Original Research Article

Ameliorative potentials of *Annona muricata* (Linn) on Sodium Fluoride-induced Toxicity on Haematology indices and Fecundity of Adult Male Wistar Rats

Abstract

Aim: Ameliorative potentials of *Annona muricata* (Linn) on Sodium fluoride-induced toxicity on haematology indices and fecundity of adult male Wistar rats. **Methods**: Eighty-five (85) adult male Wistar rats were divided into 17 groups of 5 rats each. NaF (10 mg/kg) + fruit juice, ethanol stem bark and leaf extracts of *A. muricata* at five different doses of 500, 1000, 1500, 2000 and 2500 mg/kg body weight were administered to the rats for 6 weeks. Blood samples were taken after 6 weeks through ocular puncture and the sera were used for testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH) Triiodothyronine (T3), Thyroxine (T4), and Thyroid Stimulating Hormone (TSH) tests, while whole blood was used for haematological parameters such as haemoglobin (Hb), packed cell volume (PCV), total white blood cell, platelets count, lymphocytes and neutrophils. The testes and epididymis of the rats were harvested for histological studies and sperm analysis such as sperm motility, viability, count and sperm head abnormality. **Results:** Administration of NaF + fruit juice, NaF + Stem bark and NaF + leaf extracts caused an increase ($p<0.05$) in epididymal sperm count, sperm motility and live spermatozoa along with simultaneous decrease in dead spermatozoa as compared to the rats of group treated with NaF alone. Result also showed a non-significant increase in haemoglobin, platelet count, lymphocyte count and decrease in neutrophil count, total white blood cell count except for the group treated with NaF and leaf extract that showed a non-significant increase, while treatment with stem bark and leaf extracts exhibited a varied effect on the packed cell volume. Histological examination showed that NaF treatment brought about severe testicular damage while treatment with the extracts ameliorated this effect. **Conclusion**: *A. muricata* fruit juice and extracts were found to increase testosterone concentration, thus validating its ameliorative potential in NaF-induced toxicity.

Keywords: Sodium fluoride, *Annona muricata* (Linn), fecundity, haematology, hormones, histology.

Introduction

Several clinical investigations and animal experiments suggest that fluoride has adverse impacts on male reproductive function producing structural and functional defects in spermatozoa, a decrease in sperm count, disturbances in the levels of reproductive hormones and reduced fertility [1, 2]. Spermatozoa undergo various processes to ultimately fertilize an oocyte, including spermatogenesis, capacitation, and the acrosome reaction. Fluoride has been shown to impair all three of these processes [3]. *In vitro* fluoride exposure at high concentrations affected certain signal pathways, such as inhibition of the cell cycle, apoptosis and proliferation [4]. Thyroid hormone disruption caused by fluoride results in abnormal function and development of testes, lowering libido, reducing sex hormones, interferes directly and indirectly with spermatogenesis, influencing steroid hormone receptors, inducing oxidative stress in testes. However, the most important mechanism by which fluoride reduces the level of testosterone is interference with steroidogenesis in the

Leydig cells. This interference has been demonstrated in several studies in which activity levels of testicular steroidogenic marker enzymes 3β-hydroxysteroid dehydrogenase (3β HSD) and 17β-hydroxysteroid dehydrogenase (17-HSD) decreased significantly in NaF-treated rats [5].

Annona muricata fruit juice has been shown to possess antibacterial, antifungal, anticancerous, antimalarial, antidiabetic, hepatoprotective, anti-inflammatory, hypotensive and immune enhancing effect [6]. Phytochemical screening of *A. muricata* leaf ethanolic extract shows the presence of saponins, triterpenoids, flavonoids, tannins, alkaloids, and cardiac glycosides [7]. *A. muricata* leaf extract is believed to stabilize blood sugar level in a normal range that is very useful for diabetic management [8]. Several types of research have shown that *A. muricata* leaf has hypoglycemic activity and revealed regeneration of pancreatic islet [9, 10, 11]. The ethanol leaf extract of *A. muricata* also is known to reduce serum uric acid level [12], contain essential oils with parasiticidal, antibacterial, antidiarrheal, rheumatological and antineuralgic properties [13, 14, 15]. The extract from *A. muricata* induced necrosis of pancreatic cancer cells by inhibiting cellular metabolism [16]. *A. muricata* leaf extract may possess anticancer properties by enhancing caspase-3 activity which is a pro-apoptosis marker [17]. The use of different parts of *A. muricata* for the treatment of these pathological disorders suggests it may possess anti-toxic properties and stimulated our interest to study its ameliorative effect on NaF- induced toxicity on haematology indices and fecundity of adult male Wistar rats.

Materials and Method

2.1 Animals

Male adult albino rats (150-250 g) were obtained from the animal house of College of Medicine, University of Nigeria, Enugu Campus. The animals were housed in steel cages within the Laboratory Animals Facility of Brain-Phosphorylationship Scientific Solution Services, No9. Ogui Road Enugu, Enugu State, 5th Floor, Right Wing, maintained and given standard feed and clean drinking water *ad libitum*. They were allowed to acclimatize for a period of four weeks before use. All animal experiments were in compliance with the National Institute of Health Guide for care and use of laboratory animal.

Collection and Extraction of Plant Materials and Fruit Juice.

Fresh stem bark, leaf and fruits of *Annona muricata* were collected from Abua, Rivers State, in March, 2017. The stem bark and the leaf were cut to pieces, dried under room temperature, ground and pulverized to coarse powder using a Hammer mill (Gallenkamp, U.S.A.). The plant materials were identified and authenticated by Mr. Alfred Ozioko of International Centre for Ethnomedicines and Drug Development Nsukka, Nigeria and deposited in herbarium with Voucher Number: Intercedd/16091. Known quantities (1.851kg) of the dried stem bark powder and 1.016 kg of the dried leaf powder were extracted with analytical grade ethanol using maceration method for 48 hours. The mixture was vacuum-filtered through Whatman No 1 filter paper and concentrated using a vacuum rotary evaporator (Eyla N-1000, Japan) to afford 97.352 g (5.257 % w/w) for stem bark extract and 126.312 g (12.432 % w/w) for leaf extract. The extractive yield was calculated using the relation: Yield (%) = [Weight of extract (g)/Weight of plant material (g)]*100.The fruit juices were used raw without concentrating it. The epicarps and the seeds of the ripen fruits were removed with hand and the mesocarps were sliced with knife into small sizes and ground with and an electric grinder into paste form. This was further sieved with muslin cloth to

remove the fibres. The filtrate was transferred into clean glass container, sealed and preserved in refrigerator at -10ºC until use.

Experimental Design

Eighty five sexually matured male adult albino rats (150-250 g) were divided into 17 groups of 5 rats each, according to their average weight, and received daily oral dose of the treatment as follows:

Group 1: Normal feed and water (positive control)

Group 2: NaF (10mg/kg) (negative control)

Group 3: NaF (10mg/kg) + Fruit Juice Extract (500mg/kg)

Group 4: NaF (10mg/kg) + Fruit Juice Extract (1000mg/kg)

Group 5: NaF (10mg/kg) + Fruit Juice Extract (1500mg/kg)

Group 6: NaF (10mg/kg) + Fruit Juice Extract (2000mg/kg)

Group 7: NaF (10mg/kg) + Fruit Juice Extract (2500mg/kg)

Group 8: NaF (10mg/kg) + Leaf Extract (500mg/kg)

Group 9: NaF (10mg/kg) + Leaf Extract (1000mg/kg)

Group 10: NaF (10mg/kg) + Leaf Extract (1500mg/kg)

Group 11: NaF (10mg/kg) + Leaf Extract (2000mg/kg)

Group 12: NaF (10mg/kg) + Leaf Extract (2500mg/kg)

Group 13: NaF (10mg/kg) + Stem Bark Extract (500mg/kg)

Group 14: NaF (10mg/kg) + Stem Bark Extract (1000mg/kg)

Group 15: NaF (10mg/kg) + Stem Bark Extract (1500mg/kg)

Group 16: NaF (10mg/kg) + Stem Bark Extract (2000mg/kg)

Group 17: NaF (10mg/kg) + Stem Bark Extract (2500mg/kg)

Blood was taken after the 6^{th} week of administration through ocular puncture. Two ml of the blood samples from each group (n=4) were collected in test tubes and put into centrifuge tubes, spun at 3000 rpm for 10 min and the serum collected for hormonal assays which include: testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH), Triiodothyronine (T3), Thyroxine (T4) hormone and Thyroid stimulating hormone. Whole bloods (2 ml) for haematological studies were placed in EDTA tubes and assayed for full blood count. The rats were sacrificed under chloroform anaesthesia after collection of blood samples. The testes and epididymis, were dissected out and rapidly fixed in buffered neutral formalin (10%) for histological studies. The epididymis were processed for epididymal sperm motility, viability, count and sperm head abnormality.

Histopathological examination

The tissues were subjected to standard routine histological procedures [18]. The slides were viewed using the light microscope and histopathological changes were observed and recorded at x400 magnification identifying both the normal and atrophied seminiferous tubules and spermatocytes.

2.3.2 Haematological studies

Determination of haematological parameters

Determination of haematological parameters such as haemoglobin concentration (Hb), packed cell volume (PCV), total white blood cell count (TWBC), platelet count, neutrophils and lymphocytes) were done using standard operative procedures[19].

Hormonal Assay

Plasma Testosterone, Follicle-stimulating and Luteinizing hormones were determined by fluorescence immunoassay (FIA) methods with commercial kits (Boditech Med Incorporated, Republic of Korea), using the ichroma machine (Boditech: BOD13303, Korea).

Sperm Analyses

Semen pH and sperm motility

Immediately after dissection, a puncture was made in the epididymis with a sterile pin. The semen smeared on the pin was rubbed on a pH paper of range 1.0-10.0. The colour change corresponds to the pH and was read from the paper. The dissected epididymis was measured and sliced into small pieces with a sterilized surgical blade and finally introduced into a beaker. The epididymal sperm samples were obtained by macerating known weight (100 mg) of cauda epididymis in physiological saline in the ratio of 1:10 weight by volume. After vigorous shaking, two drops of sperm suspension was put on a microscope slide and coverslip was placed. The numbers of progressively motile sperm cells were counted under ×40 lenses.

$$\textbf{\% Motility} = \frac{\text{No of motile spermatozoa}}{\text{Total no of spermatozoa counted}} \times 100$$

Percentage dead sperm cells

The percentage dead sperm cells was determined using “Eosin-Nigrosin one-step staining technique” [20]. A portion of the sperm suspension was mixed with equal volume of Eosin-Nigrosin stain and two (2) air-dried smears were prepared on glass slides for each sample. Dead sperm cells took up stain and appeared pinkish. Percentage dead sperm cells were calculated based on the number of dead sperm cells out of the total number of sperm cells observed.

Sperm viability

The sperm viability test was determined using “Eosin-Nigrosin one-step staining technique” [20]. A portion of the sperm suspension was mixed with equal volume of Eosin-Nigrosin stain and two (2) air-dried smears were prepared on glass slides for each sample. The slides were examined for percentage viability. Normal live sperm cells excluded the stain and appeared whitish, whereas dead sperm cells took up stain and appeared pinkish. Percentage viability was calculated based on the number of live sperm cells out of the total number of sperm cells counted.

$$\textbf{Sperm viability count} = \frac{\text{Life cell/viable cell}}{\text{Total cells (both dead \& alife)}} \times \frac{100}{1}$$

Sperm count

The dissected epididymis was measured and sliced into small pieces with a sterilized surgical blade and finally introduced into a beaker. The epididymal sperm samples were obtained by macerating this known weight of cauda epididymis in physiological saline in the ratio of 1:10 weight by volume. After vigorous shaking, two drops of sperm suspension was put on a microscope slide and cover slip was placed. Epididymal sperm count was obtained by cytometry using the improved Neubauer cytometer and was expressed as million/ml of suspension [21].

Sperm head abnormality test

A known volume of the sperm suspension was mixed with 1% eosin solution (10:1) for 30 min and air-dried smears were prepared on glass slides for each sample. The percentage of sperm head abnormality was calculated accordingly [21].

Statistical analysis

The data were analysed by (SPSS version 17.5, SPSS Inc.). Significant differences between means were determined by One-way ANOVA and regarded significant at $p < 0.05$. Results were presented as Mean ± Standard Deviation

Results

Haemoglobin Concentration, Packed Cell Volume and Platelets Count

Effects of fruit juice, ethanol extracts of stem bark and leaf of *A. muricata* on haemoglobin concentration of NaF-induced toxicity on fecundity profile of adult male Wistar rats is shown in Table 3.1. It was observed that NaF at a dose of 10 mg/kg caused a significant decrease ($p<0.05$) in Hb concentration, percentage PCV and platelets count in the adult male rats when compared with the control. However, treatment with doses above 500 mg/kg body weight of NaF + fruit juice, NaF + stem bark and leaf extracts produced significant increases in Hb concentration and percentage PCV values when compared with group 2 rats. Similarly, groups treated with NaF + 2000 mg/kg of fruit juice and NaF + 1000 mg/kg of stem bark and leaf extracts, showed significant increase ($p<0.05$) in platelet count in comparison with both the group treated with NaF alone and control group.

Table 3.1: Haemoglobin concentration, percentage packed cell volume and platelet count of control and test groups of NaF-induced toxicity in rats after 6 weeks of treatment.

Group	Hb Concentration (g/dl)	PCV (%)	Platelet count ($X10^3mm^3$)
Control	9.13±0.42	25.67±3.79	118.000±700
NaF	5.87±0.29$^{\alpha}$	18.33±0.58$^{\alpha}$	112.000±265
Stem bark extract			
10mg/kg NaF + 500	6.87±0.23	17.47±13.05$^{\alpha}$	96.000±259$^{\alpha}$
10mg/kg NaF + 1000	9.00±0.00$^{\beta}$	24.00±3.00$^{\beta}$	145.500±550$^{\alpha\beta}$
10mg/kg NaF +1500	8.27±1.33$^{\beta}$	25.67±2.08$^{\beta}$	128.333±288
10mg/kg NaF + 2000	8.20±3.20$^{\beta}$	24.00±1.00$^{\beta}$	131.333±321$^{\alpha\beta}$
10mg/kg NaF + 2500	8.87±1.50$^{\beta}$	24.33±4.93$^{\beta}$	140.333±416$^{\alpha\beta}$

Leaf extract			
10mg/kg NaF + 500	7.87±1.16	21.00±1.00	143.000±608$^{\alpha\beta}$
10mg/kg NaF + 1000	9.00±0.00$^{\beta}$	26.33±0.58$^{\beta}$	138.333±104$^{\beta}$
10mg/kg NaF +1500	7.93±0.91$^{\beta}$	23.33±3.51$^{\beta}$	126.000±105$^{\beta}$
10mg/kg NaF + 2000	7.80±1.14	23.67±3.06$^{\beta}$	128.333±189$^{\beta}$
10mg/kg NaF + 2500	7.65±0.35	23.00±1.00	132.500±175$^{\alpha\beta}$
Fruit Juice			
10mg/kg NaF + 500	9.25±0.75$^{\beta}$	28.50±1.50$^{\beta}$	129.500±500
10mg/kg NaF + 1000	8.23±1.63$^{\beta}$	24.33±4.51$^{\beta}$	137.000±192$^{\beta}$
10mg/kg NaF +1500	8.73±0.55$^{\beta}$	26.67±1.53$^{\beta}$	128.333±104$^{\beta}$
10mg/kg NaF + 2000	8.70±0.36$^{\beta}$	25.00±1.00$^{\beta}$	147.333±643$^{\alpha\beta}$
10mg/kg NaF + 2500	9.10±0.10$^{\beta}$	27.00±0.00$^{\beta}$	140.000±100$^{\alpha\beta}$

Results are expressed as Mean±SD; n=4

The mean values with **β** as superscripts across the column compared with group treated with NaF alone are considered significant ($p<0.05$). The mean values with **α** as superscripts across the column compared with control group fed with water and feed only are considered significant ($p<0.05$).

Total White Blood Cell, Neutrophil and Lymphocyte Count

Table 3.2 shows the results of the effect of fruit juice, ethanol extracts of stem bark and leaf of *A. muricata* on total white blood cell of NaF-induced toxicity on fertility profile of adult male rats. There was no significant difference in total white blood cell, Neutrophil and lymphocyte count in the group treated with 10 mg/kg of NaF when compared with the control group fed with water and rat feed only. Similar, there was no significance difference in the total white blood cell, Neutrophil and lymphocyte count in the groups treated with different concentrations of the extracts when compared with the controls.

Table 3.2: Total White Blood Cell, Neutrophil and Lymphocyte Count of control and test groups of NaF- induced toxicity in rats after 6 weeks of treatment

Group	WBC (x10^3 mm^3)	Neutrophil (%)	Lymphocyte (%)
Control	9.333±1154.70	44.67±4.51	55.33±4.51
NaF	10.200±721.11	51.43±7.51	49.57±7.51
Stem bark extract			
10mg/kg NaF + 500	09.467±838.65	44.00±5.20	55.67±4.93
10mg/kg NaF + 1000	07.000±500.00	41.00±1.00	58.50±1.50
10mg/kg NaF +1500	08.667±1755.94	37.67±2.08	61.00±1.00
10mg/kg NaF + 2000	08.800±1212.44	43.67±5.51	56.33±5.51

10mg/kg NaF + 2500	08.500±1322.88	36.00±3.31	64.00±4.00
Leaf extract			
10mg/kg NaF + 500	10.100±1000.00	39.67±17.62	58.00±14.00
10mg/kg NaF + 1000	11.333±577.35	39.00±1.00	61.00±1.00
10mg/kg NaF +1500	11.000±1000.00	37.67±2.52	62.00±2.65
10mg/kg NaF + 2000	10.00±105.57	53.33±14.15	46.00±13.86
10mg/kg NaF + 2500	53.50±425.00$^{\alpha\beta}$	39.00±0.00	60.50±0.50
Fruit Juice			
10mg/kg NaF + 500	9.20±220.00	47.50±2.50	54.50±4.50
10mg/kg NaF + 1000	10.93±105.40	47.33±15.31	49.33±13.58
10mg/kg NaF +1500	6.57±202.73	46.00±5.29	54.00±5.29
10mg/kg NaF + 2000	9.73±230.94	37.00±2.65	62.67±2.52
10mg/kg NaF + 2500	05.900±3500.00	42.50±1.50	67.00±1.00

Results are expressed as Mean±SD; n=4

The mean values with **β** as superscripts across the column compared with group treated with NaF alone are considered significant ($p<0.05$). The mean values with **α** as superscripts across the column compared with

Testosterone, FSH and LH Concentration

Effects of fruit juice, ethanol extracts of stem bark and leaf of *A. muricata* on Testosterone FSH and LH Concentration, of NaF-induced toxicity on fecundity profile of adult male Wistar rats shows that at a dose of 10 mg/kg, NaF caused a non-significant decrease ($p>0.05$) in the serum testosterone and LH concentration, and a significant decrease ($p<0.05$) in the FSH when compared with normal control group fed with water and feed only (Table 3.3). Groups treated with NaF + fruit juice, NaF + 2000 and 2500 mg/kg stem bark and Leaf extracts showed significant increase ($p<0.05$) in serum testosterone concentrations when compared with both group treated with NaF alone and control group. Groups treated with NaF + fruit juice, NaF + Leaf extracts showed significant decrease in serum FSH concentration, when compared with group tested with NaF alone. However, only the group treated with NaF + 500 mg/kg stem bark extract showed a significant increase ($p<0.05$) in serum FSH concentration, when compared with group treated with NaF alone. On the other hand, groups treated with NaF + 1000 and 2500 mg/kg fruit juice and NaF + 500 and 1000 mg/kg leaf extracts exhibited significant decrease ($p<0.05$) in serum LH concentration when compared with the control group.

Table 3.3: Testosterone, Follicle stimulating hormone (FSH) and Luteinizing hormone concentration of control and test groups of NaF-induced toxicity in rats after 6 weeks of treatment

Group	Testosterone (ng/ml)	FSH (Mlμ/l)	LH (Mlμ/l)
Control	0.827±0.06	3.54±2.62	1.93±0.05
NaF	0.740±0.06	2.83±0.79$^{\alpha}$	1.63±0.62
Stem bark extract			
10mg/kg NaF + 500	0.760±0.01	5.00±1.00$^{\beta}$	1.63±0.08
10mg/kg NaF + 1000	0.920±0.11$^{\beta}$	2.50±2.33	1.84±0.50
10mg/kg NaF +1500	0.833±0.22	1.87±0.25	1.57±0.33
10mg/kg NaF + 2000	2.093±0.37$^{\alpha\beta}$	1.61±1.12$^{\alpha}$	1.42±0.55
10mg/kg NaF + 2500	1.637±0.65$^{\alpha\beta}$	1.52±0.50$^{\alpha}$	1.56±0.65
Leaf extract			
10mg/kg NaF + 500	0.633±0.13	1.05±0.03$^{\alpha}$	0.80±0.03$^{\alpha\beta}$
10mg/kg NaF + 1000	0.817±0.03	1.05±0.13$^{\alpha}$	1.04±0.22$^{\alpha}$
10mg/kg NaF +1500	0.863±0.22	1.20±0.21$^{\alpha}$	1.13±0.06
10mg/kg NaF + 2000	0.953±0.13$^{\alpha\beta}$	1.93±0.29	1.63±0.46
10mg/kg NaF + 2500	1.850±0.84$^{\alpha\beta}$	1.33±0.50$^{\alpha}$	1.85±0.11
Fruit Juice			
10mg/kg NaF + 500	1.653±0.70$^{\alpha\beta}$	1.41±0.42$^{\alpha}$	1.45±0.84
10mg/kg NaF + 1000	1.900±0.10$^{\alpha\beta}$	1.38±0.50$^{\alpha}$	0.83±0.24$^{\alpha}$
10mg/kg NaF +1500	1.213±0.27$^{\alpha\beta}$	1.83±0.16	1.96±0.01$^{\beta}$
10mg/kg NaF + 2000	2.403±0.57$^{\alpha\beta}$	0.97±0.03$^{\alpha}$	1.34±0.58
10mg/kg NaF + 2500	1.653±0.22$^{\alpha\beta}$	1.66±0.13$^{\alpha}$	0.84±0.05$^{\alpha}$

Results are expressed as Mean±SD; n=4

The mean values with **β** as superscripts across the column compared with group treated with NaF alone are considered significant ($p<0.05$). The mean values with **α** as superscripts across the column compared with control group fed with water and feed only are considered significant ($p<0.05$).

Thyroid Hormones

Results in table 3.4 showed the ameliorative potential of fruit juice, ethanol extracts of stem bark and leaf of *A. muricata* on thyroid hormone concentration of NaF-induced toxicity on fertility profile of adult male Wistar rats. At a dose of 10 mg/kg, NaF, caused a non-significant ($p>0.05$) increase in T_3, non-significantly reduction ($p>0.05$) in T_4 and a non-significant increase ($p>0.05$) in TSH concentration respectively, when compared with normal control group. However, concomitant administration of NaF+2000 mg/kg of fruit juice, NaF+2000 mg/kg of stem bark and NaF+2500 mg/kg of leaf extracts exhibited significant increase ($p<0.05$) in serum thyroxine concentration when compared with group treated with NaF

alone and the control group. Similarly, groups treated with NaF + 2500 mg/kg fruit juice, NaF + 2500g/kg stem bark and NaF +500 - 2500 mg/kg leaf extracts exhibited significant increases ($p<0.05$) in TSH concentration when compared with both the group treated with NaF alone and the normal control group fed with water and feed only.

Table 3.4: Triiodothyronine (T_3), Thyroxine (T_4), and Thyroid Stimulating Hormone (TSH) concentration of control and test groups of NaF-induced toxicity in rats after 6 weeks of treatment

Group	**T_3** (ng/ml)	**T_4** (MIμ/l)	**TSH** (MIμ/l)
Control	0.83±0.06	4.23±0.35	0.89±0.70
NaF	0.74±0.06	4.13±0.48	0.93±0.10
Stem bark extract			
10mg/kg NaF + 500	0.43±0.03	3.60±0.10	1.16±0.15
10mg/kg NaF + 1000	0.33±0.02	5.63±1.13	1.23±0.03
10mg/kg NaF +1500	0.36±0.05	4.53±1.70	1.03±0.16
10mg/kg NaF + 2000	0.30±0.08	6.17±1.04$^{\alpha\beta}$	1.67±0.29$^{\beta}$
10mg/kg NaF + 2500	0.37±0.15	4.70±0.26	2.33±0.49$^{\alpha\beta}$
Leaf extract			
10mg/kg NaF + 500	0.26±0.06	2.60±0.10	2.15±0.15$^{\alpha\beta}$
10mg/kg NaF + 1000	0.40±0.05	3.02±0.03	1.55±0.35$^{\beta}$
10mg/kg NaF +1500	1.21±0.27	3.15±0.05	1.67±0.02$^{\beta}$
10mg/kg NaF + 2000	2.40±0.57	3.45±0.05	2.05±0.05$^{\alpha\beta}$
10mg/kg NaF + 2500	1.65±0.22	6.16±5.67$^{\alpha\beta}$	1.86±0.06$^{\alpha\beta}$
Fruit Juice			
10mg/kg NaF + 500	0.44±0.06	5.50±0.61	0.87±0.08
10mg/kg NaF + 1000	0.25±0.05	5.05±0.95	0.84±0.01
10mg/kg NaF +1500	0.42±0.08	4.30±0.30	0.85±0.01
10mg/kg NaF + 2000	0.46±0.31	8.87±3.63$^{\alpha\beta}$	0.97±0.11
10mg/kg NaF + 2500	0.45±0.31	4.10±0.10	1.96±0.06$^{\alpha\beta}$

Results are expressed as Mean±SD; n=4

The mean values with **β** as superscripts across the column compared with group treated with NaF alone are considered significant ($p<0.05$). The mean values with **α** as superscripts across the column compared with control group fed with water and feed only are considered significant ($p<0.05$)

Sperm Count and Sperm Motility

Results obtained on the fecundity profile of adult male Wistar rats of NaF-induced toxicity (Table 3.5), showed that administration of 10 mg/kg NaF caused a concentration dependent and statistically significant ($p < 0.05$) reduction in sperm count in rats in comparison with

control group. However, all the groups treated with NaF + fruit juice, NaF + stem bark and NaF + leaf extracts showed significant increase ($p<0.05$) in sperm count except the group treated with NaF + 500 mg/kg of leaf extract. Similarly, result for sperm motility showed that NaF at a dose of 10mg/kg caused significant decrease ($p<0.05$) in sperm motility in comparison with the control groups. Fruit juice at higher doses of 1500 - 2500 mg/kg, NaF + 2500 mg/kg of stem bark and NaF + 500 - 2500 mg/kg of leaf extracts caused a significant increase ($p<0.05$) in sperm motility when compared with the NaF treated group.

Table 3.5: Sperm count and Sperm motility of control and test groups of NaF-induced toxicity in rats after 6 weeks of treatment.

Group	Sperm count (x 10^6/ml)	Sperm motility (%)
Control	$960.03\pm5.00^{\beta}$	$94.00\pm4.00^{\beta}$
NaF	$207.04\pm2.00^{\alpha}$	$30.00\pm1.00^{\alpha}$
Stem bark extract		
10mg/kg NaF + 500	$240.00\pm2.00^{\alpha\beta}$	$31.00\pm1.00^{\alpha}$
10mg/kg NaF + 1000	$464.00\pm2.65^{\alpha\beta}$	$32.00\pm3.00^{\alpha}$
10mg/kg NaF +1500	$417.00\pm7.00^{\alpha\beta}$	$34.00\pm4.00^{\alpha}$
10mg/kg NaF + 2000	$592.00\pm2.00^{\alpha\beta}$	$35.33\pm3.79^{\alpha}$
10mg/kg NaF + 2500	$570.00\pm8.00^{\alpha\beta}$	$38.00\pm4.90^{\alpha\beta}$
Leaf extract		
10mg/kg NaF + 500	$126.00\pm2.00^{\alpha\beta}$	$20.00\pm1.00^{\alpha\beta}$
10mg/kg NaF + 1000	$241.00\pm1.00^{\alpha\beta}$	$26.00\pm5.00^{\alpha}$
10mg/kg NaF +1500	$211.34\pm1.34^{\alpha\beta}$	$25.00\pm2.00^{\alpha}$
10mg/kg NaF + 2000	$502.00\pm2.00^{\alpha\beta}$	$30.00\pm2.00^{\alpha}$
10mg/kg NaF + 2500	$569.00\pm4.00^{\alpha\beta}$	$31.00\pm4.00^{\alpha}$
Fruit Juice		
10mg/kg NaF + 500	$341.03\pm1.04^{\alpha\beta}$	$31.00\pm2.00^{\alpha}$
10mg/kg NaF + 1000	$634.21\pm4.21^{\alpha\beta}$	$37.33\pm6.51^{\alpha}$
10mg/kg NaF +1500	$450.00\pm5.00^{\alpha\beta}$	$40.00\pm4.00^{\alpha\beta}$
10mg/kg NaF + 2000	$694.51\pm4.51^{\alpha\beta}$	$57.00\pm3.00^{\alpha\beta}$
10mg/kg NaF + 2500	$651.52\pm1.51^{\alpha\beta}$	$41.00\pm2.00^{\alpha\beta}$

Results are expressed as Mean±SD; n=4

The mean values with **β** as superscripts across the column compared with group treated with NaF alone are considered significant ($p<0.05$). The mean values with **α** as superscripts across the column compared with control group fed with water and feed only are considered significant ($p<0.05$)

Live/viable Sperm Cells and Percentage Dead Sperm Cells

Results of live/viable sperm cells of NaF-induced toxicity on fertility profile of adult male Wistar rats (Table 3.6) showed that administration of 10mg/kg of NaF caused a significant

decrease ($p<0.05$) in the percentage of live and a significant increase ($p<0.05$) in percentage of dead sperm cells in comparison with the control group. However, treatment with NaF + 1500 - 2500mg/kg of fruit juice and NaF + 2000 - 2500 mg/kg stem bark extract caused a significant increase ($p<0.05$) in the percentage of live sperm cells and a significant decrease ($p<0.05$) in percentage of dead sperm cells when compared with the group treated with NaF alone. The leaf extract did not produce any visible ameliorative effect in the NaF-induced toxicity sperm cells.

Table 3.6: Percentage Live/Viable sperm cells and Sperm dead cells of control and test groups of NaF-induced toxicity in rats after 6 weeks of treatment

Group	Live/Viable sperm cells (%)	Sperm dead cells (%)
Control	$97.08\pm2.08^{\beta}$	$3.01\pm0.01^{\beta}$
NaF	$32.08\pm2.02^{\alpha}$	$67.62\pm7.62^{\alpha}$
Stem bark extract		
10mg/kg NaF + 500	$32.65\pm2.33^{\alpha}$	$67.25\pm2.25^{\alpha}$
10mg/kg NaF + 1000	$34.73\pm4.00^{\alpha}$	$65.17\pm5.17^{\alpha}$
10mg/kg NaF +1500	$38.90\pm1.90^{\alpha}$	$61.09\pm1.09^{\alpha}$
10mg/kg NaF + 2000	$41.66\pm1.33^{\alpha\beta}$	$58.34\pm2.34^{\alpha}$
10mg/kg NaF + 2500	$40.39\pm2.30^{\alpha\beta}$	$59.51\pm5.50^{\alpha}$
Leaf extract		
10mg/kg NaF + 500	$28.68\pm3.30^{\alpha}$	$71.32\pm1.32^{\alpha}$
10mg/kg NaF + 1000	$32.52\pm2.02^{\alpha}$	$67.28\pm3.21^{\alpha}$
10mg/kg NaF +1500	$32.04\pm3.04^{\alpha}$	$67.86\pm2.11^{\alpha}$
10mg/kg NaF + 2000	$33.15\pm3.10^{\alpha}$	$66.85\pm6.85^{\alpha}$
10mg/kg NaF + 2500	$36.07\pm4.07^{\alpha}$	$64.93\pm4.00^{\alpha}$
Fruit Juice		
10mg/kg NaF + 500	$34.78\pm4.00^{\alpha}$	$65.12\pm3.12^{\alpha}$
10mg/kg NaF + 1000	$40.43\pm1.23^{\alpha\beta}$	$59.24\pm5.24^{\alpha}$
10mg/kg NaF +1500	$43.47\pm3.40^{\alpha\beta}$	$57.54\pm2.32^{\alpha\beta}$
10mg/kg NaF + 2000	$60.92\pm5.02^{\alpha\beta}$	$49.07\pm7.07^{\alpha\beta}$
10mg/kg NaF + 2500	$47.81\pm7.01^{\alpha\beta}$	$53.18\pm3.18^{\alpha\beta}$

Results are expressed as Mean±SD; n=4

The mean values with **β** as superscripts across the column compared with group treated with NaF alone are considered significant ($p<0.05$). The mean values with **α** as superscripts across the column compared with control group fed with water and feed only are considered significant ($p<0.05$)

Epididymal Sperm pH

The results obtained showed that the administration of 10mg/kg of NaF alone and concomitant administration of NaF + fruit juice, NaF + stem bark and NaF + leaf extracts had no significant effect on epididymal sperm pH. Epididymal sperm pH result after 6 weeks of treatment was 6 for all the tested groups.

Histology Results

Photomicrographs of thin sections (5 µm) of the Testes of experimental rats harvested at the end of 6 Weeks of treatment with Fruit juice of *A. muricata* (Plate 1) and stained with H&E (400x).

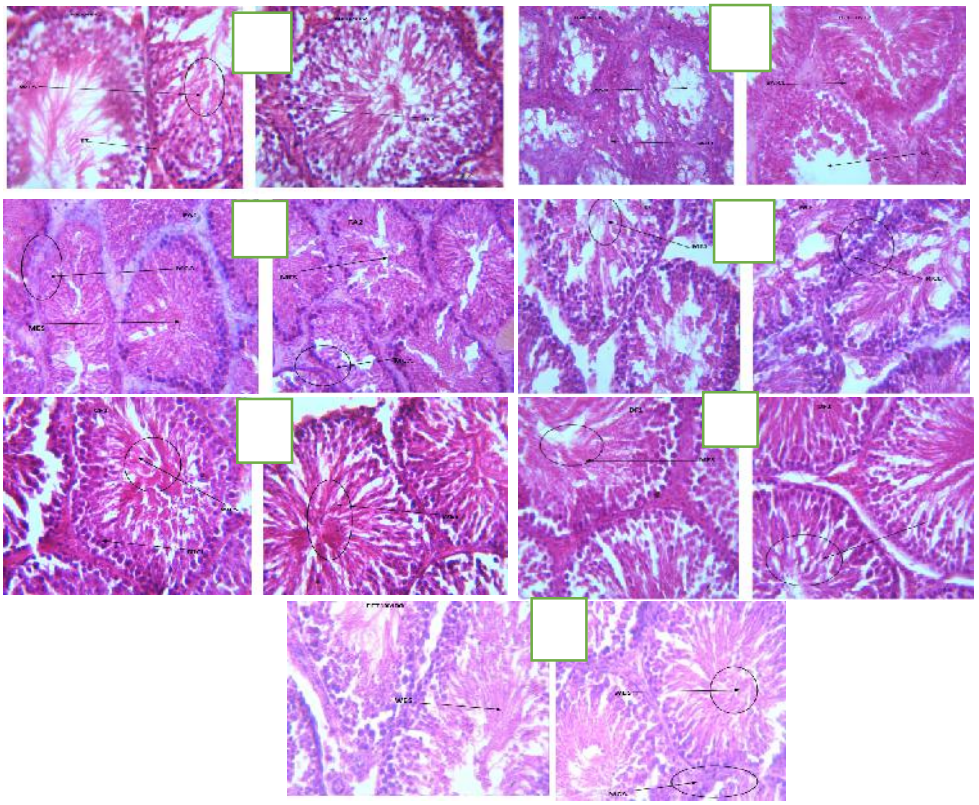

Plate 1 shows photomicrographs of a section of the testes in the control groups and rats treated with different concentrations of Fruit juice of *A. muricata*.

A – Group 1 rats, that received feed and water only, showed normal testicular micro architecture. There was normal spermatogenesis with different stages of differentiation and maturation. Seminiferous tubules were lined with interstitial cells of the Leydig and well enhanced spermatogenesis. B – Group 2 rats treated with 10 mg/kg NaF showed severe testicular damage with severe spermatogenic arrest and severe apoptosis of the interstitial cell of Leydig. The overall features are ghost like. There was lack of differentiation and maturation of spermatogenesis and there was marked infiltration in the interstitial area of seminiferous tubules. Severe spermatogenic arrest and severe apoptosis of the interstitial cell of Leydig. C – Group 3 rats treated with 10 mg/kg NaF and 500 mg/kg of Fruit juice showed mild restoration with mild enhanced spermatogenesis. However there are moderate cellular apoptosis in some areas. D – Group 4 rats treated concomitantly with 10 mg/kg NaF and treated with 1000 mg/kg of Fruit juice showed moderate restoration with moderate enhanced spermatogenesis and moderate restoration of the interstitial cells of the Leydig. E

– Group 5 rats treated concomitantly with 10 mg/kg NaF and 1500 mg/kg of Fruit juice showed moderate restoration with well enhanced spermatogenesis and interstitial cell of the Leydig appears normal. F – Group 6 rats treated concomitantly with 10 mg/kg NaF and 2000 mg/kg of Fruit juice showed moderate restoration with moderate enhanced spermatogenesis and interstitial cells of the Leydig that appears normal. G - Group 7 rats treated concomitantly with 10 mg/kg NaF and 2500 mg/kg of Fruit juice showed mild cellular apoptosis otherwise normal with well enhanced spermatogenesis.

Photomicrographs of thin sections (5 µm) of the Testes of experimental rats harvested at the end of 6 Weeks of treatment with leaf extract of *A. muricata* (Plate 2) and stained with H&E (400x).

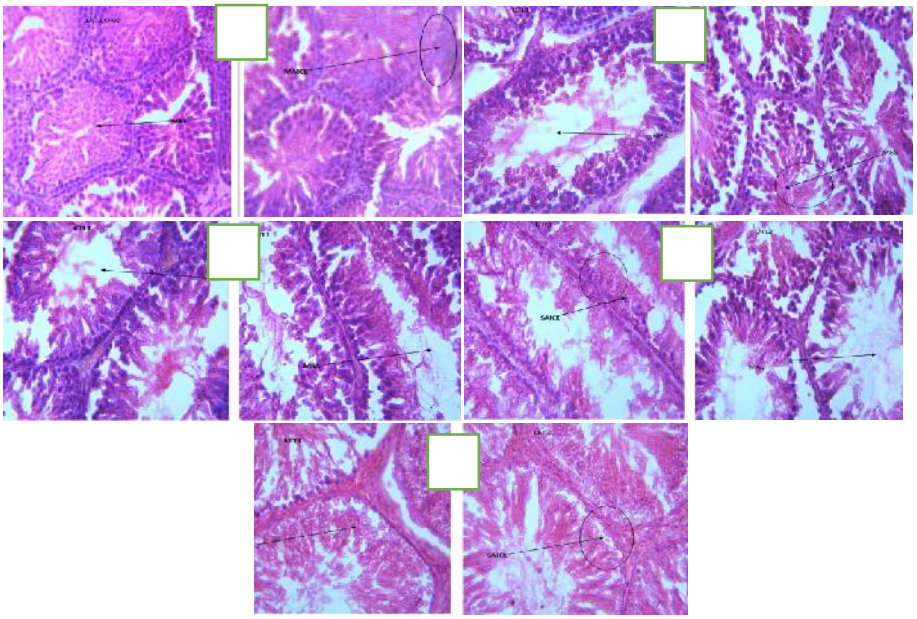

Plate 2. Photomicrographs of a section of the testes of rats treated with leaf extract of *A. muricata*

H Group 8 rats treated concomitantly with 10 mg/kg NaF and 500 mg/kg of leaf extract showed moderate regeneration with moderate enhanced spermatogenesis. However there are moderate apoptosis of the interstitial cells of the Leydig. I – Group 9 rats treated concomitantly with 10 mg/kg NaF and 1000 mg/kg of leaf extract showed moderate regeneration with moderate enhanced spermatogenesis. However there are moderate spermatogenic arrest. J – Group 10 rats treated concomitantly with 10 mg/kg NaF and 1500 mg/kg of leaf extract showed mild regeneration with moderate arrest of spermatogenesis. K – Group 11 rats treated concomitantly with 10 mg/kg NaF and 2000 mg/kg of leaf extract showed mild regeneration with moderate arrest of spermatogenesis and severe apoptosis of the interstitial cell Leydig. L – Group 12 rats treated concomitantly with 10 mg/kg NaF and 2500 mg/kg of Leave extract showed mild regeneration with severe apoptosis of the interstitial cell ledig.

Photomicrographs of thin sections (5 µm) of the Testes of experimental rats harvested at the end of 6 Weeks of treatment with Stem bark extract of *A. muricata* (Plate 3) and stained with H&E (400x).

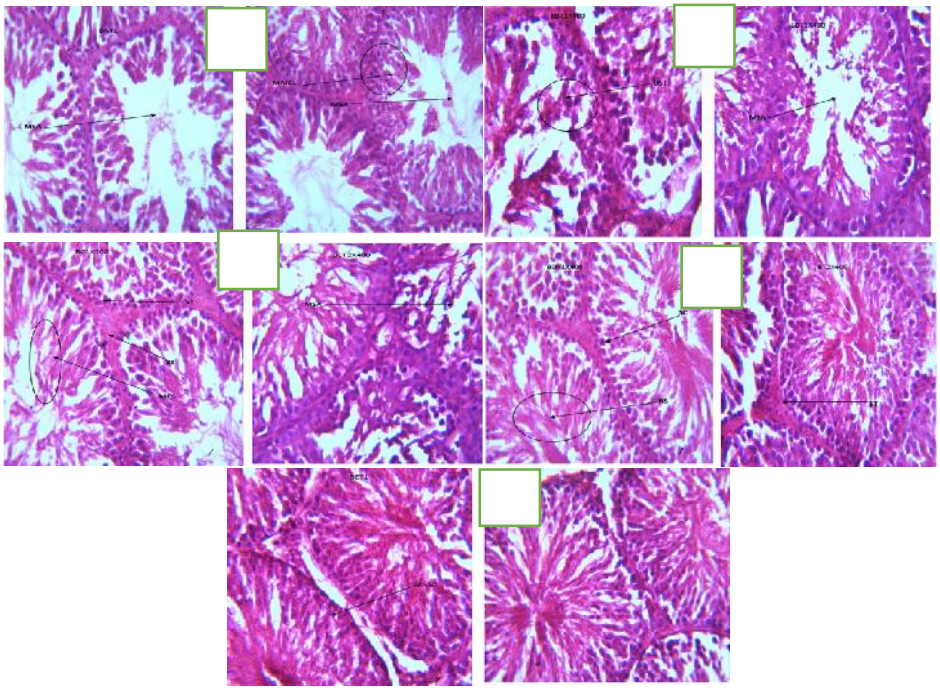

Plate 3. Photomicrographs of a section of the testes of rats treated with stem bark extract of *A. muricata*

M – Group 13 rats treated concomitantly with 10 mg/kg NaF and 500 mg/kg of stem bark extract showed mild regeneration with moderate spermatogenic arrest and mild apoptosis of the interstitial cells of the Leydig. There was mild regeneration with moderate spermatogenic arrest and mild apoptosis of the interstitial cells of the Leydig. N – Group 14 rats treated concomitantly with 10 mg/kg NaF and 1000 mg/kg of stem bark extract showed mild regeneration with moderate spermatogenic arrest and distortion of seminiferous tubules. There was mild regeneration with moderate spermatogenic arrest and distortion of seminiferous tubules. O - Group 15 rats treated concomitantly with 10 mg/kg NaF and 1500 mg/kg of stem bark extract showed moderate enhanced spermatogenesis and mild spermatogenic arrest. There was moderate spermatogenic arrest, moderate enhanced spermatogenesis and seminiferous tubules lined by sertoli cells. P – Group 16 rats treated concomitantly with 10 mg/kg NaF and treated with 2000 mg/kg of stem bark extract showed well regeneration with normal spermatogenesis and seminiferous tubules lined by sertoli cells. Q – Group 17rats treated concomitantly with 10 mg/kg NaF and 2500 mg/kg of stem bark extract showed well regeneration with mild apoptosis of sertoli cell.

Discussion

The recent findings that fluoride exposure leads to biochemical/histological alterations in male reproductive system through multiple pathways indicates that both assessment/prophylasis of chronic fluoride exposures in human populations is urgently required. Observation from this research work also indicates that sodium fluoride at a dose of 10 mg/kg caused non-significant decrease in haemoglobin concentration, platelet count, packed cell volume, and non-significant increase in neutrophil count, total white blood cell, and lymphocytes count. However, combined administration of NaF + the fruit juice and ethanol extracts of stem bark and leaf produced non-significant increase in the haemoglobin, packed cell volume and lymphocytes. The fruit juice at the concentrations of 1000 - 2500 mg/kg, and the groups treated with 500 and 1000 mg/kg of leaf extract, and 1000 and 2500

mg/kg of stem bark extract exhibited significant increase in platelet count. Reduction in haemoglobin and packed cell volume is an indication of either the destruction of red blood cells or the decreased production, which may lead to anaemia. On the contrary an increase in the count of red blood cell, haemoglobin and packed cell volume is suggestive of polycythaemia and positive erythropoiesis [22, 23]. Hence a non-significant increase or activation on haemoglobin and packed cell volume in fruit juice, stem bark and leaf extracts treated animals in comparison with the normal control is indicative of the ameliorative potential of these extracts against NaF induced toxicity. Therefore, an increased count of white blood cells and lymphocytes in NaF treated group, as observed in the present study, suggests that NaF might have compromised the immune system. This report is in agreement with [24], who reported a non-significant decrease in haemoglobin concentration of rats treated with NaF alone in comparison with the control group.

[25, 26] reported that reduced blood platelets affect the viscosity of blood, which is correlated positively to blood pressure. Concomitant administration of NaF and *A. muricata* extracts for 30 days adversely affected the count of blood platelets which may produce a positive effect on the viscosity of blood. Probably prolonged duration of the treatment may ameliorate the toxic effect of NaF [27]. Reduction in platelet count in experimental animals has been reported to indicate an adverse effect on the oxygen carrying capacity of the blood as well as thrombopoietin. Both significant and non-significant increase in platelets counts observed from the results of this study suggests that the administration of *A. muricata* fruit juice, leaf and stem bark extracts may ameliorate the disruption in the oxygen-carrying capacity of the blood caused by NaF.

The most important biochemical mechanism by which fluoride decreases the level of testosterone is its interference with steroidogenesis in Leydig cells. According to earlier research, this interference has been demonstrated, in which activity levels of testicular steroidogenic marker enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) decreased significantly in NaF-treated rats [5, 28]. Since testicular steroidogenesis is controlled by these two rate-limiting enzymes, a decline in their activities in Leydig cells significantly decreases the production and therefore the level of testosterone. Known mechanisms by which fluoride decrease testosterone levels are; inducing changes in both structures and enzyme activities in Leydig cells and interfering with hypothalamus-hypophysis-testis axis [29]. Leydig cells require normal expression and function of epidermal growth factor receptor (EGFR), androgen receptor (AR) and G-proteins in order to synthesize testosterone. Fluoride exposure has been shown to reduce both EGFR and AR expression [30] and to interfere with G-proteins in Leydig cells. However, fluoride has been found to interfere with hypothalamus-hypophysis-testis axis [31]. The non-significant decrease in testosterone level in NaF treated group in relation to the control group reported in this study is consistent with so many previous research works which had demonstrated that the NaF toxicity leads to a decrease in testosterone, a key hormone in spermatogenesis [2, 32]. The result further reveals that concomitant administration of 10 mg/kg of NaF and extracts on testosterone levels of all the stem bark and leaf extracts treated groups exhibited concentration-dependent significant increases while groups treated with NaF and fruit juices exhibited no obvious changes. This observed increase could be attributed to the interference of their phytochemical constituent(s) on the inhibitory action of fluoride ion on steroidogenesis in Leydig cells or their antioxidant effect (properties) on free radical generation by fluoride.

A lower concentration of 500 mg/kg of stem bark extract and 1000 mg/kg of fruit juice produced a significant increase in FSH and LH concentrations respectively. This suggests that stem bark extract and fruit juice at lower doses, with its antioxidant properties ameliorated the toxicity effects of NaF on Gonadotropin hormones. Gonadotropins are luteinizing hormone and follicle stimulating hormone from the pituitary gland. Testosterone in males secreted by Leydig interstitial cells is increased under the influence of luteinizing hormone. FSH regulates the development, growth, pubertal maturation and reproductive processes of the body. Diminished secretion of FSH can result in hypogonadism. This condition is typically manifested in males as a failure in the production of normal numbers of sperm. Serum levels of FSH are decreased in anterior pituitary hypofunction, hypothalamic disorders. Serum levels of LH are decreased in pituitary hypothalamic impairment. Gonadotropin-releasing hormone stimulates the production and release of follicle stimulate hormone (FSH) and luteinizing hormone (LH) from the pituitary gland [33].

Studies have reported that fluoride affects the synthesis of thyroid hormones, which inversely impair the normal function of the male fecundity. Fluoride has been shown to increase thyroid stimulating hormone (TSH) and reduce triiodothyronine (T_3) and thyroxine (T_4) [34]. Fluoride is considered to interfere with thyroid hormone levels mainly through three mechanisms; impairing normal structures of the thyroid gland, disrupting iodine metabolism in thyroid glands and interfering with the tissue-specific metabolism of thyroid hormones. Clinch in her review pointed out that fluoride interferes with the activity of Na/K-ATPase and the sodium-iodide symporter. Since iodide uptake is facilitated by the combined actions of the Na/k-ATPase and the sodium/iodide symporter [35], a decrease in the activities of these enzymes caused by fluoride would reduce the uptake of iodide in the thyroid gland and subsequent production of thyroid hormones. High fluoride intake has also been shown to inhibit the activity of thyroid peroxidase [36]. Since thyroid peroxidase is an enzyme which is essential for the production of thyroid hormones, decreased activity of thyroid peroxidase caused by fluoride would also lead to reduced thyroid hormone synthesis [35]. Hypothyroidism is known to be associated with impotence and decreased libido since thyroid hormone affect brain chemistry involved in sexual arousal, which in turn stimulates the autonomic nervous system and affects many other hormones necessary for energy [37]. There is a correlation between hypothyroidism and low serum testosterone concentration. Also, type 2 iodothyronine deiodinase which regulates the tissue-specific conversion of T_4 to the genomically active T_3 is predominantly expressed in elongated spermatids, suggesting that thyroid hormone might have a direct effect on spermatogenesis [38, 39]. It is an established fact that T_3 regulates the maturation and growth of testis, controlling Sertoli cell and Leydig cell proliferation and differentiation during testicular development in rats and other mammal species [40]. However, our observations on the effect of NaF on thyroid hormone agree with the previous research that indicated that fluoride increases TSH but reduces T_3 and T_4 [41]. Fluoride is considered to interfere with thyroid hormone levels mainly through three mechanisms; impairing normal structures of the thyroid gland, disruptive iodine metabolism in thyroid glands and interfering with the tissue-specific metabolism of thyroid hormones [42]. Several studies reveal that fluoride can directly damage the structures of thyroid follicles, resulting in the following abnormalities; flattened follicle epithelial cells, reduced cytoplasm [43]. These structural disruptions by fluoride will disrupt the synthesis of thyroid hormones in the thyroid follicles [44].

Once fluoride crosses blood-testis membrane barriers that protect spermatogenesis, after a prolonged exposure, it causes lack of maturation and differentiation of spermatocytes,

476 fragmentation of spermatozoa in the epididymis, and even cessation of spermatogenesis
477 [45]. The present investigation was carried out to explore the effects of fluoride (10mg/kg
478 NaF) and the possible ameliorative role of concomitant administration of fruit juice, leaf and
479 stem bark ethanol extract on the seminal characteristic of adult male Wistar rats. The sodium
480 fluoride treatment caused a substantial significant decrease in epididymal sperm motility,
481 progressive sperm motility, sperm concentration and live spermatozoa (%) along with a
482 simultaneous increase in dead spermatozoa (%) as compared to the rats of the control
483 group. Findings from this research work agree with [46, 47] who reported that exposure to
484 high concentrations of NaF leads to decreased sperm count, sperm motility, sperm survival
485 and increase in sperm abnormalities. The most important consequence of these fluoride
486 exposures is changes in the structure and functional behaviour of spermatozoa, disruption of
487 spermatogenesis and disturbance of multiple hormone systems that impact male fecundity.

488 **Conclusion**

489 The histopathologic findings in the present study justify the finding reported from cauda
490 epididymal spermatozoa analysis. It might be concluded that NaF at 10 mg/kg caused
491 potential reproductive cytotoxicities leading to significant alterations in testicular tissue,
492 altered semen characteristics, various morphological abnormalities in spermatozoa,
493 haematological parameters, nephrons and hepatocytes membrane permeabilities.
494 Concomitant administration of the fruit juice, ethanol stem bark and leaf extracts of *A.*
495 *muricata* for a period of 6 weeks resulted in significant prophylactic amelioration in all
496 parameters altered. Therefore, fruit juice, ethanol extracts of stem bark and leaf of *A.*
497 *muricata* therapy could be beneficial for the amelioration of fluoride-induced toxicity in male
498 reproductive system and fertility in general.

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