

In Vitro Safety & Quality Analysis on Three Species of Tongkat Ali Plants & Their In Vivo Elevation of Testosterone in Fowls

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ABSTRACT – *Eurycoma longifolia*, *Polyathia bullata*, and *Stema tuberosa* are three species of plants sharing the synonym of "Tongkat Ali" and commonly known as Tongkat Ali Putih, Tongkat Ali Hitam, and Tongkat Ali Merah, respectively. However, less study has been done on its effectiveness to increase testosterone by *P. bullata* and *S. tuberosa* compared to *E. longifolia*. In this study, all three types of Tongkat Ali were compared for their testosterone elevations in fowls. The roots of the plants were encapsulated and given to fowls. Before being tested on animals, the capsules' content was analyzed on a few safety and quality parameters, i.e., pH and moisture tests, heavy metal content, microbial load, and steroid presence. A total of 12 mg of each Tongkat Ali powdered material included in a capsule and given to 14 fowls for 30 days. The outcome showed an increase in testosterone in fowls with the highest value of 9.73 ± 1.20 nmol/L obtained by *P. bullata*, followed by *E. longifolia* and *S. tuberosa*, 7.70 ± 0.59 nmol/L, and 6.25 ± 0.70 nmol/L, respectively compared to the control of only 4.08 ± 0.85 nmol/L. The results showed that *P. bullata* and *S. tuberosa* were safe to consume (in vitro) and able to increase testosterone level in the fowls (in vivo). Thus, it also provide added information on the less studied Tongkat Ali plants and their potential as an aphrodisiac plants.

ARTICLE HISTORY

Received: 5th Feb 2023

Revised: 10th Apr 2023

Accepted: 12th June 2023

KEYWORDS

Tongkat Ali
Aphrodisiac
Testosterone
Fowls

INTRODUCTION

In Malaysia, there are three different type of Tongkat Ali that are famous for its aphrodisiac ability which are *Eurycoma longifolia* (Tongkat Ali Putih), *Polyathia bullata* (Tongkat Ali Hitam), and *Stema tuberosa* (Tongkat Ali Merah) [1]. The differences in their malay name is due to their colour of the roots as shown in the Figure 1. For *E. longifolia*, the colour of the roots are pale and whitish, or almost pale yellow meanwhile for *P. bullata*, the roots colour are darker and blackish and lastly for *S. tuberosa*, the roots colour are reddish or brownish [2, 3]. Interestingly, their botanic names differ from one to the other, however, they are all commonly called simply as Tongkat Ali due to their aphrodisiac use for men claimed by the indigenous people. Aphrodisiacs defined as a substance said to elevate the sexual desires of a man. Both males and females can benefit from the use of aphrodisiacs, but more focused on males, as their properties tend to increase testosterone levels rather than estrogen levels [4]. Most of the aphrodisiac studies were usually focusing on *E. longifolia* or, in combination with *P. bullata* and only very few studies on *S. tuberosa*. Studies have showed that *E. longifolia* able to increase serum testosterone levels in humans, as well as treat erectile function disorders, and improve sperm production [5, 6]. Compared to *E. longifolia*, the more readily available species, *P. bullata*, and *S. tuberosa* are the less popular option in research studies on aphrodisiac plants even though they are being sold widely and with many returning customers. Hence, there are minimal studies and research papers available to verify and justify the claims that state these species (*P. bullata* and *S. tuberosa*) have the component that elevates aphrodisiac properties.



Figure 1. Roots of (a) *E. longifolia*, (b) *S. tuberosa* and (c) *P. bullata*.

Due to *P. bullata*'s aphrodisiac properties, often this plant has been combined with other plants claimed to be aphrodisiacs, including the two other Tongkat Ali plants, *E. longifolia* and *S. tuberosa*, to become products of premixed

coffee, capsules or tablets and other beverages or preparations [7]. Under such conditions, it has not been possible to ascertain whether the action of the products is due to this plant or merely due to the more established libido and testosterone boosting plant of *E. longifolia* since the latter has been well studied both clinically and in laboratory environments. For instance, a study enrolled by 105 male aged 50–70 years. In this study, the subjects were given 100 mg and 200 mg extracts of *E. longifolia* for 12 weeks. The results showed that there was a significant increase in the total testosterone levels at week 12 in both group compared to placebo. Cortisol levels significantly decreased in 200 mg group, while muscle strength significantly increased in both groups [8]. The roots of Tongkat Ali plants may not be a mere pseudosexual enhancer, since it has been well-known that this plant has been sold to repeat buyers and users worldwide for many years and constantly been used to be studied or consumed [7]. In Malaysia, the roots of the Tongkat Ali plants are obtained from the wild by the diligent search of the indigenous people. The wild roots are known with better benefits with varying parameters compared to the cultivated ones depending on the soils and environment [9]. Therefore it is important for the roots of the Tongkat Ali plants to be evaluated and compared on their quality once processed. Moreover, since there are lack of studies regarding *P. bullata* and *S. tuberosa*, therefore it is important to evaluate each of Tongkat Ali plants in terms of their safety and quality analysis and their capabilities in elevating testosterone level for this study.

MATERIALS & METHODS

The roots of Tongkat Ali plants were bought from indigenous people from Perak. The roots were cut into chips and dried in the oven. The dried chips of Tongkat Ali plants were ground until it turn into powdered form. The samples were sent to the Central Laboratory, Universiti Malaysia Pahang (UMP) for quality analysis of pH and moisture test, heavy metal content and microbial analysis. The samples also were sent to Pusat Racun Negara, Universiti Sains Malaysia for steroid detection test. All the analysis were to ensure the Tongkat Ali plants are safe to be consumed on these parameters before given to the fowls.

pH, moisture content, heavy metal and microbial analysis

For pH test, a total of 50 g of Tongkat Ali powder was weighed and mixed with 500 ml of deionized water for the extraction process consisting of 5 hours under reflux. Then, the Tongkat Ali extracts obtained were filtered to remove any residues and 50 ml from the filtered extract was used to measure its pH. The test method used was in house method by using Mettler Toledo SevenEasy S20 pH meter to obtain the respective pH of the samples.

For moisture content analysis, a total of 5.0 g of Tongkat Ali powder was needed by using MB Series of moisture balances from OHAUS. A moisture balance is a device that uses loss on drying method, also called a thermogravimetric principle, to determine moisture content of a sample. Moisture content was measured using direct measurement by removing the moisture to get the water content of the various Tongkat Ali, and the weight loss was measured using equation 1:

$$\text{Moisture content (\%)} = \frac{W2-W3}{W2-W1} \times 10 \quad (1)$$

W1 = weight of a container with lid

W2 = weight of a container with lid and sample before drying

W3 = weight of a container with lid and sample after drying

A total of 5.0 g Tongkat Ali dried powder was used for heavy metal analysis. The test was done by using inductive coupled plasma mass spectrometry (ICP-MS), Agilent 7850 ICP-MS. It was used to measure certain elements such as arsenic, mercury, cadmium, and lead in biological fluids. To prepare solution samples, the dried Tongkat Ali samples were sonicated in 3 M hydrochloric acid, then added with 5% hydrogen fluoride and added with hydrochloric acid again and dissolved with distilled water. After the sample solution obtained, it was injected into the nebulizer of ICP-MS.

For microbial analysis, it was done using Tongkat Ali powdered materials. A simple microbe test was carried out using the streak plate method. This method was performed by dissolving 1 g of sample in 10 ml of autoclave deionized water. Then, dilution was prepared at a concentration of 0.1 mg/ml of sample for the streak plate. The streak plate was carried out in laminar flow to avoid any contamination. All materials that were used in the microbe test were autoclaved for two hours. The inoculating loop used for streaking was flamed on a Bunsen burner for sterilization technique. The sample was streak on the readymade XLD agar, MSA agar, and EMB agar for *Salmonella* sp., *Staphylococcus* sp., and *E. coli*, respectively. The agar was incubated for 24 hours and 48 hours at 37 °C and was observed after 24 hours and 48 hours.

Steroid detection test

For steroid detection test, a total of 10 g of Tongkat Ali powdered materials was sent to the Pusat Racun Negara at Universiti Sains Malaysia for corticosteroid determination using Gas Chromatography-Mass Spectrometry (GC-MS), PerkinElmer Clarus 600 Mass Spectrometer. GC-MS detects complex organic and biochemical mixture of steroidal hormones such as dexamethasone, betamethasone, cortisone, hydrocortisone, prednisone, prednisolone,

methylprednisolone, and triamcinolone. GC-MS combines gas chromatography and mass spectrometry to identify different steroidal substances in Tongkat Ali.

Elevation of Testosterone in Fowls (in vivo)

After satisfied with all the safety analysis done on the Tongkat Ali plants, the raw powdered material were formulated into capsules form without additional compounds or excipient. The dosage of Tongkat Ali used only 12 mg (calculate based on the weight of chicken) per capsule. A total of 14 fowls were used in this study by dividing 4 fowls for each Tongkat Ali plants group and another 2 fowls in control group. The weight of the fowls was ranged from 1.6 kg to 2.0 kg. The dosing done twice a day (morning and evening) given one capsule each time by oral feed. For testosterone elevation in blood, a volume of 5 ml of blood were collected at the end of 30 days of treatment with Tongkat Ali capsules. The blood was drawn from the control, and test fowls and the blood samples were sent to Gribbles Pathology Diagnostic Laboratory to be tested for testosterone. The approval for the animal testing achieved with IACUC of University Malaysia Pahang (UMPIACUC/2021/01).

RESULTS & DISCUSSIONS

Results of this research cover the evaluation of pH, moisture content, heavy metal, steroid detection test, and microbial analysis. These analyses were considered essential to be performed before conducting tests of in vivo. All the results of these parameters tested are as given.

pH and moisture content

The pH measured for all three types of Tongkat Ali extracts dissolved in water and filtered were found mildly acidic, as shown in Table 1. An extremely acidic or basic extract can be detrimental to the health of a person consuming it continuously as supplements for a long duration. Nevertheless, Ginseng extract had recorded with pH 5.0 and was popularly being consumed by the public without any known adverse effects [10]. An overly acidic plant includes *Terminalia catappa* and the plant's leaf is used to lower the aquarium water's pH to heal bacterially infected fish [11, 12] and due to its extreme pH, *T. catappa* is often used externally rather than internally [13]. Low pH is known to cause denaturation of metabolic enzymes in the cells; potentially, it can cause cell death [14].

Table 1. pH and moisture of tongkat ali

Parameter	<i>E. longifolia</i>	<i>P. bullata</i>	<i>S. tuberosa</i>
pH	5.9 ± 0.08	5.1 ± 0.22	6.0 ± 0.20
Moisture (%)	4.58 ± 0.31	6.73 ± 0.12	3.74 ± 0.26

n=4 with S.D included

The moisture content indicates the absorption of water through the sample. The higher the moisture contents, the more water absorption can lead to plant material spoilage [15]. According to Biotropic Malaysia Berhad (a company renowned for producing Tongkat Ali capsules such as the patented *Nu Prep* product), packed products should maintain less than 8% water to ensure microorganism spoilage is managed. The water content in the extracts of Tongkat Ali was removed by sublimation process of the frozen sample in freeze-dryer to ensure the moisture content is less than 5% w/w [16]. *E. longifolia*'s moisture content was 4.58%, while for *S. tuberosa* and *P. bullata* were 3.74% and 6.73%, respectively, considered adequately within the safe levels.

Heavy metal content

Results analysis of heavy metal tests was performed and tabulated in Table 2. The heavy metal content in Tongkat Ali is considered as low, which is less than 0.1 ppb for arsenic, cadmium, and lead, meanwhile 0.005 ppm for mercury. National Pharmaceutical Regulatory Agency (NPR), an agency in the Ministry of Health, Malaysia, responsible for monitoring the registration of herbal product and pharmaceutical, stated that the limitation for heavy metal metals as arsenic are (<10.0 ppb), cadmium (<0.5 ppb), lead (<0.5 ppb) and mercury (<0.3 ppm) [17].

Table 2. Heavy metal content

Parameter	<i>E. longifolia</i>	<i>P. bullata</i>	<i>S. tuberosa</i>
Arsenic (ppb)	ND	ND	ND
Cadmium (ppb)	ND	ND	ND
Lead (ppb)	ND	ND	ND
Mercury (ppm)	ND	ND	ND

ND: Not Detected (less than 0.1) except ND for mercury is less than 0.005

There is a common belief that herbal medicines are of natural origin and safe to consume [18]. According to Butthongkomvong [19], there was no detection of toxic metals such as lead and arsenic in *E. longifolia*. Higher containment of mercury may affect male fertility as it was found to induce sperm abnormality in humans [20]. Arsenic may be present in some of the herbal plants. Arsenic may penetrate the plant via contaminated soil and water. Besides, arsenic has a toxic effect on cellular respiration and may accumulate and disrupt vital organs' functions in the human

body, such as the brain, kidney, and liver [21]. Excessive cadmium with considerable toxicity causes a destructive effect on most organs systems. It may cause sudden cardiac death, peripheral arterial disease, and myocardial infarction as it accumulates in the aorta wall [22].

Lead may cause lethargy, seizures, coma, death, and, if chronic, affect adults' sterility [23]. No records on heavy metal toxicity are available on either of the three types of Tongkat Ali plants except a *P. bullata* report with 0.53-2.35 ppm of mercury was found in 26% of products of Tongkat Ali Hitam capsules [18]. Plants commonly found with high heavy metals contents are due to them being grown close to contaminated soils or polluted air. An example of such conditions is expected from plants harvested near industrial areas known for their high pollution [24]. The three types of Tongkat Ali roots in the present study were safe from trace amounts of heavy metals, as shown in Table 2. The results obtained were as expected since all the Tongkat Ali plants were purchased from the same indigenous people, scavenging them from Malaysia's wild jungle that are far from any contamination. Plants commonly found with high heavy metals contents are due to them being grown close to contaminated soils or polluted air. An example of such conditions is expected from plants harvested near industrial areas known for their high pollution [25, 26].

Microbial analysis

A microbial analysis is essential to determine the quality and safety of the capsule content. Total aerobic plate count, yeast and mould count, and bile tolerant gram-negative also were tested for all Tongkat Ali powders. Table 3 tabulates the total aerobic microbial count and total yeast and mould count detected beyond permissible levels in *P. bullata* however in *E. longifolia* and *S. tuberosa*, less than 1×10^1 cfu/g were detected. Several studies found that some herbal plants contained a wide variety of microbial contaminants [27]. According to Stottmeister et al. [28], 22% of plant herbs failed to comply with traditional medicines' quality requirements. Thus, accordingly a decontamination step was necessary to be included to remove the microbes found present. The sample of *P. bullata* alone was eliminated of these microbial contaminations by permissible gamma radiation exposure. Table 4 tabulates the result after the procedure.

Table 3. Microbial detection within Tongkat Ali powder

Parameter	<i>E. longifolia</i>	<i>S. tuberosa</i>	<i>P. bullata</i>
Total aerobic microbial count (cfu/g)	$< 1 \times 10^1$	$< 1 \times 10^1$	6.5×10^3
Total yeast and mould count (cfu/g)	$< 1 \times 10^1$	$< 1 \times 10^1$	2.25×10^3
Bile tolerant gram negative (cfu/g)	$< 1 \times 10^1$	$< 1 \times 10^1$	Absent
Detection of <i>E. coli</i>	Absent	Absent	Absent
Detection of <i>Staphylococcus aureus</i>	Absent	Absent	Absent
Detection of <i>Salmonella</i> sp	Absent	Absent	Absent

Table 4. Microbial test within *P. bullata* powder after decontamination step

Parameter	<i>P. bullata</i>
Total aerobic microbial count (cfu/g)	ND (<10)
Total yeast and mould count (cfu/g)	ND (<10)
Bile tolerant gram negative (cfu/g)	Absent
Detection of <i>E. coli</i>	Absent
Detection of <i>Staphylococcus aureus</i>	Absent
Detection of <i>Salmonella</i> sp	Absent

Consequently, there was an absence of *E. coli*, *Staphylococcus aureus*, and *Salmonella* sp detection in the capsule's raw materials of *P. bullata*. Therefore, according to the NPRA, the resulting product should not contain microbial contamination such as *E. coli*, *Staphylococcus aureus*, and *Salmonella* sp. The accepted level for microbial load for the traditional preparation of capsules are total aerobic microbial count ($< 2 \times 10^4$ cfu/g), total yeast and mould count ($< 2 \times 10^2$ cfu/g), bile tolerant gram-negative ($< 1 \times 10^2$ cfu/g), and with no detection or absence of *E. coli*, *Staphylococcus aureus* and *Salmonella* sp [17]. The safety and quality results can be concluded as low or permissible criteria for pH, moisture, steroids, and microbial load, as indicated in the standards specified by NPRA guidelines [17]. Although the microbial load was initially an issue, the inclusion of an additional step of decontamination was able to remove any fastidious pathogens. Assuming the fowls are continuously given capsules containing pathogenic microbes, this may cause septicemia, blood poisoning caused by infection of many gram-positive or gram-negative aerobes or anaerobes [29].

Steroid detection test

Table 5 tabulates the steroid analysis of all types of Tongkat Ali plants. Based on Table 5 there is no steroids such as dexamethasone, betamethasone, cortisone, hydrocortisone, prednisone, prednisolone, methylprednisolone, and triamcinolone were found in the *E. longifolia*, *S. tuberosa*, and *P. bullata* samples. The steroid detected were corticosteroid classes with different strengths [30]. The limitation of detection (LOD) from the Table 5 refers to the lowest concentration detected by the GC-MS [31] and none of the harmful synthetic steroids was found in either *E. longifolia*, *S. tuberosa*, or *P. bullata*.

Table 5. Steroid analysis of Tongkat Ali plants

Parameter	LOD (ng/l)	Tongkat ali plants
Dexamethasone	10	ND
Betamethasone	10	ND
Hydrocortisone	10	ND
Cortisone	5	ND
Prednisone	10	ND
Prednisolone	5	ND
Methyl Prednisolone	5	ND
Triamcinolone	40	ND

LOD: Limit of Detection; Tongkat Ali plants of *E. longifolia*, *S. tuberosa*, and *P. bullata*; ND: Not detected

The steroid is induced naturally and with adequate testosterone to increase body mass in animals and humans through muscle growth rather than fat. Steroids are regularly found to promote nitrogen retention, with the increased body mass and muscle growth in castrated male cattle and farm animals that are unable to produce hormones *de novo* [32]. However, excessive steroid inclusion levels may cause abnormal liver functions such as bromsulphalein retention and elevation of plasma levels of glutamic pyruvic transaminase, glutamic oxaloacetic transaminase, alkaline phosphatase, and bilirubin. Even worse, the continued intake of steroids may cause bile canals and jaundice, and prolonged use of steroids may cause the reproductive system's inability to generate its testosterone naturally [33]. Therefore, a test for steroidal hormones is necessary to gauge their harmful content [34].

Elevation of Testosterone in Fowls (in vivo)

As shown in Table 6, testosterone detected in the serum of fowls given *E. longifolia*, 7.70 ± 0.59 nmol/L was found to have almost twice the control's value, 4.08 ± 0.85 nmol/L. In comparison, *P. bullata* showed higher than *E. longifolia* with 9.72 ± 0.07 nmol/L but required dosing up to 60 days. Furthermore, *S. tuberosa* also showed an elevation of testosterone levels compared to control. Hence through these results, *P. bullata* provided evidence of stimulating testosterone hormone within 60 days of dosing without any untoward circumstances compared to *E. longifolia* and *S. tuberosa* requiring only 30 days.

Table 6. Testosterone Elevation if Fowls Fed with EL, ST and PB Tongkat Ali plants

Parameters	Control	<i>E. longifolia</i>	* <i>P. bullata</i>	<i>S. tuberosa</i>
Testosterone (nmol/L)	4.08 ± 0.85	7.70 ± 0.59	9.72 ± 0.07	6.25 ± 0.70

Data were expressed as mean \pm S.D. * no testosterone elevated in 30 days testing but only after 60 days

The current study showed *P. bullata* and *S. tuberosa* were able to elevate testosterone in fowls compared to control. These findings are new for both *P. bullata* and *S. tuberosa*. Many studies have already proven the more popular *E. longifolia* capabilities in boosting testosterone. Additionally, *E. longifolia* was found to have increased the libido of male rats and decreased the hesitation time for male rats towards female rats [35]. In another study, *E. longifolia* has been used as the main plant and it showed an increased of the serum testosterone level and improved of sexual activity in males [36]. Such findings indicate Tongkat Ali might stimulate testosterone synthesis and increase the natural release of free testosterone by its extract's ability to bind to sex hormone-binding globulin [8].

CONCLUSION

This study investigated the aphrodisiac ability of three plants of Tongkat Ali. Several basic safety and quality evaluations were performed on the capsules before performing the in vivo study. The analysis were done on pH, moisture content, heavy metal, steroid test, and microbial and overall resulted within the safe limits. The capsules containing *E. longifolia*, *P. bullata* and *S. tuberosa* were fed twice a day to fowls. The results concluded, fowls fed with *P. bullata* showed the highest testosterone increase, followed by *E. longifolia* and *S. tuberosa* with 9.7 nmol/L, 7.7 nmol/L, and 6.25 nmol/L, respectively, with controls averaging 4.08 nmol/L. However, *P. bullata* provided evidence of stimulating testosterone hormone within 60 days of dosing while *E. longifolia* and *S. tuberosa* required only 30 days. This outcome is in tandem with the information given by indigenous people stating *P. bullata* requires more time to take effect compared to the other two Tongkat Ali plants.

ACKNOWLEDGEMENT

The authors would like to acknowledge the contributions of Post-Graduate Research Scheme (PGRS) grant awarded by Universiti Malaysia Pahang (UMP) with project code of PGRS220377 and titled The Isolation of Bioactive Protein of Tongkat Ali for the Elevation of Testosterone Hormone in Male. Some supplementary fundings were also provided by the FRGS grant (reference code: FRGS/1/2022/STG01/UMP/02/1) awarded by the Ministry of Education having title: Investigations Of Protein From The Lesser Known Tongkat Ali Plants Of *Stema Tuberosa* And *Polyalthia Bullata* For Their Potentials In Improving Men's Health.

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