

## **Ameliorative Effects of Hydromethanolic Extract of *Citrullus lanatus* (Watermelon) Rind on Semen Parameters, Reproductive Hormones and Testicular Oxidative Status Following Nicotine Administration in Male Wistar Rats**

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**Summary:** The present study examines the possible ameliorative effects of the hydromethanolic extract of *Citrullus lanatus* rind (HECL) on some reproductive function and oxidative indices of the testes in male Wistar rats following administration of nicotine. Twenty male rats were assigned into four groups: Group A to D of five rats each. Group A served as control and received 2ml/kg body weight of 10% extract vehicle; Group B received 1mg/kg body weight of nicotine; Group C were co-administered 1mg/kg body weight nicotine and 500 mg/kg body weight of HECL and Group D received only 500mg/kg body weight of HECL. The drugs and extracts were administered orally to the rats for 42days; blood samples were collected by direct cardiac puncture for determination of serum concentrations of testosterone, Follicle Stimulating Hormone and Luteinizing Hormone. The testes were also harvested for determination of semen parameters: motility, morphology, viability and count and testicular tissue processed for superoxide dismutase and malondialdehyde concentration. Compared to Group A control rats, administration of HECL significantly increased sperm count and reproductive hormone concentrations amongst Group B rats ( $p < 0.05$ ). Treatment with nicotine caused a significant reduction in the levels of all reproductive hormones with significant diminution of some sperm parameters: motility, morphology and viability; and decrease in superoxide dismutase and increase in malondialdehyde concentration amongst Group B rats compared to Group A control rats ( $p < 0.05$ ). Co-administration of HECL with nicotine to Group C rats apparently reversed the effects of nicotine resulting in significant increases in sperm count and the reproductive hormones concentration as compared to Group A control rats ( $p < 0.05$ ). Amongst Group D rats, the extract also caused a significant increase in superoxide dismutase concentration and a significant decrease in malondialdehyde concentration compared with the Group A control rats ( $p < 0.05$ ). The findings suggest that the hydromethanolic extract of *Citrullus lanatus* rind possibly ameliorates the deleterious effects of nicotine on some reproductive indices in male Wistar rats.

**Keywords:** *Citrullus lanatus*; nicotine, superoxide dismutase, lipid peroxidation, semen; reproductive hormones.

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### **INTRODUCTION**

Although, nicotine is a naturally occurring alkaloid found in a wide variety of plants (Doolittle et al., 1995) the principal source of human nicotine exposure is through tobacco use, nicotine replacement therapy such as transdermal nicotine patches and nicotine containing gum (Heisheman et al., 1994). Nicotine can cross biological membranes including the blood brain barrier and once absorbed, is extensively metabolized by the liver to a number of major and minor metabolites (Snyder et al., 1993; Cashman et al., 1992; Neurath, 1994; Crooks and Godin, 1988; Godin and Crooks, 1986; Booth and Boyland, 1971; Kyerematen et al., 1990). Nicotine is considered as the primary chemical in tobacco responsible for engendering tobacco use and dependence (Di Chiara, 2000, Harvey

et al., 2004). Several adverse effects of nicotine leading to various diseases and pathological conditions in humans have been described (Hammer and Mitchell, 1979, Christensen et al., 1984, Wilkins et al., 1982, Pullan et al., 1994). Nicotine along with cotinine (a nicotine metabolite with a longer half-life) adversely affects spermatogenesis, epididymal sperm count, spermatocyte motility and fertilizing potential (Aydos et al., 2001). Oral administration of nicotine in male rats have been associated with testicular degeneration and disorganization of the cytoarchitecture, decreased serum testosterone, reduced semen characteristics and impaired fertility (Oyeyipo et al., 2010; Oyeyipo et al., 2011). In female rats, nicotine administration have been reported to significantly reduce the weights of visceral organs

including the ovary, kidney, pituitary and uterus while increasing the weights of the heart and liver with appearance of cartilaginous cells in the heart and deposition of adipose around the portal veins: associated necrosis, congestion, fibrosis, follicular and endometrial degeneration were also observed in the brain, pituitary, kidney, ovary and uterus respectively (Iranloye and Bolarinwa, 2005).

*Citrullus lanatus* (watermelon) is one of the major under-utilized fruits grown in the warmer part of the world. The juice or pulp from watermelon is consumed by humans, while the rind and seeds are major solid wastes (Ahmed, 1996; Lewinsohn et al., 2005; Bawa and Bains, 1977). The rind is utilized for products such as pickles and preserves, as well as for extraction of pectin (Leong and Shui, 2002; Gyamfi et al., 1999). The therapeutic effect of watermelon has been reported and has been ascribed to its composition of a number of antioxidant compounds; amongst them, citrulline and lycopene have been demonstrated to play prominent roles (Melo et al., 2006; Minotti and Aust, 1987). The rind has recently been evaluated as a wheat flour substitute for cake making (Al-Sayeed and Ahmed, 2013) and is recommended in both alcoholic poisoning and diabetes (Duke and Ayensu 1985; Jiyun et al., 2011). Furthermore, the rind has been shown to contain alkaloids, saponin, cardiac glycosides, flavonoids, phenol, moisture, lipid, protein, fiber and carbohydrates (Erukainure et al., 2010; Erhirhie and Ekene, 2013). The possible ameliorative effects of the methanolic extract the rind of *Citrullus lanatus* on some semen parameters and reproductive hormones following lead acetate induced toxicity (Kolawole et al., 2014), following aspirin induced gastric ulceration (Kolawole et al., 2016a) and in alloxan induced diabetes (Kolawole et al., 2016b) in male Wistar rats has recently been reported from our laboratory.

As part of a series of studies exploring the potential beneficial effects of the rind of *Citrullus lanatus* the present report describes the possible ameliorative effects of the hydromethanolic extract of the rind on some semen parameters, reproductive hormones and indices of testicular oxidative status following the administration of nicotine using male Wistar rat as models.

## MATERIALS AND METHODS

**Plant material and preparation of extracts:** Fresh plant and fruits of watermelon were obtained from the community market in Elele, Rivers State, Nigeria. The plant materials were identified and authenticated by Dr. Chimezie Ekeke of the Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria. Voucher specimens were also deposited with the herbarium number UPH/V/1214. The rinds were peeled off from the whole fruit washed thoroughly, sun-dried and milled into a fine powder. The method of extraction employed is percolation

(Adesanya et al., 2011). 24g of the powdered sample was soaked in a beaker containing 100ml of 98% methanol for a period of 48 hours and then filtered with a Whatman No. 1 filter paper size. The volume of filtrate obtained was 150ml before concentration; the filtrate was subsequently concentrated using a rotary evaporator. The weight of residue obtained was 8.5g.

**Determination of median lethal dose (LD<sub>50</sub>):** Acute toxicity study (LD<sub>50</sub>) was determined using the method described by Lorke 1983. The (LD<sub>50</sub>) of the extract was found to be greater than 2000mg/kg body weight.

**Nicotine preparation:** Nicotine hydrogen tartrate with product number 36733-1G (99% nicotine); was obtained from Sigma Chemical Corporation (Sigma Aldrich, St. Louis, Mo, USA). Nicotine stock solution was prepared at concentration of 1mg/ml and stored in foil-wrapped glass bottle 4°C for no longer than ten days before use.

**Experimental procedure:** Twenty male Wistar rats were used for this study. The rats weighed between 170g and 200g; were divided into four groups: Groups A to D of 5 rats each. The rats in each group were placed in separate cages in the Animal House of Madonna University, Nigeria under natural day and night cycles. The rats had free access to normal rat chow and tap water *ad libitum*. They were allowed two weeks of acclimatization to their environment and subsequently treated as follows:

**Group A:** Control group. Rats in this group were given 2ml/kg body weight 10% of extract vehicle.

**Group B:** Nicotine only group. Rats in this group were given 1mg/kg body weight of nicotine as described by Oyeyipo et al., 2010.

**Group C:** Nicotine + extract group. Rats in this group were co-administered 1mg/kg body weight of nicotine and 500mg/kg of hydromethanolic extract of *Citrullus lanatus* rind.

**Group D:** Extract only group. Rats in this group were given 500mg/kg body weight of hydromethanolic extract of *Citrullus lanatus* rind.

The hydromethanolic extract of *Citrullus lanatus* rind, nicotine and extract vehicles were administered to the rats daily using a gastric cannula. All the rats were treated for a total of 42 days. On day 43, blood samples were collected from chloroform anesthetized rats through direct cardiac puncture. The blood samples were placed in heparinized sample bottles, centrifuged at 1500rpm for 5 minutes and plasma obtained for assay of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH). The animals were then sacrificed and the testes immediately harvested for determination of semen parameters: motility, morphology, viability and count. The testes were then homogenized and the homogenate used for the assay of superoxide dismutase (SOD) and

malondialdehyde (MDA) concentration. All animal experiments were conducted in accordance with the National Institute of Health Guide for Care and Use of Laboratory animals (Pub No. 85-23 revised 1985) (NRC, 1985).

**Determination of plasma concentrations of the reproductive hormones:** The plasma concentrations of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) were determined by the enzyme-linked immunosorbent assay (ELISA) technique using commercially available kits. The hormonal kits used for the assay was obtained from Monobind Inc. Lake Forest, CA, USA. Samples were run in the same assay to avoid inter-assay variations and determined on the same day of collection of blood samples.

**Determination of semen parameters:**

**Motility:** This was as described previously by Kaur and Bansal, 2004. Briefly, the caudal epididymis was identified and its content carefully expressed into 1ml of normal saline at room temperature. One drop of the semen suspension was charged into a Makler counting chamber and the number of motile and non-motile spermatozoa counted in ten random fields. The number of motile spermatozoa was then expressed as a percentage of the total number of the counted spermatozoa (Mahaneem *et al.*, 2011).

**Sperm morphology:** This was determined by smearing a drop of the stained semen suspension obtained during determination of sperm count on a glass slide; the smear was allowed to dry and subsequently examined under the light microscope at X400 magnification. For each sample, 200 spermatozoa were carefully observed and the percentage of total abnormalities of the spermatozoa head and total abnormalities of the spermatozoa tails were determined as described by Narayana *et al.*, 2005.

**Sperm viability:** To determine viability, fluid from the caudal epididymis was carefully dropped on a slide and mixed with a drop of 0.5% eosin solution. After 2 minutes, the slide was examined under a light microscope at X40 magnification. The percentage of viable (unstained) and non-viable spermatozoa (stained red) was determined as described by Cheesbrough (2006).

**Sperm count:** This was determined as described earlier by Narayana *et al.*, (2005) with minor modifications. Briefly, the caudal epididymis was carefully separated from the testis and minced in 2ml of normal saline followed by filtration through a nylon mesh. The suspension was then stained with 2% eosin in normal saline. The spermatozoa heads were counted using a Neubauer haematocytometer. Chamber counts for the sperm head in eight chambers

(except the central chamber) were averaged and expressed as the number of sperm per caudal epididymis (Mahaneem *et al.*, 2011).

**Determination of testicular superoxide dismutase (SOD) and malondialdehyde (MDA) activity:** The levels of SOD activity was determined by the method of Misra and Fridovich (1972). This involves inhibition of epinephrine autoxidation in an alkaline medium at 480nm in a UV vial spectrophotometer. For the determination of specific activity of SOD in homogenate sample of testes tissue, the rate of autoxidation of epinephrine was noted at 30 seconds' intervals in all groups. The enzyme activity was expressed in arbitrary units considering inhibition of autoxidation, as 1 unit of SOD specific activity. The determination of malondialdehyde (an index of lipid peroxidation) activity was as previously described (Kolawole *et al.*, 2016). Briefly, 2ml of thiobarbituric acid (TBA) reagent and 1ml of trichloro acetic acid (TCA) were mixed with 2ml of homogenate of testes tissue. The mixture was heated at 60°C for 20 minutes and then cooled before centrifugation at 400rpm for 10 minutes. The absorbance of the obtained supernatant was read at a wavelength 540nm.

**Statistical Analyses:** Significant differences were determined using the one-way analysis of variance (ANOVA) followed by the LSD post hoc tests. A p value <0.05 was considered statistically significant. The results are presented in Tables 1 and Figures 1, 2, 3 and 4. All data were expressed as mean  $\pm$  standard error of mean (SEM).

## RESULTS

Table 1 shows the effects of hydromethanolic extract of *Citrullus lanatus* rind on some semen parameters of male Wistar rats when administered alone (Group D) and when co-administered with nicotine (Group C). It also shows the values of the semen parameters in Group A control and in rats administered nicotine only (Group B). Results obtained indicate that administration of nicotine alone in Group B rats generally caused a diminution of sperm quality with significant reductions in percentages of motile and viable cells and corresponding significant increases in percentages sluggish cells, cells with tail defects, non-viable cells as compared to Group A control rats ( $p < 0.05$ ). Reductions in sperm counts were however, not significant ( $p > 0.05$ ). Compared to Group A control rats, co-administration of nicotine with the hydromethanolic extract of the rind of *Citrullus lanatus* in Group C rats generally caused an improvement in all the sperm parameters; however, significant differences were seen only for sperm count, viable cells and cells with tail defects ( $p < 0.05$ ) when compared to Group A control rats. However, despite the significant improvement in sperm counts, there

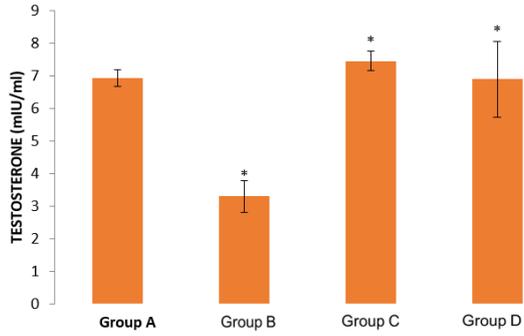


Fig. 1: Effect of the hydromethanolic extract of the rind of *Citrullus lanatus* on serum testosterone concentration in male Wistar rats following nicotine administration.

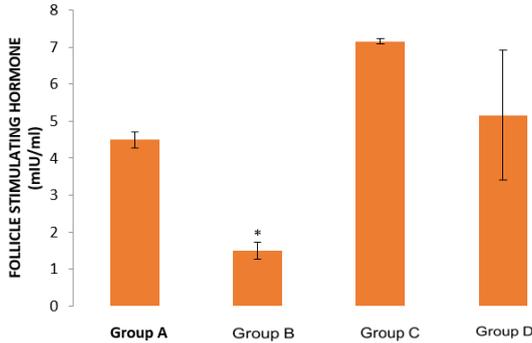


Fig. 2: Effect of the hydromethanolic extract of the rind of *Citrullus lanatus* on Follicle Stimulating Hormone concentration in male Wistar rats following nicotine administration.

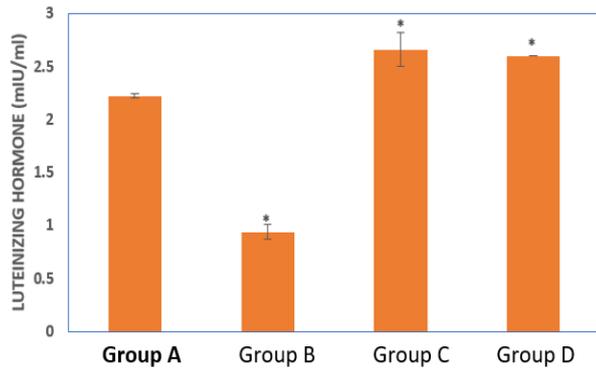


Fig. 3: Effect of the hydromethanolic extract of the rind of *Citrullus lanatus* on Luteinizing Hormone concentration in male Wistar rats following nicotine administration.

were significantly higher cells with tail defects and significantly lower percentage viable cells amongst Group C rats compared to Group A control rats ( $p < 0.05$ ). Group D rats were found to have significantly lower values of cells percentage motile cells, higher value of percentage with tail defects, non-

viable cells and sperm counts as compared to Group A control rats. Values of these parameters are as shown in the Table 1.

Figures 1, 2 and 3 are results of plasma concentration of testosterone, Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH) respectively for all groups. Generally, administration of nicotine to rats in Group B caused a significant reduction in the value of each hormone compared to Group A control rats ( $p < 0.05$ ). However, administration of the hydromethanolic extracts of the rind of *Citrullus lanatus* caused significant increases in the values of each of the hormone as seen amongst rats in Group D ( $p < 0.05$ ).

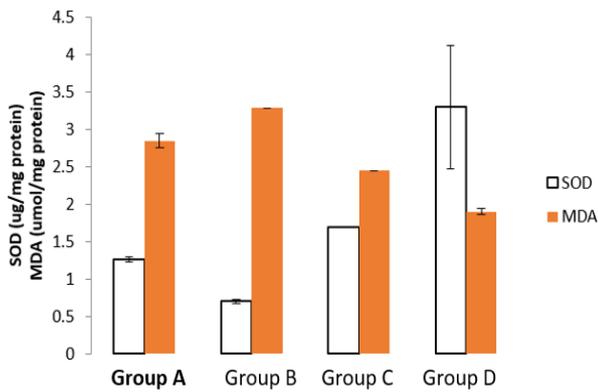
Similarly, co-administration of both nicotine and the hydromethanolic extract of the rind of *Citrullus lanatus* to rats in Group C caused significant increase in the plasma levels of all the reproductive hormones compared to Group A control rats ( $p < 0.05$ ). The pattern of changes in values of reproductive hormones are as shown in Figures 1, 2 and 3.

Figure 4 shows the results of assay of superoxide dismutase and malondialdehyde in testes tissue for all Groups of rats. Administration of nicotine caused a significant reduction in the value of superoxide dismutase in Group B rats as compared to Group A control rats ( $p < 0.05$ ). However, administration of the hydromethanolic extracts of the rind of *Citrullus lanatus* to rats in Group D caused a significant increase in the concentration of superoxide dismutase ( $p < 0.05$ ). Similarly, co-administration of nicotine and the hydromethanolic extract of the rind of *Citrullus lanatus* to rats in Group C caused significant increase in the superoxide dismutase concentration as compared to Group A control rats ( $p < 0.05$ ). Administration of nicotine only to rats in Group B caused a significant increase in the levels of malondialdehyde compared to Group A control rats ( $p < 0.05$ ). Although, administration of the hydromethanolic extracts of the rind of *Citrullus lanatus* to rats in Group D caused significant decreases in the malondialdehyde concentration ( $p < 0.05$ ); co-administration of both nicotine and the hydromethanolic extract of the rind of *Citrullus lanatus* to rats in Group C similarly caused a significant decrease in the malondialdehyde levels, as compared to Group A control rats ( $p < 0.05$ ); however, the decrease was less than that observed for Group D rats.

Table 1: Effect of hydromethanolic extract of the rind of *Citrullus lanatus* extract on some semen parameters of male albino Wistar rats following nicotine administration

Groups	Sperm Motility (%)			Abnormal sperm morphology (%)		Sperm viability (%)		Sperm count (%)
	Actively motile	Motile	Sluggish	Head defect	Tail defect	Viable	Non-viable	
A	74.00±2.44	87.00±1.22	13.00±1.22	1.20±0.49	1.80±0.09	90.6±0.97	9.40±2.19	18.20±1.95
B	27.00±1.22*	50.00±0.00*	23.00±1.22*	0.60±0.20	2.20±0.01*	60.00±1.00*	40.00±2.24*	12.20±1.71
C	58.00±4.89	67.00±4.41	9.20±0.49	1.40±0.21	4.20±0.73*	84.40±0.98*	15.60±2.19	28.20±0.20*
D	53.00±12.00*	60.00±12.2*	7.00±2.00	1.80±0.48	4.40±0.24*	95.60±0.24	4.40±0.54*	32.40±5.14*

All values=Mean ± SEM; \* significantly different from values of Group A control at  $p < 0.05$



**Fig. 4:** Effect of the hydromethanolic extract of the rind of *Citrullus lanatus* on testicular tissue super oxide dismutase and lipid peroxidation activities in male Wistar rats following nicotine administration.

## DISCUSSION

Expectedly, the observed diminution of semen parameters and reduced reproductive hormone concentrations following the administration nicotine seen in the present study is consistent with most of the previous reports (Yamamoto et. al., 1998; Aruldas et. al., 2005; Seema et.al., 2007; Jana et. al., 2010; Oyeyipo et. al., 2011; Oyeyipo et. al., 2013). Several reasons have been advanced for these detrimental effects of nicotine on reproductive function. For instance, increased production of reactive oxygen species (ROS) especially testicular (cellular) hydrogen peroxide and hydroxyl radicals (Aruldas et. al., 2005; Bandopadhyay et. al., 2008) may account for these effects of nicotine on semen parameters: Increased free radicals and ROS has been reported to cause lipid peroxidation of spermatozoa membrane with possible destruction of sperm DNA (Anto et. al., 2016). Further, the observed reduction in testosterone, FSH and LH concentrations could also contribute: testosterone the principal androgen plays an important role in several aspects of sexual maturation including behavior, spermatogenesis, differentiation and maintenance of the accessory sex organs (Ojeda and Urbanski, 1994). The synthesis and release of androgens is dependent on the pituitary gonadotropins: FSH and LH. Both gonadotropins are essential for testicular functions including spermatogenesis. LH is the main tropic regulator of Leydig cell function without which androgen production is impaired. The reduced levels of these gonadotropins could also contribute to the observed adverse effects of nicotine seen in the present study. Oyeyipo et al. (2010) further ascribed the decrease in serum testosterone level of rats treated with nicotine to disruption of testicular cytoarchitecture consequently adversely affecting Leydig cell number leading to decreased serum testosterone secretion. The results obtained from this study is consistent with the report of Oyeyipo et al., 2013 of a significant decrease in serum testosterone level in rats treated with varying

doses of nicotine. Perhaps, the reduced gonadotropin hormone secretion seen in the present study following nicotine administration may be from a possible depressive effect on the hypothalamic neural mechanisms essential for the release of Gonadotropin Releasing Hormone (GnRH) (Reddy et al., 1995; Didia et. al., 2000). This eventually could lead to disturbances in the secretion of pituitary gonadotropins essential for both spermatogenesis and steroidogenesis (Aydos et.al.,2001). Co-administration of nicotine and the hydromethanolic extract of the rind of *Citrullus lanatus* caused some improvements in the semen parameters with increases in testosterone and gonadotropins secretions. The ameliorative effect of the extract may possibly be due to its content of phenols and flavonoid which serve as potent antioxidants (Eruikainure et al., 2010). The rind has been shown to be rich in some antioxidant vitamins especially tocopherol and ascorbic acid (Edwards et al., 2003; Johnson et al., 2013).

Expectedly, testicular malondialdehyde concentration was also increased following administration of nicotine: this finding is consistent with previous reports (Husain et al., 2001; Yao et al., 2006). An elevated malondialdehyde concentration is direct evidence of toxic processes induced by free radicals (Sieja and Talerczyk, 2004). Enhanced level of tissue lipid peroxides in nicotine treated rats has been shown to be accompanied by a significant decrease in the levels of ascorbic acid, tocopherol, glutathione, glutathione peroxidase, superoxide dismutase and catalase (Kalpana et al., 2004). In the present study, the increase in testicular tissue malondialdehyde levels indicates a nicotine induced oxidative stress; suggesting an increased generation of free radicals in testicular tissue following exposure to nicotine. Increased production of free radicals or decreased function of the defense system play an important role in nicotine toxicity (Peltola et al.,1994). Hydrogen peroxide is not a particularly reactive product, but it may be reduced to the highly reactive metabolites: hydroxyl radicals or single oxygen (Peltola et al., 1992). Lipid peroxidation as evaluated by malondialdehyde value significantly increases during accumulation of hydrogen peroxide in a concentration-dependent manner (Garcia et al., 2005). Nicotine apparently may potentiate this oxidative stress in the testes of experimental animals. In the present study, lipid peroxidation in nicotine treated rats was accompanied by depletion of the concentration of antioxidant enzyme superoxide dismutase. Superoxide dismutase rapidly converts superoxide anion to less toxic hydrogen peroxide. The principal mechanism of hydrogen peroxide toxicity is thought to involve the generation of highly reactive hydroxyl ion radical through its interaction with  $Fe^{2+}$  in the Fenton reaction (Sewerynek et al., 1995). Presumably, the increase

superoxide dismutase concentration found in the present study could cause rapid conversion of hydrogen peroxide to water thus preventing hydrogen peroxide accumulation and availability to promote a shift towards lipid peroxide production. The significantly increased superoxide dismutase concentration in nicotine treated rats caused by the extract could be attributed to its phytochemical constituents especially flavonoids that have ability to act as antioxidants (Eruikainure et al., 2010). The flavones and catechins are perhaps some of the most powerful flavonoids for protecting the body against damage by reactive oxygen species (Sodipo et al., 2000). Antioxidant activity of any food source significantly increases with the presence of a high concentration of total phenol and flavonoid (Jayaprakasha et al., 2001). Therefore, the high phenolic and flavonoid contents of the watermelon rinds strongly suggest a high antioxidant potential. Another mechanism by which the extract could enhance superoxide dismutase concentration may be due to the presence of vitamins especially ascorbic acid and tocopherol; both vitamins possess antioxidant properties (Traber and Stevens, 2011). Ascorbic acid has been demonstrated to protect against some of the deleterious effects of nicotine in the lungs of experimental rat models (Maritz and van Wyk 1997); and have been reported to be significantly high in the pulp, seed and rind of *Citrullus lanatus* (Johnson et al., 2013). Low dietary intake of antioxidant vitamins such as ascorbic acid and tocopherol increases the risk of disease, whereas high dietary intake is apparently protective in function (Neunteufl et al., 2000). Furthermore, tocopherol is an effective antioxidant converting superoxide dismutase and lipid peroxy radicals to less reactive forms (Valk and Hornstra, 2000).

In conclusion, the present study reports that administration of the hydromethanolic extract of the rind of *Citrullus lanatus* fairly ameliorates the deleterious effects of nicotine administration on testicular and reproductive function in male Wistar rats. The beneficial effects of the extract could be attributable to its constituents. Our results are preliminary and could benefit from further studies.

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**Conflicts of interest:** The authors declare no conflict of interests.

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