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Research article

Protective Effects of Aqueous Extract of *Citrullus lanatus* Fruit on Reproductive Functions and Antioxidant Activities in Arsenic-treated Male Wistar Rats

***Daramola O.O, Oyeyemi W.A, Beka F.U, Ofutet E.A**

Department of Physiology, Igbinedion University, Okada. Edo State, Nigeria

ABSTRACT

Arsenic trioxide (As2O3), a known male reproductive toxicant induces it effects majorly through oxidative stress. Citrullus lanatus fruit is widely consumed for its high medicinal values and antioxidant capacities. This study investigated the effects of aqueous extract of Citrullus lanatus fruit (AECL) on reproductive functions and antioxidant activities in arsenic-treated male Wistar rats. Thirty male Wistar rats (150-190 g) were grouped into six and treated as follows; Control, 3 mg/kg As2O3, 100 mg/kg AECL, 200 mg/kg AECL, As2O3+100 mg/kg AECL and As2O3+200 mg/kg AECL. All administration was done orally for thirty days. Caudal sperm, serum hormone levels and testicular antioxidant activities were evaluated. Decreases ($p<0.05$) in sperm concentration, morphology, viability, and motility were observed in As2O3 group, however, AECL co-administered with As2O3 significantly reversed these effects. Follicle stimulating hormone decreased ($p<0.05$) in As2O3 group compared with control while significant increase was observed in groups co-treated with AECL and As2O3 relative to As2O3 group. There was an increase ($p<0.05$) in malondialdehyde level in As2O3 group compared with control while a decrease ($p<0.05$) was observed in groups co-treated with AECL and As2O3 compared with the As2O3 group. Superoxide dismutase activities decreased ($p<0.05$) in As2O3 group compared with control but increased ($p<0.05$) in 200 mg/kg AECL+As2O3 group relative to As2O3 group. Immature spermatids were observed in the seminiferous tubules of As2O3 group, while AECL improved the histology when compared with As2O3 group. The results of this study suggest that aqueous extract of Citrullus lanatus provides protection for sperm cells against arsenic-induced oxidative stress.

Keywords: *Eidolon helvum, dental formula, dental abnormalities, attrition*

*Author for correspondence: *E-mail*: *ooore324@yahoo.com; Tel:+234-8038169338*

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INTRODUCTION

Inorganic arsenic is an environmental toxicant and a sulfhydryl-reactive metalloid that is abundantly present in the earth crust (Ikuko *et al*., 2005; Akter *et al*., 2005). Humans and animals are normally exposed to chronic low-dose arsenic ingestion through contaminated water (Huff *et al*., 2000; Kim *et al*., 2011). Exposure to arsenic leads to various health hazards among which is impairment of male reproductive function such as the suppression of spermatogenesis and androgenesis (Pant *et al*., 2004). Methylation of arsenic induces oxidative stress, which is the major mechanism through which arsenic impairs male reproductive functions (Hayakawa *et al*., 2005). Arsenic methylation requires oxidation by antioxidant enzymes such as glutathione reductase, glutathione peroxidase and catalase thereby depleting the levels of these enzymes that play important role in preventing oxidative stress (Alves and de Meis, 1987; Manna *et al*., 2008). The body, therefore, requires enhancement of its endogenous antioxidants to defend against oxidative stress induced by arsenic in order to compensate for depletion of the antioxidant enzymes during metabolism of pro-oxidants, this can be provided in the diet.

Citrullus lanatus (water melon) is a fruit belonging to the family of Cucurbitaceae. It has been reported to be rich in flavonoids, Phenols, lycopene, β-carotene, vitamins B, C and E all of which are potent antioxidants (Edwards *et al*., 2003; Choudhary *et al*., 2015). It is also known to contain high amounts of arginine and citrulline both of which contribute to the production of nitric oxide, a vasodilator that plays an important role in penile erection (Cormio *et al*., 2011).

Mungule *et al*. (2014) have reported that ethanol extract of Citrullus lanatus had aphrodisiac effects on rat models. Also, the methanol extract of the rind has been reported to improve spermatogenesis and reproductive hormone concentrations in lead-treated male Wistar rats (Kolawole *et al*., 2014).

One of the mechanisms of arsenic action in inducing male reproductive damage is through oxidative stress. The antioxidant capacity of Citrullus lanatus fruit is well documented (Edwards *et al*., 2003; Choudhary *et al*., 2015). The aim of this study is to investigate the reproductive functions and antioxidant effects of aqueous extract of Citrullus lanatus fruit in arsenic-treated male Wistar rats.

MATERIALS AND METHODS

Plant material: Fruits of *Citrullus lanatus* fruit were obtained from a local market in Okada, Edo State, Nigeria. The fruit was washed, cut opened and thereafter, about 300 g of the flesh was separated from the rind and seeds. It was then blended until liquefied using Flourish electrical blender CA/BD912, sieved using a filter paper size 125 mm. The filtrate was taken to Energy Center, University of Benin, Nigeria for freeze drying. A pink colour, wax shaving aqueous extract of Citrullus lanatus (AECL) obtained weighed 19 g and was stored at 4 °C.

Chemicals: Arsenic trioxide and all other chemicals used for this study were analytical grade and products of Sigma-Aldrich, United Kingdom.

Experimental Animals: Thirty (30) male Wistar rats (150 to 190 g) were purchased from the Animal House, Igbinedion University Okada. They were kept in well ventilated plastic cages and were maintained under standard laboratory conditions. They were acclimatized for two weeks and allowed to have free access to feed and drinking water.

Experimental Design: The animals were randomly distributed into six groups of five animals each. Both As2O3 and AECL were orally administered for 30 days. The grouping and administration were as follows; Group 1 (control) was administered distilled water, Group 2 was treated with 3 mg/kg As2O3, Groups 3 and 4 were treated with 100 and 200 mg/kg AECL respectively and Groups 5 and 6 were treated with As2O3 co-administered with100 and 200 mg/kg AECL respectively.

Tissue Collection: At the end of the 30 days administration, blood sample was collected by cardiac puncture for hormones assay. The epididymis was collected and used for sperm analysis. The testes were collected. One testis was homogenized, and used for malondialdehyde (MDA), glutathione reductase (GSR), catalase (CAT) and superoxide dismutase (SOD), and glutathione peroxidase (GPX) assays while the second testis was preserved in Bouin's fluid for histology.

Sperm Motility and Concentration: The caudal epididymis was cut and washed in 2 ml of phosphate buffer saline (pH 7.4). A Neubauer's counting chamber was prepared and filled appropriately with the sperm sample. The sperm motility and concentration were analyzed with the aid of computer assisted sperm analyzer (JH-6004 CASA).

Viability: One drop of prepared sperm sample was mixed with equal volume of eosin and nigrosin suspension and allowed to air dry and examined microscopically. Viable spermatozoa remained unstained while non-viable spermatozoa stained red. A total of 100 spermatozoa were counted in at least 10 randomly selected fields. The numbers of viable and non-viable spermatozoa were noted and percentage viability was calculated (Cheesbrough *et al*., 2000).

Morphology: A thin smear of the sperm sample was made on a clean slide, fixed with 95% ethanol and allowed to air dry. The fixed slide was then sequentially immersed into different concentrations of ethanol and appropriate stains namely Harris haematoxylin, G-6 orange stain and EA-50 green stain for one minute. The slide was then examined microscopically at a magnification of 1000×, and 200 sperm were assessed. The sperm abnormalities were expressed as percentages (Cheesbrough *et al*., 2000).

Hormone Assay: The enzyme linked immunosorbent assay technique was used for follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone estimation using kits (Calbiotech Inc. California). The procedure for the estimation of serum concentration of each hormone was carried out according to the kits' manual.

Antioxidant Assays:

The testicular supernatant was used for Lipid peroxidation and antioxidant assays

Malondialdehyde (MDA) Assay: This was done according to the method of Rice-Evans *et al*. (1986). The principle is based on the reaction of Malondialdehyde, a product of lipid peroxidation with thiobarbituric acid to give a red species that can be detected at 535 nm.

Superoxide dismutase (SOD) Assay: This was estimated according to the method of Misra and Fridovich (1972). The principle is based on rapid auto-oxidation of adrenaline in aqueous solution to adrenochrome due to the presence of superoxide anions. The concentration was determined with a spectrophotometer at 420 nm.

Catalase (CAT) Assay: This was determined according to the method of Aebi (1984). Upon the addition of 30 mM H2O2 in 50 mM of phosphate buffer (pH 7.4) to sample, it is converted to oxygen and water. This action was stopped after three minutes by the addition of 1mL of H2SO4 to the mixture, followed by 7.0 mL of KMnO4. Catalase (CAT) activity was estimated by a decrease in absorbance of H2O2 at 520 nm.

Glutathione Reductase (GSR) Assay: This was done according to the method of Teitze (1969), the method utilized the principle of colour development caused by Ellman's reagent. The sample was precipitated with trichloroacetic acid for 5 mins and centrifuged, Ellman's reagent (5, 5'dithio (bis) nitrobenzoic acid in sodium citrate) and phosphate buffer was added to the supernatant and the standard. The colour developed was read at 412 nm using a spectrophotometer.

Enzymatic Assay of Glutathione Peroxidase (GPX): This principle was based on the potential ability of pyrogallol to donate an electron to hydrogen peroxide in the presence of glutathione peroxidase contained in the sample under investigation. Pyrogallol is the oxidized to purpurogallin and its absorbance is then measured at 420 nm. The result was recorded in mg protein/ml enzyme (Chance and Maehly, 1955).

Testicular Histology: The left testicle was harvested and immediately fixed in Bouin's fluid for at least 5hrs. Each sample was dehydrated using ascending grades of alcohol. It was cleared with two changes of xylene, embedded in paraffin wax, trimmed, nicked and sectioned using a microtome and stained using hematoxylin and eosin (H&E) for the purpose of determining the general morphology.

Statistical Analysis

The data from each group was expressed as mean ± standard error of the mean (mean ± SEM). The means of different groups were compared using one-way analysis of variance (ANOVA) followed by waller-Duncans post hoc test. p-value was taken to be significant when it is < 0.05.

RESULTS

As shown in Figure 1, there was a significant decrease in sperm concentration of groups treated with arsenic only, arsenic co-treated with 100 and 200 mg/kg AECL when compared with control group. However, a significant increase ($p < 0.05$) was observed in arsenic group co-treated with 200 mg/kg AECL when compared with arsenic group.

Figure 2 shows that there was a significant decrease ($p < 0.05$) in sperm motility and progressive motility but an increase in sperm non-progressive motility in the arsenic treated group compared with control, while there was an increase ($p < 0.05$) in sperm motility and progressive motility but a decrease ($p < 0.05$) in non-progressive motility in the arsenic group co-treated with 100 and 200 mg/kg AECL when compared with arsenic treated group only

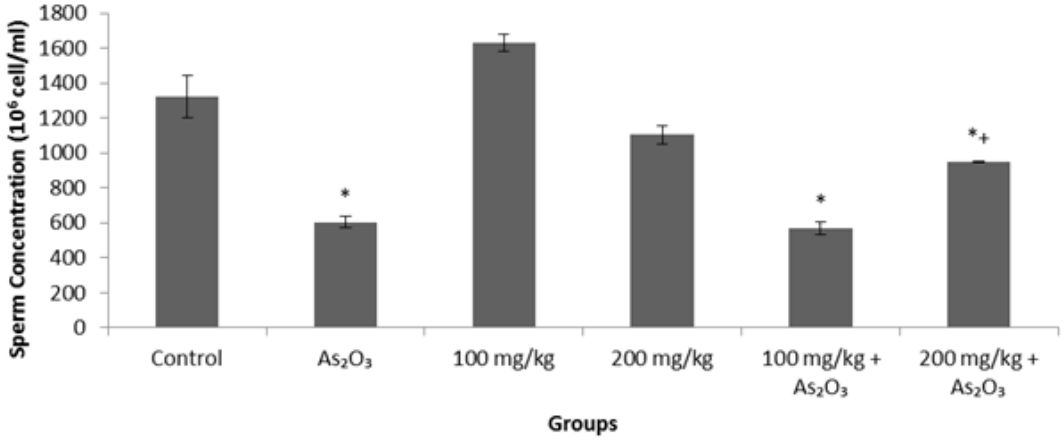

Figure 1:
Effect of Aqueous Extract of *Citrullus lanatus* (AECL) on Sperm Concentration in Arsenic (As_2O_3) Treated Wistar Rats
Bars are expressed as Mean ± SEM (n=5), $*,^{+}p < 0.05$ were considered significant compared to control and arsenic groups respectively.

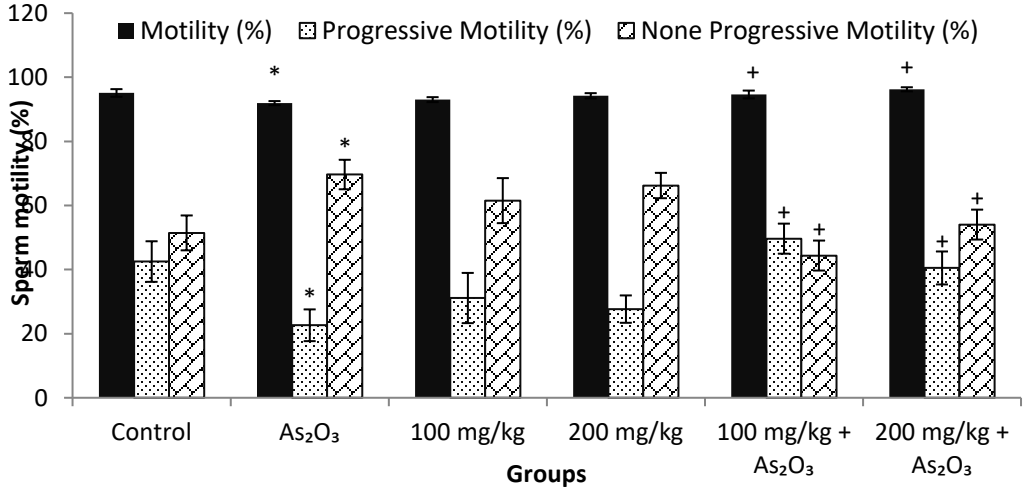

Figure 2:
Effects of Aqueous Extract of *Citrullus lanatus* (AECL) on sperm motility, progressive motility and none progressive motility in Arsenic (As_2O_3) Treated Wistar Rats.
Bars are expressed as Mean ± SEM, (n=5), $*,^{+}p < 0.05$ were considered significant compared to control and arsenic groups respectively.

Table 1:
Effects of Aqueous Extract of *Citrullus lanatus*(AECL) on Serum Concentrations of Testosterone FSH and LH in Arsenic Treated Wistar Rats

	Testosterone (ng/ml)	FSH (ng/ml)	LH (ng/ml)
CONTROL	4.23 ± 0.76	3.10 ± 0.48	33.73 ± 7.56
As_2O_3	2.65 ± 0.20*	1.77 ± 0.40*	19.02±1.36*
100 mg/kg	3.55 ± 0.58	2.65 ± 0.26	26.13 ± 1.83
200 mg/kg	4.20 ± 0.49	2.28 ± 0.41	42.13 ± 8.93
100 mg/kg + A_s2O_3	2.72 ± 0.50*	2.56 ± 0.48$^+$	19.19±2.48*
200 mg/kg +A_s2O_3	2.80 ± 0.44*	2.42 ± 0.40$^+$	19.31±1.01*

*Values are expressed as Mean ± SEM. n=5, *, $^+P < 0.05$ were considered significant compared to control and arsenic (A_s2O_3) groups respectively.*

Figure 3 shows that there was a significant decrease ($p < 0.05$) in normal sperm morphology and sperm viability in the group treated with arsenic only when compared with the control group, there was also a significant decrease ($p < 0.05$) in normal sperm morphology in the arsenic group co-treated with 100 mg/kg AECL when compared with the control. However, normal sperm morphology was significantly improved ($p < 0.05$) in the group co-treated with arsenic and 200 mg/kg AECL when compared with the arsenic only group. Sperm viability was also significantly increased ($p < 0.05$) in the arsenic groups co-treated with either 100 or 200 mg/kg AECL relative to arsenic group.

Table 1 shows that there was a significant decrease ($p < 0.05$) in serum testosterone and LH concentrations in the groups treated with arsenic only and arsenic with either 100 or 200 mg/kg AECL when compared with the control group. There was also a significant decrease ($p < 0.05$) in FSH concentration in the arsenic treated group only when compared with the control group.

The photomicrographs in Plate 1 show that the group treated with arsenic only showed atrophied tubules with sloughed germ cells with no maturation stages, the seminiferous tubule appeared abnormal when compared with the control group. The arsenic groups co-treated with 100 and 200 mg/kg AECL showed an improvement in their histology with normal sperm maturation stages and normal interstitial space when compared with arsenic only group

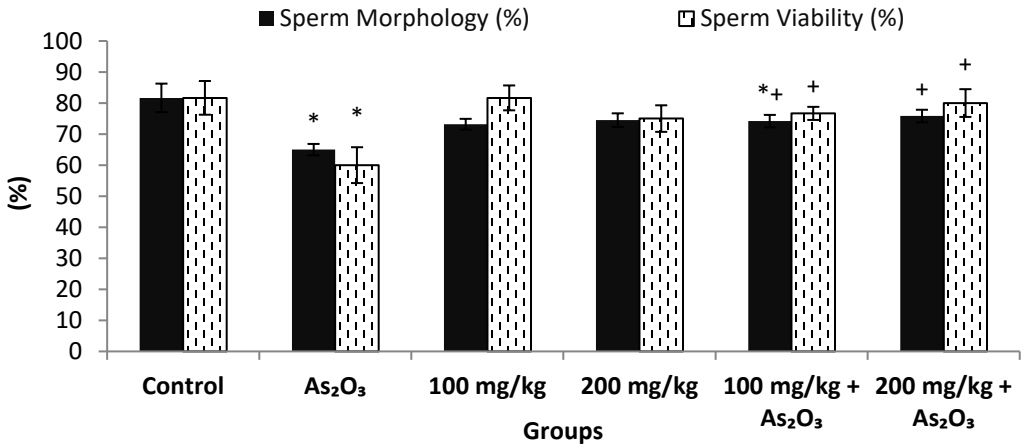

Figure 3:
Effects of Aqueous Extract of *Citrullus lanatus* (AECL) on sperm morphology and viability in Arsenic (As_2O_3) Treated Wistar Rats.
*Bars are expressed as Mean ± SEM, (n=5), *, $^+p < 0.05$ were considered significant compared to control and arsenic groups respectively.*

Table 2:
Effects of Aqueous Extract of *Citrullus lanatus* (AECL) on Redox Status and Antioxidant Enzyme Levels in Arsenic Treated Wistar Rats

	MDA (10^{-3}) (nmol/mg protein)	GPX (u/mg protein)	GSR (u/min/mg protein)	CAT (units/min/mg protein)	SOD (units/mg protein)
CONTROL	9.60 ± 1.04	3.71 ± 0.55	0.31 ± 0.05	0.82 ± 0.16	4.17 ± 0.43
As_2O_3	13.04 ± 0.50*	3.07 ± 0.30	0.14 ± 0.03*	0.96 ± 0.21	2.50 ± 0.11*
100 mg/kg	7.40 ± 0.68	2.82 ± 0.49	0.17 ± 0.05*	0.89 ± 0.05	5.07 ± 1.16
200 mg/kg	11.37 ± 0.83	3.38 ± 0.57	0.36 ± 0.04	1.09 ± 0.16	3.87 ± 0.36
100 mg/kg + As_2O_3	4.30 ± 1.45^{*+}	3.91 ± 0.50	0.14 ± 0.05*	1.56 ± 0.44^{*+}	2.99 ± 0.29
200 mg/kg + As_2O_3	6.07 ± 0.84^{*+}	3.86 ± 1.26	0.20 ± 0.06$^+$	0.96 ± 0.17	4.16 ± 0.48$^+$

*Values are expressed as Mean ± SEM. n=5, *, $^+P < 0.05$ were considered significant compared to control and arsenic (As_2O_3) groups respectively*

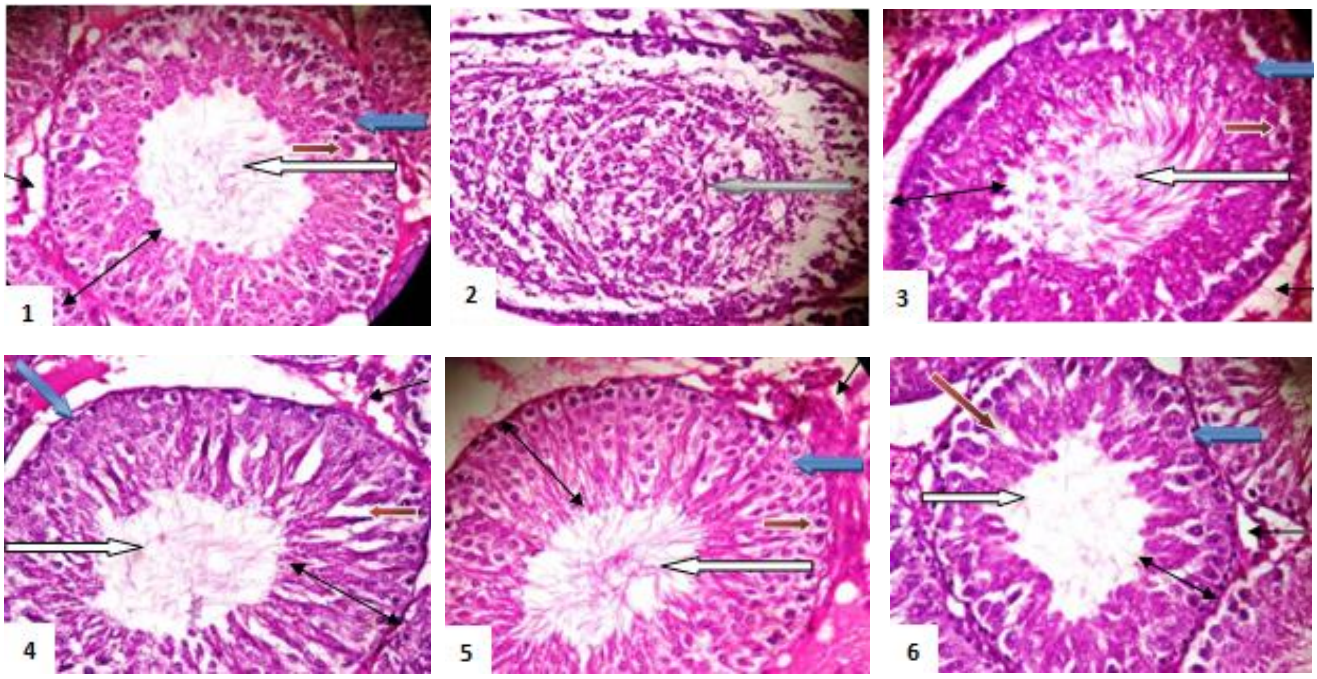

Plate 4:
Effects of Aqueous Extract of *Citrullus lanatus* (AECL) on Testicular Histology in Arsenic (As_2O_3) Treated Wistar Rats (Hematoxylin and Eosin stained × 400).
(1) control **(2)** As_2O_3**(3)** 100 mg/kg **(4)** 200 mg/kg **(5)** 100 mg/kg + As_2O_3 **(6)** 200 mg/kg + As_2O_3. Blue arrow shows normal spermatogonia layer, the red arrow indicates normal Sertoli cells, spanned arrow indicates normal germ cell layer with normal maturation stages, white arrow indicates lumen, black arrow indicates sloughed germ cells within the seminiferous tubule lumen and slender arrows indicates normal Leydig cell

Table 2 shows a significant increase ($p < 0.05$) in MDA level in the arsenic group when compared with control group, while it significantly decreased in the arsenic groups co-treated with 100 or 200 mg/kg AECL when compared with arsenic group ($p < 0.05$). A significant decrease ($p < 0.05$) was observed in the testicular GSR level in the groups treated with arsenic only, 100 mg/kg AECL and arsenic co-treated with 100 mg/kg AECL when compared with the control group. There was a significant increase ($p < 0.05$) in the testicular catalase level in the arsenic group co-treated with 100 mg/kg AECL when compared to both control and arsenic groups. There was also a significant decrease ($p < 0.05$) in the SOD level in the arsenic treated group only when compared with the control, while it significantly increased in arsenic group co-treated with 200 mg/kg AECL

DISCUSSION

The present study describes the effects of oral administration of aqueous extract of *Citrullus lanatus* fruit on sperm parameters, reproductive hormones and testicular antioxidant enzymes in arsenic treated male Wistar rats. In this study, the observed decrease in sperm concentration in the groups treated with arsenic is in agreement with the report of Pant *et al.* (2004). Arsenic has been reported to accumulate in different organs and the testis cytosol is said to have the highest amount of methylating activity (Aposhian, 1997; Healy *et al*., 1998). A methylation threshold hypothesis has been proposed (Hopenhayn-Rich *et al*., 1993), stating that after exposure to inorganic arsenic, methylation capacity begins to decline after a certain level, thus increasing the toxic effects of inorganic arsenic. The hypothesis might have led to decreased sperm count. Arsenic is known to cause compromised integrity of sperm cell membrane, depressed spermatogenic functions and subsequently, low sperm count by inducing oxidative stress (Hayakawa *et al*., 2005). The ability of 200 mg/kg AECL to ameliorate the effects of arsenic may be due to its free radical scavenging activity and its ability to reduce and chelate transitional metals (Puntel *et al*., 2005; Oboh and Rocha, 2007; Oseni and Okoye, 2013; Adetutu *et al*., 2015).

The decrease in sperm motility observed in the arsenic treated group may be due to the accumulation of arsenic in epididymis where the sperm matures and acquires motility. It has been documented that arsenic normally accumulates in the epididymis and has high affinity for binding with sulfhydryl groups on proteins due to its electrophilic affinity (Workings *et al.*, 1985). Hence, the observed decrease in sperm motility may be due to increase accumulation of arsenic in the epididymis which may interact with proteins responsible for sperm motility e.g., cysteine rich secretory protein (CRISP) family and beta defensin family. The two dosages of AECL were able to ameliorate the observed decrease in sperm motility in the arsenic treated groups. This may be due to its phenolic content (Oseni and Okoye, 2013). Natural polyphenols are capable of removing free radicals and chelating metal catalyst, activating antioxidant enzymes and reducing alpha-tocopherol radicals and inhibiting oxidases. The improvement in sperm motility in groups treated with AECL may be due to the prevention of arsenic accumulation

in the epididymis. The phenol content of AECL may chelate arsenic and enhance its excretion (Oseni and Okoye, 2013). The phenols might have also activated the endogenous antioxidant enzymes present in the epididymis.

The current observed decrease in sperm viability and morphology in the group treated with arsenic only is similar to Zubair *et al*. (2014) report. Arsenic produces an increased generation of reactive oxygen species which may interact with polyunsaturated fatty acids of spermatozoa, causing peroxidation which may lead to deformities and reduction of viability (Das *et al*., 2009). It was however observed in this study that both 100 and 200 mg/kg of AECL ameliorated the effects of arsenic on sperm viability and morphology. *Citrullus lanatus* fruit contains high amount of Vitamin C (Choudhary *et al*., 2015), It has been reported that vitamin C protects human spermatozoa against endogenous oxidative damage by neutralizing hydroxyl, superoxide and hydrogen peroxide radicals and preventing sperm agglutination (Fraga *et al*., 1991). Therefore, it is possible that the Vitamin C content of AECL helped to ameliorate the production of peroxidation thus leading to improvement in morphology and viability of spermatozoa of AECL treated rats.

The observed decrease in testosterone, luteinizing hormone and follicle stimulating hormone in the groups treated with arsenic in this study is in consonance with the reports of Sakar *et al*. (2003) and Ali *et al.* (2013). Arsenite is known to adversely affect the production of testosterone by disrupting the hypothalamic-pituitary-testicular axis (Jana *et al*., 2006) through oxidative stress and inducing cellular toxicity (Blair *et al.,* 1990; Chang *et al.,* 2007). The suppression of LH led to the observed decrease in serum testosterone concentration and in turn decreased sperm concentration in the arsenic treated groups. The effect of arsenic on FSH was however cushioned by 100 and 200 mg/kg of AECL, this may have also contributed to the improved sperm concentration observed in the arsenic groups treated with 200 mg/kg of AECL. The exogenous antioxidants present in *Citrullus lanatus* may have largely contributed to the improved FSH level and sperm concentration level in the arsenic group treated with 200 mg/kg of AECL. It is possible that a better result could have been achieved with a higher dosage of AECL.

The increase in MDA level observed in the group treated with arsenic is in agreement with the report of Fouad *et al*. (2015). It has been reported that arsenic induces morphologic changes in mitochondrial integrity leading to uncontrolled random formation of superoxide anion radical and subsequently increased formation of peroxyl radicals (ROO•), superoxide anion radical (O_{-2}), singlet oxygen ($^1O_2$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), dimethyl arsenic radical [(CH3)2As•], (Valko *et al*., 2005; Flora *et al*., 2007). Also, metabolism of arsenic in tissues leads to depletion of cellular oxidant defence such as glutathione (Hopenhayn-Rich *et al*., 1993; Cohen *et al*., 2006). In addition to the above stated, arsenic can inhibit the activity of GSR by interacting with critical thiol groups in GSR molecules (Cunningham *et al.,* 1994; Styblo *et al.,* 1997). The combination of above mechanisms of arsenic results in an increased sensitivity of cells to arsenic toxicity (Valko *et al*., 2005; Cohen *et al*., 2006). This mechanism of arsenic toxicity may have resulted in the depletion on GSR and SOD observed in groups treated with arsenic in this study. The ability of both dosages of AECL to decrease MDA level may be due to its metal chelating capacity (Oseni and Okoye, 2013; Adetutu *et al*., 2015) and the presence of lycopene in its phytochemical constituent. *Citrullus lanatus* is reported to have a high lycopene content (Kang *et al*., 2010), a lipophilic antioxidant that is present in high concentration in the testis and in the seminal plasma (Agarwal and Sekhon, 2010). Its lipophilic nature enables it to accumulate in cell membranes and lipoproteins, thus exerting a more noticeable effect on components of such a cell (Rao *et al*., 1999). It also traps free radicals and halts the propagative chain reactions (Bohm *et al*., 2003), reducing the ROS burden and alleviating oxidative stress, thus preventing oxidative damage to lipids, proteins and DNA (Rao *et al*., 2006; Palozza *et al*., 2012).

The reduction observed in SOD level in the arsenic treated group in this study is similar to the report of Banerjee *et al*. (2009). A decrease in the activity of SOD can be owed to an enhanced superoxide production during arsenic metabolism (Searle and Wilson, 1980). However, the increase in SOD levels observed in the group treated with arsenic and co-administered with 200 mg AECL is similar to Banerjee *et al*. (2009), they reported that vitamin C ameliorated the toxic effects of arsenic in the liver and spleen of mice. Vitamin C a low molecular weight compound is a potent antioxidant that is capable of protecting the testis against oxidative stress due to increased generation of free radicals such as H_20_2. The presence of Vitamin C in *Citrullus lanatus* may have been responsible for the increased level of SOD observed in the group treated with arsenic and AECL.

In the present study, it was observed that arsenic caused sloughing off of germ cells in seminiferous tubule and subsequent degeneration of spermatogenic element in the group treated with arsenic only, this is similar to the report of Pachnanda and Singh (2012). Arsenic may have compromised the antioxidant status of the testis which helps to maintain its integrity in order to carry out spermatogenic and androgenic functions. The increase in malondialdehyde level in the arsenic only treated group may be responsible for the observed distortion of cytoarchitecture of the testes. The observed normal appearance in the histology of other experimental groups may be due to the ability of AECL to prevent lipid peroxidation within the testes as observed in this study.

In conclusion, the protective effects of aqueous extract of *Citrullus lanatus* fruit against arsenic toxicity observed in the sperm profile, testicular histology, and FSH concentration in this study may be due to antioxidant activities of *Citrullus lanatus*. However, a further study needs to be done to be able to ascertain the mechanism by which arsenic causes sloughing off of immature spermatids and possibly, the dosage of AECL could be increased in subsequent studies to see if it will ameliorate the reduction in LH and testosterone caused by arsenic.

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