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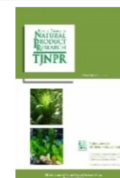

Original Research Article

Glycoproteins Isolated from Tongkat Ali Plants Capable to Elevate Testosterone in both *in vitro* and *in vivo* Experiments

Jaya Vejayan[1]*, Fatinah Ahmad[1], Sharifah A.T. Said[1], Srikumar Chakravarthi[2] and Barani Karikalan[3]

[1]*Faculty of Industrial Sciences and Technology, Universiti Malaysia Pahang Al-Sultan Abdullah, Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang, Malaysia.*
[2]*SEGi University & Colleges, No. 9, Jalan Teknologi, Taman Sains Selangor, Kota Damansara, PJU 5, 47810 Petaling Jaya, Selangor, Malaysia.*
[3]*Faculty of Medicine, MAHSA University, Saujana Putra, Jenjarom 42610, Selangor, Malaysia.*

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ABSTRACT

Tongkat Ali, a common name used to identify at least three plants, *Eurycoma longifolia*, *Polyathia bullata*, and *Stema tuberosa*, is recognized for its aphrodisiac bioactive constituent attributed to glycoproteins, particularly in *E. longifolia*. This study explores the presence and bioactivity of glycoproteins in *P. bullata* and *S. tuberosa*, employing similar extraction methods to *E. longifolia*. The crude extracts from all three plants were isolated, and glycoproteins were separated using lectin affinity chromatography. The glycoprotein yields were determined, and the protein characterization was conducted using Sodium Dodecyl Sulphate Gel Electrophoresis (SDS PAGE). Consequently, through both *in vitro* and *in vivo* experiments, the bioactivity of the isolated glycoproteins was tested for their ability to elevate testosterone levels. *In vitro* studies conducted on approximately 4000 TM-3 Leydig cells revealed that treatment with 50 µg/mL of glycoproteins extracted from *P. bullata* and *S. tuberosa* resulted in a notable increase in testosterone concentration by 45% and 48%, respectively, compared to the untreated cells after 72 h. Moreover, in groups of mice administered with the isolated glycoproteins for 20 days, testosterone concentrations increased by over 50% compared to the control groups. Despite the relatively low yields of glycoproteins, with only 0.26% and 0.21% present in the roots of *P. bullata* and *S. tuberosa*, respectively, their effectiveness was evident. This highlights that the aphrodisiac bioactive constituents, are glycoproteins, within all three Tongkat Ali plants.

Keywords: Protein; sugar moiety; herbal; steroidal hormone; mouse intercourse

Introduction

Polyalthia bullata and *Stema tuberosa* are the two lesser-known Tongkat Ali plants apart, distinct from the more widely studied and commercially sold *Eurycoma longifolia*, also known simply as Tongkat Ali. However, the use of these three Tongkat Ali plants interchangeably under the common name "Tongkat Ali" can lead to confusion and pose challenges in authentication in herbal products.[1] *P. bullata*, *S. tuberosa*, and *E. longifolia* are respectively referred based on the colour of their roots as Tongkat Ali Hitam (with black roots), Tongkat Ali Merah (with red roots), and Tongkat Ali Putih (with roots either white or yellow).[2] Like numerous other plants such as *Tribulus terrestris* (Gokshura), Ginkgo biloba, *Psoralea corylifolia*, *Sida cordifolia* (Country Mallow), *Crepidium acuminatum*, *Malaxis acuminata*, *Desmotrichum fimbriatum* (Orchids), *Roscoea purpurea* (Kakoli), and *Withania somnifera* (Ashwagandha), all three Tongkat Ali plants are utilized in traditional medicine for their reputed abilities to boost sexual desire, arousal, and sexual performance.[3,4]

*Corresponding author. E mail: jayavejayan@yahoo.com
Tel: 60166063804

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S. tuberosa, a member of the Rubiaceae family, has previously been referred to as *Jackiopsis ornata* and is renowned as an aphrodisiac plant found in secluded regions of China, Indonesia, Thailand, and virgin rainforests in Peninsular Malaysia.[5] Beyond its aphrodisiac properties, *S. tuberosa* is also believed to possess healing properties for various lung diseases such as pneumonia and bronchitis.[6]
Despite its potential therapeutic benefits, the lack of research on *S. tuberosa* has resulted in a shortage of specific knowledge regarding its active compounds. However, studies conducted over 30 days have demonstrated that *S. tuberosa* increases sexual excitement in domestic cocks and stimulates the male reproductive organ, resulting in an increase in testosterone levels by approximately 55% compared to untreated fowls.[6]
P. bullata, a woody medicinal plant native to Peninsular Malaysia and Southern Thailand, holds a distinguished status in traditional medicine owing to its multifaceted healing properties. Its leaves, blossoms, and roots have been harnessed for generations to address an array of ailments. Renowned for its therapeutic versatility, *P. bullata* is revered for its aphrodisiac, anticancer, antidiabetic, antioxidant, anti-inflammatory, anti-parasitic , antimicrobial, anti-hypertensive, and dermatological benefits, as well as its efficacy against liver conditions and cytotoxic effects.[7,8,9] It contains phytochemicals such as lipids, alkaloids, flavonoids, and triterpenoids, with the therapeutic significance strongly correlated with the presence of clerodane diterpenoids and alkaloids.[10] Notably, studies have addressed *P. bullata*'s ability to increase blood testosterone concentrations and enhance mating behaviours in fowls within a 60-day timeframe, indicating its potential as a natural aphrodisiac.[11] Additionally, human clinical trials have demonstrated its efficacy in improving erectile function and boosting blood testosterone levels.[12]

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Previous studies have demonstrated that the glycoprotein isolated from *E. longifolia*, using lectin affinity chromatography, possesses the remarkable ability to elevate testosterone levels.[1,13] These findings suggest that the unidentified glycosylated protein within *E. longifolia* may serve as the key bioactive constituent responsible for its aphrodisiac effects. However, the specific protein remains un-sequenced related to its primary amino acids sequence. In contrast, less is known about the bioactive constituents associated with the aphrodisiac properties of *S. tuberosa* and *P. bullata*. While evidence suggests the presence of proteins in both species, it has not been definitively established whether these proteins are glycosylated types of protein or glycoproteins.[14,15] Given the acknowledged aphrodisiac properties of all three Tongkat Ali plants, attributed to their testosterone-elevating capabilities, and considering the isolated glycoprotein from *E. longifolia* as a potential aphrodisiac bioactive constituent, there is an opportunity to conduct a similar effort on *P. bullata* and *S. tuberosa*.

Materials and Methods

Collection and Identification of Plant Samples

The *S. tuberosa* and *P. bullata* plants have been verified by a botanist on 8th February 2021 at the University of Malaya and deposited as vouchers with numbers HI1445 and HI1446, respectively. In the Perak state in Malaysia, located at GPS coordinates 4.71370 N and 100.94480 E, on 6th February 2021, local indigenous residents have been involved in the trade of 10 kg each of roots from *S. tuberosa* and *P. bullata*, respectively. To process this sun dried roots, a specialized piece of machinery known as the Tongkat Ali Root Cutting Machine (Ruian Hanboo Machinery Co., Ltd, China) was employed initially to slice the roots into smaller chips. Subsequently, these chips underwent grinding to obtain a finely powdered form before extraction.

Extraction of Tongkat Ali Plants

The extraction process mirrored that utilized for *E. longifolia* whereby 50 g of powder from each plant variant was mixed with 500 mL of distilled water[1]. Following a five-hour reflux boiling process, the resulting liquid underwent filtration, utilizing Whatman No. 1 coarse filter paper to eliminate any residual substances. It was then freeze-dried and carefully stored for subsequent applications. The percentage yield (w/w) was then determined by weighing the dried crude extracts.

Lectin Affinity Chromatography for Glycoprotein Isolation

To conduct lectin affinity chromatography (LAC) for the isolation and purification of glycoproteins, several materials were indispensable: sterile syringes, deionized water, methyl-α-D-mannopyranoside (Sigma-Aldrich, US), and HiTrapTM Con A-Sepharose 4B columns (Cytiva, US). LAC harnesses the binding affinity of immobilized lectins to carbohydrates present glycoproteins, allowing for their selective isolation.[16,17] Importantly, LAC operates under mild conditions, preserving the biological activity of glycoproteins.

A manual approach was adopted for the purification process. Initially, a syringe filled with deionized water was connected to a HiTrap™ Con A-Sepharose 4B column to initiate column equilibration. The column was then equilibrated by adding one column volume of deionized water. Subsequently, the Tongkat Ali extract (comprising fresh extracts of *S. tuberosa* and *P. bullata*) was sequentially applied to the equilibrated column using a syringe attached to the Luer connector. A controlled flow rate of 0.5 mL/min was maintained throughout this process, with the eluent being discarded. To eluate the glycoproteins, a solution containing 0.5 M methyl-α-D-mannopyranoside was passed through the column, using a volume equivalent to one column volume. Following elution, the column was rejuvenated by washing with another column volume of deionized water. The process was repeated several times on the column to collect the glycoproteins in bulk for an entire extraction of 50 g dried roots. Finally, to separate the glycoproteins from *P. bullata* and *S. tuberosa* from any remaining methyl-α-D-mannopyranoside residues, a spin column (Pall Corporation, US) with a 10 kDa cut-off was employed. The resulting glycoproteins, free from the mannose residues, were freeze-dried and weighed to determine their percentage weight/weight (% w/w).

Protein Characterization Techniques

Determining the Purity of the Glycoprotein using Size Exclusion Chromatography (SEC)

To assess the purity of the glycoprotein obtained from the LAC, size exclusion chromatography was performed using an AKTA Start system (GE Healthcare, US) equipped with five HiTrap™ desalinating columns (Cytiva, US) connected in series. The UV detector of the AKTA Start system was set to 280 nm, and 1 mL of the freeze-dried glycoprotein dissolved in water (concentration of 3 mg/ml was injected at a flow rate of 1 mL/min, with water serving as the eluent. Chromatographic data were analysed using UNICORNTM Start 1.1 software from GE Healthcare, US.

Determining the Molecular Weight of the Glycoprotein Using SDS PAGE

SDS-PAGE was performed using BioRad Laboratories' vertical slab gel technique to determine the molecular mass and purity of the glycoprotein fractions extracted from LAC.[1] A mixture comprising 15% resolving gel and 8% stacking gel was cast into 1 mm thick glass plates, with wells formed using combs. A total of 200 µL of sample buffer was used to denature 20 µg of the sample, and 8 µL of this mixture was loaded into each well. Electrophoresis was run at 120 V for 1 h and 30 min. Bromophenol blue served as the tracking dye in the gel, while a protein ladder sourced from Takara (Takara Bio Inc., US) was employed as a reference. Protein bands were stained using Coomassie Brilliant Blue staining solution (PhastGel Blue R, GE Healthcare, US). The gel was treated with the staining solution and agitated for approximately 40 min. Subsequently, a 10% acetic acid solution (Merck KGaA, DE) was used to de-stain the gel until the background became transparent.

In vitro Evaluations Using TM-3 Leydig Cells

Cell Culturing and Experimentations

The TM-3 Leydig cell line (American Type Culture Collection, ATCC® CRL-1714™, US) was utilized for cell culturing and experimentation,[1] following a modified version of the method outlined by Said *et al*.[18] Briefly, cells were cultured in growth media under 5% CO_2 and 37 ºC for 24 h. Once the cells reached 70% – 85% confluency, after about 72 h with media changes every 24 h, they were trypsinized, the trypsin was neutralized, and the cells were centrifuged to obtain the free cells. The cells were re-constituted in fresh media and briefly cultivated to promote further growth. Subsequently, a sample concentration of 50 µg/mL was introduced to each well of a 96-well plate, with 4000 cells in each well (cell count determined using a haematocytometer, Dolphin, China). After a 72-h incubation period (with a dose of 50 µg/mL added every 24 h along with media changes), testosterone levels were determined using an ELISA kit (Elabscience Biotechnology Inc., US). The selected cell morphology was observed using an inverted phase-contrast microscope (CKX41, Olympus, JP).

Determining the Testosterone Concentration using ELISA

The testosterone concentration was determined using the Testosterone ELISA kit (Elabscience, US), following the manufacturer's instructions. A standard curve for testosterone was generated (Figure 1), exhibiting a high R^2 coefficient value of 0.999, with a closeness of fit of nearly 1. The absorbance readings at 450 nm were obtained using a Tecan Infinite M200 Microplate Reader (Tecan Trading AG, Switzerland) and converted into corresponding testosterone concentrations using the generated standard curve.[18] Subsequently, Equation 1 was applied to the data to assess the tested samples' ability to increase testosterone levels compared to untreated cells.

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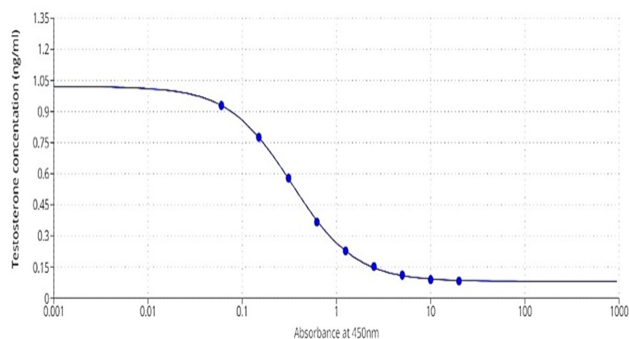

Figure 1: Standard curve using AAT online curve calculator (AAT Bioquest, 2024)[19] at 450 nm

Equation 1:

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

In vivo Investigations in Mice

Experimental Animal and Dosing

The study employed ICR mice weighing between 25 and 35 g. Animal ethical permission was obtained from the Institutional Animal Care and Use Committee (IACUC) of University Malaysia Pahang Al-Sultan Abdullah, with the approval number UMPIACUC/2021/01(extension). A total of 12 mice were included in the study, with four animals assigned to each of the three groups (control, *P. bullata*$_{\text{glycoprotein}}$, and *S. tuberosa*$_{\text{glycoprotein}}$). For 20 days, each glycoprotein from Tongkat Ali plants was orally administered at a dose of 2.5 mg, calculated based on the overall average weight of mice, using an oral gavage (unbranded, China). The administration was carried out in the morning and evening ("bis in die" b.d.) in 1 mL of distilled water. Mice in the control group received 1 mL of distilled water.

Determining the Sexual Mating Behaviours

The experimental procedure involved pairing male test subjects with adult female mice for a duration of 8 h, employing a modified approach.[20] The twelve male mice (four mice each for controls, *S. tuberosa*$_{\text{glycoprotein,}}$ and *P. bullata*$_{\text{glycoprotein}}$) were paired with 12 female mice, with each pair housed in separate cages for observation. Before pairing, the mice were allowed 5 min to become accustomed to the examination chambers. The entire 8-h observation period was recorded using cameras and later analysed for three sexual behaviours: ejaculations, intromission, and mounts. If ejaculation did not occur, testing proceeded until the female returned to the male in pacing tests. The frequency of ejaculations, intromissions, and mounts was recorded. Intromissions were further classified if they were terminated by the female actively pushing the male away. Researchers conducted measurements of sexual behaviours and compared them to those of untreated male controls to ascertain their statistical significance.

Blood Collection and Determination of Testosterone Concentration

The mice were anesthetized using a ketamine-xylazine cocktail (10 mg/kg xylazine and 125 mg/kg ketamine) purchased from the UKM Animal House (Universiti Kebangsaan Malaysia). Blood was collected via cardiac puncture, up to a maximum of 0.9 mL. To obtain the blood serum, the collected blood samples were allowed to clot at room temperature for 2 h and then centrifuged at 1000 x g for 20 min at 4 °C. The supernatant, or serum, was collected for testosterone determinations using an ELISA kit, following a similar method to the *in vitro* evaluations conducted earlier.

Histology on Selected Organs

After meticulous dissection, the liver, kidney, and testicles were quickly immersed in 5 mL of 10% formalin and left overnight. The tissue processing and grading procedures followed established guidelines.[21] After the tissues were cut, they were treated with alcohol and graded xylene before being embedded in blocks of paraffin wax. The tissues were then sectioned into 5-micron pieces using a Leica microtome. These sections were mounted on glass slides and stained with haematoxylin and eosin. The produced slides were examined using a Nikon Eclipse TS100 light microscope to assess the inflammation, congestion, and grading in each group.

Statistical Analysis

Using Microsoft Office Excel 2010, an ANOVA was used to examine the data. Through analysis of variance, the statistical significance of the data was determined, with differences considered significant at ($p<0.05$). The average ± standard deviation is used to represent all the data.

Results and Discussion

Extraction and Isolation of the Glycoproteins

The extractions of crude extracts of *P. bullata* and *S. tuberosa*, followed by the isolation of their glycoproteins, were conducted similarly to the method applied for the more commonly studied Tongkat Ali plant, *E. longifolia*.[11] The chromatograms achieved after performing lectin affinity chromatography (LAC) are depicted in Figure 2, with the second peaks indicating the targeted ligands, denoted as the glycoproteins, eluted using an eluent containing appropriate sugar methyl-α-D-mannopyranoside. LAC is a technique used to isolate and separate glycoproteins by using immobilized lectins. This method employs different immobilized lectins with varying affinities for different sugar substrates to separate a variety of complexes bound to glycans.[17]

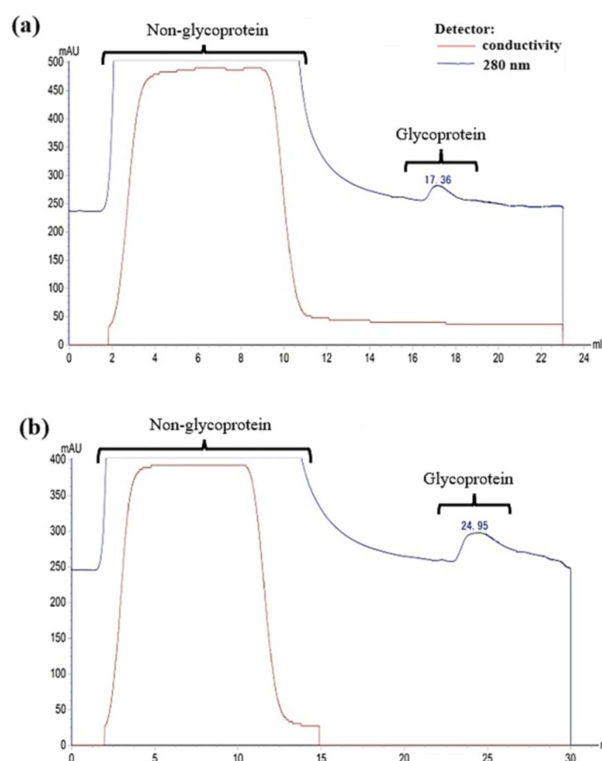

Figure 2: LAC chromatograms of *P. bullata* **(a)** and *S. tuberosa* **(b)** extracts. Blue lines indicate UV 280 nm detection, while red lines indicate conductivity, which is high at peaks containing charged ions

The lectin of the Con A-Sepharose 4B column binds to the conjugated sugar of the glycoprotein in the Tongkat Ali samples. The broad first peaks observed indicated unbound molecules in the samples, generally consisting of phytoconstituents such as alkaloids, glycosides, steroids, saponins, tannins, and flavonoids, which are abundantly found in

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plants.[22] Table 1, generated after several hot water extractions and LAC runs on the crude extracts, clearly shows the low presence of glycoproteins in *P. bullata* and *S. tuberosa*, as summarized in the table. The total amount of glycoprotein obtained in a single run was insufficient for *in vitro* testing, even though only a concentration of 50 µg/mL of glycoprotein was required for each sample. As indicated in Table 1, the total amount of glycoprotein in both types of Tongkat Ali plants did not exceed 0.5% w/w yield. Hence, repeated runs were necessary, especially for the possibility of testing at the *in vivo* level using the smallest mammal possible, i.e., a mouse.
The purity of the glycoprotein fractions was assessed by injecting them into a size exclusion column (SEC), and noticeably, single peaks were achieved (Figures 3c and 3d) compared to the crude extracts of *P. bullata* and *S. tuberosa*. The extracts were observed with two peaks, peak 1 (containing higher molecular weight compounds) with a shorter retention time compared to peak 2 (containing low molecular weight compounds) for *P. bullata* and *S. tuberosa*, as shown in Figures 3a and 3b, respectively. Gel filtration, also known as size exclusion chromatography (SEC), is a technique used to separate molecules based on size. It involves filtering through a gel matrix, facilitating smaller molecules to pass through the pores of the gel and slowing down their flow through the column. In comparison, larger molecules are excluded from the pores and eluted in the void volume of the column. As a result, molecules are eluted in decreasing molecular weight and are separated by size as they pass through the column.[23,24] Therefore, peak 2 (Figure 3) likely represents organic compounds found abundant in these plants and indicated as peak non-glycoprotein observed in Figure 2 (LAC chromatograms). Peak 2 (Figure 2) belonged to glycoprotein and was able to be separated with high purity due to its specific affinity towards LAC. Glycoproteins are macromolecules with high molecular weights compared to the low molecular weight organic compounds or phytochemicals generally found in plants.

This was confirmed by conducting SDS-PAGE on the glycoprotein fractions isolated from the two Tongkat Ali plants, which showed molecular weight between 10 to 15 kDa (Figure 4). The SDS-PAGE findings also revealed a single protein band, leading to the conclusion that there were no other protein contaminants. Said *et al.*[18] discovered that *S. tuberosa* shared the same molecular weight of protein as *E. longifolia*. The presence of glycoproteins in both *P. bullata* and *S. tuberosa* was crucial, especially with evidence emerging that the glycoprotein isolated from *E. longifolia* contributed to testosterone boosting.[25] Thus, sufficient glycoproteins were extracted from both Tongkat Ali plants to evaluate their potential to increase testosterone in the TM-3 Leydig cell culture (*in vitro*) and mice (*in vivo*).

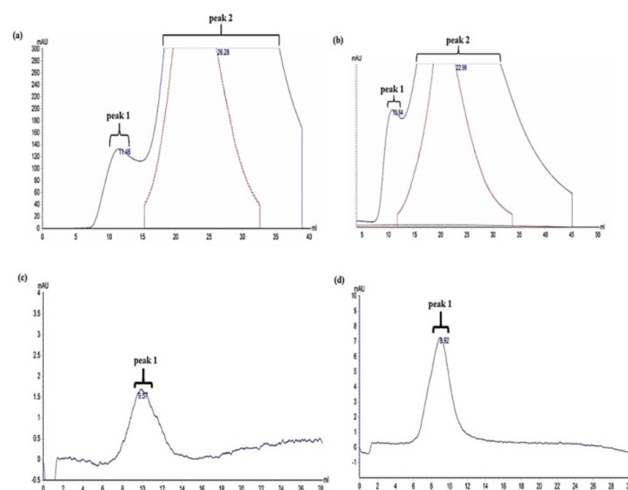

Figure 3: SEC chromatograms of crude extracts **(a)** & **(b)** and glycoproteins **(c)** & **(d)** of *P. bullata* and *S. tuberosa*, respectively

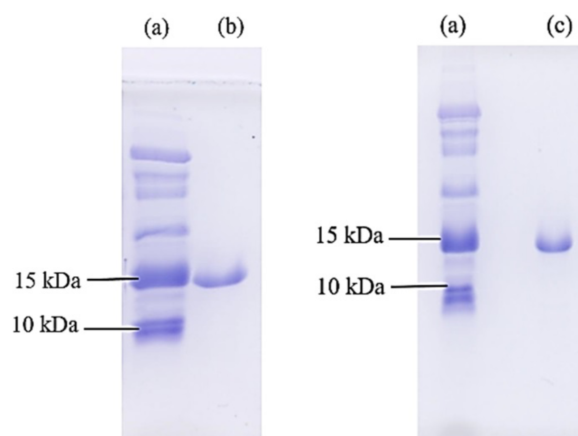

Figure 4: SDS PAGE of glycoproteins in *P. bullata* (b) and *S. tuberosa* (c) with low molecular weight marker as standard (a)

Table 1: The yield of Tongkat Ali plant extracts and glycoproteins

Plants	Mass of dried powdered roots (g)	Volume obtained after extraction and residue removals (mL)	Average mass (mg) of glycoprotein after freeze-drying	Glycoprotein yield (% w/w) in 100 g of Tongkat Ali plant root (mg)
P. bullata	50	465.5 ± 7.5	131.79 ± 4.89	0.26 ± 0.01
S. tuberosa	50	450 ± 11.3	101.81 ± 14.67	0.21 ± 0.03

n = 4, mean ± S.D included

In vitro Evaluations
In vitro studies on testosterone synthesis often utilized TM-3 Leydig cells because these cells, located in the testicular interstitial region, are the primary producers of testosterone. Research has demonstrated the critical function these cells play in testosterone synthesis.[26] In a controlled *in vitro* environment, TM-3 Leydig cells are useful for studying the regulation of testosterone synthesis and the effects of various stimuli on this process. A study using TM-3 Leydig cells for testosterone production showed that treatment with the glycoprotein of *E. longifolia* could stimulate testosterone release.[25]
Figures 5a and 5b demonstrate the 400-times enlarged inverted phase contrast image of the healthy morphology of TM-3 Leydig cells at 0 h (4000 cells per well) and after 72 h, respectively. Figures 5c to 5f depict the morphological alterations in the cells after exposure to the various substances at a concentration of 50 µg/mL at 0 h (4000 cells per well) and after 72 h. After 72 h, all treatments maintained confluency and normal cell growth. The amount of extracellular testosterone released after 72 h was measured using the enzyme-linked immunosorbent assay (ELISA). Table 2 indicates that *S. tuberosa* had higher levels of testosterone in both its crude and isolated glycoprotein, with values of 2.51 nmol/L and 2.97 nmol/L, respectively. According to an ANOVA statistical analysis, the testosterone concentrations in the four groups differed significantly ($P < 0.05$) from the control group. The percentage of testosterone released, determined from highest to lowest, was in the order of glycoprotein *S. tuberosa* (48.30%), glycoprotein *P. bullata*

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(45.01%), crude of *S. tuberosa* (25.16%), and crude of *P. bullata* (22.56%).

In vivo Evaluations

For 20 days, male mice were given a dosage of isolated glycoproteins from *S. tuberosa* and *P. bullata* twice daily. The mice were monitored for changes in their weight observed for their sexual conduct after being paired with females, and selected tissues were examined after 20 days.

Table 2: Optical density, testosterone concentration, and testosterone increase (%)

Samples	Optical density (450 nm)	Testosterone concentration (nmol/L)	Testosterone increase (%) compared to control
Controls	0.31 ± 0.003	2.00 ± 0.01	-
P. bullata	0.20 ± 0.05	2.45± 0.78	22.56 ± 1.24
S. tuberosa	0.19 ± 0.01	2.51 ± 0.67	25.16 ± 1.91
P. bullata$_{glycop}$	0.11 ± 0.004	2.90 ± 0.02	45.01 ± 1.04
S. tuberosa$_{glycop}$	0.10 ± 0.003	2.97 ± 0.02	48.30 ± 0.89

n = 3, mean ± S.D include; *P. bullata*$_{glycop}$ and *S. tuberosa*$_{glycop}$ = isolated glycoproteins from the respective plants

On days 1, 10, and 20, the mice's weights were recorded for each of the three groups. After day 10, the mice in the *P. bullata* group showed a decrease in weight followed by an increase on the 20th day. The percentage of weight loss compared between day 0 and day 20 was approximately 8%. Such loss of weight in the experimental animals may be attributed to the adverse effects of the stimulant administered to the animals, which in this case is the glycoprotein isolated from *P. bullata*. However, according to Vejayan *et al.*[11], in an investigation on fowls given *P. bullata* dried powdered root preparations, weight gain was not observed until 60 days into the test. Therefore, based on their findings and the results achieved in Table 3, where the initial weight slightly decreased on day 10, followed by an increase on day 20, it can be assumed that the adverse effects exerted by the glycoprotein on the weight of the mice are not severe. The animals were adapting throughout the dosing period. However, a similar conclusion cannot be made for glycoprotein in the *S. tuberosa* group, as displayed in Table 3. There was a loss in weight of approximately 5%, with downward decreases in each instance; the weight was measured on days 0, 10, and 20 compared to controls, achieving a steady increase in weight with a final weight gain of 2.5%.

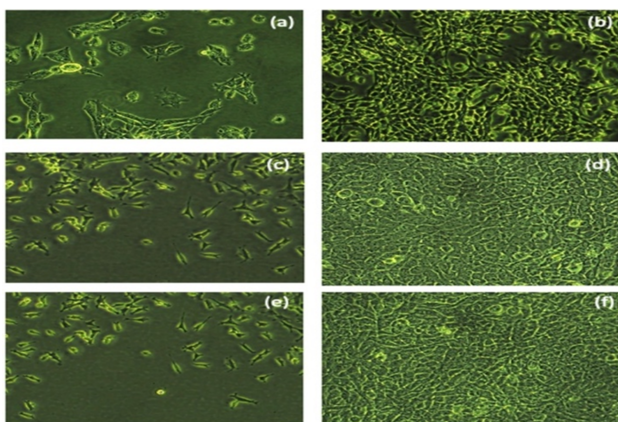

Figure 5: The morphology of TM-3 Leydig cells without treatment (control) at **(a)** 0 h and **(b)** 72 h; cells incubated with glycoprotein of *P. bullata* at **(c)** 0 h and **(d)** 72 h and with glycoprotein of *S. tuberosa* at **(e)** 0 h and **(f)** 72 h under magnification of 400X

3: The weight changes of mice

Samples	Average weight (g) ± S.D			% Gain /Loss (±)
	0th day	10th day	20th day	
Control	33.13 ± 0.52	33.78 ± 0.71	33.95 ± 0.67	+ 2.5
P. bullata$_{glycop}$	32.96 ± 2.97	29.39 ± 3.83	30.29 ± 2.48	- 8.1
S. tuberosa$_{glycop}$	33.54 ± 3.96	32.11 ± 3.22	31.73 ± 2.14	- 5.4

n = 4, mean ± S.D include

The results of the histology gradings performed on the chosen organs showed that all treatments were safe. The kidney, liver, and testicles of the mice were removed after humanely sacrificing the mice. The histological grading (Table 4) and result interpretation (details not included) were conducted by a skilled pathologist, with selected photomicrographs of kidneys, livers, and testes shown in Figure 6. Overall, the dosage administered for a short period of 20 days was concluded to be safe for the kidneys, livers, and testes.

Intromissions and mountings are two prelude coital acts performed by male rats with a female before ejaculation.[27] Table 5 presents a comparative analysis of the frequency of these two sexual mating behaviours in male control mice treated with glycoproteins of *P. bullata* and *S. tuberosa*. Upon placing the female mice in the male mice cage, every male mouse in each group exhibited fascinating behaviour towards the female, including following and smelling her. The males were observed following the females around, frequently sniffing and smelling their partners before progressing to intromissions or mountings. Mice administered with the glycoprotein of *S. tuberosa* over 20 days exhibited the highest frequencies of mounting and intromission. Overall, test mice showed higher mounting and intromission frequencies than those observed in the control group. In this study, based on statistical analysis using ANOVA, the glycoproteins from the *P. bullata* and *S. tuberosa* groups revealed non-significant differences ($P \geq 0.05$) compared to the control for the mounting and intromission activities. Additionally, only one mouse was able to achieve ejaculation during the short duration of observations; this mouse belonged to the *P. bullata* group, and a sperm plug was clearly visible on the female mating partner. It is worth noting here the simulation with Tongkat Ali is targeted at the male mouse alone and expected to have no direct influence towards the female mouse's readiness toward a successful ejaculation.

The testosterone concentration was determined using an ELISA kit from 50 µL of blood serum. There was almost a similar percentage increase in testosterone between glycoproteins-treated *P. bullata* and *S. tuberosa* groups, with the latter showing slightly higher at about 56.44% compared to the control group (Table 6). An ANOVA statistical study showed that the *S. tuberosa* glycoprotein group connected to testosterone concentrations differed significantly ($P < 0.05$) from the control. Combining the results obtained for the frequencies of sexual behaviours as well as the increase in testosterone for *in vitro* and *in vivo* experiments, it was evident that the glycoprotein isolated from *S. tuberosa* had a slight advantage over the glycoprotein from *P. bullata*.

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Table 4: Histopathology of kidney, liver, and testis

Tissue	Group (n = 4 mice)	Inflammation	Oedema	Congestion	Necrosis
Kidney	C1 to C4	-	-	-	-
	PB1 to PB4	-	-	- to +	-
	ST 1 to ST4	-	-	- to +	-
Liver	C1 to C4	-	-	-	-
	PB 1 to PB4	- to +	-	- to +	-
	ST 1 to ST4	- to +	-	- to +	-
Testis	C1 to C4	-	-	-	-
	PB 1 to PB4	-	-	-	-
	ST 1 to ST4	-	-	-	-

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

Table 5: Mounting, intromission, and ejaculation

Samples	Frequencies of sexual behaviours observed		
	Mounting	Intromission (female terminate)	Ejaculation
Control	14.0 ± 1.15	3.0 ± 0.15	-
P.bullata$_{glycop}$	17.0 ± 0.58	5.0 ± 0.58	1
S.tuberosa$_{glycop}$	20.5 ± 1.26	10.0 ± 1.15	-

n = 4, mean ± S.D include

Table 6: Optical density, testosterone concentration, and testosterone increase

Sample	Absorbance (nm)	Testosterone concentration (nmol/L)	Testosterone increase (%) compared to control
Controls	3.19 ± 1.78	0.93 ± 0.20	-
P.bullata$_{glycop}$	1.37 ± 0.82	1.46 ± 0.40	55.99 ± 4.27
S.tuberosa$_{glycop}$	1.27 ± 0.43	1.46 ± 0.27	56.44 ± 2.86

n = 4, mean ± S.D

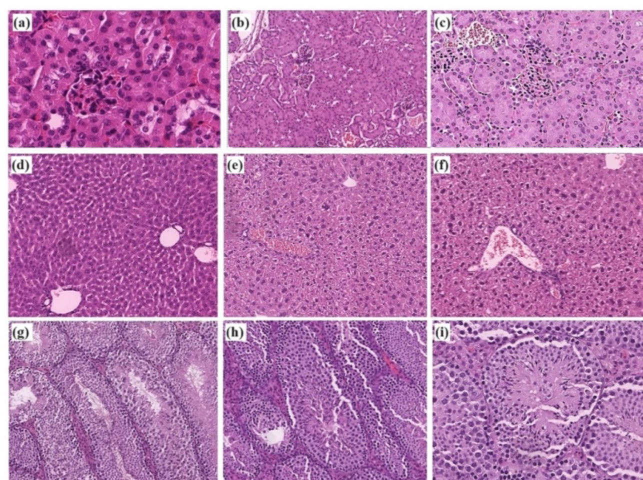

Figure 6: Histology of the selected kidneys, livers, and testes in each group. Row 1, kidney: (a) control (40X magnifications), (b) *P. bullata* (10X), (c) *S. tuberosa* (20X). Row 2: liver (d) control (10X), (e) *P. bullata* (10X) (f) *S. tuberosa* (20X). Row 3: testis (g) control (10X), (h) *P. bullata* (10X) (i) *S. tuberosa* (20X) groups.

Conclusion

Tongkat Ali plants, such as *E. longifolia*, *S. tuberosa*, and *P. bullata*, have previously been established as testosterone boosters. Additionally, the bioactive constituent from *E. longifolia*, which is able to increase the frequencies of sexual mating behaviours and elevate testosterone, has been identified as a glycoprotein. In the current study, glycoproteins isolated from the lesser-studied plants *S. tuberosa* and *P. bullata* also successfully elevated testosterone in both *in vitro* and *in vivo* experiments. Both plants worked well as sexual stimulants during the 20-day dosage period, with *S. tuberosa* performing slightly better than *P. bullata*. Although *S. tuberosa* caused a reduction in weight during the 20-day acute studies, histological evaluations did not reveal any adverse effects on the examined organs, including the kidneys, liver, and testes. Consequently, it was established that all treatments were safe, primarily based on the histopathology gradings conducted on the selected organs. However, it was advised that ongoing assessments be conducted to ascertain the overall safety of these therapies.

Conflict Of Interest

All authors hereby declare no conflict of interest in deriving the data of this study leading toward the publication of this article.

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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