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Disturbances in Calcium and Zinc Homeostasis During Testicular Damage Induced by *Citrus aurantifolia* Juice in Wistar Rats

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Summary: Infertility rate is high globally and in Nigeria. The reported spermicidal activity of *Citrus aurantifolia* juice (CAJ) and its popular consumption may be a contributing factor to the rise in male infertility. This study examined the effects of CAJ on testis and evaluated the role of calcium and zinc in these effects. Twenty-eight male rats (200-220g) were grouped into four (n=7). Group I (control) received 0.5ml normal saline, while groups II, III and IV received 600mg/kg, 900mg/kg and 1200mg/kg of CAJ, respectively, orally for 35 days. Sperm analysis, testicular histology, testicular zinc and calcium concentrations were evaluated. The results showed a significant decrease ($P < 0.001$) in body weight and gonad-somatic index (GSI) of the rats in group IV. No sperm cells were found in the sperm samples of all the treatment groups in contrast to control. There was a significant decrease ($P < 0.001$) in zinc concentration of group III and IV animals and a significant increase ($P < 0.001$) in testicular calcium content of group III and IV animals. Derangement of testicular cyto-architecture, shrinkage or complete destruction of seminiferous tubules as well as absence of spermatogenic cells were observed in the treatment groups. It was concluded that CAJ induced a destructive effect on testes of rats as evidenced by damaged testicular tissue, reduced gonado-somatic index, azospermia and disruption in testicular electrolyte homeostasis. It was concluded that CAJ caused hypercalcaemia and hypozincaemia in the testicular tissue of the treated rats. Concurrently, CAJ also caused damage to testicular histology, azospermia and decreased GSI. *Citrus aurantifolia* juice should be consumed with caution due to its potential to cause infertility in males.

Keywords*: Citrus aurantifolia* juice, Sperm quality, Infertility, Gonado-somatic index, Testicular histology

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INTRODUCTION

Infertility rate is high in Nigeria and the male factor may account for about 40-50% of all the infertility cases (Uadia and Emokpae, 2015). A critical factor causing infertility in males is low sperm quality. Falling sperm count and the rise in male infertility has led to an increase interest in the nutritional factors that influence the development and quality of sperm. Recent researches have shown *Citrus aurantifolia* juice (CAJ) to have spermicidal activity (Okon *et al.*, 2014). The widespread usage of CAJ as food and traditional medicine (Aprioku and Briggs, 2018) and its spermicidal activity calls for concern because this situation may contribute to the development of male infertility among the consumers.

Lime (*Citrus aurantifolia*), a polyembryonic plant belonging to the family Rutaceae, is widely grown in sub-tropic and tropical region of the world (Niththep *et al.*, 2016), and forms an important part of diet as a component of commonly used beverages and for medicinal purposes (Aprioku and Briggs, 2018). The fruits are globose to ovoid berry of about 3 - 6 cm in diameter and sometimes have apical papilla. It is highly acidic and fragrant (Patil *et al.* 2009), yellow when ripe but usually picked green commercially (Enejoh *et al*., 2015). Lime fruit contains an array of bioactive and nutritional constituents which include flavonoid, limonoid, alkaloid, ascorbic acid, tannins, saponin, reducing sugars, cardiac glycosides, citric acid and amino acid (Bakare *et al*., 2009).

Many useful properties of CAJ have been reported, including anti-proliferative and immuno-modulatory effect on activated human lymphocytes (Gharagazloo *et al.*, 2001), antimicrobial activity against respiratory tract infections and cholera (Adeleye 2003), anti-oxidant activity (Boshtam *et al.*, 2011). In contrast, other studies reported negative effects of CAJ. *Citrus aurantifolia* juice reduces the number of ova shaded

and causes irregularity in the histology of the ovaries and uterus in female rats (Bakare *et al.,* 2012). The juice also exhibits anti-fertility effect by disrupting the estrous cycle in Wistar rats (Aprioku and Briggs, 2018). Intra-vaginal douching with CAJ showed a destructive effect on fetal development and female reproductive histology (Solomon *et al*., 2014).

Despite research reports on the biological effects of CAJ, including the aforementioned, there is paucity of literature on its effect on testicular biology. In particular, it is not known how CAJ affects testicular calcium and zinc concentrations. Given the diverse nature of its phytochemical content, it is conceivable that CAJ may affect the homeostasis of some important electrolytes such as calcium and zinc in the testicular tissue, as one of the mechanisms of its effects. Calcium is abundant in seminal fluid and plays an important role in sperm activities such as hyper-activation, chemotaxis, capacitation, and the acrosomal reaction, all of which are essential for successful fertilization and normal male fertility (Polina *et al*., 2014; Qi *et al.,* 2007; Kwon *et al*., 2013; Kwon and Park, 2013; Shukla *et al.,* 2013). Zinc is a micronutrient required for the action of more than 200 metallo-enzymes (Jinxiang *et al.,* 2014). It is a very important mineral for male fertility. Zinc is found in high concentration in male sex organs and sperm (Oliveira *et al*., 2004). It is also necessary for making the outer membrane and tail of the sperm (Awadallah *et al.,* 2003). Deficiency of zinc can impair spermatogenesis and decrease serum testosterone level (Wong *et al.,* 2002). It conserves genomic integrity in the sperm head and tail (Tuerk and Fazel, 2009).

This study examined the effects of CAJ on testicular calcium and zinc concentrations and some testicular and sperm parameters.

MATERIALS AND METHODS

Preparation of crude *Citrus aurantifolia* juice and acute toxicity study

Fresh *Citrus aurantifolia* (lime) fruits were obtained during the rainy season from a local farm in the outskirts of Kano city and authenticated by a botanist and given herbarium accession number- BUKHAN 0028. The fruits were properly washed and sliced after removing the rind. Juice was extracted using a juicer and filtered through a filter paper and pH was determined using a pH meter. Fresh CAJ was prepared everyday of administration.

To obtain the weight of solute in a given volume of CAJ, 120ml of ultra-filtered fresh CAJ was collected into a clean pre-weighed container, dried in an oven at a temperature of 40 ^{o}C. Actual weight of solute was obtained by subtracting weight of container from total weight. Concentration of solutes present in 120ml of CAJ was calculated thus:

Concentration = mass/volume

Median lethal dose (LD_{50}) was estimated in the rats using Lorke's method (1983). This method has two phases. Phase 1 involved nine adult male rats, which were divided into three groups (n=3). Each group of animals were administered different doses (10, 100, 1000mg/kg) of CAJ and observed for 24 hours for signs of toxicity or death. In phase 2 three animals were administered higher doses (1600, 2900 and 5000mg/kg) of CAJ and similarly observed.

Phytochemical screening of secondary metabolites in *Citrus aurantifolia* juice

The extracted CAJ was screened for alkaloids, flavonoids, saponins, steroids, anthraquinones, combined reducing sugars (Sofowora, 1993), tannins (Trease and Evans, 1989) and cardiac glycosides (Parekh and Chanda, 2007) using previously described methods.

Experimental animals and study design

Animals were housed in plastic cages and fed on standard feed and water *ad libitum.* They were maintained under standard conditions. Animal house was well ventilated at room temperature under natural day/night photoperiodicity.

A total of twenty eight adult male Wistar rats weighing between 200 - 220g were subdivided into four groups (n=7). The animals received either normal saline (control), 600mg/kg body weight, 900 mg/kg body weight or 1200mg/kg body weight of CAJ juice for 35 days. Administration was carried out by oral gavage daily using metal cannula.

At the end of the treatment, rats were weighed and then sacrificed after anaesthesia by intraperitoneal injection of 40mg/kg ketamine. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. Both testes and epididymis were carefully removed and weighed using an electronic analytical and precision balance (BA 210S, d=0.0001- Sartoriusen GA, Goettingen, Germany). Left testis of each rat was homogenized in 2 ml of physiologic saline using a homogenizer. The homogenate was then centrifuge at 10000g for 15 minutes. The supernatant was used for determination of zinc and calcium concentrations.

Sperm count determination

Spermatozoa in the left epididymis were counted by a modified method of Yokoi and Mayi (2003). Briefly, the epididymis was minced with scissors in 5 ml physiologic saline, placed in a rocker for 10 minutes, and allowed to incubate at room temperature for 2 minutes. The supernatant was then diluted 1:100 with solution containing 5 g sodium bicarbonate and 1 ml formalin (35%). Total sperm number was determined by using the new improved Neuber counting chamber (haemocytometer). Approximately 10 µL of the diluted sperm suspension was transferred to each counting chamber of the haemocytometer and allowed

to stand for 5 minutes. This chamber was then placed under a binocular light microscope using an adjustable light source. The ruled part of the chamber was focused and the number of spermatozoa counted in five 16-small squares. The sperm concentration was calculated then multiplied by 5 and expressed as [X] x 10^6/ml, where [X] is the number of spermatozoa in a 16-small square.

Determination of sperm progressive motility

Sperm progressive motility was determined by the method of Sonmez *et al.* (2005) and slightly modified. Accordingly, the fluid obtained from the left cauda epididymis with a pipette was diluted to 0.5 ml with Tris buffer solution. A drop of the solution was placed on a pre-warmed microscopic slide. A cover slip was lowered on to the sample avoiding formation of air bubble. The slide was examined using light microscope at a magnification of x 400. In evaluating motility, sperm cells were classified as non-motile and progressively motile. A progressively motile sperm swims forward in an essentially straight line, whereas a non-progressively motile swims but in an abnormal path, such as in tight circles. Motility estimates were performed from three different fields in each sample. The mean of the three estimations was used as the final motility score.

$$\% \text{ of motile sperm} = \frac{\text{Number of motile sperm}}{\text{Total number of sperm}} \text{X100}$$

Determination of sperm morphology

The sperm cells morphology was determined with the aid of light microscope at x 400 magnification as described by Sonmez *et al*., 2005. The fluid obtained from the left cauda epididymis was pipetted and diluted to 0.5 ml with Tris buffer solution. It was further diluted 1:20 with 10% neutral buffered formalin. Five hundred spermatozoa were examined for morphological abnormalities such as rudimentary tail, round head and detached head (Atessahin *et al.*, 2006). The result was expressed as percentage of abnormal spermatozoa to morphologically normal spermatozoa.

Measurement of testicular zinc concentration

Zinc concentration was determined in testicular tissue homogenate as described by Eliason (2003). 1000µl of reagent was mixed with 50µl of sample and standard, respectively. The mixture was allowed to incubate for 8 minutes at 28 ºC. Standard was taken against the reagent blank. Sample absorbance was measured at a wavelength of 560nm using colorimeter.

Measurement of testicular calcium concentration

The reagent provided in the kit were prepared and then stabilized for three days at 25ºC. 25µl of reagent blank, standard, testes homogenate were pipetted into three test tubes, 1ml of working reagent was then added into each of the test tubes. It was allowed to stand for 50 minutes after which absorbent of the sample and standard was taken against the reagent blank at a wave length of 570nm using colorimeter.

Histological study of testicular tissue

The testicular biopsies were fixed with 10% formol-saline, dehydrated with ascending grade of alcohol (70%, 90% and 95%) cleared with toluene, infiltrated with molten paraffin wax at a melting point of 56 ºC. Sections of 5µ thickness were cut on a rotary microtone. Sections were floated out on clean microscopic slides, to prevent detachment from slides during staining procedure. They were then dried for 2 hours at 37 ºC. Slides were stained with haematoxylin and eosin and then passed through ascending concentrations of alcohol (20-100%). A permanent mounting medium was put on the tissue section. A thin cover slip was placed on the covering mounting medium and underlying tissue sections were allowed to dry and later observed using DIALUX Research microscope. Photomicrographs were taken in bright field at x 400 (Awioro, 2010).

Determination of gonado-somatic index

Gonado-somatic index (GSI) was calculated as described by Caldeira *et al*. (2010) and Silva *et al*. (2014) by dividing the average of the weight of the (right and left) testicles by the live weight of the rat before sacrifice. The result was then multiplied by one hundred.

$$\text{GSI} = \frac{\text{Average weight of testes}}{\text{Live weight of rat}} X\ 100$$

Statistical Analysis

Obtained data were expressed as mean ± SEM and compared using one-way analysis of variance (ANOVA) and Scheffe *post hoc* test on SPSS Statistics version 20.0 (SPSS Inc., Il, U.S.A). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Preliminary phytochemical screening and acute toxicity study

The result of the preliminary phytochemical screening of CAJ revealed the presence of alkaloids, flavonoids, tannins, saponins, anthraquinones, reducing sugars, steroids and cardiac glycosides. There were no signs of toxicity or death in both phase I and II of the acute toxicity study.

Effect of *Citrus aurantifolia* juice on body weight and gonado-somatic index of rats

Table 1 shows the result of the body and organs weight respectively, the weights were recorded after the period of the treatment. There was a significant decrease ($P<0.05$) in body weight and GSI of the group that receive the highest dose (1200mg/Kg) of CAJ in comparison with the control, a similar decline was observed (though not statistically significant) in the moderate (900mg/Kg) and the low (600mg/Kg) doses administered CAJ, respectively.

Table 1: Effect of *Citrus aurantifolia* juice administration on bodyweight (g) and gonado-somatic index of rats.

Treatments	LBW (g)	LT (g)	RT (g)	AWT(g)	GSI(%)
Normal saline	265.57±4.11[a]	1.48±0.01[a,b,c]	1.48±0.01[a,b,c]	1.49	0.55
600 mg/kg	243.00±8.26	1.15±03.05[a]	1.15±0.03[a]	1.13	0.46
900 mg/kg	237.04±7.31	1.27±0.03[b]	1.23±0.03[b]	1.32	0.46
1200mg/kg	232.53±5.73[a]	0.18±0.03[c]	0.24±0.02[c]	0.19	0.28

Values with similar superscripts in the same column are significantly different. Mean ±S.E.M, n=7. BW= live bodyweights, LT=left testis, RT=right testis, AWT=average weight of testes sand GSI=gonado-somatic index.

Table 2: Effect of ***Citrus aurantifolia*** **juice** administration on the sperm count, morphology and motility in rats

Treatments	Sperm count (x 10^6/ml)	% normal morphology	% motile cells
Normal saline	77.14 ± 4.00	52.29 ± 11.5	60.14 ± 4.98
600 mg/kg bw	0.00	NA	NA
900 mg/kg bw	0.00	NA	NA
1200 mg/kg bw	0.00	NA	NA

Mean ±S.E.M, n=7. NA = not available (no sperm cells were present in the sample). CAJ = *Citrus aurantifolia* juice

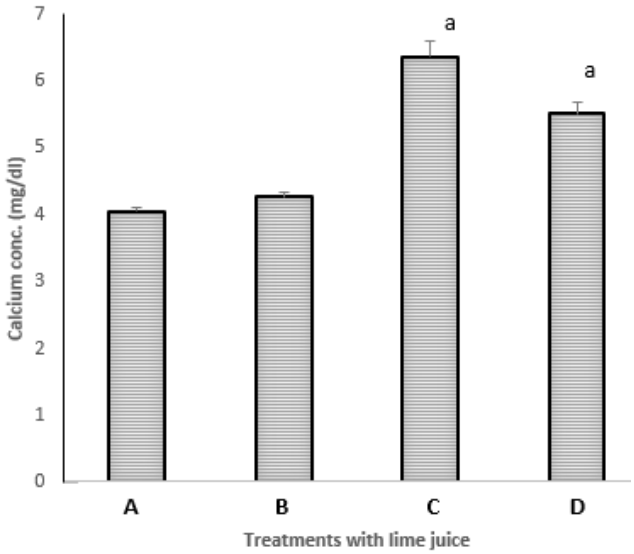

Figure 1: Effect of *Citrus aurantifolia* juice administration on testicular calcium concentration in rats. A= Control, B= 600 mg/kg bw, C= 900 mg/kg bw, D= 1200 mg/kg bw. a = statistically significant (0.001) compared to normal saline group.

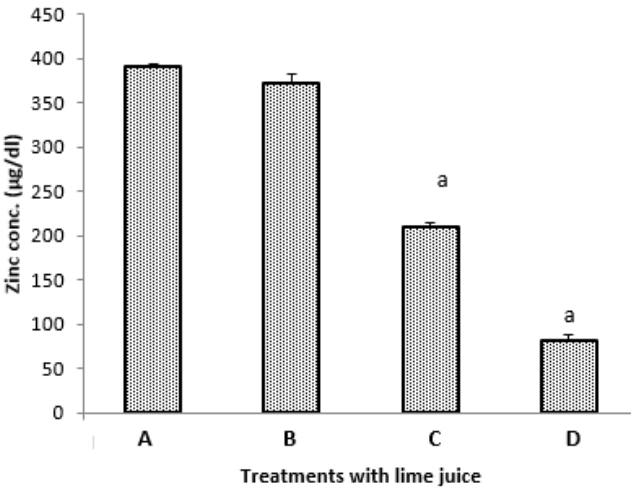

Figure 2: Effect of *Citrus aurantifolia* juice administration on testicular Zinc concentration in rats. A= Control, B= 600 mg/kg bw, C= 900 mg/kg bw, D= 1200 mg/kg bw. a = statistically significant (0.001) compared to normal saline group

Effect of *Citrus aurantifolia* juice on testicular calcium and zinc concentrations in rats

The result of testicular levels of calcium and zinc were presented on figure 1 and 2, respectively. There was a significant decrease ($P<0.05$) in the level of testicular zinc concentration in the 900 mg/kg bw and 1200 mg/kg bw administered groups when compared with the control, while testicular calcium concentration increased significantly ($P<0.05$) in these groups compared to control. There was no statistical significance ($p > 0.05$) difference in lower dose administered group in both testicular calcium and zinc concentrations.

Effect of fruit extract of lime on testicular histology of rats

Results of histological studies of control and treated rats are illustrated using photomicrographs on plate I (A – D). Examination of the slides from testicular tissue of control rats (A) showed normal histological findings- essentially normal and undisturbed pattern and shape of seminiferous tubules, with spermatozoan seen at different stages of development. The testicular tissue of the animals that received 600mg/kg of CAJ (B) revealed slight changes in tubular shapes with decrease in diameter and an increase in the length of seminiferous tubules. There was also disorganized testicular tissue architecture with no spermatogenic cells seen. For the rats that received 900mg/kg of CAJ (C), testicular tissue showed degeneration and shrinkage of the seminiferous tubules with no sperm cells seen in all stages of development. The shape of tubules changed from normal round to an irregular elongated shape. Examination of testicular tissue of rats that received 1200mg/kg of CAJ (D) showed total destruction of the seminiferous tubules and disappearance of testicular architecture with no spermatogenic cell seen.

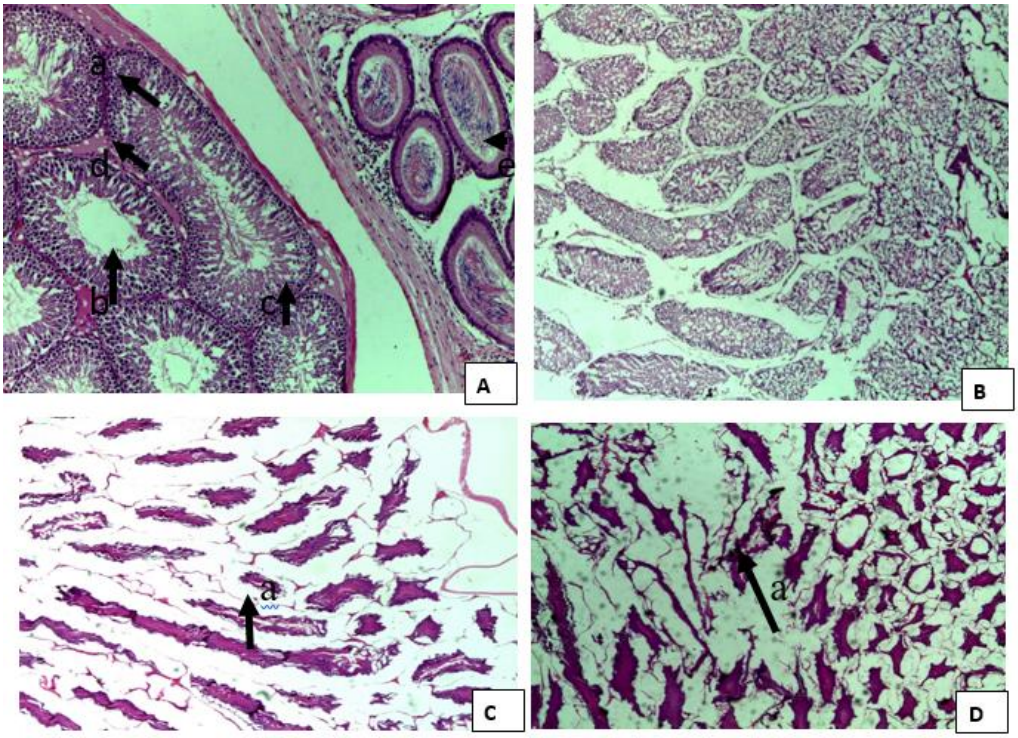

Plate I: Photomicrograph of testicular tissue of rats treated with CAJ (H&E ×400). Note the essentially normal and undisturbed pattern and shape of seminiferous tubules, with spermatozoa seen at different stages of development (A); mild changes in tubular shapes with decreased diameter and an increased in the length of seminiferous tubules (B); distortion of testicular architecture, degeneration and shrinkage of the seminiferous tubules with no sperm cells seen in all stages of development (C); total destruction of the seminiferous tubules and disappearance of testicular architecture with no spermatogenic cells seen (D). a = seminiferous tubules, b = lumen of seminiferous tubules, c = spermatocytes, d = Leydig cells, e= released spermatozoa.

DISCUSSION

Preliminary phytochemical screening of crude lime juice revealed the presence of different phytochemical - non-nutrient bioactive compounds that are produced by plants. Phytochemicals especially polyphenols constitute a major group of compounds that act as primary antioxidants (Giuseppe *et al*., 2007). Flavonoids, which cannot be synthesized by humans, are important components of most plants (McCullough *et al*., 2012; Shashank and Abhay 2013) and are known to possess anti-hypertensive, antioxidant, anti-anxiolytic, anti-inflammatory, anti-cholesteronemic and antimicrobial activities (Liu *et al*., 2014).

The findings of this study is in conformity with the work of Enojoh *et al*. (2015) who, using high-performance lipid chromatography (HPLC) and gas chromatography mass spectrometry, demonstrated that *Citrus aurantifolia* contained flavonoids identified from the plant. A similar study carried out by Nwankwo *et al.* (2015) showed presence of tannins, alkaloids, saponins and flavonoids, with tannins and saponins well known as important plant metabolites (Nwanko *et al.* (2015).

Acute toxicity is the adverse biological effects occurring after oral or dermal administration of a single dose of a substance, or multiple doses given within a short period of time. The toxicity studies in animals are commonly used to assess the potential health risk in humans by intrinsic adverse effect of phytochemicals in the extracts (Oyedemi *et al* 2010). These adverse effects may cause significant alterations in the levels of biomolecules metabolites derangement of histomorphology of tissues and organs (Yakubu *et al* 2009). Changes in general behaviour, weights of body and internal organs are critical parameters for the evaluation of the effect of a compound, such changes are often the first signs of toxicity (Carol 1995).

In this present acute toxicity study, lime juice at a doses of 10mg/kg, 100mg/kg, 1000mg/kg, 1600mg/kg, 2900mg/kg and 5000mg/kg orally given to the rats showed neither sign of toxicity nor death. This result is in line with that of Chunlarathanaphom *et al*. (2007) who showed that acute and sub-chronic toxicities of the water extracts from the roots of *Citrus aurantifolia* in both male female rats did not produce any sign of toxicity or mortality. Another study by Akhtar (2013) showed that the doses above 3.5 g/kg were toxic to rats, so also was the methanol extract of the peels in mice.

The present study demonstrated that *Citrus aurantifolia* juice decreased GSI in a dose-dependent manner, with the highest dose (of 1200mg/kg) having a significant effect. This finding is in line with the reports of Aseth *et al* (1995). Gonadosomatic index indicates calculation of gonads mass relative to body weight and is an important parameter in reproduction (Franca *et al*., 1998). Gonadosomatic index predicts the rates of sperm production as well as sperm function

in a given specie (Gomendio *et al.*, 2006; Adebayo *et al*., 2009).

There is a direct correlation between the testes weight and sperm production. Testis weight primarily reflects the total volume of the seminiferous tubules and its main components. Heavy loss of testicular cells was reported to be a major cause of testicular weight loss in rats (Naganatura *et al.,* 2008). This detrimental effect predisposes the animals to reduction in sperm count which may lead to infertility. In this study, lime juice may have had a toxic effect on the testicular tissue leading to decreased testicular weight relative to the body weight. Similar result was reported previously by Aseth *et al*. (1995). This effect may be due to disturbance in normal regulation of spermatogenesis through a fall in testosterone concentration following reduction in density of Leydig cells (Komili *et al*., 2015). The observed reduction in testes weight tallies with the zero sperm count described in this study.

The loss of body weight observed in this study might be as a result of interruption of metabolism of essential nutrients for health and normal body growth as reported by others (Marija *et al* 2008), and is in line with previous studies (Bakare *et al.,* 2012; Dosephine *et al.,* 2015).

Administration of CAJ induced damage on the testicular tissue, which increased with the increase in dose from absence of spermatozoa, mild distortion of seminiferous tubules to complete destruction of seminiferous tubules and testicular architecture. This effect will predispose the animal to infertility. These changes could be the result of oxidative damage induced by some constituents of the juice such as flavonoids, tannins and alkaloids as determined by the phytochemical screening of the juice in this study. Oxidative stress induced by these substances could result in damage to the cell membranes of the spermatozoa, seminiferous tubules and other testicular cells (Bahorun *et al*., 2006; Halliwell and Gutteridge, 2007; Azza *et al.,* 2010). As reported in this study, CAJ also induced disturbances in calcium and zinc homeostasis. This could cause disturbances in the fluid and electrolyte milieu of the testicular tissue with the resultant destructive histological changes.

Citrus aurantifolia juice abolished sperm cells in the rats in all the treatment doses, in contrast to what was observed in the control animals. No cells were seen to evaluate morphological defects and motility. Testicular function is assessed in parts by analysis of spermatic indices including sperm count, motility and morphology (Zinaman *et al* 2000, Eliason *et al* 2003). These parameters indicate the quality and functionality of sperm, thus, very vital for male fertility. The observed azospermia caused by CAJ in this study gives a clear indicator of the potential of CAJ to induce male infertility.

Several plant extracts and their active constituents have been reported to enhance reproductive process whereas some others act to antagonize the process by adversely affecting the hormonal, testicular and spermatogenic functions. The spermicidal effect of juices of natural products especially lime could be due to one of their characteristics which is acidity (Bakare *et al*., 2009). Lime juice contain high amount of organic acids like citric and coumaric acid (Patil *et al.* 2009) and as testicular milieu is highly sensitive to most chemicals, the destructive effect of CAJ in this study may be attributed partly to the acid constituent of the juice.

The fact that the juice contain high level of pro-oxidants like flavonoids, saponins, anthraquinones, alkaloids, tannins suggest that prolonged administration of the crude lime juice for the period of thirty five consecutive days may lead to oxidative damage due to free radicals (FR) and reactive oxygen species (ROS) generated by the metabolites, presumably by destroying testicular germ cells either due to membrane damage or macromolecular degradation, which resulted in significant decrease in the sperm count and testicular weight (Bahorun *et al*., 2006; Halliwell and Gutteridge, 2007). Alkaloids (such as nicotine) were previously reported to cause testicular degeneration (Jorsarrau *et al* 2008; Azza *et al.,* 2010). The alkaloid content of lime juice as reported in this study may have contributed to the observed destructive effects. Further studies will provide further insight.

Administration of CAJ for 5 consecutive weeks in this study has simultaneously increased calcium and decreased zinc concentrations. Zinc plays an important role in the process of cell growth as a co-factor for both DNA-RNA polymerase activities. Zinc is important for maintenance & regulation spermatogenesis and sperm motility (Sonoko *et al.,* 2009). Lack of zinc causes a decrease in ribonucleic acid (RNA), deoxynobneclric acid DNA and protein activity in the testes of rats (Chealth *et al.,* 1995). Previous studies have reported that high concentration of Zn is detectable in testes and that Zn deficiency inhibited spermatogenesis and caused sperm abnormality (Hidiroglou *et al*., 1984; Merker *et al.,* 1997). Zinc is also essential for the maintenance of germ cells, the progression of spermatogenesis, stabilization of the cell membrane and regulation of capacitation, acrosome reaction and sperm motility (Chandel Chand, 2014). Its deficiency leads to gonadal dysfunction, decreases testicular weight, and causes shrinkage of seminiferous tubules (Zeng *et al.,* 2013). The zinc deficiency state reported in this study may explain the mechanism of azospermia and histological changes here reported following lime juice administration. This is in support of the role of zinc in the destructive effect caused by lime juice on the testis.

The increase in calcium concentration reported in this study is in line with previous findings (Xia *et al.,* 2007; Marquez *et al.,* 2008). Calcium triggers multiple physiological events in spermatozoa, such as hyper-activation, chemotaxis, capacitation, and the acrosomal reaction, all of which are essential for successful fertilization (Ren *et al.,* 2001; Bohmer *et al.,* 2005; Krichok *et al.,* 2006; Qi *et al.,* 2006; Marquz *et al.,* 2008) and some of which are pH-dependent (Ho and Suarez, 2001). It was reported that a potential functional interaction exists between the sperm proteins and Ca^{2+} permeable channel proteins, thus modulating the Ca^{2+} influx mechanism (Kwon *et al.,* 2013; Kwon and Park, 2013; Shukla *et al.,* 2013) and playing a vital role in adjusting male fertility. It is evident that optimum calcium concentration is essential for normal sperm function and male fertility; and a state of increased calcium concentration as induced by lime juice in this study has provided evidence to support the role of calcium in the normal function and dysfunction of spermatozoa. Testicular hypercalcaemia is, therefore, suggested as one of the mechanisms of lime juice-induced testicular damage.

Our data indicate that CAJ caused hypercalcaemia and hypozincaemia in the testicular tissue of the treated rats. Concurrently, CAJ also caused damage to testicular histology, azospermia and decreased GSI. *Citrus aurantifolia* juice should be consumed with caution due to its potential to cause infertility in males.

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