in vivo activity in animal models. The induction of reversible male infertility in experimental animals and humans resulting from treatment with medicinal plants and their products had drawn the attention of researchers over the years (Salman and Adesokan, 2008). Male reproductive capacity was found to be deficient in no less than 50% of infertile couples evaluated according to a study of the World Health Organization (WHO, 2000). Several conditions can interfere with spermatogenesis and reduce sperm quality and production. Many factors such as drug treatment, chemotherapy, toxins, air pollution and insufficient vitamin intake may have harmful effects on spermatogenesis and the normal production of sperm (Mosher and Pratt, 1991). The plants are selected based on their ethnobotanical use. In fact, many indigenous plants have been reported to be effective in male fertility regulation (Noumi et al., 1998; Khaki et al., 2009; Kada et al., 2012). In traditional medicine of Cameroon, *Nymphaea lotus* Linn. (Nympheaceae) is used for treatment of male sexual disorders and neuropsychiatric conditions. The present study was undertaken to evaluate the effect of *N. lotus* flowers extract on spermatogenesis of rats at an aim of developing a male fertility regulating agent of plant origin.

Materials and methods

Chemicals

Testosterone enanthate (Androtardyl®) was purchased from Sigma Chemicals (USA).

Preparation of aqueous extract of Nymphaea lotus flowers

Fresh flowers of *Nymphaea lotus* were collected in Yaounde, Cameroon. Botanical identification was done at the Cameroon National Herbarium (HNC) in Yaounde in comparison with the specimen N°8647/HNC. The flowers were cut into small pieces, air-dried and powdered using an electric grinder. The dried powder (150g) was infused in 3l of boiled water. The resultant extract was filtered and the filtrate was evaporated at 45°C. The yield of the extraction was 12.66 % (w/w in term of dried material). This extract was administered by gavage at two different doses of 75 and 150 mg/once per day for 55 days. These doses were chosen based on indications of the traditional practitioner.

Repartition and treatment of animals for the fertility test

The University of Yaounde I Committee approved the animal protocol for animal experimentation and the experiments were performed according to the Principles of Laboratory Animal Care (National Institute of Health guideline; publication no. 86-23, revised 1984). A total of 24 adult male Wistar rats (160-200 g) were obtained from our colony, raised at room temperature (23°C) with a natural light-dark cycle (12/12 h) and maintained at standard laboratory rat diet and tap water given ad *libitum*. The male rats were randomly assigned into four equal groups (n = 6 per group) and orally administered in the following manner: distilled water (10 ml/Kg), testosterone enanthate (5 mg/Kg), aqueous extract of *N. lotus* at the dose 75 mg/Kg and aqueous extract of *N. lotus* at the dose of 150 mg/Kg.

Distilled water and aqueous extract of the plant were given once daily whereas testosterone was injected intramuscularly once a week. The dose of testosterone has been chosen according to previous studies (Yassin et al., 2006; Lee et al., 2010).

From day 47 of treatment, each male was cohabited overnight with two receptive females (non-ovariectomized) until the 55th day and this was followed by the examination of the vaginal smear each next morning (7am). The following reproductive parameters were then computed according to the method of Yakubu and Afolayan (2009):

Index of libido = (mated/number paired) × 100;

Quantal pregnancy = (number of pregnant animals/number mated) × 100 and %Fertility was calculated using the following formula: %success = [pregnant females / paired females] ×100; indices of mating and fertility success.

Relative weight and biochemical analysis of androgens dependent organs

After 55 days of treatment, the animals were sacrificed by cervical decapitation and testis, seminal vesicles, epididymis, muscle levator ani and prostate glands were carefully removed and the weight of each organ was determined with a sensitive electronic balance.

Biochemical analysis

Fructose levels were determined in seminal vesicle following protocols described in a WHO manual

(OMS, 1993). Colorimetric method (Richmond, 1973) was used to determine the level of cholesterol in serum and testis. Total proteins in serum and sexual organs (testis and epididymis) were determined using colorimetric methods described by Gornal et al. (1949) and Bradford (1976), respectively. Calcium in serum was determined using colorimetric method provide with the commercial kit assay (Fortress Diagnostic, United Kingdom).

Histological analysis

After measuring the weight of both testes, a routine paraffin fixation for the testicular and epididymis tissues was performed to determine histological changes in the testes following treatment with distilled water, testosterone enanthate or aqueous extract of *N. Lotus*. The tissues were first fixed in Bouin liquid for two week, followed by a dehydration procedure using a series of graded alcohol mixtures. The dehydrated tissue was then immersed in xylene for two hours and 30 minutes. Tissues were then embedded in paraffin and were cut at a thickness of 5µm. The tissues were mounted on slides and stained by immersing them in Mayer hematoxylin solution. The slides were rinsed under running tap water to remove excess hematoxylin. The slides were dipped in alcohol, eosin solution and then dehydrated through a series of graded alcohols. Finally, the tissues were mounted under a synthetic resin. Microscopic evaluation of the slides was undertaken and variations in histoarchitecture were recorded (Chauhan and Dixit, 2008).

Testicular Fertility Index

The specimen of testis cut into 5µm thickness, prepared on slide after staining with hematoxylin-eosin have been examined by one examiner and the mean tubular diameter (MTD) was evaluated. Thirty tubules with round circular configuration were selected and the tubular diameter for each were measured using a light microscope Olympus linked to a computer (Compaq nx9010) where all images were transferred and analysed with an image analysis program (Image J Version 1.32) for calculation of the MTD (Lee et al., 2010).

Epididymal sperm motility, viability and counts

Epididymal sperm counts were performed to assess the rats' reproductive status. The caudal epididymal tissue was cut into small pieces and diluted with 10ml of NaCl 0.9%. A small aliquot of the diluted tissue was placed on a slide and examined using a light microscope. We used a Malassez's cell to count the number of spermatozoids in five randomly selected quadrants. The different sperm parameters are calculated with the formula:

$$\text{Sperm count} = \frac{X \times df \times 10^6}{4}$$

%Viability = (Alive sperm/Dead sperm) × 100
%Mobility = (Mobile sperm/Total number of sperm) × 100
X = sperm count in 4 randomly selected quadrants of the Malassez's cell
df = dilution factor (20)

Statistical analysis

One-way analysis of variance (ANOVA) followed by post-hoc Student-Newman-Keuls multiple comparison test was performed using GraphPad Instat software version 3.10. A probability of $P < 0.05$ was accepted as significant.

Results

Effects of *N. lotus* on some indexes of fertility

The treatment with *N. lotus* during 55 days increased by 83.33% the index of libido in animals receiving the dose of 75 mg/kg versus 50% in animals receiving only distilled water. Testosterone injection at the dose of 5 mg/kg once a week during 8 weeks provoked a decrease of the libido index compared to control (16.67 vs 50). We observed no pregnancy nor descendant in female mated with male rats receiving testosterone whereas *N. lotus* treated animals of both doses showed high percentage (100%) of quental pregnancy as well as elevated number of descendant (39 and 28 respectively for *N. lotus* 75 and *N. lotus* 150 mg/kg) compared to control (Table 1).

Effect of *N. lotus* on organs/body weight ratio

The treatment with *N. lotus* aqueous extract during 55 days induced a dose dependent enhancing effect on sexual organs relative weight. We observed at the dose of 75 mg/kg a significant elevation of the relative weight of testis ($P<0.05$), levator ani muscle ($P<0.01$) and penis ($P<0.05$) compared to control whereas testosterone increased significantly the relative

Table 1: Indexes of fertility

	Control	Testosterone	*N. lotus* 75	*N. lotus* 150
Libido index (%)	50	16.67	83.33	50
Quental pregnancy (%)	100	0	100	100
Number of descendant	23	0	39	28

Table 2: Relative weight of androgens dependent organs

Relative weight of androgens dependent organs	Control	Testosterone	*N. lotus* 75	*N. lotus* 150
Testis	1.09 ± 0.15	2.16 ± 0.21 *	1.86 ± 0.26 *	1.27 ± 0.20
Epididymis	0.43 ± 0.02	0.50 ± 0.06	0.53 ± 0.08	0.45 ± 0.05
levator ani muscle	0.51 ± 0.08	0.58 ± 0.06	0.48 ± 0.08	0.47 ± 0.04
Prostate	0.11 ± 0.03	0.24 ± 0.05 **	0.21 ± 0.5 **	0.10 ± 0.05
Seminal vesicles	0.43 ± 0.05	0.56 ± 0.03	0.47 ± 0.08	0.33 ± 0.01
Penis	0.13 ± 0.01	0.16 ± 0.01	0.19 ± 0.06 *	0.14 ± 0.02

Each value represents the mean ± SEM of group; **P < 0.01, *P < 0.05 compared to distilled water treated group.

weight of testis (P<0.05) and prostate gland (P<0.01) compared to control. Others organs remain unaltered by the treatments (Table 2).

Effects of *N. lotus* on caudal epididymis sperm count, viability and Motility

Concerning semen analysis, it was found that oral administration of *N. Lotus* extract at 75 mg/Kg and 150 mg/kg for 55 days to adult male rats induced significant increase of the sperm motility, sperm count and viability (Fig. 1).

Sperm motility

Figure 1A shows the percentages of motile sperms from all groups, which were obtained by microscopic examination at room temperature (37°C). *N. Lotus*-treated rats presented a higher percentage of motile sperms at the dose of 75 mg/kg compared to control group (P < 0.001) as well as in animals treated with *N. lotus* at the dose of 150 mg/kg (P<0.01), whereas no significant changes were observed in animals receiving testosterone at the level of 5 mg/kg.

Epididymal sperm count

Epididymal sperm counts were markedly decreased in testosterone-treated rats as compared to control (P < 0.05, Fig. 1B). In contrast, rats that were treated with *N. lotus* at the both doses showed a significant increase in sperm counts as compared to testosterone treated animals (P < 0.05).

Sperm viability

Moreover, we observed a considerable increase (32.41%) in sperm viability from 50.11 ± 7.81% in the control up to 66.35 ± 34.78% in animals treated with *N. lotus* at the dose of 75 mg/kg whereas animals treated with the dose of 150 mg/kg showed an increase by 29.59% (Fig. 1C).

Effects of *N. lotus* on some biochemical parameters of androgens-dependent organs

The administration of *N. lotus* aqueous flowers extract induced significant increase (P<0.05) of the total cholesterol concentration in the serum of animals treated particularly at the dose of 150 mg/kg, whereas those receiving the dose of 75 mg/kg exhibited an increase by 56.90% compared to control receiving distilled water (Table 3). Testis cholesterol concentrations were significantly reduced (P<0.05) in animals treated with testosterone and plant extract at both doses in comparison to control. The administration of *N. lotus* induced at the dose of 150 mg/kg a significant increase of serum proteins level whereas the lower dose of extract as well as

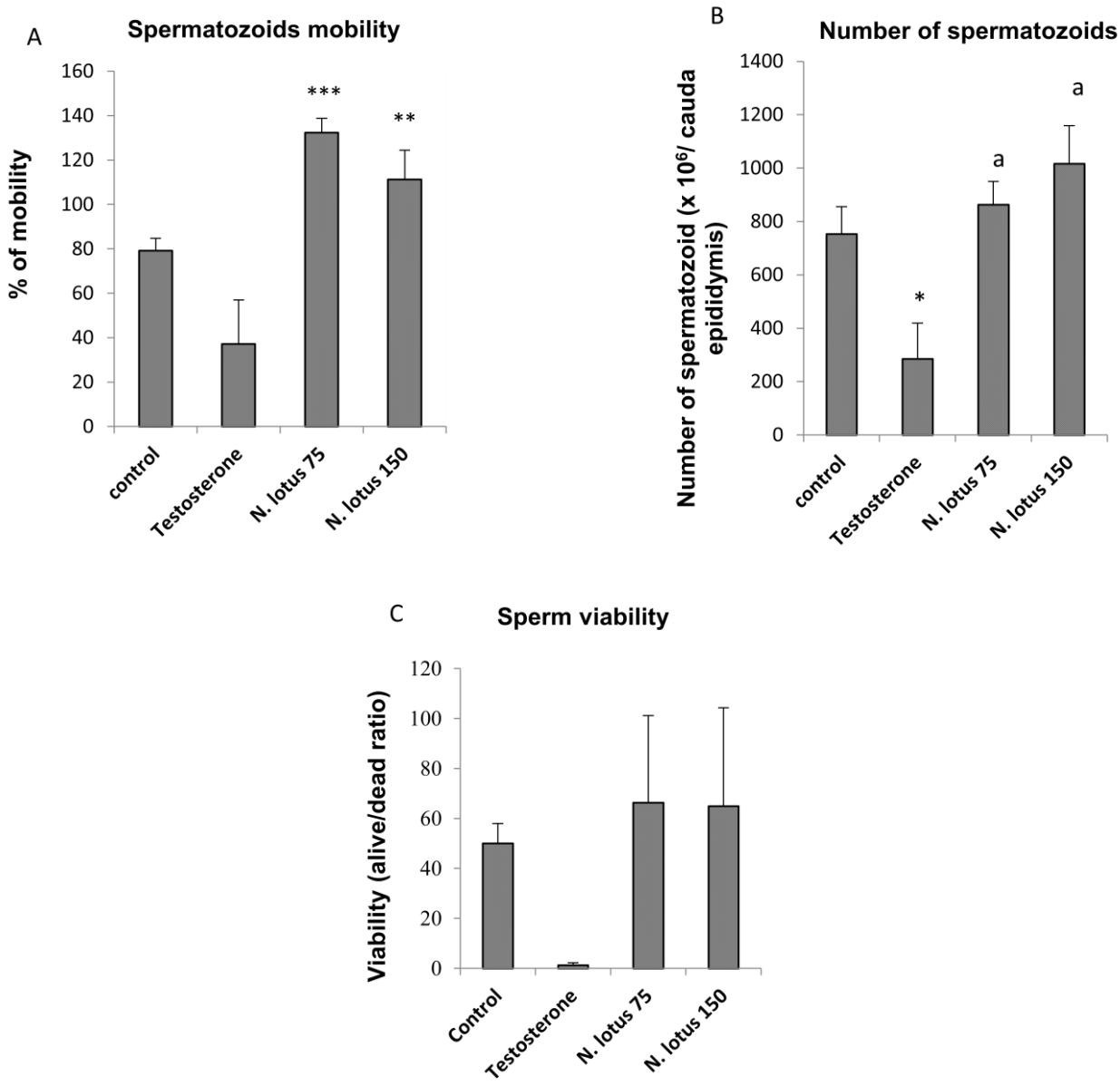

Fig.1. Effects of *N. lotus* aqueous extract on spermatozoids mobility (A), number of spermatozoids (B) and sperm viability (C).
The bars are shown as the mean ± SEM **P*<0.05, ***P*<0.01, ****P*<0.001 as compared to control; [a] *P*<0.05 as compared to positive control (Testosterone).

testosterone enanthate do not change significantly this concentration. Besides, *N. lotus* at the dose of 75 mg/kg enhanced significantly by 107.69% (*P*<0.01) the amount of proteins in testis whereas testosterone enhanced it by 110.26% in comparison to control. The two groups treated with *N. lotus* at the doses of 75 and 150 mg/kg presented a significant increase of the fructose (*P*<0.001; *P*<0.01 respectively) in comparison to control. The administration of the plant extract for 55 consecutive days also enhanced significantly (*P*<0.001; *P*<0.01) the fructose levels in seminal vesicles respectively at the doses of 75 and 150 mg/kg. Meanwhile, calcium concentration in serum remained in normal ranges after the different treatments.

Effects of *N. lotus* after 55 days of treatment on histopahological profile of testis and epididymis
Control

The presence of thick collagenous connective tissue is observed and there are seminiferous tubules embedded in interstitial connective tissue. Leydig cells and blood vessels are observable in testis. Spermatogenetic cells forming a stratified epithelial and sperms are often found in clusters embedded in cytoplasm of sertoli cells. There is no sign of

Table 3: Concentration of some biochemical parameters of androgens-dependent organs

	Total cholesterol		Total protein			Fructose	calcium
Groups	Serum (mg/dl)	Testis (ng/g)	Serum (mg/dl)	Testis (mg/g)	Epididymis (mg/g)	Seminal vesicle (mg/g)	Serum (mg/dl)
Control	34.48 ± 3.21	47.7 ± 4.59	1.80 ±0.14	0.39 ±0.10	0.58 ± 0.04	0.71 ± 0.07	7.85 ± 0.37
Testosterone	35.81 ± 5.70	23.35 ± 2.50*	2.01± 0.07	0.82± 0.16	0.61 ± 0.03	0.65 ± 0.10	7.23 ± 0.54
N. lotus 75	54.10 ± 5.31	27.40 ± 2.08*	2.11 ±0.10	0.81± 0.08**	0.73 ± 0.05	0.20 ± 0.02***	7.23 ± 1.02
N. lotus 150	53.71 ± 7.71*	24.11 ± 4.40*	2.19± 0.04*	0.35± 0.03	0.58 ± 0.03	0.32 ± 0.06**	9.08 ± 1.12

Each value represents the mean ± SEM of group; ***P < 0.001, **P < 0.01, *P < 0.05 compared to distilled water treated group

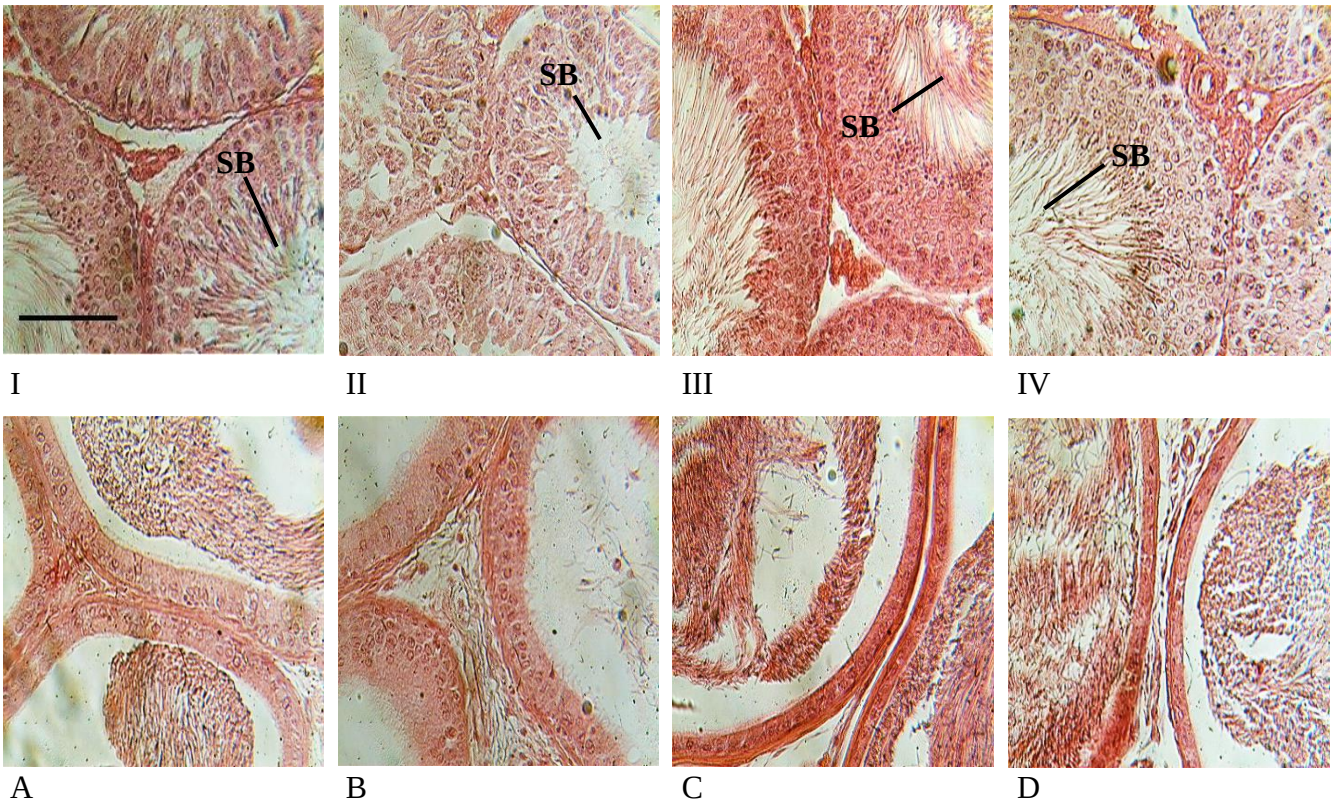

Fig.2. Histomorphological analysis of testis and epididymis of animals treated with *N. lotus* aqueous extract
I-IV: Section of seminiferous tubules showed the different spermatozoids stages, from rat testis receiving respectively distilled water (I); testosterone enanthate (II) and extract at the doses of 75 mg/kg and 150 mg/kg (III and IV) (H&E X400).
A-D: Section of epididymal caudal showed the concentration of mature sperms account from group rat receiving respectively distilled water (A); testosterone enanthate (B) and extract at the doses of 75 mg/kg and 150 mg/kg (C and D)(H&E X 400). SB=sperms bundles. The bar represents 0.4 mm at 400X.

Table 4: Histomorphometric analysis of seminiferous tubules

Seminiferous tubules	**Control**	**Testosterone**	***N. lotus* 75**	***N. lotus* 150**
Epithelium height (µm)	0.51 ± 0.04	0.38 ± 0.01**	0.35 ± 0.01**	0.37 ± 0.004**
Area (µm^2)	192.10 ± 15.79	119.58 ± 7.88***	216.21 ± 6.62	251.52 ± 5.04**
Mean tubular diameter (µm)	1.91 ± 0.15	1.83 ± 0.11***	1.81 0.03***	1.74 ± 0.05***

Each value represents the mean ± SEM of group; ***P < 0.001, **P < 0.01, *P < 0.05 compared to distilled water treated group

inflammation (Fig. 2I).

Testosterone

Histoarchitecture of testis of testosterone treated group show a different profile to aqueous treated group. An alteration of the germ cells proliferation is observable and leydig cells are meagerly present in the testis. Very few sperm bunches are observed in the epididymal tubules lumen as such as cellular damage of tubular elements, but there is no evidence of inflammation (Fig. 2II and 2B).

***N. lotus* 75 mg/kg**

Cross section of testis revealed, in the animal treated with extract of *N. Lotus* at the dose of 75 mg/kg, presence of good seminiferous tubules with uniform arrangement of numerous sertoli cells. The interstitial stroma is full of blood and lymph vessels and contains distinct group of interstitial cells of leydig in the testis and meaningfully sperm bunches compared to controls in the epididymal lumen (Fig. 2III and 2C). There is no sign of inflammation.

***N. lotus* 150 mg/kg**

The transverse section of testis treated with extract at the dose of 150 mg/kg reveled highly populated seminiferous tubules closely connected and embedded in the interstitial connective tissues. A group of leydig cells present in the interstitial sertoli cells is well differentiated and highly populated with producing a group of sperms. A group of sperms thread like on the center of epididymis is observed clearly. There is no sign of inflammation (Fig. 2IV).

Effects of *N. lotus* on histomorphometric parameters of seminiferous tubules

The administration of the both doses of *N. lotus* aqueous flowers extract during 55 days induced a significant decrease (P<0.01) of the epithelium height of the seminiferous tubules, as well as observed in testosterone-treated animals (P<0.01) in comparison to animals receiving distilled water (Table 4). However, the aqueous extract induced a dose dependent increase of the seminiferous tubules area compared to control. The dose of 150 mg/kg provoked a significant increase (P<0.01) of this area, whereas the dose of 75 mg/kg exhibited an elevation up to 12.55% compared to control. Contrarily, the administration of testosterone once a week at the dose of 5 mg/kg induced a significant reduction (P<0.001) of this area. Seminiferous tubular diameter was significantly increased in extract-treated (P<0.001) groups compared to distilled water control as well as in testosterone (P<0.001) treated animals (Table 4).

Discussion

The empiric claim of *Nymphaea lotus* flowers as an aphrodisiac has encouraged investigation into its potential as an androgenic agent. Considering the fact that male sexual behavior depends on the circulating levels of testosterone in the blood, testosterone enanthate was used as standard referent to validate androgenic properties. Distilled water, which did not affect the testosterone levels, and ultimately sexual or aphrodisiac activity in adult male rats was used as negative control also for comparison purpose. The evaluation of biochemical parameters of testes/body weight ratio, concentrations of testicular secretory constituents like total protein and cholesterol, can give useful information on the androgenic potential of chemical compounds and plant extracts. These parameters can also be used to evaluate normal functioning capacity of the testes (Kamtchouing et al., 2002; Watcho et al., 2004).

Treatment with the aqueous extract of *Nymphaea lotus* Linn. flowers influenced the parameters of the treated animals in a dose-dependent manner. Both 75 mg/kg and 150 mg/kg significantly affected androgenic indicator values, compared to control. Meanwhile we observed at the end of treatment, a significant increase in certain sexual organ (penis, testis) relative weight. This increase may either indicate inflammation or an increase in the secretory ability of the organ while a reduction in the parameter may imply cellular constriction. However as evidenced by histomorphological analysis, no evidence of inflammation in any of the tested groups have been reported. Therefore, the increase in the relative organs weight observed following the administration of the plant extract might be attributed to increased secretory activity of the testes. This hypothesis is supported by the decrease in the concentration of testicular cholesterol and the elevation in the concentration of testicular protein.

Effectively, an increase in the concentration of cholesterol in testes may reflect reduced conversion

of cholesterol to testosterone (Vijaykumar et al., 2004). Thus the decreasing cholesterol rate observed in the testes of extract-treated male rats compared to control clearly indicated an increased conversion of cholesterol into testosterone. A constant supply of cholesterol is required for the synthesis of steroid hormones (Das and Dasgupta, 1997) and its requirement for normal testicular activity has been well established (Watcho et al., 2004). Similarly, increased protein concentrations have been reported to enhance sperm maturation that is an important component of androgenicity (Gupta et al., 2004). Testicular proteins are one of the constituents that ensure the maturation of spermatozoa (Kasturi et al., 1995). Increased weight and high protein concentration of the testes observed in animals treated with the plant extract indicates enhancement of testicular growth and androgenic activities. Androgens also have important effects on bone *in vivo*, possibly by direct activation of the androgen receptors in osteoblasts or boosting calcium fixation. To test this hypothesis, calcium level in blood has been evaluated, but no significant changes were observed between different groups of animals.

Seminal fructose content is an important parameter for evaluating the normal sexual functioning in male, as fructose is consumed during fructolysis for providing energy to immotile spermatozoa (Sharma et al., 2009). Treatment with the aqueous extract at both doses decreased seminal fructose content. Some authors reported that after ejaculation, the spermatozoa in a process named fructolysis consume fructose. At higher sperm counts, the process will be stronger resulting in a low seminal fructose concentration (Gonzales, 2001), suggesting that high fructose concentrations observed in animals treated with *N. lotus,* have been released to supply motile sperm in energy after ejaculation in the fertility test. Furthermore, lumen size of seminiferous tubules is increased and an increase in vascularization is observable in extract treated group, these effects are considerably restrained in the testosterone treated group compared to *N. lotus* treated groups. The leydig cells observed as well as cytoplasm was highly stained with eosin in the group of animals treated at the dose of 75 mg/kg compared to controls. Comparison of paleness between stained control slide and the extract group testis suggests a proper differentiation and vascularization of the spermatids and spermatogonia (Sharma et al., 2009).

The proliferation was evidently more perceptible in case of extract-treated group animals as compared to distilled-water control. Differences in various stages of spermatogenesis were observable in both extract-treated groups, confirming the efficacy of extracts of *N. lotus* in spermatogenic activity as indicated by the huge amount of spermatozoids in groups treated with plant extract. This improvement of *in vivo* sperm count confirmed an improved spermatogenic activity of the test extract. Surprisingly, the testes of the testosterone-treated animals revealed arrest of spermatogenesis and complete abolition of fertility as evidenced by the low percentage of quental pregnancy and the number of descendant numbers observed in this study. The observed increase in testicular protein following extract administration may be the result of testosterone-like action in extract treated male rats.

Contrarily, reduced testicular and epididymal protein content observed in testosterone treated animals, may be correlated with absence of spermatozoa in the lumen (Zhen et al., 1995) as evidenced by the photomicrograph of testes in this group. This low androgenic potential of testosterone observed in this study could be explained by the duration of the treatment. Testosterone has long half-life and a long lasting biotransformation period. The repeated injection of testosterone at 5 mg/kg once a week resulted in to a negative retro control of spermatogenesis or a saturation of the testosterone receptors in all androgens dependent organs. Thus, the antireproductive effect of testosterone observed in this study, was not due to a general toxicity but due to an increase in pre-implantation losses resulting from oligozoospermia, impairment of sperm motility and reduction in the fertilizing potential of spermatozoa or viability (Ratnasooriya and Jayakody, 2000). Effectively, injections of conventional testosterone fatty acid esters (enanthate, cypionate, decanoate and propionate) have been reported to have an effective duration of action of 1 to 2 weeks (Yassin et al., 2006).

On the other hand, the increasing libido index, the improvement of relative weight of the androgen dependent organs and all others fertility parameters observed after 55 days of treatment with aqueous extract of *N. lotus*, as well as the increased number of offspring related to the increased sperm count,

motility and viability (Moundipa et al., 1999), confirm aphrodisiac, reproductive and androgenic-like effects of our plant extract.

Conclusion

From these findings, it was concluded that *N. lotus* flowers have androgenic and reproductive properties justifying its empirical uses.

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Conflict of interest

The authors declare that they have no competing interests.

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