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Original Research Article

In Vitro and *In Vivo* Evaluations of *Smilax myosotiflora* (Ubi Jaga) Compared to the Well-Known Aphrodisiac Plant of *Eurycoma longifolia* (Tongkat Ali)

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ABSTRACT

Smilax myosotiflora and *Eurycoma longifolia* are recognized as aphrodisiac medicinal plants in Malaysia. *E. longifolia* is widely purchased and extensively studied for its aphrodisiac effects, unlike *S. myosotiflora*. There has been some uncertainty regarding whether *S. myosotiflora* is comparable to *E. longifolia* in its aphrodisiac properties. In this study, both plants were extracted using a water distillation method under reflux, followed by clarification, freeze-drying, and yield comparison. The extracts were then subjected to *in vitro* experiments using TM-3 Leydig cell cultures. *In vivo* experiments involving mice were administered oral doses of 6 mg of each extract twice daily for 20 days. The results indicated that the extraction yield for *E. longifolia* was higher at 7.43% (w/w) compared to 5.06% (w/w) for *S. myosotiflora*. In the *in vitro* experiments, Leydig testicular cells treated with 50 µg/mL of *E. longifolia* for 72 hours exhibited an 83.93% increase in testosterone concentrations compared to untreated controls. Additionally, testosterone levels increased by 94.61% in mice treated with *E. longifolia* compared to control mice. However, no increase in testosterone was reported in either the *in vitro* or *in vivo* experiments conducted with *S. myosotiflora*. Both *S. myosotiflora* and *E. longifolia* were confirmed to enhance libido, as indicated by increased frequencies of mounting and intromission in pairing experiments with treated males and females. In conclusion, while both plants can enhance libido, *S. myosotiflora* did not demonstrate the ability to boost testosterone during the short duration of the treatments.

Keywords: *Ubi* Jaga, Tongkat Ali, Testosterone, Aphrodisiac, Libido, Medicinal plants.

Introduction

Smilax myosotiflora belongs to the family Smilacaceae and is a herbaceous climber found on small trees and bushes at altitudes up to 1,400 m.[1] It is commonly found in lowland evergreen forests and is characterized by a tough, rigid yet smooth, slender stem, lanceolate leaves, and pale-green or white flowers.[1] *S. myosotiflora* produces irregularly shaped, hard tubers and rhizomes that are slightly sweet in taste and odor.[1] Locally known as "Ubi Jaga," it has a reputation as an aphrodisiac among the Malays and indigenous populations. Traditionally, the rhizome of *S. myosotiflora* is consumed with betel.[2] The tuber is taken orally as a decoction for virility and back pain problems.[3] The leaves and fruit are used in the treatment of syphilis.[4] Extracts from this plant have also been shown to be effective against bacterial infections in humans.[5] *S. myosotiflora* contains phytochemicals such as stigmasterol, sitosterol, campesterol, alkaloids, coumarins, flavonoids, saponins, and tannins.[6,7] A study demonstrated that extracted samples of *S. myosotiflora* increased penile erection rates during one hour of observation.[8] Additionally, an 18% increase in epididymal sperm count was observed when treated with 400 and 800 mg/kg of *S. myosotiflora* extracts.[9]

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This study confirmed that orally ingesting the extract of *S. myosotiflora* enhances sexual behavior and fertility in male rats.[9] *S. myosotiflora* was also found to boost fertilization ability and sexual behavior indices in male rats and significantly increase sperm density.[10] These studies suggest that *S. myosotiflora* is an aphrodisiac plant, although the bioactive compound responsible for this activity has not yet been identified. Rats treated with *S. myosotiflora* have shown to have higher testosterone levels.[11] However, a later investigation discovered that rats' testosterone levels were lower than those of controls.[12] These contradictory findings highlight the uncertainty surrounding *S. myosotiflora's* capacity to raise testosterone levels similarly to *E. longifolia*. Known for its aphrodisiac properties, *E. longifolia*, sometimes locally referred to as "Tongkat Ali Putih," has been shown in both *in vitro* and *in vivo* experiments to raise testosterone levels.[13,14] A glycoprotein has been shown to be the bioactive component in *E. longifolia* that increases testosterone.[15] Recently, lectin affinity chromatography was used to isolate this glycoprotein quickly and precisely.[14] A 4.3 kDa bioactive peptide was found in both *S. myosotiflora* and *E. longifolia* by research employing the SELDI-MS method.[16] These results point to the possibility that *S. myosotiflora* contains a protein. Therefore, in contrast to *E. longifolia*, this work aims to verify the existence of protein in *S. myosotiflora* and assess how well its crude extract raises testosterone levels both *in vitro* and *in vivo*.

Materials and Methods

Plant Collection and Identification

On February 8, 2021, 30 kg of *Eurycoma longifolia* roots and *Smilax myosotiflora* rhizomes were purchased in bulk from indigenous people residing in Perak, Malaysia (GPS coordinates: 4.724305, 100.951893). Herbarium specimens of *E. longifolia* (HI1445) and *S. myosotiflora* (HI1447) were deposited at University of Malaya.[13] After being sun-

dried, the roots and rhizomes were dried at 45°C in a laboratory convection oven (OFA-110-8, Isotherm® Forced Convection Laboratory Oven, Esco Technologies Pty Ltd, SG). They were then chopped into smaller pieces and ground into a coarse powder using a blender (SUS304, Electric Powder Grinder, Suzhou Donggang Precision Technology Co., Ltd., CN).

Extraction
Each powdered plant material was extracted by aqueous distillation under reflux, similarly done previously for *E. longifolia*.[17,18] Briefly, a round bottom flask filled with 50 g of the root material was mixed with 500 mL of water. Next, the mixture with the round bottom flask connected to a condenser with a cooling jacket (attached to running tap water) was heated (EB 103, Mtops Heating Mantle, KR) to boiling for five hours. Once completed, the insoluble debris was separated from the soluble extract using Whatman No. 1 filter paper. The clarified extract was consequently dried and preserved by a freeze-dryer and weighed to calculate its % w/w.

Bradford assay
The same technique was used to measure the total protein in *E. longifolia* and the total protein in *S. myosotiflora*.[19] In total, 0.1 mL of the freeze-dried extract that was dissolved in 0.25% w/v water was combined with 1.0 mL of Bradford reagent [prepared using Coomassie Brilliant Blue G-250 (Bio-Rad Laboratories, US), 95% ethanol (absolute for analysis EMSURE® ACS, ISO, Reag. Ph Eur., Merck, US) and 85% (w/v) phosphoric acid (Phosphoric acid 85% for analysis EMSURE® ACS, ISO, Reag. Ph Eur., Merck, US)]. Using a spectrophotometer (Genesys 10s UV-Vis, Thermo Scientific, US) calibrated to a wavelength of 595 nm, absorbance measurements were collected after mixing and letting the mixture sit at room temperature for 20 minutes. Based on the absorbance values, a standard curve was created using Bovine Serum Albumin (BSA stock, 2 mg/mL concentration, molecular biology grade, ThermoFisher, US) to determine the protein content. A similar process for the *S. myosotiflora* extract was utilized to generate BSA solutions with different concentrations (0 to 10 µg), followed by mixing with 1.0 mL of Bradford reagent to create the standard curve.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
The freeze-dried extract of *S. myosotiflora* was subjected to SDS-PAGE to determine the molecular weight and presence of protein. The SDS-PAGE process was comparable to the previously mentioned procedures.[13] The electrophoresis apparatus utilized was a 15% homogenous gel and glass plates spaced one millimeter apart from Bio-Rad Laboratories in the US. About one hour and thirty minutes was spent with the power supply unit's (1645070, PowerPac Universal Power Supply, Bio-Rad Laboratories, US) specifications set to 120 V. The *S. myosotiflora* extract was ran next to a low molecular weight ladder (Takara Bio Inc., US) in different wells. Following the run's conclusion, the gel was stained with Coomassie Brilliant Blue (PhastGel Blue R, GE Healthcare, US) and repeatedly de-stained with 10% acetic acid (anhydrous for analysis EMSURE® ACS, ISO, Reag. Ph Eur., Merck, US) while being shaken to reveal the protein bands.

In vitro evaluations with testicular cell cultures
Cell cultures of TM-3 Leydig cells (CRL-1714TM cell types, acquired from American Type Culture Collections, ATCC, US) were grown to confluency.[13,14] Each well was approximated with 4000 cells using a hemocytometer (Neubauer, CN). After treating each well with 50 µg/mL of the plant extracts for 72 hours, the quantities of testosterone were measured using an ELISA kit (Elabscience Biotechnology Inc., US). Using a Tecan, CH ELISA microplate reader (Infinite M200 Pro), absorbances at 450 nm were measured to create a testosterone standard curve for the kit. The testosterone concentration in the test samples was determined using this standard curve. In comparison to the untreated control wells, the ultimate outcomes were presented as a percentage of testosterone release.

In vivo evaluations
Animal selection and treatments
ICR mice (30–40 g) were used for this experiment following approval from the university's Institutional Animal Care and Use Committee (IACUC), with approval No.: UMPIACUC/2021/01 (extension). Three groups were established: a control untreated group and two treated groups receiving *S. myosotiflora* and *E. longifolia* extracts, respectively, with four mice in each group. The treatment protocol was similar to previous work on *E. longifolia*, with modifications to the dosages.[13] The mice were dosed for 20 days with 6 mg of *S. myosotiflora* and *E. longifolia* extracts dissolved in water, administered orally at a volume of 1 mL via a feeding gavage. The dosing frequency was twice daily. All husbandry conditions were maintained according to Institutional Animal Care and Use Committee (IACUC) approval.

Sexual mating behaviours
After the 20th day, treated male mice were paired with females in a cage equipped with closed-circuit TV to observe the frequencies of their sexual behavior over a period of 24 hours.[20] The test males were first introduced to the cages for 30 minutes before introducing their partners to allow for acclimatization. Three parameters of sexual behavior—intromissions, mountings, and ejaculations—were carefully recorded and tabulated without averaging, as done previously.[19] The frequencies of these sexual behaviors were statistically compared to those of the control mice.

Drawing blood and measuring the quantity of testosterone
Upon dosing for 20 days and completing the sexual mating experiments, the procedure of sedating the animal to draw the blood was performed to measure the changes in blood testosterone concentration. Cardiac puncture was performed under sedation caused by the ketamine-xylazine mixture, and 0.8 mL of blood was extracted. The ketamine-xylazine cocktail was given at a dose of 125 mg/kg of ketamine and 10 mg/kg of xylazine. It was acquired from UKM Animal House (Universiti Kebangsaan Malaysia, Malaysia). The blood sample was centrifuged at 1000 × g for 20 minutes at 4 °C after being left to coagulate for two hours at room temperature to separate the serum. The blood serum or supernatant was used in an ELISA experiment. This experiment also used the same kind of ELISA kit utilized for the *in vitro* investigations.

Histopathology of certain organs
The kidneys and livers were dissected, and then they were immersed in 5 milliliters of 10% formalin (10% Neutral Buffered Formalin, Epredia, US) and left overnight. The tissues underwent the same processing and grading procedures as previously delineated.[21] Using graded xylene (for analysis EMSURE® ACS, ISO, Reag. Ph Eur., Merck, US) and alcohol, the tissues were cut and examined before being embedded in paraffin wax blocks. Sections were cut at 5 microns using a Leica microtome, put on glass slides, and stained with hematoxylin (for microscopy, Merck, US) and eosin (for microscopy, Sigma-Aldrich, US). Using a light microscope (Nikon, Eclipse TS100), the produced slides from each group were inspected for inflammation and congestion before being assessed.

Statistical analysis
Data collected for the mating observations and biochemical parameters were evaluated by one-way analysis of variance (one-way ANOVA) from Microsoft Excel version 10 by comparing data of each sample and control. Statistical significance of data was assessed by analysis of variance with four replications (n=4) for each sample, and differences were considered significant at ($p<0.05$).

Results and Discussion

Extraction yield
To extract the bioactive compounds from *S. myosotiflora* rhizomes and *E. longifolia* roots, a solid-liquid extraction method was employed using deionized water as the solvent.[17] Table 1 shows the extraction yield for *S. myosotiflora* and *E. longifolia* after freeze-drying, indicating

that the yield percentage of the water extract for *S. myosotiflora* was lower than that for *E. longifolia*. Water extraction is commonly chosen because it preserves the structure and allows the isolation of high-molar-mass compounds such as hemicelluloses, although it generally provides comparatively lower yields.[22] Additionally, water extraction was used for these aphrodisiac plants because they were traditionally consumed as decoctions for their purported benefits, necessitating a standardized methodology for all tested plants.[11,23] Freeze-dried plant samples efficiently remove water while preserving bioactive components, such as antioxidant compounds.[24] Polar proteins are particularly well-extracted using aqueous solvents. Previously, it was demonstrated that the glycoprotein from *E. longifolia* was isolated from its polar water extract and shown to elevate testosterone in *in vitro* tests using TM-3 Leydig cells.[13,14] Consequently, in the current study, extraction for both *S. myosotiflora* and *E. longifolia* was performed using hot distillation under reflux. The results for *S. myosotiflora* water extract indicated the presence of protein by the Bradford assay and quantitated as indicated in Table 2. The protein was also determined to have a single band, as shown in Figure 1. Similarly, the *E. longifolia* water extract also produced a single band of protein in SDS-PAGE, though with a lighter molecular weight of approximately 20 kDa.[19]

Table 1: Percentage of yield of extracts after freeze-drying

Root samples	Mass of solute (g)	Mass after removing solute and freeze-dried (g)	Yield percentage (% w/w)
S. myosotiflora	50	2.53 ± 0.08	5.06 ± 0.11
E. longifolia	50	3.72 ± 0.10	7.43 ± 0.12

n=4, mean ± S.D included

Table 2: Yield of total protein in *S. myosotiflora* rhizomes

Absorbance	Protein concentration	Yield of total protein (% w/w)
0.05 ± 0.03	12.68 ± 2.31	2.58 ± 0.61

n=4, mean ± SD included

In vitro evaluations

For a duration of 72 hours, 50 µg/mL of extracts of *S. myosotiflora* and *E. longifolia* were applied to TM-3 Leydig cells. During this time, the cells continued to exhibit normal shape and proliferation while still having an epithelial-like appearance. The morphology of Leydig cells under control and following a 72-hour treatment with extracts from *S. myosotiflora* and *E. longifolia* is shown in Figure 2.

A standard curve was produced using the enzyme-linked immunosorbent assay (ELISA) (Figure 3). Table 3 shows that, compared to the control, *E. longifolia* had the greatest testosterone concentration and percentage of testosterone release, measured 9.99 nmol/L (83.93%). According to ANOVA, there was a significant difference (P-value < 0.05) in the testosterone levels between *E. longifolia* and the control group.

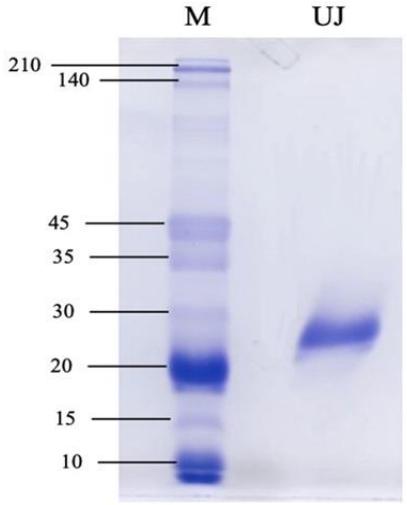

Figure 1: SDS PAGE for *S. myosotiflora* extract. M (low molecular weight marker); UJ lane loaded with the *S. myosotiflora* water extract showing a single band between 20 to 30 kDa.

When *E. longifolia* was dosed for 48 and 96 hours, it was discovered to stimulate testosterone production in similar TM-3 cells. When the extract was dosed for 72 hours, comparable repeatable action was reported.[25] For *S. myosotiflora* extract, the testosterone concentration and percentage of testosterone release were lower, measuring 5.38 nmol/L (-0.88%), nearly equivalent to the control's. ANOVA analysis for *S. myosotiflora* indicated no significant difference (P-value > 0.05) compared to the control. Although the percentage for *S. myosotiflora* was negative, this is not considered suppressive of testosterone production, as slight measurement errors are generally expected. Thus, the outcome suggests that *S. myosotiflora* has similar testosterone-producing capabilities to the untreated controls. It is concluded that *S. myosotiflora* did not significantly elevate testosterone concentration compared to *E. longifolia* in the TM-3 cells.

In vivo evaluations

Weight of mice and its histology

Twelve male mice were used in this experiment, with three groups: extracts from *S. myosotiflora*, *E. longifolia*, and a control group. For 20 days, the plant extracts were given orally by gavage twice a day at a dose of 6 mg/mL, whereas the control group was given water only. On the first day and the twentieth day, mice were weighed.

Table 4 shows the weight gain difference between the 20th day and the initial day. The *E. longifolia* and control groups exhibited weight gain by the 20th day, whereas the *S. myosotiflora* group did not. According to ANOVA, the testosterone concentration in *E. longifolia* was not significantly different (P-value > 0.05) compared to the control. A previous study demonstrated that the weights of the prostate, seminal vesicles, and body weight of rats significantly increased after orally administering *E. longifolia* extract for ten days.[26] In contrast, another study reported that rats treated with *S. myosotiflora* extract showed a decrease in weight within the first three weeks, followed by an increase in the fourth week.[27] Figure 4 presents photomicrographs of liver and kidney tissues at ten times magnification under a light microscope. Histological examinations of liver and kidney tissues from 3 out of 4 mice treated with *S. myosotiflora* (UJ1 to UJ3) were graded as moderate, mild, or unaffected for inflammation and congestion. No edema or necrosis was observed in the graded tissues, and none of the tissues exhibited severe histological damage.

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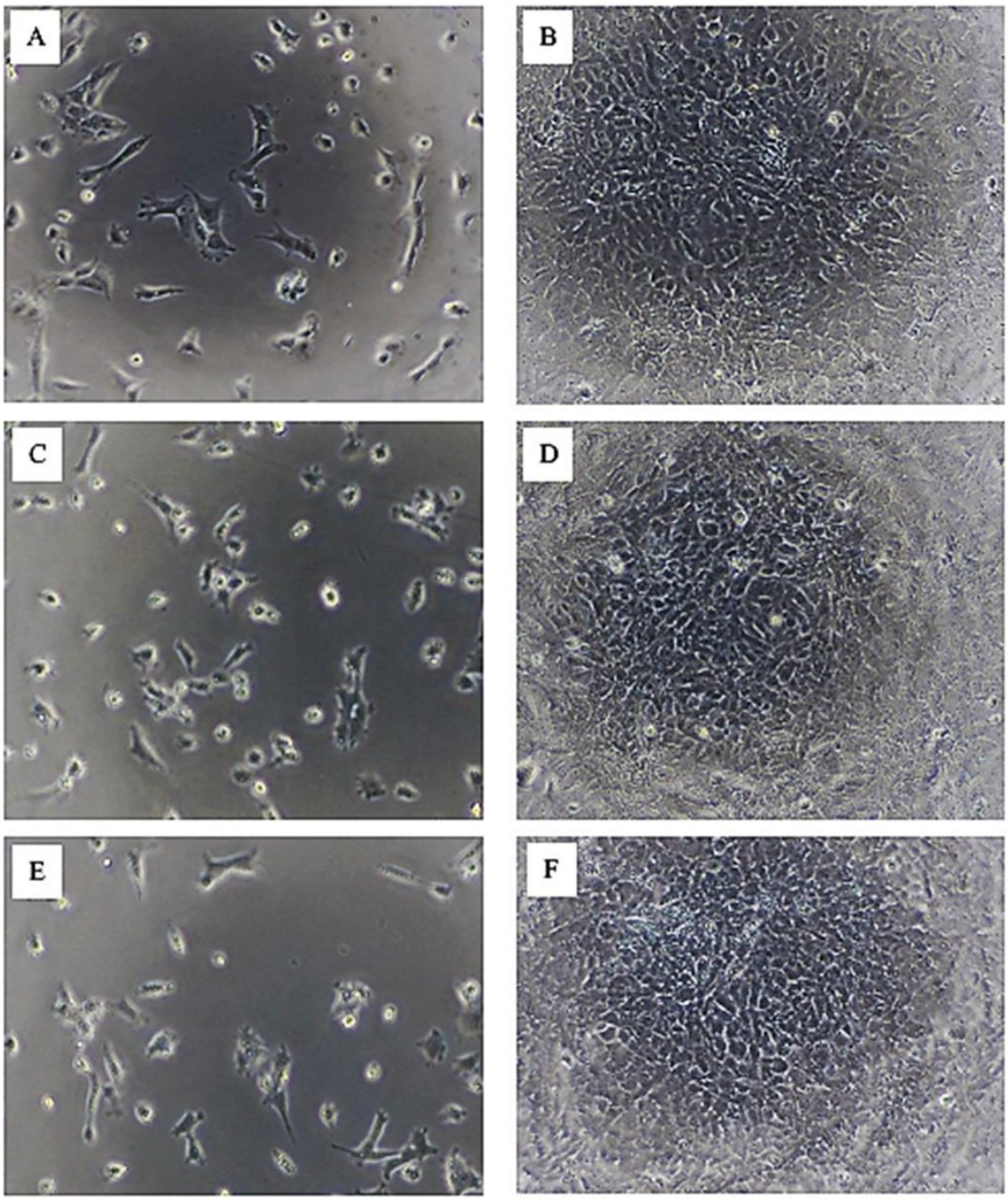

Figure 2: Morphology of TM-3 Leydig cells without treatment (control) at (A) 0 hour and (B) 72 hours, cell incubated with *E. longifolia* at (C) 0 hour and (D) 72 hours and with *S. myosotiflora* at (E) 0 hour and (F) 72 hours under magnification of 400X using inverted phase contrast microscope (CKX41 Olympus, JP)

A study indicated that male rats receiving *S. myosotiflora* orally for 70 days did not experience any harm.[9] The methanol extract of *S. myosotiflora* showed no cytotoxic activity, with LC_{50} values exceeding 200 µg/mL for all liver cancer cells.[28] Furthermore, the LC_{50} of *S. myosotiflora* methanolic extract was reported to be greater than 1,000 µg/mL in brine shrimp assays.[10]

Sexual behaviour frequency

The coital activity of an average male rat consists of three parts: ejaculation, intromission, and mounting. A male assuming a copulatory position—dorsally and from behind—without placing his penis within the female's vagina is known as mounting.[29] Pelvic thrusts, or hindquarter thrusting actions, are mostly connected to mounting. When the penis penetrates the vagina during a mount, this is known as intromission. The hindquarter thrusting actions in mice are associated with intromission. Intromission is usually followed by a brief phase of genital auto-grooming.[30] After many mountings and intromissions, the male will ejaculate, or forcefully expel semen from its body. A sperm plug is deposited in the vagina to block further intromission by other males until the sperm has had time to fertilize the ovum.[31]

Table 5 compares the frequencies of sexual mating behaviors in male mice treated with crude extracts of *E. longifolia* and *S. myosotiflora*. The observation involved 12 males paired with a new female each time in separate cages. Observations were conducted for 24 hours, and all behaviors were recorded. Ejaculation was rare among the treated groups, although sperm plugs were observed in the female mice.

Table 3: Percentage of testosterone release

Samples	Absorbance (450 nm)	Testosterone concentration (nmol/L)	Testosterone release compared to control (%)
Control	0.36 ± 0.02	5.43 ± 0.11	NA
S. myosotiflora	0.35 ± 0.02	5.38 ± 0.10	-0.88
E. longifolia	0.26 ± 0.01	9.99 ± 0.14	83.93

n=4, mean ± S.D included; NA: not applicable

Table 4: Weight of mice

Samples	Average weight (g)		Difference in weight (20th day – initial day)
	Initial day	20th day	
Control	33.27 ± 0.98	35.58 ± 0.99	+2.31
S. myosotiflora	35.89 ± 1.02	34.13 ± 0.91	-1.75
E. longifolia	32.82 ± 0.87	33.07 ± 0.88	+0.25

n=4, mean ± S.D included, + = weight gain, - = weight loss

A 24-hour observation period was deemed sufficient for the animals to become comfortable with each other and achieve ejaculation. Mice that achieved ejaculation were observed to refrain from further sexual encounters with the female. Typically, a prolonged period of genital auto-grooming follows ejaculation, and the male enters a phase of sexual inactivity, which can extend up to 24 hours in mice.[32]

Upon introduction to the female, each male mouse in all groups displayed notable behaviors, including following and smelling the female. Table 5 indicates that mice treated with *E. longifolia* exhibited a higher frequency of mounting and intromission than the *S. myosotiflora* and the control groups. An increase in libido, or sexual desire may explain this. A study on rats showed an increase in sexual activity following treatment with *E. longifolia* extract.[26] Statistical analysis using ANOVA revealed no significant differences (P-value > 0.05) in the frequencies of mounting and intromission between the treatment groups and the control.

Table 5: Sexual behaviour frequencies

Samples	Total frequencies observed		
	Mounting	Intromission	Ejaculation
Control	5	2	-
S. myosotiflora	8	4	1
E. longifolia	9	5	1

Total frequencies observed for n=4 mice without averaging

Table 6: Testosterone release when compared with control (%)

Samples	Absorbance (450 nm)	Testosterone concentration (nmol/L)	Testosterone release when compared to control (%)
Control	0.30 ± 0.04	8.11 ± 0.26	-
S. myosotiflora	0.30 ± 0.04	8.03 ± 0.25	-1.02
E. longifolia	0.12 ± 0.01	15.78 ± 0.43	94.61

n=4, mean ± S.D included

Blood testosterone levels

Blood was extracted and an ELISA kit was used to measure the testosterone content, referencing the earlier testosterone standard curve (Figure 3). As detailed in Table 6, the testosterone concentration in the blood serum of mice treated with *E. longifolia* extract was the highest among all groups, at 15.78 nmol/L (94.61%). In contrast, *S. myosotiflora* showed a negative percentage release of testosterone (-1.02%), nearly identical to the testosterone release observed in the control mice. ANOVA analysis indicated that the testosterone concentration in the *E. longifolia* group differed significantly (P-value < 0.05) from the control group, whereas the testosterone concentration in the *S. myosotiflora* group was not significantly different (P-value > 0.05) compared to the control. Several studies have demonstrated the efficacy of *E. longifolia* in enhancing testosterone levels. *E. longifolia* has been found to elevate serum testosterone levels in humans, address erectile function issues, and improve sperm production.[33,34] Furthermore, all three Tongkat Ali plants—*S. tuberosa* (Tongkat Ali Merah), *E. longifolia* (Tongkat Ali Putih), and *P. bullata* (Tongkat Ali Hitam)—contain various glycoproteins that have been shown to increase testosterone in both *in vitro* and *in vivo* tests.[14,35] In contrast, *S. myosotiflora* did not elevate testosterone levels either in the 72-hour TM-3 Leydig cell cultures or in the group of mice treated twice daily for 20 days. However, this does not definitively rule out any aphrodisiac activity of *S. myosotiflora*, as it did increase sexual behavior frequencies as shown in Table 5, suggesting an improvement in libido in mice. It is also not conclusive that *S. myosotiflora* cannot boost testosterone compared to untreated controls. Recent studies have reported that *S. myosotiflora* increases testosterone in rats.[2,36] These studies involved treating rats for 28 and 60 days using less polar methanolic extraction, whereas the current study employed hot water distillation under reflux, which extracts polar

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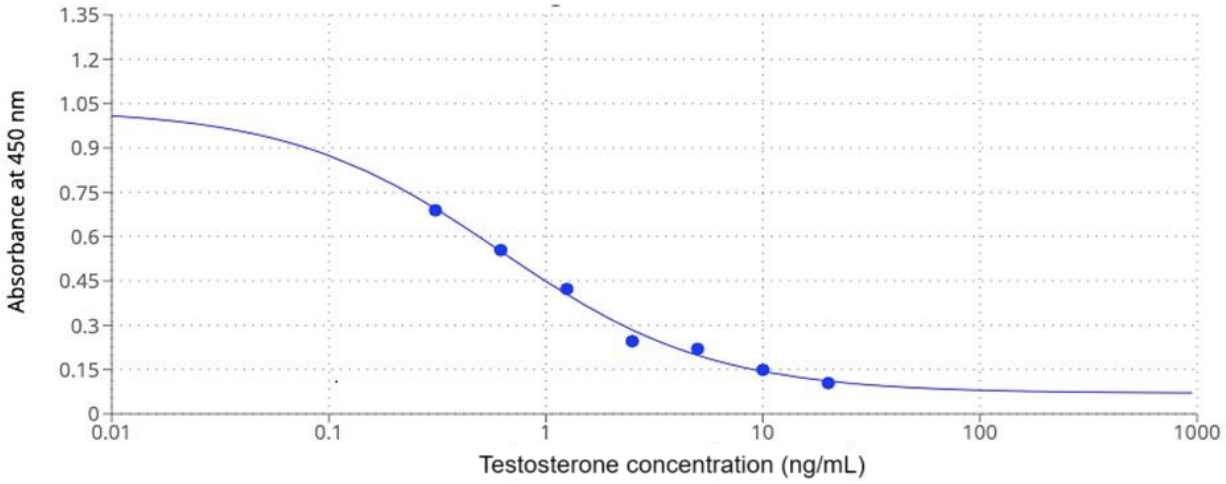

Figure 3: Standard curve for various testosterone concentrations at 450 nm absorbance using online curve calculator (AAT Bioquest, 2021)

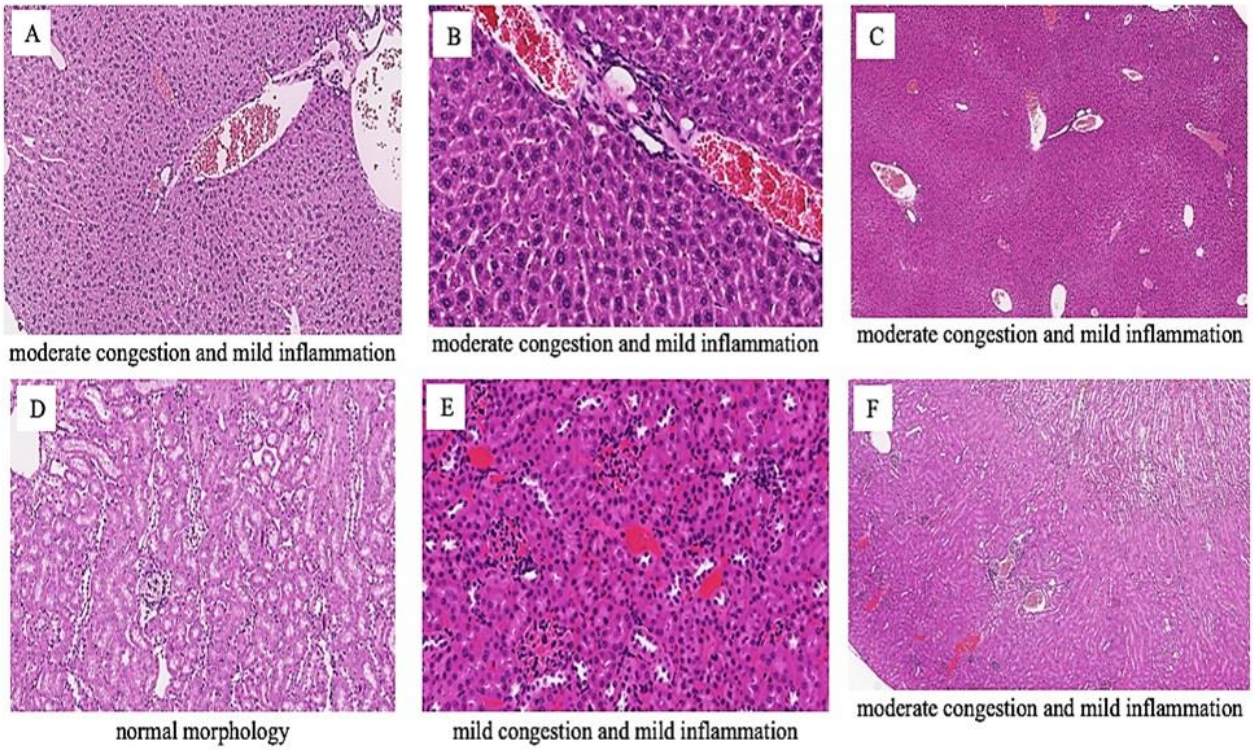

Figure 4: Photomicrograph and grading (normal, mild or moderate with none severe) of liver tissue: A) UJ1, B) UJ2 and C) UJ3 and kidney tissues: D) UJ1, E) UJ2 and F) UJ3 of mice treated with *S. myosotiflora*

constituents more effectively. Additionally, the duration of treatment in this study (20 days) was shorter than those studies. Nonetheless, *E. longifolia* appears to have a more rapid effect on boosting testosterone than *S. myosotiflora*, which may require a longer dosing duration to achieve similar effects.

Conclusion

Both *S. myosotiflora* and *E. longifolia* were extracted using water under reflux and examined for testosterone-boosting properties *in vitro* and *in vivo*. Similar to previous studies, *E. longifolia* was shown to elevate testosterone levels in treated TM-3 Leydig cell cultures and in a group of mice dosed twice daily for 20 days. In contrast, *S. myosotiflora* did not exhibit similar potential to elevate testosterone in male mice. However, it did produce higher sexual behavior frequencies compared to untreated controls during the 24-hour observation period following pairing with females. Based on these observations and the available literature, it is suggested that the aphrodisiac effects of *S. myosotiflora* are not likely due to testosterone boosting, particularly within the 20-day timeframe studied, unlike *E. longifolia*. While *S. myosotiflora* was safe for consumption at the tested dose and duration, further chronic studies of at least six months are recommended to determine its long-term safety conclusively.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that they will bear any liability for claims relating to the content of this article.

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