (Fig. 6A). The transversal section of epididymis also exhibited a normal architecture with a lot of spermatozoa in the lumen (Fig. 6B).

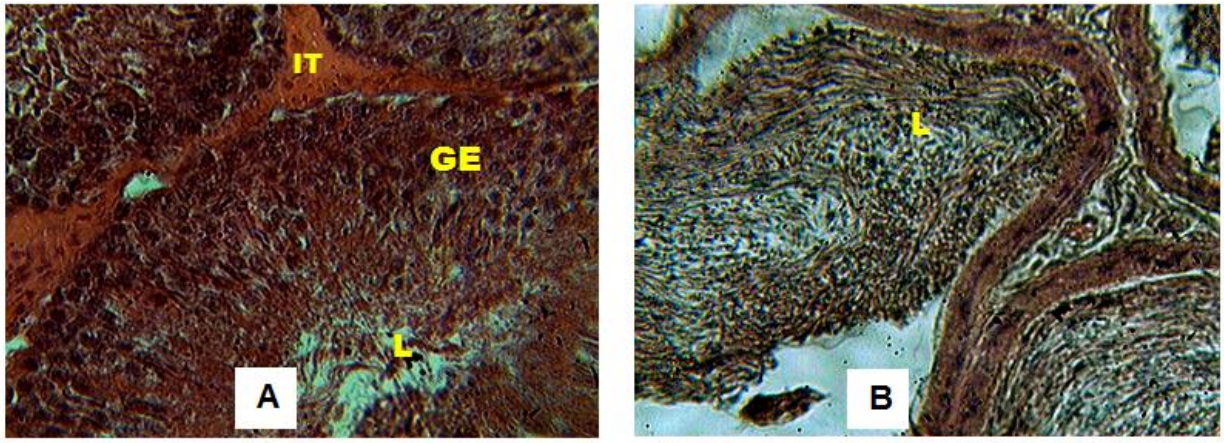

Fig. 6: Microphotography of testis (A) and epididymis (B) sections of rats treated with distilled water. (H&E., 400X). L: lumen; IT: interstitial tissue; GE: germinal epithelium.

The testis of aluminium chloride treated group showed an impairment of the spermatogenesis process and a disorganised germinal epithelium in the seminiferous tubules which had no spermatozoa in the lumen. There was degeneration and necrosis in some regions of the germinal epithelium. A reduction of the interstitial tissue has also been observed (Fig. 7A). The lumen of epididymal tubules contained immature nucleated spermatocytic cells (Fig. 7B). Nevertheless, a partial recovery was noticed after the withdrawal of the treatment with aluminium chloride. The spermatogenesis seemed to be taking place in the seminiferous tubules (Fig. 7C) and some spermatozoa were observed in the lumen of epididymis (Fig. 7D).

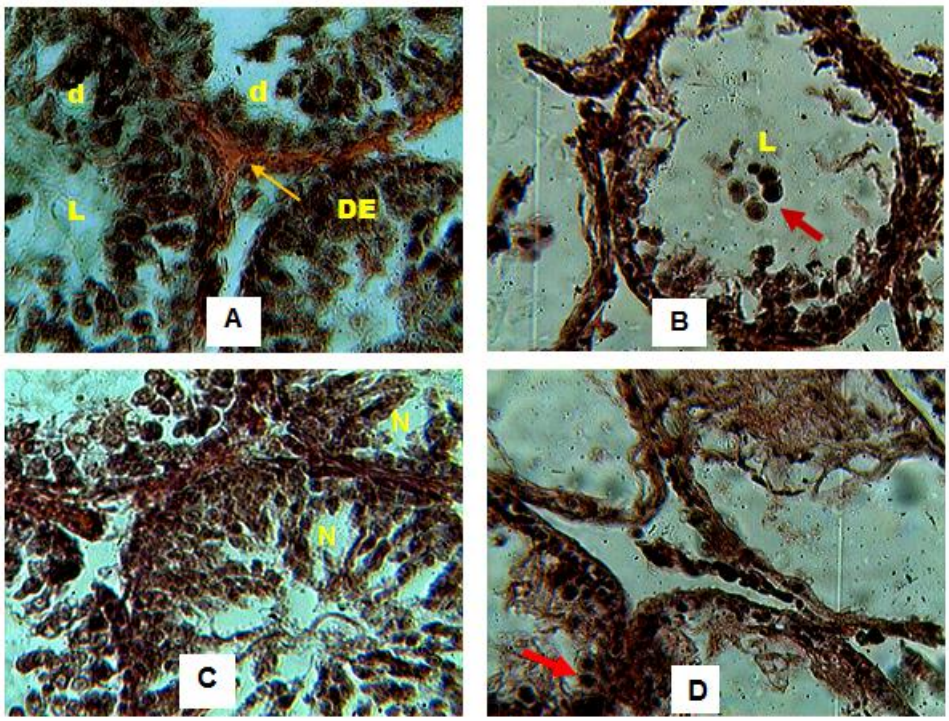

Fig. 7: Microphotography of testis (A) and epididymis (B) sections of rats treated with aluminium chloride, with partial recovery observed in the testis (C) and the epididymis (D). (H&E., 400X). L: lumen; immature spermatocytes (red arrow); DE: disorganised germinal epithelium; diminished interstitial tissue (yellow arrow); d: degeneration and necrosis zones (N).

The microphotographs of testis and epididymis of rats treated with the plant extract at the two doses and vitamin C presented normal tissue architecture similar to the group which received distilled water. The ongoing spermatogenesis was normal in the testis, as well as the interstitial tissue. Degeneration and necrosis zones of germinal epithelium tended to diminish. The lumen of the seminiferous tubules and the epididymal lumen were full of spermatozoa (Fig. 8, Fig. 9 and Fig. 10).

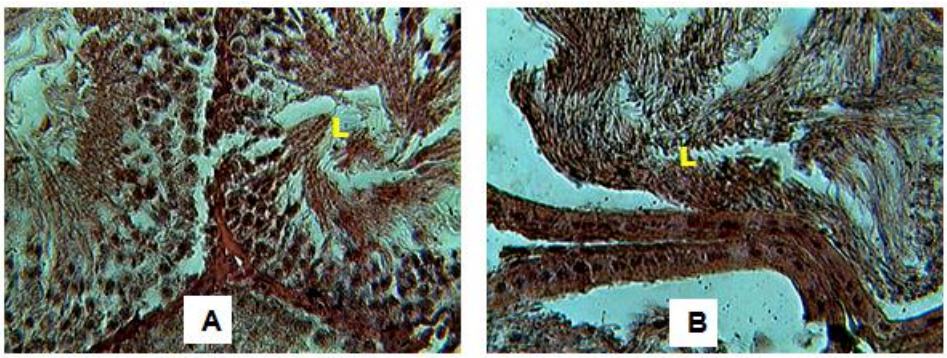

Fig. 8: Microphotography of testis (A) and epididymis (B) sections of rats treated with *Piper umbellatum* at the dose of 75 mg/kg. (H&E., 400X). L: lumen.

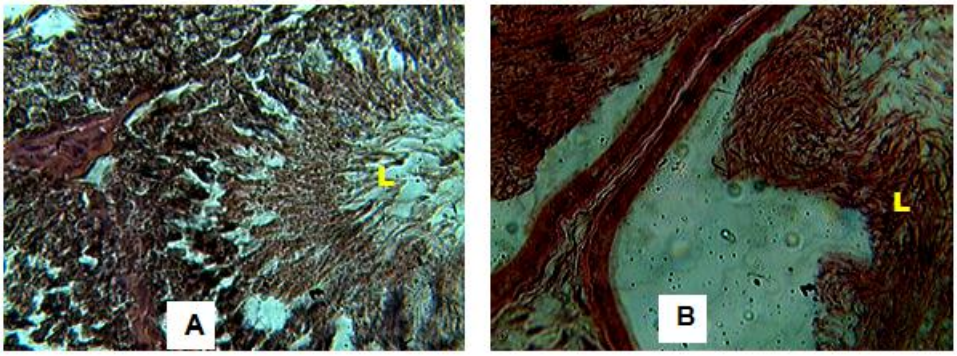

Fig. 9: Microphotography of testis (A) and epididymis (B) sections of rats treated with *Piper umbellatum* at the dose of 150 mg/kg. (H&E., 400X). L: lumen.

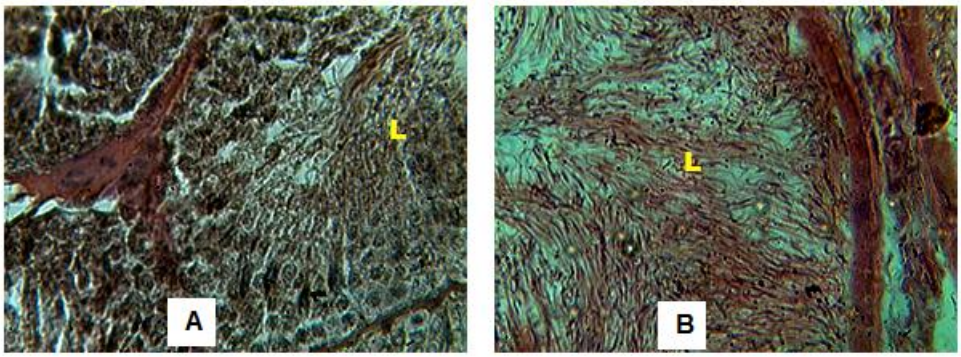

Fig. 10: Microphotography of testis (A) and epididymis (B) sections of rats treated with vitamin C at the dose of 30 mg/kg. (H&E., 400X). L: lumen.

DISCUSSION

The present study was undertaken to evaluate the effects of *Piper umbellatum* on some fertility parameters of male rats which previously received a daily intraperitoneal injection of $AlCl_3$, for 35 days. $AlCl_3$ induced a decrease of relative weight of the testis, epididymis, seminal vesicles and the ventral prostate. As aluminium accumulates in testis, $AlCl_3$ may have deleterious effects on these reproductive organs through cell degeneration and cell death, reduced tubule size, spermatogenic arrest and inhibition of steroid hormones biosynthesis by Leydig cells.[30,31] This therefore results to testes, epididymis and sexual glands atrophy. These effects were reversed by a 60-days treatment with *Piper umbellatum* leaves extract. The plant extract may stimulate tissue regeneration and so may have a positive impact on these organs weight. The increase of sexual organs relative weight is also probably a consequence of an increase in functional activity of these organs leading to increase in their secretory ability.[32] Knowing that these organs are androgen-dependent organs, the plant extract may also increase the availability of androgens, therefore promoting their normal functioning.

The significant decrease in serum total cholesterol in $AlCl_3$-group suggests a great perturbation of cholesterol metabolism. In fact, the biosynthesis of cholesterol takes place in many organs of the body like the intestines and gonads. The liver is the major site of cholesterol synthesis in mammals.[33] So, the administration of aluminium chloride might impair the synthesis of cholesterol in the liver, as this substance affects the normal functioning of the liver. This can thus result to the decrease cholesterol level in the serum. The results showed that cholesterol significantly increased in the testis of $AlCl_3$-group and decreased in the other groups. Once synthesised, the excess of liver's own needs of cholesterol is exported into the blood flow and can be transported to the testes where it serves as the precursor of steroid hormones.[33,34] The intraperitoneal injection $AlCl_3$ may have adverse effects on the interstitial tissue of the testes, making its cells unable to synthesize properly testosterone from cholesterol, thus resulting to accumulation of cholesterol in the testis. On one hand, the plant extract might alleviate impairment of cholesterol metabolism perturbation, especially in the liver, due to aluminium chloride injection and stimulate its synthesis. On the other hand, *Piper umbellatum* leaves extract might protect interstitial Leydig cells against aluminium chloride negative effects, thus, stimulating an efficient steroidogenesis in the testis.

Results obtained in our study suggest that $AlCl_3$ may impair not only endocrine but also exocrine testicular function. Our results indicated significantly decreased sperm count, motility and viability in $AlCl_3$ treated group. Rats treated with *Piper umbellatum* leaves extract exhibited a higher sperm count, motility and viability. Motility is one of the most important features of fertile spermatozoa, widely used as an indicator of sperm function. Sperm motility is an important attribute, because it is readily identifiable and reflects several structural and functional competence, as well as essential aspects of spermatozoa metabolism.[35] Eosin-staining is an easy assay which gives information on necrospermia levels in sperm. This method assesses whether the sperm membrane is intact or disrupted involves examining a percentage of viable sperm by a stain exclusion assay. Integrity of the plasma membrane is shown by the ability of a viable cell to exclude the dye, whereas the dye will diffuse passively into sperm cells with damaged plasma membranes.[35] So, the plant extract might restore and stimulate the spermatogenesis process altered by $AlCl_3$ injection in the testis, and ameliorate sperm quality in extract treated group. Our results suggest that *P. umbellatum* leaves extract may have pro-fertility effects.

The pro-fertility effects of *Piper umbellatum* extract can also be confirmed by the results obtained during the libido test. The plant extract increased in a significant manner the number of mounts and intromissions. Significant increases in mount and intromission frequencies suggest that the plant enhances sexual performance and libido. These parameters are considered as indices of libido and potency.[36]

Testicular and epididymal proteins are required for spermatogenesis and sperm maturation.[37] Thus, the significant reduction in the concentration of testicular and epididymal proteins in $AlCl_3$-treated rats can lead to impaired sperm maturation. The restoration of the concentration of testicular and epididymal proteins following the administration of *Piper umbellatum* leaves extract could enhance sperm maturation.

Gamma glutamyl transferase (GGT) is an enzyme marker of Sertoli function and a key enzyme of gamma glutamyl cycle which catalyses the transfer of gamma glutamyl group between peptides and amino acids and play a role in the absorption, the synthesis and the transport of amino acids and proteins.[38] Sertoli cells provide a mechanical support to germinal cells and a favourable environment for their differentiation. GGT is also involved in the secretion of the fluid into seminiferous tubules which carries the spermatozoa into the rete testis.[39] The significant decrease in gamma-glutamyl transferase in $AlCl_3$ treated rats could result in Sertoli dysfunction and consequently alteration in spermatogenesis. The increase observed of GGT activity in plant extract treated rats might be the sign of stimulation of Sertoli cells. The ability of the plant extract to reverse the decrease mediated by $AlCl_3$ treatment indicates its capability to enhance spermatozoa production.

Environmental toxicants are known to induce reproductive toxicity by perturbing the pro-oxidant and antioxidant balance leading to oxidative stress.[40] The imbalance between production of reactive oxygen species (ROS) and antioxidant mechanism causes oxidative stress in spermatozoa.[41] Spermatogenesis and Leydig steroidogenesis are vulnerable to oxidative stress. Therefore, oxidative stress has been shown to play an important role in causing male infertility by inducing defects in sperm functions.

In our study, aluminium chloride treatment decreased the activities of enzymatic defence mechanisms: superoxide dismutase (SOD), catalase (CAT) in the testes and the epididymis. This clearly indicated an imbalance between pro-oxidant and antioxidant system, in favour of the pro-oxidant one, leading to an oxidative stress state in these organs.

SOD is considered as the first line of defence against deleterious effects of oxygen reactive species in the cell and plays a prominent role in protecting spermatozoa against lipid peroxidation. SOD catalyses the dismutation of superoxide anion into hydrogen peroxide and oxygen, and scavenges both intracellular and extracellular superoxide radical. The reduction in the activity of SOD causes a rise in the level of superoxide anion, which inactivates catalase activity.[42] Catalase detoxifies both intracellular and extracellular hydrogen peroxide to water and oxygen. The decreased activities of SOD and catalase in the testes and epididymis of aluminium chloride treated animals observed in the present study may reflect the inability of these organs to eliminate superoxide anion and hydrogen peroxide produced by the influence of aluminium chloride. This reduction might also reflect the adverse effect of aluminium chloride on antioxidant system in the testis and epididymis of rats.[43] In plant extract treated groups, the increase of antioxidant enzymes observed may indicate an enhancement of the capacity to scavenge free radicals, thus limiting adverse effects of $AlCl_3$ on teste and epididymis, therefore protecting the spermatogenesis process.

Levels of MDA were higher in $AlCl_3$-group than in plant extract and vitamin C groups. Free radicals attack membrane lipids and induce formation of MDA, the end product of lipid peroxidation. Spermatozoa membrane is rich in polyunsaturated fatty acids and their cytoplasm is poor in scavenging enzymes.[44] Thus, elevated MDA in $AlCl_3$-group indicates enhanced lipid peroxidation due to oxidative stress. In the plant extract treated groups, the concentration of MDA was decreased at the two doses. *P. umbellatum* extract may have protective effects on the testes and the epididymis, against lipid peroxidation induced by $AlCl_3$. The decrease of MDA levels and the increased activities of antioxidant enzymes in plant extract treated groups suggest that the extract contained secondary metabolites with antioxidant properties, which alleviated the oxidative stress state induced by aluminium chloride.

Previous phytochemical analysis carried out on *Piper umbellatum* leaves[45] showed that it contained flavonoids and saponins and were in accordance with our own phytochemical screening. Flavonoids are phenolic compounds with known antioxidant activity, through radical scavenging, inhibition of the production of reactive oxygen species or inhibition of lipid peroxidation.[46] It has been reported that steroids and saponins constituents found in many plants possess fertility potentiating properties.[47] Saponins essentially enhance the biosynthesis of endogen androgens.[48] Saponins can also exert antioxidant properties.[49] So,

the stimulation of the antioxidant system in plant extract treated groups could be due to the presence of flavonoids and the saponins have beneficial effects on fertility. Considering libido test results, the bioactive agents of the plant extract may also exert an aphrodisiac effect on different levels, in a synergic manner.[50]

Histopathological examination of rats treated with $AlCl_3$ showed apparent alteration in the testis, where aluminium chloride induced marked degeneration and necrosis of germ cells lining seminiferous tubules. Our results were in agreement with two previous studies of $AlCl_3$ effects' on the rat's testes.[21,51] The lumen of the epididymis of the negative control group contained immature spermatocytes, as observed in an experiment on the protective effect of grape seed extract against experimental aluminium toxicosis in male rat.[52] These histopathological changes in the testes and the epididymis in the negative control group might useful to confirm the fact that aluminium chloride is a testes and epididymis toxicant. It affects the synthesis of testosterone in the testes and the normal functioning of the testes, epididymis and the sexual glands, which are all androgen-dependant organs. Treatment with *Piper umbellatum* extract showed noticeable improvement in these histopathological alterations induced by $AlCl_3$ in testis and epididymis sections. These findings are in favour of the argument that our extract protects germ and interstitial Leydig cells, thus stimulating steroidogenesis and spermatogenesis processes, and indirectly, fertility.

CONCLUSIONS

The results obtained in the present study were in accordance with previous ones on aluminium reproductive toxicity. Its adverse effects were observed on testicular and epididymal functions. The negative control group exhibited lower fertility parameters than normal rats. The treatment with the plant extract alleviated these adverse effects of aluminium chloride. So, our study confirms the beneficial effects of *Piper umbellatum* leaves extract against infertility induced by aluminium chloride and justify the empirical use of the plant for the treatment of male infertility in the Centre region of Cameroon.

ACKNOWLEDGMENTS

The authors acknowledge the laboratory chief of inorganic chemistry and the laboratory chief of vegetal biology for providing necessary facilities to carry out this study successfully. The authors also acknowledge all the animal physiology laboratory team for their great support in any step of this study.

CONFLICT OF INTEREST

None declared.

FUNDING SOURCE

For the conduct of this study, authors did not receive any specific grant from any funding agency in the public or commercial sectors.

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