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COMPARATIVE EVALUATION OF SOLVENT EXTRACTS OF *AZANZA GARCKEANA* FRUIT PULP ON HORMONAL PROFILES, SPERMIOGRAM AND ANTIOXIDANT ACTIVITIES IN RABBIT BUCKS

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Abstract: The study investigated the comparative influence of different extraction solvents on spermiogram, hormonal profiles and antioxidant activities in rabbit bucks. Adult New Zealand White rabbit bucks (n=18), with average live weight of 1.2±0.03 kg and aged 10-18 mo were fed *ad libitum* on a commercial diet. They were administered five different *Azanza garckeana* (AG) fruit pulp extracts at 500 mg/kg via oral gavage, comprising control group (Con), crude (AG Cr), methanol (AG M), n-hexane (AG H), ethyl acetate (AG E) and aqueous (AG AQ) for four weeks. The extracts improved the spermiogram in rabbit bucks administered methanol (AG M) and the reaction time was significantly (*P*<0.05) lower in AG E group when compared to other groups. The ejaculate volume, sperm motility, pH and sperm concentration were significantly (*P*<0.05) higher in the AG M group when compared to the other groups. There was a significant (*P*<0.05) increase in concentrations of blood testosterone, follicle-stimulating hormone and luteinising hormone in methanol extract group (AG M). While the glutathione and malondialdehyde concentrations were (*P*<0.05) lower, catalase and superoxide dismutase activities were significantly (*P*<0.05) higher in the groups administered methanol extract (AG M). It was concluded that AG M extracts of AG pulp elicited the best response in spermiogram, hormonal concentrations and antioxidant activities in New Zealand White rabbit bucks. Its use as the extraction solvent is recommended.

Key Words: antioxidants, *Azanza garckeana*, hormonal profiles, rabbit bucks, spermiogram.

INTRODUCTION

Rabbit (*Oryctolagus cuniculus*) is defined as a mini/micro-livestock animal with a very high reproduction potential. One of its unique abilities is that it serves as a flexible financial reserve and as a good laboratory animal, which allows for scientific studies and periodic sample (blood and semen) collections ante mortem, unlike other laboratory animals. This, by implication, means that results obtained while using rabbit as a research model can be extrapolated to other animals and even humans (Subasinghe *et al.*, 2021). Antioxidant administration has been linked to the prevention of diseases caused by oxidative stress. Plants and their various extracts are been extensively used in animals because natural antioxidants have fewer adverse effects than synthetic antioxidants. Antioxidants obtained from plants may promote the reproductive health of male animals (Agarwal and Prabakaran, 2005; Nantia *et al.*, 2009). Although plant-derived antioxidants are of great benefits in the treatment of oxidative stress, there are some challenges to be resolved, particularly with the type and procedure of extraction. This is because many distinct effects

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Figure 1: *Azanza garckean*a (goron tula).

have been obtained from the same plant when different extraction procedures were used, apparently due to the fact that different bioactive chemicals are extracted by different solvents from the same plant (Azwanida, 2015). As a result, plant-derived bioactive chemicals, which often contain antioxidants, are known as “double-edged swords” in animal reproduction, as they exert both favourable and harmful effects on spermatogenesis, semen qualities, hormonal profiles and antioxidant parameters (Zhoung and Zhou, 2013). The alternative technique of employing plants extracts as antioxidants in animals has recently been proven to be successful and widely used (Maroyi and Cheikh-Youssef, 2017). The most effective constituents responsible for antioxidative properties of plants are phenolic compounds, including flavonoids, hydrolysable tannins and phenolic terpenes (Gupta and Sharma, 2006; Carlsen *et al.*, 2010). The antioxidant properties of phenolic compounds are attributed to their structure and particularly their ability to donate a hydrogen ion to the peroxy radical, generated as an outcome of lipid peroxidation (Bisby *et al.*, 2008). Oxidative stress is one of the major underlying causes that interfere with spermatogenesis to reduce sperm quality and production, and even cause infertility (Boonsorn *et al.*, 2010). This is because the elevated reactive oxygen species (ROS) count generated during oxidation damages the spermatozoa deoxyribonucleic acid (DNA), resulting in increased cell death and poor reproductive rate (Kaur and Bansal, 2003). Flavonoid-containing plants exhibit antioxidant, androgenic and anti-infertility activities, and have been extensively used in the management of animal reproductive diseases (Middleton *et al.*, 2000; Dobrzyñska *et al.*, 2004; Purdy *et al.*, 2004). In addition to natural herbaceous plants, some fruit and vegetable extracts possessing antioxidant properties are beneficial in improving animal reproduction. A very good example of such plants is *Azanza garkeana* (AG), locally called ‘goron tula’ (Hausa, Nigeria). It is also known as snot apple, Azanza, tree hibiscus, quarters, wild hibiscus and African chewing gum (English). The plant is widely grown in Tula, Kaltungo Local Government Area of Gombe State, Nigeria (latitude 9° 48’51’’N, longitude, 11° 18′ 32″E and altitude of 610 m). It is also found in Kankiya, Katsina State (latitude 12° 32′ 57″N and longitude 7° 49′ 31″E) and the Daggish Kali highlands, Michika Local Government Area of Adamawa State (Edward *et al.*, 2021). In tropical Africa, AG is one of the most popular multi-purpose fruit trees. It is distinguished by edible fruits and plant parts utilised as herbal medicines and plant goods, which are sold in local markets to obtain cash for the home (Glew *et al.*, 2005).

AG is very rich in antioxidants, scavenging ROS that damage cell membranes and DNA (Capasso, 2013). It also demonstrates antioxidant properties in the reproductive system of the males. Snot apple fruit contains nutrients, minerals and phytochemical compounds, such as flavonoids, steroids, triterpenes, saponins, phenols and tannins that are beneficial for human and animal health (Maroyi and Cheikh-Youssef, 2017). To assess the importance of AG in male rabbit nutrition, more research is needed. Since AG has been reputed in folklore medicine to exert positive effects on the reproductive capacity of males, this study may provide some scientifically beneficial pieces of information that may be of value in the efforts to increase the efficiency and productivity of male rabbits, including their growth, fertility, hormonal profiles and antioxidant parameters.

The aim of the research was to assess and compare the effects of different methods of extraction of AG pulp on spermiogram, hormonal profiles and antioxidant properties in New Zealand White rabbits bucks.

ANIMALS, MATERIALS AND METHODS

Ethical approval

Approval for this study was sought from the Ahmadu Bello University Committee for Animal Use and Care (ABUCAUC) with the approval number: ABUCAUC/2021/062.

Study area

The research was conducted at the Department of Theriogenology and Production, Faculty of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria.

Plant material

Azanza garckeana (AG) ripe fruits were sourced during February-April, 2021, which coincided with the harvest season in the Tula area of Kaltungo Local Government Area, Gombe State, Nigeria. A sample was sent to the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria for authentication and identification, with voucher n°.: ABU07276. The authenticity of the plant was also confirmed by comparing it with the features available in databases (http://www.theplantlist.org/' and 'http://www.ipni.org/).

Sequential crude extraction

Reagents and chemicals including methanol, n-hexane and ethyl acetate used for extraction of the plant material were of analytical grade, obtained from Sigma Chemical Company, St. Louis, MO, USA. All other chemicals used were also of analytical grade and were prepared in distilled water.

The whole AG nut was rinsed under clean running water and the seeds were removed. The pulp was air-dried for two weeks. The dried material was pulverised into a coarse powder using a grinder mill. Exactly 5 kg of the plant material was extracted with n-hexane, ethyl acetate and methanol successively, using Soxhlet apparatus at 50°C. Extraction with n-hexane was carried out until solvent became clear to obtain the n-hexane extract. The solvent was recovered, concentrated and finally dried to a constant weight on a rotary evaporator. Thereafter, it was stored in an air-tight

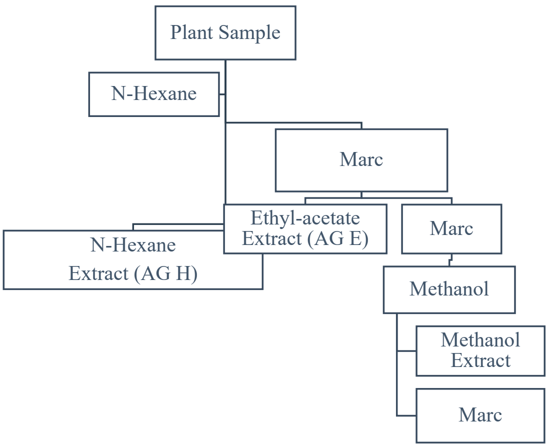

Figure 2: Schematic representation of a serial exhaustive extraction of *Azanza garckeana* in a Soxhlet apparatus.

container for subsequent use. The marc was extracted again with ethyl acetate and methanol solvents and the extract was treated in the same manner as in n-hexane extract (Wu *et al.*, 2013).

For the preparation of aqueous extract, 1 kg of pulverised pulp was air-dried in the shade for two weeks after being washed with running water. The aqueous extract was prepared by cold maceration of 1 kg of powdered pulp in 3500 mL of distilled water for 72 h. Then, the extract was filtered, concentrated and dried *in-vacuo* (yield 67 g), and the residue was stored in a refrigerator at 2-8°C for use in subsequent experiments.

The weight of the extract was recorded and the percentage extract yield was computed using the formula below:

$$\% \text{ Yield} = \frac{\text{Weight of the Extract (g)}}{\text{Weight of Dried powdered sample (g)}} \times 100$$, Tsado, *et al.* (2015).

Phytochemical analysis and acute toxicity test of AG pulp extract

The phytochemical constituents of AG pulp extract were screened and those present were quantified using standard methods as described by (Ogbu *et al.*, 2020).

Phytochemical analysis

The AG fruit pulp extracts were screened for the presence of secondary metabolites like alkaloids, saponins, flavonoids, phenols and tannins according to standard tests described (Yadav *et al.*, 2013). The specific tests for the phytochemicals were done as follows:

Test for alkaloids (Wagner's test): 10 mg of each of the extracts were dissolved in 1 mL of distilled water. To this solution three drops of Wagner's reagent were added. The presence of alkaloids was confirmed by the formation of a reddish-brown coloured solution (Ogbu *et al.*, 2020).

Tannins test (lead acetate and ferric chloride test): For the lead acetate test, 0.1 gm of each of the extracts was dissolved in 2 mL of distilled water. Then, 1 mL of each of the solutions was taken and 0.5 mL of 1% lead acetate was added to it. Formation of yellowish precipitate was observed for the presence of tannins. For the ferric chloride test, 0.5 mL of 5% ferric chloride solution was added to the same solution used for the lead acetate test, and the development of dark bluish or black colour was observed for the presence of tannins (Wadood *et al.*, 2103).

Test for flavonoids (alkaline reagent or NaOH test): The extracts (0.3 g) of each of the preparations were dissolved in 2 mL of distilled water. To these, 3 drops of 20% sodium hydroxide solution were added. An intense yellow colour was formed which turned colourless with the addition of 3 drops of 20% hydrochloric acid, which indicated the presence of flavonoids in each of the extracts. Besides, a lead acetate test was performed. To the same solution used above 3 drops of 10% lead acetate were added and the formation of yellow precipitate was observed for the presence of flavonoids (Ogbu *et al.*, 2020).

Test for saponins (foam test): About 0.3 g of each of the extracts was taken and dissolved in 20 mL of distilled water. After vigorous shaking, the formation of persistent foam observed for 30 min was taken as an indication for the presence of saponins.

Test for phenols (ferric chloride test): 10 mg of each of the extracts was dissolved in 1 mL of water. Half a mL of 5% ferric chloride solution was added to it and the development of deep blue or black colour was taken as an indicator for the presence of phenols.

Test for steroids (Liebermann-Burchard test): About one-half gram (0.5 g) of each of the extracts was dissolved in 0.5 mL dichloromethane to produce a dilute solution. To this solution 0.5 mL of acetic anhydride was added, followed by 3 drops of concentrated sulphuric acid. Formation of a blue-green colouration indicated the presence of steroids (Yadav *et al.*, 2014).

Acute toxicity Test: The acute toxicity profile of AG pulp extract was performed in two phases for each of the extracts following a previously described method (Lorke, 1983). Briefly, out of the 30 rabbit bucks used in the acute toxicity study, 15 rabbit bucks divided into three groups of five rabbit bucks each were used in phase I. Rabbit bucks in groups 1, 2, and 3 received 10, 100 and 1000 mg/kg body weight (b.w.) AG pulp extracts, respectively. The rabbit bucks were monitored for 24 h. From the result of phase I, higher doses were chosen for phase II. In this phase, the remaining 15 rabbit bucks were divided into three groups of five rabbit bucks each: groups 4, 5 and 6 received 1600, 2900 and 5000 mg/kg b.w. AG pulp extracts, respectively. They were observed for 24 h for lethality or any morphological and behavioural signs of toxicity, including dullness, changes in eyes and fur appearance/colour, hyperactivity, changes in feeding patterns, sedation and mortality.

Eighteen apparently healthy New Zealand White rabbit bucks (*Oryctolagus cuniculus*), 10-18-mo-old with b.w of 1.20-2.00 kg, were used for the study. The bucks were sourced from rabbit farms within Zaria and environs. They were screened and treated against endoparasites and bacterial infection before the beginning of the experiment. The bucks were housed in standard rabbit cages, one buck per cage (40×50×35 cm). They were all given access to water and standard rabbit feeds *ad libitum.* The rabbit bucks were allowed to acclimatise for 14 d before the onset of the study. The feed was sourced from Labar Feed Mills, Zaria, Nigeria.

Experimental diet

The proximate analysis of the diets (Table 1) was carried out according to the American Organisation of Analytical Chemists method (AOAC, 2012). The diet was of isonitrogenous and isocaloric values.

Experimental design

Rabbit bucks were randomly divided into six groups, with three animals in each group: 1) Control treated with normal saline (2 mL/kg). 2) Rabbit bucks treated with crude extract of AG pulp (500 mg/kg b.w.). 3) Rabbit bucks treated with methanol extract of AG pulp (500 mg/kg b.w.). 4) Rabbit bucks treated with n-hexane extract of AG pulp (500 mg/kg b.w.). 5) Rabbit bucks treated with ethyl acetate extract of AG pulp (500 mg/kg b.w.). 6) Rabbit bucks treated with aqueous extract of AG pulp (500 mg/kg b.w.).

Semen quality

Semen was collected weekly for 4 wk. Thus, 72 ejaculates were obtained during the study. Ejaculates were collected using an artificial vagina maintained at 45°C to 46°C and a teaser doe.

The reaction time (RT) is the time between introducing the teaser doe into the male's cage and the time that it takes the buck to sniff, groom, mount and ejaculate. It was measured in seconds. A stopwatch was used to determine the libido. The three-colour categories of milky, creamy and colourless, designated 1, 2 and 3, respectively were used for scoring the semen as described by Rekwot *et al.* (1997).

Semen was kept at 35°C in a water bath for examination immediately after collection. The volume of each ejaculate was recorded after removal of the gel mass. Fresh semen (two drops) was placed on a warm slide and covered with a cover slip (20×20 mm) to determine mass motility. Mass motility from at least three fields was examined at 37°C under a microscope with phase-contrast optics, at 40×, and assessed from 0-100%. The estimate of mass activity was based on the vigour of the wave motion. This was assessed on a 0-5 scoring system. Scores from least active (+0=10-20%) to most active (+5=90-100%) were given to the wave motion of the spermatozoa according to the intensity of swirling bands. A weak eosin solution was used at a rate of 1:99 before counting the cells for the evaluation of sperm concentration (×10^6/mL) according

Table 1: Composition of experimental diet.

Feedstuff Composition (%)	
Maize	30.16
Groundnut cake	28.12
Rice offals	35.32
Vitamin premix	0.5
Palm oil	1.0
Bone meal	4.0
Methionine	0.4
Salt	0.5
Total	100
Proximate Composition	
Dry matter	89.50
Crude protein (CP)	16.81
Ether extract (EE)	1.27
Crude fibre	8.65
Nitrogen free extract (NFE)	53.96
Ash	7.20
ME (kcal/kg)	2640.42

Metabolisable energy was calculated according to formula of Pauzenga (1985): ME= 37×% CP+81×% EE+35.5×% NFE.

to Smith and Mayer (1955) using the improved Neubauer haemocytometer slide (GmbH1Co., Hamburg, Germany). The total sperm output was estimated by multiplying the volume of the ejaculate and the concentration of the sperm.

Blood collection

Exactly 3 mL of blood was collected weekly from the marginal ear vein of each buck using a 27 G needle and placed immediately on ice in heparinised tubes. Serum was collected from blood by centrifugation (Spectrafuge 6C from Deeksha Technologies Srirampura, Bengalaru, Karnataka.) at 3000× *g* for 5 min and kept at a temperature of 25-26°C (Attia and Kamel, 2012).

Blood hormonal profiles

The testosterone, follicle-stimulating hormone (FSH) and luteinising hormone concentrations in plasma were measured using immunoassay commercial kits (Biosource-Europe S.A., Nivelles, Belgium). The absorbance in each well was read at 450 nm wavelength in a microplate reader and plotted against the concentration of standard T in ng/mL on linear graph paper, to determine the concentrations (Oda and Waheeb, 2017).

Antioxidant biomarker assessment

Reduced glutathione (GSH): Reduced glutathione concentration was measured according to Ellman (1959) as described by Rajagopalan *et al.* (2004). It was based on the reaction between 5, 5-dithiobis nitrobenzoic acid (DNTB) and reduced glutathione (GSH).

To 150 µL of serum (in phosphate-buffered saline, pH 7.4), 1.5 mL of 10% trichloroacetic acid was added and centrifuged at 1500 *g* for 5 min. Exactly 1 mL of the supernatant was treated with 0.5 mL of Ellman's reagent and 3 mL of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm. The quantity of reduced glutathione GSH was obtained from the graph of the GSH standard curve. The GSH concentration was expressed as IU/mg serum protein concentration.

Catalase: Catalase (CAT) activity was determined by the method described by Beers and Sizer (1952). The ability of one unit of CAT to decompose 1.0 µmole of H_20_2 per min at pH 7.0 at 25°C, while the H_20_2 concentration falls from 10.3 mM to 9.2 mM. The rate of disappearance of H_20_2 is followed by observing the rate of decrease in the absorbance at 240 nm (Stern, 1937).

Evaluation of serum lipid peroxidation

Lipid peroxidation generates peroxide intermediates which upon cleavage release malondialdehyde (MDA). The concentration of MDA serves as an index of intensity of lipid peroxidation, a product which reacts with thiobarbituric acid (TBA). The reaction yields a complex colour which absorbs light at 535 nm and can be measured.

Exactly 150 µL of serum was treated with 2 mL of 0.37% TBA solution-15% trichloroacetic acid solution-0.25 N HCl reagent (1:1:1 ratio) and placed in water bath at 90°C for 60 min. The mixture was cooled and centrifuged at 3000× *g* for 5 min and the absorbance of the pink supernatant (TBA-MDA complex) was measured at 535 nm. The concentration of MDA formed was calculated using the molar extinction coefficient of 1.56×10^{-5}cm $1M^{-1}$.

MDA concentration (nmol/mg protein)=Absorbance of sample/$1.56\times10^{-5}\times$protein concentration (mg).

Superoxide dismutase

Superoxide dismutase (SOD) activity was determined by the method described by Fridovich (1989). The ability of SOD to inhibit auto-oxidation of adrenaline at pH 10.2 formed the basis of this assay.

Exactly 0.1 mL of serum was diluted in 0.9 mL of distilled water to make 1:10 dilution. An aliquot mixture of 0.2 mL of the diluted solution was added to 2.5 mL of 0.05 M carbonate buffer. The reaction was started with the addition of 0.3 mL of 0.3 mM adrenaline. The reference mixture contained 2.5 mL of 0.05 M carbonate buffer, 0.3 mL of 0.3 mM adrenaline and 0.2 mL of distilled water. The absorbance was measured over 30 s up to 150 s at 480 nm.

Increase in absorbance per minute=(A_2-A_1)/2.5.

% inhibition=100–[(increase in absorbance for sample/increase in absorbance of blank)×100].

One unit of SOD activity was the quantity of SOD necessary to elicit 50% inhibition of the oxidation of adrenaline to adrenochrome in 1 min. The SOD activity was expressed in IU/mg serum protein concentration.

Statistical analyses

Data was expressed as mean±standard error of mean and subjected to repeated-measures one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Values of $P<0.05$ were considered significant. The analyses were carried out using GraphPad Prism version 5.0 for windows 2003 from GraphPad Prism Software, San Diego, California (www.graphpad.com).

RESULTS

Tannins, flavonoids, saponins, phenols and tannins were found in varying quantities all the extract. (Table 2).

The extract yield in grams of different extracts of AG fruit pulp are shown in Table 3. Methanol extract of the AG fruit pulp produced higher extract yield of 546.22 g when compared to the ethyl acetate extract and n-hexane, which registered 82.11 g and 65.26 g extract, respectively.

There was no mortality recorded up to the 5000 mg/kg b.w. dose of the five different extracts of AG pulp administered via oral gavage. In addition, there were no significant b.w. or behavioural changes within 24 h of acute toxicity study. The observations suggested that the five different levels of AG pulp extract were safe for consumption. The n-hexane extract caused continuous diarrhoea.

There was no significant difference in semen colour across the various groups. However, rabbit bucks administered AG M and AG Aq exhibited the overall best semen colour (mostly creamy), when compared to the control and other groups (Table 4).

The overall mean reaction time (RT) was significantly ($P<0.05$) lower in the AG E (66.33±25.90 s) and AG M (72.50±11.71 s) extracts, when compared to the RT of bucks in the group administered AG H (170.08±15.86 s) (Figure 3). The ejaculate volume was significantly ($P<0.05$) higher among the groups administered extract than in the control group at second, third and fourth weeks of the experiment (Figure 4). The AG Aq (0.63±0.15 mL) extract had the highest semen volume at week 2, while the AG M was the highest, and the volume was significantly ($P<0.05$) higher in week three (0.90±0.10 mL) compared to other groups. The overall mean ejaculate volume was highest in the bucks administered AG M (0.77±0.12 mL) when compared to the control group (0.31±0.05mL). There was a significant difference ($P>0.05$) between the means within the extract groups.

The sperm concentrations varied significantly across various groups. At week 1, the concentration in AG E ($110.00±21.70×10^6$/mL) and AG AQ ($152.00±39.30×10^6$/mL) were significantly ($P<0.05$) higher than in other groups (Figure 5). At week 2, there was a significant ($P<0.05$) increase in sperm concentration between different groups when compared to the controls. The overall mean sperm concentration significantly ($P<0.05$) increased in AG M ($113.68±12.10×10^6$/mL) group, when compared to that of the AG H group ($77.40±14.21×10^6$/mL).

Table 2: Mean±standard error of mean of quantitative phytochemical composition of *Azanza garckeana* fruit pulp.

Phytoconstituents	AG Cr (w/w) (%)	AG M (w/w) (%)	AG H (w/w) (%)	AG E (w/w) (%)	AG Aq (w/w) (%)
Tannins	20.00±0.45^a	14.90±0.15^b	25.63±0.35^c	23.22±0.09^d	20.03±0.09^a
Flavonoids	21.09±0.68^a	25.50±0.12^b	2.10±0.01^a	15.01±0.36^c	19.23 ±0.28^d
Saponins	5.43±0.67^a	18.90±0.43^b	3.29±0.42^c	5.35±0.67^a	24.02±0.02^d
Phenols	34.32±2.34^a	36.52±0.11^a	10.03±0.76^b	25.34±0.32^c	30.01±0.21^d
Alkaloids	15.18±0.11^a	19.00±0.65^b	5.11±0.84^c	10.78 ±0.15^d	10.15±0.58^d

^{a,b,c,d,}Means along rows with different superscript letters are significantly ($^aP<0.05$, $^bP<0.01$, $^{cd}P<0.001$) different, n=3.

Table 3: The AG fruit pulp extract yield in grams of different extracts of AG fruit pulp.

Extract	Yield (g)
Crude	359.93
Methanol	546.22
n-Hexane	65.26
Ethyl acetate	82.11
Aqueous	482.25

The progressive sperm motility significantly (P<0.05) varied within the groups administered extract when compared to the control group within the weeks. Bucks exposed to AG M (70.00±11.02%) extract had the significantly (P<0.05) highest overall mean sperm motility, whereas AG H (57.50±3.11%) extract group had the lowest (Figure 6). There was no significant (P>0.05) difference in the mean pH values across the various groups (Figure 7). However, the rabbit bucks administered AG H (6.00±0.00) and AG E (6.33±0.33) had significantly (P<0.05) lower pH (acidic) semen at weeks 1 and 4, respectively.

There was a significant (P<0.05) increase in mean testosterone concentrations in all the extract groups compared to that of the control group (Figure 8). The rabbit bucks administered AG M extract (1.76±0.77 ng/mL) had the highest overall mean testosterone concentration when compared to either the AG E (0.77±0.24 ng/mL) or the control group (0.84±0.29 ng/mL).

The LH concentration fluctuated significantly (P<0.05) across the groups (Figure 9). The LH concentrations for AG Cr in weeks 1 (2.05±0.29 ng/mL) and 2 (2.03±0.25 ng/mL) were significantly (P<0.05) higher than the remaining groups. At week 3, the LH concentrations in AG M (2.15±0.15 ng/mL) and AG E (2.09±0.18 ng/mL) extract groups were significantly (P<0.05) higher than the other groups. At week 4, the LH concentration of control bucks (1.53±0.30 ng/mL) was significantly (P<0.05) lower when compared to the groups administered different extracts. The overall mean LH concentration was significantly (P<0.05) lower in the control group than in all the groups that were given the extract. The highest LH concentration was recorded in the rabbit bucks administered AG M (1.92±0.07 ng/mL).

The follicle-stimulating (FSH) hormone concentration was significantly (P<0.05) higher in all the groups administered the extract, when compared to the control group (Figure 10). At week 1, the concentration of AG Cr (1.38±0.73 ng/mL) was significantly (P<0.05) higher than those of the control (0.61±0.06 ng/mL) and AG M (0.67±0.06 ng/mL) groups. However, at weeks 3 (0.93±0.12 ng/mL) and 4 (1.36±0.16 ng/mL), the AG M group recorded a significant (P<0.05) increase in FSH concentrations when compared to other groups.

The GSH concentration was significantly (P<0.05) higher in the AG Cr (1.06±0.21 U/L) and AG M (0.94±0.15 U/L) groups than in the AG H (0.37±0.08 U/L) and AG AQ group (0.08±0.11 U/L) (Figure 11). The CAT activity was significantly (P<0.05) lower in the AG H group (6.29±1.88 U/L) when compared to the control group (11.10±2.87 U/L) at week 1. At week 2, the AG E (10.80±1.14 U/L) group had a significantly (P<0.05) higher CAT activity than the AG H (5.53±1.71 U/L) group. The mean CAT activity significantly (P<0.05) decreased in the group administered AG H (6.29±1.88 U/L) when compared to the AG E group (10.36±2.14 U/L) and the other groups. However, bucks administered AG M had the greatest overall mean CAT activity (10.53±2.16 U/L) (Figure 12). The mean malondialdehyde (MDA) concentration was significantly (P>0.05) higher in the control bucks (11.40±1.23) when compared to the AG M group (4.73±0.176) at week 3. The overall mean of MDA concentration was significantly (P<0.05) lower in the AG M (5.52±0.37 nMol/L/protein) and the extract groups when compared to the control group (10.59±1.47 nMol/L/protein) (Figure 13). The SOD activities fluctuated in the various groups throughout the four-week

Table 4: Effect of extract on color (n=3) of rabbits' semen.

Week	Control	Crude	Methanol	n-Hexane	Ethyl acetate	Aqueous
1	2	2^a	2^a	2	2	2^a
2	2	2^a	3^b	2	2	3^a
3	2	2^a	2^a	2	2	2^a
4	2	3^b	3^b	2	2	3^b

Values are expressed as mean±standard error of mean.

[a,b]Means along the rows with different superscript letters are significantly different ([a]P<0.05; [b]P<0.01), n=3.

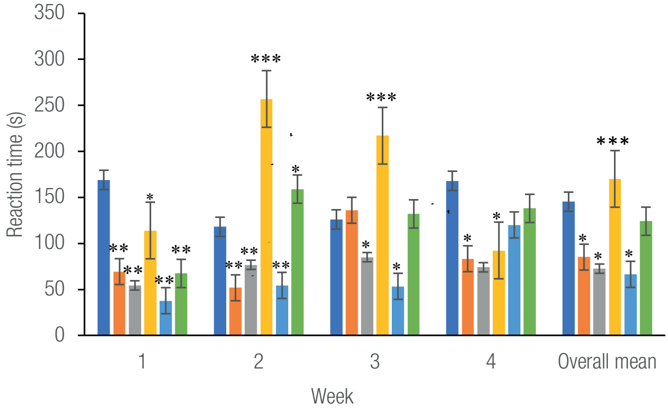

Figure 3: Effect of treatment on reaction time of rabbit bucks. Values are expressed as mean±standard error of mean.**P*<0.05, ***P*<0.01, ****P*<0.001 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

period (Figure 14). However, the SOD activity was significantly (*P*<0.05) higher in the AG M group (7.52±0.82 U/L), compared to the group administered AG Cr (5.97±0.98 nMol/L/protein).

DISCUSSION

The phytochemical analyses showed that the extracts contained phytochemicals exerting beneficial effects. Phytochemicals are physiologically active in plants, which in addition provide health benefits as sources of micronutrients and macronutrients in animals and humans (Hasler and Blumberg, 1999). Flavonoids are polyphenolic compounds synthesised by plants and they exhibit broad biological and pharmacological activities. They contain spermatogenic, aphrodisiac and antioxidant properties, exhibiting antimicrobial, cytotoxic, anti-inflammatory, anti-cancerous, oestrogenic, anti-allergic and hematopoietic actions (Itodo *et al.*, 2022). They are compounds that protect biological systems against the damaging effects of oxidative processes on the body's macromolecules (Atmani *et al.*,

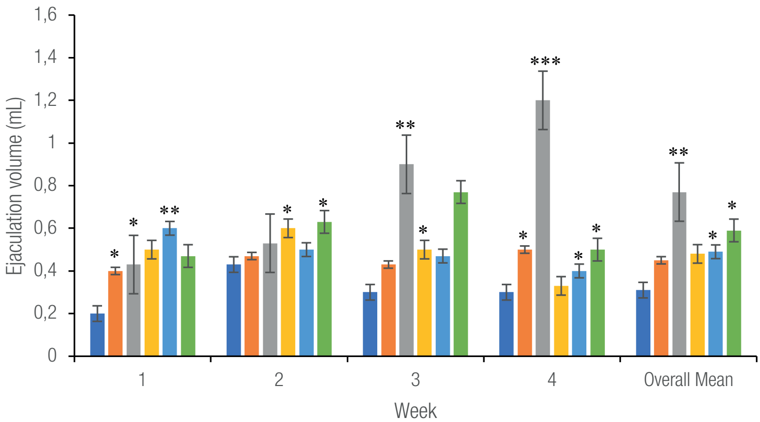

Figure 4: Effect of treatment on ejaculate volume of rabbit bucks. Values are expressed as mean±standard error of mean.**P*<0.05, ***P*<0.01, ****P*<0.001 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

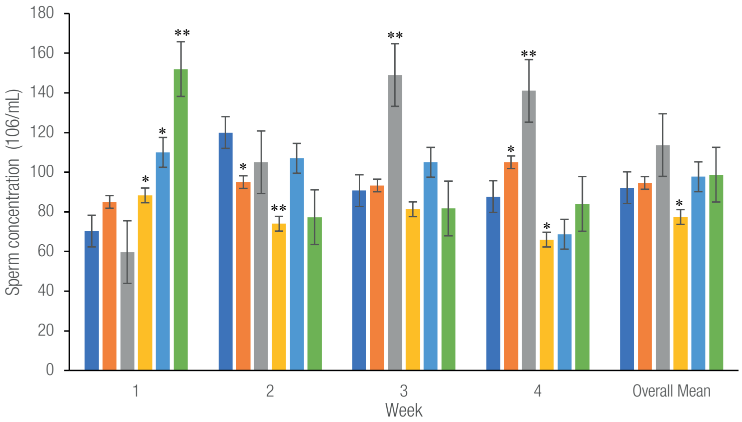

Figure 5: Effect of treatment on sperm concentration of rabbit bucks. Values are expressed as mean±standard error of mean. *P<0.05, **P<0.01 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

2009). Saponins are well-known for their anti-inflammatory, haemolytic, cholesterol-binding and bitter effects. Tannins are described as plant-derived antinutrients because they precipitate proteins, block digestive enzymes and reduce vitamin and mineral availability in the body (Ryszard, 2007).

Alkaloids have many pharmacological effects, such as antihypertensive, antimalarial and anti-carcinogenic capabilities (Saxena *et al.*, 2013). AG pulp, shown to contain a very high quantity of phenols and flavonoids, has very high steroidogenic and antioxidant properties. The phytochemical compounds identified in the fruit are the bioactive constituents of the plant. This finding suggests that the AG fruit may be a valuable reservoir of bioactive compounds of substantial medicinal importance (Itodo *et al.*, 2022). The reproductive and antioxidant activities of *Azanza garckeana* fruit extracts varied, depending on the type of extract used.

The results suggest the suitability of polar solvents for the extraction of antioxidant compounds from plant materials, particularly AG fruit pulp. The study also reveals that phytochemical substances extracted in polar solvents exhibited stronger antioxidant activity, reducing characteristics and free-radical scavenging activity than those extracted in non-

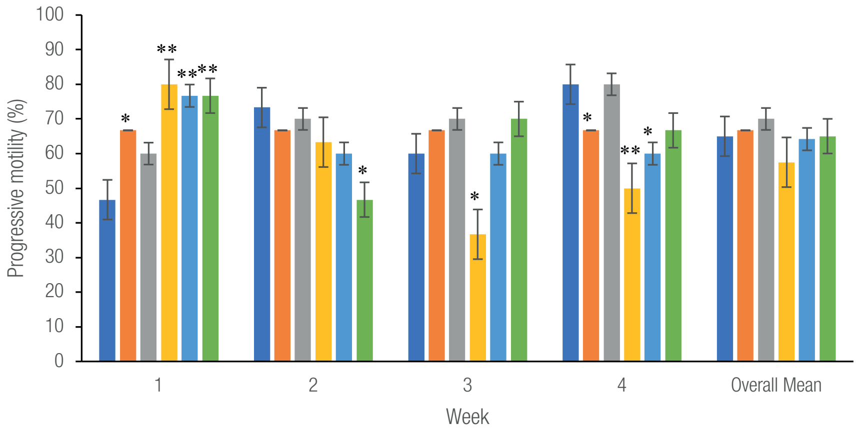

Figure 6: Effect of treatment on progressive motility (n=3) of rabbit bucks. Values are expressed as mean±standard error of mean. *P<0.05, **P<0.01 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

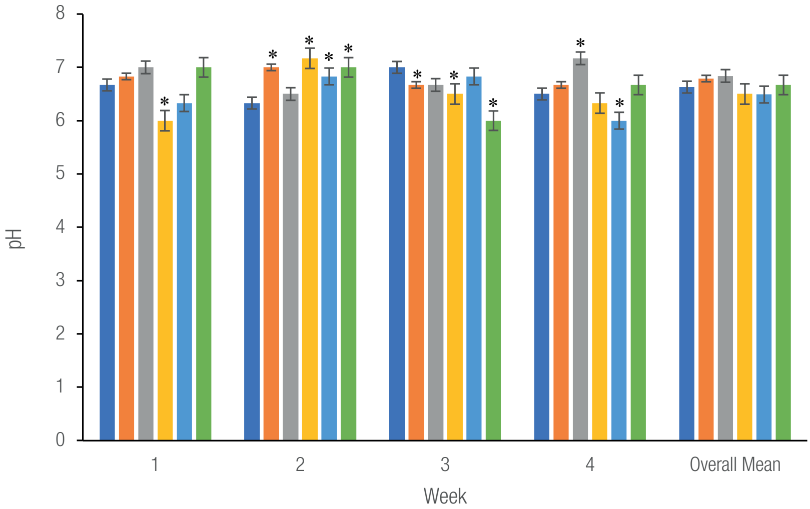

Figure 7: Effect of treatment on pH of rabbit semen. Values are expressed as mean±standard error of mean. **P*<0.05 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

polar solvents (Tijjani *et al.,* 2007; Lawal *et al.,* 2014; Yusuf *et al.,* 2018; Yusuf *et al.,* 2020a). The phytochemical composition of the AG M and ethyl acetate fruit pulp reported in this current study is in agreement with the findings of Yusuf *et al.,* 2020a and Yusuf *et al.,* 2020b, who analysed the methanol and ethyl acetate extracts of AG pulp. However, Jacob *et al.,* 2016 reported lower levels of the phytochemical compounds, and the differences recorded may be as a result of the location of the AG plant (Katsina State) and the process of extraction.

The present findings reported that methanol extracts of AG fruit pulp had the highest total extract yield in grams when compared to the ethyl acetate and n-hexane extracts. This outcome is in agreement with the findings of Yusuf *et al.,* 2020a that reported higher percentage extract yield for air-dried AG M fruit pulp and shaft when compared to the ethyl acetate extract. This may due to the fact that non-polar solvents like hexane and ethyl acetate have lower yield when compared to more polar solvents such as methanol.

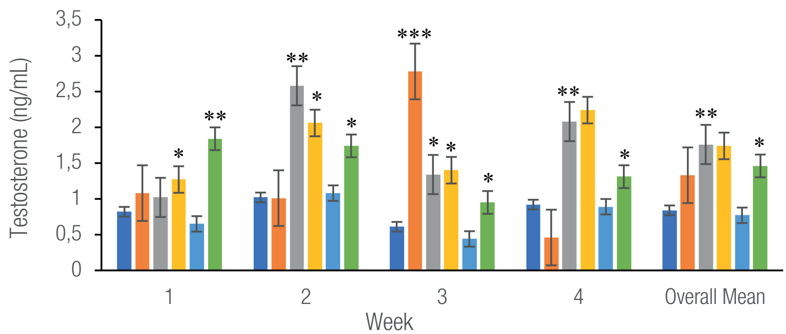

Figure 8: Effect of treatment on testosterone in rabbit bucks. Values are expressed as mean±standard error of mean. **P*<0.05, ***P*<0.01, ****P*<0.001 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

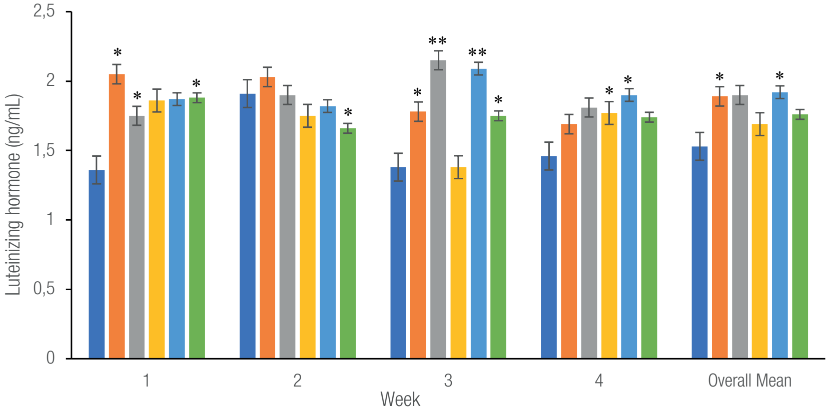

Figure 9: Effect of treatment on luteinising hormone in rabbit bucks. Values are expressed as mean±standard error of mean. *P<0.05, **P<0.01 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

There was no mortality recorded up to 5000 mg/kg b.w. dose of the AG pulp. In addition, there were no significant b.w. or behavioural changes within 24 h of acute toxicity study. The observations suggest that AG pulp is safe for consumption. However, caution should be taken when using the n-hexane extract because of its ability to cause diarrhoea during a prolong period of administration, possibly as a result of the oily consistency of the extract, which may be responsible for increased colonic movement (Dikko *et al.,* 2016).

The significantly lower RT obtained in the AG M group when compared to the various groups may be the result of the increase in the amount of phenols in the methanolic extract and the high polarity of methane in extracts. This result is in agreement with the findings of Yusuf *et al.* (2020a), who compared the phenolic constituents of both methanolic (34.32±2.34) and ethyl acetate (25.34±0.32) extracts and showed that methanol extract had more phenols than ethyl acetate. The present result is also in agreement with the findings of Yusuf *et al.* (2020b), who compared the effect of air-drying and sun-drying on the phytochemical composition of both the pulp and shaft of AG. The authors

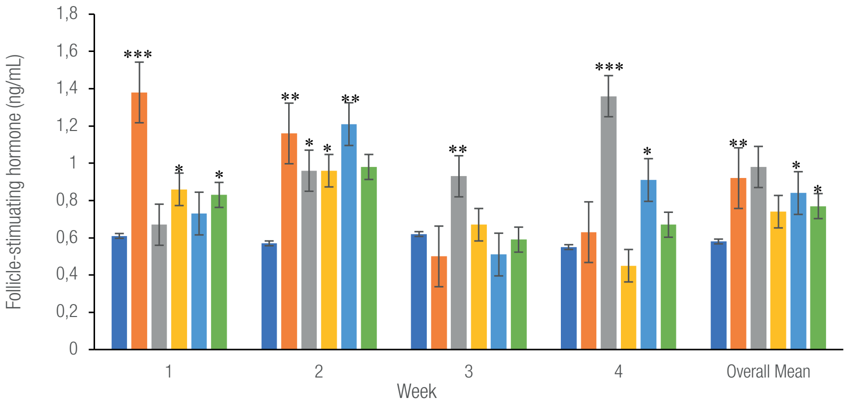

Figure 10: Effect of treatment on follicle-stimulating hormone in rabbit bucks. Values are expressed as mean±standard error of mean. *P<0.05, **P<0.01, ***P<0.001 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

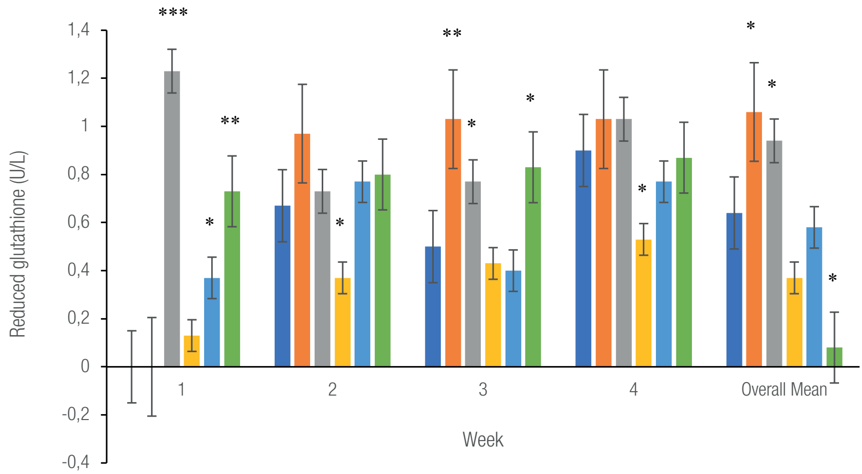

Figure 11: Effect of treatment on reduced glutathione concentration in rabbit bucks. Values are expressed as mean±standard error of mean. **P*<0.05, ***P*<0.01, ****P*<0.001 are significantly different when compared to control, *n*=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

found that the air-dried methanol extracts had higher composition of phenol levels than the ethyl acetate extracts. However, the result was in contrast with the findings of Nkafamiya *et al.* (2015), who reported the absence of phenols in AG pulp. Conversely, the AG H extract group had a significantly high RT, which could be indicative of the presence of anti-aphrodisiac factors. This is because hexane has a lower polarity than the other groups (Zhoung and Zhou, 2013).

The bucks administered AG M and AG Aq extracts had mostly creamy semen colour. Rabbit bucks administered AG M and AG Aq extracts had the overall best semen colour when compared to other treatment groups. The results show that rabbits fed with different extracts had better semen colour when compared to the control. Therefore, the control group had poor semen colour when compared to the treatment groups. This could be due to the presence of antioxidant in various quantities in all the AG pulp extracts, which were lacking in the control group. This finding is in agreement with the results of Yusuf *et al.*, (2020b) and Maroyi and Cheikh -Youssef (2017) who reported that

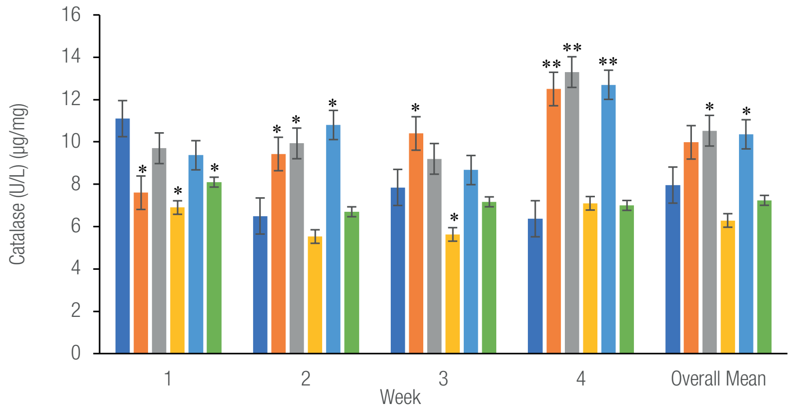

Figure 12: Effect of treatment on catalase activity in rabbit bucks. Values are expressed as mean±standard error of mean. **P*<0.05, ***P*<0.01, ****P*<0.001 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

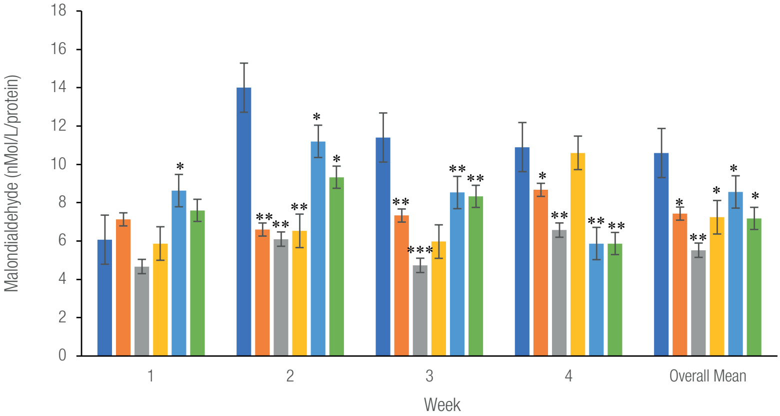

Figure 13: Effect of treatment on malondialdehyde concentration in rabbit bucks. Values are expressed as mean±standard error of mean. **P*<0.05, ***P*<0.01 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

AG metabolites have very high levels of antioxidants that helped improve the spermiogram of the bucks treated with the extracts.

The AG M had an overall increase in both sperm concentration and motility. The finding may be the result of increased phenolic and flavonoid contents in AG M extracts, responsible for increased antioxidant and spermatogenic effects. Studies have shown that treatments with antioxidants improve steroidogenesis through enhancement of the Leydig cell and endocrine function and, consequently, increasing circulating concentrations of testosterone, which stimulates spermatogenic functions (Meli *et al.*, 2020). The present finding is in agreement with the result of Adienbo *et al.* (2013) in rabbits, who reported increased sperm motility and concentration of hydro-methanolic extract of Ethiopian pepper (*Xylopia aethiopica*). The reason for the reduced sperm motility and concentration in the AG H extract group could be as a result of decreased phenol and flavonoid contents. The finding is in agreement with the results of Truong *et al.* (2019) and Ngo *et al.* (2017), who reported low phenolic and flavonoid components in normal hexane solvent when used for extraction. Hexane extract contains very high numbers of alkaloids, which have been reported

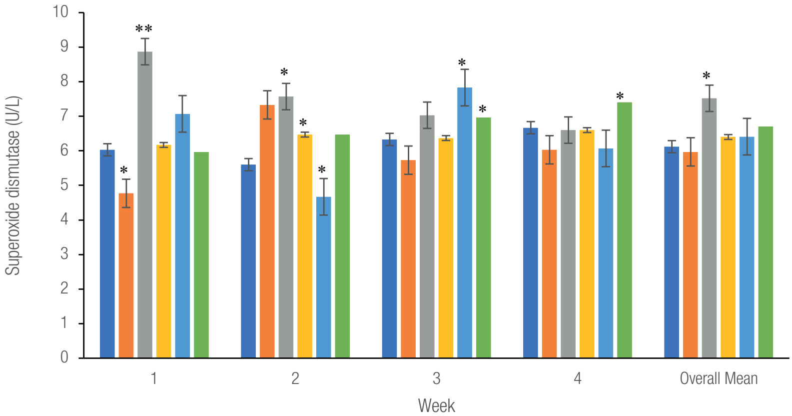

Figure 14: Effect of treatment on superoxide dismutase activity in rabbit bucks. Values are expressed as mean±standard error of mean. **P*<0.05, ***P*<0.01 are significantly different when compared to control, n=3. ■ Control, ■ Crude, ■ Methanol, ■ n-Hexane, ■ Ethyl acetate, ■ Aqueous.

to also possess bioactive potentials, releasing metabolites which bind to cell molecules and cross link DNA to induce cytotoxicity (Chang *et al.*, 2018).

The serum concentrations of testosterone, LH and FSH fluctuated over the weeks in rabbit bucks. However, bucks administered AG Cr and AG M extracts had increased serum hormonal concentration when compared to other experimental groups. The hormones have been reported to play a significant role in the improvement of male reproductive functions (Abo-elsouda *et al.*, 2019). The result shows that AG pulp contained an appreciable quantity of bioactive components, including polyphenols such as flavonoids, which may reduce oxidative stress and harmful biological activities (Maroyi and Cheikh-Youssef, 2017; Edward *et al.*, 2021). Studies have shown that treatments with antioxidants improve steroidogenesis through the enhancement of the Leydig cell, endocrine function and, consequently, increasing circulating levels of testosterone, which stimulate spermatogenic functions (Zhoung and Zhou, 2013). The present report lends support to earlier reports on the use of the plant in folklore medicine for the enhancement of fertility (Maroyi, 2011; Ochokwu *et al.*, 2015). It has been documented that AG pulp contains L-DOPA (L-3,4-dihydroxyphenyl alanine) as its active substance, which improves male reproductive function in animals (Maroyi, 2012). In addition, L-DOPA has been demonstrated to promote gonadotropin (GnRH) secretion, which in turn stimulates the secretion of FSH and LH from the anterior pituitary gland (Singh *et al.*, 2013). Elevated serum levels of FSH and LH stimulate spermatogenesis processes through testosterone production. Therefore, it may be inferred from the findings of the present studies that concentrations of L-DOPA in AG Cr and AG M were, apparently, sufficient to stimulate the secretion of GnRH from the hypothalamus; thereby resulting in higher serum levels of testosterone, LH and FSH. In addition, the higher levels of the reproductive hormones observed among rabbit bucks in AG Cr and AG M may be attributed to the levels of flavonoids, phenols, saponins and tannins present in AG Cr and AG M. (Yusuf *et al.,* 2020b). The compounds have been documented as increasing testosterone level through hypothalamo-pituitary-testicular axis stimulation in animals (Ahangarpour *et al.*, 2017), and a similar mechanism is reasonably proposed in this study. The reduction in the concentrations of the three hormones in the AG H group may be attributed to a severe reduction or total absence of active spermatogenic and steroidogenic constituents in the n-hexane extract of AG pulp. This is because n-hexane is a non-polar solvent when compared to methanol (Iloki-Assanga *et al.*, 2015; Nguyen *et al.*, 2018; Yen *et al.*, 2018).

The present research also buttressed the point that AG fruit pulp contains antioxidant activities that can be used to ameliorate oxidative stress and biomarker changes in bucks. The concentration of reduced glutathione, MDA was measured as a product of lipid peroxidation and the activities of antioxidant enzymes SOD and CAT in the blood. The present findings are in agreement with the works of Hamden *et al.* (2008) and Shinkut *et al.* (2020), who reported that oxidative stress reduces serum antioxidant capacity, manifested in decreased activities of the antioxidant enzymes, superoxide dismutase, catalase and glutathione peroxidase. ROS-scavenging enzymes, such as superoxide dismutase and catalase, are the first-line cellular defence enzymes against oxidative injury. For the effective elimination of ROS in intracellular organelles, the equilibrium between the enzymes is critical (Yeh *et al.*, 2007). This level of oxidative stress was reflected in reduced sperm counts, as well as a decrease in sperm viability (Ghosh *et al.*, 2002).

The current findings on oxidative stress were consistent with the results of Aybek *et al.* (2008), who obtained similar findings in terms of serum SOD activity and MDA concentration. Shinkut *et al.* (2020) observed a marked increase in the SOD, CAT and GPx activities in rabbit bucks, supplemented with garlic (*Allium sativum*). The mammalian sperm plasma membrane, which is rich in polyunsaturated fatty acids, can easily be damaged by the reaction between ROS, such as OH· and polyunsaturated fatty acids (Alvarez and Storey, 1995). This mechanism is widely known as the lipid peroxidation reaction (Agarwal *et al.*, 2003) and indirectly measured as MDA concentration, which is the end-point reaction product of lipid peroxidation (Baumber *et al.*, 2000; Saleh *et al.*, 2015).

In the present study, bucks treated with AG Cr and AG M extracts had a greater oxidative stability, denoted by increased concentration of serum total antioxidant capacity and decreased concentration of serum MDA. Total antioxidant capacity is an indicator for the availability of reducing agents in blood plasma, and, thus, the ability of plasma to scavenge ROS produced from oxidation processes (Enechi *et al.*, 2021).

CONCLUSION

The solvents utilised had a significant impact on the extraction yield, chemical component concentration, and physiological activity. Methanol was the most effective extraction solvent, resulting in the highest antioxidant and *in vitro* spermatogenic activities. Methanol was the best solvent for bioactive compound extraction from AG fruit pulp. It is a promising antioxidant and spermatogenic agent, which may be valuable to nutraceutical and pharmaceutical industries.

Declaration of interest: The authors declare that there are no conflicts of interest.

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