



## The Ranking of Tongkat Ali Plants to Boost Testosterone Hormone Evaluated in both *In vitro* and *In vivo* Experiments

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### ABSTRACT

In this study, the Tongkat Ali plants were evaluated for effect on testosterone concentrations and their order in boosting the steroidal hormone from the highest to lowest ranked. *Eurycoma longifolia* (EL), *Stema tuberosa* (ST), and *Polyalthia bullata* (PB) are collectively referred to as “Tongkat Ali”. The roots were dried and powdered, then extracted with water under reflux. Size-exclusion chromatography was utilized to isolate the protein fraction, which was subsequently characterized using the Bradford Assay and SDS-PAGE. LC-MS was used to test for the presence of natural testosterone within the Tongkat Ali plants. For *in vitro* and *in vivo* evaluations, each plant extract was treated with TM-3 Leydig cells (50 µg/mL) for 72 hours and administered in mice (6 mg/mL) twice/day for 20 days. The extraction of EL, ST, and PB yielded 0.74%, 0.46%, and 0.34% w/w of total protein, respectively. SDS-PAGE analysis revealed a single band between 10 and 15 kDa. *In vitro* evaluations showed that extracts of EL, ST, and PB increased testosterone secretion by 56.02 nmol/L (41.1% compared to the untreated controls), 40.49 nmol/L (18.65%) and 36.99 nmol/L (10.93%), respectively. In the *in vivo* studies, EL extract showed the highest testosterone concentration at 3.85 nmol/L (51.18% compared to the untreated controls), followed by ST with 3.35 nmol/L (43.95%) and lastly, PB at only 1.88 nmol/L (9.1%). Tongkat Ali plants boosted the male hormones in both *in vitro* and *in vivo* studies, with the order being EL>ST>PB.

**Keywords:** *Eurycoma longifolia*; Leydig cells; mice; root

### Introduction

Tongkat Ali is widely recognized as a prominent medicinal herb that has gained global popularity. In Malaysia, National Pharmaceutical Registry Agency (NPRA) registered products of Tongkat Ali and other herbals included within their labels the approval numbers of MAL#####TC (##, product specific code number; MAL short for Malaysia while T indicating it is an herbal category product and C, the product manufactured by a Good Manufacturing Practices (GMP) certified factory. It is mostly known for its aphrodisiac properties and has been consumed traditionally by men. An aphrodisiac is a substance that increases libido or sexual desires when consumed.<sup>1</sup> Aphrodisiac plants can be consumed naturally or made into products to stimulate sexual desires, primarily for men.<sup>2</sup> While any individual of any gender can use aphrodisiacs, they are often marketed towards men due to their role in increasing testosterone rather than oestradiol hormone levels. Maintaining a normal range of blood testosterone is vital for promoting libido and sexual performance of men. Studies have shown that Tongkat Ali can improve men's sexual desires and performances by elevating testosterone levels in men.<sup>3,4</sup>

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However, most research on Tongkat Ali has focused on one type of plant, *Eurycoma longifolia*, commonly called Tongkat Ali Putih in Malaysia. It is worth noting that two other types of Tongkat Ali have also been used traditionally for their aphrodisiac properties. According to the indigenous people, these two types of Tongkat Ali known are *Polyalthia bullata* (Tongkat Ali Hitam) and *Stema tuberosa* (Tongkat Ali Merah), both of which have been harvested and used for their aphrodisiac functions.<sup>5, 6</sup> Although all three plants are commonly known as Tongkat Ali, they belong to different genera and have roots with distinctive natural colors. Interestingly, their common names in Malay (the national language of Malaysia) are descriptive of the color of their roots: Tongkat Ali Putih (Putih meaning white), Tongkat Ali Hitam (Hitam referring to black), and Tongkat Ali Merah (Merah meaning red), respectively for *E. longifolia*, *P. bullata* and *S. tuberosa*.<sup>7</sup> In Indonesia, Tongkat Ali is referred to as Pasak Bumi.

*E. longifolia* is an abundant and widely available species, making it the preferred choice in research compared to *P. bullata* and *S. tuberosa*. This preference is evident in the literature on the less-studied *P. bullata* and *S. tuberosa* as aphrodisiacs. In *P. bullata*, the presence of protein has been reported, with the detection of a 4.3 kDa peptide using SELDI MS.<sup>8</sup> However, for *S. tuberosa*, there is still no scientific research reporting bioactive protein. Previously, in *E. longifolia*, a protein, more specifically a glycosylated protein, was extracted and demonstrated to increase testosterone in TM-3 ATCC® CRL-1714TM treated cells.<sup>9</sup> Thus, this study aims to provide evidence of the protein presence in Tongkat Ali plants and, additionally, to assess the effectiveness of crude extracts from the three Tongkat Ali plants in boosting testosterone levels through both *in vitro* and *in vivo* treatments.

## Materials and Methods

### Sampling and extraction of plant roots

*Eurycoma longifolia* Jack, *Polyalthia bullata* King, and *Stema tuberosa* (also known as *Jakiosopsis ornata* Wall.) roots were obtained from indigenous people living in Gambang, Pahang (GPS coordinate: 3°43'35.9 "N 103°08'57.7" E). Previously on 8<sup>th</sup> February 2021, specimens of *E. longifolia*, *S. tuberosa*, and *P. bullata* were placed at the herbarium of Universiti Malaya and tagged with the voucher numbers HI1445, HI1446, and HI1447, respectively.<sup>10</sup> The roots obtained were cut chips, then placed in a convection oven at 50 °C for drying. Subsequently, the dried Tongkat Ali chips were blended into a powder. Following the previously established extraction procedures, 10 % (w/v) of the powdered materials were mixed and heated under reflux for five hours before being filtered using Whatman No.1 filter paper.<sup>9</sup> The filtered crude extract was freeze-dried, weighed to determine the % w/w, and stored at -20 °C until further use.

### Size exclusion chromatography

To achieve better peak resolution, size exclusion chromatography (SEC) was performed using a series of 5 units of HiTrap™ desalting columns (Cytiva, US) attached to an AKTA Start system (GE Healthcare, US). After extraction, 1 mL of the sample was injected at a 1 mL/min flow rate, with water as the eluent and a UV detector set to 280 nm. For the Tongkat Ali plants, only deionized water was used as the mobile phase, as the plant root extract is known to be robust and not easily denatured, making the use of a buffer unnecessary.<sup>9</sup> The columns were performed on the ÄKTA™ Start with a flow rate of 1 mL/min, and the eluted protein fraction from the system was collected and freeze-dried. The chromatography results were analyzed using UNICORN™ Start 1.1 software (GE Healthcare, US).

### Protein quantification and characterization

#### Bradford assay

Protein content in the Tongkat Ali roots was quantitated using Bradford assay.<sup>10</sup> A volume of 100 µL from a 0.2 % (w/v) solution of the SEC-derived protein fractions was thoroughly mixed with 1 mL of Bradford reagent. The preparations were then incubated for 20 minutes at room temperature before measuring the absorbance using a Genesys 10s UV-Vis spectrophotometer (Thermo Scientific, US) set to a 595 nm wavelength. A 2 mg/ml stock solution of Bovine Serum Albumin (BSA) purchased from ThermoFisher, US, was diluted to produce concentrations of 10, 8, 6, 4, 2, and 0 µg to create a standard curve. The BSA standard curve was utilized to determine the concentration in the samples.

### Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis (SDS-PAGE)

To determine the molecular weight and purity of the protein fractions isolated from SEC, SDS-PAGE was performed using a vertical slab gel system from BioRad Laboratories, US, similar to previous work.<sup>9</sup> The glass plates have a thickness of 1 mm, and the resolving gel was cast with a homogeneity of 15 %. Electrophoresis was conducted at a voltage of 120 V for 1.5 hours. A stained protein ladder from Takara (Takara Bio Inc., US) was included in the gel. Protein bands were detected using a staining solution of Coomassie Brilliant Blue (PhastGel Blue R, GE Healthcare, US). The gel was soaked in the staining solution for approximately 40 minutes while gently shaken. De-staining procedures were repeated until the gel became transparent. At this point, a 10% acetic acid solution (Merck KGaA, DE) was used.

### Test for testosterone in the plant extracts

This test detected the presence of natural testosterone in the extracts. A total of 10 g of *E. longifolia*, *S. tuberosa*, and *P. bullata* extracts were sent to the Central Laboratory, University Malaysia Pahang Al-Sultan Abdullah for testosterone detection using Liquid Chromatography-Mass Spectrometry (LC-MS). The column employed was the Acquity UPLC-HSS T3 (Waters Corp., US), which separated ions based on their mass-to-charge (m/z) ratios. The unique mass spectrums obtained were then compared with the theoretical mass of testosterone available in the mass spectra libraries (Waters UNIFI-scientific library).

Additionally, 10 µl of a 50 ng/ml testosterone standard (purchased from Sigma-Aldrich, DE) was injected into the LC-MS instrument using the same parameters, and its m/z ratio was obtained for comparison with the extracts.

### In vitro studies

In vitro investigations of Tongkat Ali extracts in TM-3 Leydig cells. The TM-3 Leydig cell line (American Type Culture Collection, ATCC® CRL-1714TM, US), isolated from mouse testes, was selected as the cultured cell type, consistent with previous work on Tongkat Ali.<sup>9</sup> The complete medium consisted of DMEM/F-12 media (Nacalai Tesque, JP), which is a 1:1 ratio of Dulbecco's Modified Eagles Medium and F-12 Ham's nutrient mixture, 2.5 % Fetal Bovine Serum (Thermo Scientific, UK), 5 % Horse serum (Thermo Scientific, UK) and supplemented with 1% penicillin/streptomycin (Nacalai Tesque, JP) were prepared for the cell cultivation. A total of 1 mL of cryopreserved cell solution (BioVerde Inc., JP) was pipetted into a sterile 25 cm<sup>2</sup> tissue culturing flask, along with 4 mL of growth media. The flask was then incubated at 37 °C and 5 % CO<sub>2</sub> for 24 hours. The cells were grown to a confluence of 70 % for 2 to 3 days, with the culture medium replaced every 24 hours. Subsequently, the depleted media in the flask was removed every two days, and 5 mL of fresh complete medium was added.

Sub-culturing was carried out once the cells reached around 70 - 85% of confluency. The cells were washed with 4 mL of D-PBS (Nacalai Tesque, JP), and the depleted media was aspirated and discarded using a sterile pipette. Then, 1 mL of a 10x diluted 0.53 mM EDTA (Nacalai Tesque, JP) trypsin (0.25% (w/v)) solution was added and allowed to stand for 5 min. Subsequently, 1mL of growth medium was added to the flask to inactivate the trypsin, followed by centrifugation at 730 x g for 5 min. After discarding the supernatant, the cells were resuspended in 1 mL of the growth medium, and 0.25 mL of this cell suspension was added to tissue culture flasks containing fresh complete medium with supplements. The flasks were supplemented with 4.75 mL of growth media and were incubated for further growth. The cells were incubated with a 50 µg/mL sample concentration. Testosterone levels were measured using an ELISA kit (Elabsience Biotechnology Inc., US) after a 72-hour incubation period. The cell morphology was observed using an inverted phase-contrast microscope (CKX41, Olympus, JP).

### Determination of testosterone concentration using ELISA

The method followed the instructions from the Testosterone (T) ELISA kit (Elabsience, US). A testosterone standard curve was constructed according to the manufacturer's protocol. Absorbances obtained at 450 nm using a microplate reader (Infinite M200 Pro manufactured by Tecan, CH) were converted into corresponding testosterone concentrations using the plotted testosterone standard curve. After that, the results were analyzed to assess the testosterone-boosting activity of the tested plants using Equation 1.

Equation 1:

Testosterone release (%) =

$$\frac{(\text{Sample testosterone conc.} - \text{Control testosterone conc.}) \times 100}{\text{Sample testosterone conc.}}$$

### In vivo studies in mice

#### Animal selection

The mice used in this study were healthy ICR mice with weights ranging from 25g to 40g. Animal ethics approval was obtained from IACUC of University Malaysia Pahang Al-Sultan Abdullah, with approval number UMPIACUC/2021/01. A total of 16 mice were used, with four mice in each group (Control, extracts of *E. longifolia*, *P. bullata*, and *S. tuberosa*). Dosage method: Tongkat Ali plant extracts were administered orally via gavage in the morning and evening ("bis in die" b.d.) daily for 20 days at a dosage of 6 mg in 1 ml of distilled water, while the control group received 1 ml of water.

### Sexual mating behavior

Testing involved pairing male and female mice for 8 hours, following a modified protocol based on Johansen *et al.*<sup>11</sup> The observation was conducted with 20 males (4 mice from each group: Control without

treatment and groups treated with *E. longifolia*, *P. bullata*, and *S. tuberosa* paired with 20 females, each pair observed in separate cages. Males were given 5 minutes to acclimatize to the testing chambers before introducing the female. All behavioral tests were recorded on camera and analyzed using a conventional event recording program. The test was terminated if no intromissions were detected within the first 20 minutes. Otherwise, testing continued until ejaculation occurred or until the female returned to the male after ejaculation in pacing tests. Mounts, intromissions, and ejaculations were all counted. Only female-terminated intromissions were further categorized, with female termination as the female pushing the male away. The sexual behaviors were quantified and evaluated for statistical significance compared to untreated controls.

#### Blood collection and determination of testosterone concentration

A total of 1.0 mL of blood was obtained from the sedated mice via heart puncture using a ketamine-xylazine cocktail (125 mg/kg ketamine and 10 mg/kg xylazine), procured from the UKM Animal House (Universiti Kebangsaan Malaysia). The blood sample was allowed to clot for 2 hours at room temperature and then centrifuged at 1000 x g for 20 minutes at 2-8 °C to obtain the blood serum. The supernatant (blood serum) was collected for ELISA analysis. The method and the type of ELISA kit used were the same as those employed for the *in vitro* investigations by TM-3 Leydig cells.

#### Histology on selected organs

The kidney, liver, and testis were meticulously dissected and immersed in 10% formalin (5 mL) overnight. The tissue processing and grading procedures were in accordance with Haleagrahara *et al.*<sup>12</sup> The tissues were sliced, processed with graded xylene and alcohol, and then embedded in paraffin wax blocks. The tissues were sliced to 5 microns, and the sections were placed on glass slides using a Leica microtome and stained with hematoxylin and eosin. The prepared slide was examined under a light microscope (Nikon, Eclipse TS100) to assess all groups' inflammation, congestion, and grading.

#### Statistical analysis

The data collected were analyzed using ANOVA with Microsoft Office Excel 2010. The analysis of variance determined the statistical significance of data, and differences were considered significant at (p<0.05).

## Results and Discussion

#### Isolation of the protein fractions and their characterizations

The Tongkat Ali roots were extracted using a solid-liquid extraction method, commonly called leaching, using distilled water as the

solvent. This boiling process was carried out for an extended duration to allow the biomolecules or phytochemicals in the root powder to leach into the extraction solvent.<sup>13</sup> This extraction method was chosen due to its widespread use in preparing aphrodisiac herbs.<sup>14,15</sup> The total extracted solution was filtered to remove plant debris and subsequently freeze-dried to determine the net weight of the dry extract, as illustrated in Table 1. Consequently, the total protein yield in the plant roots was calculated by conducting the Bradford assay on the first peaks accumulated from size exclusion chromatography.

(Figure 1).

**Table 1:** Quantity of extracts and protein in Tongkat Ali roots

Plant Sample	Percentage of extract (% w/w)	*Yield of protein (% w/w)
<i>E. longifolia</i>	7.433 ± 0.12	0.735 ± 0.057
<i>P. bullata</i>	3.120 ± 0.09	0.340 ± 0.056
<i>S. tuberosa</i>	5.715 ± 0.11	0.455 ± 0.043

n=4; mean ± S.D included\* yield of total protein in 100 g roots was achieved by calculating the protein concentration in each Tongkat Ali plant extract, based on a protein standard curve constructed using various BSA concentrations, following the Bradford assay

Size exclusion chromatography (SEC), also known as gel permeation chromatography, separates the macromolecules in plants from their smaller constituents, typically those with a molecular weight of less than 1 kDa.<sup>16</sup> This entropically controlled separation method segregates molecules based on their hydrodynamic molecular volume or size. Size exclusion chromatography was employed to purify the plant root extracts and remove undesired non-protein components and small-sized compounds.<sup>17</sup> The results depicted in Figure 1 (A, B, and C) reveal that Tongkat Ali plants showed the first peak (blue line), which corresponds to the protein, as proteins are characterized by their high molecular weight, elute as the first peak in the size exclusion chromatography.<sup>18</sup> The second peak (blue line) represents the non-protein fraction, and the conductivity peak (red line) is more pronounced for this peak compared to the first peak. The separation occurs as sample molecules pass through a bed of porous particles, with larger molecules having shorter retention times and eluting faster from the column.<sup>19</sup> The highest content, expressed as % (w/w), of the aqueous extract and protein, was found in *E. longifolia*, as shown in Table 1. A previous study also demonstrated that *E. longifolia* had the highest protein yield compared to the other Tongkat Ali plants.<sup>10</sup>

**Table 2:** Absorbance, testosterone concentration, and % testosterone release

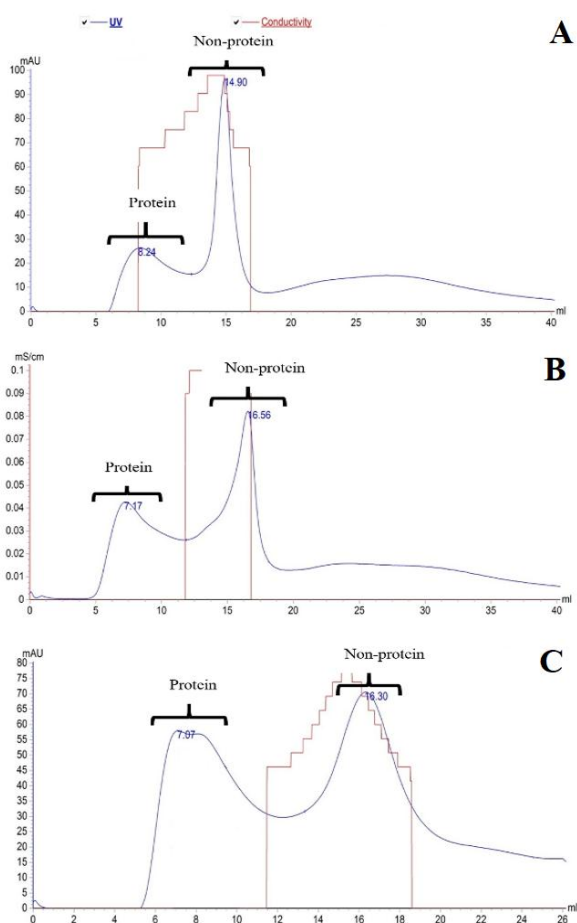
Samples	Absorbance (450 nm)	Testosterone concentration (nmol/L)	Testosterone release (%)
Control	0.217 ± 0.02	32.943 ± 0.77	-
<i>E. longifolia</i>	0.143 ± 0.01	56.018 ± 1.38*	41.192 ± 0.35
<i>P. bullata</i>	0.199 ± 0.05	36.985 ± 0.78*	10.930 ± 0.76
<i>S. tuberosa</i>	0.186 ± 0.01	40.495 ± 0.67*	18.649 ± 0.54

n=4, mean ± S.D included. Data were analyzed using one-way ANOVA for testosterone concentration. \* Showed a statistically significant difference (p<0.05) vs. Control.

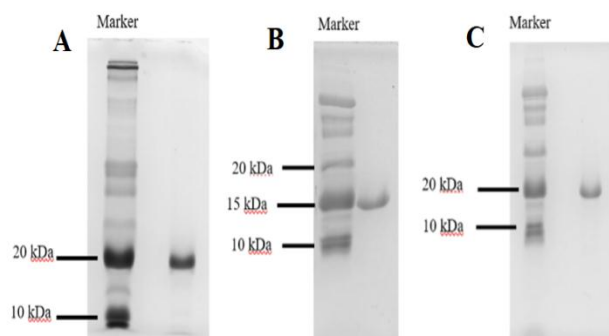
**Table 3:** Sexual behavior frequencies

Sexual Behavior	Sexual Frequency Observed on Males			
	Control	<i>E. longifolia</i>	<i>P. bullata</i>	<i>S. tuberosa</i>
Frequency of Mounting	2.5 ± 0.71	7.5 ± 2.12*	4.5 ± 0.71	3.5 ± 2.12
Frequency of Intromission (female terminate)	1 ± 0	4 ± 0.71*	3.5 ± 0.71	1.5 ± 0.71
Ejaculation	-	-	-	-

n=4, mean ± S.D included. One-way ANOVA analyzed data for frequency of mounting and frequency of intromission. \* Showed a statistically significant difference (p<0.05) vs. Control.



**Figure 1:** Chromatograms of (A) *E. longifolia*, (B) *P. bullata*, and (C) *S. tuberosa* extracts separated by using SEC.



**Figure 2:** The results of SDS PAGE for (A) *E. longifolia*, (B) *P. bullata*, and (C) *S. tuberosa* with low molecular weight marker as the protein standard

Based on the result presented in Figure 2 of the SDS-PAGE analysis, single protein bands in the lower molecular weight regions were observed for all the Tongkat Ali plants. Protein bands from extracts of *E. longifolia* and *S. tuberosa* were found at approximately 20 kDa when compared to the protein ladder. However, for *P. bullata*, the band appeared lower at approximately 15 kDa. Hence, the Tongkat Ali protein fractions isolated using SEC displayed a single band with a low molecular weight of less than 20 kDa. The intensity and location of these bands varied, indicating the presence of proteins with different molecular weights and concentrations in the plants. The presence of *E. longifolia*'s protein was supported by Sambandan *et al.*<sup>20</sup> who identified bioactive peptides with a molecular weight of 4.3 kDa in purified components of *E. longifolia* aqueous extracts.

Additionally, Vejayan *et al.*<sup>15</sup> studied the *E. longifolia*'s protein contents found in Pahang and, using SDS-PAGE observed a single protein band with an approximate molecular weight of 10 kDa. The presence of protein in the Bradford assay and SDS-PAGE indicates the evidence of macromolecules. Generally, plants contain only trace amounts of soluble protein compared to animal samples. The most abundant organic constituents in plants are secondary metabolites such as quassinoids, flavonoids, tannins, and alkaloids.<sup>21, 22</sup> Protein in Tongkat Ali plants is not a novel finding; *E. longifolia* has been shown to contain protein in its roots.<sup>23, 24, 15</sup> In the study conducted by Asiah *et al.*<sup>8</sup> they successfully detected a 4.3 kDa protein marker using SELDI-MS in both *E. longifolia* and *P. bullata*. In their US patent, Sambandan *et al.*<sup>20</sup> claimed that *E. longifolia* contained a 4.3 kDa protein or polypeptide. Furthermore, the protein isolated from *E. longifolia* has been demonstrated to elevate testosterone in TM-3 ATCC® CRL-1714TM.<sup>9</sup> Hence, the only Tongkat Ali plant yet to be proven to contain protein is *S. tuberosa*.

#### Testosterone in the extracts

The detection of testosterone was performed using LC-MS, and no testosterone was found in the Tongkat Ali plants. Firstly, the observed mass-to-charge ( $m/z$ ) ratios for the detectable compounds in the Tongkat Ali samples were compared to the theoretical  $m/z$  ratios from the mass spectral library of Waters UNIFI-scientific library. The theoretical  $m/z$  for testosterone is expected to be 289.21. However, from the list of identified compounds obtained (list not included), no similar  $m/z$  was found. Secondly, the retention time (RT) for testosterone is expected to be at 16.23 min (Figure 3A). Still, no peak was detected in all three Tongkat Ali plants, which exhibited similar retention times as shown in Figure 3 (B, C, and D).

Hence, it can be concluded that the increase in testosterone is not directly related to the presence of steroidal hormones in the Tongkat Ali plants but rather attributed to other components of the plants. Previously, *E. longifolia* has been proven to increase testosterone in men.<sup>5</sup> This role in increasing testosterone levels will likely contribute to its aphrodisiac potential in men. Additionally, the presence of glycosylated proteins in the plant has been shown to exert aphrodisiac effects.<sup>20, 9</sup> Interestingly, the protein was also found in *P. bullata* and suggested to be its bioactive aphrodisiac constituents.<sup>8</sup> However, no such reports of glycoprotein or protein were found in *S. tuberosa* other than those observed in the current study.

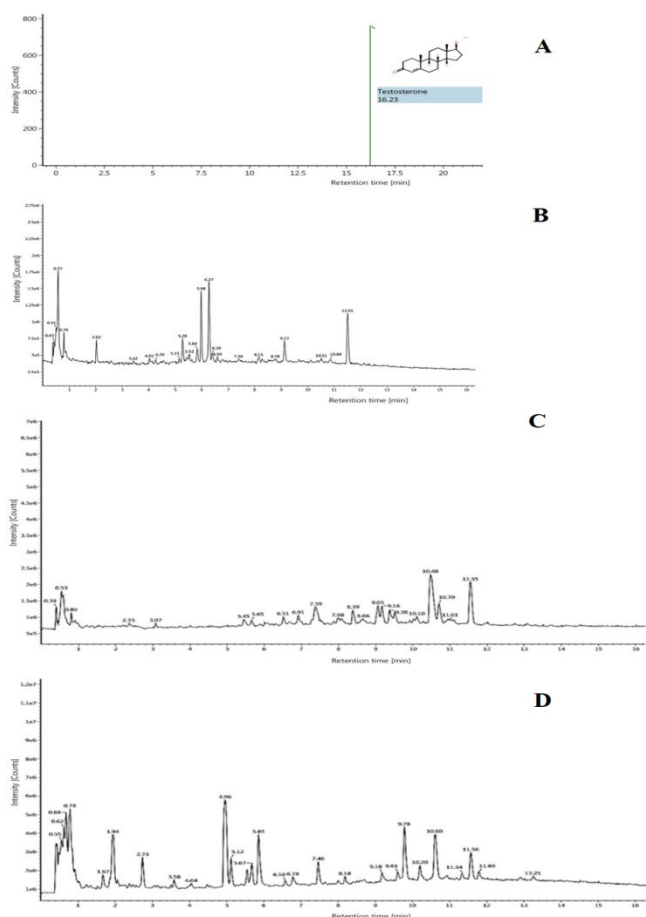
#### In vitro evaluations

##### Effects of the plant extracts on TM-3 Leydig cells

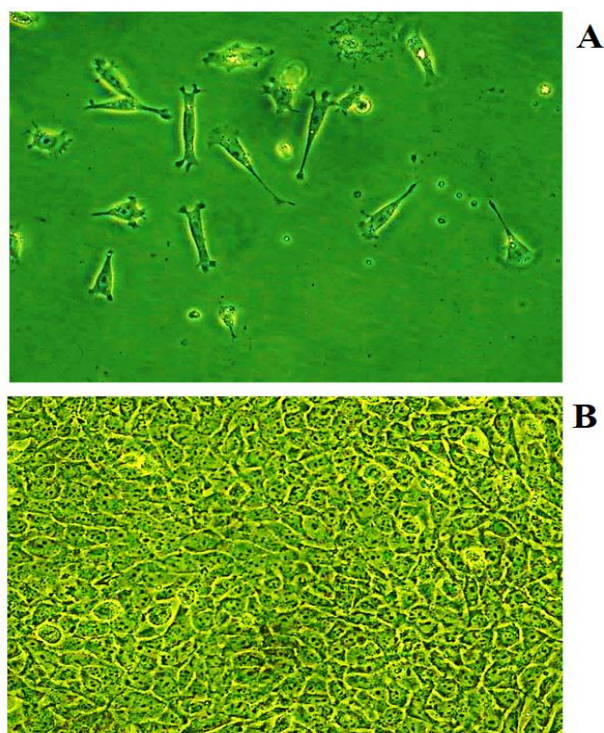
Figure 4 (A) shows healthy morphology for the TM-3 Leydig cells after one day of growth, as observed under an inverted phase-contrast microscope. Subsequently, after five days of culturing (Figure 4B), TM-3 Leydig cells remained healthy and increased to 90% confluency. Therefore, the cells were introduced to the Tongkat Ali samples. All the treatments involving the plants were found to support healthy cell growth and achieved full confluency after 72 hours (figures not included).

##### Determination of testosterone concentration using enzyme-linked immunosorbent assay (ELISA)

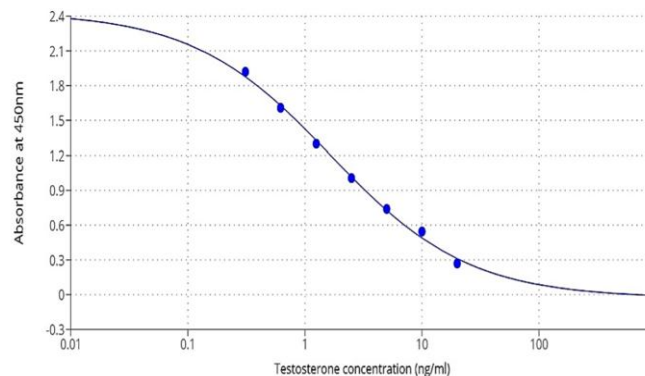
The enzyme-linked immunosorbent assay (ELISA) was performed to generate a standard curve (Figure 5), and the absorbances (Table 2) were used to determine the testosterone concentrations. *E. longifolia* achieved the highest testosterone concentration at 56.018 nmol/L, as shown in Table 2. Based on statistical analysis using ANOVA, the testosterone concentrations for all three samples showed significant differences ( $p < 0.05$ ) compared to the Control. Calculating the percentage of testosterone release compared to the untreated wells (Control) using the determined testosterone concentrations was possible. The order, ranked from the highest to the lowest, was *E. longifolia* (41.19%), *S. tuberosa* (18.65%), and *P. bullata* (10.93%). Previously, TM-3 Leydig cells were observed to secrete more testosterone after receiving *E. longifolia* doses for 48 and 96 hours.<sup>25</sup> However, no studies have reported testosterone elevations in testicular cells grown in the *P. bullata* and *S. tuberosa* laboratories.



**Figure 3:** LCMS chromatograms of (a) standard testosterone ( $C_{19}H_{28}O_2$ ) achieved a retention time of 16.23 min, (b) *E. longifolia*, (c) *P. bullata* and (d) *S. tuberosa*.



**Figure 4:** (A) Healthy TM-3 Leydig cells after 24 hours and (B) 120 hours observed under 1000 x magnification.



**Figure 5:** Absorbances were measured at 450 nm and plotted as a standard curve using an online curve calculator (AAT Bioquest, 2021).

#### *In vivo effects of plant extracts*

##### *Sexual mating behavior*

Mounting, intromission, and ejaculation are the three elements that constitute the usual male coital behavior in rodents' sexual actions.<sup>26</sup> The male mice treated with the Tongkat Ali plants were observed in their mounting, intromission, and ejaculation behaviors compared to untreated control mice (Table 3). No ejaculation occurred during the short observation time, and no sperm plugs were observed in the female mice paired with all groups. When the female mice were introduced to the male mice cage, all the male mice in each group exhibited interesting behavior towards the female mice, such as smelling and following the females around. The mice treated with the *E. longifolia* extract showed the highest frequency of mounting and intromission. Based on statistical analysis using ANOVA, only the *E. longifolia* group showed significant differences ( $p < 0.05$ ) in the mounting frequency and intromission frequency when compared to the Control. A study on rats showed a high frequency of sexual activities when treated with *E. longifolia* extract.<sup>27</sup>

##### *Testosterone levels determined using enzyme-linked immunosorbent assay (ELISA)*

A total of 50  $\mu$ L of the serum was used to determine the testosterone concentration using an ELISA kit. The previous standard curve (Figure 5) served as a reference. The testosterone release (%) was calculated using the testosterone concentration obtained compared to the untreated wells (Control). As shown in Table 4, the crude extract *E. longifolia* has the highest testosterone concentration and testosterone release (%) at 3.845 nmol/L (51.18%), followed by *S. tuberosa* at 3.349 nmol/L (43.95%), and lastly *P. bullata* 2.065 nmol/L (9.1%). Similar to the *in vitro* studies, the three Tongkat Ali plants were ranked from the highest testosterone release to the lowest, i.e., in the order of *E. longifolia*, *S. tuberosa*, and *P. bullata*. Based on statistical analysis using ANOVA, all three groups showed no significant difference ( $p > 0.05$ ) in testosterone concentration compared to the Control. Although there were no significant differences *in vivo*, *E. longifolia* was the only group that showed a significant difference in both *in vitro* and sexual behavior evaluations compared to the control group.

Among the three Tongkat Ali species, *E. longifolia* has conclusively been established as an aphrodisiac. In humans, *E. longifolia* has been shown to boost serum testosterone levels, treat erectile dysfunction, and improve sperm production.<sup>28, 3</sup> Animal studies have demonstrated an increasing trend in serum testosterone levels when rats were fed with *P. bullata*.<sup>29</sup> *P. bullata* required a longer duration of up to 60 days to increase testosterone in fowls compared to *E. longifolia* and *S. tuberosa*, which showed an increment in testosterone within 30 days.<sup>6</sup> Chan *et al.*<sup>30</sup> also state that the quassinoid molecule Eurycomanone can increase testosterone. However, how Eurycomanone has been isolated was unclear to conclude with certainty that this quassinoid compound is exerting the claimed bioactivity.<sup>10</sup> Furthermore, a US patent mentioned the peptide (glycopeptide) having a molecular weight of about 4.3 kDa isolated from an aqueous extract of *E.*

*longifolia* using size exclusion chromatography (SEC) and high-performance liquid chromatography.<sup>20</sup> The patent claimed the glycoprotein to be the bioactive compound for its aphrodisiac activity.

#### Histology of kidney, liver, and testis

The mice were sacrificed to obtain their kidney, liver, and testis. An expert pathologist performed the histological grading and interpretation of results, as shown in Table 5.

Based on Figure 6, the kidneys in the control group had sufficient well-spaced glomeruli and proper tubule orientation. There was no inflammation or edema in the interstitium. There were no casts or bleeding in the empty tubules. For *E. longifolia* and *P. bullata*, the kidneys in the groups showed normal morphology and scattered zones with a few interstitial inflammatory cells and congested vessels (in the circle). Furthermore, for *S. tuberosa*, the kidneys in these groups had appropriate glomeruli that were well-spaced and had proper tubule orientation. The interstitium was unremarkable, with moderate irritation in some regions. There were no casts or bleeding, and the tubules were empty. Based on Table 6, the dosage given was considered safe for the kidneys.

For the liver (Figure 7), the control group showed normal morphology. The hepatic sinusoids were well positioned, and the central vein was visible inside the healthy parenchyma, comprised of hepatocytes with prominent round nuclei and an abundance of cytoplasm. In the case of *E. longifolia*, the livers in this group were predominantly of normal morphology. EL1 and EL3 showed focal areas of central venous congestion (in the circle) and periportal dispersed inflammation, implying that the dosage provided induced hepatic alterations suggestive of drug metabolism but not severe enough to cause necrosis or harm. Moreover, the livers in the *P. bullata* group were predominantly of normal morphology. PB1 showed focal areas of congestion of the central vein (in the circle) and periportal scattered inflammation but no necrosis or damage. As for *S. tuberosa*, the livers

in this group showed normal morphology. The central vein was large, and the hepatic sinusoids were well organized inside healthy parenchyma composed of hepatocytes with prominent round nuclei and plentiful cytoplasm. Focal areas of dilated central veins with mild congestion were noted. Generally, based on Table 6, the dosage given was still considered safe for the liver.

The testis histology (Figure 8) in the Control, *P. bullata*, and *S. tuberosa* groups revealed abundant seminiferous tubules sized at 150-200 microns and containing adequate cellularity of spermatogonia, spermatids, spermatozoa, primary spermatocytes, and secondary spermatocytes. There is no sign of inflammation or fibrosis. In the case of *E. longifolia*, the testis had larger seminiferous tubules, measuring around 200-250 microns, than the control group. Compared to the Control, the tubules showed increased cellularity with an increase in all cells, including spermatogonia, spermatids, spermatozoa, primary spermatocytes, and secondary spermatocytes. Spermatogonia were noticeable in the lumen's core, with long tails and densely grouped. There is no sign of inflammation or fibrosis. The dosage given to the mice was considered safe for the testis.

Although *P. bullata* is a less potent aphrodisiac than *E. longifolia* and *S. tuberosa*, overall *in vivo* test findings demonstrated that it is sufficient to be claimed as a testosterone booster. In the brief period of dosing, i.e., 20 days, all plants functioned effectively as a sexual stimulant, with *E. longifolia* ranking higher than *S. tuberosa*. Even though *P. bullata* was not as effective as the other two, and it might require a longer time to show its effectiveness. A study showed that *E. longifolia* and *S. tuberosa* elevated testosterone in fowls after 30 days of dosing, while *P. bullata* required almost 60 days of dosing to show elevation in testosterone.<sup>6</sup> Overall, based on the results obtained, it is clear that all three varieties of Tongkat Ali can boost testosterone, and their aphrodisiac effects are related to testosterone. Additionally, all treatments were observed to be safe in the histology gradings done on their selected organs

**Table 4:** Absorbance, testosterone concentration, and % testosterone release in mice serum

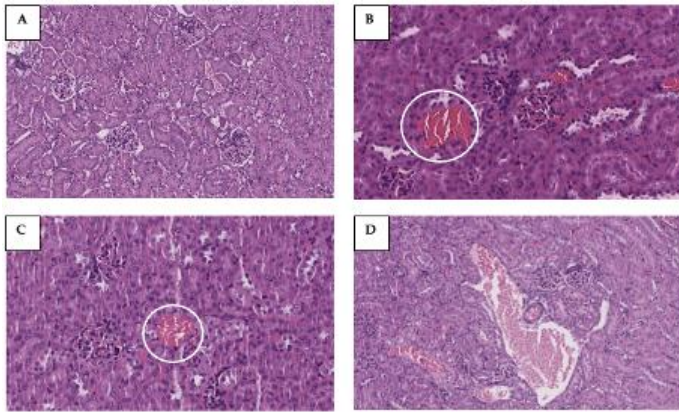
Samples	Absorbance (450 nm)	Testosterone concentration (nmol/L)	Testosterone release (%)
Control	1.215 ± 0.36	1.877 ± 1.4	-
<i>E. longifolia</i>	1.130 ± 0.74	3.845 ± 3.62	51.183 ± 4.15
<i>P. bullata</i>	1.193 ± 0.2	2.065 ± 0.88	9.104 ± 0.72
<i>S. tuberosa</i>	1.176 ± 0.72	3.349 ± 3.64	43.953 ± 1.33

n=4, mean ± S.D included. Data were analyzed by one-way ANOVA for testosterone concentration. None showed a statistically significant difference (p<0.05) vs. Control.

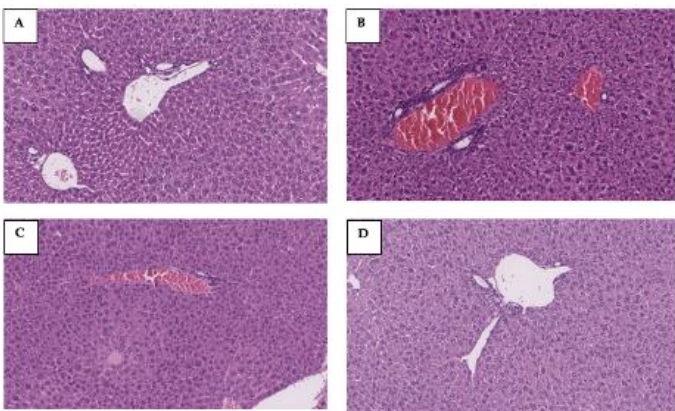
**Table 5:** Histopathological evaluations of kidney, liver, and testis

Tissue	Group (n = 4 mice)	Inflammation	Oedema	Congestion	Necrosis
Kidney	C1 to C4	-	-	-	-
	EL1 to EL4	-	-	- to +	-
	PB1 to PB 4	- to +	-	+	-
	ST1 to ST 4	- to +	-	- to +	-
Liver	C1 to C4	-	-	-	-
	EL1 to EL4	- to +	-	- to +	-
	PB1 to PB 4	- to +	-	- to +	-
	ST1 to ST 4	-	-	- to +	-
Testis	C1 to C4	-	-	-	-
	EL1 to EL4	-	-	-	-
	PB1 to PB 4	-	-	-	-
	ST1 to ST 4	-	-	-	-

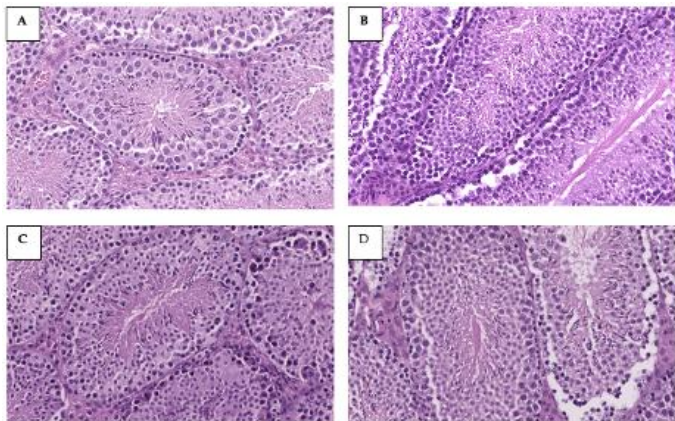
Mouse: Control (C), *E. longifolia* (EL), *P. bullata* (PB) and *S. tuberosa* (ST); Grading: (-) = normal, (+) = mild, (++) = moderate, (+++) = severe



**Figure 6:** Histology of kidney of selected mouse for each sample. (A) Control, (B) *E. longifolia*, (C) *P. bullata*, (D) *S. tuberosa* groups under 20X magnifications



**Figure 7:** Histology of liver of selected mouse for each sample. (A) Control, (B) *E. longifolia*, (C) *P. bullata*, (D) *S. tuberosa* groups under 20X magnifications



**Figure 8:** Histology of testis of selected mouse for each sample. (A) Control, (B) *E. longifolia*, (C) *P. bullata*, (D) *S. tuberosa* groups under 20X magnifications.

## Conclusion

The three plants were tested for testosterone using LC-MS and showed no natural testosterone, likely due to stimulants found within these plants instead. The first peaks isolated using SEC showed the presence of protein, and its quantity was determined using techniques of SDS-PAGE and Bradford Assay, respectively. Previous research showing the ability of a protein extracted from *E. longifolia* to increase testosterone suggested the need to investigate the protein fractions in the other two plants for their bioactivity. The plants' extracts tested for

their *in vitro* and *in vivo* testosterone boosting showed positive activities. They enabled the ranking of *E. longifolia*, *P. bullata*, and *S. tuberosa* for their testosterone elevation potentials. None of the three plants were found to exert untoward effects, either classified as moderate or severe in the histopathological assessments of kidney, liver, and testicular tissues. To conclude, all three varieties of Tongkat Ali plants could increase testosterone naturally, and none were acutely detrimental to health.

## 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 any liability for claims relating to the content of this article will be borne by them.

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