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Glycoprotein Isolated from *Eurycoma longifolia* (Tongkat Ali) is Capable of Boosting Testosterone Levels in Leydig cells

Jaya Vejayan[1]*, Yasmin A.C. Yahya[1], Sharifah A.T. Said[1], Aini Norhidayah[1], Venugopal Jayarama[1], Normaiza Zamri[1], Halijah Ibrahim[2]

[1]*Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang. Lebuhraya Tun Razak, Pahang, 26300 Kuantan, Malaysia*
[2]*Institute of Biological Sciences, University of Malaya, 50603 Kuala Lumpur, Malaysia*

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ABSTRACT

Eurycoma longifolia (Tongkat Ali) is renowned for its aphrodisiac potential, and its active constituent has been presumed to be a protein and more likely a glycosylated protein. In this study, the ability of the glycoprotein to increase testosterone hormone levels was investigated. The dried root powder of the plant was extracted using water under reflux. The protein fraction was separated using size-exclusion chromatography and subjected to SDS-PAGE analysis. Thereafter the protein fraction was isolated from its glycoprotein using a lectin column. Finally, TM-3 Leydig cells were treated with the isolated glycoprotein fraction (50 µg/mL). The extraction yielded 14.3% w/w protein and the SDS PAGE analysis showed a single band at approximately 20 kDa. Treatment of TM-3 Leydig cells with the glycoprotein fraction for 72 hours demonstrated an increase in testosterone levels by almost 100% (0.36 ± 0.03 nmol/L) in comparison to the untreated cells (0.18 ± 0.05 nmol/L). The findings suggested that the glycoprotein in *Eurycoma longifolia* root can be easily isolated because its sugar moiety can bind to a lectin affinity column. Moreover, this glycoprotein was shown to have testosterone-boosting activity. These findings identified the glycoprotein as the bioactive constituent associated with its aphrodisiac properties.

Keywords: Protein, Leydig cells, Testosterone, Aphrodisiac.

Introduction

Tongkat Ali is a famous plant used traditionally by indigenous people in Malaysia and neighbouring countries due to its aphrodisiac properties. Traditional Malaysian herbal practitioners often utilize aphrodisiac plants to treat sexual dysfunction.[1] There are several plant species grouped as Tongkat Ali that can be differentiated through their root colour. The most popular species is *Eurycoma longifolia,* with a yellow root. There are at least two other types of Tongkat Ali, *Polyalthia bullata* (black roots) and *Stema tuberosa* (red roots).[2-4] In previous studies, various bioactive components in *E. longifolia* were identified, isolated, and characterized.[5] Most commonly, phytochemicals were identified from the roots but sometimes also from the stems, leaves, or bark. Due to its essential phytochemicals, there are numerous therapeutic effects exerted by this medicinal plant. Plants are in high demand and sourced from the wild mostly for marketing as herbal products worldwide.[6] Although many studies have been conducted on this species, few chemical constituents have been identified and verified in many research focusing on aphrodisiac activities. There is a need to conclusively identify the active constituent or multiple active constituents that act synergistically in order to isolate pure compounds responsible for the testosterone boosting effect of *E. longifolia*. Subsequently, this information would enable the development of pharmaceutical drugs.[5]

*Corresponding author. E mail: jayavejayan@ump.edu.my
Tel: +6016-6063804

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Unlike commercialized plant extract, a drug synthesized with only the bioactive compound or refined fraction allows the maximum dose to be given without any hindrance from the other compounds present in the plant extract. Evidence from other studies suggested the presence of protein and confirmed that the protein containing sugar moieties exhibited aphrodisiac effects.[7,8] The slower than usual migration of the protein in SDS-PAGE due to the glycosylation resulted in the 14 kDa band rather than the expected band of 4.3 kDa as determined by SELDI-MS or MALDI-TOF MS. Affinity chromatography can be used for specific and rapid separation of this glycosylated protein from *E. longifolia.*

Aphrodisiac plants, either alone or in a mixture taken regularly can arouse sexual desire or libido and sustain average or above-average sexual performance while alternatively treating erectile dysfunction and infertility in men or women.[9-11] The measurement of testosterone levels is indicative of the presence of aphrodisiac potentials in men. Testosterone is a prerequisite for normal spermatogenesis.[12] Leydig cells are the primary cells for testosterone hormone production and secretion.[13,14] *E. longifolia* was demonstrated to increase testosterone *in vitro* and *in vivo*.[1,15-19] In the current study, glycoproteins in *E. longifolia* were isolated explicitly using an affinity column and evaluated for their testosterone boosting effect.

The field of proteomics emphasizes the importance of studying proteins beyond proteins alone, i.e., those with post-translational modifications or conjugated proteins. One of such focus is on glycoproteins that play a major role as antibodies, with their glycosylation structure determining their functions and structural alterations to their sugar moieties may possibly cause diseases.[20] The rapid and precise isolation of glycoprotein from mixtures, for example by lectins, is becoming increasingly relevant, especially since most biological therapies currently used are glycoproteins.[21]

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Materials and Methods

Sample preparation
E. longifolia roots were obtained as dried chips sourced and processed by indigenous people residing in the forest of Perak state, Malaysia. Fresh samples were also sourced by the indigenous people. Voucher specimens were deposited at the herbarium of the University of Malaya, Malaysia, with voucher number HI1447. Palm leaves (*Elaeis guineensis*) were handpicked from the palm oil estate of Felda Lepar Hilir, Pahang state, Malaysia. Both plant materials were oven-dried at 50°C before being pulverized into finely powdered materials using a blender.

Extraction of plant roots and leaves
The extraction was similar to procedures performed previously.[22] Briefly, a total of 200 g of each plant powder was mixed with 2 L of water. The mixture was boiled under reflux for five hours and filtered with coarse filter paper to remove residues before being freeze-dried and then stored for further use.

Protein quantification
A modified Bradford micro-assay was performed to determine the amount of protein in *E. longifolia* crude extract using 2 mg/mL bovine serum albumin (BSA) purchased from Thermo Scientific (UK) for the standard curve.[23]

Size Exclusion Chromatography
A HiTrap™ desalting column (GE Life Sciences, US) containing the sorbent G-25 Sephadex was able to elute organic molecules with smaller molecular weights. The protein sample in the *E. longifolia* crude extract was pre-fractionated by size exclusion chromatography, and the absorbance was determined at 280 nm using an Akta Start system (GE Life Sciences, US). A total of 6 units of the 5 mL HisTrap™ desalting columns were consecutively connected to Akta Start (GE Life Sciences, US). In each run, a total of 0.15 g of crude was dissolved in 1 mL of water. The sample solution was then loaded into a sample loop. The column was calibrated with a 1 mL/min flow rate with a six-column volume of water. The elution representing the peak of interest was then freeze-dried.

SDS-PAGE Analysis
SDS-PAGE was performed following a previous protocol with a few modifications.[20] Electrophoresis was briefly carried out on a 15% resolving gel with a stacking gel of 8% with wells made from a comb. The run duration was approximately 1 h and 30 min with bromophenol blue as the tracking dye on a mini vertical slab gel system (BioRad Laboratories, US) with the voltage set at 120 V. A total of 20 µg of protein was denatured with 200 µL of sample buffer. Then, from these sample preparations, 5 µL was loaded onto each well. A stained protein ladder (Takara Bio, US) was included. Staining was performed using Coomassie brilliant blue solution (PhastGel Blue R, GE Healthcare, US) with 10% acetic acid solution for destaining.

Affinity column using lectin
An empty column with dimensions of 0.7 × 2.5 cm (1 mL) was loaded with the Sepharose gel material Con A Sepharose® 4B (GE Life Sciences, US). The protein fraction from size exclusion chromatography was spun down using a 50 mL ultrafiltration spin column (Macrosep Advance Centrifugal Devices with Omega Membrane 1K, Pall Corporation, USA) to concentrate the sample further as suggested by the manufacturer's instructions. The pellet containing the protein fraction was re-constituted in 1 mL of water and loaded into the lectin column that had been packed with Con A Sepharose® 4B. The initial step consisted of washing with water to remove the non-glycosylated protein (discarded). Subsequently, to elute the desired glycosylated protein portion, 50% methyl-α-D-mannopyranoside (Sigma-Aldrich, US) solution was used. An Akta Start system was used for all the column running processes, which were performed at room temperature.

TM-3 Leydig cell culturing
TM-3 cells and mouse-derived Leydig cells (ATCC, US) were cultured at 37°C in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium/nutrient mixture F-12 medium (Nacalai Tesque, JP), 5% horse serum (Thermo Scientific, UK), 2.5% foetal bovine serum (Thermo Scientific, UK), and 1% penicillin-streptomycin (Nacalai Tesque, JP). The cells were allowed to reach 80% confluence before passaging.
Cell passaging was carried out according to previously described method with slight modifications.15 Briefly, the culture media was discarded, and cells were washed with 4 mL of Dulbecco-PBS (Nacalai Tesque, JP). Next, 1 mL of 10 × diluted 0.25% (w/v) trypsin 0.53 mM EDTA (Nacalai Tesque, JP) was added, and the cells were incubated for 5 min at 37°C with 5% CO2 until the cells were detached. Two millilitres of complete medium were added and centrifuged at 750 × g for 5 min at 37°C, and the supernatant was discarded. The cell pellet was resuspended in 1 mL of complete medium, and 0.25 mL of the suspension was transferred into other 25 cm2 cell culture flasks. Then, 4.75 mL of complete medium was added.

Cell morphology and viability assessments
The cell morphology for the initial 72 hours of incubation time was observed and recorded with an inverted phase-contrast microscope (CKX41, Olympus, JP). A thiazolyl blue tetrazolium bromide (MTT) assay (Sigma-Aldrich, US) was carried out as described previously with slight modifications.[24] Briefly, once the cells reached passage 10, they were treated with trypsin, centrifuged, resuspended in 1 mL of complete fresh medium, and then counted using a haemocytometer. The cells were seeded at a concentration of 4 × 103 cells/well onto a 96-well plate and cultivated for 24 - 48 h before dosing the cells with 50 µg/mL *E. longifolia* crude extract, *E. longifolia* glycoprotein fraction, and palm leaf extract for 72 h. All the samples were prepared in D-PBS and filtered through a sterile 0.22 µm nylon syringe filter membrane (Merck, Germany). Non-treated cells and MTT were kept as controls and blanks, respectively. Hereafter, the culture media was removed, and 20 µL of MTT solution and 180 µL of DMEM/F-12 (serum-free media) were added to each well. Then, the plates were incubated for 3 h, after which the culture medium was removed. Next, a total of 200 µL of DMSO was pipetted into each well to dissolve any formazan crystals. Upon mixing in a microplate shaker and reader, the absorbance was read at a wavelength of 560 nm, additionally setting 670 nm as a reference. The samples were tested in five replicates. The percentage of cell viability was determined as follows:

$$\text{Cell viability (\%)} = \{(\text{ODtreated cells})/(\text{ODcontrol})\} \times 100$$

Evaluation of testosterone production
Cells (4×10^3 cells/well) were seeded in a 96-well plate, treated with various samples at 50 µg/mL, and incubated at 37°C with 5% CO_2 for 24 h. After 72 h, testosterone production was assessed using a testosterone ELISA kit (Elabscience, US) following the manufacturer's instructions. The following formula was used to calculate the percentage testosterone secretion:

$$\%\ \text{testosterone secretion} = \frac{\text{Test}_{\text{with sample}} - \text{Control}_{\text{without sample}}}{\text{Test}_{\text{with sample}}} \times 100$$

Statistical analysis
A two-tailed T-test was performed with Excel 2016, assuming unequal variance between sample groups; toolpak analysis with probability of $p < 0.05$ was considered to be significant. All data are represented as average ± standard deviation.

Results and Discussion

Water Extraction of the Plant
Solid-liquid extraction, also known as leaching, was used in the extraction of the plant roots. The plant root powders were boiled in water under reflux for five hours and then filtered. The extract, once freeze-dried, appeared brownish and fluffy. This method was chosen because it is the traditional preparation method of this aphrodisiac plant.[25] Freeze-drying the filtrate helps to preserve the plant sample for

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a longer time owing to the existence of phenolic compounds, lipids, and plant pigments because moisture induces contamination. Such a method of extraction under an aqueous solvent is also ideal for extracting polar proteins. The total yield of protein was 14.31%, as shown in Table 1. Overall, the protein content determined in *E. longifolia* depends on the extraction method used, batch-to-batch variations, and location of sampling.[26]

Isolation of Glycoprotein Fraction using Stepwise Chromatography

Size-exclusion chromatography (SEC) was applied to remove the microconstituents (approximately less than 1 kDa in molecular weight) from the macromolecules of this plant. Separation was attained by differential elimination of the sample molecules' packing material from the pores during passage through a bed of porous particles. After the sample has been applied, the molecules of interest eluted as the first peak, as they are larger than the pores and cannot diffuse into the beads. Deionized water without any buffer was used as the mobile phase because this plant root extract is easy to handle.

As shown in Figure 1, the first peak (blue line) detected using the UV wavelength of 280 nm is the protein peak. This assignment is based on evidence that the macromolecule (protein ideally having a molecular weight of more than 1 kDa) is eluted first in SEC. Additionally, the first peak does not show any conductivity (red line), and protein macromolecules generally do not contribute to conductivity. Consequently, SDS-PAGE performed on the fraction of the first peak resulted in a single band, as shown in Figure 2. Hence, the first peak was determined to be the protein fraction, and the second peak was the non-protein fraction. To generate this chromatogram separation curve, 6 units of 5 mL HisTrap™ columns were connected in parallel and utilized to increase peak resolution. This observation was also supported by a study whereby a purified peptide with a molecular weight of 4300 Daltons found in this plant increased testosterone levels.7 The clear band obtained in this study has a molecular weight of 20 kDa (Figure 2). This finding is somewhat similar to the study conducted in 2013 in which a protein band with a molecular weight of 21.1 kDa was found in an *E. longifolia* sample from Pahang, Malaysia, as determined by SDS-PAGE.[26] Other research findings show that EL proteins are in different molecular weight bands, namely, 40 kDa and 10 kDa.[22] In all studies, the molecular weight varied due to unexplained reasons; however, it was generalized to contain similar proteins based on the presence of only a single band observed in SDS-PAGE. The protein fraction was further refined to glycoprotein using Con A-Sepharose 4B (GE Life Sciences, US) as a stationary phase, whereas 50% methyl-α-D-mannopyranoside solution was used as a mobile phase in the lectin affinity column. As shown in Figure 3, the first 2 peaks were identified to be unbound molecules. These unbound molecules were likely from the non-protein fraction overlapping in SEC (as shown in Figure 1) or other non-glycosylated proteins. The bound fraction consisting of glycoprotein was eluted in 50% methyl-α-D-mannopyranoside solution.

Cell morphology and viability

TM-3 Leydig cells exhibited normal cell viability and morphology and retained their epithelial-like morphology after incubation with 50 μg/mL crude *E. longifolia* extract and glycoprotein (Figure 4A to 4C) as well as *E. guineensis* extract (figure not included) for up to 72 hrs. The MTT test showed that all of the samples exerted a positive effect on the viability of TM-3 cells, except for *E. longifolia*, which had a slight viability reduction of 14% (Figure 5) compared to the control. The cells treated with *E. longifolia* after 48 hours (Figure 4B) and 72 hours (Figure 4C) exhibited characteristics of cell rounding, shape irregularity, and cell shrinkage, similar to apoptosis.[27] The *E. longifolia* water extract contains microconstituents (approximately less than 1 kDa in molecular weight) of quassinoids or lactones such as eurycomanone (a major compound), 9-methoxycanthin-6-one, canthin-6-one, eurycomalactone, and longilactone and was previously demonstrated to exhibit cytotoxicity against different epithelial cancerous cell lines.[28-34] The cytotoxicity may extend to normal cells. Hence, the crude *E. longifolia* extract may have constituents that reduce TM-3 Leydig cell viability at a 50 μg/mL extract concentration. Such an outcome is expected in crude extracts that contain various components other than the bioactive constituent alone.

Table 1: Estimated percentage of yield in extract and protein of *E. longifolia* root

Mass of root powder (g) (before extraction)	Mass of freeze-dried sample (g) (after extraction)	Yield of extract (% w/w)	Yield protein in extract* (% w/w)
200	12.75 ± 2.31	6.38 ± 1.89	14.31 ± 3.25

* This result was % w/w of protein in the crude extract determined using Bradford assay[21]. All results represent 4 replicates.

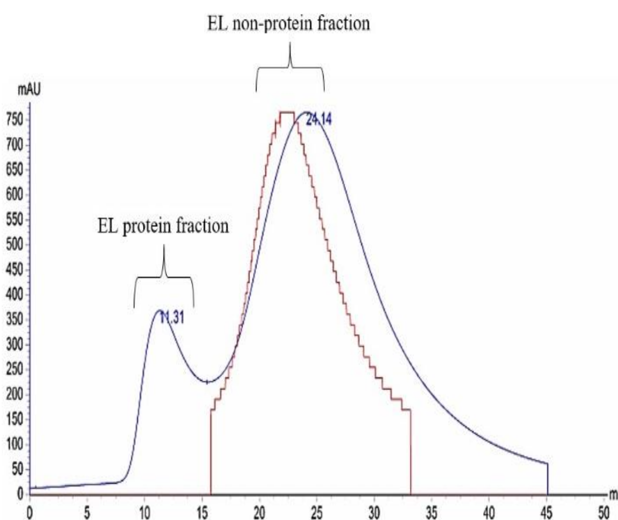

Figure 1: Size exclusion chromatogram of *E. longifolia*

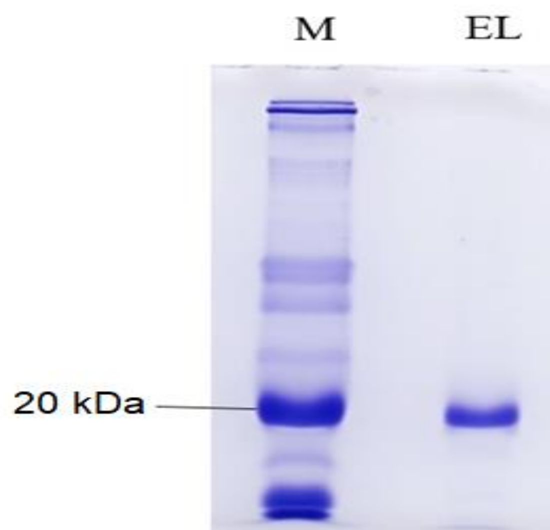

Figure 2: SDS-PAGE gel analysis of the low molecular weight marker (M) and protein fraction of *E. longifolia* (EL). The estimated molecular weight for the protein band of approximately 20 kDa is shown.

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Evaluation of testosterone production

TM-3 Leydig cells were used for testosterone production by treating the cells for 72 hours. As indicated in Figure 6, the *E. longifolia* crude extract treatment had the highest percentage increase in testosterone secretion, i.e., approximately 350% (0.81 ± 0.02 nmol/L), followed by its glycoprotein, 99% (0.36 ± 0.03 nmol/L). There was no activity (the testosterone level estimated to almost identical to that of the control, 0.18 ± 0.05 nmol/L) observed in the randomly selected *E. guineensis* (palm leaf) extract, as there was no previous report of aphrodisiac or testosterone boosting activity for this plant. Hence, the ability to increase testosterone levels is not expected to be common in plants, unlike other biological activities such as antioxidant activity. In contrast, the testosterone boosting activity obtained for *E. longifolia* has been previously reported.[15] A patent registered in the USA identified a bioactive component isolated consecutively by several size exclusion columns followed by reversed-phase HPLC, and it was found to be a peptide capable of increasing testosterone levels.[7] Additionally, it was presumed to be a glycopeptide containing xylose, glucose, and fucose moieties. Due to the specific binding, the current work confirmed that the glycoprotein bound to the lectin in the affinity column. Our method shortens the time to isolate the glycoprotein compared to the patent embodiment due to few stepwise chromatography techniques used and, more importantly, particular use of affinity chromatography.

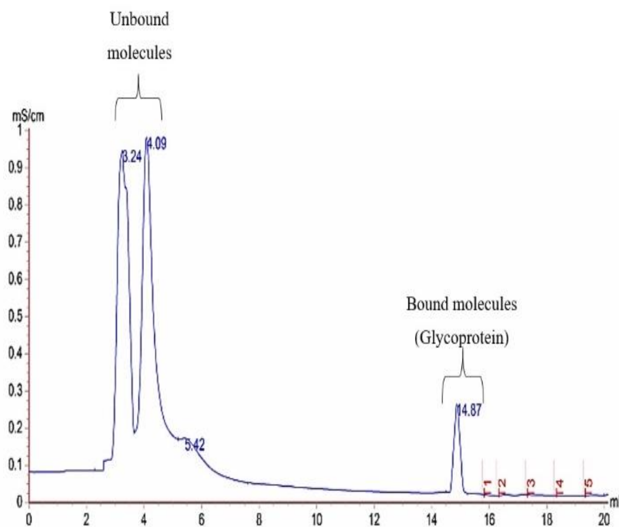

Figure 3: Chromatogram of lectin affinity chromatography of the protein fraction in *E. longifolia*

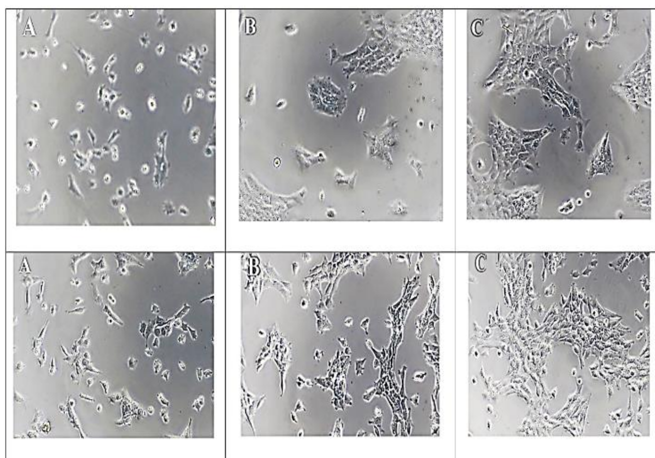

Figure 4: Inverted phase contrast observations of TM-3 Leydig cells incubated with *E. longifolia* (upper images): **A** (24 hours, low confluency), **B** (48 hours, intermediate confluency) and **C** (72 hours, high confluency); glycoprotein (lower images): **A** (24 hours, low confluency), **B** (48 hours, intermediate confluency) and **C** (72 hours, high confluency) in a 96-well plate at magnifications of 400X.

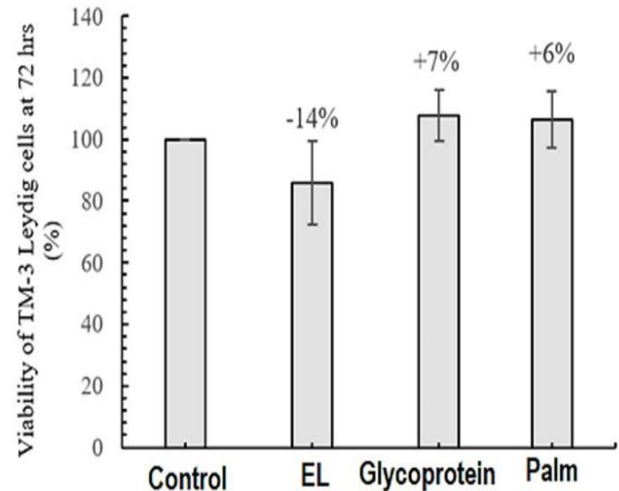

Figure 5: The effect of different plant extracts on TM-3 Leydig cell viability *in vitro* with respect to the control (cells grown without any incubation with plant extract) at a concentration of 50 µg/mL for 72 hours determined with the MTT assay. Symbol [+] denotes increase or decrease [-], and the statistical significance of each test compared to the control was at P > 0.05 (i.e., statistically insignificant). Data are shown as replicates, and the error bar is the standard deviation of the mean. EL = *E. longifolia*; Palm= *E. guineensis.*

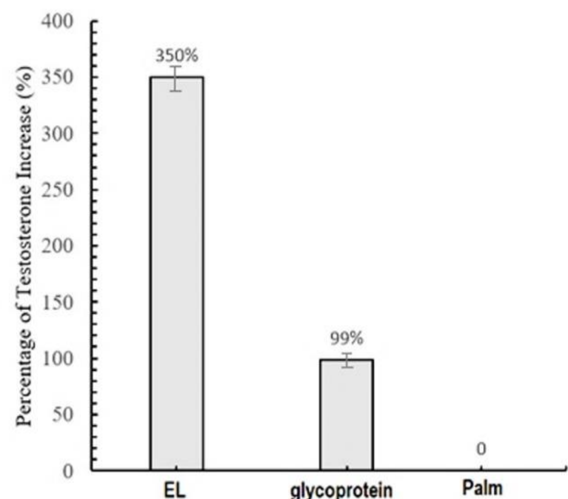

Figure 6: The percentage testosterone secretion of different samples on TM-3 Leydig cells at a concentration of 50 µg/mL for 72 hours compared to the control (without incubation with any plant extract). Data are shown as replicates, and the increase are significant (P < 0.05) compared to the control. EL = *E. longifolia*; Palm = *E. guineensis*.

Conclusion

The lectin column isolated the glycoprotein due to its specificity in binding the sugar moiety. In addition, size exclusion chromatography allowed an initial clean-up phase to separate the abundant non-proteins generally found in plants from the protein constituents. Consequently, the contaminant-free glycoprotein demonstrated testosterone-boosting ability in Leydig TM-3 cells. The resulting purified bioactive glycoprotein may potentially be a lead compound for drug development to treat men with low testosterone.

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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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