

Full Length Research Paper

Preliminary study on the antioxidant effect of *Kigelia africana* fruit extract (Bignoniaceae) in male Sprague-Dawley rats

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Testicular germ cells as well as epididymal maturing spermatozoa are endowed with enzymatic and non-enzymatic scavenger systems to protect lipid peroxidation damage. A number of pathologies and systemic challenges can lead to an antioxidant/pro-oxidant disequilibrium. With increasing interest in herbal therapies for management of some fertility-related and inflammatory conditions, we investigated the antioxidant effect of *Kigelia africana* fruit extract (KAFE) on normal rats. KAFE showed a non-dose dependent elevation in testicular catalase ($p < 0.05$), significant decline in malondialdehyde ($p < 0.001$) and an up-regulation of glutathione ($p < 0.001$) levels. Seminal parameters were also enhanced by KAFE with the lower dose producing better effects. Male infertility is frequently accompanied by increased testicular or seminal fluid oxidative stress. This result provides further scientific basis for the use of KAFE in the treatment of male infertility.

Key words: *Kigelia africana*, catalase, sperm count, malondialdehyde, glutathione, body weight.

INTRODUCTION

Knowledge has been built for decades on the use of herbal medicinal products and extracts in the treatment of human diseases (Iwalewa et al., 2007). In fact, ethno-medicinal plant-use data in many forms has been heavily utilized in the development of formularies and pharmacopoeias, providing a major focus in global health care as well as contributing substantially to the drug development process (Graham et al., 2000). A number of factors linked to imbalance in antioxidant/pro-oxidant system have been implicated in the development of male infertility, including varicocele, environmental pollutants, smoking and cancer. Quite a number of men have been linked with idiopathic infertility which presents with significantly high reactive oxygen species levels and lower antioxidant levels than normal healthy fertile counterparts (Cocuzza et al., 2007). In man, a balance called oxidative stress status normally exist between the production of free radicals and the antioxidant scavenging

system in the male reproductive tract (Sikka et al., 1995).

The human spermatozoa however produce a small amount of reactive oxygen species that play significant role in many sperm physiological processes like capacitation, hyperactivation and sperm-oocyte fusion (Sies, 1993; Lewis et al., 1995). But this must be strictly maintained to keep a small amount necessary for normal sperm function.

Kigelia africana (Lam.) Benth. belongs to the family of *Bignoniaceae* and has a wide geographical distribution in west and central Africa. The tree grows on riverbanks, wet areas along streams and on floodplains of Nigeria, Cameroon, Kenya, Guinea and Senegal. It can also be found in open woodland from KwaZulu-Natal to Tanzania, Chad, Eritrea, South Africa and Namibia (Ogbeche et al., 2002; Abioye et al., 2003; Owolabi et al., 2007; Owolabi and Omogbai, 2007). The tree is widely grown as an ornamental plant in tropical regions for its decorative flowers and unusual fruit hence the name 'sausage tree' (Roodt, 1992).

The fruit pulp is fibrous and pulpy containing numerous hard seeds and tends to be inedible to humans as well as being poisonous when unripe. However, several species

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of mammals eat the seeds, e.g., baboons, bush pigs, monkeys, porcupines, savannah elephants, giraffes and hippopotamus (Owolabi and Omogbai, 2007). The seeds are dispersed via their dung. In Malawi, during famine the seeds are roasted and eaten. Brown parrots and brown-headed parrots also eat the seeds (del Hoyo et al., 1997; Owolabi and Omogbai, 2007). In Kenya, the roasted seeds mixed with beer cause enlargement of sexual organs (Kokwaro, 1976). In South eastern Nigeria, the fruits and flowers are mixed with alcohol or water and used by traditional healers for fertility treatment among women and men of child bearing age (Ogbeche et al., 2002). Previous studies of the fruits showed some anti-inflammatory effects (Picerno et al., 2005; Owolabi and Omogbai, 2007), anticancer activity (Houghton et al., 1994; Jackson et al., 2000; Picerno et al., 2005) and hepatoprotective effect (Olaleye and Rocha, 2007, 2008). However, there is no report to our knowledge on the antioxidant properties of the fruit of this plant on testicular tissues in any experimental protocol. We therefore studied the effect of this extract on testicular antioxidant, antioxidant enzymes and malondialdehyde which are reliable indices for oxidative stress.

MATERIALS AND METHODS

Plant materials

The matured ripe fruits were collected based on ethno-pharmacological information from the forest in Badagry, Lagos. The botanical identification of the plant and fruits was done at the Forestry Research Institute of Nigeria, Ibadan where a voucher specimen number- FHI/08257 was recorded for ease of identification. The fruits were washed, cut into small pieces; air-dried and ground into powdery form using a grinding machine. The powdered fruit was weighed and kept for further phyto-chemical analysis and extraction.

Extraction of plant material

The extraction was done using the Soxhlet apparatus with methanol as the solvent as described in Abioye et al. (2003). 1.15 kg of the powdered fruits was packed into the thimble of the Soxhlet apparatus containing 1 litre of methanol for extraction. At the end of the extraction process, the extract was further dried in an oven regulated at 38°C and the yield which was 115.33 g (representing 10.03%) was stored in sterile universal containers and kept in the freezer regulated at 4°C prior to use.

Drugs and chemicals

All chemicals were standard laboratory reagents and solvents of Analar grade and were obtained from registered distributors.

- Ketamine was obtained from Rotex Medica, Trittau, Germany.
- 5, 5'-dithiobisnitro benzoic acid (DTNB) is made by Sigma Aldrich Inc., St. Louis, MO, USA (Batch D8130-5G).

Acute toxicity test (LD₅₀)

Forty male Sprague-Dawley rats (200 ± 20 g) were obtained from

the Animal House of the College of Medicine of the University of Lagos, Nigeria. The animals were randomly divided into eight groups of 5 animals per group. The animals were fed with pelleted rat chow and had free access to drinking water but were starved for 12 h prior to testing. The extract was orally administered in doses of 100, 400, 800, 1600, 3200, 6400, 12800 mg/kg to the rats according to the group. Group one was given distilled water (10 ml/kg) p.o as control. General symptoms of toxicity and mortality were observed for 24 h for any sign of delayed toxicity (Lorke, 1983).

Animal treatment

All experimental procedures were approved by the Departmental Ethical Committee of the College of Medicine, University of Lagos. Thirty, 10 - 12 weeks old male Sprague-Dawley rats weighing 180 – 200 g were used for the experiments. The animals were obtained from the Animal House of the College of Medicine, University of Lagos. They were kept in the Animal Room of the Department of Anatomy under standard conditions of temperature (27 - 30°C), with a 12-h light: 12-h dark cycle to acclimatize for two weeks prior to the commencement of the experiment. All animals were allowed unrestricted access to water and commercial rat pellets (Pfizer Feeds PLC, Ikeja) and properly housed in wire mesh cages. The experiment was divided into phases I and II, lasting 4 and 8 weeks respectively. Animals were randomly divided into 6 groups; Groups A, B, and C was for phase I while groups D, E and F was for phase II. Each group comprised of five rats per group. Groups A and D served as control rats and received 1 ml normal saline. Group B animals received 100mg/kg KAFE while group C received 500 mg/kg KAFE daily for 4 weeks. Group E animals were administered KAFE 100 mg/kg daily for 8 weeks. Group F animals were given KAFE 500 mg/kg daily for 8 weeks. All treatment was administered orally through a metal oro-pharyngeal cannula.

Sample collection

At the end of the experiment, animals were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg) (Camara et al., 2008) following which laparotomy was done and the testes were dissected out for further analyses. The caudal epididymes of each animal was minced in a bottle containing 1 ml of Ham's F 10 Stock Solution. Sperm count and motility was carried out after sperm swim up into the solution.

Testicular weight (TW) and testicular volume (TV)

TW was measured by an electronic balance, while the TV was estimated by water displacement. The two testes of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation. Values are expressed in g and cm³ for TW and TV respectively.

Testicular catalase activity

Catalase was assayed colorimetrically at 620 nm and expressed as μmoles of H₂O₂ consumed/min as described by Sinha (1972). The reaction mixture (1.5ml) contained 1.0ml of 0.01M pH 7.0 phosphate buffer, 0.1ml of tissue homogenate and 0.4ml of 2M H₂O₂. The reaction was stopped by the addition of 2.0ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio) (Rukkumani et al., 2004).

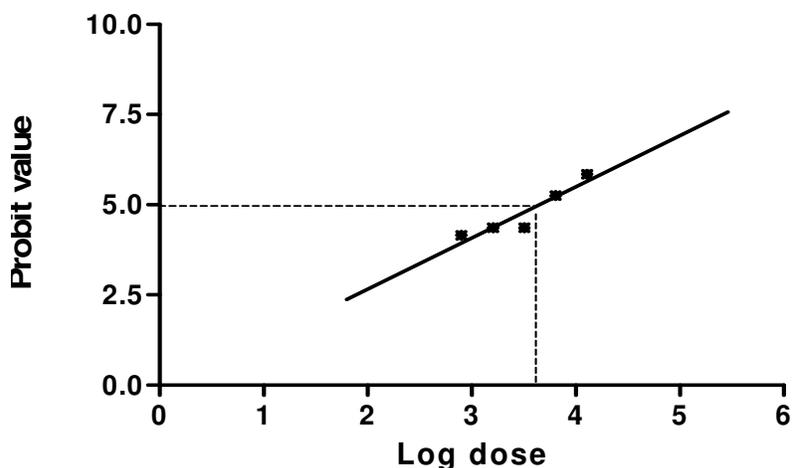


Figure 1. Log dose response curve of the methanolic extract of *Kigelia africana* fruit extract.

Testicular glutathione levels

Reduced glutathione (GSH) was estimated in testicular tissue by the method of Ellman (1959). To the homogenate 10% trichloro acetic acid (TCA) was added and centrifuged. 1.0 ml of supernatant was treated with 0.5 ml of Ellmans reagent (19.8 mg of 5, 5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.1% sodium nitrate) and 3.0 ml of phosphate buffer (0.2 M, pH 8.0). The absorbance was read at 412 nm (Rukkumani et al., 2004).

Testicular Malondialdehyde

Testicular malondialdehyde (MDA) levels were determined using the modified thiobarbituric acid (TBA) method of Buege and Aust (1978). MDA reacts with thiobarbituric acid to give a red compound absorbing at 532 nm. The stock reagent contains 2 ml 15%w/v trichloroacetic acid, 0.375%w/v thiobarbituric acid and 0.25mol/L hydrochloric acid. A 0.5 g testicular tissue sample was homogenized in 5 ml of 0.15 M KCl and the homogenate centrifuged at 1000 g for 10 min in a Uniscope laboratory centrifuge and the supernatant collected. An aliquot of 2 ml of the stock reagent was added to 1 ml of testicular homogenate supernatant and mixed thoroughly and placed in an Equitron water bath (80 - 90°C) for 15 min. It was then cooled and the flocculent precipitate removed by centrifugation at 1000 g for 10 min and the absorbance of the supernatant determined with a spectronic spectrophotometer at 532 nm against blank containing all the reagents. Concentration of malondialdehyde was calculated using the molar absorptivity coefficient of malondialdehyde which is $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Histopathological analysis

The testes were removed and fixed in 10% neutral buffered formalin for at least 24 h. Tissues were processed for microscopic examination using a standard protocol and 3 μm thick paraffin sections were stained with hematoxylin and eosin for view in an Olympus® microscope.

Statistical analysis

Results were expressed as means \pm standard deviation and subjected to statistical analysis using the Student's t-test to comp-

are differences between groups and the control and analysis of variance (ANOVA) with the Turkey's post-hoc test. The significance level considered was $p < 0.05$.

RESULTS

Phytochemical screening

The phytochemical screening done revealed that KAFE contain the following active constituents; alkaloids, flavonoids, tannins, cardiac glycosides, cyanogenic glycosides, anthraquinone glycoside, saponins, antho-cyanosides (anthocyanin pigment) and reducing compounds.

Acute toxicity studies

The LD_{50} was estimated from a log-dose curve to be 3,981.07 mg/kg (Figure 1).

Body weight

Statistically significant increase in the body weight was recorded in groups A ($p < 0.05$), B ($p < 0.01$) and C ($p < 0.001$) respectively. This corresponds to 4.56, 8.28 and 7.44% increase accordingly after 4 weeks. All animals recorded increases in body weight that varied according to the groups. The decreasing orders in % increase are as follows: E>D>F corresponding to 18.56, 14.90 and 12.78%, respectively after 8 weeks (Figure 2).

Testicular weight (TW)

A statistically significant increase ($p < 0.001$) in TW was observed in groups B and E after 4 and 8 weeks compared

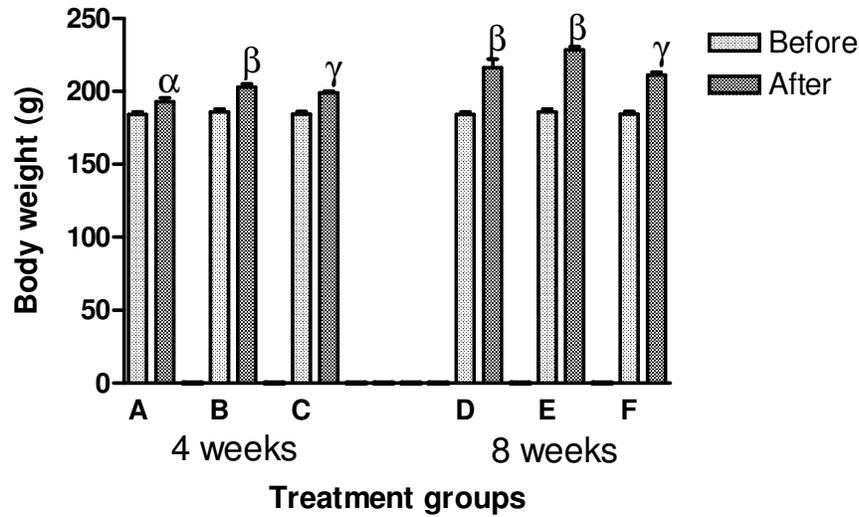


Figure 2. Effect of KAFE on body weight (g) of Sprague-Dawley rats after 4 and 8 weeks Key: α : $p < 0.05$; β : $p < 0.01$; γ : $p < 0.001$.

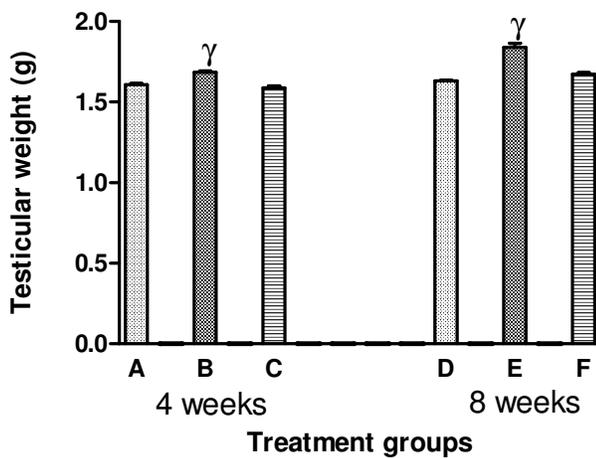


Figure 3. Effect of KAFE on testicular weight (TW) Sprague-Dawley rats after 4 and 8 weeks. Key: γ : $p < 0.001$.

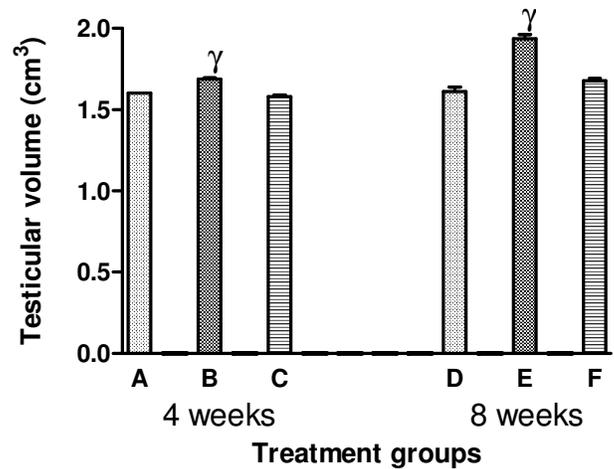


Figure 4. Effect of KAFE on testicular weight (TW) Sprague-Dawley rats after 4 and 8 weeks. Key: γ : $p < 0.001$.

with the control. In Group F, TW was high but not significantly after 8 weeks (Figure 3).

Testicular volume

TV was significantly high in groups B and E ($p < 0.001$) after 4 and 8 weeks respectively. However, group C was high but not significant when compared with the control (Figure 4).

Semen analysis

After 4 weeks, a significant increase ($p < 0.001$) in the sperm count of rats in group B was recorded. Sperm

motility in groups B was high but not statistically significant compared with control. The sperm count in group C was higher than control but this was not statistically significant but there was a significant decline in motility ($p < 0.05$) (Figures 5 and 6). After 8 weeks however, sperm count in group E was high compared with the control. The sperm motility of all groups was above 50%. Though group E was higher than control the results were not statistically significant (Figures 5 and 6).

Testicular glutathione levels

Glutathione levels after 4 weeks was high in B and C but not significant statistically compared with control.

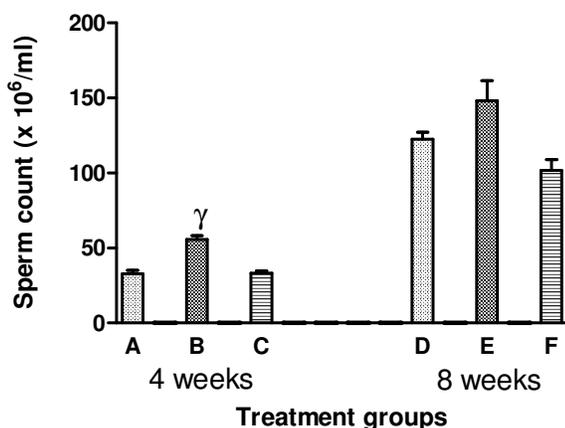


Figure 5. Effect of KAFE on sperm count of Sprague-Dawley rats after 4 and 8 weeks Key: Y: $p < 0.001$.

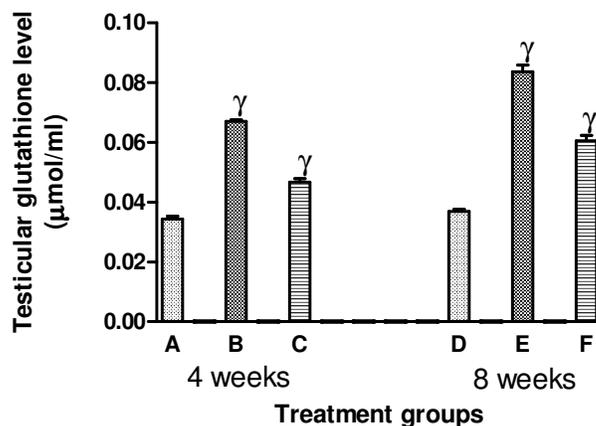


Figure 7. Effect of KAFE on testicular glutathione level in Sprague-Dawley rats after 4 and 8 weeks. Key: Y: $p < 0.001$.

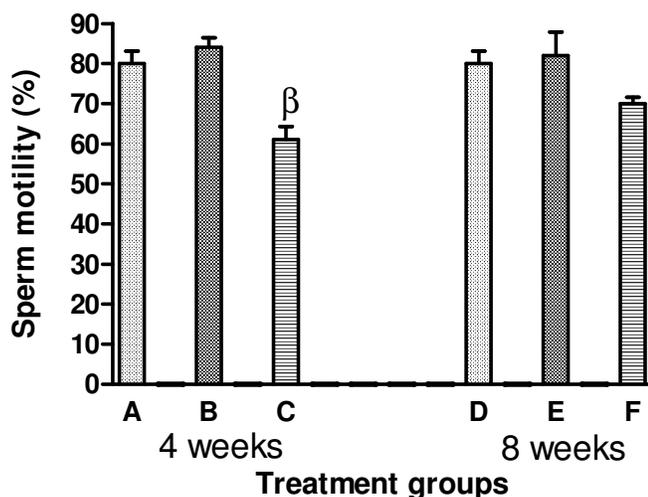


Figure 6. Effect of KAFE on sperm motility of Sprague-Dawley rats after 4 and 8 weeks. Key: β: $p < 0.01$.

Glutathione levels in groups E and F were significantly higher ($p < 0.001$) compared with the control after 8 weeks (Figure 7).

Testicular malondialdehyde levels

The MDA level in groups B and C were significantly lower than controls after 4 weeks likewise groups E and F after 8 weeks ($p < 0.001$) (Figure 8). Of note however is the higher reading for MDA for the 8 week groups as against those of 4 weeks.

Testicular catalase activities

Testicular catalase activity was higher in group B compared to the control but it was not significant.

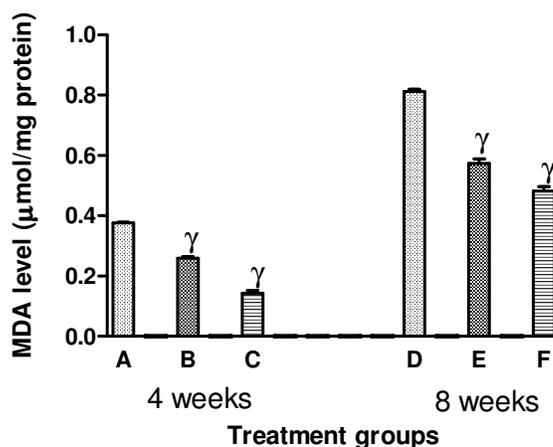


Figure 8. Effect of KAFE on MDA level in Sprague-Dawley rats after 4 and 8 weeks. Key: γ: $p < 0.001$.

Catalase activity was significantly lower in group C after 4 weeks. By 8 weeks, catalase activity was significantly increased in group E compared to control. Group F was significantly lower (Figure 9).

Histological studies

After 4 weeks of treatment, microscopic examination of the testes of control rats (group A) showed no deleterious changes in the testicular capsule. The seminiferous tubules were longitudinal, elongated, regular and uniformly spaced. All cells of the spermatogenic series were present, organised and stratified. The interstitial spaces (containing Leydig cells) were normal. The seminiferous tubules of rats treated with 100 mg/kg KAFE (B) showed increased spermatogenesis with abundance of spermatogonia, spermatids and immotile spermatozoa in the lumen. Histological sections of group C (500 mg/kg

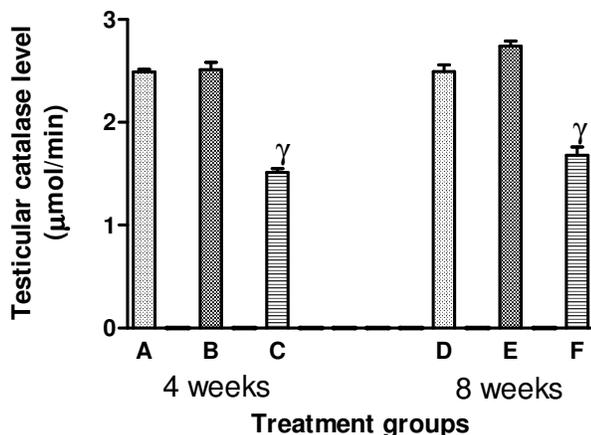


Figure 9. Effect of KAFE on testicular catalase activity (µmol/min) Sprague-Dawley rats after 4 and 8 weeks. Key: γ: $p < 0.001$.

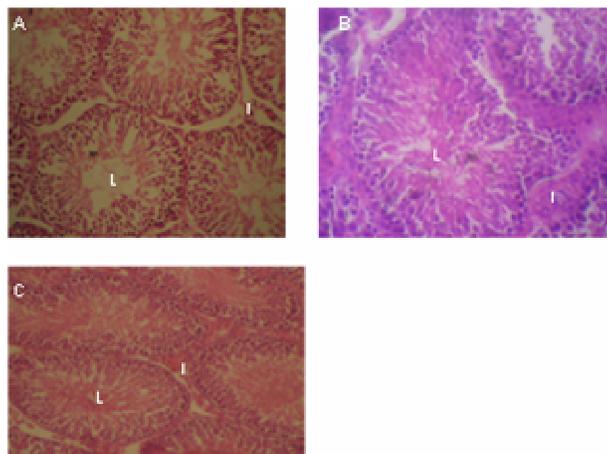


Figure 10. Histological sections of testes of Sprague-Dawley rats after 4 weeks H&E x200; I is interstitium; L is lumen of seminiferous tubule. Sections from treated groups (B & C) are essentially normal as in control (A).

KAFE) showed uniformly spaced and elongated seminiferous tubules with all the cells of the spermatogenic series present but there were reduced spermatids in some of the tubules when compared with the control. There was also stromal vascular congestion (Figure 10).

Histological sections after 8 weeks from control rats (D) showed normal seminiferous tubules that are uniformly spaced and cut transversely, containing all the cells of the spermatogenic series. The interstitium is essentially normal. Cytoarchitecture of testis of group E (KAFE (100 mg/kg) animals' shows qualitative increase in cell layer thickness (suggestive of increased spermatogenesis). Interstitial spaces were devoid of any pathological finding compared to control. Histological sections of rats treated with KAFE (500 mg/kg) (F) showed also normal seminiferous tubular architecture and contents with few

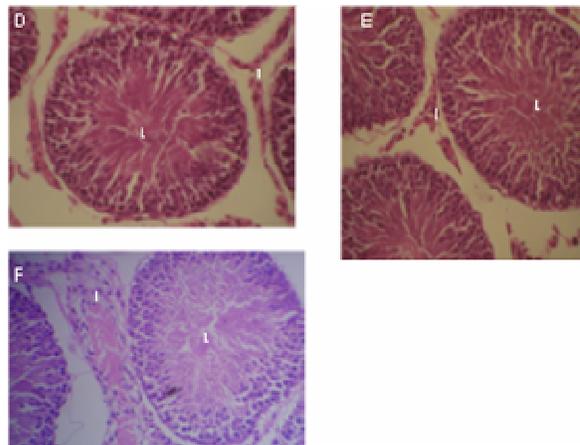


Figure 11. Histological sections of testes of Sprague-Dawley rats after 8 weeks. H&E X400, I is interstitium; L is lumen of seminiferous tubule. Sections from control (D) and E (KAFE 100 mg/kg) are normal while sections from F (KAFE 500 mg/kg) shows widened interstitial spaces.

focal degenerative changes in some seminiferous tubules when compared with the control. The interstitial spaces were normal or slightly increased (Figure 11).

DISCUSSION

Testicular oxidative stress (TOS) is known to play a role in a number of conditions detrimental to male fertility. Indices for derangements in oxidant status of the animals that were assayed in the various experimental groups shed more light on possible role(s) of KAFE. Induced peroxidation in testicular tissues was assessed by determining the production of thiobarbituric acid reactive substances (TBARS), mostly malondialdehyde. Testicular MDA levels was lower than controls in the short term group and significantly reduced ($p < 0.001$) in animals treated with KAFE in long term group.

It is known that the lipid composition of the sperm membrane exert a significant effect upon the functional quality of spermatozoa (Zalata et al., 1998). Data on sperm count/motility from our results showed a corresponding increase of both parameters in group B (KAFE 100 mg/kg) at both short and long term durations. The higher dose of KAFE was not as effective as the lower dose on the parameters. The reason(s) for the higher levels of MDA in this experiment are not completely understood, but may be suggestive that KAFE may not be as effective in higher doses and at chronic administration. Also, the significant increase in absolute TW/TV by KAFE in the absence of any pathology suggests increased androgen biosynthesis especially when viewed alongside the significant increase in body weight. It is equally possible that this may be due to increased sperm content in the testes all pointing towards a favourable balance between oxidant-antioxidant

equilibrium.

The fertilising function of spermatozoa could explain why this cell is provided with a fragile but very active membrane that can be destabilised and activated (Lenzi et al., 2000). PUFA are in fact one of the main targets of free radical damage and an inverse relationship between lipid peroxides and sperm mobility has been established (Aitken, 1991) *in vivo* and *in vitro*.

Testicular catalase activities greatly mirrored the antioxidant enzyme system in the animals. Catalase and superoxide dismutase are the main antioxidant enzymes in seminal plasma that prevent increases in reactive oxygen species concentration thus protecting the sperm cells against damage (lipid peroxidation) (Kawakami et al., 2007). Sperm plasma membranes have high content of polyunsaturated fatty acids (PUFA) thereby making them sensitive to damage by free radicals resulting in loss of membrane integrity (Suzuki and Sofikitis, 1999). Low catalase activities closely relates to low motility of ejaculated spermatozoa (Kawakami et al., 2007) and this can be gleaned from the results on sperm count/motility vis-à-vis testicular catalase which indicate that the later was enhanced by KAFE 100 mg/kg at both short and long term durations.

The high concentration of PUFA in sperm cells with the relative paucity of scavenger mechanism and the virtual absence of cytoplasm in comparison with other cells is however compensated by the powerful antioxidant system in seminal plasma. Seminal plasma is known for abundance of catalase, superoxide dismutase and glutathione peroxidase activities in addition to high concentration of antioxidants such as ascorbic acid and glutathione (Daunter et al., 1981). In this experiment however, a higher dose of 500 mg/kg KAFE was not as effective as the lower dose of 100 mg/kg. This adjudged with the similar observation on MDA levels imply that higher doses of KAFE may be detrimental to antioxidant enzyme activities in the testis but may be effective in selectively increasing endogenous antioxidants (like glutathione).

Glutathione (GSH) is an important cellular reductant and the most abundant intracellular thiol involved in the protection against free radicals, peroxides and other toxic components. In addition to serving as a substrate for glutathione related enzymes, glutathione acts as a free radical scavenger, a generator of α -tocopherol and plays an important role in the maintenance of protein sulphhydryl groups (Rudin et al., 2003; Rukkumani et al., 2004). It is the sulphhydryl group of GSH that is essential for its antioxidant activity against reactive oxygen species in cells (Durgo et al., 2007). KAFE at both doses induced an up-regulation in GSH levels especially at the long term duration and this may be attributed to its inherent flavonoid content. There is supporting evidence that some flavonoids can elevate intracellular basal level of GSH thereby allowing better tolerance of free radicals (Durgo et al., 2007). Flavonoid activities depend heavily on their antioxidant and chelating properties.

Bearing in mind that sperm morphology, count and motility are highly associated with the production and activity of free radicals and antioxidant enzymes (Krishnamoorthy et al., 2007), we are likely to agree that the ability of KAFE to up-regulate GSH, lower MDA levels and enhance testicular catalase activities does confer a strong antioxidative role to its components. However, the lower dose of 100 mg/kg was more effective than the higher dose of 500 mg/kg and the reason for this is not known. This may suggest that KAFE does not have a dose-dependent effect on the various parameters examined.

In conclusion, this work provides a rationale for the use of this plant in folk medicine for treatment of inflammatory disorders and male infertility where oxidative stress plays very crucial roles. However, further studies is needed to elucidate the precise mechanism responsible for the differential effects of higher dose of KAFE on lipid peroxidation marker (MDA) and catalase activity especially at the long term. This would shed light on its application as adjunct/or prophylactic treatment in these conditions.

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