Therapeutic and fertility restoration effects of *Ionidium suffruticosum* on sub-fertile male albino Wistar rats: effects on testis and caudal spermatozoa

Kuppusamy Chenniappan and Kadarkari Murugan

Department of Zoology, Bharathiar University, Coimbatore, India

**ABSTRACT**

**Context:** *Ionidium suffruticosum* (L.) Ging (Violaceae) is an important medicinal plant widely used as a herbal traditional medicine in Ayurveda for the treatment of infertility. Currently, little pharmacological information is available on its male fertility properties following prolonged use.

**Objective:** To investigate *I. suffruticosum* leaf extracts for male fertility parameters.

**Materials and methods:** The ethanol lyophilized fraction was administered orally on carbendazim-induced sub-fertility rats (250 mg/kg body weight for 28 days). The effects of fractions on rat’s fertility parameters i.e., body and testes weight, sperm motility, sperm vitality, epididymal sperm counts, its morphology, enzyme and antioxidant stress and histopathology were studied and compared with clomiphene citrate.

**Results:** The sub-fertile male rats treated with *I. suffruticosum* leaf extract increased the body weight of 7 g, testis weight of 97 mg, increased cauda epididymal sperm counts of 34.2 x 10^6 sperm/mL, motility of sperm 46% and vitality 28% also increased and normal sperm morphology also improved up to 32%. The carbendazim-treated group showed loss in body weight of 33 g, testis weight of 851 mg, decreased epididymal sperm counts of 15 x 10^6 sperm/mL, with sluggish motility and a highly significant fall in the live sperms of about 57%.

**Discussion and conclusion:** The leaf fraction of *I. suffruticosum* increased the testicular weight, spermatogenesis, sperm counts, lessened sperm agglutination, and increased testicular oxidative biomarkers, SOD, and CAT. This study therefore supports the usage of *I. suffruticosum* in traditional medicine for infertility.

**Introduction**

In the recent times, there has been serious concern about the deterioration of human semen quality. It is generally believed that the counts of viable sperm in the normal human ejaculate have decreased to half over a period of about 50 years (Carlson et al. 1992; Bradbury 1997) and the causes of male infertility due to oligosperma or azoosperma are also on the increase (Jager et al. 1992; Bradbury 1997) and the causes of male infertility due to oligosperma or azoosperma are also on the increase (Jager et al. 1992; Bradbury 1997). On access into non-target organisms including man, it can manifest effects disrupting the microtubules. As microtubules constitute an important aspect of male reproduction, maintaining cytoskeletal support of the Sertoli cell (Nakai et al. 1995) and other epithelial cells, the ectoplasmic specialization of the sertoli cell into germ cell associations (Nakai et al. 1995) and other epithelial cells, the ectoplasmic specialization of the sertoli cell into germ cell associations (Nakai et al. 1995) and cell division apparatus in male germ cells (Nakai & Hess 1997), carbendazim could be a serious male reproductive toxicant.

Traditional knowledge related to the use of natural resources including medicinal plants has been recognized as one of the important assets inherited through generations by the local communities. The tribal communities such as Gowlis Kaani, Kunabhis and Siddis inhabiting the Western Ghats region are one of the richest knowledge systems on tribal medicines in India; the treatment of their diseases is almost entirely confined to herbal medicines. Several herbals are known to potentiate the male reproductive function and increase the sperm counts.

*Eagle marmelos* (L.) Correa (Rutaceae) is a popular plant, with rich medicinal properties (Bhattacharya 1986), the leaves of this plant are used to treat spermatorrhea (Bhattacharya 1986). *Eurycoma longifolia* Jack (Simaroubaceae) root extract treated rats retained a higher level of sexual activity and also showed
The aqueous extracts of *Cynomorium coccineum* (L) (Cynomoriaceae) and *Withania somnifera* (L) Dunal (Solanaceae) showed direct spermatogenic influence on the seminiferous tubules of the immature rats as well as serum levels of testosterone. *Withania somnifera* is recommended and used to treat for impotence or seminal debility, as an aphrodisiac and as an invigorator (Sankaran 1984). *Withania somnifera* roots have many steroidal lactones, which have structural similarities with that of androgens (Tripathy et al. 1996). Yohimbine a drug obtained from *Rauvolfia* species is also reported as having a stimulatory effect on spermatogenesis in rats (Rastogi & Malhotra 1990).

*Ionidium suffruticosum* Ging (Violaceae) is a sporadic, rare, ephemeral, ethnomedicinal herb (Deshpande 2006) widely used in traditional healers to treat the diseases such as jaundice, male sterility (Kheraro & Bouquet 1950; Senthil Kumar et al. 2013), diabetes (Sarita et al. 2004), malaria (Soh & Benoit-Vical 2007), urinary tract infections and water retention (Pushpagadan & Atal 1984) and also used as a tonic. The fruits are antitode for scorpion-sting. The leaves are sub-sessile, linear to oblanceolate, 1.5–2.0 × 0.08–0.3 inches, entire or serrate, flowers solitary, axillary, red, spurred, fruit a small sub globose capsule containing ellipsoid, longitudinally striate, yellowish white seeds. The leaves and tender stalks are demulcent and used as a decoction or electuary, in conjunction with oil, employed in preparing cooling liniment for the head. The plant is a seasonal perennial herb and is widely distributed in Africa, Madagascar, Sri Lanka, China, New Guinea, tropical Australia and India. In nature, the plants appear for only few months from June to September (monsoon season). The roots and few basal stem stocks remaining in the soil regenerate only during rainy seasons and soon after the rainy season the plants dry and disappear. As less research was carried out on male fertility, an attempt was made in search for the traditionally used medicinal plants i.e., *I. suffruticosum*, to cure male impotency. It would be important to confirm the fertility effects of *I. suffruticosum* in carbendazim-induced sub-fertile male albino rats and compared with that of standard fertility drug clomiphene citrate.

**Materials and methods**

**Plant extracts preparation**

The leaves of *I. suffruticosum* were collected locally from the foothills of the Western Ghat’s area adjacent to Bharathiar University, Coimbatore, Tamil Nadu, India in mid-2008. Botanic identification was first conducted in the field and further confirmed by P. Mahendran, Scientist, the Botanical Survey of India, Tamil Nadu Agricultural University Campus, Coimbatore, Tamil Nadu, India. The voucher herbarium specimen was deposited at the departmental General Library, School of Life Sciences, Bharathiar University, Coimbatore, India. The leaves were washed with double-distilled water and were shade dried at room temperature. The dried parts were powdered with the help of an electric blender. Crude extractions from the plants were performed in the medicinal chemistry laboratory of the School of Life Sciences, Bharathiar University, Coimbatore, India. The powdered dried leaves (50g) were subjected to ethanol in a Soxhlet apparatus (Borasil, Mumbai, India) for 72 h (Saxena et al. 1994), and the solvents were removed from the plant extracts in a vacuum rotary evaporator at 80°C under reduced pressure yielded a semi-solid materials of 11 g. Chloroform and diethyl ether (1:3, v/v) were added to semisolid materials. The resulting mixture was stirred using a rotary (120 rpm) shaker (Gerhardt, Königswinte, Germany) at 20°C for 5 h. The content was later filtered through Whatman grade 1 filter paper. The filtrate was again passed through a round filter paper of 90 mm diameter with the help of mini filter pump machine. Fraction of organic filtrate collected this way were pooled together, kept in wide mouth amber bottles. The organic solvent was then removed with the help of a rotary evaporator and afterwards subjected to lyophilization procedure. The lyophilization was done using a freeze dryer (Labconco Corporation, Kansas City, MO).

**Experimental animals**

Healthy adult male albino rats (Wistar strain) weighing 150–180 g, procured from Tamil Nadu Veterinary and Animal Sciences University Chennai, were used in this investigation. The animals were housed in well-ventilated polyurethane cages at normal room temperature, fed with standard rat pellet diet and clean drinking water *ad libitum*. Handling of the animals was done in accordance with the Guide for Care and Use of Laboratory Animals, Bharathiar University; this work was approved by the ethical committee for using animals (no. 721/02/R/CPCS EA).

**Clomiphene citrate**

The fertility effects of *I. suffruticosum* leaf extracts were compared with a minimal dose (0.03 mg/kg) of clomiphene citrate 2-p-(2-chloro-1, 2-diphenylvinyl) phenoxy] triethylamine dihydrogen citrate, clomiphene citrate 25 mg Fertyl-M Tablets, Ar-Ex Laboratories Pvt Ltd, Goregaon (E), Mumbai, India. Stock solutions of 25 mg/mL is prepared and test solutions are prepared accordingly by adopting $V_1C_1 = V_2C_2$ approach.

**Carbendazim**

Carbendazim (methyl 2-benzimidazole carbamate) (Bavistin, BASF-India Ltd, Mumbai) was purchased from a local agrochemical supplier. The product powder contains carbendazim at 50% strength (w/w).

**Carbendazim treatment**

Bavistin (carbendazim) was suspended quantitatively in refined sunflower oil in such a way as to prepare 100 mg powder in 1 mL oil (50 mg carbendazim active compound in 1 mL). Each animal weighing about 150 g received Bavistin at a single bolus dose of 800 mg/kg body weight (i.e., 400 mg carbendazim active compound per kg body weight) and the control rat received equal quantity of the sunflower oil only.

Experimental design was done as below. The albino rats were divided into four groups and each groups comprising of five animals.

- **Group-1:** Served as control. One group received 1 mL of water orally as vehicle and another group received plant extract of *I. suffruticosum* by using an intragastric catheter (IGC) for 28 days.
- **Group-2:** Carbendazim-induced sub-fertile male rats. They received Bavistin (carbendazim) at a single bolus dose of 800 mg/kg (i.e., 400 mg carbendazim active compound per kg body weight) through oral route by using an IGC.
- **Group-3:** Carbendazim-induced sub-fertile male rats treated with the leaf extracts of *I. suffruticosum* orally by using an IGC (250 mg/kg/day) for 28 days.
Group-4: Carbendazim-induced sub-fertile male rats treated with clomiphene citrate (0.3 mg/kg/day) orally by using an intragastric catheter for 28 days.

All groups are maintained for 28 days with food and water ad libitum.

Harvest and preparation for analysis

The rats were weighted before and after the treatment. At the end of the treatment, the rats were subjected to anaesthesia and the testes were removed and washed in physiological saline. The right testis was weighted in an electronic balance and data for each group were used to calculate the respective means and the standard deviation. Slices of left testis were fixed in Bouin’s fluid and used for histological analysis. The cauda epididymis from the right side was transferred on to a cavity slide. After washing in physiological saline of 0.9% NaCl, the cauda was incised at two or more places allowing the semen to ooz out. The semen was used for the several spermatological analyses.

Spermatological studies

The cauda epididymal duct on one side was exposed and incised. The sperm oozing out from the incision was quickly sucked into a capillary tube up to 0.05 mL mark. It was diluted 200 times using phosphate buffer saline. After thorough mixing, the dilute semen was used for the spermatological analysis.

Sperm motility assessment

A drop of dilute sperm was transferred with help of Pasteur pipette on to a cover glass. The cover glass was inverted over the cavity slide to obtain a hanging drop. The edges of the cover slip were sealed using Vaseline. The hanging drop preparation was observed under a compound microscope at 450 magnification. The preparation was observed at regular intervals in such a way as to find the duration, in minutes, of the motility of the last motile sperm. For each animal, two separate hanging drop preparations were made, and motility was assessed by two independent observers. The data for each animal were used to obtain the average.

Sperm vitality

Sperm vitality is assessed by adding one drop of eosin stain Y and one drop of Nigrosine in an Eppendorf tube. To this, one drop of semen was added and mixed using a Pasteur pipette. Drop of the mixture was placed on a micro slide and covered with coverslip and observed for at least 200 spermatozoa under microscope and considered as stained spermatozoa as dead and unstained as alive.

Sperm counts and morphology

Sperm counts were made according to Gopal and Shah (1985), briefly diluted and thoroughly mixed semen was transferred to improved Neubauer counting chamber and a cover slip was overlaid. The Neubauer chamber was observed under a compound microscope and sperm in Central Square were counted. The central square has 25 large squares. The volume of each of the 25 squares is 0.1 mL. The sperm counts were calculated using the following formula:

\[ = \frac{(\text{Number of sperm in 25 squares}/25) \times 10 \times \text{dilution factor} \times 1000}{\text{sperm/mL}} \]

Sperm morphology was observed adopting Eosin–Nigrosin methods. One or two drops of Eosin–Nigrosin stain and a drop of semen were separately placed on the one end of a clean warm microscopic glass slide. The semen and the stain were mixed well and drawn out with the edge of another slide which served as a spreader so that, a thin film was and air dried and was observed under microscope. At least 200 spermatozoa from different fields of the slide were examined for their morphological features.

Sperm agglutination

To find the agglutination, a drop of diluted semen was smeared on a clean slide, fixed in ethanol and stained with the Leishman stain. Then, the slide was examined under 450 × to find the incidence of sperm agglutination.

Oxidative stress and antioxidant enzyme assays

The antioxidant and oxidative stress of carbendazim-induced sub-fertile rats were measured. The lipid peroxidation (LPO) was estimated by adopting methodology of Ohkawa et al. (1979). Superoxide dismutase (SOD) activity was measured using methods of Rest et al. (1977). Catalase (CAT) activity was determined from the method of Aebi et al. (1974) by measuring the rate of decomposition of hydrogen peroxide. Briefly, the testis and tissues were homogenized in 0.1 M phosphate buffer and centrifuged at 18,000 RPM for 30 min and the supernatant was utilized for biochemical analysis.

Histological analysis

The histology of the testis was studied adopting the paraffin method by Humason (1979) and sections were observed under microscope and photographed at 100×, 200× and 450× magnification.

Statistical analysis

The statistical data were analyzed using Student’s t-test (Olsen et al. 1995) and expresses as mean ± Standard Error of Mean (SEM) and calculated as:

\[ \text{SEM} = \frac{\sqrt{\sum x^2 - (\sum x^2/n)}}{n(n-1)} \]

where \( x \) = individual observations, \( n \) = number of observation, value was calculated by the following formula and compared by the table value of 5% and1% levels of significance.

\[ T = \frac{X_1 - X_2}{S\sqrt{(1/n_1 + 1/n_2)}} \]

where

\[ S = \sqrt{\sum x_1^2 - (\sum x_1^2/n_1) + \sum x_2^2 - (\sum x_2^2/n_2)} \]

\( n_1 \) and \( n_2 \) denotes the number of observed value in the classes being compared (Olsen et al. 1995).
The following levels of significance were used. The values falling in $p < 0.01$ levels were considered significant data and $p > 0.01$ are non-significant data. One-way ANOVA with post hoc Tukey’s HSD Test Calculator with the Scheffé multiple comparison tests was used.

Results

**Effect of I. suffruticosum on body and testis weight in carbendazim-induced sub-fertile male rats after 28 days of the treatment**

**Body weight**

The results of the body and testicular weight of the experimental animals are shown in Table 1. The body weight of the control rats (group 1) were 164 g. After 28 days, there was a normal weight gain in the body weight of 8–9 g leading to 172 g (saline) to 174 g (extract). But, in the carbendazim induced sub-fertile rat in the group 2, there was a significant loss in the body weight of about 33 g when compared to its initial weight of 158 g. In group 3, rats treated with I. suffruticosum have shown a loss in the body weight of about 24 g from the initial weight of 156 g to the final weight of 132 g. In group 4, the rats treated with clomiphene citrate had a loss in the body weight of about 30 g from the initial weight, when compared to carbendazim-induced sub-fertile group 2 rats, the remaining treated groups 3 gained a slight gain in the body weight and are statistically significant ($p < 0.01$).

**Weight of the testis**

The weights of the right testis of the group 1 rats were 949 mg/100 g of body wt. The weights of the right testis of the group 1 rats were 1248 mg/100 g of body wt. When compared to group 2 treated animals, the remaining group 3 rats have the testicular weight was 906 mg/100 g body wt. When compared to group 2 rats and are statistically significant.

| Experimental group                | Body weight (g) | Testis weight (mg/kg body wt) ± SEM | Caudal epididymal sperm counts × 10^6 sperm/ml ± SEM |
|----------------------------------|----------------|-------------------------------------|------------------------------------------------------|
| Group 1: Control (saline only)   | 164 ± 0.6      | 1248 ± 1.4                          | 40.50 ± 0.5                                          |
| Control (extract only)           | 164 ± 0.7      | 1258 ± 1.2                          | 49.0 ± 0.7                                           |
| Group 2: Carbendazim-induced subfertile male rats | 158 ± 0.7 | 851 ± 0.3                           | 15.40 ± 0.3                                          |
| Group 3: Carbendazim-induced subfertile male rats treated with I. suffruticosum | 156 ± 0.4* | 948 ± 0.4*                          | 49.60 ± 0.5*                                         |
| Group 4: Carbendazim-induced subfertile male rats treated with Clomiphene citrate | 150 ± 0.7 | 906 ± 0.3                           | 45.50 ± 0.4                                          |

Each value is Mean ± SEM of five animals.

*Significantly different ($p < 0.05$) from the control group by Dunnett’s test.

Scheffé multiple comparison tests used to identify the pairs of treatments are significantly different from each other. Control saline vs Group 3, control saline vs Group 4, Group 3 vs Group 4 are statistically insignificant $p > 0.01$.

**Effect of I. suffruticosum on cauda epididymal sperm counts in carbendazim-induced sub-fertile male rats after 28 days of treatment**

The caudal epididymal sperm counts of the experimental animals are shown in Table 1. The group 1 control rats having normal epididymal sperm counts of about 40.50–49.0 × 10^6 sperm/mL. But, in group 2 carbendazim-induced sub-fertile rats, the counts significantly decreased to about 15 × 10^6 sperm/mL and the difference compared with the control group was highly significant. In the group 3 carbendazim-induced sub-fertile rats treated with I. suffruticosum have observed a tremendous increase in the sperm count of about 49 × 10^6 sperm/mL. In group 3 rats, epididymal sperm count was comparatively higher than that of other groups. In group 4 rats treated with clomiphene citrate the epididymal sperm counts were 45 × 10^6 sperm/mL. The increased sperm counts were observed in group 3 than that of group 1 and group 2 rats and are statistically significant.

**Effect of I. suffruticosum on cauda epididymal sperm motility and pattern in carbendazim-induced sub-fertile male rats after 28 days of the treatment**

Cauda epididymal sperm motility and pattern are shown in Table 2. In the control rats, the cauda epididymal sperm motility and pattern (Min) 55.17 ± 0.4

| Experimental groups                  | Cauda epididymal sperm motility and pattern (Min) |
|--------------------------------------|--------------------------------------------------|
| Group 1: Control (saline only)       | 55.17 ± 0.4                                       |
| Control (extract only)               | 60.12 ± 0.7                                       |
| Group 2: Carbendazim-induced subfertile male rats | 10.16 ± 1.8                                     |
| Group 3: Carbendazim-induced subfertile male rats treated with I. suffruticosum | 56.20 ± 0.8*                                    |
| Group 4: Carbendazim-induced subfertile male rats treated with Clomiphene citrate | 57.11 ± 0.2                                     |

Each value is Mean ± SEM of five animals.

*Significantly different ($p < 0.05$) from the control group by Dunnett’s test.

Scheffé multiple comparison tests used to identify the pairs of treatments are significantly different from each other. Control saline vs Group 3, control saline vs Group 4, Group 3 vs Group 4 are statistically insignificant $p > 0.01$.  

Each value is of Mean ± SEM of five animals.

*Significantly different ($p < 0.05$) from the control group by Dunnett’s test.
exhibited rapid and progressive motility and it lasted for about 55.17 to 60.12 min. The group 2 rats treated with carbendazim alone, the sperms exhibited very sluggish motility for about 10.16 min. During investigations, we have observed a tremendous change in the pattern and duration of motility in group 3 *I. suffruticosum*-treated rats which last for about 56.20 and the pattern was rapid and progressive. Group 3 rats treated with *I. suffruticosum*, the duration of the motility and the pattern was highly significant when compared to group 2 treated animals. In group 4 animals treated with clomiphene citrate sperms exhibited rapid and progressive motility with duration of about 57.11 min. The group 3 experimental animals’ sperm motility and pattern was highly significant compared with that of group 2 animals treated with carbendazim.

**Effect of I. suffruticosum on cauda epididymal sperm vitality in carbendazim-induced sub-fertile male rats after 28 days of treatment**

The results of the sperm vitality in experimental animals are shown in Table 3. In group 1 control rats, the live sperms were about 83–91% and the dead sperms were 16–96%. After carbendazim treatment in group 2 rats, a highly significant fall in the live sperms of about 57% and a highly significant rise in the number of dead sperms of about 43%. In group 3 rats treated with *I. suffruticosum*, a highly significant rise in the percentage of live sperms to about 85% when compared to group 2 rats. In group 4 rats treated with clomiphene citrate, the percentage of live sperms were 83%. In group 3 and 4, the sperm vitality was superior to that of group 2-treated animals and are statistically significant.

**Table 3. Effect of Ionidium suffruticosum on Cauda epididymal sperm vitality and spermmorphology in Carbendazim induced subfertile male rats after 28 days of treatment.**

| Experimental groups | Sperm vitality | Sperm morphology |
|---------------------|----------------|-----------------|
|                     | Live sperms (%) | Dead sperms (%) | Normal sperms (%) | Abnormal sperms (%) |
| Group-1: Control (saline only) | 83 ± 0.6 | 17 ± 0.5 | 93 ± 0.5 | 7 ± 0.8 |
| Control (extract only) | 91 ± 0.5 | 9 ± 0.3 | 97 ± 0.5 | 3 ± 0.3 |
| Group 2: Carbendazim induced subfertile male rats | 57 ± 0.4 | 43 ± 0.4 | 58 ± 0.5 | 42 ± 0.5 |
| Group 3: Carbendazim induced subfertile male rats treated with *I. suffruticosum* | 85 ± 0.4* | 15 ± 0.4* | 90 ± 0.7* | 10 ± 0.3* |
| Group 4: Carbendazim induced subfertile male rats treated with clomiphene citrate | 83 ± 0.4 | 17 ± 0.3 | 87 ± 0.4 | 13 ± 0.7 |

Each value is of Mean ± SEM of 5 animals.

*Significantly different (p < 0.05) from the control group by Dunnett’s test.

Scheffe multiple comparison tests used to identify which of the pairs of treatments are significantly different from each other. Control saline Vs Group 4 in live sperms, Control saline Vs Group 3, Control saline Vs Group 4, Group 3 Vs Group 4 in DTT, Control saline Vs extract, Control saline Vs Group 3, Control extract Vs Group 3 in TFFAF, Control saline Vs Group 3, Group 3 Vs Group 4 in DTH, Control saline Vs extract, Control saline Vs Group 3, Control extract Vs Group 3, Control extract Vs Group 3 in COF and all groups from DTT are statistically insignificant p > 0.01 and in normal and abnormal sperms counts all groups are statistically significant p < 0.01.

**Table 4. Percentage of predominant abnormalities of sperm observed in different experimental groups.**

| Experimental Groups | Predominant abnormalities (%) |
|---------------------|------------------------------|
|                     | TFFAF | DTH  | FAS  | BMP  | DTT  | COF  | TCOF  |
| Group-1             |       |      |      |      |      |      |       |
| Saline only         | 2 ± 0.3 | 3 ± 0.3 | 1 ± 0.3 | 0 ± 0 | 1 ± 0.4 | 0 ± 0 | 0 ± 0 |
| Extract only        | 1 ± 0.3 | 0 ± 0 | 0.5 ± 0.1 | 0 ± 0 | 1 ± 0.3 | 0 ± 0 | 0.5 ± 0.1 |
| Group-2             | 10 ± 0.7 | 13 ± 0.7 | 9 ± 0.3 | 6 ± 0.5 | 2 ± 0.2 | 1 ± 0.3 | 1 ± 0.3 |
| Group-3             | 2 ± 0.3* | 4 ± 0.3* | 0 ± 0* | 1 ± 0.3* | 1 ± 0.4* | 0 ± 0* | 1 ± 0.3* |
| Group-4             | 3 ± 0.6 | 5 ± 0.3 | 1 ± 0.3 | 2 ± 0.3 | 2 ± 0.3 | 0 ± 0 | 0 ± 0 |

Each value is of Mean ± SEM of 5 animals.

*Significantly different (p < 0.05) from the control group by Dunnett’s test.

TFFAF: Sperm with head flexed, tip of the head facing away from the flagellum; DTH: Detached Head; FAS: Fusion/attachment of sperm at one or more points between two or more sperm. BMP: Broken middle piece; DTT: Detached Tail; COF: Coiling of the flagellum; TCOF: Twisting and coiling of the flagellum.

Scheffe multiple comparison tests used to identify which of the pairs of treatments are significantly different from each other. Control saline Vs extract, Control saline Vs Group 3, Control saline Vs Group 4, Control extract Vs Group 3 in TFFAF, Control saline Vs Group 3, Group 3 Vs Group 4 in DTH, Control saline Vs extract, Control saline Vs Group 3, Control extract Vs Group 3, Control extract Vs Group 3 in COF and all groups from DTT are statistically insignificant p > 0.01 and Control saline Vs Group 2, Control saline Vs Group 3, Control saline Vs Group 2, Control extract Vs Group 2, Group 2 Vs Group 3, Group 3 Vs Group 4 in FAS groups are statistically significant p < 0.01.
Further, germinal epithelial cell masses in a scattered manner admixture with the sperms. In group 3 rats treated with *I. suffruticosum*, a significant rise in the percentage of normal sperms to about 90% (Figure 1(A)) and the improvement over group 2 carbendazim-treated rats were significant. In group 4 rats treated with clomiphene citrate, the percentage of normal morphology was 90% as shown in Figure 1(B). The groups treated with *I. suffruticosum* and clomiphene citrate, the percentage of normal sperms was higher than that of group 2 carbendazim-induced untreated rats.

**Effect of *I. suffruticosum* on cauda epididymal sperm agglutination in carbendazim-induced sub-fertile male rats after 28 days of treatment**

Sperm agglutination in experimental animals treated with carbendazim and *I. suffruticosum* is carefully observed. The rate of sperm agglutination in the group 1 control was very minimal with 1–5%. In the carbendazim-treated group 2 rats, there was a significant rise in the sperm agglutination of about 32% and is shown in Figure 1(C). In the *I. suffruticosum* treated group 3 rats; there was a significant fall in the level of sperm agglutination of about 9% as shown in Figure 1 when compared to untreated group 2 rats. In the clomiphene citrate-treated group 4 rats, the sperm agglutination was 11%.

**Effect of *I. suffruticosum* on malondialdehyde and antioxidant enzyme levels in carbendazim-induced sub-fertile male rats after 28 days of treatment**

The results are presented in Table 5; there was a significant increase in malondialdehyde (MDA) levels of testes about 42.3 nmol/mL in animals treated with carbendazim for 28 days, which is significantly higher compared to control groups, which showed 27.9 nmol/mL. In the three group treated with *I. suffruticosum* showed decreased levels of MDA in testes of about 33.6 nmol/mL compared with carbendazim-induced untreated group rats. The clomiphene citrate treated rat groups also showed decreased levels of MDA of 32.8 nmol/mL but not significant with that of *I. suffruticosum*-treated rat groups.

The carbendazim-treated animals showed decreased testicular catalase (CAT) activity of 2.1 µmol/S/mL and superoxide dismutase (SOD) activities of 88.7 µ/ml compared with control groups of 4.2 µmol/S/mL and 136.2 µ/ml for CAT and SOD, respectively. The experimental rat group 2 treated with *I. suffruticosum* showed significant increased activity of CAT 3.8 µmol/S/mL and SOD 131.3 µ/ml, which is greater than the standard fertility drug clomiphene citrate CAD and SOD activities of 3.6 µmol/S/mL and 133.1 µ/ml, respectively.

**Effects of *I. suffruticosum* on the histopathological changes of the testes in the carbendazim-induced sub-fertile male rats after 28 days of the treatment**

In the control rats, the seminiferous tubules of the testes were compactly arranged, with tall seminiferous epithelium and a narrow lumen. The seminiferous epithelium consisted of tall and branched sertoli cells and germ cells belonging to different generation of the lineage spermatogonia, primary spermatocytes, secondary spermatocytes, round spermatid and elongating spermatids. The spermatids in the different steps in spermiogenesis, spermiated spermatozoa were present in the lumen. No atrophied tubules were seen in the control testes. The tubules of control rat spermatocytes were engaged in the meiotic division and cells in all the divisional stages including anaphase and telophase were noticed, and seminiferous epithelium above the level of spermatocytes was abundant with spermatids.

In the carbendazim-treated group 2 rats, the seminiferous tubules were disorganized. The loss of several pachytenant spermatocytes in the stage 3 tubules and the corresponding places appear empty are shown in Figure 1(E). In tubules, the entire mass of cells beyond spermatocytes (i.e., the round and elongating spermatids) appeared as detached and sloughing off germinal epithelium including Sertoli cells at a level from round spermatids are shown in Figure 1(G). In several tubules aggregations of sloughed material were present in the lumen. In the carbendazim-treated rats, the spermatocytes appeared in arrested cell division and cells in anaphase and telophase were totally missing, also the layer appeared as depleted. An extreme manifestation of carbendazim treatment was fibrosis of several seminiferous tubules and depletion of germinal layers in several other tubules are shown in Figure 1. The 30% atrophied tubules in the carbendazim-treated group 2 rats were observed.

In group 3 rats, the testes consisted of seminiferous tubules in a compact arrangement with tall seminiferous epithelium and a narrow lumen. The seminiferous tubules were in an organized manner and histologically were much better than the carbendazim-treated group 2 rats as shown in Figure 1(H). The seminiferous tubules invariably contained all the germ cell types in the
prescribed order and in a manner exactly comparable with that of a control rats. In *I. suffruticosum* treated rats, the seminiferous tubules recovered to a very extent and looks like the seminiferous tubule of a control rats as shown in Figure 1(J). In the seminiferous tubules, carbendazim-induced histopathological changes such as atrophy of the seminiferous tubules, degenerative epithelium, cavity formation due to killing of spermatocytes, sloughed of germinal epithelium and fibrosis were completely recovered in group 3 rats treated with *I. suffruticosum* after 28 days of treatment are shown in Figure 1(F).

We have observed seminiferous tubules as normal like control groups without any signs of atrophy condition in clomiphene
citrate-treated rats are shown in Figure 1(B). Treatment with leaf extracts of *I. suffruticosum* for 28 days resulted in normal histological features of the seminiferous tubules with compact cells arranged in an orderly manner, showing all stages of meiotic division. Clomiphene citrate completely reversed the carbendazim-induced changes in the seminiferous tubules. The histological picture of group 4 rats was better than group 2 carbendazim-treated rats by reducing atrophy of the seminiferous tubules and inhibiting the sloughing of germinal epithelium and improving the cell divisions of spermatogenesis.

**Discussion**

A lack of pharmacological and its toxicological information on medicinal plants considerably restrict their therapeutic application in ethnomedicine and ethnoveterinary medicine. Consequently, experimental pharmacokinetic and toxicological studies are paramount in the development of botanical products as safe and efficacious drugs (Wu et al. 2000). Changes in body weight and testicular weight rate are important parameters in prolonged experimental toxicity studies on effects of drugs and chemicals on laboratory animals. The results suggested that the untreated control group rats had a significant normal body weight gain, but the carbendazim-administered male albino rats showed a significant decrease in the body weight by inhibiting mitotic division. Edward and Keith (1986) reported that these fungicides are able to bind microtubules and thereby inhibit mitosis. Such reduction in the mitotic division caused reduction in the body weight of our experimental animals treated with carbendazim.

The carbendazim-induced sub-fertile rats treated with traditional medicinal plant *I. suffruticosum* showed a little or marginal body weight gain after 28 days of treatment, compared with the groups treated with carbendazim alone. This may be attributed to the curative effect of *Ionidium* sp. by reducing the damage caused by carbendazim and improving the mitotic division. The treatment of carbendazim-induced sub-fertile rats with *I. suffruticosum*, improved the testicular damage caused by carbendazim, further it induced the significant decrease in LPO and increased the testicular oxidative biomarkers, SOD and CAT. We found that carbendazim treatment caused a significant decrease in the testicular weight of about 400 mg/100 g body weight. Nakai et al. (1995) observed that the carbendazim exposure caused such decrease in the testicular weight of about 490 mg after 70 days of treatment. Gray et al. (1990) reported that these fungicides cause long-term atrophy in the testis. When carbendazim is administered orally to mammals, produces various adverse effects on male reproduction, such as sloughing off germ cells (Gray et al. 1990), inhibition of germ cell division (Tyrkiel 1984), seminiferous tubular atrophy (Gray et al. 1990), and alterations in hormone concentrations (Goldman et al. 1989). The decrease in the testicular weight in our studies may be due to the effect of carbendazim on the inhibition of germ cell division, seminiferous tubule atrophy, alteration in hormone concentration and sloughing off germ cells.

The carbendazim-induced sub-fertile male rats treated with leaf extract of *I. suffruticosum* showed a significant testicular weight gain of about 100 mg after 28 days of treatment. The gain in the testicular weight may be due to the effect of curative property of the *I. suffruticosum* on carbendazim-induced male testicular dysfunction by improving the mitotic division thereby increasing the spermatogenesis by preventing the sloughing off the germ cells and by enhancing the secretion of male sex hormone. One of the testicular effects of the carbendazim is the reduction of the testicular blood flow (Nakai et al. 1995). Tripathy et al. (1996) reported that *Withania somnifera* accustomed to remove blocks in the blood flow to the testis. Our experimental plant *I. suffruticosum* may also have such property by enhancing the blood flow to the testis and thereby increasing the testicular weight. We have observed that the gain in the testicular weight was more in *I. suffruticosum*-treated animals than that of clomiphene citrate-treated rats. So, the *I. suffruticosum* leaf extract showed a superior effect on male reproductive organs than the clomiphene citrate drugs. The experimental rat groups treated with standard male fertility drug clomiphene citrate, showed a slight gain in the testicular weight, compared to the group 2 carbendazim–treated rats. The gain may be due to the fact that by enhancing male sex hormone secretions. Weniger et al. (2004) reported that low plasma level of testosterone in rats caused decreases in the testicular weight. Clomiphene citrate stimulates the production of LH and thereby stimulating the production of male sex hormone testosterone.

The cauda epididymal sperm counts in control group rats were normal, but in group 2 carbendazim-treated rats, the sperm counts were significantly decreased. We have observed in group 3 carbendazim-induced sub-fertile male rats treated with *I. suffruticosum*, the cauda epididymal sperm counts were tremendously increased. Such a tremendous increase of sperm counts was due to the curative effect of *I. suffruticosum* leaf extract on carbendazim-induced damages in relation to spermatogenesis as well as increased the activities of acid and alkaline phosphatases and LDH of cauda epididymis, thereby increased the epididymal sperm counts. In the Indian traditional systems of medicine such

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**Table 5. Effect of *Ionidium suffruticosum* on malondialdehyde and antioxidant enzyme levels in Carbendazim induced subfertile male rats after 28 days of treatment.**

| Experimental groups | MAD nmol/ml | CAT μmol/S/ml | SOD μ/ml |
|---------------------|-------------|---------------|-----------|
| Group 1: Control (saline only) | 27.9 ± 0.5 | 4.2 ± 0.6 | 136.2 ± 0.4 |
| Control group (extract only) | 28.2 ± 0.3 | 4.4 ± 0.3 | 138.3 ± 0.5 |
| Group 2: Carbendazim induced sub-fertile male rats treated with *Ionidium suffruticosum* | 42.3 ± 0.5 | 2.1 ± 0.1 | 88.7 ± 0.3 |
| Group 3: Carbendazim induced sub-fertile male rats treated with clomiphene citrate | 33.6 ± 0.2* | 3.8 ± 0.2* | 131.3 ± 0.5* |
| Group 4: Carbendazim induced sub-fertile male rats treated with clomiphene citrate | 32.83 ± 0.3 | 3.6 ± 0.3 | 133.1 ± 0.5 |

MAD: Malondialdehyde; CAT: Catalase; SOD: Superoxide dismutase.

Each value is of Mean ± SEM of 5 animals.

*Significantly different (p < 0.05) from the control group by Dunnett’s test.

Scheffé multiple comparison tests used to identify which of the pairs of treatments are significantly different from each other. Control saline Vs Control extract, Group 3 Vs Group 4 in MAD, SOD are statistically insignificant and in CAT Control saline Vs Group 2 and Control extracts Vs Group 2 are statistically significant p < 0.01.
As Siddha, Ayurveda and Unani, *I. suffruticosum* is used as tonic, diuretic, demulcent and aphrodisiac (Arun & Sonappanavar 2011). The leaves and roots of *I. suffruticosum* are used in bowel complaints of children and fruits are used to treat scorpion sting. This plant shows a great medicinal value on male fertility by increasing the sperm counts.

After 28 days of treatment with *I. suffruticosum* leaf extract on carbendazim-induced sub-fertility group 3 rats, increased the cauda epididymal sperm counts which can be attributed to an increasing spermatogenesis by preventing the sloughing off germ cells and by enhancing the secretion of male sex hormone testosterone levels. Similarly, Nakai et al. (2002) reported that the presence of alkaloids, steroidal lactones and flavonoids are directly influenced on the fertility enhancing property of *I. suffruticosum*. These alkaloids, flavonoids and steroidal lactones have an influencing role on spermatogenesis and increased production of male sex hormone testosterone. From our results, we have inferred that the alkaloids and flavonoids present in the leaf extract of *I. suffruticosum* may have a stimulating role on micro-tubule polymerization and increasing the formation of mitotic spindle by binding to tubulin, which thereby increases the spermatogenesis. Our results are similar to Tripathy et al. (1996) who reported that *Withania somnifera* has certain steroidal lactones with structural similarities to androgens, which stimulated the enzymes related to steroidogenesis, such as glucose 6-phosphate dehydrogenases (Yu et al. 2009). Our experimental plant *I. suffruticosum* also has steroidal lactones which behaved the same as androgens, thereby increased spermatogenesis, which ultimately led to tremendous increase of cauda epididymal sperm counts.

Rastogi and Malhotra (1990) reported that the phytochemical drug yohimbine obtained from the plant *Rauvolfia* stimulated spermatogenesis by increasing the mitotic activity of spermatogenic cells. From the results, it is evident that the standard fertility drug clomiphene citrate on carbendazim-induced sub-fertility group 4 rats enhanced the epididymal sperm counts significantly. Many previous reports suggested that clomiphene citrate has a strong influence on the hypothalamus hypo physical testicular axis. It stimulated the hypophysis for releasing more gonadotropin by which there is faster and higher release of FSH and LH occurs, results in an elevated endogenous testosterone level. Clomiphene citrate normalized the testosterone level and the spermatogenesis process within 10–14 days of treatment, and had a direct influence on the hypothalamus and hypophysis, thus regenerating the entire regulating cycle and shows a pronounced ability to stimulate ovulation.

The untreated control group on cauda epididymal sperm motility was normal and the pattern was very rapid and progressive, but the carbendazim-treated rats showed very poor sluggish motility. In group 3 rats treated with *I. suffruticosum*, the duration of motility and progressive pattern were highly significant. Such an improved motility was due to conducive effect of *I. suffruticosum* by providing normal microenvironment in the cauda epididymis. Further attributed to the fact that the presence of alkaloids, steroidal lactones and flavonoids which acted on microtubular apparatus of the flagellum. The group 4 rats treated with clomiphene citrate showed the sperm motility was normal with a rapid and progressive pattern. Such improved motility of the sperm was due to its action over hypothalamo-hypophysial gonadal axis and by increasing the secretion of male sex hormone.

Our investigations on morphology of sperms showed that the group 1 control rats showed 92% of sperms with normal morphology. The group 2 carbendazim-induced untreated rats showed a very abnormal cauda epididymal sperm morphology with different kinds. The major abnormalities were flexed head, detached head, attached sperms, broken tails, curved or coiled tail etc., further germinal epithelial cell masses containing cells in a scattered manner or as a compact mass in admixture with sperms. Various abnormalities of the cauda epididymal spermatozoa strongly indicated the impact of carbendazim through an epididymal route as the spermatozoa were complete as far as the developmental changes acquired in testis are concerned. The origin of the abnormalities can be explained following Nakai et al. (2002), which reported that treatment of rats with a single bolus dose of 400 mg/kg weight of carbendazim resulted in decrease in testicular sperm head counts between days 8 through 16, and increase in the incidence of abnormal sperm in the epidymis with 10% of the spermatozoa having the head separated from flagellum.

Treatment of carbendazim resulted in significant decrease in the epididymal sperm counts (control, 21.88 ± 0.78 \times 10^6 per mL; treated 13.14 ± 0.11 \times 10^6 per mL), and inhibition of motility of the spermatozoa. In the control rats 93–95% of the spermatozoa possessed normal morphology, while as in carbendazim-treated rats only 55–61% had normal morphology and in about 15% spermatozoa, the head was flexed in such a way that the pointed tip of the head was either facing away from the flagellum or facing the flagellum. About 10% of the spermatozoa had the head detached from the flagellum (Akbarsha et al. 2001).

The breaking away of head from flagellum and flexion of head of the sperm appear to occur due to impact of the chemical at the neck or connecting piece of the flagellum. The main components of the connecting piece are the basal plate (the point at which implantation fossa lies), capitulum and the segmented columns. Fine filaments traversing the narrow region between the capitulum and basal plate presumably are responsible for attaching the capitulum of the flagellum to the basal plate of the head (Baccetti 1984). It has been reported that the critical protein at the connecting piece is Ankyrin (Hecht et al. 1984). This could be perceived that carbendazim disrupts the protein also, as much as disrupting tubulin, causing the breaking away of the head from the flagellum. A lesser impact at this point would cause head to flex or flexion itself may be a step towards the breaking away.

The curved or coiled nature of the flagellum may be sought in the microtubule support of the axoneme of the sperm. The microtubules are composed of \( \alpha - \) and \( \beta - \) tubulins (Hecht et al. 1984). Microtubules are undoubtedly the established targets for carbendazim action, any disruption caused to microtubules of the sperm axoneme, and curvature/coiling of the flagellum. Agglutination or fusion or attachment of sperm may be explained in the light of imminent changes in the surface proteins. It has been conclusively showed that the spermatozoa, during their epididymal maturation are altered with respect to the surface proteins. The epithelium of the epididymis, particularly the principal cells of the initial segment and caput, secrete several proteins some of which get translocated on to the spermatozoa. Also, the changing luminal microenvironment along the ductuli efferentes and ductus epididymidis contributes to the change in the sperm surface proteins (Robaire et al. 1988).
Spermatozoa that retain extra cytoplasm are inhibited motility (Akbarsha et al. 2003). The retention of the CD by cauda epididymis sperm of carbendazim-treated rats could be impairing of the process of shedding of cytoplasmic droplet and subsequently inhibited motility (Akbarsha et al. 2001).

In group 3 rats treated with I. suffruticosum sp., the normal sperm morphology was significantly increased. The 90% increase of normal sperm morphology was due to the curative property of our experimental plant I. suffruticosum on increased sperm counts with improved morphological features. The occurrence of normal sperm morphology in clomiphene citrate-treated groups was observed. The treatment of clomiphene citrate clearly prevents the toxic effects of carbendazim-induced damages in testis and sperms to a great extent.

The results suggested that, the group 1 control rats showed very minimal sperm agglutination compared to group 2 carbendazim-induced untreated rats. The higher percentage of agglutination is the result of changes in the surface proteins. The spermatozoa are altered during their epididymis maturation in respect to surface proteins. The epithelium of the epididymis, particularly the principal cells of the initial segment and caput, secrete several proteins, some of which translated on to the spermatozoa. Also, the changing luminal microenvironment along the ductuli efferentes and ductus epididymis contributes to the change in the sperm surface proteins (Robaire & Hermo 1998), therefore it is reasonable to speculate that carbendazim affects the epididymis epithelium towards secretion of proteins or luminal microenvironment, affecting change in the sperm surface proteins rendering them to stick together in small to large numbers and over short to long distances (Eddy & Brien 1994). The I. suffruticosum treated rats showed a minimal sperm agglutination because of the curative property on the epididymal epithelium, and regulating the secretion of epididymal cell protein. There by maintaining normal sperm surface proteins and maintaining a normal pH in the environment.

Carbendazim-treated animals showed an increased LPO, which can be mirrored by the increased level of MDA. Subsequently marked decrease in the level of testicular CAT and SOD activity. The results were further supported by Metwally et al. (2011) and reported that carbendazim-treated animals showed a significant change in MDA levels and significant decrease in the activity of SOD and glutathione peroxidase (GPx) in testis of rat. Similarly Rajeswary et al. (2007) reported that Leydig cellular activities of carbendazim-treated animals antioxidan enzymes SOD, GR (glutathione reductase), CAT, GPx, GST (glutathione-S-transferase), G-6-PDH (glucose-6-phosphate dehydrogenase), γ-GT and non-enzymatic antioxidants, such as GSH (reduced glutathione), and vitamins C, E were significantly diminished, whereas LPO and ROS were markedly elevated. Similarly, the results of the present work indicated that carbendazim induced oxidative stress in treated experimental animals. This can be evidenced by increased level of LPO and decreased activity levels of CAD and SOD. Hamdy et al. (2010) reported that carbendazim administration caused testicular dysfunction with an increase in MDA and reduction in SOD and GPx activity.

Treatment of carbendazim-induced sub-fertility rats with I. suffruticosum decreased the testicular damage caused by carbendazim, further it induced the significant decrease in LPO and increased the testicular oxidative biomarkers, SOD and CAT. The results can be comparable to Huo et al. (2011) reported that Glyceria glabra aqueous extracts had protective effects against CCl4-induced toxicity in rats and returned the increased LPO and decreased GSH, and antioxidant enzymes levels bring back to their normal control levels. I. suffruticosum leaf extracts have been shown to be a potent free radical scavenger and stimulating activities of antioxidant enzymes. This finding is in agreement with Al-Olayan et al. (2014) Punica granatum fruit juice showed significant elevation in testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), depleted by the injection of CCl4. Activity levels of endogenous testicular antioxidant enzymes; superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and glutathione reductase (GR) and glutathione (GSH) contents were increased while lipid peroxidation (LPO) and nitric oxide (NO) were decreased with Punica granatum fruit juice. Moreover, degeneration of germ and Leydig cells along with deformities in spermatogenesis induced after CCl4 injections were restored with the treatment of pomegranate juice.

**Histopathological changes**

In the control group rats, the seminiferous tubules were compactly arranged. The seminiferous tubules consisted of tall seminiferous epithelium and a narrow lumen. The epithelium in the different tubules was at different stage of spermatogenic cycle and testes were without atrophied seminiferous tubule. In the group 2 carbendazim-treated rats showed significant histological changes such as loss of pachytene spermatocytes, sloughing off germinal epithelial cells, arrested cell division, spermatid and germinal layer depletion and fibrosis an atrophy of the seminiferous tubules. The selective loss of pachytene spermatocytes from the seminiferous epithelium probably reflects a direct effect on carbendazim toxicity to the germ cells. Breslin et al. (2013) reported that the carbendazim prevents polymerization of tubulin and prevents the formation of fresh microtubules, the metaphase chromosomes fail to separate and the cell division arrested and sloughing off the related cell types in the seminiferous epithelium and 30% of the atrophied seminiferous tubules were observed in the present study. Nakai et al. (1995) reported that carbendazim treatment in rats resulted in a 48% of atrophied seminiferous tubules after 70 days of exposure.

Further, carbendazim directly acts on the excrrent ducts of the testis, primarily in the ductuli efferentes and resulted in occlusion of these ductules. These occluded ductules produced a fluid pressure that causes seminiferous tubular swelling, loss of germ cells and eventual atrophy of the testis. In the group 3 treated with I. suffruticosum, the histological observations of testes were normal. After 28 days of treatment, the seminiferous epithelium and a narrow lumen are compactly arranged. Further, the epithelium in the different tubules was at different stage of spermatogenic cycle and the testis without atrophied seminiferous tubule. The pachytene spermatocytes were increased.

The presence of various compounds in I. suffruticosum such as flavonoids, steroidal lactones and alkaloids exerted a curative role on the carbendazim-damaged pachytene spermatocytes and regulating meiotic division. The extract had a role in enhancing polymerization of tubulin and had a role in the formation of fresh microtubules which resulted in enhanced normal cell division, subsequently prevented sloughing off seminiferous epithelium. Further, the treatment of I. suffruticosum decreased the percentage of atrophy on ductuli efferentes by preventing the occlusion and regulating the normal sperm transport and pressure in the seminiferous epithelium. In the group 5 rats, treated
with clonimic citrate, the histopathological observations were normal. The various histopathological damages caused by carbendazim treatment were minimized and showed a normal impression of the seminiferous tubules. An increase in the number of pachytene spermatocytes, a reduction in the rate of sloughing off germinal epithelial cells and a reduction in the percentage of atrophied seminiferous tubules were observed.

Conclusions

Our results demonstrate that the leaf extracts of *I. suffructicosum* possess potent therapeutic fertility properties as well as curative properties against the chemical-induced damages in male reproductive system. The extract having a role in enhancing the levels of male sex hormone testosterone, increasing the testicular weight, increasing spermatogenesis, increasing sperm, counts lessening sperm agglutination by maintaining normal pH in testicular micro environment and increasing the testicular oxidative biomarkers, SOD and CAT. This study therefore supports the usage of *I. suffructicosum* in traditional medicine for infertility.

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Disclosure statement

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