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ANTIOXIDANT AND AMELIORATIVE EFFECTS OF SINGLE DAILY DOSE OF AQUEOUS EXTRACT OF *Azanza garckeana* ON FORMALIN-INDUCED TESTICULAR TOXICITY IN MALE RATS

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ABSTRACT

The objective of this study was to evaluate the antioxidant and ameliorative effects of the aqueous extract of *Azanza garckeana* on formalin-induced testicular toxicity in rats. Acute toxicity and phytochemical analyses of the extract were conducted using standard procedures. Reproductive parameters and antioxidant enzymes were determined using standard methods. The acute toxicity test showed that the LD_{50} of the extract is greater than 5,000 mg/kg, while the phytochemical analyses revealed the presence of abundant flavonoids with moderate presence of alkaloids and tannins. Semen analyses showed a significant increase concentration at 125 mg/kg compared to normal and formalin groups ($p < 0.05$). There was an increase in testosterone level at 125 mg/kg compared to formalin group ($p < 0.05$). The antioxidant analyses revealed a significant increase activity of SOD at 125 mg/kg, and GSH and catalase at 250 mg/kg in formalin-induced toxicity compared to normal control ($p < 0.05$). On the other hand, there was a decrease in concentration of MDA at 125 mg/kg. In conclusion, this investigation revealed the anti-oxidant and ameliorative effects of *Azanza garckeana* that may serve as scientific justifications of its ethno-medicinal use in male infertility.

KEYWORDS: ***Azanza garckeana*; Testosterone; Antioxidant; Gonadosomatic index; Infertility; Toxicity.**

INTRODUCTION

Reproductive health, a priority global health area and a problem of impaired fecundity, has been a concern for a very long time and is also a significant social problem affecting about 8-12 % of the global population [1]. Among all reported infertility cases, approximately 40-50 % attributed to male factor alone, and as many as 5 % of all men will exhibit sub-optimal sperm parameters [1, 2].

Various sperm indices such as the count, motility and viability are highly susceptible to free radicals or reactive oxidative species (ROS) which are known to reduce fertility in men [3]. Free radicals life-threatening attacks to the body's different organs can cause arterial occlusion and induction of oxidative stress which subsequently cause serious damage to tissues. Meanwhile, testicular tissues and sperm cells are highly predisposed to activity of free radicals and oxidative stress through constant cell division, cell competition for oxygen and low oxygen pressure due to weakened vessels and high fatty acids.

Certain xenobiotics such as formalin and some environmental toxins such as lead or cadmium have been shown to contribute to testicular oxidative stress with consequent disturbances in spermatogenesis [4, 5]. This toxic effect occurs due to chronic accumulation of these chemicals in some organs of the body. Furthermore, since the body's antioxidant system including superoxide dismutase, catalase, and glutathione peroxidase produced in the body are not able to neutralize all the free radicals, the use of antioxidant supplements is occasionally recommended to fight against the

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adverse effects of oxidative stress, thus enhancing spermatogenesis and fertility [6].

Azanza garckeana is an important food and herbal medicine plant naturally found in Tropical Africa. It is reported to contain multiple classes of compounds such as alkaloids, amino acids, flavonoids, antioxidants, cyanogenic glycosides, saponin and tannins [7]. Various parts of the plant are believed to possess some diverse medicinal properties and are traditionally used to manage diseases throughout its distributional range. *A. garckeana* leaves, stem, root or the ripe fruits are taken orally as remedy for infertility and liver problems in some African countries including Botswana, Kenya, Tanzania, Malawi, Namibia, Zimbabwe and Nigeria [7]. Interestingly, *Azanza garckeana* is reported as possessing significant potential pro-fertility profiles in fish [8].

Infertility has been reported as one of the major health and social problems and quite a significant proportion of the male population are at risk [9]. Closely linked to this, is the impact of oxidative stress on spermatogenesis which has been shown to play a major role in the pathophysiology of male infertility, in addition to inaccurate diagnosis and lack of effective treatment associated with poor outcomes as suggested by some reports [10, 11]. It is on the basis of these acclaimed traditional uses of *Azanza garckeana* that our investigation was based.

MATERIALS AND METHODS

Laboratory Animals

Thirty (30) adult albino male rats weighing between 125 and 208 g were purchased from the Animal Experimental Unit of the University of Jos. They were approved and certified for the experiment by the Committee on use of experimental animal protocol of the Department of Pharmacology and Toxicology, University of Jos under the certificate number F17.00379. They were then handled under ethical conditions for the use and care of laboratory animals [12]. The animals were fed with standard solid nutritional pellets and water *ad libitum* until the commencement of the experiment.

Preparation of extract

Fruit pulp of *A. garckeana* was bought in Tula Wange Market, Kaltungo Local Government Area of Gombe state, Nigeria. Some samples of the plant leaves were taken to the Department of Pharmacognosy, University of Jos, for identification and authentication. The pulps were removed, washed and carefully crushed in small pieces to enhance drying. They were then dried under shade in the laboratory. Thereafter, they were grounded to a fine powder and extracted according to the method described by Adegboye *et al* [13]. 765 g of the powdered pulp was extracted continuously with distilled water in a Soxhlet extractor for 25 hrs at 55 ^{0}C. The extract was evaporated to dryness in a vacuum evaporator at 50 ^{0}C until a constant yield of 365.4 g (47.76 %) was obtained after repeated weighing.

Phytochemical analysis

The phytochemical screenings were carried out using standard method for each constituent that include alkaloids, saponins, glycosides, and carbohydrates [14], tannin [15], anthraquinones [16], steroids [17] and terpenes [18].

Acute toxicity test

The acute toxicity of *Azanza garckeana* was determined as described by Lorke [19]. Briefly, three groups each consisting of 3 animals were fasted for 12 hours before the test and administered the extract orally at the dose of 10; 100 and 1000 mg/kg respectively. The mice were observed over a period of 24 hours for morbid signs and mortality. In the second phase, another set of three animals were divided into three groups of one animal each and administered 1600; 2900 and 5000 mg/kg of the extract respectively, and thereafter observed for 24 hours for mortality.

Treatment of animals

The thirty adult albino male rats were randomly divided into six groups of five rats each. Rats in the first group (I) were considered as the normal control and were administered normal saline (1 ml/kg) by the oral route every morning daily for 40 days. In similar manner, animals in groups 2 received formalin alone by the IP route (10 mg/kg) while groups 3 and 4 received in addition to formalin, the extract at doses of 125 and 250 mg/kg respectively by the oral route. Mice in groups 5 and 6 were administered the extract alone at doses of 125 and 250 mg/kg respectively by the oral route for same duration of 40 days. The weights of each mouse in the respective groups were determined periodically at 10-day intervals until the end of the 40-day treatment.

Determination of organ weights and serum testosterone levels

The micro well enzyme-linked immunoassay (ELISA) method as described by Braide [20], and Gan and Patel [21] was used. The principle of this method is based on competitive binding of the gonadotropins on immobilized specific antibody. It

allows the detection of very small quantities of antigens such as protein peptides and hormones in a fluid sample. It utilizes enzyme-labelled antigens and antibodies to detect the biological molecules. The antigen is allowed to bind a specific antibody which is itself subsequently detected by a secondary enzyme-coupled antibody. A chromogenic substrate for the enzyme yields an antigen that allows for quantitative measurement based on such colorimetric readings.

Following the daily administration of the drug and extract, and 24 hours after the last dose, the animals were anesthetized using chloroform and blood samples obtained through cardiac puncture. The blood samples from each mouse were collected in a non-heparinized tube and allowed to stand for 3 hours in iced water. Thereafter, it was centrifuged at 3000 rpm for 10 minutes to separate the serum from clots and the serum was collected and stored at -20 ^{0}C for two days. Bioassay using the micro well ELISA method was carried out for the testosterone.

To determine the weight of the selected organs which include the testes, epididymis, liver, heart and kidney, the animals were dissected and the organs careful isolated and the fats removed. Thereafter, the weight of each organ was carefully measured using a mettle weighing balance. For the testes and epididymis, in addition to the weight, the length and width were also determined using a Vernier calliper. All organ weights were reported as percentage of body weight and the weights were used to calculate the Gonadosomatic index (GSI) and fecundity potentials.

Semen assay

The methods of Bavister and Andrews [22] and that of Robb *et al*. [23] were used with little modifications. Following the last dose of the daily administrations of the extract and normal saline, the mice were allowed to rest for 24 hours. Thereafter, the distal caudal epididymis was dissected and placed in an equilibrated 2 ml medium in a petri dish. A needle was used to release the sperm cells from the caudal epididymis into the medium and the sperm cells were allowed to swim out into the medium for about 4 minutes. Sperm concentrations were then determined by physical count under the electronic microscope at a magnification of x400. Thereafter, the solution was added to the culture for sperm motility assay. The rapid motility was determined after 4 hours as the cut-off time.

Evaluation of antioxidant enzymes

Determination of malondialdehyde (MDA) concentration

This was determined by measuring the levels of MDA produced during lipid peroxidation according to the method described by Varshney and Kale [24]. An aliquot of 400 microliter (µL) of the testes sample was mixed with 1.6 ml of tris-KCl buffer to which 500 µL of 30 % TBA was added. Then 500 uL of 0.75 % TBA was added and placed in a water bath for 45 minutes at 80 ^{0}C. This was then cooled in an ice and centrifuged at a revolution of 3000 g for 5 minutes. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. Lipid peroxidation expressed as MDA formed/mg protein was computed with a molar extinction coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$.

Determination of glutathione (GSH) activity

An aliquot of the testes homogenate was de-proteinated by the addition of an equal volume of 4 % salicylic acid. This was centrifuged at 4000x g for 5 minutes. Thereafter, 0.5 ml of the supernatant was added to 4.5 ml of Ellman reagent. A blank was prepared with 0.5 ml diluted precipitating agent and 4.5 ml of Ellman reagent. Reduced GSH level is proportional to the absorbance at 412 nm.

Determination of catalase activity

Catalase activity was determined by the method of Claiborne [25]. Hydrogen peroxide (2.95 ml) solution was pipette into a 1cm quartz cuvette and 50 uL of the sample added. This was done to reduce the dilution of the samples (done according to other protocols whereby hydrogen peroxide was prepared separately in distilled in 100 ml of water) and the buffer was also prepared separately. The mixture was rapidly inverted to mix and placed in a spectrophotometer. Change in absorbance was read at 240 nm for every 5 min. Calculation of the catalase activity was done.

Determination of superoxide dismutase (SOD)

The level of SOD activity was determined by the method of Misra and Fridovich [26]. 0.2 ml of the sample was diluted in 0.8 ml of distilled water to make a 1:5 dilution. An aliquot of 0.2 ml of the diluted sample was added to 2.5 ml of 0.05 M Carbonate buffer to equilibrate in the spectrophotometer and the reaction started by the addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 ml buffer, 0.3 ml of adrenaline and 0.2 ml of water. The increase in absorbance at 480 nm was monitored every 30 secs for 150 seconds. Calculation of the increase in absorbance

per minute and percentage inhibition was calculated for each test sample.

Statistical analysis

Data were collected and expressed as Mean ± SEM. They were analysed statistically using student's t-test and the two-way ANOVA on the IBM SPSS 23 Statistical Software Program. The level of statistical significance was placed at p = 0.05, using the Bonferroni Post Hoc test.

RESULTS

Acute toxicity test

The acute toxicity test revealed that the LD_{50} of the aqueous extract of *Azanza garckeana* is greater than 5,000 mg/kg. There was no lethality in any of the groups.

Phytochemical composition

Results for the phytochemical screening of the aqueous extract of *Azanza garckeana* fruit pulp are presented on Table 1. The results revealed the presence of flavonoids in high quantity, followed by alkaloids and tannins. Steroids and cardiac glycosides were present in much lower quantity, while saponins, terpenes and anthraquinones were absent.

Effect of *Azanza garckeana* on body weight of rats

The results of the effect of daily administration of *Azanza garckeana* extract on body weight are presented on Table 2. The results indicate that concurrent administration of formalin, 10 mg/kg and extract at a dose of 250 mg/kg significantly decreased body weight compared to baseline and control group ($p < 0.05$). However, the extract alone at 250 mg/kg induced significant increase in body weight compared to baseline ($p < 0.05$).

Effect of *Azanza garckeana* on weights of liver, kidney and heart of rats

The results indicated that administration of the extract alone at doses of 125 and 250 mg/kg caused significant decrease in the weight of the heart compared to normal control group ($p < 0.05$). Similarly, concurrent administration of formalin 10 mg/kg and the extract at the dose of 125 mg/kg caused a significant decrease only on the weight of the heart compared to the control ($p < 0.05$) (Table 3).

Effect of *Azanza garckeana* on weight, testicular length and epididymis of rats

The result showed that concurrent administration of the extract at 250 mg/kg with formalin caused a significant decrease on testicular weight and length ($p<0.05$) compared to the control group, while no significant effect on the weight of epididymis ($p > 0.05$) (Table 4).

Effect of *Azanza garckeana* on testosterone and gonadosomatic index (GSI)

The results demonstrate an increase in testosterone level in the group that received extract alone at 125 mg/kg and those co-administered with formalin at same dose of extract (125 mg/kg) compared to control group 2 (formalin alone) with $p<0.05$. Animals treated with formalin 10 mg/kg alone had the lowest concentration of testosterone and this was significantly lower compared to control ($p < 0.05$) (Table 5).

Effect of *Azanza garckeana* on semen parameters of rats

The results indicated that there is significant increase in sperm concentration in animals treated with the extract only at a dose of 125 mg/kg compared to normal control and formalin group ($p < 0.05$). Similarly, sperm vitality was highest from animals in the groups treated with the extract at both doses when induced with formalin toxicity ($p > 0.05$). Furthermore, motility was highest in animals treated with the extract alone at 125 mg/kg compared to normal and formalin control groups, and lowest in the group that concurrently received formalin 10 mg/kg the extract at 250 mg/kg ($p > 0.05$) (Figures 1 & 2).

Effect of *Azanza garckeana* on antioxidant enzymes

The result demonstrates that co-administration of formalin with extract at a dose of 125 mg/kg caused a significant increase in SOD activity compared to normal control groups ($p < 0.05$). Similarly, the highest concentration of MDA was seen in the testicles of animals in the group induced with formalin toxicity and concurrently treated with the extract at the dose of 250 mg/kg while the lowest MDA concentration was seen in animals induced with formalin toxicity and treated with 125 mg/kg ($p > 0.05$). Catalase activity was highest in testicles of animals from the group induced with formalin toxicity but concurrently treated with the extract at 250 mg/kg ($p > 0.05$) Figures 3, 4 & 5).

Table 1: Phytochemical composition of aqueous extract of *Azanza garckeana* fruit pulp

Constituents	Composition
Alkaloids	+ +
Saponins	–
Tannins	+ +
Flavonoids	+ + +
Carbohydrate	+ + +
Steroids	+
Terpenes	–
Anthraquinones	–
Cardiac glycosides	+

Key:
+ = Low.
++ = Moderate.
+++ = High.
- - = None.

Table 2: Effect of 40-day *Azanza garckeana* treatment on body weight (g) of rats

Treatment	DAY				
	Baseline	10	20	30	40
Normal Saline 1 ml/kg	159.70 ± 9.91	172.70 ±4.5.58	173.80 ± 4.55	144.92 ± 7.30	169.70 ± 5.30
Formalin, 10 mg/kg	166.57 ± 4.56	166.7 ±11.92	166.25 ± 12.92	143.37±11.76	143.32±11.40
A. garckeana 125 mg/kg	163.87 ±16.16	163.2 ±13.83	166.62 ± 14.45	135.97±12.46	170.50±12.23
A. garckeana 250 mg/kg	136.89 ± 7.30	162.90 ± 4.37	172.55 ± 5.98	160.40 ± 4.59	168.62±14.59
A. garckeana 125 mg/kg + Formalin 10 mg/kg	153.42 ± 4.24	161.95 ± 2.65	169.35 ± 4.27[b]	157.20 ±4.75	166.12±10.41
A. garckeana 250 mg/kg + Formalin 10 mg/kg	173.67±11.74	146.3 ±11.43[a]	143.2 ± 16.81[a]	143.45 ± 9.02	153.35±31.46

n=5
[a] = Significant effect compared to control (Normal saline) Value ($p < 0.05$).
[b] = Significant effect compared to Baseline ($p < 0.05$).

Table 3: Effect of 40-day *Azanza garckeana* treatment on some isolated organ weights of rats

Treatment	Weight (g)		
	Liver	Kidney	Heart
Normal Saline (1 ml/kg)	5.73 ± 0.30	1.24 ± 0.03	0.83 ± 0.03
Formalin (10 mg/kg)	5.67 ± 0.50	1.22 ± 0.08	0.67 ± 0.04[a]
A. garckeana extract (125 mg/kg)	5.51 ± 0.47	1.15 ± 0.11[a]	0.61 ± 0.03[a]
A. garckeana (250 mg/kg)	5.63 ± 0.10	1.18 ± 0.04	0.58 ± 0.01[a]
A. garckeana, 125 mg/kg + Formalin, 10 mg/kg	5.70 ± 0.37	1.25 ± 0.08	0.59 ± 0.04
A. garckeana, 250 mg/kg + Formalin, 10 mg/kg	5.60 ± 0.10	1.15 ± 0.01	0.59 ± 0.10

n = 5
[a] = Significant effect compared to control (Normal saline) value ($p < 0.05$).

Table 4: Effect of 40-day *Azanza garckeana* treatment on some isolated reproductive organs of rats

Treatment	Testicular Weight (g)	Testicular Length (mm)	Epididymis (g)
Normal saline 1 ml/kg	3.2 ± 0.71	20.62 ± 0.93	58.40 ± 3.70
Formalin alone (10 mg/kg)	2.64 ± 0.51	19.26 ± 1.11	54.50 ± 2.39
Azanza garckeana extract (125 mg/kg)	2.86 ± 0.37	20.68 ± 1.55	57.25 ± 2.01
Azanza garckeana extract (250 mg/kg)	3.09 ± 0.13	20.83 ± 1.45	55.75 ± 2.83
Azanza garckeana extract (125 mg/kg)+Formalin (10 mg)	2.71 ± 0.36	19.56 ± 0.91	54.25 ± 1.49
Azanza garckeana extract (250 mg/kg)+Formalin (10 mg/kg)	2.61 ± 0.46[a]	16.6 ± 2.05[a]	49.25 ± 12.23

n = 5

[a] = Significant effect compared to control (Normal saline) ($p < 0.05$).

Table 5: Effect of 40-day Treatment with *Azanza garckeana* on serum testosterone levels and gonadosomatic index

Treatment	Body Weight (g)	Testicular Weight (g)	GSI (%)	Testosterone (ng/ml)
Normal Saline (1 ml/kg)	169.70 ± 5.30	3.2 ± 0.71	1.89	5.78 ± 1.65
Formalin alone (10 mg/kg)	173.32 ± 11.40	2.64 ± 0.51	1.48	3.02 ± 1.25
Azanza garckeana (125 mg/kg)	170.50 ± 12.23	2.60 ± 0.37	1.51	8.00 ± 0.53[b]
Azanza garckeana (250 mg/kg)	168.82 ± 14.59	3.09 ± 0.13	1.86	4.20 ± 1.82
Azanza garckeana (125 mg/kg) +Formalin (10 mg/kg)	166.12. ± 31.46	1.61 ± 0.46[a]	1.63	7.00 ± 1.39[b]
Azanza garckeana (250 mg/kg) +Formalin (10 mg/kg)	153.35 ± 31.46	1.61 ± 0.01	1.18	3.62 ± 2.21

n = 5

[a] = Significant effect compared to control group ($p < 0.05$).

[b] = Significant effect compared to group 2 ($p < 0.05$).

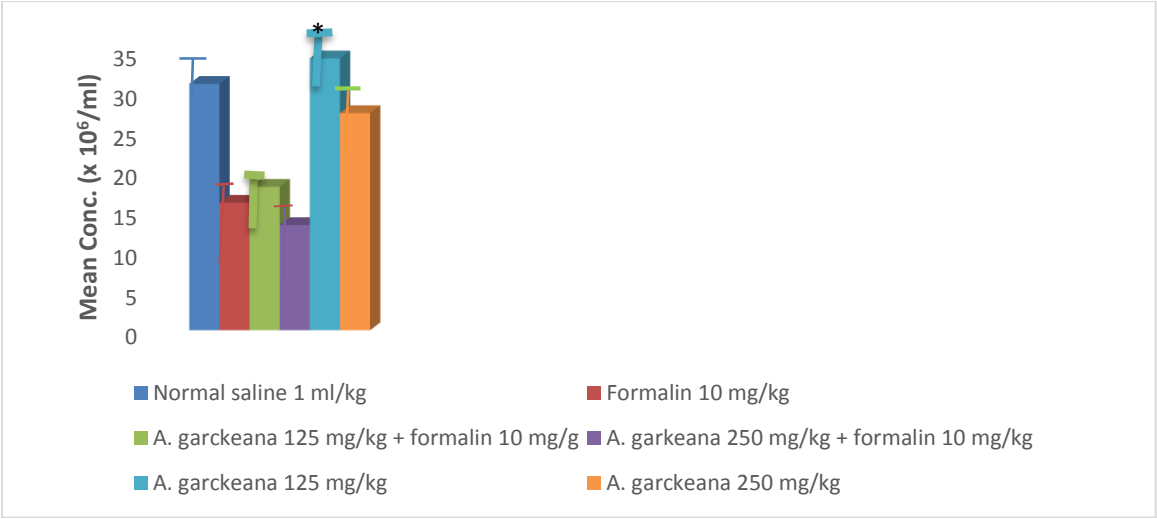

Figure 1: Effect of 40-Day treatment with *A. garckeana* on sperm concentration of rats. * = P< 0.05

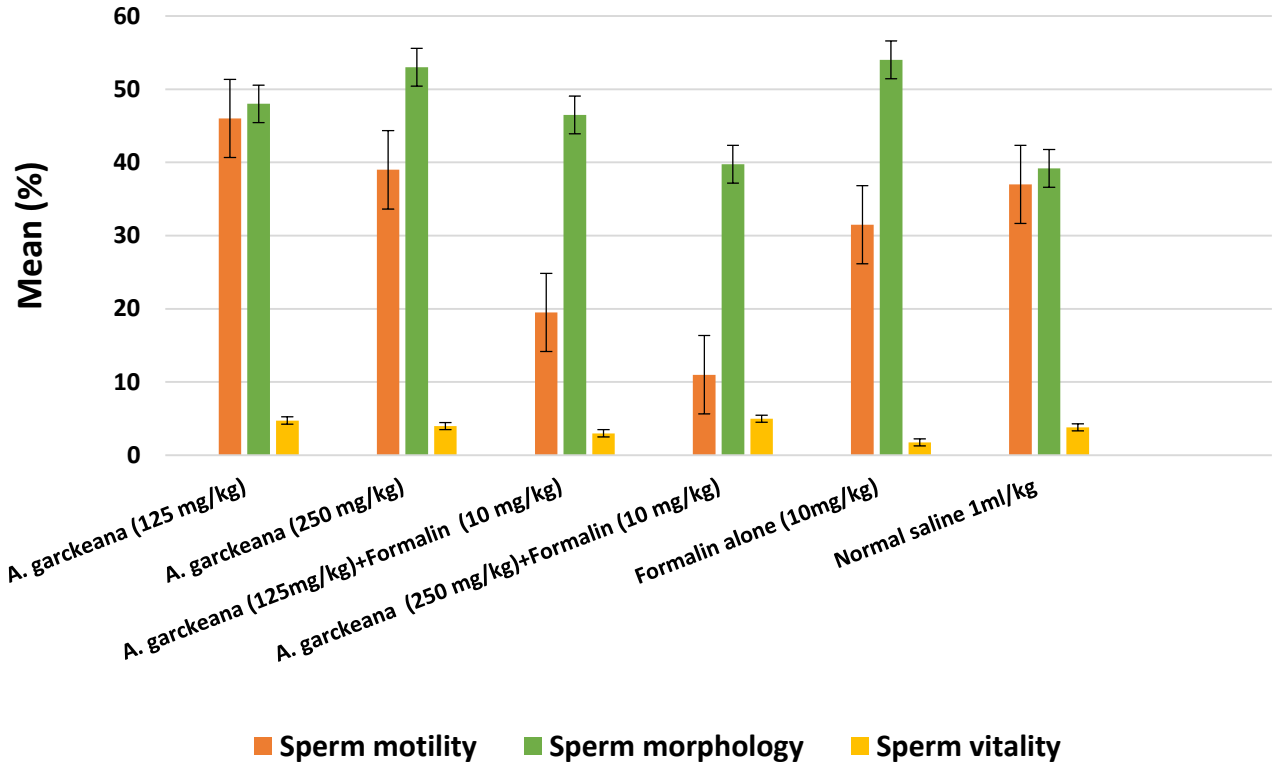

Figure 2: Effect of 40-days treatment on some sperm parameters.

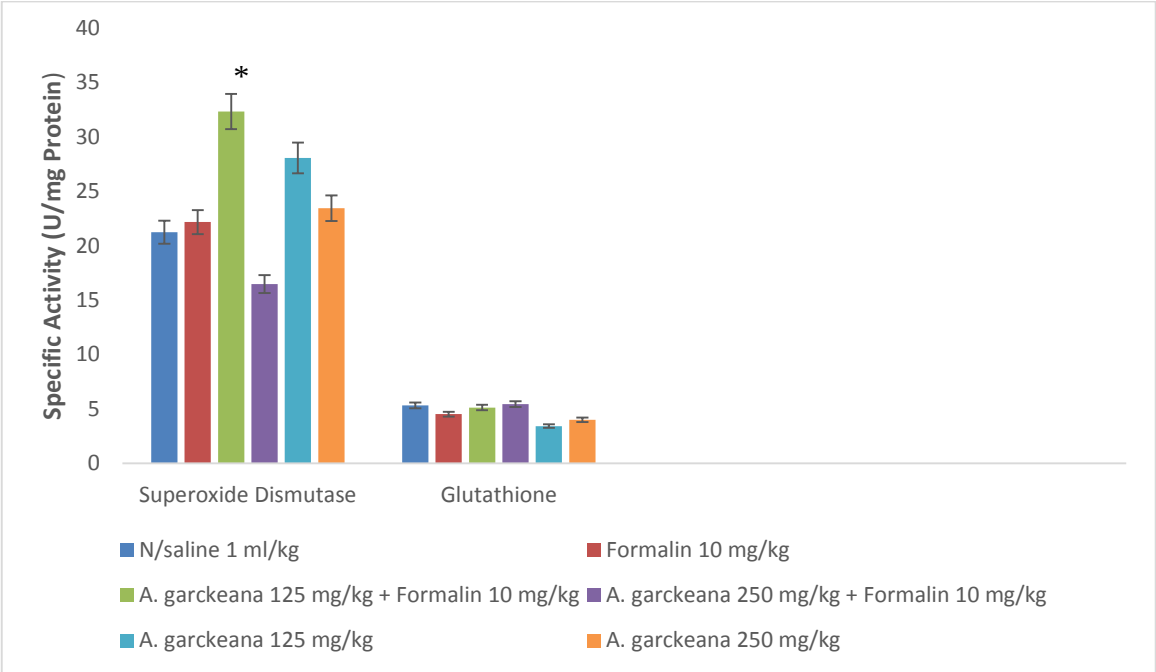

Figure 3: Effect of 40-Day treatment with *A. garckeana* on SOD and GSH enzymes in formalin-induced testicular toxicity in rats, * = $p < 0.05$.

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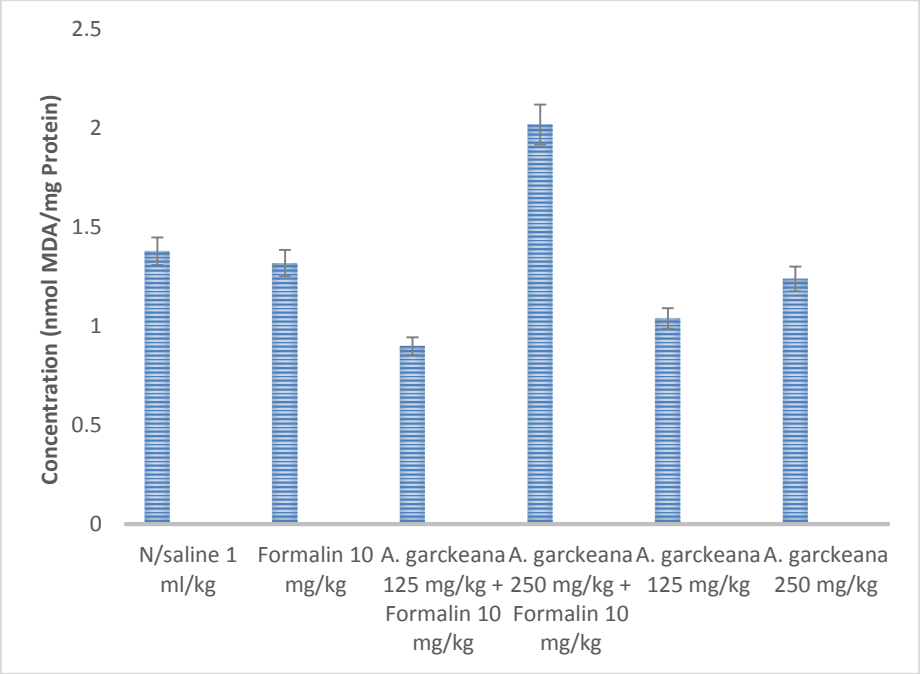

Figure 4: Effect of 40-Day treatment with *A. garckeana* on malondialdehyde in formalin-induced testicular toxicity in rats.

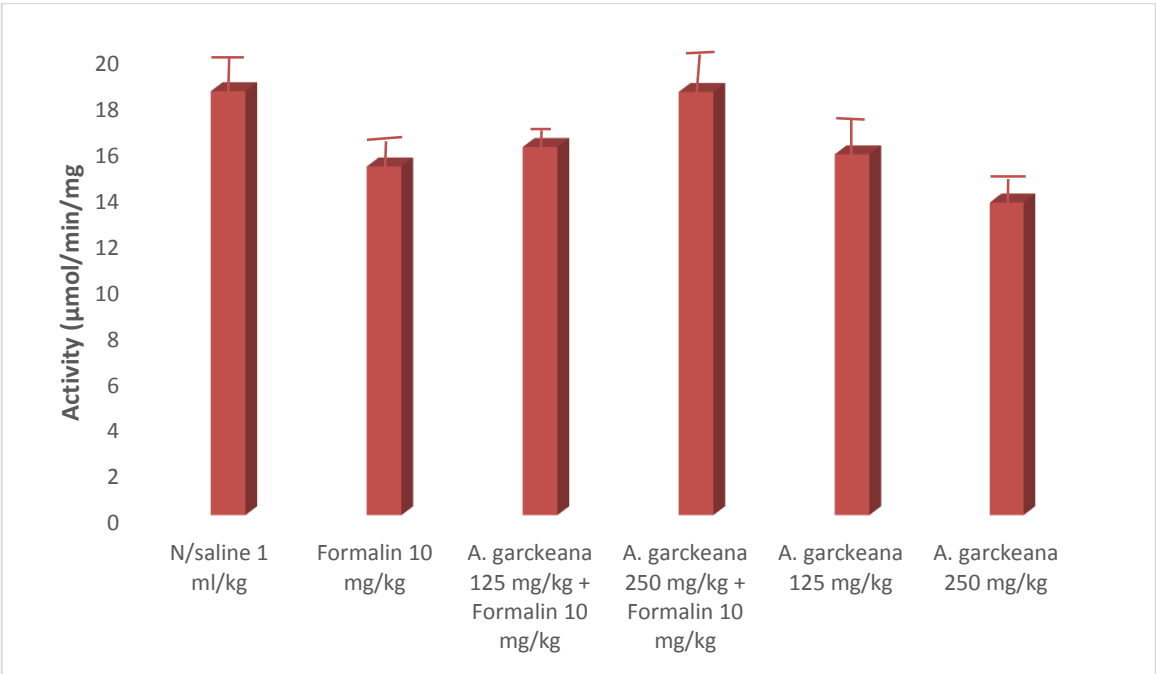

Figure 5: Effect of 40-Day treatment with *A. garckeana* extract on catalase activity in formalin-induced testicular toxicity in rats.

DISCUSSION

In this study, the acute toxicity test revealed that the LD_{50} of the aqueous extract of *Azanza garckeana* is greater than 5,000 mg/kg. There are 4 categories of toxicity with > 5000 being category 4, which is considered practically non-toxic and non-irritant [27]. The interpretation is that the drug is safe even in very high doses, in accordance with the Hodge and Sterner scale of toxicity. Results of the phytochemical screening revealed the presence of high composition of flavonoids and other phytochemicals like alkaloids and tannins which are known to possess antioxidant properties. Indeed, according to Pietta [28], the antioxidant activity of flavonoids, is due to their ability to reduce free radical formation, as well as their ability to scavenge free radicals. Alkaloids are also the major antioxidants in several natural products, and their antioxidant activities have been demonstrated in a previous study [29]. The presence of flavonoids and alkaloids in the aqueous extract of *Azanza garckeana* is therefore supportive of the antioxidant action exhibited by the extract in this study.

Analysis of organ weight in toxicology studies is an important endpoint for identification of potentially harmful effects of chemicals [30]. In this study, a significant decrease in body weight ($p < 0.05$) seen with concomitant administration of formalin and extract compared to baseline and control group is an indication of toxicity. However, the extract alone induced a significant increase in body weight compared to baseline ($p < 0.05$). In several animal experiments, scientists and local authorities have defined a body-weight reduction of 20 % or more as severe suffering and thereby as a potential parameter for humane endpoint decisions [31, 32]. The relationship between the testicular and total body weight is used to calculate the Gonadosomatic Index (GSI), which is the calculation of the testicular mass as a proportion of the total body mass. The GSI is a tool for measuring the sexual maturity and performance of animals in correlation to ovary or testicular development. This index is frequently used as reporting point in OECD test guideline which may be used as indication or evidence of potential endocrine disruption effect of chemicals in regulatory frame work [33]. The extract at a dose of 250 mg/kg was able to significantly increase the GSI compared to the formalin control group ($p < 0.05$), thereby suggesting the extract's ability to increase sexual performance.

It has been reported that repeated exposure to chemicals such as formalin can contribute to testicular oxidative stress with consequent disturbances in spermatogenesis [4, 5]. In this study, increase in the levels of testosterone and sperm concentration seen in groups administered the extract alone and those co-administered with formalin plus extract when compared to control group, suggest its ameliorative effects on the formalin induced toxicity. Oxidative stress (OS) is involved in poor semen quality and subsequent infertility in males with spinal cord injury [34].

SOD eliminates ROS by reducing superoxide to form hydrogen peroxide [35]. High levels of ROS production does lead to peroxidation of sperm acrosomal membrane and diminish acrosin activity [36]. Indeed, increased ROS levels are known to correlate with reduction in sperm motility [37, 38]. Endogenous antioxidants present in normal semen include SOD, catalase and GSH, with SOD being the most predominant. There was a significant increase in SOD activity and this is associated with decreased free radicals and a subsequent decrease in oxidative stress. This is because SOD is known to interact with the radical oxygen specie to produce hydrogen peroxide (H_2O_2) which is in turn acted upon by catalase to produce water [39]. Catalase detoxifies both intracellular and extracellular H_2O_2 to produce water and oxygen [40, 41]. Catalase activity was highest in testicles of animals from the group induced with formalin toxicity but concurrently treated with the extract.

Glutathione is a known potent antioxidant with high redox potential and it also serves as a co-factor for several oxidative stress detoxifying enzymes [42, 43]. In this study, glutathione activity was highest in the group treated with the extract. In a previous study, glutathione activity was similarly and significantly raised in auto-mechanics compared with controls ($P<0.05$) where induction of glutathione in the presence of altered oxidative stress biomarkers was reported [44].

The highest concentration of MDA was seen in the testicles of animals in the group induced with formalin and concurrently treated with the extract at the dose of 250 mg/kg while the lowest MDA concentration was seen in animals induced with formalin toxicity and treated with 125 mg/kg suggesting that higher doses of the plant extract provides better ameliorative effects.

CONCLUSION

The results obtained from this study showed that the aqueous extract of *Azanza garckeana* possesses antioxidant and ameliorate effects against formalin-induced testicular toxicity. This finding could justify its ethno-medicinal use by the locals to treat male infertility.

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