European Journal of Medicinal Plants

32(8): 23-33, 2021; Article no.EJMP.73030
ISSN: 2231-0894, NLM ID: 101583475

Effects of the Aqueous Extract of *Cnestis ferruginea* Leaves on the Male Reproductive System in Alloxan-induced Diabetic Mice

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2021/v32i830408
Editor(s):
(1) Dr. Naseem A. Qureshi, National Center of Complementary and Alternative Medicine, Saudi Arabia.
(2) Prof. Marcello Iriti, Milan State University, Italy.
Reviewers:
(1) Manoj Kumar Saurabh, India.
(2) Ramchandra Prabhakar Limaye, Bharati Vidyapeeth (Deemed to be University), India.
(3) Jeetendra Kumar Gupta, GLA University, India.
Complete Peer review History: https://www.sdiarticle4.com/review-history/73030

Original Research Article

Received 21 June 2021
Accepted 01 September 2021
Published 09 September 2021

ABSTRACT

Diabetes mellitus is the most common endocrine disease and one of the most common chronic disorders. Diabetes mellitus has been associated with impaired reproductive health, mainly in men. The present study aimed to evaluate effects of the aqueous extract of *Cnestis ferruginea* leaves on the male reproductive system in alloxan-induced diabetic mice. The determination of phenolic compounds content in the aqueous extract was performed by conventional methods. Diabetes was induced in adult male mice by intraperitoneal injection with a single dose of 220 mg/Kg body weight of alloxan. Animal's treatment with 100 and 200 mg/Kg of body weight of the aqueous extract was started 34 days after induction of diabetes. Sperm density, morphology and motility were assessed by standard methods. Serum levels of testosterone, FSH, and LH were measured. In addition, the testes were removed for histological study. Proportions of total polyphenols, flavonoids and tannins

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in the aqueous extract of *Cnestis ferruginea* were *5260.32 +/- 26 mg EAG /100g, 1384.43 +/- 4 mg EQ /100g and 7380.95 +/- 121 mg EC /100g* respectively. 100 and 200 mg/Kg of body weight doses induced highly significant (*P < .001*) reductions of *68%* and *71%* in blood glucose levels respectively, significant (*P < .05*) increases in testes weight of *9%* and *10%* respectively, highly significant (*P < .001*) increases in testosterone, pituitary gonadotropins and sperm parameter levels. In addition, regeneration of seminiferous tubules and interstitial cells was observed. *Cnestis ferruginea* leaves are rich in phenolic compounds. These compounds have anti-diabetic and fertilizing activities on the male reproductive system of diabetic mice.

Keywords: Cnestis ferruginea; reproductive system; diabetes mellitus; alloxan.

1. INTRODUCTION

Diabetes mellitus is the most common endocrine disease and one of the most common chronic disorders [1]. It is a condition characterized by chronic hyperglycemia [2]. This persistent hyperglycemia causes oxidative stress in most tissues if left unregulated. Oxidative stress mainly causes cell loss in tissues, leading to organ dysfunction [3]. Diabetes mellitus has been associated with impaired reproductive health in both men and women [4]. However, a recent study showed that hypogonadism is common in men with diabetes mellitus, and the prevalence is as high as 40% for type 2 diabetes [5]. Male reproductive function is a targeted physiological process mostly damaged by diabetes due to the high susceptibility of testicular microenvironment to oxidative stress [6]. Clinical studies have shown that patients with type 1 diabetes had significantly reduced motile and normal sperm counts and sperm cell concentration compared to the control group [7,8]. Results from several experimental studies have corroborated these facts [4,9,10]. Despite advances in the management of type 1 and type 2 diabetes, treatment goals are frequently not achieved. People dissatisfied with the results of conventional medicine often turn to alternative solutions. As a result, more attention is being paid to medicinal plants.

Cnestis ferruginea is a species of the Connaraceae family found in West Africa from Senegal to West Cameroon [11]. It is a lianascent, sarmentose perennial shrub 3.0-3.6 m high, sometimes with woody tendrils [12,13]. This species is traditionally used to treat diarrhea, asthenia, inflammation, bronchitis, conjunctivitis, syphilis, gum pain, wounds, dysentery, gonorrhea and diabetes mellitus [14,15,16]. Chemical compounds in the leaves, fruits, stem and roots of *C. ferruginea* have been identified in previous works [17,18,19,20]. These different parts contained sterols, polyphenols, flavonoids, tannins, quinone, alkaloids and saponosides.

Anti-diabetic activities of ethyl-acetate and methanol extracts of *C. ferruginea* leaves were demonstrated in an earlier experimental study [21]. Another study showed fertilizing effects of aqueous extract of *C. ferruginea* leaves in healthy wistar rats [22]. However, no study has yet shown that *C. ferruginea* leaves can both restore glycemic balance and correct hypo-fertility observed in diabetic subjects.

The present study aims to evaluate effects of the aqueous extract of *C. ferruginea* leaves on the male reproductive system in alloxan-induced diabetic mice.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Adult male Swiss mice were used in this study. They were 10 to 12 weeks old and weighed between 28 and 32 g. These animals were bred in the vivarium of the "École Normale Supérieure" in Abidjan (Ivory Coast). They were housed and maintained at a constant temperature of 27-29º C with a relative humidity of 65% and standard 12:12 h light-darkness cycles. They had free access to standard rodent chow and tap water ad libitum. Animals were handled according to the guidelines of the Ethical Committee on the use and care of experimental animals of the Department of Biosciences, Université Félix Houphouët-Boigny.

2.2 Plant Material

The plant material, consisting of *C. ferruginea* leaves was collected in May 2019 in the region of Buyo in Ivory Coast. A sample was authenticated at the National Floristic Center of the University of Félix HOUPHOUËT-BOIGNY on the basis of taxonomic characters and by direct comparison

with herbarium specimens No. 3974, 4327 and 15116. The fresh leaves were dried in a room at room temperature for one month. They were then pulverised to obtain a fine powder.

2.3 Preparation of the Aqueous Extract

For the preparation of the aqueous extract, 50 g of *C. ferruginea* leaves powder was added to 1000 mL of distilled water. The mixture was stirred with the blender for 9 minutes (3 stirrings of 3 minutes). The homogenate obtained was wrung out in a clean white cloth and filtered three times successively on cotton wool and Whatman paper No.1. The filtrate obtained was evaporated in an oven at 50°C until a dry extract was obtained. The weight of the dry extract of *C. ferruginea* leaves (AECF) obtained was 6.96 g, which corresponds to a yield of 13.92%.

2.4 Determination of Phenolic Compounds Content

2.4.1 Measurement of total polyphenols content

The determination of total polyphenols content in the AECF was performed according to the Folin-Ciocalteu reagent method reported by Singleton et al. [23]. A volume of 1 mL of 10% Folin-Ciocalteu reagent was added to 1 mL of extract previously contained in a test tube. After three (3) minutes, a volume of 1 mL of 20% (w/v) sodium carbonate (Na2CO3) is added to the mixture. Finally, the mixture is made up to 10 mL with distilled water and the whole is placed in the dark for 30 min. The optical density reading is taken at 745 nm against a control prepared under the same conditions but containing methanol instead of the extract. The calibration curve is performed with a stock solution of gallic acid 0.1 mg/mL under the same conditions as the tests. The results are expressed as mg gallic acid equivalent.

2.4.2 Measurement of total flavonoids content

The flavonoid content was determined according to the method reported by Meda et al. [24]. A volume of 0.5 mL of extract is introduced into a test tube. To the contents of the tube are successively added 0.5 mL of distilled water, 0.5 mL of 10% (w/v) aluminium chloride, 0.5 mL of 5% (w/v) sodium acetate and finally 2 mL of distilled water. The mixture is left for 30 min in the dark at room temperature. The absorbance was then measured at 415 nm against a blank. The flavonoid concentrations are determined at the end by referring to a calibration curve made with quercetin 0.01 mg/mL. The results are expressed as milligram quercetin equivalent per 100 g dry matter (mg QE/100 g).

2.4.3 Measurement of tannins content

The determination of tannins was carried out according to the method described by Bainbridge et al [25]. A volume of 1 mL of extract was introduced into different test tubes, to which 5 mL of 1% (w/v) vanillin prepared in 70% (v/v) sulphuric acid was added. The absorbance of the solution is measured at 500 nm after 30 min in the dark against a blank. The tannin content is obtained with the aid of a standard range using a catechol stock solution (0.2 mg/mL) made under the same conditions as the tests.

2.5 Induction of Diabetes

Animals were deprived of food for 16 hours. Diabetes was induced by intraperitoneal injection of a single dose of 220 mg/Kg of body weight (BW) of alloxan (ALX) dissolved in isotonic solution (0.9% NaCl). Animals developed diabetes after 3 days. Mice with a blood glucose level of 3 g/L (clinical diabetes ≥1.26 g/L) or higher were selected for the study. Treatment of the animals with the test products started on day 34 after ALX injection, i.e. after one cycle of spermatogenesis.

2.6 Experimental Design

Test products were administered to the mice daily by gavage in 0.5 mL for 40 days. Four (4) groups of six (6) mice were formed. Control groups 1 and 2, consisting of healthy and diabetic animals respectively, were given distilled water while groups 3 and 4 were given 100 and 200 mg/Kg of BW of AECF respectively. At the end of the experiment, animals were anesthetised in order to collect spermatozoa for analysis of sperm parameters. Blood samples were collected in dry tubes for the determination of serum testosterone, FSH, and LH concentrations. In addition, testes were removed, weighed and fixed in 10% formalin for histological study.

2.7 Blood Glucose Level and Body Weight Measurements

Animals were fasted prior to the determination of blood glucose level and body weight. Values

were recorded weekly. Blood glucose estimation was performed with an On Call® Extra test strip meter (USA). Blood samples were collected from the tail end of the mice.

2.8 Sperm Parameters

2.8.1 Sperm collection

Animals were anesthetised with ether. The tail of the left epididymis was collected by opening the scrotum, and then dilated in 5 mL of 0.9% NaCl previously incubated in a water bath at 36 °C. Thus the spermatozoa diffused into the solution [26].

2.8.2 Sperm motility

A fine drop of epididymis macerate was placed and spread lightly on a slide previously maintained at 36°C. The slide was mounted on a light microscope (Olympus CX31RBSF, Philippine) at ×100 magnification. The sperm were filmed with an AmScope camera (London, United Kingdom). Motile and immobile sperm were subsequently counted on 5 random fields and the percentage of motile forms was determined [22].

2.8.3 Sperm cell concentration

A drop of epididymis macerate was collected and deposited on a Malassez cell and covered with a coverslip. The sperm count was performed under a light microscope (magnification ×400). The number of sperm per mm^3 was estimated by the following formula [27]:

$$N = \frac{X \times fd \times 10^6}{4}$$

X: Number of sperm counted in 5 grids of the Malassez cell; fd: Dilution factor (20); N: Number of sperm per mm^3

2.8.4 Sperm morphology

Sperm morphological abnormalities include fusion, isolated heads and deformed heads and/or tails [28]. Two hundred (200) sperm were examined in liquid medium on 3 random fields. The percentage of normal sperm was calculated [29].

2.9 Serum FSH, LH and Testosterone Measurements

Pituitary gonadotropins (FSH and LH) and testosterone were determined using the Hitachi 902 (Japan) ELFA (Enzyme Linked Fluorescent Assay) technique.

2.10 Histological Study

Testicles were removed and fixed in 10% formalin. After 72 hours they were dehydrated and cleared in alcohol and toluene baths respectively. They were impregnated and embedded in paraffin. The whole set was cut at 5 µm with a microtome (Leica RM2125 RTS, Germany). The resulting sections were stained in Harris haematoxylin and eosin solutions respectively. Mounting them using Eukitt allowed their good readability under a light microscope (Olympus CK41SF, Philippines) [22]. The installation of a camera connecting the microscope to a computer allowed image taking via AmScope 3.7 software (London, United Kingdom).

2.11 Statistical Analysis

Different values obtained were expressed as the mean followed by the standard error of the mean (M+/-SD). The significance of differences observed between different tests groups is assessed by analysis of variance (ANOVA) of the Turkey-Kramer multiple comparison test using GraphPad Prism 7.03 software (California, USA).

3. RESULTS

3.1 Phenolic Compounds Content

Table 1 shows the Phenolic compounds content of the AECF. The proportions of total polyphenols, flavonoids and tannins were 5260.32 +/- 26 mg EAG /100g, 1384.43 +/- 4 mg EQ /100g and 7380.95 +/- 121 mg EC /100g respectively.

Table 1. Content of total polyphenols, flavonoids and tannins in AECF

Phenolic compounds	Total polyphenols (mg GAE /100g)	Flavonoids (mg QE /100g)	Tannins (mg CE /100 g)
Contents	5260,32 +/- 26	1384,43 +/- 4	7380,95 +/- 121

Quoted values are Means+/-SD of triplicate measurements
GAE: Garlic Acid Equivalent. QE: Quercetin Equivalent; CE: Catechol Equivalent

3.2 Changes in Blood Glucose Concentration and Body Weight

Fig. 1 shows the time course of basal blood glucose levels of animals in the different experimental groups. Highly significant reductions (*P < .001*) of 68% and 71% in blood glucose levels were observed in groups treated with l00 and 200 mg/Kg of BW of AECF respectively compared to the diabetic control. Groups treated with l00 and 200 mg/Kg of BW of AECF and the normal control gained significantly 20%, 22% and 35% of BW respectively at the end of the experiment. There were no significant differences between these values. In contrast, a weight loss of 14% was observed in the diabetic control (Fig. 2).

3.3 Testicular Weight and Serum FSH, LH and Testosterone Levels

Animals' treatment with AECF induced significant (*P < .05*) increases in testicular weight of 9% and 10% respectively for 100 and 200 mg/Kg of BW compared to the diabetic control (Fig. 3). Highly significant increases (*P < .001*) in serum testosterone and pituitary gonadotropins levels were also observed in these groups compared to the diabetic control. Indeed, serum levels of pituitary gonadotropins and testosterone in AECF-treated groups were 3-fold and 2-fold higher than in the diabetic control, respectively (Table 2).

3.4 Sperm Parameters

The AECF (l00 and 200 mg/Kg of BW) induced significant (*P < .05*) increases of 153% and 168% in sperm count compared to the diabetic control (Fig. 4). Also, these doses induced highly significant (*P < .001*) increases of 26% and 27% of normal sperm and 40% and 43% of motile sperm, respectively, compared to the diabetic control. However, normal and motile sperm counts of AECF-treated groups were comparable to the normal control (Figs. 5 and 6).

3.5 Histological Study

Fig. 7 shows cross sections of testes from different experimental groups. Seminiferous tubules of normal control mice were intact. Different stages of spermatogenesis were observed. The interstitial tissue was present. In contrast, seminiferous tubules of diabetic control animals were atrophied. There was also an increase in inter-tubular spaces, loss of interstitial tissue and degeneration of seminiferous tubules. In AECF-treated groups, seminiferous tubules were intact, but their diameters were smaller than in normal control. Some interstitial cells were also present.

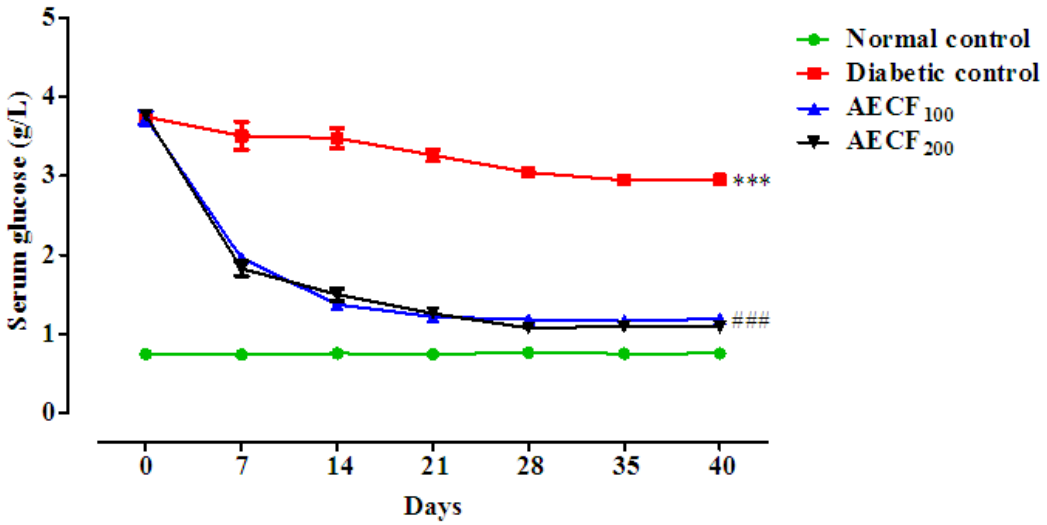

Fig. 1. Effect of AECF on serum glucose level in ALX-induced diabetic male mice during the experiment

*(*) Comparison with Normal control; (#) Comparison with Diabetic control, (Mean+/-SD), (n = 6). *p< .05, ** p<.01, *** p<.001; # p< .05, ## p<.01, ### p<.001*

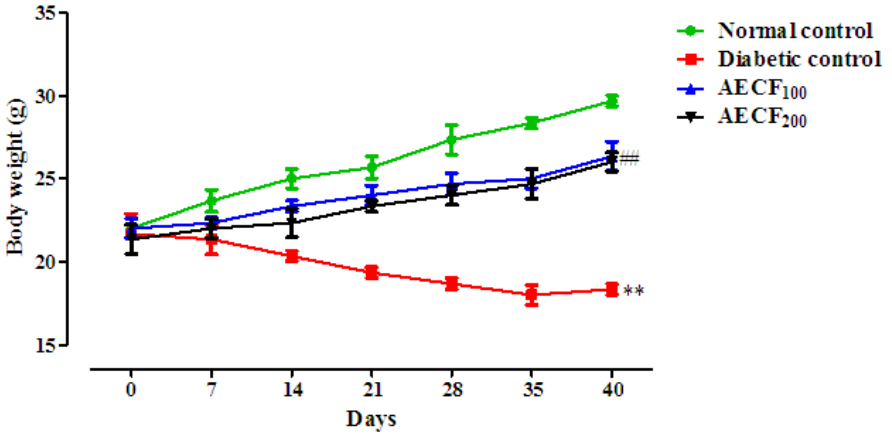

Fig. 2. Effect of AECF on body weight in ALX-induced diabetic male mice during the experiment

*(*) Comparison with Normal control; (#) Comparison with Diabetic control, (Mean+/-SD), (n = 6). *p< .05, ** p<.01, *** p<.001; # p< .05, ## p<.01, ### p<.001*

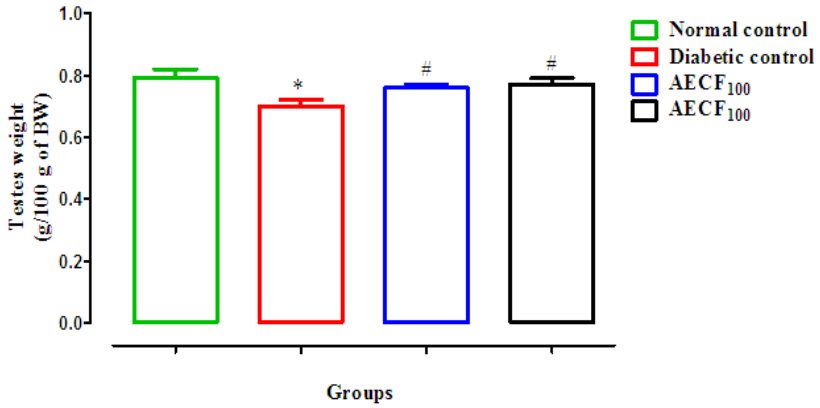

Fig. 3. Effect of AECF on testes weight in ALX-induced Diabetic male mice

*(*) Comparison with Normal control; (#) Comparison with diabetic control, (Mean+/-SD), (n = 6). *p< .05, ** p<.01, *** p<.001; # p< .05, ## p<.01, ### p<.001*

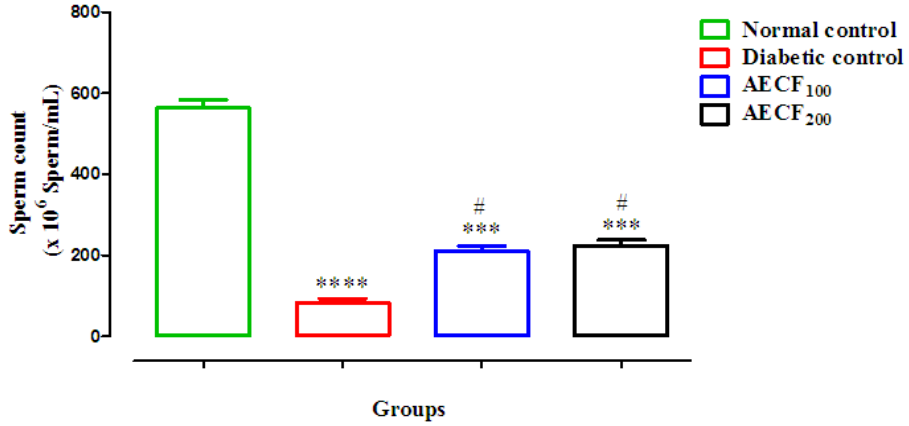

Fig. 4. Effect of AECF on sperm count in ALX-induced Diabetic male mice

*(*) Comparison with Normal control; (#) Comparison with Diabetic control, (Mean+/-SD), (n = 6). *p< .05, ** p<.01, *** p<.001; # p< .05, ## p<.01, ### p<.001*

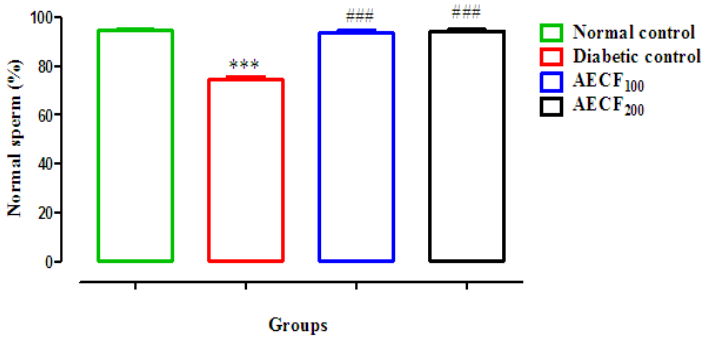

Fig. 5. Effect of AECF on sperm morphology in ALX-induced Diabetic male mice

*(*) Comparison with Normal control; (#) Comparison with Diabetic control, (Mean+/-SD), (n = 6). *p< .05, ** p<.01, *** p<.001; # p< .05, ## p<.01, ### p<.001*

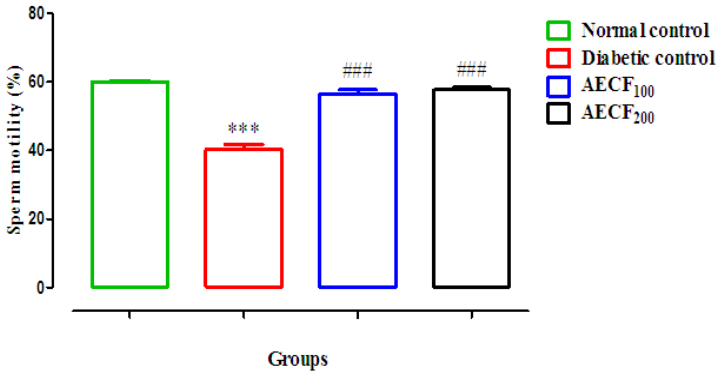

Fig. 6. Effect of AECF on sperm motility in ALX-induced Diabetic male mice

*(*) Comparison with Normal control; (#) Comparison with Diabetic control, (Mean+/-SD), (n = 6). *p< .05, ** p<.01, *** p<.001; # p< .05, ## p<.01, ### p<.001*

4. DISCUSSION

Several studies have shown that diabetes mellitus impairs reproductive function, mainly in men. In this study, results showed elevated blood glucose levels, significant decreases in body and testicular weights, plasma testosterone, FSH and LH levels, normal and motile sperm counts and sperm density in the diabetic control. In addition, the histological study showed that seminiferous tubules of testes in this group were atrophied. There was also an increase in inter-tubular spaces, loss of interstitial tissue and degeneration of seminiferous tubules. However, treatment with AECF induced a very significant decrease in blood glucose levels in diabetic animals. *C. ferruginea* leaves therefore have anti-diabetic properties. These results corroborate those of Adisa et al. who observed a significant reduction in basal blood glucose levels in diabetic rats after 10 days of treatment with 250 mg/Kg of BW of methanol and ethyl acetate extracts of *C. ferruginea* leaves [21]. In addition, animals treated with AECF showed significant increases in body and testicular weights, plasma testosterone, FSH and LH levels, normal and motile sperm counts and sperm density. In addition, AECF induced regeneration of seminiferous tubules and interstitial cells. These beneficial biological effects of AECF are attributable to its constituent bioactive compounds. Indeed, bioactive compounds are primary or secondary metabolites that exhibit specific biological effects in addition to being functional food ingredients at low concentrations [30]. These compounds have antioxidant, anti-inflammatory, anti-carcinogenic, anti-diabetic effects and may be protective against various diseases and metabolic disorders. In this study, proportions of total polyphenols, flavonoids and tannins in AECF were 5260.32 +/- 26 mg EAG /100g, 1384.43 +/- 4 mg EQ /100g and 7380.95 +/- 121 mg EC /100g respectively. AECF is therefore rich in phenolic compounds. Contents of total polyphenols and flavonoids in the aqueous extract of *C. ferruginea* fruits were studied by Ita [31]. This author found contents of 2550 mg EAG /100g and 960 mg EQ /100g for

total polyphenols and flavonoids respectively. These values show that *C. ferruginea* leaves would be richer in phenolic compounds than its fruits. Naturally, fruits of a plant are richer in phenolic compounds than its leaves. These results can be explained by the difference in geographical areas and/or by the difference in extraction methods.

Anti-diabetic activities of polyphenols and flavonoids have been widely demonstrated. Indeed, numerous experimental studies showed that treating animals with these compounds or a diet rich in phenolic compounds prevented and cured diabetes mellitus [32,33,34]. Anti-diabetic effects of these compounds may be related to inhibition of carbohydrate digestion through inhibition of salivary and pancreatic ά-amylase and ά-glucosidase in the brush border of the small intestine, inhibition of glucose uptake, stimulation of insulin secretion, and protection of pancreatic β-cells against glucotoxicity. They can also suppress glucose release from the liver and enhance glucose uptake in peripheral tissues by modulating intracellular signalling [35].

Previous work indicated that oxidative stress damages sperm nuclear and mitochondrial DNA [9,36,37]. Spermatozoa are highly vulnerable to oxidative attack as they lack significant antioxidant protection due to the limited volume and restricted distribution of cytoplasmic space in which an appropriate armoury of defensive enzymes is housed [38]. *C. ferruginea* leaves have the ability to repress this oxidative stress given their high content of phenolic compounds. Polyphenols have antioxidant activity and can inhibit the formation of advanced glycation products [39]. Indeed, it is well established that antioxidant activity is positively correlated with polyphenol structure [40].

In addition, fertilizing activities of *C. ferruginea* leaves have been studied [21,41]. These authors had shown that *C. ferruginea* leaves potentially improve the reproductive health of rats of both sexes by increasing and enhancing the functions of hormone-dependent reproductive organs through the stimulation, synthesis and release of pituitary gonadotropins and sex steroids. These biological actions are attributable to flavonoids as they are said to have the capacity to boost the level of androgens and thus blood testosterone level. Thus, anti-diabetic activities of leaves of this plant associated with its antioxidant and fertilizing activities would explain the convincing results obtained in diabetic mice.

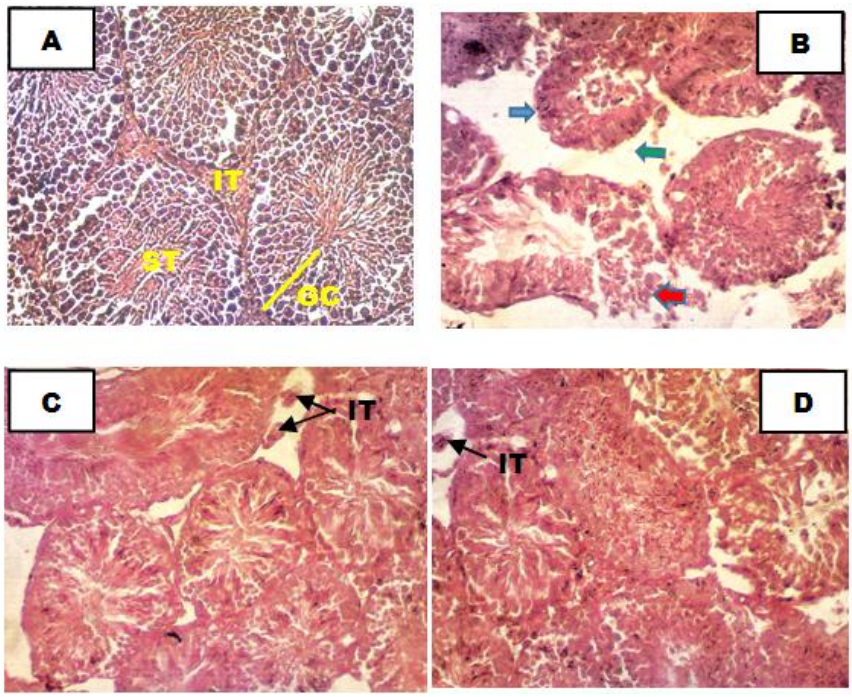

Fig. 7. Cross section of testicular tubules in experimental groups

A: Normal control; B: Diabetic control; C: Treated 100 mg/Kg of BW; D: Treated 200 mg/Kg of BW. ST: Seminiferous tubules; IT: interstitial tissue; GC: germ cells; Tubular atrophy (blue arrows); Increase of intercellular space (green arrows); Degeneracy of seminiferous tubules (red arrows); Magnification: ×400; Hematoxylin and Eosin staining

5. CONCLUSION

In conclusion, *C. ferruginea* leaves have the capacity to remedy the male reproductive system disorders observed in diabetic subjects. This is due to its anti-diabetic and fertilizing activities. The high content of phenolic compounds in *C. ferruginea* leaves would be largely responsible for these activities.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

Animals were handled according to the guidelines of the Ethical Committee on the use and care of experimental animals of the Department of Biosciences, Université Félix Houphouët-Boigny.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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