FABAD J. Pharm. Sci., 48, 2, 265-278, 2023
Doi: 10.55262/fabadeczacilik.1263949
RESEARCH ARTICLE

Toxicological Evaluations of *Smilax myosotiflora* Methanol Extract and its Effect on Testosterone Level of Male Rats in Subacute Study

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SUMMARY

S. myosotiflora A. DC., the horny little devil, is a tropical creeping plant which popularly consumed as a male aphrodisiac, energy booster, and lumbago reliever in the old traditional medicine. The scientific studies showed that the plant able to increase sexual behaviors and testosterone levels in male rats. However, its toxicity effect still remained unknown. Therefore, this study aimed to investigate the toxicity effects of S. myosotiflora methanol extract (SMME) through in vitro and in vivo studies. The SMME was subjected to the brine shrimp lethality test (BSLT) to determine the LC50. Acute and subacute toxicity studies according to the Limit Test of OECD guidelines no. 425 and 407 were carried out through oral gavage accordingly. It was found that the LC50 of SMME was 674.4ppm while its LD50 via acute test was more than 5000 mg/kg. Neither sign of toxicity nor significant difference in food intake, weight gain, gross necropsy, hematological and biochemical analyses, and histological evaluation were recorded between the subacute of control and treated groups except the levels of AST and testosterone in male and sodium and triglycerides in female rats. The increase of testosterone in male rats might occur through a specific pathway as the SMME did not increase the hormone level in the female's. According to Globally Harmonized System (GHS) classification, SMME in this study can be classified as Category 5 (Safe) and nontoxic. Data from this study can be served as a primary predictive guide for future research in assessing the efficiency and safety of S. myosotiflora consumption for human trials.

Key Words: *Smilax myosotiflora, aphrodisiac, acute, subacute, toxicity, BSLT.*

Smilax myosotiflora Metanol Ekstresinin Toksikolojik Değerlendirmeleri ve Subakut Çalışmada Erkek Sıçanların Testosteron Düzeyine Etkisi

ÖZ

S. myosotiflora A. DC., popüler bir erkek afrodizyağı, enerji artırıcı ve geleneksel tıpta bel ağrısını giderici olarak tüketilen tropikal bir sürünücü bitkidir. Bilimsel çalışmalar, bitkinin erkek sıçanlarda cinsel davranışları ve testosteron seviyesini artırabildiğini göstermiştir. Bununla birlikte, toksisitesi hala bilinmemektedir. Bu nedenle, bu çalışmada S. myosotiflora metanol ekstresinin (SMME) toksisitesinin in vitro ve in vivo çalışmalarla araştırılması amaçlanmıştır. SMME, LC50'yi belirlemek için tuzlu su karidesi ölüm testine (BSLT) tabi tutuldu. Akut ve subakut toksisite çalışmaları OECD yönergelerinin 425 ve 407 nolu testleri ile oral gavaj kullanılarak gerçekleştirildi. SMME'nin LC50'sinin 674.4ppm olduğu, akut toksisite testi ile LD50'sinin ise 5000 mg/kg'dan fazla olduğu bulundu. Subakut kontrol ve tedavi edilen gruplar arasında erkek sıçanlarda AST ve testosteron ve dişi sıçanlarda sodyum ve trigliseritler dışında, ne toksisite belirtisi ne de gıda alımı, kilo alımı, makroskopik otopsi, hematolojik ve biyokimyasal analizler ve histolojik değerlendirmelerde anlamlı fark kaydedilmedi. SMME kadınlarda hormon seviyesini artırmadığı için, erkeklerde testosteron artışı, belirli bir yolakla gerçekleşiyor olabilir. Küresel Uyumlaştırılmış Sistem (GHS) sınıflandırmasına göre SMME, bu çalışmada Kategori 5 (Güvenli) ve toksik olmayan olarak sınıflandırılabilir. Bu çalışmadan elde edilen veriler, insan denemeleri için S. myosotiflora tüketiminin etkinliğini ve güvenliğini değerlendirmede gelecekteki araştırmalar için birincil tahmin kılavuzu olarak kullanılabilir.

Anahtar Kelimeler: *Smilax myosotiflora, afrodizyak, akut, subakut, toksisite, BSLT.*

Received: 12.03.2023
Revised: 28.06.2023
Accepted: 05.07.2023

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INTRODUCTION

In the history of Greek, Aphrodite was referred to a daughter of Zeus which embodied the meaning of goddess of love, beauty, allure, and procreation. Today, adapting from the word, aphrodisiac is defined as a substance or component that can boost sexual desire or increase sexual enjoyment. Its comprehension has been widened to include any material that is able to aid sexual function or improve the systems of sexual operation either in males or females (Sharma et al., 2017). The substance is designated to any form which able to increase sex performances, for example foods, vitamins, beverages, or natural/chemical compounds. Inability to perform in the intimate event may create huge problems and socially, and physiologically affects the one and their partners (Capogrosso et al., 2021; Sharma et al., 2016). For men, this indicates that they may potentially have the male sexual dysfunction (MSD) problem, one of the most common health threats other than heart and diabetes diseases among them (Andrea et al., 2021). The growing incidences and the demand for better therapeutic drugs on the men's sexual incompetence issue has led to the discoveries of natural substances from aphrodisiac plants, organisms, or microbes as an alternative to treat the MSD. Natural constituents from aphrodisiac plants received a great deal and global attention from scientists as they are cheaper, more accessible, lesser toxic, have no physical suffer and safer than synthetic drugs or other conventional clinical treatments. Among these natural and traditional love potions is a plant from *Smilax* sp., *Smilax myosotiflora* A. DC., which known as the 'horny little devil'.

S. myosotiflora is a plant that habited throughout the tropical climate regions in Southeast Asia (SEA), such as Peninsular Malaysia, the Indonesian Island of Java, and southern Thailand. The plant was popularly consumed by the indigenous people and local folks as a male aphrodisiac, energy booster, and lumbago reliever in the old medicinal practice in the regions (Nurraihana et al., 2016; Rao et al., 2016). It also was widely used by the ancient medicine practitioners to treat rheumatism, diabetes, syphilis, fever, sore throat, and virility (George et al., 2010; Lin, 2005; Ong & Azliza, 2015). The leaves are dark green, heart-shaped, and deciduous from 5-15cm long, while the tubers are dark brown rough surface, irregular round shape, and slightly sweet in odor. The tuber was reported to be the most functional part of the *S. myosotiflora* plant as it composed many bioactivities, especially as a male sexual enhancer and other medicinal benefits (Chyang et al., 2018; Dasuki et al., 2012; George et al., 2010; Mustaffar Bakri, 2013; Rahman et al., 2010; Wan Ghazali et al., 2016; Wan et al., 2013). Previously, it was found to have a comparable peak of protein to *E. longifolia*, *Rafflesia sp*., and *Labisia pumila*, which is responsible to increase the expression of testosterone levels in the Leydig cells, the 4.3kDa peptide (A. Osman et al., 2007). This finding was relatable to the *in vivo* test elsewhere, where the intake of the plant was able to significantly elevate the level of testosterone in male rats (Hilmi et al., 2015; Hoon et al., 2005; Wan et al., 2013, 2016)commonly known as ubi jaga in Malaysia, from the family of Liliaceae. The optimum dose of *S. myosotiflora* as an aphrodisiac was found between 400-800mg/kg where aqueous and methanol were the most active forms (Hilmi et al., 2015; Hoon et al., 2005; Wan et al., 2013).

Despite its broad use in folk medicine and significant contributions to male reproductive studies, data on its toxicity effect remains unknown. Thus, this study aimed to investigate the toxicity effects of *S. myosotiflora* methanol extract (SMME) through acute and subacute tests *in vivo*. The cytotoxicity profile of the plant was also evaluated *in vitro* through the brine shrimp lethality test (BSLT). The findings from this study can be a primary predictive guide for future research in assessing the efficiency and safety of *S. myosotiflora* consumption for human trials.

MATERIALS -METHODS

Sample Preparation

Tubers of *S. myosotiflora* were collected from the Titiwangsa range in the state of Perak, Malaysia (5°29'31.6"N, 101°26'26.6"E) from May to August

2018 with the help of the aboriginal people. The plant material was identified by a botanist and was deposited at the Herbarium Universiti Kebangsaan Malaysia Bangi of the Faculty of Science and Technology, Universiti Kebangsaan Malaysia, with an authentication no. PIIUM0018-1. Tubers were washed under running water and dried in a circulating air oven at 50°C for approximately five days. The grinding process was performed on the dry material using a power grinder machine (Golden Bull, Malaysia) to obtain the powdery sample. Plant powder was subjected to Soxhlet extraction, where methanol was the solvent using a 1:10 ratio. The methanolic solution was filtered and concentrated in a rotary evaporator under reduced pressure continued with oven-drying to yield the sticky paste of SMME. The SMME was tightly sealed in 4°C storage prior to future use.

Toxicity Test in vitro - BSLT

BSLT is one of the effective *in vitro* assays to evaluate the cytotoxicity substance using a biological model, *Artemia salina*. The assay was conducted according to Laurentius et al. (2018) with slight modifications. Briefly, the cysts were initially hatched in the artificial seawater for 48 hours. A set of SMME concentrations (1000, 800, 600, 400 , and 200ppm) was prepared using distilled water and 1% DMSO as the emulsifier. Later, 10 active brine shrimps were transferred into each 6-well plate before 2.5mL of an SMME concentration, and seawater were added to every well. Each SMME concentration was prepared in triplicate in three independent experiments. Controls were set by 10 brine shrimps in 5mL seawater as the negative and 10 brine shrimps with 2.5mL of pure ethanol and seawater as the positive. Using a magnifying glass, the dead shrimps were counted after 24 hours of incubation. The percentage of mortality was calculated for every concentration by summing the dead over total brine shrimp tested. The median lethality concentration (LC_{50}) of SMME was determined by plotting a graph of mortality percentage against SMME concentrations using the linear regression method. The obtained data explained the concentration of the SMME, which can cause to half death of total shrimps in a certain time exposure.

Toxicity Test in vivo - Acute , and Subacute Tests

In this study, the *in vivo* toxicity tests were performed according to the Limit Test of the Organization of Economic Co-Operation and Development (OECD) guidelines no. 425 (Up-and-down Procedure) for acute toxicity and Limit Test of no. 407 (Repeated dose 28-days) for subacute toxicity (OECD/OCDE, 2008a, 2008b). The animal ethic of the tests was obtained from Universiti Sains Malaysia (USM) Institutional Animal Care and Use Committee (Approval no.: USM/IACUC/2018/113-936) while the rats were supplied and placed in the Animal Research and Service Centre (ARASC), USM, Malaysia. In the tests, healthy, nulliparous with 8-10 weeks old of Sprague Dawley rats were first acclimatized for a minimum of five days and were maintained under the standard laboratory condition at ambient temperature 22±2°C and relative humidity of 60-70%. The photoperiod was consistent throughout the study, with 12 hours light and 12 hours dark. Their food supply was freely accessible, where a standard rodent diet pellet and reverse osmosis (RO) water *ad libitum* were given. Rats were grouped in 2-3 per standard cage filled with wood shaving as the bedding.

In the acute toxicity through the Limit Test method, five female rats received a single dose of 2000mg/kg SMME on their first day and were observed within a fortnight for any sign of toxicity or mortality. The test proceeded with a new batch of five female rats treated with a single dose of 5000mg/kg and monitored within the same period accordingly. In the Limit Test of subacute, 12 rats of both sexes which were randomly selected into control and treated groups, were treated with distilled water or 1000mg/kg SMME daily for 28 days. Weight gain, food intake, signs of toxicity, and mortality of the rats were recorded during the period. Then, all rats were anesthetized and humanely sacrificed on the

following day through intraperitoneal injection of pentobarbital for blood collection (only for subacute rats), and dissection of organs such as liver, kidney, heart, spleen, brain, lungs and sex organs to perform the gross examination and relative organ weight calculation.

Hematological , and biochemical analyses

The blood samples collection taken through cardiac puncture were subjected to the heparin lithium tubes for hematological test and the non-EDTA coated tubes for biochemical analyses at a commercial laboratory. Other than the normal biochemical parameters such as total protein (TP), albumin (ALB), aspartate transaminase (AST), aminotransferase (ALT), total bilirubin (TBIL), triglyceride (TG), total cholesterol (TC), creatinine (CRE), and so forth, the reproductive hormones namely testosterone, estradiol, and progesterone were also evaluated.

Histological analysis

After euthanasia, the liver, kidney, and testis from the subacute rats were preserved in a fixation medium of 10% buffered formalin for histopathological examination. The organs were routinely processed, embedded in paraffin wax, sectioned into 3–5µm with a microtome and stained with hematoxylin and eosin (H&E) stain according to a standard laboratory method. Finally, the stained sections were analyzed for any modifications in the morphology of particular tissues under a microscope fluorescence and an image analyzer (Olympus, Japan).

Statistical analysis

Data was first determined for their normality using D'Agostino-Pearson Omnibus and, or Shapiro-Wilk normality tests. All values were expressed as the mean ± standard deviation (SD), and the results were analyzed statistically by One-way Analysis of Variance (ANOVA) or Kruskal Wallis test followed by Mann-Whitney when not normally distributed using statistical software GraphPad PRISM Version 6.0 by GraphPad Software Incorporated Company, California. $P < 0.05$ compared to control was considered to be statistically significant.

RESULTS AND DISCUSSION

BSLT is an early cytotoxicity screening of the bioactive compounds in plant extracts using the *A. salina* as a bioindicator. Figure 1 displayed the graph of mortality percentage of the brine shrimp over SMME concentrations with an equation y = 0.1075x-22.5 and $R^2 = 0.938$. The mortality percentage in the graph was the average number from triplicates in the three independent experiments. According to the graph, the mortality percentage would increase if the SMME concentration increased. At the maximum of SMME concentration, 1000 ppm, 90% of the brine shrimp was killed. From the graph, it was found that the LC_{50} of crude methanol extract of *S. myosotiflora* was 674.4 ppm.

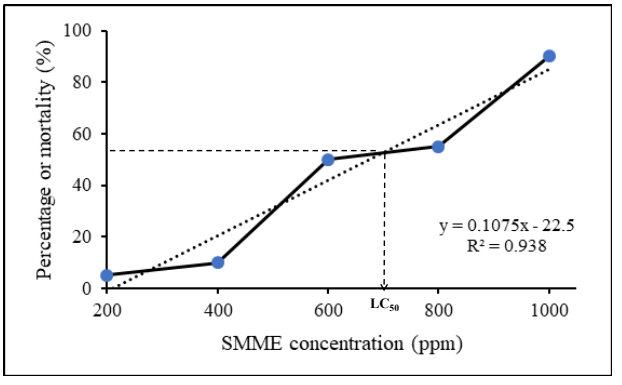

Figure 1. Mortality percentage of *A. salina* over SMME concentrations in BSLT toxicity study. *Abbreviation*: LC_{50} - Median lethality concentration, SMME - *S. myosotiflora* methanol extract.

The acute toxicity effect of the SMME was determined as per OECD guideline no. 425, where the Limit Test single doses of 2000 and 5000 mg/kg were applied. At the high doses of SMME administration, the female rats did not induce any lethality or mortality effect till their necropsy day. All rats displayed normal behavior and no visible signs of toxicity; for example, salivation, aggression, rising furs, and writhing were noticed throughout the study, except half of 5000 mg/kg SMME-treated rats experienced frequent head rubbing in the first few hours. The color stools and urine of all rats were also normal. Food and water consumption in all groups were perceived as normal within the period. Through the gross necropsy, no pathological abnormalities were discovered in the groups. On top of that, no significant difference was noted in the relative organ weight (ROW) in all groups (Table 1). According to the OECD guideline no. 425, since no irreversible or remarkable toxicity or mortality effect occurred in the treated rats at 2000 mg/kg and 5000mg/kg dosages, hence, the median lethality dose (LD_{50}) of the SMME was greater than 5000 mg/kg.

Table 1. Absolute and relative organ weight of the female rats in acute toxicity test.

Organs	Control		2000mg/kg		5000mg/kg	
	AOW (g)	ROW (%)	AOW (g)	ROW (%)	AOW (g)	ROW (%)
Brain	2.32 ± 0.16	0.98 ± 0.08	1.98 ± 0.15	0.84 ± 0.05	1.94 ± 0.23	0.88 ± 0.13
Thyroid	0.51 ± 0.14	0.22 ± 0.07	0.34 ± 0.19	0.21 ± 0.00	0.47 ± 0.04	0.21 ± 0.02
Trachea	0.25 ± 0.14	0.10 ± 0.05	0.16 ± 0.03	0.07 ± 0.01	0.17 ± 0.08	0.08 ± 0.04
Lungs	2.13 ± 0.47	0.89 ± 0.15	11.3 ± 0.23	0.78 ± 0.12	1.47 ± 0.28	0.66 ± 0.14
Thymus	0.40 ± 0.08	0.17 ± 0.04	0.47 ± 0.03	0.20 ± 0.01	0.45 ± 0.17	0.21 ± 0.08
Heart	0.93 ± 0.06	0.39 ± 0.03	0.87 ± 0.11	0.37 ± 0.03	0.91 ± 0.07	0.41 ± 0.03
Stomach	2.41 ± 0.39	1.02 ± 0.15	2.72 ± 1.37	1.00 ± 0.37	2.07 ± 0.43	0.93 ± 0.19
Intestines	18.09 ± 3.32	8.08 ± 1.83	13.4 ± 3.45	6.41 ± 0.76	17.33 ± 1.42	7.80 ± 0.60
Spleen	0.85 ± 0.16	0.37 ± 0.06	0.63 ± 0.20	0.30 ± 0.03	0.61 ± 0.04	0.28 ± 0.03
Liver	9.36 ± 1.97	3.94 ± 0.79	8.29 ± 1.05	3.55 ± 0.55	8.09 ± 1.50	3.68 ± 0.63
Adrenals	0.07 ± 0.00	0.03 ± 0.00	0.05 ± 0.02	0.03 ± 0.00	0.06 ± 0.01	0.03 ± 0.01
Kidneys	2.36 ± 0.48	0.97 ± 0.14	1.52 ± 0.41	0.72 ± 0.07	1.60 ± 0.15	0.72 ± 0.05

Values are expressed as mean ± SD; n = 5-6 rats per group. No significant difference between the organs of control and SMME-treated groups in AOW or ROW. P-value < 0.05 is considered significantly different. *Abbreviations*: AOW - absolute organ weight, ROW - relative organ weight.

In the subacute test, the Limit Test of the OECD guideline no. 407 was implemented. The daily oral administration of SMME at a high dose, 1000 mg/kg, for 28 days did not induce any symptoms of toxicity either in male or female rats. No deaths or obvious clinical signs were found in any groups throughout the study. None of the rats showed signs of toxicity on their skins, fur, eyes, sleep, salivation, diarrhea, and behavior. Overall, their daily food intake, and body weight gain in the male and female groups were found no significant difference between the control and treated rats (Figures 2). The average daily food intake for control and treated groups in male were 14.4 and 17.9g/day, while in the female were 16.1 and 17.1g/day accordingly. The body weight gain in male control rats was 15.3%, while for the treated group was 18.5% during the interval. For females, the body weight gain was 14.0% in the control group and 14.2% for the treated group. The gross observations have revealed no abnormalities either in the control or treated groups. The ROW recorded on the necropsy day also did not show any significant difference between both

groups of genders, as displayed in Table 2, except there were significant differences on the absolute weight of thyroid in male and trachea in female groups. Meanwhile, in the study, the cervix, uterus body, uterine horns, oviduct, and ovary were weighed as a set of female sex organs, and they were found to have no significance between the control and treated groups. Concurrently, no significant difference was also displayed in the sex organs of male rats namely, the vesicle, penile, and testis.

The effects of the subacute oral administration of the SMME on the hematological and biochemical parameters are represented in Table 3. The hematological test was evaluated through full blood count, while the biochemical test was determined by renal function test, liver function test, and lipid profile. All of the tested hematological parameters were within comparable range in both genders. The SMME also caused no significant effect on the biochemical parameters except for the sodium and triglycerides of female rats, where they were detected significantly higher in the treated rats. Meanwhile, the treated male rats demonstrated a significantly reduced in the enzyme of AST but a significantly increased in testosterone levels ($P < 0.0001$). Likewise, the thyroid-stimulating hormone (TSH) and follicle-stimulating hormone (FSH) levels were lower than 0.008mIU/L and 0.3IU/L in the control and treated male rats. There were no significant differences in the testosterone, oestradiol, and progesterone hormone levels among female rats, with the TSH value being equal in both groups, 0.006 ± 0.002 mIU/L.

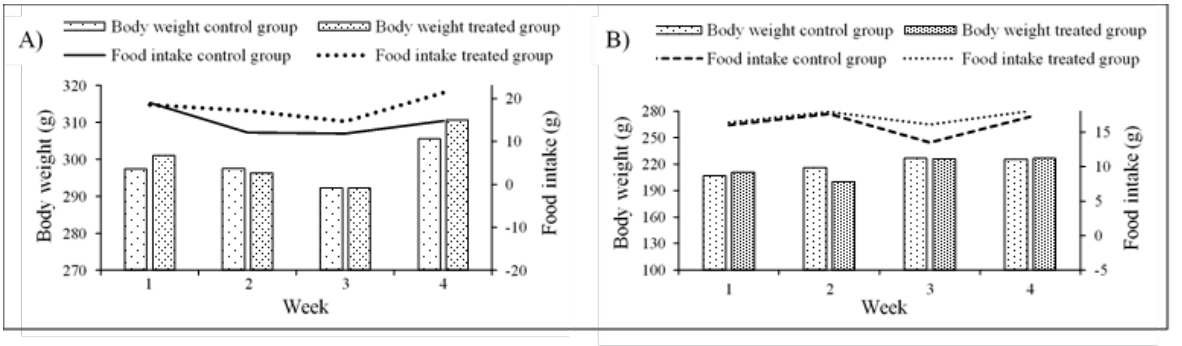

Figure 2. Body weight and food intake in male (A) and female (B) groups in the subacute test.

Table 2. AOW and ROW of male and female rats in the subacute test.

Organs	AOW (g)				ROW (%)			
	Control (M)	Treated (M)	Control (F)	Treated (F)	Control (M)	Treated (M)	Control (F)	Treated (F)
Brain	2.25 ± 0.09	2.25 ± 0.20	2.29 ± 0.18	2.22 ± 0.18	0.75 ± 0.06	0.76 ± 0.10	1.02 ± 0.12	0.98 ± 0.08
Thyroid$^{\alpha}$	0.89 ± 0.19	0.71 ± 0.16	1.04 ± 0.25	0.84 ± 0.34	0.29 ± 0.06	0.24 ± 0.05	0.46 ± 0.12	0.37 ± 0.15
Trachea$^{\beta}$	0.42 ± 0.36	0.29 ± 0.13	0.30 ± 0.02	0.25 ± 0.04	0.14 ± 0.14	0.10 ± 0.04	0.13 ± 0.01	0.11 ± 0.02
Lungs	2.23 ± 0.83	2.14 ± 0.81	2.84 ± 0.26	2.45 ± 0.66	0.73 ± 0.28	0.71 ± 0.24	1.26 ± 0.26	1.07 ± 0.27
Thymus	0.45 ± 0.08	0.45 ± 0.18	0.66 ± 0.59	0.42 ± 0.14	0.15 ± 0.03	0.15 ± 0.05	0.30 ± 0.15	0.18 ± 0.05
Heart	1.24 ± 0.17	1.15 ± 0.20	1.02 ± 0.19	0.94 ± 0.16	0.41 ± 0.03	0.39 ± 0.04	0.45 ± 0.07	0.41 ± 0.04
Stomach	3.00 ± 1.02	3.47 ± 1.23	2.63 ± 0.86	3.08 ± 0.48	1.01 ± 0.44	1.16 ±0.33	1.18 ± 0.42	1.65 ± 0.39
Intestines	7.25 ± 1.03	7.02 ± 1.21	19.79 ± 1.76	19.69 ± 2.08	7.25 ± 1.03	7.02 ± 1.21	8.77 ± 0.48	8.63 ± 0.62
Spleen	1.00 ± 0.08	0.92 ± 0.15	0.83 ± 0.15	0.76 ± 0.11	0.33 ± 0.02	0.31 ± 0.03	0.37 ± 0.06	0.33 ± 0.03
Liver	12.18 ± 0.78	12.08 ± 2.30	8.51 ± 0.86	8.27 ± 1.41	4.07 ± 0.50	4.05 ± 0.40	3.77 ± 0.28	3.60 ± 0.33
Adrenals	0.05 ± 0.01	0.06 ± 0.02	0.10 ± 0.14	0.08 ± 0.01	0.007 ± 0.001	0.007 ± 0.003	0.02 ± 0.03	0.02 ± 0.002
Kidneys	3.01 ± 0.54	2.70 ± 0.49	1.59 ± 0.70	1.65 ± 0.24	0.50 ± 0.11	0.46 ± 0.07	0.40 ± 0.06	0.36 ± 0.02
Vesicle	2.19 ± 0.64	1.78 ± 0.52	-	-	0.71 ± 0.18	0.60 ± 0.16	-	-
Penile	0.39 ± 0.14	0.36 ± 0.24	-	-	0.13 ± 0.05	0.13 ± 0.08	-	-
Testis	7.79 ± 1.78	6.08 ± 0.69	-	-	2.38 ± 0.54	1.97 ± 0.34	-	-
Female sex organs	-	-	1.20 ± 0.29	1.50 ± 0.86	-	-	0.53 ± 0.14	0.66 ± 0.38

Values are expressed as mean ± SD; n = 5-6 rats per group. *Abbreviations*: M - male, F - female, AOW - absolute organ weight, ROW - relative organ weight, $^{\alpha}$ - Significant difference in AOW of male groups, $^{\beta}$- Significant difference in AOW of female groups. P-value < 0.05 is considered significantly different.

Table 3. Effects of SMME on hematological and biochemical parameters in subacute toxicity study.

Parameters	Male		Female	
	Control	Treated	Control	Treated
Full Blood Count				
Hemoglobin (g/L)	143.00 ± 22.11	142.70 ± 4.08	136.40 ± 9.24	137.80 ± 6.62
RBC (x10^12 L)	8.53 ± 1.54	8.19 ± 0.29	7.84 ± 0.59	8.04 ± 0.43
PCV (L/L)	0.48 ± 0.10	0.45 ± 0.03	0.46 ± 0.03	0.45 ± 0.02
MCV (fL)	56.00 ± 3.16	55.17 ± 2.40	58.40 ± 2.30	56.50 ± 1.05
MCH (pg)	16.67 ± 0.82	17.33 ± 0.82	17.60 ± 1.34	17.33 ± 0.52
MCHC (g/L)	302.00 ± 28.02	317.00 ± 24.60	298.60 ± 16.9	303.70 ± 8.1
RDW (%)	19.75 ± 3.42	19.08 ± 1.86	16.70 ± 2.08	16.72 ± 1.30
White cell (x10^9L)	4.70 ± 3.47	5.25 ± 2.59	4.08 ± 1.03	3.67 ± 1.41
Neutrophils				
%	28.33 ± 13.97	34.33 ± 15.85	17.20 ± 4.9	16.33 ± 4.37
x 10^9 L	1.50 ± 1.27	1.93 ± 1.31	0.72 ± 0.33	0.62 ± 0.31
Lymphocytes				
%	64.50 ± 14.63	59.50 ± 14.38	72.00 ± 7.21	72.50 ± 7.12
x 10^9 L	2.85 ± 2.04	3.07 ± 1.57	2.92 ± 0.63	2.70 ± 1.11
Monocytes				
%	4.67 ± 3.32	3.50 ± 2.43	8.80 ± 4.44	8.00 ± 4.90
x 10^9 L	0.28 ± 0.26	0.15 ± 0.12	0.38 ± 0.22	0.27 ± 0.19
Eosinophils				
%	2.33 ± 2.33	2.67 ± 3.67	1.80 ± 0.84	2.83 ± 0.98
x 10^9 L	0.07 ± 0.05	0.08 ± 0.08	0.06 ± 0.05	0.10 ± 0.06
Platelet (x10^9 L)	489.80 ± 370.9	832.50 ± 403.3	723.20 ± 401.3	834.00 ± 329.4
Renal Function Test				
Sodium (nmol/L)$^{\beta}$	144.60 ± 3.91	142.00 ± 1.89	140.00 ± 1.41	142.00 ± 0.89
Potassium (mmol/L)	6.13 ± 1.49	5.17 ± 0.73	4.92 ± 0.84	4.72 ± 0.70
Chloride (mmol/L)	106.80 ± 12.26	102.10 ± 2.13	101.80 ± 1.30	100.80 ± 1.47
Urea (mmol/L)	8.04 ± 1.52	7.83 ± 1.16	7.28 ± 1.41	8.02 ± 1.36
Creatinine (µmol)/L	44.36 ± 8.57	38.28 ± 8.61	42.40 ± 11.67	36.67 ± 5.72
Uric acid (mmol/L)	0.17 ± 0.10	0.09 ± 0.05	0.06 ± 0.01	0.07 ± 0.03
Liver Function Test				
Total protein(g/L)	61.67 ± 4.41	58.00 ± 4.47	59.40 ± 4.40	62.67 ± 2.58
Albumin (g/L)	35.00 ± 2.53	33.00 ± 0.00	34.20 ± 1.92	35.50 ± 1.64
Globulin (g/L)	26.67 ± 3.01	25.00 ± 4.47	25.20 ± 2.78	27.17 ± 1.60
Albumin/Globulin ratio	1.33 ± 0.14	1.34 ± 0.22	1.36 ± 0.11	1.30 ± 0.09
Alkaline phosphatase (U/L)	164.50 ± 80.02	141.60 ± 43.88	158.20 ± 58.53	170.50 ± 47.45
AST (U/L)$^{\alpha}$	197.00 ± 40.08	144.40 ± 14.26	177.60 ± 54.98	178.30 ± 98.95
ALT (U/L)	99.33 ± 34.63	62.20 ± 13.83	63.40 ± 12.10	60.83 ± 14.70
Lipid Profile				
Total Chol. (mmol/L)	1.65 ± 0.26	1.58 ± 0.29	1.54 ± 0.17	1.70 ± 0.17
Triglycerides (mmol/L)$^{\beta}$	0.70 ± 0.31	0.54 ± 0.19	0.56 ± 0.09	0.77 ± 0.10
HDL Chol. (mmol/L)	0.47 ± 0.18	0.39 ± 0.07	0.46 ± 0.07	0.50 ± 0.14
LDL Chol. (mmol/L)	0.86 ± 0.27	0.94 ± 0.21	0.82 ± 0.14	0.84 ± 0.02
Total Chol/ HDL Ratio	3.75 ± 0.92	4.14 ± 0.80	3.39 ± 0.27	3.53 ± 0.60
Testosterone (nmol/L)$^{\alpha}$*	5.62 ± 3.61	21.40 ± 8.70	0.53 ± 0.23	0.48 ± 0.22
Estradiol (pmol/L)	-	-	151.00 ± 30.55	122.8 ± 57.00
Progesterone (nmol/L)	-	-	26.88 ± 17.90	48.77 ± 27.68

Values are expressed as mean ± SD; n = 5-6 rats per group. *Abbreviations*: M - male, F - female, P-value < 0.05 is considered significantly different. $^{\alpha}$ - Significantly different in male groups, $^{\beta}$- Significantly different in female groups, *- P-value < 0.0001, RBC-Red blood cell, PCV-Polycythemia vera, MCV-Mean corpuscular volume, MCH-Mean corpuscular hemoglobin, MCHC-Mean corpuscular hemoglobin concentration, RDW-Red cell distribution width, AST-Aspartate transaminase, ALT-Alanine transaminase, Chol.-Cholesterol, HDL-High density lipoprotein, LDL-Low density lipoprotein.

The histological evaluation of liver, kidney, and testis (the male) from rats of the subacute test was featured in Figure 3. Those photomicrographs represented the organs of both genders. The histology of the liver manifested normal architecture of visible central vein, hepatocyte, and sinusoids with no apparent congestion, inflammation, or cytoplasmic inclusion in all groups. While the kidney showed a normal histological structure of glomerulus, Bowman's space, and convoluted tubules in control and treated groups. For testis, the spermatogenesis process was normal in male rats where spermatogonia, spermatocytes, and spermatids exhibited normal arrangement in the respective stages. The seminiferous epithelium of SMME-treated rats appeared similarly to the control's. Normal features of Sertoli cells were also observed in the 1000mg/kg SMME-treated group.

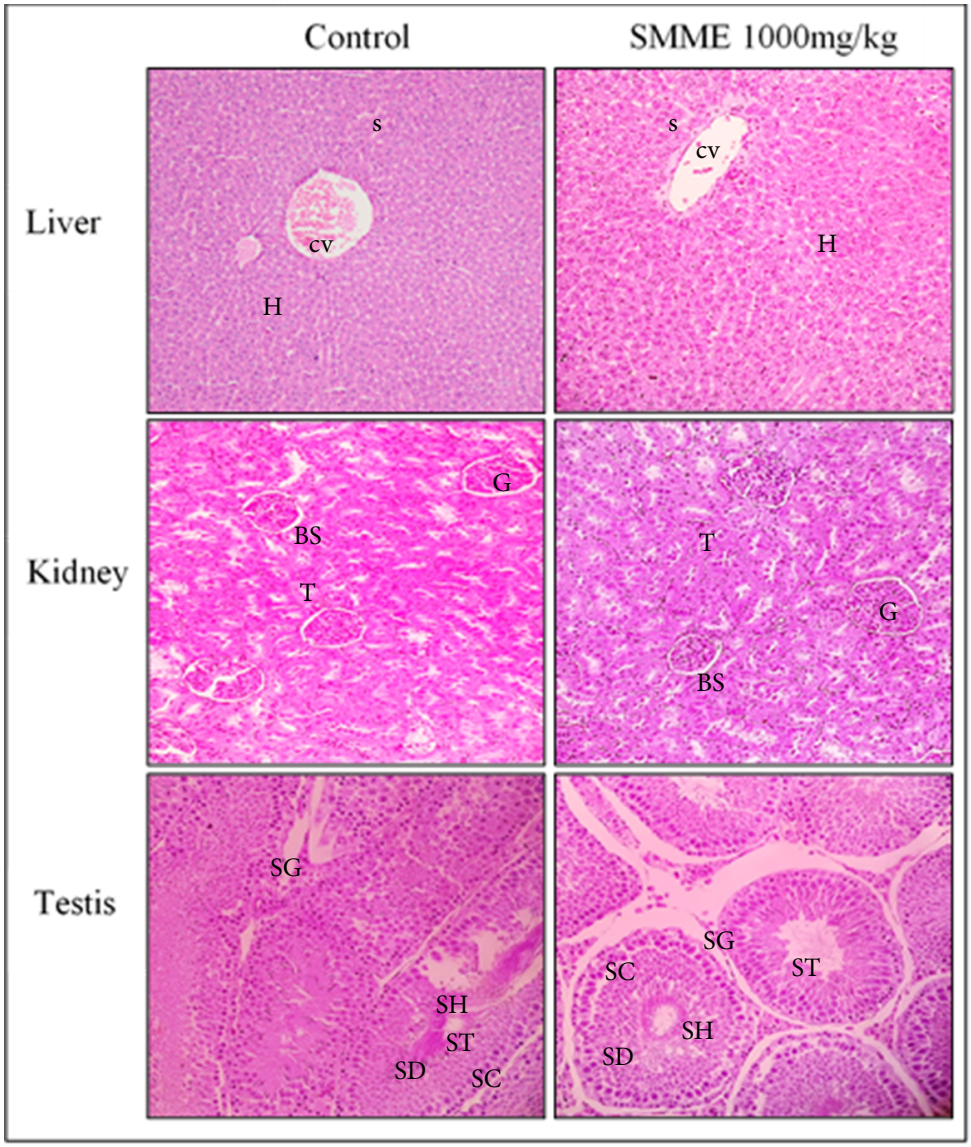

Figure 3. Histological result of liver, kidney, and testes under magnification 200x from subacute toxicity test where all tissues displayed normal architecture and morphologies. *Abbreviations*: S-Sinusoids, CV-Central vein, H-Hepatocytes, G-Glomerulus, BS-Bowman's space, T-Renal tubules, SC-Spermatocytes, SG-Spermatogonia, SD-Spermatids, SH-Sperm heads, ST-Sperm tails.

Aligned with the up-trend research and applications of medicinal plants to treat numerous medicinal issues and diseases, the toxicology studies are supposed to be along to ensure the safety use of the plant, especially when it relates to the reproductive organ system. This study has evaluated the toxic effects of horny little devil, a male aphrodisiac plant, in methanol extract through *in vitro* and *in vivo* studies. BSLT is regarded as an effective method to assess the cytotoxicity levels of substances due to its cost-effectiveness, time efficiency, and the no animal euthanasia needed. The determination of the LC_{50} through *in vitro* studies such as the BSLT will provide the first hint in the toxicology profile of the particular substance, including the *S. myosotiflora*. The value of LC_{50} from BSLT is defined as the concentration of a particular compound that can cause 50% mortality of the brine shrimps. For the SMME in this study, the LC_{50} was found to be 674.4ppm where this was classified as moderate toxic (LC_{50}: 500-1000ppm) according to Meyer's classification (Meyer et al., 1982). While based on the United State Environmental Protection Agency (US EPA) (United State Environmental Protection Agency, n.d.), SMME was considered as 'Practically nontoxic' since its LC_{50} was more than 100ppm; hence not harmful to the aquatic organism, which in this study was referred to the brine shrimps. Previously, the LC_{50} of SMME was reported to be greater than 1000mg/mL in a study by Wan et al. (2016).

Rodents including rats, were among the most common and primary predictive models of human effect in the toxicity assessments via acute and subacute tests through the OECD guidelines. In this study, both tests were performed using the Limit Test of acute no. 425 and subacute no. 407. The application of the Limit Test method can be applied efficiently for assessing particular substances which anticipated low toxicity risk based on the evidence derived from previous studies or experiments. Priorly, *S. myosotiflora* caused no changes in general behavior, sperm morphology, and pregnancy outcome after the SMME treatments in the paternal rats (Ahmad et al., 2014; Hilmi et al., 2015). Elsewhere, the LC_{50} of *S. myosotiflora* in petroleum ether, and ethyl acetate extracts also revealed high values ranging from 2900 to 4800 ppm (H. Osman et al., 2001). Hence, the Limit Test was executed to assay the acute and subacute toxicity profiles of *S. myosotiflora* in this study. As seen in the acute toxicity test, the SMME in 2000 and 5000mg/kg dosages did not exhibit any unusual behavior, mortality, morbidity, or adverse clinical signs in the treated rats. The AOW and ROW were also normal between the groups. According to the GHS, SMME could be classified under Category 5 (Safe) as its LD_{50} was higher than 5000mg/kg; thus, it considered as a nontoxic substance. Other aphrodisiac plants which reported to be in the same category were *Gardenia aqualla* (Mahmudul Hasan et al., 2018), *Pseudopanax arboreus* (Besong et al., 2018)we evaluated the effects of the leaf-aqueous extract of P. arboreus on the sexual behavior of normal male rats. The present study was designed to assess the effects of the leaf-methanolic extract of P. arboreus on amitriptyline-induced sexual dysfunction in male rats. Sexually impaired male rats were randomly divided into 4 groups of 8 rats each. Group 1 received 10 ml/kg distilled water, while group 2 was given 6 mg/kg Viagra. Groups 3 and 4 received 46.5 and 93 mg/kg of the leaf-methanolic extract, respectively. Female rats were made receptive by ovariectomy and subsequent hormonal treatment. Sexual behavior parameters were monitored on days 1, 7, 14, and 21 by pairing each male to a receptive female. The extract-treated rats registered significant decrease in mount latency (ML, *Nymphaea lotus* (Mahmudul Hasan et al., 2018), and *Cassia sieberiana* (Evenamede et al., 2019).

Meanwhile, all the elements measured in the subacute, including food intake, body weight gain, ROW, hematological and biochemicals analyses, and histopathology evaluation, were found to be normal, except there were significantly different in the AST and testosterone of the male group and significantly increased the sodium and triglycerides in the female treated group. AST is one of the biomarker enzymes

which monitor the structural integrity and damage in the liver (Mahmudul Hasan et al., 2018). The normal range of the AST in rats is 50-150 IU/L (Patrick & Villano, 1998). Thus, high AST enzyme in the blood of the male control group could be a sign of damaged in the liver caused by ischemic or toxic. Through this study, SMME has significantly helped to diminish the toxic compounds and restore the level of AST back to the normal range in the treated rats. For female rats, the level of AST was considerably normal and comparable between the control and treated groups, as its standard value is slightly higher. Meanwhile, the level of an essential male hormone, testosterone, was significantly increased in the SMME male-treated rats (P-value < 0.001) but not in female's. The remarkable increase of testosterone only in males is a great sign that the SMME may work selectively to increase the testosterone level through specific pathways, for example, the reproductive system while no detrimental effect to the liver and kidney occurred. This finding is aligned with the former studies (Dasuki et al., 2012; Wan Ghazali et al., 2016; Wan et al., 2013) and proved that *S. myosotiflora* is a potent male aphrodisiac and might be a promising agent to treat the MSD problem.

For female rats, the sodium was significantly increased in the SMME-treated group. However, the increment of the sodium was not considered as an adverse effect of the substance since the value was still within the normal range, 135-145 nmol/L. Simultaneously, the level of triglycerides exhibited a significant increase in the female rats treated with SMME. In response to excessive energy intake, the body stores surplus energy as triglycerides in adipose tissue. In the case of SMME treatment, the extract likely served as an energy source for the rats, resulting in elevated triglyceride levels in the treated group compared to the control group. Due to potential differences in energy requirements between gender, thus, female rats accumulated more triglycerides leading to a higher level in the treated female rats. Nevertheless, these stored triglycerides can be hydrolyzed into fatty acids to meet future energy demands. This situation explained the use of *S. myosotiflora* in traditional medicine as an energy booster where the plant might contain high-calorie content. Meanwhile, the treatment of *S. myosoriflora* in methanol extract form did not induce any abnormalities in the histopathology of liver, kidney, and testis rats. All examined organs appeared with the normal architecture of the respective organs. Some variations were spotted; however, they were very minimal and also detected in the control group. The histopathological results in the study have strengthened overall findings that *S. myosotiflora* is nontoxic and can be further investigated in human trials prior to the development of *S. myosotiflora*-based drug. It is recommended to perform other toxicity evaluations, for instance, genotoxicity, carcinogenicity, and teratogenicity studies in order to scrutinize and validate the safety profile of the *S. myosotiflora* plant.

CONCLUSION

The *in vitro* and *in vivo* toxicity evaluations of the SMME were carried out in the present study. Through the *in vitro* test of BSLT, the LC_{50} of SMME was 674.4ppm hence it was classified as 'Practically nontoxic' according to the US EPA. Meanwhile, the LD_{50} of SMME from the acute toxicity test *in vivo* was revealed to be more than 5000mg/kg and categorized as Category 5 (Safe). The SMME did not exhibit any treatment-related adverse effects in the subacute toxicity test after the behavior, food intake, weight gain, gross necropsy, hematological, biochemical, and histological analyses appeared no significant changes between the control and treated groups. SMME increased the testosterone level of males but not in female rats has shown that the plant was considerably safe after those *in vitro* and *in vivo* toxicity tests and can be further investigated for the application as a male aphrodisiac and a potent drug related to male testosterone deficiency provided more clinical research were carried out in the future.

ACKNOWLEDGEMENT

The authors would like to thank Universiti Sains Malaysia for the financial support through the Research Universiti Initiative (RUI) with grant no. RUI/1001/8012209. Many appreciations also to the staff of the Science Lab Management Unit of School of Health Sciences and the Central Research Laboratory of School of Medical Sciences, USM, for all the help given during the experiments.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTION STATEMENT

RAR: Experimenting, preparing the study text, reviewing the text, statistics analysis, interpretation of the data, literature research & etc. DSD: Developing hypothesis & reviewing the text. MDS: Developing hypothesis, literature research & reviewing the text. BNBB: Experimenting, interpretation of the data & technical supports. NFA: Experimenting & technical supports. WRWI: Developing hypothesis & reviewing the text.

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