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Comparison of Three Aphrodisiac Plants (*Eurycoma longifolia, Polyalthia bullata* and *Stema tuberosa)* Synonymous with Tongkat Ali

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ABSTRACT

The indigenous people of Malaysia pioneered the use of Tongkat Ali for its aphrodisiac purposes. They gave at least three plants the common name of Tongkat Ali, including *E. longifolia*, *P. bullata* and *S. tuberosa*. Since *E. longifolia* is the most widely used root of the three, it has undergone extensive research compared to *P. bullata* and *S. tuberosa*. Proteins found in *E. longifolia* have been proven to be the aphrodisiac bioactive constituent. Parameters such as pH level, moisture content, heavy metal content and microbial load were compared between all three roots after aqueous extraction under reflux, followed by quantitative protein assay, SDS PAGE and HPLC. The pH and moisture content of the extracts were within the acceptable ranges of 5% to 6% and 3% to 7% (w/w), respectively; additionally, no heavy metals were found. Microbes, which were initially detected, were undetectable once a decontamination step was introduced during the treatment. The protein yield for *E. longifolia*, *P. bullata* and *S. tuberosa* were 0.014%, 0.008% and 0.006% (w/w), respectively, and the SDS PAGE provided a single band within the range of 10 to 20 kDa molecular weight. The HPLC of Eurycomanone, a common quassinoid compound, was found in *E. longifolia* but not in the other two Tongkat Ali. In conclusion, the three plants investigated possess several physicochemical differences but share the same protein, likely contributing to their aphrodisiac activity.

***Keywords*:** Protein, HPLC, Eurycomanone, root, aphrodisiac, Tongkat Ali

Introduction

Tongkat Ali is a popular herbal plant in Malaysia and is related to at least three different plant species. The distinctiveness of Tongkat Ali as a medicinal plant may have developed throughout time through local Malay and indigenous folk who claimed a plant with roots resembling the shape of the male genitalia to possess aphrodisiac properties. The three most commonly utilised Tongkat Ali plants are *Eurycoma longifolia*, *Polyalthia bullata* and *Stema tuberosa*.[1]

The most popular of the three, *E. longifolia*, is commonly known by various Malay names such as Tongkat Ali Putih or Pasak Bumi Putih due to its roots and bark having yellow or almost white colour. This plant, similar to the other two Tongkat Ali plants, is endemic to South East Asian countries with a tropical climate and not known to grow wild in other countries. *E. longifolia* belongs to the Simaroubaceae family and has very few branches and thinly growing shrubs up to 10 metres tall. Traditionally the plant has been used for many remedies and is now popularly sold worldwide as supplements in the form of capsules, tablets, pre-mix beverages, majun (an Indonesian medicinal infusion), bottled drinking water concoctions, or simply in raw dried chipped form.[2]

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The Tongkat Ali market presence in the US is considered safe by the general public as Federal Drug Agency (FDA) regulates its use less than many newer aphrodisiacs due to its long-acceptable presence in the market.[3] Although the plant is recognised for its century-long use in many ailments in Malaysia, it is most popularly used in the Western world, notably to support the active lifestyles of males, who are keen to develop muscle over fat, revive libido of ageing men, improve sexual performance and cure specific sexual dysfunctions.[4]

P. bullata is frequently referred to as Tongkat Ali Hitam or Pasak Bumi Hitam due to the deeper hue of its roots and bark. *P. bullata* belongs to the Annonaceae genus of flowering plants. The plant can grow up to 2–3 metres, primarily in the lowlands of Peninsular Malaysia and the primary and secondary forests of Sabah (a state of Malaysia located within Borneo island).[5] *P. bullata* is another aphrodisiac herb historically utilised predominantly in Malaysia.[6] The root extract of *P. bullata* is often ingested as a decoction by most consumers and is readily accessible on the global market. It is also taken to increase sexual desire and as a general tonic for males.[7]

Stema tuberosa is the least known and studied of the three Tongkat Ali plants. *S. tuberosa* was previously known as *Jackia ornata* or *Jackiopsis ornate*.[8] Interestingly, very few insignificant studies were made on the aphrodisiac potential. *S. tuberosa* has a distinctive red-coloured root, thereby known to the locals as Tongkat Ali Merah, Pasak Bumi Merah, Tongkat Haji Samat, and Ajisamat or spelt instead as Haji Samat.[9] It belongs to the *Stemonaceae* family of flowering plants and grows in sandy hills with altitudes below 400 metres as well as on flat secondary forests.[8, 10] This plant is very rarely sold on western E-commerce platforms. If found, it is usually sold in its original form of dry-cut chips and often only stated as Tongkat Ali with no mention of *S. tuberosa* or Tongkat Ali Merah; hence consumers may confuse it with the other Tongkat Ali plants.[1, 11] Similar to the other two Tongkat Ali, *S. tuberosa* is also sold for its aphrodisiac potential.

Therefore, due to their commercial significance as aphrodisiacs, the three Tongkat Ali plants are investigated to compare their properties to

avoid vague or inadequate labelling by herbal product manufacturers using only 'Tongkat Ali'.[11]

Materials and Methods

Collection of plant samples

The *E. longifolia*, *P. bullata* and *S. tuberosa* plants were first authenticated by a botanist on 8th February 2021 at the University Malaya herbarium with voucher numbers HI1447, HI1446 and HI1445, respectively. The roots of *E. longifolia*, *P. bullata*, and *S. tuberosa*, weighing 10 kg each, were procured from the indigenous people of Perak state in Malaysia (GPS coordinates: 4.7137^0 N, 100.9448^0 E). The roots were first cut into smaller pieces to be made into chips using a commercially sold equipment known as Tongkat Ali Root Cutting Machine (Ruian Hanboo Machinery Co., Ltd, China). Next, the chips were finely ground using a blender before extraction.

Extraction

The extraction was done by reflux using distilled water with total dissolved solids of less than 0.5 ppm, as previously conducted in past studies.[12] A total of 200 g of the powdered plant material were extracted into 1 L of water by boiling under reflux for 5 hours. Once completed, the residues were filtered using Whatman No.1 filter paper and the pH level of the filtrate was measured, followed by freeze drying to obtain the crude extracts. The dried crude extracts were then weighed to determine the yield.

Protein Concentration

The protein concentration in the crude extracts was determined using the Bradford technique with few modifications.[13, 14] Briefly, two mg of extract were dissolved in 1 mL of water, and then 100 µL of the sample solution was mixed with 1 mL of Bradford reagent. After incubating at room temperature for 20 minutes, the absorbance was read at 595 nm using a spectrophotometer (Thermo Scientific™ Genesys™ 10S Vis Spectrophotometer, USA). A total of 2 mg/mL stock solution purchased from Pierce™ Bovine Serum Albumin (BSA) of Thermo Fisher Scientific (USA) was diluted to create different concentrations of BSA. The measured absorbance was then used to construct a standard curve to estimate the protein concentrations in the Tongkat Ali extracts.

Moisture, heavy metal and steroid

The moisture content was directly measured by removing the moisture from 1 g of Tongkat Ali powdered materials by drying, and the weight loss was measured and calculated. In determining heavy metals and steroids, a total of 500 mL filtrate obtained during the extraction process was sent to the Central Laboratory, Universiti Malaysia Pahang and the Toxicology Laboratory, National Poison Centre of Malaysia, Universiti Sains Malaysia, respectively.[15,16] Heavy metal analysis was then conducted using Inductive Coupled Plasma Mass Spectrometry (ICP-MS), Agilent 7850 ICP-MS for the detection of four selected elements: arsenic, cadmium, lead, and mercury. Overall, 8 steroids (dexamethasone, betamethasone, hydrocortisone, cortisone, prednisone, prednisolone, triaimcinolone and testosterone) were detected using the Gas Chromatography Mass Spectrometry (GCMS), PerkinElmer Clarus 600 Mass Spectrometer.

Microbial analysis

The Central Laboratory of UMP conducted a microbial analysis of the Tongkat Ali powdered materials using the streak plate method.[17] This technique was done by dissolving one gram of powdered plant material in 10 mL of autoclaved water. Then, the streak plate was diluted at a concentration of 0.1 mg/mL of material. The sample was streaked using a sterile inoculating loop on the readymade Xylose Lysine Deoxycholate agar (XLD agar), Mannitol Salt Agar (MSA agar) and Eosin Methylene Blue (EMB agar) for *Salmonella* sp., *Staphylococcus* sp., and *E. coli*, respectively. The agar plates were incubated for 24 and 48 hours at 37^0C and observed for the colony forming units (cfu). For the decontamination step, 5 kg of powdered material from each plant was sent to the Agensi Nuklear Malaysia to undergo radiation sterilisation using gamma irradiation not exceeding 10 kGy.[18] Once the decontamination procedure was completed, the microbial test was then repeated to observe for cfu formation.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The technique used is similar to the protocol previously conducted to separate proteins found in Tongkat Ali.[14] Briefly, the resolving gel was placed in 15% homogenous gel in a Mini Vertical Slab Gel System (BioRad Laboratories, US) set with a continuous voltage of 120 V. The amount of protein loaded onto each well was 20 µg for the Tongkat Ali plants based on the concentration of protein determined by the Bradford assay earlier.[13] The molecular weight marker used was purchased from Takara Bio, US, and the staining of the gel was performed using Coomassie brilliant blue solution (PhastGel Blue R, GE Healthcare, US) with 10% acetic acid solution for de-staining.

High-Performance Liquid Chromatography (HPLC)

The HPLC analysis was conducted similarly to the previous method using equipment from Waters, US, with a photodiode array detector and XBridge column (Supelcosil 5 µm, 250 mm x 4.6 mm).[12] The mobile phase was programmed to run with an isocratic elution of water: acetonitrile (85:15) and 1 mL/min flow rate. For the sample analysis, all three Tongkat Ali plant extracts were prepared at a 5 mg/mL concentration and filtered using a 0.45 µm syringe filter before loading 10 µL into the HPLC. The Eurycomanone (ChromaDex, US) compound was prepared at a 2.5 mg/mL concentration.

Quantitative data analysis

Tests were conducted in quadruplicates, and the findings were displayed as means and standard deviations calculated using the 2019 version of Microsoft Excel.

Results and Discussion

Table 1 displays the exterior structure of the three Tongkat Ali roots. In Malaysia, the three plants are locally known as Tongkat Ali Putih, Tongkat Ali Hitam, and Tongkat Ali Merah based on the colour of their natural root bark, which is faint yellow or almost white for *E. longifolia*, black for *P. bullata*, and red for *S. tuberosa*.[9] In the Malay language, putih, hitam and merah means white, black and red, respectively. The root chips shown in Figure 1 are produced using the Tongkat Ali Root Cutting Machine. The *E. longifolia* chips have the most bitter taste, followed by a slight bitterness for the *P. bullata chips*, while the *S. tuberosa*. chips are generally not bitter. The bitterness in *E. longifolia* is expected due to reports of the high content of quassinoid, a bitter compound in this plant.[19,20] Eurycomanone is one of the most abundant quassinoids in *E. longifolia*. Its harsh flavour discourages individuals who cannot endure the taste of drinking the decoction after boiling and removing the root chip remnants. Alternatively, most customers prefer the root preparations to be in capsules instead.

The extraction method for the Tongkat Ali plants is similar to previously reported methods. Meanwhile, the local Malays and indigenous people have practised boiling the roots in water in preparing the decoction for generations.[20] Table 2 shows the yields after the aqueous extraction under reflux. The three plants were found to contain protein, with the yields calculated almost double in *E. longifolia* compared to the other two plants. The presence of protein in the plants is crucial, especially with evidence emerging in which the protein contributed towards the testosterone-boosting abilities of *E. longifolia* and also in the other two lesser studied Tongkat Ali plants of *P. bullata* and *S. tuberosa*.[12, 21, 22] The glycosylated protein or glycoprotein that was isolated from *E. longifolia* was able to elevate testosterone in the TM-3 Leydig cell culture.[14] In addition, *P. bullata* showed the presence of peptide mass signal at 4.3 kDa using SELDI-MS similar to the one found in *E. longifolia*.[23] However, unlike the other two Tongkat Ali plants, there is no evidence of protein in *S. tuberosa*.

The three plants were tested to be mildly acidic and had low moisture content (Table 3). Heavy metals and steroids were not found to be present based on the equipment's limit of detection (LOD). Most countries have an acceptable pH range for medicinal herbal preparations between 5 to 7.[24] Ginseng extract has a pH of approximately 5.0 and is

popularly consumed without known adverse effects.[25] A very acidic herb used as a supplement can affect cell function and denaturation of functional proteins within the body.[26] Certain extremely acidic plants, such as *Terminalia catappa* or tropical almond, with a pH range of 2 to 5 (depending on their concentration), are beneficial for decreasing the pH of aquarium water to cure infectious skin illnesses in fish.[27] Due to its extreme pH, *T. catappa* is often used externally only.[28]

There are several considerations by regulatory bodies for any herbal plants before being commercialised as traditional supplements, including pathogenic microbes, heavy metals, pesticides, mycotoxins, illegal substance adulterations and misidentification, either on purpose or accidental.[29] The Tongkat Ali plants were found without any detectable heavy metals, as shown in Table 3. In one study, Ang and co-workers tested a total of 100 *E. longifolia* health goods with a cold vapour atomic absorption spectrophotometer and found that 36% of the products contained 0.52–5.30 ppm of mercury, which is beyond the allowed limit.[30] Another study on *P. bullata* on its products sold in Malaysia showed worrisome results, whereby many of the tested products contain heavy metals beyond the permitted limit.[31] Commonly, wild plants are known to have lower levels of heavy metals than cultivated ones. Plants commonly found with high heavy metals contents are due to them being grown close to contaminated soils or polluted air.[32] An example of such conditions is expected from plants harvested near industrial areas known for their high pollution.[33] All three Tongkat Ali roots used in the current study were sampled by the indigenous people from the wild away from industrial activities, hence limiting pollution possibilities.

Table 1: Visuals and tastes of Tongkat Ali roots

Plant samples	Root visuals	Taste of raw chips upon chewing
E. longifolia	 Cross section of root (yellow bark) with diameter of 4 cm x 3 cm. Also included its shaved chips.	Strong lasting bitterness
P. bullata	 Cross section of root (black bark) with diameter of 7 cm x 5 cm. Also included its shaved chips.	Mildly bitter followed by tasteless
S. tuberosa	 Cross section of root (red bark) with diameter of 4 cm x 3.5 cm. Also included its shaved chips.	Non-bitter and tasteless

Table 3 highlights that none of the eight steroids (dexamethasone, betamethasone, hydrocortisone, cortisone, prednisone, prednisolone, triaimcinolone and testosterone) tested were found in the plants. The only steroid detected were of the corticosteroid class with varying strengths.[34] Dexamethasone and betamethasone are potent anti-inflammatory steroidal drugs used in managing acute and chronic pain together with 50 times the strength of hydrocortisone.[35] If found with these types of steroids, the consequences can be detrimental to the consumers of Tongkat Ali. Steroids, either natural (e.g. testosterone) or synthetic, increase body mass in animals and humans through muscle growth rather than fat. Steroid treatments are regularly found to promote nitrogen retention, with increased body mass and muscle growth in castrated male cattle and farm animals that are unable to produce hormones by *de novo*.[36] However, excessive steroid inclusion might result in abnormal liver functions, such as bromsulphalein retention and an increase in plasma levels of glutamic oxaloacetic transaminase, bilirubin, glutamic pyruvic transaminase, and alkaline phosphatase.[37] Even worse, the continued intake of steroids may cause bile canals and jaundice, and prolonged use of steroids may even cause the reproductive system's inability to generate intratesticular testosterone.[38] Therefore, a test for steroidal hormones is necessary to gauge their harmful content.[39] Interestingly, none of the three plants were found with testosterone, suggesting they have organic compounds capable of producing de novo testosterone in a person consuming them instead. Previously, the three Tongkat Ali plants were shown to elevate testosterone in fowls.[40, 1] Exogenous, bioidentical or synthetic testosterone therapy, either as tablets, skin patches, or injections, may lead to various side effects such as acne breakouts, aggressive behaviour, and an increase in heart muscle mass that can cause heart attacks.[41]

Table 4 shows the various microbial determinations on the dried plant materials. The three varieties of Tongkat Ali had less than 10 cfu/g of total aerobic microbial count, yeast and mould, and bile-tolerant gram-negative bacteria. According to several studies, herbal plants have a range of microbiological contaminants.[42, 43] According to Ang and co-workers, 22% of plant herbs failed to comply with traditional medicines' quality requirements.[30] The accepted level for microbial load for the traditional preparation of capsules are total aerobic microbial count (<2 x 10^4 cfu/g), total yeast and mould count (<2 x 10^2 cfu/g), bile tolerant gram-negative (<1 x 10^2 cfu/g), and with no detections or absence of *E. coli*, *S. aureus* and *Salmonella sp*.[44] Thus, a decontamination step was necessary to be included to remove the microbes found. The samples were eliminated of the microbial contaminations by permissible levels of gamma radiation exposure of not exceeding 10 kGy.[45]

The evaluations for pH, moisture, heavy metal, steroids, and microbial load can be considered low or within the permissible limit as indicated in the standards specified by the National Pharmaceutical Regulatory Agency (NPRA) guidelines.[44] Although the microbial load was initially an issue, especially for *S. tuberosa*, an additional decontamination step could remove any present pathogens. Assuming a person is continuously given capsules containing pathogenic microbes, this may eventually cause septicaemia, blood poisoning caused by gram-positive or gram-negative aerobes or anaerobes infection.[46]

Upon determining the protein concentration by the Bradford assay, the SDS PAGE analysis was then conducted, as shown in Figure 1, to characterise the protein in all three plants. In previous works, *E. longifolia* was already established with a low molecular weight protein of approximately 20 kDa.[14]

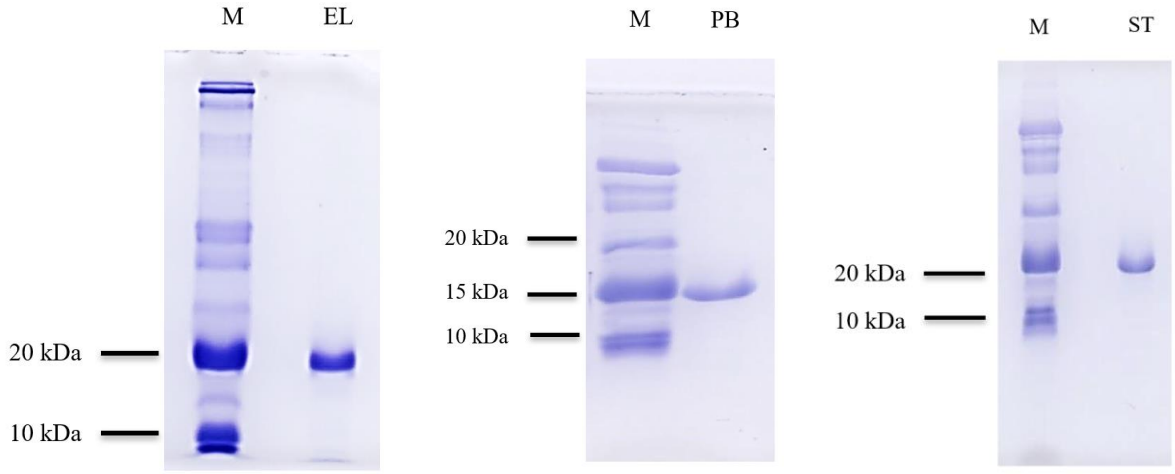

Figure 1: SDS-PAGE results with bands between 10 to 20 kDa molecular weight range. EL: *E. longifolia*; PB: *P. bullata*, ST: *S. tuberosa* and M = molecular weight marker. Staining by Coomassie blue stain.

Table 2: The yield of plant extracts after freeze dry

Plant Sample	Mass of freeze-dried sample (g)	Average yield of dry extract in root (% w/w)	*Protein concentration (µg/ml)	Average yield of protein in root (% w/w) x 10^{-2}
E. longifolia	12.70 ± 0.15	6.34 ± 0.07	28.62 ± 6.5	1.36
P. bullata	11.62 ± 0.11	5.88 ± 0.02	15.86 ± 4.41	0.75
S. tuberosa	8.75 ± 0.16	4.38 ± 0.08	13.01 ± 4.06	0.62

n=4 ± S.D included; mass of plant materials used = 200 g; *values determined by the modified Bradford assay[13]

Table 3: Determination of pH, moisture, heavy metals and steroids

Plant Sample	Parameters			
	pH	Moisture (%) w/w	*Heavy metals	Steroids
E. longifolia	5.9 ± 0.08	4.58 ± 0.31	-absent-	-absent-
P. bullata	5.1 ± 0.22	6.73 ± 0.12	-absent-	-absent-
S. tuberosa	6.0 ± 0.20	3.74 ± 0.26	-absent-	-absent-

*Arsenic, cadmium and lead detection up to 0.1 ppb while mercury to 0.005 ppm.

Table 4: Microbial detection within Tongkat Ali before and after decontamination step

Plant Sample	Decontamination step	Total aerobic microbial count (cfu/g)	Total yeast and mould count (cfu/g)	Bile tolerant gram negative (cfu/g)	**E. coli, S. aureus* & *Salmonella spp.*
E. longifolia	-no-	< 10	< 10	< 10	-absent-
	-yes-		-absent-		-
P. bullata	-no-	< 10	< 10	< 10	-absent-
	-yes-		-absent-		-
S. tuberosa	-no-	6.5 x 10^3	2.25 x 10^3	-absent-	-absent-
	-yes-		-absent-		-

Note: cfu determined after 24 hours and 48 hours incubations for bacteria and non-bacteria, respectively. *Detection of *E. coli*, *S. aureus* and *Salmonella spp.* were done separately for each plant.

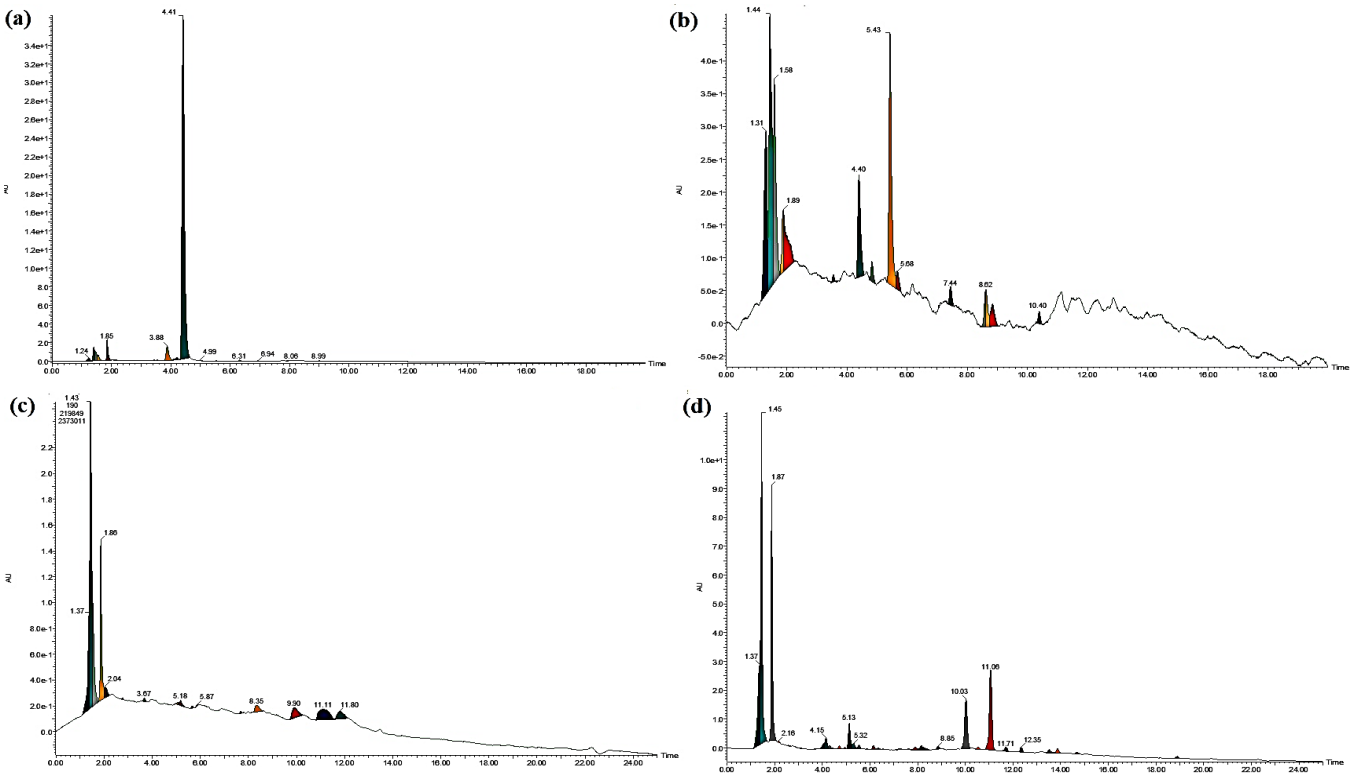

Figure 2: HPLC profiles for Eurycomanone standard and Tongkat Ali plants. HPLC profiles for (a), Eurycomanone standard; (b), *E. longifolia*; (c) *P. bullata* and (d) *S. tuberosa*.

Interestingly, both *P. bullata* and *S. tuberosa* as well found with single bands at 15 kDa and 20 kDa of low molecular weights, respectively. Other than for *E. longifolia*, such outcomes have not yet been reported for the other Tongkat Ali plants. It is expected that if the SDS PAGE analysis on samples originated from animals or microbes, the number of bands will be numerous and may even be concentrated. However, due to their low abundance of protein within plants, the outcome in performing SDS PAGE is usually found to have no protein bands or achieve similar results to that for the three Tongkat Ali plants in this study. Similarly, in previous works, the protein in *E. longifolia* was isolated and shown to elevate testosterone in TM-3 leydig cells.[14] Additionally, due to using a lectin affinity column to purify the protein in *E. longifolia*, the protein was further proven to be glycosylated or glycoprotein.

In Figure 2, a prominent peak with a retention time of 4.4 minutes belonging to Eurycomanone was achieved using HPLC. A peak with a similar retention time was observed in the HPLC profile of *E. longifolia* extract but not in the other 2 plants. *E. longifolia* contains Eurycomanone, a member of the quassinoids family, as one of its primary constituents.[47] Manufacturers of Tongkat Ali herbal capsules often use Eurycomanone as the standard in their quality control.[21] Therefore, it is clear this compound distinctively belongs to *E. longifolia* and products manufactured from the other two Tongkat Ali cannot be authenticated and quantitated on the content of Eurycomanone. Previously, two markers i.e. Eurycomanone and a protein marker referred to as marker A (detected using two-dimensional electrophoresis) were used to authenticate some 50 Tongkat Ali selected products. The results identified almost half of the products to be found without the presence of either of the markers, concluding them as fake products.[12] It is possible those products, without the presence of the markers, were manufactured using the other lesser-studied Tongkat Ali plants of *P. bullata* or *S. tuberosa*.

Conclusion

The three investigated Tongkat Ali plants clearly show variances in their botanical and organic contents, even though they share a common name and aphrodisiac potential. All three plants were evidently found with protein based on their protein yields and SDS PAGE bands. Differences in the HPLC profile highlighted that only *E. longifolia* has the presence of Eurycomanone, making the compound a useful marker to authenticate and differentiate this plant from *P. bullata* and *S. tuberosa*.

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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