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Evaluations of cytotoxicity of *Smilax myosotiflora* and its effects on sexual hormone levels and testicular histology in male rats

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ABSTRACT

Objective: To investigate the cytotoxicity of *Smilax myosotiflora* (*S. myosotiflora*) methanolic extract and its effects on sexual hormone levels and testicular histology in male rats.

Methods: The cytotoxicity of *S. myosotiflora* methanolic extract was investigated by employing brine shrimp lethality assay. Forty eight male rats were randomly divided into four groups (Groups I–IV) of 12 each. Rats in Group I were administered with 0.5 mL of distilled water (vehicle), whilst Groups II, III and IV received 200, 400 and 800 mg/kg of the methanolic extract of *S. myosotiflora* in 0.5 mL of the vehicle, respectively. Male rats treated with continuous daily dosing were killed and necropsied after a total dose period of 60 days. Sexual hormones were assayed and histological examination of testes was performed according to standard methods.

Results: *S. myosotiflora* extracts did not produce any cytotoxicity to brine shrimp in all concentrations tested. Serum testosterone level was significantly higher in rats treated with high dose of *S. myosotiflora*. Testicular histology showed normal architecture with all stages of spermatogenesis in all experimental groups.

Conclusions: The present work confirmed that *S. myosotiflora* extract improves reproductive functions, without any cytotoxic activity and produces no histological changes to the testes.

1. Introduction

The use of medicinal plants has played a crucial role in health and disease management for many centuries. It is estimated that around 35 000 to 50 000 species of plants worldwide are applied for medicinal intentions [1]. One of such botanicals claimed to have therapeutic properties is *Smilax myosotiflora* (*S. myosotiflora*). It is a herbaceous climber from the family Smilacaceae and locally known as "ubi jaga". This plant is widely distributed in Peninsular Malaysia, Southern Thailand and Indonesia [2].

According to folk medicines of Malaysia, *S. myosotiflora* is used for several purposes. The decoction of *S. myosotiflora* tubers has been consumed to strengthen male energy and intensify sexual performance [3]. To obtain the best result, the decoction is mixed with *Eurycoma longifolia* [4]. It has been previously used in cases of skin ailments including wounds, inflammations, boils and ulcers [5]. In addition, the leaves and fruits of *S. myosotiflora* are used for treating syphilis and rheumatism [6].

Recent scientific evidences revealed that the prosexual activity of *S. myosotiflora* is probably because of the presence of 4.3 kDa bioactive peptide. This protein has been recognized as an aphrodisiac marker [7]. In addition, oral ingestion of *S. myosotiflora* extract has been proven to improve sexual behavior and fertility in male rats [8]. The extract also exhibited a strong antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay [9]. Despite long record of usage for various purposes, little information on *S. myosotiflora* toxicity is available. Therefore, the present

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study was aimed to explore the cytotoxicity of *S. myosotiflora* methanolic extract by brine shrimp lethality assay, and its possible effects on sexual hormone levels and testicular histology in male rats.

2. Materials and methods

2.1. Plant collection and extraction

S. myosotiflora plants were collected from area of Sungai Sok, Kelantan, Malaysia. The plants were authenticated by Dr. Rahmad Zakaria, botanist of Herbarium Unit, Universiti Sains Malaysia (voucher No. 11397). The tuber parts were dried at 50 °C in the oven for 2–3 days until a constant weight was obtained. The dried tubers were grinded into fine powder (100 g) and then extracted with 300 mL methanol using Soxhlet apparatus at 60–70 °C for 42 h [10]. The extract was concentrated through vacuum using rotary evaporator at 40 °C, and the final yield was 7.12 g.

2.2. Brine shrimp lethality assay

The cytotoxic activity of the plant was evaluated using brine shrimp lethality assay where 8 graded doses (1 000, 500, 250, 125, 62.5, 31.2, 15.6 and 7.8 μg/mL) were used. The assay was done according to the procedure of Ullah *et al*. with some modifications [11]. Simply, brine shrimp eggs were collected from local market and hatched with properly aerated filtered seawater for 48 h. After hatching, 10 active nauplii were drawn through a dropper and placed in Petri dishes containing 5 mL of seawater and 5 mL of each extract dilution. The control for this assay was 10 mL of seawater containing 10 brine shrimps. The numbers of survivors were counted after 24 h. The experiment was performed in triplicate. The percentage of mortality was then determined. LC_{50} value was obtained from the best-fit line by plotting concentration versus percentage of mortality.

2.3. Animals

Forty eight adult male Sprague-Dawley rats of proven fertility, 3 months old and weighing 250–270 g were supplied by Animal Research and Service Centre, Universiti Sains Malaysia, Kelantan, Malaysia. The animals were caged at a temperature of (22 ± 1) °C with a reversed light: dark cycle (light from 800 to 2 000 h) and relative humidity of 50% ± 5%. Male rats were fed with excess standard animal feed and water *ad libitum* was available. The rats were allowed to acclimatize for one week prior to the experiment. All experimental procedures on rats were conducted in accordance to Universiti Sains Malaysia Guide for the Care and Use of Laboratory Animals and approved by Animal Ethics Committee [PPSG/07(A)/044/(2009)(50)].

2.4. Experimental design

The animals were randomly divided into four groups (Groups I–IV) of 12 each. Group I were orally administered with 0.5 mL of distilled water (control), whilst Groups II, III and IV received 200, 400 and 800 mg/kg of the methanolic extract of *S. myosotiflora* in 0.5 mL of vehicle, once daily for 60 days [12]. All rats were sacrificed under ether anesthesia 24 h after the termination of the respective treatment schedule.

2.5. Hormonal assay

Blood samples were collected from the inferior vena cava and were allowed to clot for 10 min at room temperature. Subsequently, the blood was centrifuged at 3 000 r/min for 10 min and the supernatant (serum) was stored at −20 °C until use. Testosterone, follicle stimulating hormone (FSH) and luteinizing hormone (LH) radioimmunoassay test kits were the products of DRG Diagnostics GmbH, Germany. Serum samples were assayed by using the procedure described by DRG Diagnostics. This was based on the principle of radioimmunoassay of competitive binding between the sample serum and the standards for a constant amount of the antisera [13].

2.6. Testicular histology

The left testis was cut into small pieces (5 mm × 5 mm) and fixed in Bouin's solution for 48 h. Testes tissues were washed through graded concentrations of ethanol saturated with lithium carbonate. Then, the samples were dehydrated, cleared and embedded in paraffin wax. Serial transverse sections were cut at 5 μm thicknesses by a rotary microtone, mounted on clean slides, hydrated and stained with Mayer's hematoxylin and eosin (H&E). The sections were examined using light microscope to investigate the spermatogenesis process [14]. Besides, tubular differentiation index (TDI), seminiferous tubular diameter (STD) and seminiferous epithelial height (SEH) were also measured [15].

2.7. Statistical analysis

Data were expressed as mean ± SEM. One-way ANOVA was used to test the significant difference between mean values of all. Percentage was analyzed by using *Chi*-square test. A value of $P \leq 0.05$ was considered as statistically significant. The statistical analyses were performed using SPSS software (version 20.0).

3. Results

3.1. Brine shrimp lethality assay

The extract of *S. myosotiflora* was evaluated for brine shrimp lethality at different concentrations (Table 1). All experiments were done in triplicate and the mean result was noted. The LC_{50} of the test samples after 24 h was determined by a plot of the percentage of the dead nauplii against the logarithm of sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. From the assay, LC_{50} value of methanolic extract of *S. myosotiflora* was not determined since all of the concentra-

tions did not cause 50% mortality of nauplii. Moreover, the extract also did not induce any lethal effects to nauplii at all concentrations. However, based on the finding of the statistical analysis and mathematical equation, the LC_{50} was deduced as more than 1 000 μg/mL.

Table 1
Cytotoxic activity of methanol extracts of *S. myosotiflora* on brine shrimp nauplii.

Concentrations (μg/mL)	Number of survivors (after 24 h)			Total number of survivors	Mortality (%)
	Trial 1	Trial 2	Trial 3		
1 000.0	10	10	10	30	0
500.0	10	10	10	30	0
250.0	10	10	10	30	0
125.0	10	10	10	30	0
62.5	10	10	10	30	0
31.5	10	10	10	30	0
15.6	10	10	10	30	0
7.8	10	10	10	30	0

3.2. Sexual hormone levels

The levels of hormones testosterone, LH and FSH of all groups were shown in Table 2. The methanolic extract of *S. myosotiflora* after 60 days of treatment was able to significantly increase the level of testosterone at the dose of 800 mg/kg. In contrast, the levels of LH and FSH remained unaffected with the treatment of *S. myosotiflora* in comparison with the control group.

Table 2
Levels of sexual hormones in control and *S. myosotiflora* treated groups.

Parameters	Control	*S. myosotiflora* extracts		
		200 mg/kg	400 mg/kg	800 mg/kg
Testosterone (nmol/L)	1.68 ± 0.14	2.65 ± 0.46	2.68 ± 0.47	3.41 ± 0.47*
LH (IU/L)	1.26 ± 0.19	1.64 ± 0.38	1.98 ± 0.29	2.30 ± 0.31
FSH (IU/L)	1.12 ± 0.23	1.42 ± 0.33	1.98 ± 0.15	2.08 ± 0.28

ANOVA test was used. Values were expressed as mean ± SEM. *: $P \leq 0.05$ compared with control group.

3.3. Histologic examination

Results from histological studies showed normal seminiferous tubules in all experimental groups (Figure 1). The seminiferous epithelium of *S. myosotiflora* treated rats had similar appearance as the control ones. Spermatogonia, spermatocytes and spermatids exhibited normal arrangement in different stages of spermatogenesis (Figure 1A). Normal features of Sertoli cells which were closely associated with the seminiferous tubules basement membrane (Figure 1B, C) and Leydig cells in the interstitium were also observed in *S. myosotiflora* treated groups at all doses (Figure 1D). Table 3 shows a summary of spermatogenesis patterns in the control and *S. myosotiflora* treated rats. The extract at various doses did not cause any significant variations in the percentage of tubular differentiation, when compared to the control group. Also, there were no significant differences observed in the diameter of seminiferous tubules and seminiferous epithelia height.

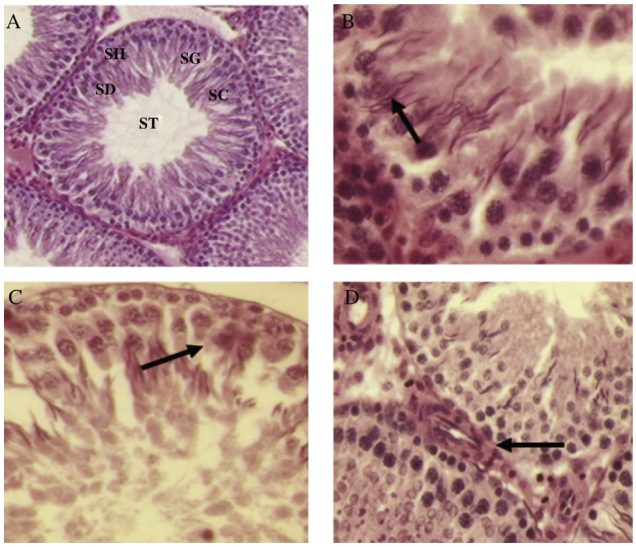

Figure 1. Histology of testes of *S. myosotiflora* treated rat.
A: Seminiferous tubule showed normal characteristics with different stages of spermatogenesis including spermatids (SD), spermatocyte (SC), spermatogonia (SG), sperm head (SH) and sperm tail (ST) (H&E 100×); B and C: Seminiferous tubule showed Sertoli cells with no apparent histological lesions; D: The interstitial tissue was closely interacted with blood vessels and Leydig cells (H&E 400×).

Table 3
Effects of *S. myosotiflora* treatment on TDI, STD and SEH.

Parameters	Control	*S. myosotiflora* extracts		
		200 mg/kg	400 mg/kg	800 mg/kg
TDI^X (%)	100	100	100	100
STD^Y (μm)	257.25 ± 3.43	260.81 ± 4.32	261.16 ± 3.98	262.12 ± 4.01
SEH^Y (μm)	92.42 ± 3.11	92.57 ± 2.85	93.45 ± 2.97	93.39 ± 3.12

^{X}: *Chi*-square test. Values were expressed as mean of percentage, $n = 100$ of seminiferous tubules per rat. ^{Y}: ANOVA test. Values were expressed as mean ± SEM.

4. Discussion

S. myosotiflora has been found to have several medicinal properties [16]. However, the toxic potentials of methanolic extract of this herb have not been investigated despite its use in folk medicine as a sexual-enhancing agent. This raises a concern on its safety and implications for its use as medicines. An agent that produces adverse effect in experimental animal studies is assumed to pose a similar threat to humans [17].

The brine shrimp lethality assay has been extensively used in the primary screening of the crude extracts as well as the isolated compounds to evaluate the toxicity towards brine shrimps, which could also provide an indication of possible cytotoxic properties of test materials [18]. Furthermore, there is a positive correlation between cytotoxicity and antitumor activity towards the brine shrimp. The inhibitory effects on cancer cells might be due to the toxic compounds present in the active fraction that possess ovicidal and larvicidal properties [19]. The results of this work showed that the methanolic extract of *S. myosotiflora* demonstrated no cytotoxic activity against brine shrimp in all concentrations tested. According to Bhatti *et al.* [20], crude plant extract was toxic if it has LC_{50} value less than 1 000 μg/mL; while it was non-toxic if the LC_{50} value is greater than 1 000 μg/mL. Study by Dasuki *et al.* further supported our findings; it was observed that

S. myosotiflora extract also exhibited no inhibitory effects on several human tumor cell lines [9].

Testosterone, LH and FSH are hormonal markers of androgenicity [21]. Testosterone is the main male gonadal hormone produced by the interstitial cells of Leydig in the testis. It has been documented that critical level of testosterone is required for the maintenance of normal sexual desire, nocturnal penile tumescence and non-erotic penile erections. A certain concentration of testosterone is also required for the initiation and maintenance of spermatogenesis and for the stimulation of growth and function of the prostate and seminal vesicles [22]. So, any change in the level of testosterone would have direct effects on spermatogenesis, including a decrease in sperm counts [23]. LH and FSH are called gonadotropins because they stimulate the gonads and testes in males. LH produced in the anterior pituitary is called gonadotrophs. In the testes, LH binds to receptors on Leydig cells, stimulating synthesis and secretion of testosterone [24]. FSH has a stimulatory effect on the testes and is thus essential for normal reproduction. It is required for the initiation and maintenance of spermatogenesis and for quantitatively normal spermatogenesis in pubertal rats [25].

The results of the current work showed that animals received 800 mg/kg of methanolic extract of *S. myosotiflora* exhibited a significant increase in serum testosterone level. However, the LH and FSH levels remained statistically unaffected even though they showed the tendency to increase in dose dependent manner. As reported in our previous studies, treatment of *S. myosotiflora* was able to improve the fertilizing ability and sexual behavior indices, as well as promote a significant increase in sperm density of male rats [8,26]. Therefore, the continued administration of the plant extract for 60 days which led to the significant increase in serum testosterone may be responsible for the marked improvement on libido and spermatogenic activity of the male rats.

Histopathological evaluation of the testes is considered the most sensitive assessment to see the effects of harmful substances on spermatogenesis and a good indicator for reproductive toxicity [27]. It is also a useful indicator of testicular damages, which can provide information on toxicity and on target sites including target cell, show the extend of toxicity and indicate the potential of recovery from the effects of toxicity [28]. Sections taken from the testes of *S. myosotiflora* treated rats showed no gross abnormalities at all doses. In order to confirm these findings, measurement of TDI, STD and SEH were performed. Treatment of *S. myosotiflora* did not show any overt changes on the TDI, STD and SEH parameters.

Adverse effects of any deleterious compounds on spermatogenesis are manifested by five mechanisms, including alteration in the level of the reproductive hormones such as FSH, LH and testosterone, increase in capillary permeability of testicular blood vessels which result in generalized edema and testicular disruption [29], toxicity to Sertoli cell resulting in sloughing and reducing spermiogenesis, decreased spermatogenesis in the absence of apparent Sertoli cell toxicity and chromosomal aberrations and unscheduled DNA synthesis, sometimes accompanied by cytotoxicity [30]. In our investigations, there was no evidence of disruption in spermatogenesis and all stages of spermatogenesis were clearly seen.

Thus, we conclude that the methanolic extract of *S. myosotiflora* did not have the cytotoxic effect on the brine shrimp nauplii. The extract could also be promoted as sexual booster since it demonstrated an increment on testosterone level in treated rats. In addition, histological results of the testes in *S. myosotiflora* treated rats showed the integration of the seminiferous tubule walls and normal spermatogenesis.

Conflict of interest statement

We declare that we have no conflict of interest.

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