



**METHANOL EXTRACT OF SQUIRREL NUT (*CURCULIGO PILOSA*) AMELIORATED SOY ISOFLAVONE-INDUCED REPRODUCTIVE CHANGES OF ADULT MALE WISTAR RATS**

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**ABSTRACT**

The study evaluated parturition rate, pup weight and litter sizes of pups sired by adult male Wistar rats treated with methanol extract of *Curculigo pilosa* (CP) rhizome and/or soy isoflavones (ISF), as well as the histological changes of testis of the treated rats. Thirty-two adult male rats were divided into 4 groups (I to IV) comprising of 8 rats each (n = 8). Group I (control) received 1% carboxyl methyl cellulose (CMC). Groups II and III were administered 1000 mg/kg CP and 100 mg/kg ISF, respectively, while group IV received combination of CP + ISF by oral gavage for 54 days. On day 55, four male rats from each group were sacrificed to harvest testis for histological studies using Haematoxylin and Eosin, and examined under light microscope at x 400 magnification. The remaining 4 males were each paired with 4 untreated female rats each for further reproductive studies. All male rats were removed from the groups after 21 days of pairing. Pregnancy was detected using physical examination. Number of pups/litter and weights were taken and recorded within 24 hours of each parturition. Parturition rate was 100% in the control group (1% CMC), 75% in the 1000 mg/kg CP and the CP + ISF treated groups, and 25% in the 100 mg/Kg ISF 54 days post treatment. Treatment for 54 days with 1000 mg/kg CP recorded the highest significant average pup body weight ( $P < 0.05$ ;  $7.950 \pm 0.11$  g) followed by CP + ISF (IV;  $6.471 \pm 0.11$  g), then 100 mg/kg CP (III;  $6.057 \pm 0.20$  g), and lastly the control group (I;  $5.689 \pm 0.06$  g). Administration of 100 mg/kg ISF for 54 days caused moderate to severe histological changes in the testes of treated rats in the form of seminiferous tubular atrophy, disorganized germ cell layers with severe vacuolations. The germ cell layers were normally arranged and the spermatozoa, the Leydig cells within the interstitial spaces, and the associated blood vessels were also normally arranged in the CP and CP+ISF-treated groups. This study demonstrated the beneficial pro-fertility and antioxidant effects of methanolic extract of *C. pilosa* administered at 1000 mg/kg/day, or its ameliorative benefit when co-administered with 100 mg/kg ISF. The administration of 100 mg/kg of soy isoflavones exerted deleterious pro-oxidant effects on the reproduction of adult male Wistar rats.

**Keywords:** *Curculigo pilosa*, Histoarchitecture, Male Wistar rats, Parturition rate, Soy isoflavone, Testis

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**INTRODUCTION**

Several conditions interfere with spermatogenesis, sperm quality and output [1, 2, 3]. They include infectious and non-infectious diseases, drug treatments, toxins, insufficient vitamin intake as well as environmental factors [4, 5, 6]. Treatment is sometimes with hormonal and antioxidant supplementation [7], although scientifically acceptable evidence of these pharmacological treatments is limited [8, 9]. Complementary and alternative medicines have also received a lot of attention for treatment of different diseases, including male infertility [10]. Herbal remedies have been reported to alleviate male infertility, irrespective of the aetiology of the disorder [11, 12]. Several plants have been tested for their possible fertility regulatory properties [13]. Some of the plants that are extensively used are aphrodisiacs,

which are administered to relieve sexual dysfunction [14], or as fertility-enhancing agents [15]. They boost nutritional value, thereby improving sexual performance and libido [16, 17]. *Curculigo pilosa* (Schumach. and Thonn.) Engl. commonly referred to as squirrel nuts, belongs to the family *Hypoxidaceae* [18]. It is an herbaceous plant with stout, erect rhizomes bearing a cluster of grass-like leaves of 60 cm long and yellow flower which shoots up to 20 cm at the end of the dry season. Although the genus *Curculigo* includes some 20 species of tropical origin, the rhizome of *C. pilosa* was the first African species to be described of the genus [19]. The plant is called ‘Epakun’ in Yoruba, and ‘Doyar kurege’ in Hausa [20]. In Nigeria, it is commonly found in Mubi (Adamawa State), Abuja, and Igboho and Erin-Odo (both in Oyo State). Its rhizome possessed medicinal properties, and it is used as food. Sometimes the plant is traditionally

used to manufacture infant food and beer in Burkina Faso [21]. Among its medicinal uses are as purgative, and for treatment of diabetes mellitus [22, 23], hernia, genital infections [24], liver damage, hypertension [25, 26], and infant illnesses in South-Western Nigeria [27]. The plant is also used to treat radical-related pathological damages [28]. *Curculigo pilosa* is reported to be rich in iron, potassium and zinc [29]. Although most investigations only document the medicinal effects of *C. pilosa*, there is paucity of information on its reproductive potentials, including its possible role in the management of reproductive disorders. Isoflavones, belonging to a class of phytoestrogens, are natural compounds described as angiogenesis inhibitors. They are the most common secondary metabolites of the clovers (*Trifolium* species) and soybean (*Glycine max* L) [30, 31]. Soybean is the most common source of isoflavones in human food (majorly genistein and daidzein; 57% and 37%, respectively), as it is widely used as dietary supplement in both human and animal diets [32, 33], being the most important protein source used in farm animal feeds, alongside fishmeal that in turn yields animal protein for human consumption [34]. Its edible products such as milk, yoghurt, flour and curd (popularly known as *Awara*, or *Beske*, in Nigeria) have been proved to be nutritionally healthy [35, 36]. Soy milk is an alternate of dairy animal milk due to the fact that it is less expensive, despite having high-quality protein, and is important especially for people who are allergic to cow milk [37].

## MATERIALS AND METHODS

### Study area

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

### Ethical approval

The protocol for handling and caring for the animals used in this study was created by the Ahmadu Bello University Committee on Animal Use and Care (ABUCAUC), and approval number ABUCAUC/2022/072 was issued.

### Collection and identification of plant

Fresh rhizomes of *Curculigo pilosa* Gaertn were purchased from Bode market in Ibadan, Oyo State, Nigeria. The rhizomes were transported to Zaria in paper bags for adequate aeration. The rhizomes were taxonomically identified, authenticated, and given a voucher number (ABU022398) at the herbarium,

Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Nigeria.

### Methanol extraction

Desiccated powdered rhizome (100 g) was soaked in one litre of 70% methanol at room temperature (25°C) for 72 hours before being filtered. Under vacuum, the filtrate was allowed to dry, evaporate and concentrated to a solid mass over water bath with a shaker at 50°C (Gesellschaft Fur Laboratory, model 1083, Germany). The concentrated extract was kiln-dried at 40°C for three days, followed by 48 hours at room temperature. Extract was kept at -20°C until it was used. The method of (38) was used to assess the acute toxicity of the *C. pilosa* rhizome extract.

### Preparation of concentration of extract

Both the extract and supplement were separately placed in 1% carboxymethyl cellulose (CMC). The 1% CMC was made by dissolving 1 gram of CMC granules in 100 mL of distilled water. And for the highest stock culture of 400 mg/mL, 4 g of *C. pilosa* methanol extract was dissolved in 10 mL of 1% CMC using the serial dilution method, followed by serial dilution with 1% CMC to produce various concentrations. The tubes holding the different concentrations were properly labelled and used right away [39].

### Experimental animals and management

Adult male Wistar rats with average weight of 220 g were acquired from the Department of Veterinary Physiology Animal House, A. B. U., Zaria, Nigeria. The rats were kept under a 12-hour light/12-hour dark cycle in well-ventilated plastic cages in the experimental Animal House, Department of Veterinary Parasitology and Entomology, A. B. U., Zaria. Two weeks prior to the start of the research, they were acclimated to the laboratory setting. Water and pelleted feed were provided *ad-libitum*. The pelleted feed was made from commercially prepared poultry growers' mash (Vital Feeds Ltd.) and maize bran in a 2:1 ratio.

### Experimental design

Thirty-two adult male rats were divided into four groups (I to IV) comprising of 8 rats each (n = 8). Group I received 1% CMC, and served as the control. Groups II and III were administered oral doses of *C. pilosa* methanol extract (CP) 1000 mg/kg/day and soy isoflavones (ISF) 100 mg/day, respectively [40, 41]. The doses of CP and ISF — 1000 mg/kg CP and 100 mg/kg ISF — were co-administered to Group IV. Body weights were taken for each rat and was used to calculate the volume of 1% CMC, or extract, or isoflavones administered to each rat daily. All administrations were performed by oral gavage between 7:00 and 8:00 a.m. for 54 days.

**Sacrifice of animals**

Treatment ceased on day 54; at completion of a cycle of spermatogenesis [42], after which four rats from each group were sacrificed on day 55 by chloroform inhalation. The right testes were harvested from the sacrificed rats for histological studies. Four female rats, each of proven fertility (previously given birth once) were introduced to the remaining four male rats (control and treated) in each group. This is to create

new groups comprising four male rats and 4 female rats each (Table 1). Mating was confirmed with camcorder placed in front of each cage overnight for 14 days, and by vaginal swab viewed for presence of spermatozoa. All male rats were removed from all cages 21 days post-pairing. Pregnancy in the female rats were detected using physical examination such as increased body weight, bulging of abdomen (pear-shaped) and abdominal palpation to detect beaded uterus.

**Table 1:** The newly-formed groups on day 55

S/No	Group	n	Male Treatment
1	I	n = 4M+4F	1% CMC
2	II	n = 4M+4F	1000 mg/kg CP
3	III	n = 4M+4F	100 mg/kg ISF
4	IV	n = 4M+4F	1000 mg/kg CP + 100 mg/kg ISF

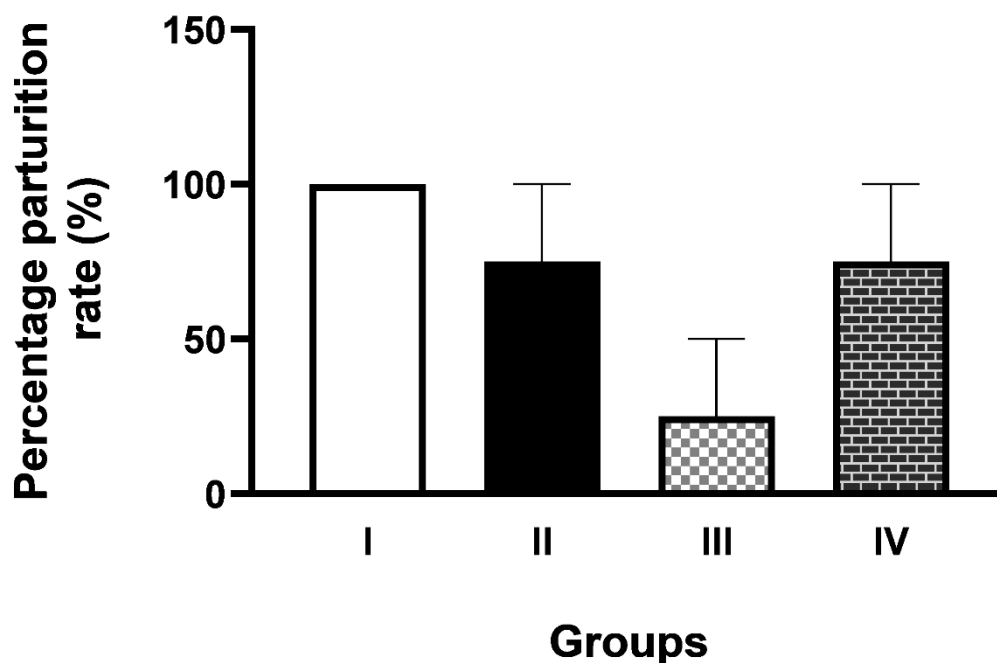
Key: CMC = Carboxymethyl cellulose; CP = *Curculigo pilosa*; ISF = Soy isoflavones; M = Male; F = Female

**RESULTS AND DISCUSSION**

**Effect of *Curculigo pilosa* and soy isoflavones administration to adult male Wistar rats on parturition rate**

Group I (control; 1% CMC) recorded the highest parturition rate of 100% (4/4; Figure 1). The first set of pups recorded in group I were littered on day 22 post-pairing of the female and the male rats (D22). Recorded days of parturition for the four females in group I were D22, D25, D29 and D44 post-pairing. Group II (1000 mg/kg CP) recorded 75% (3/4), group III (100 mg/kg ISF) had 25% (1/4), and group IV (CP + ISF) recorded 75% (3/4) parturition rate each. The first set of litters recorded in group II started from day 33 post-pairing (D33). The parturition days recorded for the 3 females in group II were D33, D38 and D43 post-pairing. Day 44 post-pairing (D44) was recorded for the only parturition in group III. Then, group IV (also 75%) had its first set of litters recorded on D35. The days of parturition recorded for the three females in group IV were D35, D39 and D47 post-pairing.

The current study is an indication that soy isoflavones administration can cause decrease in conception rate and prolong gestation length in female Wistar rats mated with exposed male Wistar rats. This is in agreement with the reports of Glover & Assinder [43], who reported transient reduction in fecundity in the female rats mated with adult male rats exposed to diet of high phytoestrogen content. An effect which coincides with reduced expression of epididymal androgen receptor (AR) and estrogen receptor- $\alpha$  (ER $\alpha$ ), indicating disrupted epididymal function. It was proposed that the reduction in fecundity resulted from reduced steroid-regulated antioxidant protection in the epididymis, leading to oxidative damage and loss of sperm function. These changes were similar to those induced by genistein in the adult rat prostate [44]. Also, Nurdiana *et al.* [45] reported that the disruption of spermatogenesis and infertility by isoflavones is attributed to the phytoestrogen compounds present in soybean which can down regulate mRNA from FSH receptors, inhibin  $\alpha$ , INH $\beta$ B, ABP, and transferrin in Sertoli cells.



**Figure 1:** Parturition rates of female Wistar rats mated with adult male rats treated with methanol extract of *Curculigo pilosa* and soy isoflavones.

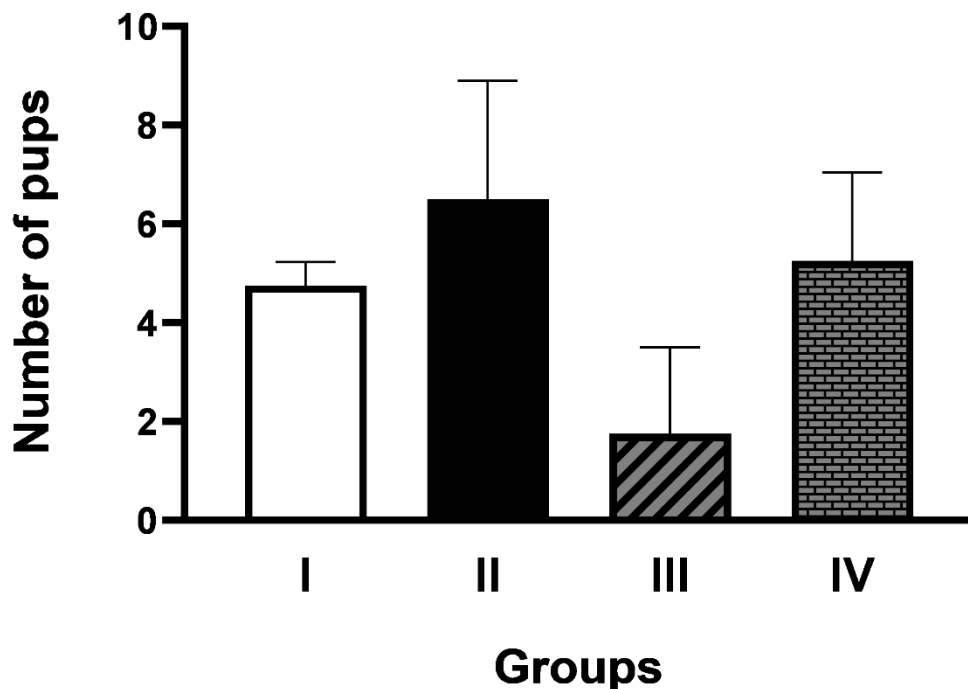
**Key:** I = 1% CMC; II = 1000 mg/kg CP; III = 100 mg/kg ISF; IV = 1000 mg/kg CP+100 mg/kg ISF; CMC = carboxymethyl cellulose; CP = *Curculigo pilosa*; ISF = soy isoflavones

#### Effect of *Curculigo pilosa* and soy isoflavones administration to adult male Wistar rats on litter size

Group I (control; 1% CMC) had the highest parturition rate (100%), but all the four females had total of 19 pups. The recorded mean number of pups was  $4.750 \pm 0.48$ , while three females in group II (1000 mg/kg CP) littered a total of 26 pups, with average of  $6.500 \pm 2.39$ . Only one female in group III (100 mg/kg ISF) had seven pups, and the recorded average number of pups was  $1.750 \pm 1.75$ . The average number of pups in group IV (CP + ISF) was  $5.25 \pm 1.79$  as 21 pups were delivered by three females. Group II recorded the highest average litter size, followed by group IV, then group I, and lastly group III (Figure 2). There were significant ( $P < 0.05$ ) differences observed between group III and the control and other treatment groups.

The highest average litter size obtained in the 1000 mg/kg CP-treated group IV of course may be

attributed to the pro-fertility quality of its phytoconstituents such as phenolics, flavonoids, terpenoids, saponin, tannins, steroids and cardiac glycosides. These are all antioxidant constituents of the plant having positive influence on the reproductive indices [46]. They have been reported to prevent lipid peroxidation which is attributed to their ability to boost the testicular non-enzymatic and enzymatic antioxidants in order to effectively scavenge for free radicals [46, 47, 48], as evidenced in the overall sperm count, motility and viability, and decreased abnormal sperm morphology in this study. And the higher number of pups recorded in group IV (CP + ISF) than that recorded in group III (100 mg/kg ISF) is indicative of CP to cushion the negative effect of ISF. In offspring whose parents were fed genistein, [49] reported reduction in litter size in the 500ppm group of the F2 generation, together with significant decreasing dose trends for litter size in the F1 and F3 generations.



**Figure 2:** Average litter size of female Wistar rats bred with adult male rats treated with methanol extract of *Curculigo pilosa* and soy isoflavones. <sup>a,b</sup> = values with error bars with different letters are significantly ( $P < 0.05$ ) different.

**Key:** I = 1% CMC; II = 1000 mg/kg CP; III = 100 mg/kg ISF; IV = 1000 mg/kg CP+100 mg/kg ISF; CMC = carboxymethyl cellulose; CP = *Curculigo pilosa*; ISF = soy isoflavones

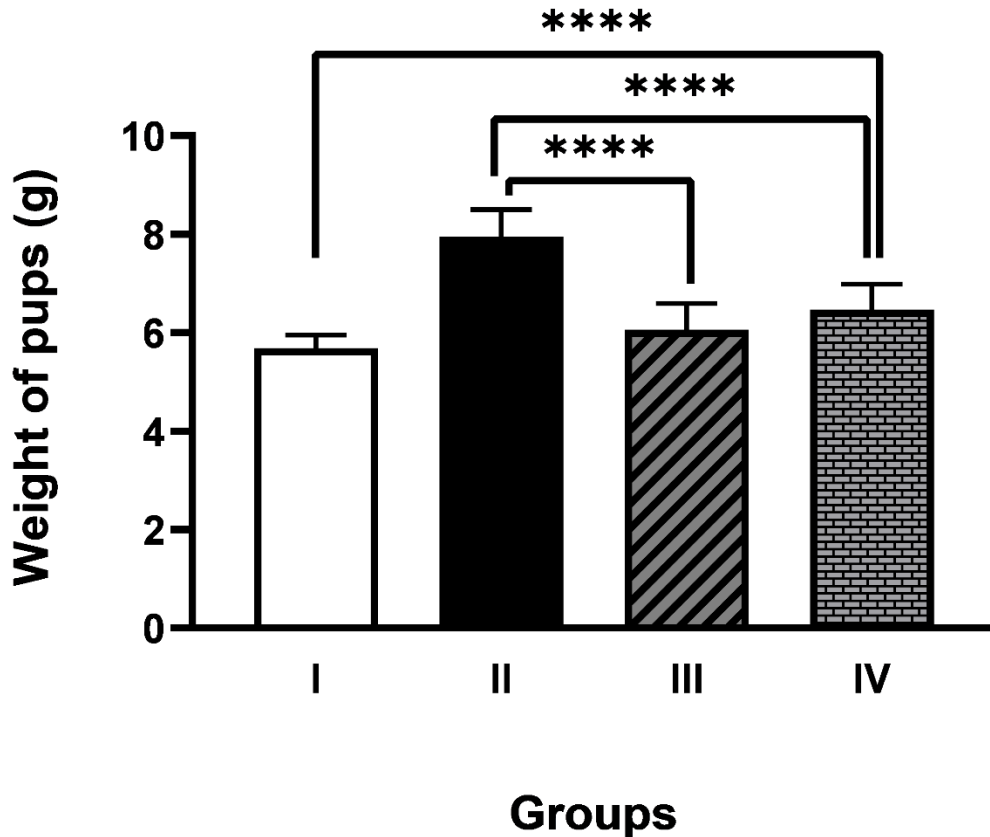
#### Effect of *Curculigo pilosa* and soy isoflavones administration to adult male wistar rats on weight of pups

As shown in Figure 3, the body weight recorded for pups in groups I, II, III and IV were  $5.689 \pm 0.06$  g,  $7.950 \pm 0.11$  g,  $6.057 \pm 0.20$  g and  $6.471 \pm 0.11$  g, respectively. Group II recorded the highest significant ( $P < 0.05$ ) pup body weight compared to groups I, III and IV. There was also a significant ( $p < 0.05$ ) difference between the recorded average weight of pups in group I and that recorded in group IV.

This study recorded the lowest average body weight of pups in group III (100 mg/kg ISF-treated sire), and the highest average body weight of pups in group II (1000 mg/kg CP-treated sire) of significant difference. Soy isoflavone supplementation have been reported to be beneficial for reduction of body weight in obese and, or diabetic menopausal and post-menopausal women, and in animal models [50, 51, 52, 53]. And studies have shown that micronutrients from both parents have an impact on the epigenetic programming of the offspring, and can have long-lasting effects on the health of the offspring up till in

adult life [54]. The effect on weight reduction may be attributed to soy isoflavone ability to dose-dependently reduce food intake, and its chronic daily administration (14 days only) was able to reduce liver fat content, accompanied by high leptin and low adiponectin, thereby affecting transcription factors and lipogenic enzymes, particularly the pivotal enzyme in obesity, stearoyl co-enzyme A desaturase 1 [51]. It has been shown that soy isoflavones administered to male Wistar rats can cause DNA methylation leading to generation effect that can result in damping the growth of their offspring [55]. Disruption of growth can be caused by phytoestrogen compounds in soybean extracts which can affect growth genes [56], suggesting that isoflavones in the soy attenuate the body mass and overall growth performance [57]. Previously reported phytochemical studies proved that *C. pilosa* contains enzymes like amylase, cellulase, lipase, alkaline phosphatase, carboxypeptidase [58], which are involved in the digestion of dietary carbohydrates and lipids. These enzymes positively affect the digestion of ingested food, thus making the substances readily

available for absorption, and thus promote body weight gain.



**Figure 3:** Average weight of pups of Wistar rats bred by adult male rats treated with methanol extract of *Curculigo pilosa* and soy isoflavones. \*\*\*\* =  $P < 0.0001$

**Key:** I = 1% CMC; II = 1000 mg/kg CP; III = 100 mg/kg ISF; IV = 1000 mg/kg CP+100 mg/kg ISF; CMC = carboxymethyl cellulose; CP = *Curculigo pilosa*; ISF = soy isoflavones

#### Effect of *Curculigo pilosa* and Soy Isoflavones Administration to Adult Male Wistar Rats on Histo-morphological Changes of testis

Group I treated with 1% CMC on day 55: There were normal histoarchitecture of the spermatogonia and Sertoli cells resting on the basement membrane. As shown in Plate I, spermatozoa were present in the lumen of some normal tubules with their tails extending into the middle of the lumen. The spermatocytes and spermatids were also normally distributed above the spermatogonia within the Sertoli cells. Some epithelia appeared shorter with less tails seen, extending into the lumen of the tubules. There were clear separations between the spermatogonia and spermatocyte beddings, indicating spermatozoa evacuation with delayed spermiogenesis. Leydig cells

were normally arranged in the interstitium, but with thick-walled associated blood vessels.

Group II treated with 1000 mg/kg methanol extract of *C. pilosa* on day 55: The section of a rat testis in this group showed normal histological architecture (Plate II). The spermatogonia and Sertoli cells were normally arranged over the basement membrane, and the spermatocytes and spermatids were normally arranged over them. The spermatozoa had their tails extended into the lumen of the tubules. The Leydig cells and associated blood vessels were also arranged within the interstitial spaces.

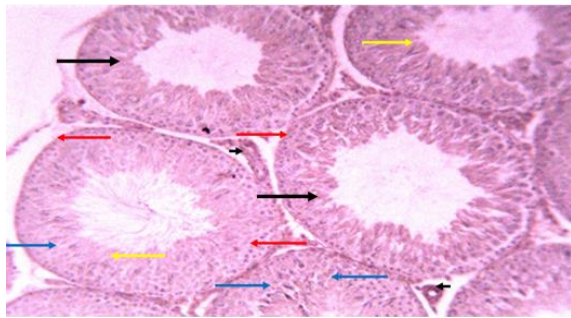
Group III treated with 100 mg/kg of soy isoflavones on day 55: Fifty-five days post-treatment, the testis of a rat treated with 100 mg/kg ISF showed severe degeneration and depletion of germ cell organisation, characterized by tubular dissolution, vacuolation of Sertoli cell cytoplasm (micro-vacuolation) and other germ cells (macro-vacuolation),

as well as disorganisation and loss of germ cells. The presence of spermatid retention and maturation depletion in some tubules was observed. There were widened interstitial spaces with lymphocytic infiltrations plus loss of most Leydig cells (Plate III).

Group IV: Treated with 1000 mg/kg *Curculigo pilosa* + 100 mg/kg of soy isoflavones: Plate IV shows the histoarchitecture of the testis of a rat in group IV (co-administration of CP and ISF (1000 mg/kg CP + 100 mg/kg ISF)) on day 55. Seminiferous tubules showed normal Sertoli cells and germinal cells arrangement along the basement membrane comprising of the spermatogonia, spermatocytes and spermatids. Spermatozoa arranged within the lumen had their tails

extending into the lumen. The Leydig cells appeared normal within the interstitial spaces.

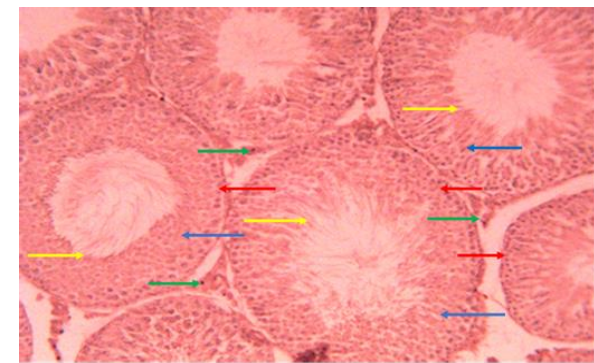
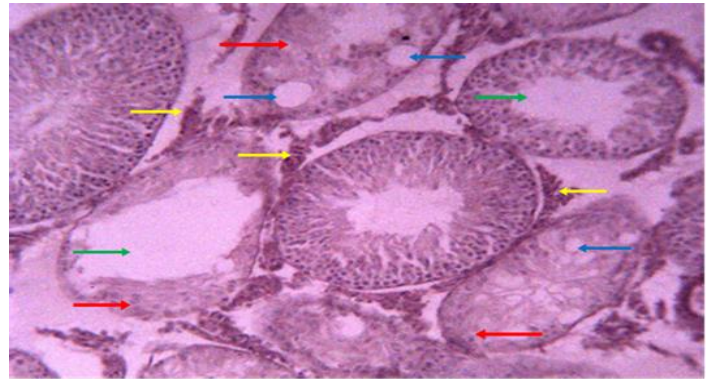
Mineral composition of CP, can promote sperm quality and can have an extension effect on speed of the growth process in offspring. The pro-fertility activity of CP could also be as a result of Curculigoside ( $C_{22}H_{26}O_{11}$ ), which is the major bioactive component of *Curculigo pilosa*, *C. orchioides*, *C. sinensis* and others [59, 60]. Curculigoside has been reported to drastically decrease caspase-3 activity and p53 mRNA expression, both of which are known to be important in  $H_2O_2$ -induced cell apoptosis [61].



**Plate I:** Transverse section of testis of a control rat (1% CMC) stained with H and E on day 55.

Note: Normal spermatogonia and Sertoli cells on basement membrane (red arrow), and spermatocytes and spermatid (blue arrow) above them. Shorter epithelium and less spermatozoa in some tubules (black arrow). Normal tubules with spermatozoa with tails extending into the lumen (yellow arrow). Normally arranged Leydig cells in the interstitial space with thick-walled blood vessels were seen (short black arrow).

spermatocytes and spermatids (blue arrow), and spermatozoa in the lumen of tubules with extended tails into the lumen (yellow arrow). Normally arranged Leydig cells and associated blood vessels in the interstitial spaces (green arrow).

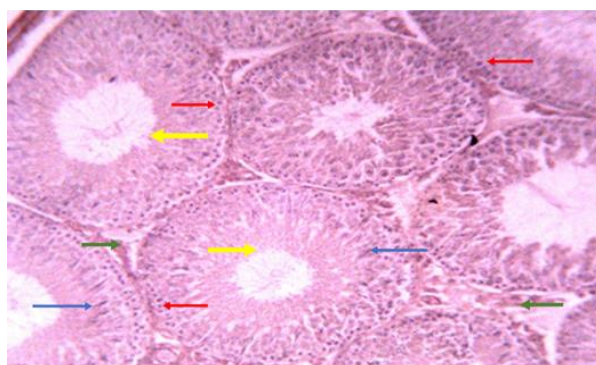


**Plate II:** Cross section of the testis of a rat in group II stained with H and E (1000 mg/kg CP) on day 55.

Note: Normal arrangements of spermatogonia and Sertoli cells (red arrow), normal distribution of

**Plate III:** Cross section of testis of a rat in group III (100 mg/kg) stained with H and E 55 days post-treatment.

Note: Severe degeneration and depletion of germ cells (red arrow), severe vacuolations (blue arrow) and azoospermia (green arrow). Widened interstitial spaces plus lymphocytic infiltration, and loss of most Leydig cells (yellow arrow) were observed.



**Plate IV:** Testis of rat in group IV (1000 mg/kg CP + 100 mg/kg ISF) stained with H and E on day 55.

Note: Normal histological architecture. The spermatogonia and Sertoli cells were normally arranged over the basement membrane (red arrow), and the spermatocytes and spermatids normally arranged over them (blue arrow). The spermatozoa have tails extended into the lumen of the tubules (yellow arrow). The Leydig cells and associated blood vessels were arranged within the interstitial spaces (green arrow).

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