R&D&C EFFORTS OF FOREST HERBALS OBTAINED FROM INDIGENOUS PEOPLE INTO NPRA APPROVED COMMERCIAL CAPSULES

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ABSTRACT

This study was initiated as UMP-community technology solution platform with a grant bearing number: UIC170904. The technology is the R&D&C efforts by a small group of university researchers in assisting the community of the indigenous people at Kampung Bukit Cermin, Perak. Based on the feedback given by the indigenous community an herbal product compromising mixture of three Tongkat Ali plants been selected and worked upon. It is believed by Malay and indigenous traditional practitioners that all three types of Tongkat Ali have aphrodisiac properties. *Eurycoma longifolia* or known as Tongkat Ali is widely known for their aphrodisiac properties. However, many researchers only focused on Tongkat Ali Putih to be proven with aphrodisiac properties with less or absolutely no emphasis to Tongkat Ali Hitam and Merah. This study provides some account on the procedures of utilizing a contract manufacturer, TPM Biotech Sdn Bhd, in registering the product with NPRA. R&D on the three plants were concurrently done and established the presence of protein within them. Up to now, the compounds that responsible for its aphrodisiac properties not yet studied. Glycoprotein was suspected to be the protein that responsible for the aphrodisiac capabilities in enhancing testosterone in male. This study is to detect the presence of protein in all three types of Tongkat Ali crude extracts. SDS PAGE analysis showed the presence of protein bands in all three types of Tongkat Ali crude extracts. The protein content in Tongkat Ali Putih, Hitam and Merah were calculated by Bradford Assay analysis as 0.61 w/w %, 1.67 w/w % and 2.01 w/w % respectively. Also investigated was the aphrodisiac efficacy and the safety of the plants in fowls. The blood samples of the chicken were collected and send to a Diagnostic Laboratory in Kuantan, Pahang to be evaluated. The result shown that chicken that consumed Tongkat Ali Putih have the highest testosterone level compared to others. Tongkat Ali Merah ranked second while Tongkat Ali Hitam results were in doubt and required repeat. Additional test was also conducted which were the important safety biomarkers for liver. The result indicated that there were mild damages on the liver biomarkers of the chicken by Tongkat Ali Putih with the other types of Tongkat Ali found to be at safe levels. Besides that, four different sexual mating behavior observations conducted shown with positive sexual mating activities on all chicken tested. Chicken that consume Tongkat Ali Putih have the highest frequency of sexual mating activity compare to others. While chicken that consumed Tongkat Ali Merah and Hitam have a somewhat similar frequency of sexual mating activities. The overall analysis showed that Tongkat Ali Putih can be ranked first with the most positive aphrodisiac properties followed with Tongkat Ali Merah and lastly Tongkat Ali Hitam (pending a repeat on its *in vivo* investigation). It was also concluded due to higher dose used i.e. 12mg of the Tongkat Ali Putih per capsule, it is likely provided some acute safety issues and hence required lowering of this dose in order to assess further in the chronic studies (more than 6 months). In overall conclusion, the study provided an example of an initiative to develop herbal product with the efforts provided by the indigenous people, researchers and industry. This can be useful platform for other researchers to mimic in developing more such products from the rich Malaysian biodiversity.

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LIST OF SYMBOLS AND ABBREAVIATIONS

Kg	Kilogram
mg	Miligram
m	Meter
cm	Centimeter
mm	Millimeter
min	Minute
kDa	Kilodalton
°C	Celsius
SS	Sum of square
df	Degree of freedom
MS	Mean square
F	$\frac{\text{Mean square between group}}{\text{Mean square within group}}$
P-value	Probability - value
F crit	Value obtain from F Distribution Table
SD	Standard deviation
U/L	Units/Litre
TA	Tongkat Ali
ALT	Alanine transaminase
AST	Aspartate aminotransferase
GGT	Gamma-glutamyl transpeptidase
RC	Rooster control
RT	Rooster test

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The traditional medicinal plant, *Eurycoma longilofia* Jack is commonly used as herbal medicine in many countries. This traditional plant is mostly found in Southeast Asian countries such as Indonesia, Vietnam, Thailand and Malaysia. In Malaysia, *Eurycoma longilofia* Jack is commonly called as "Tongkat Ali". While in Indonesia known as "Pasak Bumi" and in Vietnam known as "Cay Ba Binh" (Chen *et al*, 2014).

Besides *Eurycoma longifolia* Jack, there are three other plant species also known as "Tongkat Ali" which is *Polyathia bullata*, *Entomophthora apiculata* and *Stema tuberosa*. Some discrepancies been found related to the botanical names of these latter types of Tongkat Ali. *E. longifolia* is a shrub tree with green colour long leaves that will grows up to 10 meter in height. The flowers are dioecious and the shape of leaves are pinnate shape. The fruit is ovoid-shaped and when its ripe, the colour change to dark red or dark brown (Effendy, Mohamed, Muhammad, Mohamad and Shuid, 2012).

The plant extract, from the root has been used (traditionally) by men to increase their testosterone levels. In addition, *E. longifolia* is also used as antiulcer, cytotoxic, antimalarial, antipyretic and aphrodisiac. The *E. longifolia* is extremely popular because of its aphrodisiac property due its capability to stimulate the production or section of androgen hormones, especially testosterone (Bhat and Karim, 2010).

The aphrodisiac properties of *E. longifolia* have been extensively studied since the 1980s. *E. longifolia* become a famous herbal medicine in Asia due to its high aphrodisiac properties. Many products have been development based on *E. longifolia* and commercialized either in the capsule form, chips form or in the pre-mix coffee.

Most researchers have tested the aphrodisiac effects of Tongkat Ali on male rats. A research by Ang *et al* (2004), found that *E. longifolia* increased the levator ani muscle, when compared with the control (untreated) in the uncastrated intact male rats and testosterone-stimulated castrated intact male rats. The male rats were dosed for 12 successive weeks with water, methanol, butanol and chloroform fractions of *E. longifolia* Jack. The result showed that all the fractions increased the levator ani muscle, when compared with the control (untreated) in the testosterone-stimulated castrated intact male rats and uncastrated intact male rats. Thus, the pro-androgenic effect as proved by this study promote the traditional use of this herbal plants as an aphrodisiac (Ang and Cheang, 2001).

In another research done by Ang *et all* (2001) showed that *E. longifolia* produced a dose-dependent increase in sexual performance of the treated animals which is male rats. Further result proven that *E. longifolia* help the growth of both ventral prostate and seminal vesicle as compared with control. The current study also gives further evidence of the folk use of *E. longifolia* as an aphrodisiac (Ang, Cheang and Yusof, 2000).

Recently, Tee *et al*. (2017) reported that a purified extract of *E. longifolia* known as dichloromethane subfraction-I was able to antagonize angiotensin-converting enzyme and angiotensin II that induced contraction which caused corpus cavernosum relaxation via inhibition of angiotensin II type I receptor. Dichloromethane subfraction-I also increase bradykinin-induced relaxation through inhibition of angiotensin-converting enzyme. This discovery could be progressed into an alternative form of therapy for erectile dysfunction without the attendant reported side effects of phodphodiesterase type 5 inhibition (Tee, Hoe, Cheah and Lam, 2017)

Interestingly, although *E. longifolia* or Tongkat Ali Putih been extensively studied for its aphrodisiac activities, not much can be said related to the other two types of Tongkat Ali. Both Tongkat Ali Merah and Hitam as they known locally are said to be useful for their aphrodisiac benefits by indigenous people as well as anyone continuous using them more than once. Whether the aphrodisiac claims are pseudo effects or accurate can only be proven scientifically.

1.2 Problem Statement

Tongkat Ali is a synonym used by local Malays and indigenous people in Malaysia to relate on at least three plants believed traditionally being aphrodisiac. The three plants are *Eurycoma longifolia* (Tongkat Ali Putih), *Stema tuberosa* (Tongkat Ali Merah) and *Polyalthia Bullata* (Tongkat Ali Hitam). These plants are all been sold by the indigenous people at Kampung Cermin, Perak. Currently some 10-15 families in this village rely on sourcing the jungle for medicinal plants, mildly processing them and selling at the booth stands of R&R Sungai Perak, Perak. Previously these booth stands sold capsules of powdered herbs together with whole parts and dried chips of the plants. However, as the capsules were not registered to National Pharmaceutical Regulatory Agency (NPRA), Ministry of Health of Malaysia, the regulatory officers confiscated the existing capsules and the indigenous people were advised not to sell anymore products without the MAL number. In order to assist this group of society to sell to a wider customer pool as well as in achieving NPRA registration, a small group of scientists in Universiti Malaysia Pahang decided to obtain the Tongkat Ali roots from the indigenous people to be developed into registered product for sales. The knowledge obtained from the indigenous people are invaluable related to product development. The information led to the decision of formulating herbal product compromising of three types of Tongkat Ali mixed at the ratio sold by them for some 20 years. Hence it is expected once the product been registered and sold officially, the indigenous families of this village to benefit in being the continuous provider of authentic Tongkat Ali roots. In Malaysia the law states only indigenous people been given rights to harvest the jungle for protected medicinal plants such as Tongkat Ali.

In recent times, many of the medicinal plants sold marred of being fake products. Some scrupulous Tongkat Ali manufacturers known to mix roots of other plant in place of Tongkat Ali whenever unable to obtain the original plant from the wild. Occasionally stem of the Tongkat Ali rather than the more potent root been used as the active ingredient for the capsules. While others desperate to obtain quick sales encouraged to adulterate the Tongkat Ali roots with prescription drugs such as generic drugs of Tadalafil or sildenafil. Such acts may taint the reputation of Tongkat Ali as a useful aphrodisiac plant. To deter future decline of the excellent market of this plant effort required to be conducted to study the aphrodisiac capabilities of all three types of Tongkat Ali

scientifically. Currently only *E. longifolia* had been proven extensively as a testosterone booster at *in vitro* and *in vivo* including clinical trial.

1.3 Research Objectives

1. To initiate discussion and sign letter of intent (LOI) with indigenous families in Kampung Bukit Cermin for continuous purchase of authentic Tongkat Ali roots.
2. To appoint a suitable contract manufacturer to assist in matters of registering to NPRA and later for production of market ready Tongkat Ali capsule product.
3. To determine scientifically the aphrodisiac efficacies and safety uses of capsules formulated from each type of Tongkat Ali.

1.4 Scope of Study

In the initial stages to fulfill objective 1, a spokesperson within the 10-15 indigenous families was sorted out. Madam Aida bt Yun cooperated well towards this objective until LOI been signed. Also a good pricing of the roots mutually beneficially for both parties achieved.

As for objective 2, upon discussing with a number of contract manufacturers finally decided on TPM Biotech Sdn Bhd. All matters pertaining to the appointment dealt and completed. The process of obtaining MAL number for the product is a lengthy process and may take more than a year time for approval. Currently still waiting for approval.

It is imperative to conduct proper experimentation to gauge the safety and efficacy of the herbs prior to being marketed. Although NPRA do not request results proving the efficacy of the plants used for the product it is the responsibility of the researchers of this project to investigate the traditional claims of the three types of Tongkat Ali plants. The *in vitro* investigation to be conducted by utilizing leydig cells (cells of the testes responsible to secrete testosterone). The experimentation required many requisites and expertise to be first in place before can be done. Due to some evidences suggested the presence of protein in *E. longifolia* and possibly responsible as

the active constituent in exerting the aphrodisiac effects, investigations in characterizing proteins in all three Tongkat Ali plants initiated. Lastly, *in vivo* studies conducted on the plants using fowls. There are three main approaches for the in vivo research which are preparation of Tongkat Ali capsule, feed the rooster with the capsules and laboratory analysis. The study will adhere to animal use in ethical and humane norms by obtaining with Institution Animal Care and Use of UMP. The ethic number is UMPIACUC/2018/01.

CHAPTER 2

LITERATURE REVIEW

2.1 Taxonomy and Classification

2.1.1 Tongkat Ali Putih

Many countries utilized and applied herbal medicinal plants for thousands of years. *Eurycoma longifolia* or more popularly known as "Tongkat Ali" is an herbal medicinal plant of South-East Asian origin. It is indigenous to the rainforests of Myanmar, Indonesia, Thailand, and Malaysia (Bhat and Karim, 2010).

Table 2.1: The taxonomy and classification of *Eurycoma longifolia*

Kingdom	Plantae
Order	Sapindales
Family	Simaroubaceae
Genus	*Eurycoma*
Species	*longifolia*

Source adapted from Bhat and Karim (2010)

Besides *Eurycoma longifolia* jack, there are other species also known as "Tongkat Ali" which is *Polyalthia bullata*, *Entomophthora apiculata* and *Stema tuberosa*. The species had been differentiated by the colour of its root, namely "Tongkat Ali Putih", "Tongkat Ali Merah" and "Tongkat Ali Hitam". Some discrepancies been found related to the botanical names of these latter types of Tongkat Ali.

E.longifolia is more preferred and used than other species. Tongkat Ali also known as "Long Jack" and "Malaysia Ginseng". Pasak Bumi, Payung Ali, Bedara Pahit, Setunjang Bumi, Pokok Syurga, Cay ba binh, Ian-don, Jelain and Penawar Pahit are several popular name of this plant. *E.longifolia* height can reach to 15-18m and stance fruits after nearly 2-3 years of cultivation. This plant might take up to 25 years to completely matured.10-15 inches long with 10-30 leaflets, pinnate shape and spirally arranged are the characteristic of the leaves. The fruit are 2-3 cm long, green in colour and change to dark red once ripening (Bhat and Karim, 2010).

(a) *Eurycoma longifolia* Plant
Source adapted from photo © 2011 MedicalHealthGuide.

(b) Root
Source adapted from Euryco® photo (www.javaplant.co.id)

Figure 2.1: (a) plant of *Eurycoma longifolia* and (b) root of the *Eurycoma longifolia*

2.1.2 Tongkat Ali Hitam

Table 2.2: The taxonomy and classification of *Polyathia bullata*

Kingdom	Plantae
Order	Magnoliales
Family	Annonaceae
Genus	*Polyalthia*
Species	*bullata*

Source adapted from https://www.gbif.org/species/3156441

Polyalthia bullata or known as Tongkat Ali Hitam is a small tree from family Annonaceae and can be found in Peninsular Malaysia and Borneo. According to Burkill (1966), there is no precise information of it as a medicine but the name Tongkat Ali suggests that it has aphrodisiac property (Asiah, Nurhanan and Ilham, 2007).

Polyalthia bullata,locally known as Tongkat Ali Hitam, is a shrub with a height of 2-3 m. The young twigs are covered with stiff golden hairs. Leaves are papery or thinly leathery. Early growth of the plant is known to be slow but becoming more rapid at a latr.r stage' The plant is characterised by a single straight trunk' Flowering occurs twice ayear and just before the new leaves appear.

It is dispersed as main canopy tree, shrub or as understory tree in lowland forests in Peninsular Malaysia and Sabah. Sometimes it can be discover in lower montane forests up to an altitude of 1,200 m. Tongkat Ali Hitam also grows well in evergreen and monsoon forests both on well-drained or poorly-drained terrains (Chee and M, 2013).

	
(a)*Polyalthia bullata* plant Source adapted from https://www.google.com/search?q=tongkat+ali+hitam&source=lnms&tbm=isch&sa=X&ved=0ahUKEwjd3OT95JraAhUaSo8KHf5LADQQ_AUICigB&biw=1366&bih=613#imgrc=RgO7-YYpaasTeM:	(b) Root of *Polyalthia bullata* Source from Dr Jaya Vejayan (2018)

Figure 2.2: (a) plant of *Polyalthia bullata* and (b) root of the *Polyalthia bullata*

2.1.3 Tongkat Ali Merah

Table 2.3: The taxonomy and classification of Tongkat Ali Merah

Kingdom	Plantae
Order	Gentianales
Family	Rubiaceae
Genus	*Jackia*
Species	*ornata*

Source adapted from http://arctos.database.museum/name/Jackia%20ornata

Some discrepancies been found related to Tongkat Ali Merah scientific name. Some sources referred them as *Stema tuberosa* while others used the name of *Jackia ornata.* Among those three types of Tongkat Ali, the Tongkat Ali Merah is claimed by indigenous people to be with the most aphrodisiac benefits. It grows in the forest on the slope area. The leaves are the biggest among the Tongkat Ali types, smooth, and soft (Sourced adapted from http://petua.whatta.org/khasiat-tongkat-ali-merah/). Unfortunately, no further scientific information was able to be obtained related to this plant.

(a) *S. tuberosa* Plant
Source adapted from https://www.bio-asli.com/herb/tongkatalimerah.asp?id=

(b) Root of *S. tuberosa*
Source from Dr Jaya Vejayan (2018)

Figure 2.3: (a) plant of *S. tuberosa* and (b) root of the *S. tuberosa*

2.2 Chemical Compositions

There are variety of chemical constituents present in the *E. longifolia* especially at the roots part. Quassinoids, eurycomalactone, tannins, eurycomanone, squalene derivatives, eurycolactone, and biphenylneolignans are the common chemical composition detected in *E. longifolia* (Effendy et al., 2012).

The bitter taste of the plant is because of the presence of quassinoid. This plant rich with secondary metabolites with a large number of quassinoid were located in the roots. Eurycomanone, and its derivative, eurycomanol were located most in the root (Hajjouli *et al*., 2014). The root extract has been reported to induce aphrosidiac properties, increase sperm quality and reversed oestrogen-induced infertility (Low, Choi, Wahab, Das and Chan, 2013). However, it is strange to know only of the late eurycomanone been implicated to contain or proven with aphrodisiac capabilities especially much been studied on *E. longifolia* for years. Eurycomanone is one of the major compound of *E. longifolia*.

In addition, Tada *et al* had studied the isolation from the roots of *E. longifolia* with nearly 65 compounds and classified them by mass spectral data, one and two-dimensional nuclear magnetic resonance spectroscopy. Four quassinoids diterpenoids was recognized as the primary compound, which is eurycomalide A, eurycomalide B, 13β, 21-dihydroxyeurycomanol and 5α, 14β, 15β-trihydroxyklaineanone (Low *et al*., 2013). The other two plants of Tongkat Ali found with very little information regarding the chemical constituents contained within them.

2.3 Pharmaceutical Properties

Table 2.4: An overview of several major works on pharmacological features of each parts of Tongkat Ali (*Eurycoma longifolia)* plant.

Plant part	Activity
Stem bark, roots	Anti-plasmodial activity (antimalarial)
Leaves (herb sample)	Anti-hyperglycaemic activity
Roots	Cytotoxic
Roots	Sexual/aphrodisiac activity
Leafs	Anti-tumor
Roots	Anti-ulcer
Leafs, stem and roots	Anti-microbial

Source: Adapted from Bhat and Karim, 2010

The table shown an overview of several crucial works on pharmacological possessions, the isolated chemical component and their activity that has been evaluated on the Tongkat Ali plant by it separate parts. Its antiulcer, cytotoxic, antimalarial, antipyretic and aphrodisiac properties cause this plant to been used as medicine in the past. The plant extract, especially the roots part, is unique cause been used (traditionally) for men to help in enhancing testosterone levels. Its capability to stimulate the production or section of androgen hormones, especially testosterone was the reason for it popularity compare to other plants (Bhat and Karim, 2010).

An aphrodisiac is defined as a substance that have come from plants, animals or minerals with function to increase sexual desire or libido especially for male. Another definition of aphrodisiac is any drug or food that induce the sexual instinct, venereal desire and increases performance. Many natural substances have historically been known as aphrodisiacs like mandrake tree in Europe. While in South-East Asian origin, *E. longifolia* have been known as aphrodisiacs (Bhat and Karim, 2010).

The butanol, methanol, water, and chloroform extracts of the roots of *E. longifolia* Jack were researched using diverse tests of effectiveness of treated male rats. The results proven that *E.*

longifolia developed a dose-dependent, recurrent and gradually increase in the episodes of penile reflexes as evidenced by increases in long flips, quick flips and erections of the treated male rats during the 30 min examination period. This results provided further evidences that *E. longifolia* enhanced the aphrodisiac potency activity in treated animals. Hence, this study strengthen *E. longifolia* usage as traditional medicine especially for its aphrodisiac property (Ang and Cheang, 2001).

In 12 successive week male rats were dosing with water, methanol, butanol and chloroform fractions of *E. longifolia* Jack. Results showed that all the fractions increased the levator ani muscle, when compared with the control (untreated) in the testosterone-stimulated castrated intact male rats and uncastrated intact male rats. Thus, the pro-androgenic effect as proved by this study promote the traditional use of this herbal plants as an aphrodisiac (Ang and Cheang, 2001).

A decoction of the Tongkat Ali Hitam roots is drunk regularly to treat kidney infections. High blood pressure and diabetes can be treat by are finely pounded and taken the roots, flowers or leaves of these plants. A decoction of the roots mixed with *E. longifolia* is drunk as an aphrodisiac for men (Ong and Nordiana, 1999). Unfortunately, less or no information was found regarding the two types of Tongkat Ali due to no scientific work been carried out.

2.4 Aphrodisiac Properties

Aphrodisiac is the term originated from Greek mythology, where Aphrodite was the goddess of love and beauty. 'Aphrodisia' is the Greek word that means sexual pleasure. Aphrodisiac modern definition can differ, but is generally regarded as a substance that increase sexual pleasure and desire. Substances include minerals, vitamins, foods, other natural and synthetic chemicals (Melnyk and Marcone, 2011).

The aphrodisiacs were classified into 3 types, which are those that increase potency, sexual pleasure or libido based on their technique of action. Few of variants substances from animal and plant origin have been identified pharmacologically although it's have been used in primitive medicines of different culture to energize, improve sexual function and physical performance in men. *Eurycoma longifolia, Montanoa tomentosa, Lepidium meyenii, Crocus sativus, Casimiroa edulis, Mondia whitei* and *Panax ginseng* are several of aphrodisiacs herbal (Kotta, Ansari and Ali, 2013).

Malaysian plants such as *E. longifolia* and *Polyalthia bullata* are claimed to have aphrodisiac property and have been used in Malay traditional medicine. The aphrodisiac property of the plants is only a claim and has not been scientifically proven except for *E.longifolia* (Ang et al. 2000). A potential phytoandrogen isolated from Tongkat Ali which is 4.3 kDA, a bioactive peptide has been reported to rise the testosterone level in rat leydig cells (Sambandan *et al*. 2004). Methods of using purified bioactive peptide for treatment of sexual dysfunction and male infertility are described too (Asiah *et al*., 2007).

2.5 Chicken as Domestic Animal in Malaysia

The Red Jungle Fowl (*Gallus gallus*) is widely accepted as single ancestor of the population of all domesticated chicken. Many accepted that Red Jungle Fowl alone is adequate to account for the maternal ancestry of the domesticated chicken although other wild species of Gallus might have contributed to the domesticated chicken. Now, the improved Mediterranean type populations are the most closely related to the Red Jungle Fowl as it was the earliest chicken brought into Europe (Hillel *et al*., 2003).

Later, enormous use of selection and crossbreeding were developed for local breeds and lines in different parts of Europe. Asian breed of the Chinese and Malay types also been introduces. The present biodiversity of chicken populations due to all of these sources. Inter-crossing might have eliminate the differences among groups or breeds as the result of genetic relationships between chicken populations are not always definitive (Hillel *et al*., 2003).

Table 2.5: Total Number of Chicken Meat Production from Year 2010- 2013.

Year	Chicken				Total
	Broiler	Laying	Breeder	Village	
2010	148,304,515	42,355,738	15,107,501	11,459,713	217,227,467
2011	157,605,416	49,520,194	11,808,436	13,935,095	232,869,141
2012	170,273,718	52,762,461	11,925,339	16,195,822	251,157,340
					268,242,584

According to Department of Veterinary Services records, broiler chicken production increased from year 2010 until year 2012 about 12.9 % where the number of production from 148,304,515 increased to 170,273,718 and about 79 farms conducted the operation in year 2012.

According to the Statistics Department, it is estimated that Malaysia's population will reach almost 40 million in 2040 along with the world population will reach 9 billion. This means that the demand for food will increases for at least another 40 years. The quantity of production also shows that the demand for chicken was higher throughout the year. Thus, this situation will stimulate food prices as greater percentage of the disposable income needs to be spent on food. This has been proven by the increases in the number of chicken production from year 148 million birds in 2010 to 170 million birds in 2012, which clearly shows increased production to meet increased demand (Samsuddin *et al.*, 2015).

As of now, no record of poultry industry in Malaysia used any aphrodisiac enhancers either in drug forms (e.g. Viagra) or herbals (e.g Tongkat Ali). The use of Tongkat Ali however been studied to increase testosterone level in pigs (Rozkot *et al*., 2008).

2.6 Testosterone, ALT, AST and GGT

Carbohydrate, fat and protein metabolism require the present of testosterone as the hormones plays a key role in all of the process. The body fat composition and muscle mass in the male has been influenced by testosterone. Impaired glucose tolerance, low HDL-cholesterol, increased fat mass and reduced insulin sensitivity is related with lack of testosterone (D. M. Kelly & Jones, 2013).

Alanine transaminase (ALT) is a transaminase enzyme made by cells in the liver. ALT is one of the enzymes that help the liver break down other protein so the body can absorb them easily. Generally, ATL found inside liver cells. ALT can be released into bloodstream when the liver is damaged and this causes increase of serum ALT levels (Olsen, 2017).

Aspartate aminotransferase (AST) is an enzyme that's present in different parts of body. A highest concentration of AST can be found in liver, heart and muscles. AST also can be found in bloodstream with a small amount. A sign of health problem when the amount of AST in blood is higher than normal (Marcin, 2017).

The transfer of gamma-glutamyl functional groups from molecules to an acceptor require an enzyme known as Gamma-glutamyl transpeptidase (GGT). GGT function is to transport molecule to move around the body. It also helping the liver metabolize drugs and other toxins. GGT are present in the gallbladder, spleen, pancreas and kidney, but it's is concentrated in the liver. GGT blood levels will increase once the liver is damaged (Marcin, 2017).

One of the important diagnostic equipment used for determining causes of morbidity and mortality is phlebotomy (blood collection). The blood was drawn using the syringe from the brachial wing vein. Underside of the wing, brachial wing vein can be found between the triceps and biceps muscles. 6–7.5% is the percentage of blood volume by body weight suggested for poultry blood collection. It must not more than 1% of the body weight equivalent of blood and blood should be taken in a one collection. A minimum of 14 days for the chicken to recover before more blood can be withdraw (Kelly & Alworth, 2013).

2.7 HPLC Analysis on *E. longifolia*

High Performance Liquid Chromatography (HPLC) is known as a form of column chromatography that pumps a sample mixture or analyte in a solvent (known as the mobile phase) at high pressure through a column with chromatographic packing material (stationary phase). The sample is carried by a moving carrier gas stream of helium or nitrogen. HPLC has the ability to separate, and identify compounds that are present in any sample that can be dissolved in a liquid in trace concentrations as low as parts per trillion. Because of this versatility, HPLC is used in a variety of industrial and scientific applications, such as pharmaceutical, environmental, forensics, and chemicals.

An analytical method that was developed to identified the quassinoid content from the *E. longifolia* Jack (Tongkat Ali) collected from various sources i.e. indigenous people was HPLC with UV detection. There were several chemical compound that has been isolated as reference

standard which; eurycomanone, longilactone, 14,15β-dihydroxyklaineanone, 15β-acetyl-14-hydroxyklaineanone, 6α-hydroxyeurycoma-lactone, and eurycomalactone. Besides that, the synthesized 1β,12α,15β-triacetyleurycomanone (6, internal standard), were identified by NMR, UV, IR and MS spectroscopies. Their coefficient of variation values for 0.50-35 µg/mL (-1) concentrations of quassinoids and their retention times measured were small for both within-day and between-day. The recovery of the spiked quassinoids in *E. longifolia* samples and their detection limits at 8.5 times signal to noise ratio were 99.75-109.13% and 0.01 µg/mL (-1), respectively (Tong et al., 2015).

2.8 Guidelines of NPRA for The Labelling of Herbal Medicinal Capsule

According to NPRA, the minimum requirements that must be followed to register the health supplement or herbal capsule product are microbial load test, heavy metal and should be with the GMP certificate. In the labelling, the general guidelines should include a product name, dosage form, name of active substances, manufacturing date, expiry date, batch number, storage condition, logo, specific information, country registration number, route of administration, name and address of manufacturer and etc. to make the standardize labelling for the herbal capsule. The purpose of labelling is to differ category of product based on its function. In addition, all labels and package inserts must be in Bahasa Malaysia or English and in the same size of fonts as a rule of labelling. (Pharmaceutical et al., 1915)

Table 2.8.1 Some of the major parameters required in labelling

No.	Parameters
1	Product name
2	Dosage form
3	Name of active substances
4	Strength of active ingredient in weight

5	Expiry date
6	Manufacturing date
7	Batch number
8	Dosage
9	Storage condition
10	Registration number (MAL)
11	Name & address of product registration holder
12	Name & address of manufacture
13	Warning label
14	Contraindication/ precaution

Source: Adapted from Drug Registration Guidance Document (DRGD), National Pharmaceutical Regulation Agency (NPRA), Ministry of Health Malaysia

2.9 Quality Control Test Accordance to NPRA Guidelines

According to the NPRA, for the development of herbal medicine must be followed the quality control test; included the heavy metal test, test for uniformity of weight and microbial load test. The testing was the important to ensure the safety of the product. The limit and guidelines for each testing can be refer to Drug Registration Guidance Document (DRGD) (Pharmaceutical et al., 1915).

2.9.1 Heavy Metal Test

Table 2.9.1.1 Limit for heavy metals

Lead	NMT 10.0 mg/kg or 10.0 mg/L (10.0 ppm)
Arsenic	NMT 5.0 mg/kg or 5.0 mg/L (5.0 ppm)
Mercury	NMT 0.5 mg/kg or 0.5 mg/L (0.5 ppm)
Cadmium	NMT 0.3 mg/kg or 0.3 mg/L (0.3 ppm)

*NMT: Not More Than Source: Drug Registration Guidance Document (DRGD)

2.9.2 Test for Uniformity of Weight

To ensure the consistency of dosage units, each unit in a batch should have a drug substance content within a narrow range around the label claim (Katori, Aoyagi, & Kojima, 2001). Dosage units are defined as dosage forms containing a single dose or a part of a dose of drug substance in each unit. The term "uniformity of dosage unit" is defined as the degree of uniformity in the amount of the drug substance among dosage units. Individual weight of the capsule to be within the limit of 90 % - 110 % of the average weight.

Table 2.9.2.1 The deviation of individual net weight should not exceed the limit given

Average net weight of capsule	**Deviation (%)**	**Number of capsule**
Less than 300 mg	± 10.0	Minimum 18
	± 20.0	Maximum 2
300 mg and more	± 7.5	Minimum 18
	± 15.0	Maximum 2

2.9.3 Microbial Load Test

Herbal medicine products containing, for example, extracts and/or herbal drugs, with or without excipients, where the method of processing or, where appropriate, in the case of herbal drugs, of pre-treatment reduces the levels of organism to below stated for this category.

Table 2.9.3.1 Limit for microbial load

Microbiological control	Acceptance criteria
TAMC	NMT 5×10^4 CFU/g or CFU/mL
TYMC	NMT 5×10^2 CFU/g or CFU/mL
Bile-tolerant gram-negative bacteria	NMT 1×10^2 CFU/g or CFU/mL
Escherichia coli	Absence (1 g or 1 mL)
Salmonella	Absence (25 g or 25 mL)
Staphylococcus aureus	Absence (1 g or 1 mL)

*TAMC: Total Aerobic Microbial Count

*TYMC: Total Yeast and Mould Count

CHAPTER 3

METHODOLOGY

3.1 Collection of Tongkat Ali roots

The Tongkat Ali roots were purchased from the indigenous people in accordance to the LOI. A simple test was performed to confirm Tongkat Ali obtained from indigenous people was genuine and not fake. Briefly the shaved chip from the Tongkat Ali root was applied onto the tongue area which known to be sensitive for bitterness. The quassinoid contain in *E.longifolia* contributed the bitter taste of Tongkat Ali (Guo, Vangapandu, Sindelar, Walker, & Sindelar, 2005). Apart from *E.longifolia*, two other types of Tongkat Ali plants which are Tongkat Ali Merah and Tongkat Ali Hitam were be obtained.

The Tongkat Ali roots were cut into small size by using the saw. Aluminium foil were used as container to hold the Tongkat Ali root during drying process. Oven was used with a range of temperature between 35 to 45 °C. Next, electric grinder was used to crush the powder into granules form. Finally, a sieve size 50 Mesh which equal to 297 microns was used to standardize the size of the root powder particle size by sieving the powder form of Tongkat Ali root through it.

3.2 Registration to NPRA by TPM Biotech Sdn Bhd

The steps to register the product to NPRA for the MAL number are as shown below:

- Setting up of new company with SSM (company name: Industrial Biotech Enterprise)
- Appointment of contract manufacturing and authorization for handling regulatory submission (appointed TPM Biotech Sdn Bhd).
- TPM Biotech Sdn Bhd advised to Industrial Biotech Enterprise on step-by-step procedures of NPRA included:
    - Purchase and transfer of NPCB Quest 3 – USB security token
    - Design and label making in accordance to NPRA regulations for herbal capsules

- Testing for heavy metal and microbial load on three types of Tongkat Ali delivered to TPM Biotech Sdn Bhd.
- Answering to queries imposed by NPRA
- Waiting period of 6 months to 1 year before approval for MAL number

3.3 Characterizing the protein in Tongkat Ali plants

3.3.1 Extraction of Tongkat Ali roots

The 50 g of each Tongkat Ali Putih powder were put into 500 mL deionized water. The powdered materials were boiled under reflux for five hours, followed by filtration with Whatman No1 filter paper and the process of freeze drying.

3.3.2 Protein Quantification (Bradford Assay)

3.3.2.1 Materials

The Coomassie brilliant blue G-250 (Biorad,US), Ethanol (Merck, US), Phosphoric acid (Merck, US), deionized water, and Bovine Serum Albumin (BSA) (Thermo Scientific, UK) were obtained from Lab Technician in FIST Laboratory.

3.3.2.2 Preparation of Bradford reagent

The 100mg Coomassie Brilliant Blue G-250 was dissolved in 50ml of 95% ethanol and 100ml of 85% (w/v) phosphoric acid added. Once the dye completely dissolved, it was diluted to 1 L with deionized water. This Bradford reagent was filtered through Whatman no 1 filter paper.

3.3.2.3 Preparation of samples and standards

A total of two mg of sample extract were dissolved in 1 mL deionized water and the 100 µL of sample solution then was mixed with 1 mL of Bradford reagent and mixed well. Incubation was done where the sample solution incubated in the dark at room temperature for 20 minutes. The absorbance reading was read at 595 nm by using UV spectrophotometer. The 2 mg/mL stock

solution of Bovine Serum Albumin (BSA) was diluted in order to produce five different concentrations of BSA which were 10, 8, 6, 4, and 2 µg/mL. The protein content of Tongkat Ali roots was determined by standard curve of the BSA.

3.3.3 SDS PAGE Gel Electrophoresis

Electrophoresis was carried out using a mini vertical slab gel system, with 1 mm thickness (stacking gel 8% and resolving gel 15%). The 50mg of dithiothreitol (DTT) was added into the 1 mL sample buffer. Then, 20µg of protein sample was dissolved in 200µL sample buffer. The 5µL of the sample solution was pipetted into the sample well together with bromophenol blue which acted as a tracking dye. Low molecular weight marker also was pipetted into one of the wells in the gel which acted as a reference. Figure 3.8 below shows the molecular weight of protein marker. For 1 hour and a half, the electrophoresis was run at 120V voltage. Protein bands were detected using Coomassie Brilliant Blue Staining after the migration completed. The gel was photographed and stored in 10% acetic acid solution. Coomassie brilliant blue G-250 powder was dissolved in 10%, 2L of acetic acid solution. Then, the solution was filtered using Whatman filter paper no. 1. The heating process of staining solution was done until boiling prior to putting the gel in and the gel was put in staining solution for about 10 minutes with gentle shaking. Repeated destaining steps were carried out until the gel turned into transparent colour where the 10% of acetic acid used for destaining step.

3.4 *In vivo* efficacy testing in Fowls

3.4.1 Capsule preparations

Once quality and quantification has been verified, conventional basic capsule marker was used to formulate the capsule. The capsule was formulated from the raw material as in powder form without the other additional compounds or excipient. Additionally, in the current study, other than the capsule containing Tongkat Ali Putih (*Eurycoma longifolia)* only, another two type of capsule to be included. The capsules are Tongkat Ali Hitam and Tongkat Ali Merah. The capacity of the capsule used is 300mg. However, the dosage of Tongkat Ali being used is only 12mg

(calculate based on the weight of chicken). The capacity left being fill with the corn bran which is the food for the chicken.

3.4.2 Selecting the Animal and Preparations

The animal that was be used in this study is roosters. The reason to use chicken as our domestic animal is due to the size. Chicken have a small size compared to cow, goat, horse and others. The dosage of Tongkat Ali capsule to be used also in a small quantity. If cow or horse to be used in this research, a lot of Tongkat Ali needed to produce the capsule. Besides that, chicken easier to handle and taken care compared to other domestic animal. Mice also preferable to be used in this research as it can fulfill all the requirement. However, mice are very common and many others researchers already used mice in their study for Tongkat Ali Putih. Due to this, chicken had been selected to be used as no research about aphrodisiac supplement have been done on this animal for Tongkat Ali.

A total of 16 roosters was used which is 4 for each 4 groups (control, TA putih, TA merah and TA hitam). All the roosters selected are aged 26 - 28 weeks. This is because the fertility peaks for roosters is 30 and 40 weeks of age. (So, after a month the rooster age will reach 30 weeks. Hence expected if the Tongkat Ali capsules able to increase testosterone levels in roosters, rapid maturity achieved due to this secondary sexual hormone effects.

All the food and water needed for the roosters was given without any disruption. The different is that the tested group will be fed with Tongkat Ali capsules while the control group will consume a normal diet throughout 30 days. All procedures related to the use of chicken will be in accordance to the approved animal ethics, UMPIACUC/2018/01.

3.4.3 Dosage Method

Gastric gavage, pilling (capsules) and powdered diet are the most commonly used equipment required for oral dosing. While for voluntary consumption, the equipment used are liquid, water-soluble substances and aqueous solution bottles affixed to the caging. Capsules and pills can be given to animal larger than 150g by means of pill or balling guns. This help to record

the amount of medicated diet eaten, timing and duration of feeding sessions (Turner, Pekow, Vasbinder and Brabb, 2011).

The capsule can be put into the distal esophagus or stomach of the animals. An alternative is orogastric dosing which is the capsule placed into the tip of hollow tubing and pass the tubing. Once the tube is in place, a syringe is used to push a bolus of air into the tubing to dislodge the pill. An oil lubricant such as vegetable oil is preferred over water-based lubricants if used a gelatin capsule. This is because the water-based lubricant may adversely affect the integrity of the capsule. As the pill blocks the tube lumen, the best way to confirm correct tube placement prior to dispensing the pill is by palpation (Turner *et al.*, 2011).

Although known method of dosing in chicken are injection, spray, intraocular (eye drop) or nasal drop and treatment in feed, this study choose to deliver the capsules orally to unanesthesized animal. The animals will be given two capsules per day over a period of 30 days continuously. All other conditions were maintained ad libitum.

3.4.4 Sexual Mating Behavior Observations

A series of movement made by the roosters to court a hen apart from crowing frequently. The rooster will start to drop their wing and "dance" around the hen. The hen lowers her back if receptive. Then, the rooster mounts and grabs her by the back of the head. Balancing himself by flapping his wings, he lowers and moves his tail alongside the hen's tail and their cloacas meet. Around 100 million and 5 billion sperm where deposits into her vagina. The amount of sperm ejaculate decreases as the rooster continues his daily breeding activities (Jane Meggitt, 2018).

There a few indicators that will be used as parameters in the sexual behavior observation. Wing flapping, dance and crows are the indicators that will be observed throughout the research. The rooster will be placed with the hen every week to observe any sign of mating behavior. Weight also been included in the parameter. The rooster weight was measured at initial, 15 and 30 days to gauge any changes. Increase in muscle mass expected in roosters with elevated testosterone.

ROOSTER X	WEIGHT
Initial	
After 15 Days	
After 30 Days	

Note :

*Control roosters are those without Tongkat Ali capsules treatment

*Test roosters are those with Tongkat Ali capsules treatment

In the observation, each rooster individually exposed to healthy hens (aged approximately 30 weeks – adult period) for a duration of 25 minutes after the 30 days treatment.

BEHAVIOUR		FREQUENCY									
		1	2	3	4	5	6	7	8	9	10
Wing Flapping	Control										
	Test										
Dance around Hen	Control										
	Test										
Crows	Control										
	Test										
Pecking	Control										
	Test										

Description on the behaviours:

Wing Flapping: the chicken flapping their wing with the head held high and the chest puffed out

Dance around Hen: the chicken drop their wing and "dance" around the hen in circle, with the dropped wing held inside

Crows: noises that the chicken make to attract the hen

Pecking: the chicken will bite the hen by the back of the neck with its beak

*Source adapted from http://www.yourchickens.co.uk/care-and-advice/chicken-body-language-14494696

3.4.5 Blood Test

At the end of 30 days of treatment with Tongkat Ali capsules and after evaluating the roosters' sexual mating behavior, the blood was drawn from control and test subjects. A volume of 5 ml of blood was collected from the rooster by slaughtering. The blood that draw out after slaughtering was collected by using the beaker. A syringe was used to draw the blood collected in the beaker and then transferred into a vacutainer (obtained from Gribbles Pathology Diagnostic Laboratory). The blood samples collected from this research was send to a Diagnostic Laboratory in Kuantan, Pahang to be tested. The blood was tested on testosterone, ALT, AST and GGT.

3.4.6 Histology of Chicken Liver and Testis

Tissues like liver and testis were dissected carefully and placed into 5ml of 10% formalin overnight. The tissue was sliced, and processed through graded xylene, alcohol and then embedded in paraffin wax blocks. Parafin sections were taken at 5 microns using a Leica microtome on glass slides and were stained with hematoxylin and eosin. The prepared slides were analyzed under a light microscope (*Nikon, Eclipse TS100*) for inflammation, congestion, and grading for all the groups. This steps were performed by pathologist at Perdana University at Serdang.

Figure 3.1: Rooster's testis and liver to be tested

The tissues were fixed in 10% neutral buffered formalin and then processed in a Leica tissue processor through timed intervals of xylene, alcohol and brought to paraffin wax. A suitable sized mould was selected to fit the tissue. The mould was then partially filled with molten wax and transferred to a hotplate. The sample was placed in the mould and orientated till desired position was achieved after which the mould was transferred to a small coldplate to allow the wax to solidify checking that the desired orientation had been preserved. The cassette base was placed on top of the mould and more molten wax was added to fill the cassette. After that, the mould was transferred to a large chiller plate and left to cool. When the wax block had solidified, it was removed from the mould.

Excess wax was cleaned off the wax block with a scalpel leaving at least 2 mm all around the sample. The block was then left in the fridge until they were to be sectioned. Blocks were sectioned at 3-4 mm. Sections were removed from the microtome knife and transferred to a glass slide flooded with 30% alcohol. Sections were then placed onto a labeled frosted glass slide and drained of excess water after which they were placed directly on a hotplate at 30-35 °C. The slides were then left to dry for 5 minutes before proceeding to stain them.

Firstly, wax was removed in two changes of xylene at 1 minute each. Next, xylene was removed in 3 changes of alcohol at 30 seconds each. Sections were then rendered aqueous by immersion in tap water after which the slides were stained in Haematoxylin solution for 4-8 minutes. After staining, slides were rinsed in tap water. Consequently, the slides were differentiated in acid-alcohol for 2-3 seconds then rinsed with tap water. This was followed by dipping slides in bicarbonate solution to 'blue' them. They were then rinsed with tap water again. This was followed by 2 changes of 95% alcohol then stained in Eosin for 2 minutes. After staining, slides were rinsed with 2 changes of 95% alcohol, 30 seconds each then they were rinsed with 2 changes of absolute alcohol, 30 seconds each. Alcohol was removed with 2 changes of xylene at 30 seconds each. Slides were then kept in clearance until they were ready to be cover slipped (Haleagrahara et all., 2010).

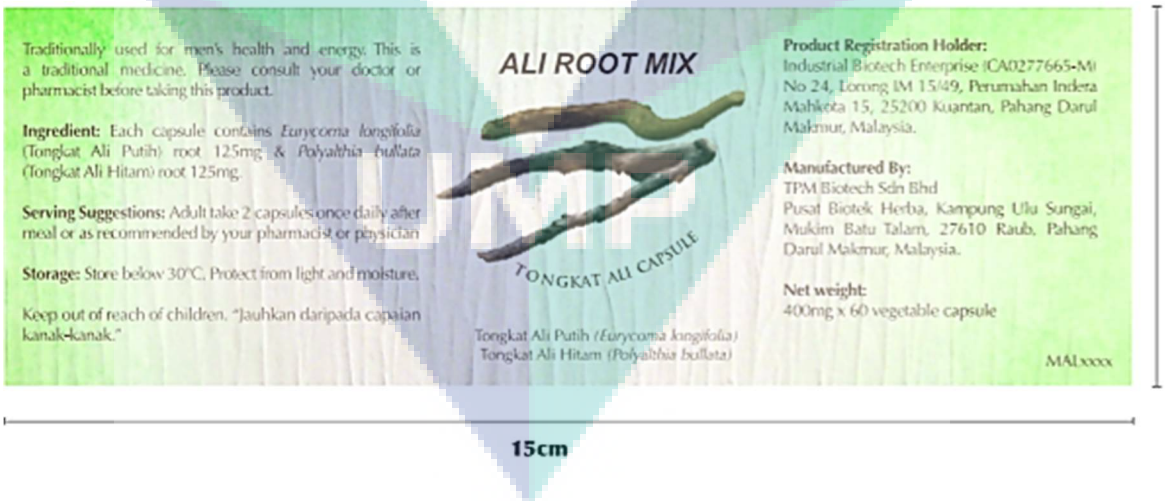
CHAPTER 4

RESULT AND DISCUSSION

4.1 Product registration

The indigenous people via their spokesperson, Madam Aida bt Yun, been encouraging in providing knowledge in the development of the Tongkat Ali product. The use of the three Tongkat Ali instead of Ubi Jaga was justified well based on information provided by the indigenous people on the price per kilogram being too expensive (RM400-600/kg). Additionally, Ubi Jaga is not an easy plant to come by in the wild. Such vital information ensured the choice of aphrodisiac plant to be the three Tongkat Ali plants and as mixtures.

The contract manufacturer, TPM Biotech Sdn Bhd, provided step-by-step guidance in the registration of the product. The label design was done by an independent hired designer. After several drafts been consulted to be changed by TPM Biotech Sdn Bhd the final version was completed as shown:



As for the testing results, TPM Biotech Sdn Bhd personal confirmed the outcome were acceptable for the heavy metal detection and microbial load by NPRA. However, NPRA was reluctant to accept the use of three types of Tongkat Ali because one of it i.e. Tongkat Ali merah was not in their database of herbal plants registered previously for MAL approval. The product

Ali Root Mix was decided to be mixture of Tongkat Ali Putih and Tongkat Ali Hitam to avoid further complication in registration.

To date the registration is at its final stages before decision made on the approval of MAL number. All medicinal products in Malaysia, including imported medicines, must be registered with the Ministry of Health Malaysia (MOH) before they can be sold or marketed to consumers. Registered medicines must carry both registration numbers (i.e. MAL20125467T) and Meditag Hologram labels on its label or packaging. A valid registration number begins with "MAL", followed by 8-digit numbers and ends with an alphabet to indicate their registration category. The categories of registered product are as follows:

A – Controlled medicines
X – Over the counter medicines (OTC)
T – Traditional medicines
N – Supplements

Consumers can verify the registration number and view other information related to the medicine at www.pharmacy.gov.my. Any medicine not listed on the website or does not have any of these features can be doubted.

Upon approval of MAL numbers for the product effort to be done for the production of its first batch. Such an effort requires further monetary investments estimated to be in the region of RM10 to RM15 for each bottles containing 60 capsules of the herbal mixture. This estimate is considered high due to the need to rely on the same contract manufacturer stated within the label. NPRA specified in their regulation the capsulation is to be done in a Good Manufacturing Practice (GMP) establishment and serially numbered hologram for both registration numbers i.e. MAL20125467T and Meditag Hologram labels is controlled by the contract manufacturer rather than by the product registration holder. In doing so the product registration holder will only provide the active herbal material to the contract manufacturer for the capsulation, bottling and labelling with sticker of the regulatory numbers. Due to this overhead in manufacturing costs the product is likely to be sold between RM60 to RM80 for best returns.

4.2 Protein in Tongkat Ali plants

4.2.1 Water Extraction of Tongkat Ali root

The water extraction of Tongkat Ali root was carried out in laboratory scale where the ground root of Tongkat Ali was boiled under reflux for 5 hours. The filtered root extract was freeze-dried to remove all moisture in order to get crude extract powder. The water extraction method was used for extraction of Tongkat Ali root because it could mimic the traditional preparation method of Tongkat Ali root.

According to the previous study from Bhat and Karim (2010), they reported that most of Tongkat Ali products in the market are available in the form of freeze-dried extract instead of chipped roots. This water extraction also could get rid of plant pigments, phenolic compound, and lipids which might contaminate the extract. Water extraction produces low yield but higher protein content compared to other precipitation methods of extraction. The higher protein content in water extraction produced a higher number of protein bands and better resolution of protein bands on polyacrylamide gel.

4.2.2 Analysis of Tongkat Ali Root Extract

4.2.1 Bradford Assay

The protein analysis of Tongkat Ali root extract and analytical method of gel electrophoresis will be discussed in this section. The protein content in the extract will be quantified by using Bradford Assay. Bradford assay is a rapid and accurate method for protein quantification due to its high sensitivity, faster and simpler which become the method of choice in many laboratories for protein analysis (Kruger, 2002).

In this protein analysis, Bovine Serum Albumin (BSA) was used which acted as a reference protein standard. The serial dilution and UV absorbance of BSA was prepared as in Table 4.1. Based on the standard calibration curve of BSA obtained (Figure 4.1), the protein content in Tongkat Ali root extract was measured which can be observed in Table 4.2.

Table 4.1: UV absorbance of BSA standard curve (595 nm)

BSA concentration (µg/mL)	Absorbance (AU)
0	0
2	0.119
4	0.232
6	0.333
8	0.416
10	0.491

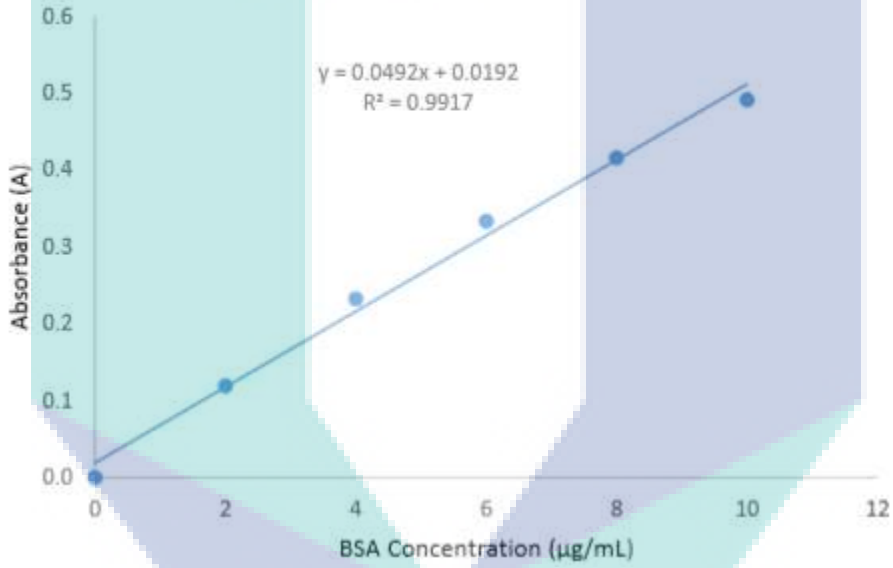


Figure 4.1: The standard curve of BSA by Bradford Assay for protein analysis in Tongkat Ali root extract.

Table 4.2: Protein content of Tongkat Ali root extracts

Sample	Absorbance (UV 595 nm)	Protein content (µg/mL)	Protein percent % (w/w)
Tongkat Ali Putih	0.079	12.16	0.61
Tongkat Ali Hitam	0.184	33.50	1.68
Tongkat Ali Merah	0.217	40.20	2.01

Based on Table 4.2, Tongkat Ali Merah has the highest protein content in the root water extract followed by Tongkat Ali Hitam and Tongkat Ali Putih as 0.04020 (0.61 w/w % of extract),

0.03350 (1.68 w/w % of extract) and 0.01216 (2.01 w/w % of extract) mg protein in 1 mL protein solution, respectively. This result is slightly different from previous studies by Aini et al. (2015), which the result obtained from their studies is Tongkat Ali Hitam has the highest protein content among all three types of plants.

4.2.2 SDS PAGE Analysis

This section is on the proteomic analysis within Tongkat Ali root extracts. In this current studies, SDS PAGE was performed to study the presence of protein molecule in the Tongkat Ali root water extracts especially in Tongkat Ali Merah and Tongkat Ali Hitam root extracts as these two plants do not have many scientific studies on its protein presences. This method is one-dimensional gel electrophoresis technique as it is a simple technique where the protein is separated based on its molecular mass (Weber et al, 1972).

The analytical result of SDS PAGE obtained below (Figure 4.2) showed the protein band was observed in Tongkat Ali Putih (*E. longifolia*) root water extracts which only one protein band appeared. Theoretically, Tongkat Ali root water extract should have the same protein band in protein profiling which can be done by SDS PAGE analysis. Based on AmershamTM ECLTM RainbowTM Marker-Low Range, the thick protein band that appeared on the gel was estimated to have molecular mass below 3.5kDa. The lane that labelled as TAP1-TAP5 were the samples of Tongkat Ali Putih with different concentration which the showed different intensity of protein band on the gel. In the previous study by Sambandan et al, (2006), a bioactive peptide with molecular weight 4.3kDa was found.

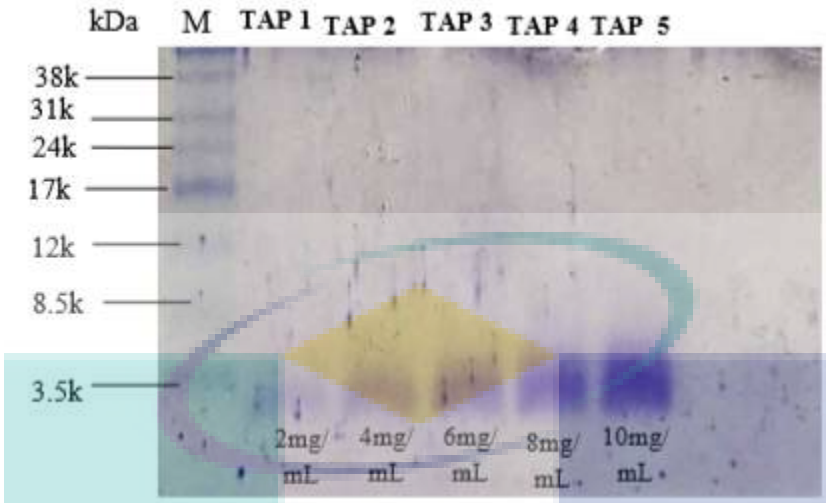


Figure 4.2: The gel of SDS PAGE analysis of low molecular weight marker (M) and Tongkat Ali Putih freeze dried extract (TAP)

It can be seen in Figure 4.3, the crude sample of Tongkat Ali Merah root water extract showed multiple protein bands at the region of higher molecular weight on the gel as this plant was higher in protein content as proved by Bradford assay analysis. The multiple bands appeared on the gel also could be due to some reasons as the protein in the extract was not purified and extracted which it may contain some non-protein constituent that will affect the appearance of protein band on the gel.

Figure 4.3: The low molecular weight marker (M) and protein bands of Tongkat Ali Merah root extract (TAM)

As we can see in Figure 4.4, the protein band that was observed on the gel were smeared which the molecular size of protein that presents in the root extract cannot be estimated properly. This result happened due to some factors that may contribute to the smear effect of the protein band. Overloading of the protein is one of the reasons that caused smeared band on the gel which the capacity of mini-gel can load protein sample commonly 20-40 microgram per well. The presence of contaminant such as lipid also could lead to smeared protein band (Mayer, 2018). In this case, it is possible the contaminants due to the non-protein or micro-constituents that present in TAH. So, clarity of the sample is needed in order to remove the contaminants and to get the better result for protein analysis. In future, troubleshooting is needed for analysis of protein profile of Tongkat Ali Merah so that the molecular weight of protein band on the gel can be measured.

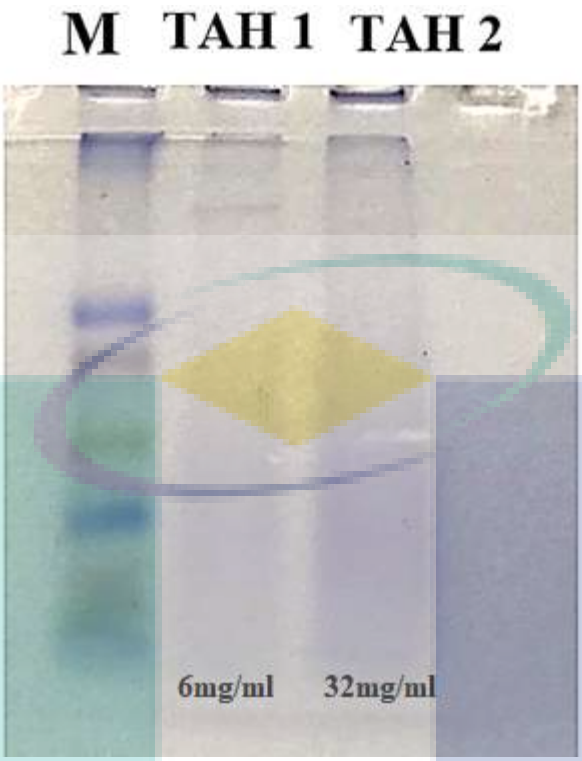


Figure 4.4: The gel of low molecular weight marker (M) and protein band of Tongkat Ali Hitam root extract (TAH)

From this SDS PAGE analysis for all three types of different plants of Tongkat Ali root water extract, it can be concluded that only Tongkat Ali Putih (*E. longifolia*) have one protein band in SDS PAGE gel which the protein band is estimated to have below 3.5 kDa in size. While, Tongkat Ali Merah and Tongkat Ali Hitam root extract showed the absence of single protein band on the SDS PAGE gel. This SDS PAGE analysis also proved that proteins are present in all three types of Tongkat Ali plants even though one of them, i.e Tongkat Ali Merah do not have enough information regarding their protein profile. SDS PAGE is only one-dimensional gel electrophoresis, so two dimensional gel electrophoresis (2-DE) can be carried out for further characterization of the protein profile in future since 2-DE cannot be carried out in this current study because there are some limitations regarding the time and cost needed.

4.3 Efficacy testing in Fowls

4.3.1 Weight of the Chicken

Table 4.3: The weight of the chicken throughout 30 days

Chicken Treatment	Initial Weight(Kg)	Gain (Kg)		End Weight(Kg)
		Day 15	Day 30	
Control 1	1.9	+0.1	+0	2.0
Control 2	1.8	+0	+0	1.8
Control 3	2.3	+0.2	+0	2.5
Control 4	2.1	+0	+0	2.1
Putih 1	1.6	+0.05	+0.15	1.8
Putih 2	1.2	+0	+0.5	1.7
Putih 3	1.4	+0	+0	1.4
Putih 4	1.2	+0	+0	1.2
Merah 1	1.65	+0.05	+0	1.7
Merah 2	1.7	+0.05	+0	1.75
Merah 3	1.35	+0.05	+0	1.4
Merah 4	1.7	+0	+0	1.7
Hitam 1	1.7	+0	+0.05	1.75
Hitam 2	1.5	+0	+0	1.5
Hitam 3	1.4	+0	+0	1.4
Hitam 4	1.3	+0	+0	1.3

According to Gheorghe *et al*., (2013), the body weight of chicken was influenced by the dietary treatment. Due to this, all the weight of the chicken was measured throughout the experiment. There is an increase of weight of chicken from day 0 to day 15. A total of 6 out of 16 of the chicken show an increase of their weight and with 0.2 kg gain as the highest weight increase recorded. While from day 15 to 30, only 3 chicken show an increase of the weight with 0.5 kg is the highest weight increase recorded. A total of 8 chicken show an increase of the weight from day 0 to day 30.

As expected, there is a weight increase occur throughout the experiment. However, the amount of weight increase is very little and does not indicate that after consuming Tongkat Ali, the weight will increase. There is also no drastic decrease or fall of weight throughout the experiment for both either control or test group. This showed that consuming Tongkat Ali does not really affect and change the weight of the chicken. In addition, the duration of the experiment was only 30 days and hence short to determine if there is significant weight change when consuming Tongkat Ali.

The main purpose to record the weight of the chicken is to evaluate benefit of consuming Tongkat Ali on the mass of chicken. However, it is apparent such effect not found under observation at day 15 or 30. The other purpose of observing the weight is to ensure none of the chicken experienced sudden or drastic loss of weight which in turn indicated a detrimental effect to the chicken and therefore requiring it to be removed from the experiment altogether. This is in accordance to the IACUC ethical approval.

4.3.2 Sexual Mating Behavior Observations

Table 4.4: The mean of sexual behaviour frequency for all chicken with different treatment

Activity	Mean of Frequency			
	Control	Putih	Merah	Hitam
Wing Flappping	4	6	5.5	5
Dance around Hen	4.25	7.25	5.5	5.25
Crows	3.5	6	5.25	5.5
Pecking	3	7	4.5	5

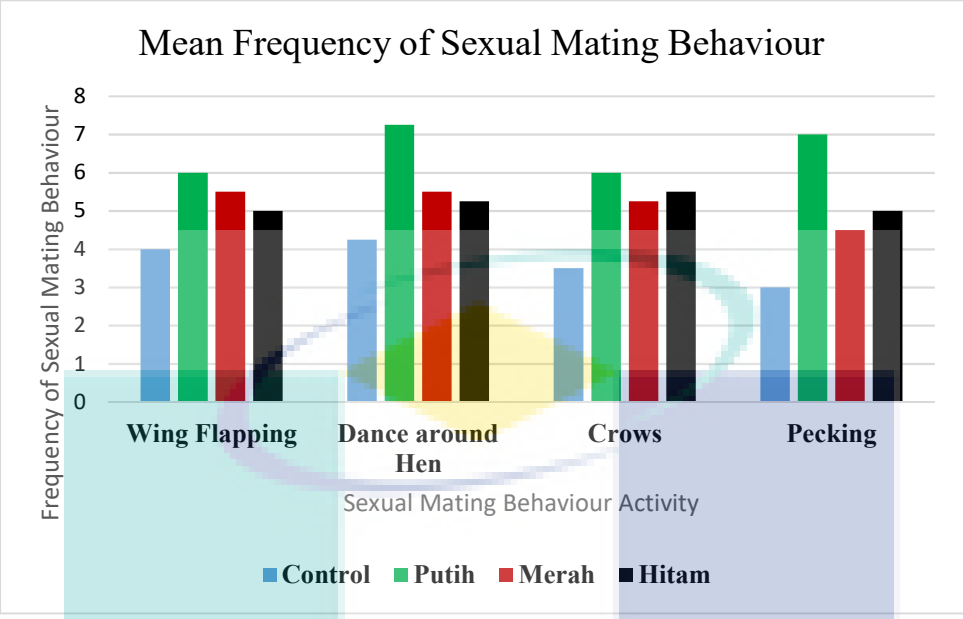


Figure 4.5: Mean Frequency of Sexual Mating Behaviour

From the table and bar graph, we can see that the control had the lowest mean of frequency for all the sexual mating activity between the range from 3 to 4.25. The highest mean of frequency for all the sexual mating activity are the chicken that consumed TA Putih with the range from 6 to 7.25. While for chicken that consumed TA Merah and Hitam had almost the same mean of frequency for all the sexual mating activity between the range from 4.5 to 5.5.

Control and Tongkat Ali Putih

Sexual Behaviour Frequency	Mean of Sexual Frequency Observed	
	Control	Putih
Wing Flapping	4	6
Dance around Hen	4.25	7.25
Crows	3.5	6
Peaking	3	7

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	14.75	3.6875	0.307292
Putih	4	26.25	6.5625	0.432292

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	16.53125	1	16.53125	44.70423	0.000543	5.987378
Within Groups	2.21875	6	0.369792			
Total	18.75	7				

Since (f_{test}=44.70)> ($f_{critical}$=5.99), H_0 is rejected

At α = 0.5, at least one mean of sexual behaviour frequency is different

H_0: The mean of sexual behaviour frequency are not different from 2 treatments

H_1: The mean of sexual behaviour frequency are different from 2 treatments

Control and Tongkat Ali Merah

Sexual Behaviour Frequency	Mean of Sexual Frequency Observed	
	Control	Merah
Wing Flapping	4	5.5
Dance around Hen	4.25	5.5
Crows	3.5	5.25
Peaking	3	4.5

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	14.75	3.6875	0.307292
Merah	4	20.75	5.1875	0.223958

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.5	1	4.5	16.94118	0.006244	5.987378
Within Groups	1.59375	6	0.265625			
Total	6.09375	7				

Since (f_{test}=16.94)> ($f_{critical}$=5.99), H_0 is rejected

At $\alpha = 0.5$, at least one mean of sexual behaviour frequency is different

H_0: The mean of sexual behaviour frequency are not different from 2 treatments

H_1: The mean of sexual behaviour frequency are different from 2 treatments

Control and Tongkat Ali Hitam

Sexual Behaviour Frequency	Mean of Sexual Frequency Observed	
	Control	Hitam
Wing Flapping	4	5
Dance around Hen	4.25	5.25
Crows	3.5	5.5
Peaking	3	5

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Control	4	14.75	3.6875	0.307292
Hitam	4	20.75	5.1875	0.057292

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	4.5	1	4.5	24.68571	0.002531	5.987378
Within Groups	1.09375	6	0.182292			
Total	5.59375	7				

Since (f_{test}=24.69)> ($f_{critical}$=5.99), H_0 is rejected

At $\alpha = 0.5$, at least one mean of sexual behaviour frequency is different

4.3.3 Testosterone Level

Table 4.5: The mean of testosterone level for all chicken with different treatment

Chicken	Testosterone Level (nmol/L)			
	Control	Putih	Merah	Hitam
1	4.8	7.1	5.6	8.2
2	4.7	8.1	5.7	1.9
3	3	7.3	6.9	1.9
4	3.8	8.3	6.8	x
Number	4	4	4	3
Mean ± SD	4.08 ± 0.85	7.7 ± 0.59	6.25 ± 0.70	4 ± 3.64

Note "x" denoting chicken with values not used for calculating mean

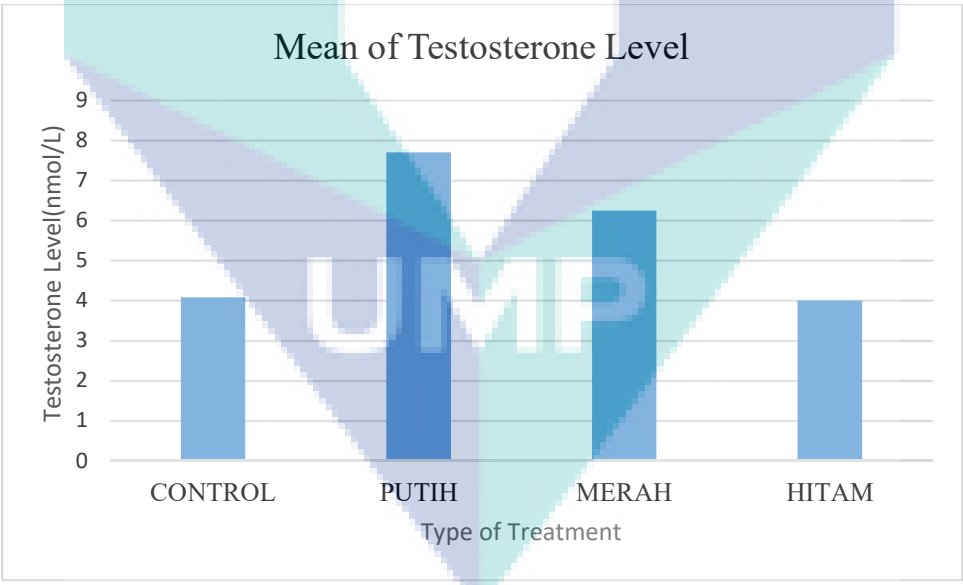


Figure 4.6: Mean of Testosterone Level

The *E. longifolia* always been popular because of its aphrodisiac property to stimulate the production or section of androgen hormones, especially testosterone (Bhat and Karim, 2010). The table and bar graph show the mean of testosterone level for each of the treatment of the chicken. Tongkat Ali Putih has the highest testosterone level with 7.7 while the second highest is Tongkat Ali Merah with 6.25. Surprisingly, the testosterone level for the normal diet (control) is almost similar to Tongkat Ali Hitam with 4.08 and 4. It is expected through this result, there is an increase of testosterone level for both Tongkat Ali Putih and Merah by comparing with the control group. However, the result of testosterone level for Tongkat Ali Hitam is not much different compared to the control group. The result was not convincing and reliable based on standard deviation (SD) value being too big of a variation. The experiment for Tongkat Ali Hitam must be repeated to come out with a conclusion either it is true or not that Tongkat Ali Hitam can really increase testosterone level. Some communications with sellers of Tongkat Ali Hitam indicated the onset of activity take a longer duration than that of 30 days. A repeat of experiment compromising duration of 60 days hence recommended.

4.3.4 ALT, AST and GGT Level

Table 4.6: Effects of different diet on biochemical parameters of liver in chicken after 30 days

Normal Diet (Control)	Number of Chicken				Mean ± SD
Plasma profile	1	2	3	4	
ALT (U/L)	8	5	12	7	8 ± 2.94
AST (U/L)	353	x	328	257	312.67 ± 49.80
GGT (U/L)	15	24	x	27	22 ± 3.51
Consume Tongkat Ali Putih	Number of Chicken				Mean ± SD
Plasma profile	1	2	3	4	
ALT (U/L)	x	28	19	x	23.5 ± 6.36
AST (U/L)	274	368	342	249	308.25 ± 55.95
GGT (U/L)	30	11	9	25	18.75 ± 10.34
Consume Tongkat Ali Merah	Number of Chicken				Mean ± SD
Plasma profile	1	2	3	4	
ALT (U/L)	6	5	5	5	5.25 ± 0.5
AST (U/L)	246	251	203	231	232.75 ± 21.58
GGT (U/L)	21	19	26	28	23.5 ± 4.20
Consume Tongkat Ali Hitam	Number of Chicken				Mean ± SD
Plasma profile	1	2	3	4	
ALT (U/L)	5	5	5	5	5 ± 0
AST (U/L)	280	305	268	357	302.5 ± 39.47
GGT (U/L)	24	20	25	19	22 ± 2.94

Note:

"x" indicates that the result adopted is out of range.

The three values observed for the biochemical parameters for ALT, AST and GGT suggested only ALT value for Tongkat Ali Putih markedly elevated in comparison to control chicken. This provided some suggestions of potential damage toward the liver over prolong use. A prolong use can only be done in chronic investigation involving duration of more than 6 months.

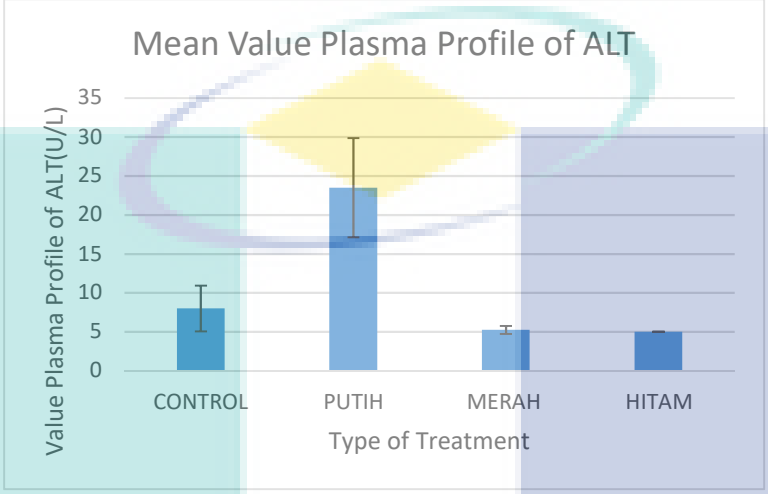


Figure 4.7: Mean Value Plasma Profile of ALT

Normal range of ALT is from 7.9 to 10.20. The bar chart show that Tongkat Ali Putih has higher value of ALT with 23.5 and much higher than control. The results Tongkat Ali Merah and Hitam for ALT are somewhat similar to control value.

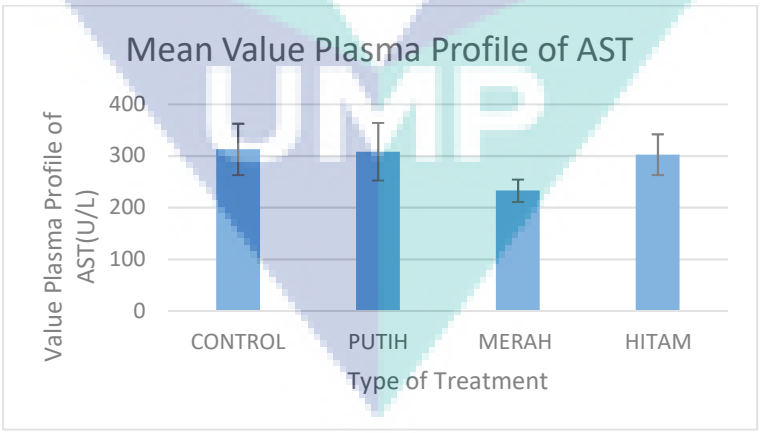


Figure 4.8: Mean Value Plasma Profile of AST

Normal range of AST is from 35.97 to 198. The bar chart show that all the AST values for the three Tongkat Ali were similar to the control value.

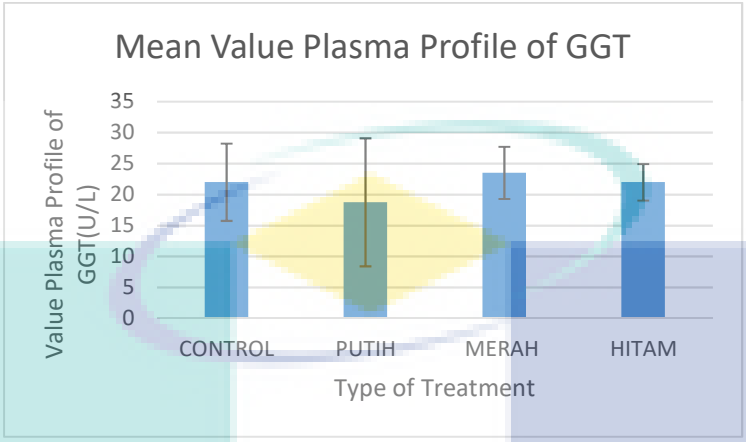


Figure 4.9: Mean Value Plasma Profile of GGT

Control value of GGT is from 19 to 22. The bar chart show that Tongkat Ali Merah has value of GGT with 23.5 and somewhat similar to control. The result of GGT are similar as well for Tongkat Ali Putih and Tongkat Ali Hitam compared to control.

Hence overall all biochemical markers for liver showed similar levels to control and with only ALT of Tongkat Ali Putih markedly elevated. Such elevation is alarming if prolong dosage of the plant.

4.3.5 Histological analysis of Liver and Testis Tissues after Treating with Tongkat Ali Putih Capsules

The prepared slides were analyzed under a light microscope (Nikon, Eclipse TS100) for inflammation, congestion, and grading for all the groups. Photomicrographs were taken by Nikon 8.1 MP camera using Nikon Eclipse software. The scoring scale used for inflammation and congestion was 0= no change, + = mild, ++ = moderate, +++ = severe.

Table 4.7: Histological Grading of Inflammation, Necrosis and Congestion

Symbol	Type of change
0	No change
+	Mild changes
++	Moderate changes
+++	Severe changes

Table 4.8: Quantitative analysis of the morphological details
(RC: Rooster control, RT: Rooster Test given dose of Tongkat Ali Putih capsules)

Group Normal	Inflammation liver	Necrosis liver	Congestion liver
RC1	-	-	-
RC2	-	-	-
RC3	-	-	-
RC4	-	-	-
RT1	-	-	-
RT2	+	-	++
RT3	+	+	++
RT4	+	-	++

There were no changes for the control rooster on their inflammation, necrosis and congestion of liver during the experiments about 30 days. But there were moderate changes on congestion liver for RT2, RT3 and RT4 which are the test roosters. Besides, there is also mild changes for inflammation of liver for tested rooster.

According to the pathologist (Perdana University), this observation is likely due to the amount of Tongkat Ali Putih in each capsule being too high and suggested to reduce for better

outcome related in improving the safety aspects. Interestingly, a similar outcome was also observed for the plasma ALT marker for Tongkat Ali Putih. The pathologist report also concluded significantly higher cellularity activity within the spermatogonia cells of the testis. This also suggesting an overdose of Tongkat Ali Putih given and hence precaution to be taken to reduce the dosage to 0.50 – 0.75 times compared to the one used in each capsule i.e. 12mg.

4.3.6 Morphological Observation of Organ under Microscope

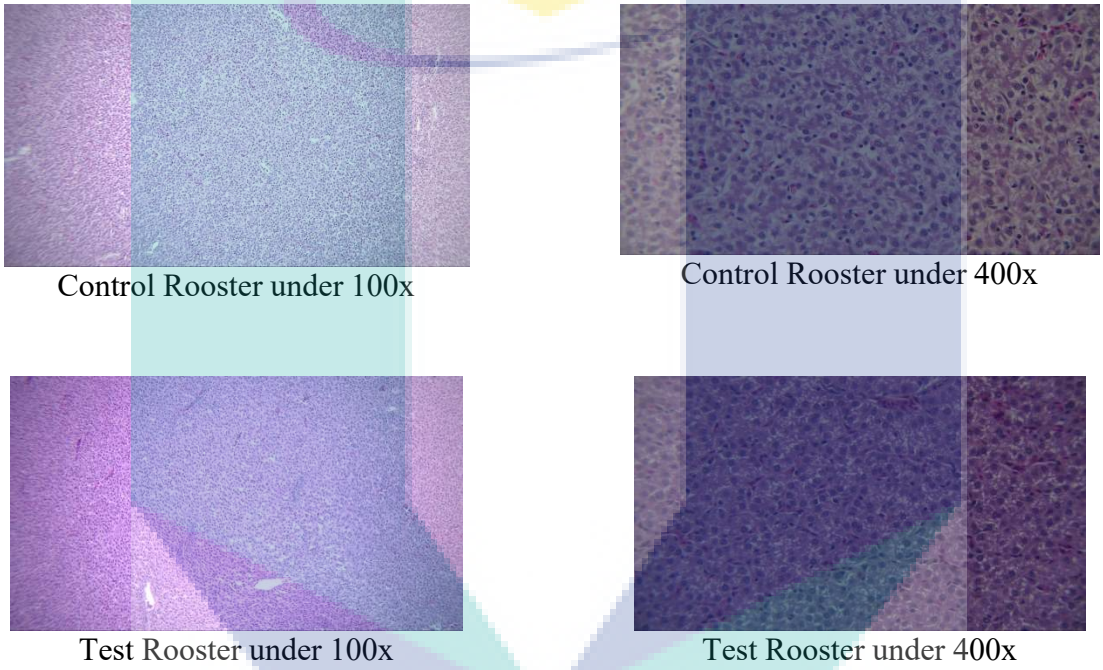


Figure 4.10: Morphological observation of liver under microscope

Observation was done on the histopathology changes in the liver in all the groups. For control rooster the liver showed normal architecture with normal central vein and well-spaced portal triads, no evidence of congestion. Hepatocytes and sinusoids appeared normal.

For test (given Tongkat Ali Putih capsules) rooster, the liver in RT 1 was of normal morphology. RT 2, 3 and 4 showed focal areas of congestion of central vein and perioral scattered inflammation, suggesting that perhaps the dosage administered was causing hepatic changes suggestive of drug metabolism, but not significant enough to cause necrosis or damage.

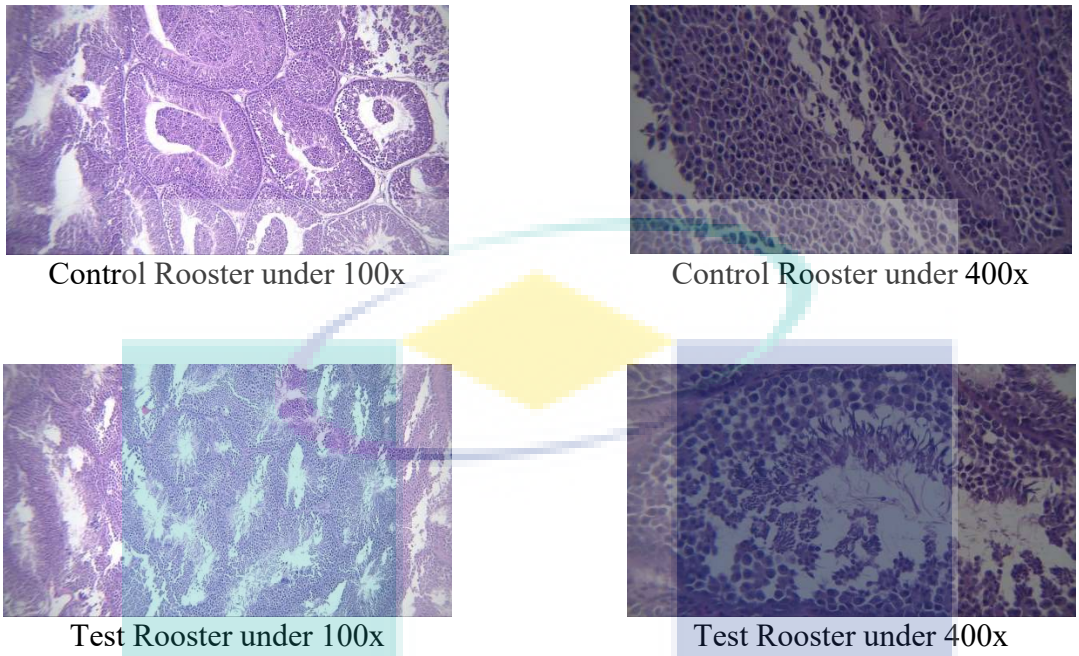


Figure 4.11: Morphological observation of testis under microscope

The testis for control rooster showed numerous seminiferous tubules, sized at approximately 150-200 microns, showing adequate cellularity of spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. There are scattered Leydig cells. No evidence of inflammation or fibrosis.

For test (given Tongkat Ali Putih capsules) rooster, the testis showed increased seminiferous tubules in all samples, sized at approximately 200-250 microns, which were larger than the control group. The tubules showed increased cellularity compared to RC group, with increase in all cells spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. The spermatogonia were prominent in the centre of the lumen with long tails and closely clustered together. No evidence of inflammation or fibrosis. This suggests that the drug administered had a direct stimulating effect on both the seminiferous tubules, and also the mitosis of the germ cells and also the maturation of the cells.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In overall the study shown the indigenous people were vital in providing information related to the choice of herbal for the aphrodisiac capsule formulation. Other than that they have been useful source for knowledge on processing and purchasing of authentic plant materials. A mutual relationship has been established between both the researchers and the spokesperson, Madam Aida in ensuring constant supply of authentic plant material in the future for production of product.

While for the purpose of attaining MAL numbers, researchers encountered numerous challenges especially in delay on the part of NPRA. It is uncertain for the reasons on the delay but was informed by contract manufacturer, TPM Biotech Sdn Bhd, it is the standard operating procedures of NPRA. Hence, the researchers have completed all pertaining steps required by NPRA with only requiring for approval. Once approved next course of action is to test the market by producing the minimum number of batch i.e. 1000 bottles.

Safety and efficacy are two important components to be studied prior to developing any herbal preparations. Although the minimal requirements of NPRA for traditional medicine stated for microbial load and heavy metal, the researchers were compelled do additional experimentations. All three plant materials provided to NPRA complied to the minimal requirements. In the additional investigations done, all three plants shown to contain protein indifferent from one to the other. Tests on animal shown as previous results, Tongkat Ali Putih able to boost testosterone and sexual behavior. Tongkat Ali Merah was also capable to do similarly but in lower level than Tongkat Ali Putih. Unfortunately, due to NPRA cautious approach on approving the inclusion of Tongkat Ali Merah, it was abandon from being mixed with the other two types of Tongkat Ali in the product development. Tongkat Ali Hitam requires repeat of its animal study due to probably insufficient duration of exposure. Lastly, Tongkat Ali Putih shown

with some safety issues that indicated the dose was too high hence suggested to be lowered accordingly. Currently the work done by this team of researchers defined under the Technology Readiness Level (TRL) is at levels 5-8 out a total of nine levels found within the TRL categorization.

5.2 Recommendation

Tongkat Ali plants are important commodity for the indigenous community. This study been useful to develop a product with the aid of a small community. It is therefore only justified this study does not stop until obtaining MAL numbers but progress further to manufacture the first batch for sales. Such an endeavor required investments from the university and will rightfully applied in the near future by the researchers of this study.

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APPENDIX
OUTPUT OF THE PROJECT

- **ISI journal:**

  CURRENT SCIENCE, VOL. 115, NO. 5, 886-894.
- **Scopus Journal:**

  JOURNAL OF BIOLOGICAL SCIENCES, VOL. 19, NO.3, 251-279.
- **Perpustakaan Wadah UMP article, Title:** Tongkat Ali: Pusaka Bumi di Persada Dunia
- **Undergraduate:**
  - Nursyazana binti Rosdi (SB14040) with thesis title: PRELIMINARY DEVELOPMENT OF TONGKAT ALI CAPSULE BY FOLLOWING THE MALAYSIA STANDARD AND NATIONAL PHARMACEUTICAL REGULATIONS AGENCY
  - NIK NUR SYAHIRAH BINTI NIK HUSAIN (SB15015) with thesis title: STUDIES ON TONGKAT ALI PROTEIN CONTENT
  - ABDUL AZIZ BIN AZHAR (SB15043) with thesis title: EFFECTS OF TONGKAT ALI HERBAL CAPSULES ON DOMESTIC ANIMALS
  - GOH NENG YAO (SB16069) with thesis title: DETERMINING THE ELEVATION OF TESTOSTERONE LEVEL BY KNOWN MALAYSIAN APHRODISIAC PLANTS IN TESTICULAR CELL LINES
  - NUR QISTINA BINTI MOHAMAD AFANDI (SB16038) with thesis title: Determining aphrodisiac and safety parameters in chicken dosed with known Malaysian aphrodisiac plants.
  - SHANGKARI NAIR A/P SIVAKUMARAN (SB16079) with thesis title: ISOLATING AND CHARACTERIZING TOTAL PROTEIN FROM KNOWN MALAYSIAN APHRODISIAC PLANTS.
- **Internship:**
  - Nursyazana binti Rosdi (IC950606065490): period of internship 5/3/2018 – 10/8/2018
- **Master students**
  - YASMIN AMIRAH BINTI CHE YAHAYA (MKT18002) with master on-going, proposal of thesis titled: DEVELOPMENT OF A PROTEIN MARKER AND ITS UTILIZATION IN DERIVING A TONGKAT ALI HERBAL PROTOTYPE PARTIALLY COMPLYING TO DRUG REGULATORY AGENCY

- **Company set up for purpose of NPRA registration:**
    - Industrial Biotech Enterprise (CA0277665-M)
- **Letter of intent**
    - Between researcher and Madam Aida binti Yun (Spoke person of the indigenous people).

Appendix: Letter of intent:

Cik Aida bt Yun
Kampung Bukit Cermin
Bendang Kering
33040, Kuala Kangsar,
Perak.

10hb Feb 2017

PER: Usaha Pengkomersialan Produk Tongkat Ali Orang Asli Kampung Bukit Cermin, Perak

Saya selaku ketua yang mencadangkan projek dengan tajuk diatas ingin mempelawa Cik Aida sebagai wakil daripada pihak Kampung Bukit Cermin dalam hal-hal urusan melibatkan saya dan pihak kampung jika projek ini mendapatkan kelulusan geran nanti. Bagi pengetahuan Cik Aida serta pihak Kampung Bukit Cermin, projek ini merupakan usaha murni kerajaan Malaysia membantu komuniti luar bandar mencapai pembangunan. Oleh yang demikian saya dan beberapa penyelidik di universiti tempatan di Malaysia ingin membantu dengan cara mengembeling kepakaran kami berkenaan Tongkat Ali untuk memasarkan akar Tongkat Ali yang tulen yang dijadikan produk dipermajukan di makmal-makmal kami dan seterusnya dipasarkan di luar negara melalui laman web.

Jika Cik Aida bersetuju dengan usaha murni ini sila tandatangan di bawah,

UBAT TRADISIONAL ORANG ASLI
DATARAN SUNGAI PERAK SEBELAH UTARA
---------- (setuju)

Aida bt Yun (Talipon: 0125542421)

Sekian terima kasih.

Yang benar,

DR JAYA VEJAYAN
Pensyarah Kanan
Fakulti Sains & Teknologi Industri
UNIVERSITI MALAYSIA PAHANG
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