EFFECTS OF *EURYCOMA LONGIFOLIA* SUPPLEMENTATION: AN EVALUATION OF CELL GROWTH, EXERCISE PERFORMANCE AND WELLBEING IN ADULT MALES

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EFFECT OF *EURYCOMA LONGIFOLIA* SUPPLEMENTATION: AN EVALUATION OF CELL GROWTH, EXERCISE PERFORMANCE AND WELLBEING IN ADULT MALES

ABSTRACT

Eurycoma longifolia is often prescribed in complementary and alternative medicine. The extract has gained a reputation for boosting sexual performance, mood, muscle mass, and strength by increasing testosterone levels in males facing low testosterone levels, stress, and lack of energy due to ageing. *E. longifolia* has demonstrated the ability to regulate testosterone production by affecting the hypothalamic-pituitary axis in animals, but this phenomenon has not been thoroughly examined in humans. Even though studies are limited, *E. longifolia* supplementation has shown improvement in exercise performance and wellbeing. This murine and human study has four primary aims: (1) to examine the cytotoxicity of *E. longifolia* on skeletal muscle cells; (2) to elucidate the effect of *E. longifolia* supplementation in terms of selected reproductive hormonal changes; (3) to compare the short-term versus long-term effects of *E. longifolia* supplementation on psychological mood state and sexual function among young participants; (4) to determine the ergogenic properties of *E. longifolia* with regard to exercise performance following supplementation among young participants.

This work's main findings show higher cytotoxicity levels in the control treatment (CON) treated with a differentiation medium compared to skeletal muscle cells (SMC) treated with *E. longifolia*. An increase in lactate dehydrogenase activity was observed from 96 to 144 hours with a 4.4, 3.8 and 3.3 (CON > 0.5 mg/ml > 1.0 mg/ml) fold increase and 4.4, 3.3 and 3.9 (CON > 1.0 mg/ml > 0.5 mg/ml) fold increase in passage 13 and 26,

respectively. While findings have shown that SMC exposure to *E. longifolia* treatment did not exhibit any toxicity, there is an increase in cell viability in *E. longifolia* treated cells.

With regard to hormonal changes, short-term supplementation of 600 mg/day showed an increase in testosterone (0.97 ng/ml, 11.1%; $p = 0.004$), while long-term supplementation showed an increase up to week 4, then a decrease (0.142 ng/ml, 2%; $p > 0.05$) at week 8. Neither short nor long-term supplementation showed any significant changes in luteinising hormone (LH) and estradiol. Findings also show that the changes in testosterone are not due to the extract influencing the hypothalamic-pituitary gland axis. In addition, the dosage did not exhibit deleterious effects on liver functions, demonstrating that supplementation *E. longifolia* for 8 weeks is safe for consumption.

When investigating duration, short-term supplementation of *E. longifolia* demonstrated an improvement in mood state domains, such as anger (5.12%) and fatigue (11.09%); long-term supplementation seemed to affect tension (8.19%) and anger (4.14%). These findings indicate that supplementation of *E. longifolia* improves certain mood state domains, indicating that this extract may be an effective remedy to improve daily mood states affected by daily stress. Meanwhile, the sexual function test was measured through self-reported data. Short-term supplementation showed improvement in the ejaculation domain (6.13%), and after long-term supplementation, the erection (25.89%) and overall satisfaction (33.33%) domains. The present study showed improvement in sexual function upon completing the *E. longifolia* supplementation programme, providing evidence of *E. longifolia* acting as an aphrodisiac, a substance that increases sexual desire, sexual attraction, sexual pleasure, or sexual behaviour.

The effect of *E. longifolia* following the 8-week supplementation showed no improvements in isokinetic strength or anaerobic power. Hence, the supplementation of *E. longifolia* alone did not exhibit any ergogenic effects among young adults, which may be due to the lack of changes in the circulating testosterone levels after 8-weeks of *E. longifolia* supplementation.

In conclusion, the cytotoxicity test demonstrated that *E. longifolia* has lower lactate dehydrogenase activity in the SMC, suggesting low toxicity levels. While current results show an increase in testosterone level after short-term supplementation, findings also provided evidence that *E. longifolia* did not influence the hypothalamic-pituitary-gonadal (HPG) axis. Supplementation of *E. longifolia* improved mood state and sexual function, providing further evidence of the use of *E. longifolia* as an adaptogen and aphrodisiac. Results also showed 600 mg of *E. longifolia* alone, the highest dose permitted at present, does not have any ergogenic effects on strength or power.

Keywords: *Eurycoma longifolia*, testosterone, mood, sexual function, strength

KESAN SUPPLEMENTASI *EURYCOMA LONGIFOLIA*: PENILAIAN PERTUMBUHAN SEL, PRESTASI LATIHAN DAN KESEJAHTERAAN DALAM KALANGAN LELAKI DEWASA

ABSTRAK

Eurycoma longifolia sering dijadikan sebagai komplementari dan ubat alternatif. Ekstrak mempunyai reputasi dalam memperbaiki prestasi seksual, mood, jisim otot, dan menambah kekuatan dengan meningkatkan paras testosteron pada individu lelaki yang menghadapi paras testosterone yang rendah, tekanan, dan kekurangan tenaga. *E. longifolia* mempunyai keupayaan untuk mengawal pengeluaran testosteron dengan mempengaruhi paksi hipotalamus pituitari aksis pada haiwan, tetapi fenomena ini belum dikajisecara teliti pada manusia. Walaupun kajian adalah terhad, suplemen *E. longifolia* menunjukkan peningkatan prestasi fizikal aktiviti dan kesejahteraan. Terdapat empat objektif utama: (1) Untuk mengkaji kesitotoksikan *E. longifolia* pada sel otot skeletal tikus; (2) Untuk mangkaji mekanisma yang mempengaruhi perubahan hormon reproduktif disebabkan oleh pengambilan *E. longifolia*; (3) Untuk membandingkan kesan jangka pendek dengan jangka panjang pengambilan suplemen *E. longifolia* terhadap mood psikologi dan fungsi seksual di kalangan individu muda; (4) Untuk mengkaji sifat ergogenik *E. longifolia* terhadap prestasi latihan di kalangan individu muda.

Penemuan utama dari penyelidikan ini menunjukkan tahap sitotoksisiti yang lebih tinggi dalam rawatan kawalan (CON) berbanding dengan sel otot rangka (SMC) yang dirawat dengan *E. longifolia*. Peningkatan aktiviti laktat dehidrogenasi (LDH) diperhatikan dari jam 96 hingga 144 dengan peningkata kali ganda sebanyak 4.4, 3.8 dan 3.3 (CON > 0.5 mg/ml> 1.0 mg/ml) dan 4.4, 3.3 dan 3.9 (CON > 1.0 mg/ml > 0.5 mg/ml) bagi *passage* 13 dan 26 masing-masing. Walaupun penemuan menunjukkan bahawa

pendedahan SMC terhadap rawatan *E. longifolia* tidak menunjukkan ketoksikan, pertumbuhan myotube diperhatikan.

Berkenaan dengan perubahan hormon, suplemen jangka pendek 600 mg/hari menunjukkan peningkatan testosteron (0.97 ng / ml, 11.1%; p = 0.004)), sementara suplemen jangka panjang menunjukkan peningkatan hingga minggu ke-4, kemudian menurun (0.142 ng/ml, 2%; $p > 0.05$) pada minggu ke-8. Kedua-dua suplemen jangka pendek dan jangka panjang tidak menunjukkan perubahan ketara dalam LH dan estradiol. Walaupun suplemen *E. longifolia* jangka pendek menunjukkan kesan yang baik dalam meningkatkan tahap testosteron, penemuan juga menunjukkan bahawa perubahan dalam testosteron bukan disebabkan oleh ekstrak yang mempengaruhi paksi hipotalamik-pituitari-gonadal (HPG). Di samping itu, dos tidak menunjukkan kesan buruk pada fungsi hati, menunjukkan bahawa *E. longifolia* selamat untuk dimakan.

Semasa mengkaji jangka masa suplementasi, suplemen jangka pendek *E. longifolia* menunjukkan peningkatan dalam domain keadaan mood seperti kemarahan (5.12%) dan keletihan (11.09%), jangka panjang mempengaruhi ketegangan (8.19%) dan kemarahan (4.14%). Penemuan ini menunjukkan bahawa suplemen *E. longifolia* meningkatkan domain keadaan mood tertentu, menunjukkan bahawa ekstrak ini mungkin berupaya untuk memperbaiki keadaan mood harian yang dipengaruhi oleh tekanan harian. Sementara itu, untuk ujian fungsi seksual, suplemen jangka pendek menunjukkan peningkatan dalam domain ejakulasi (6.13%), dan untuk jangka panjang; domain ereksi (25.89%) dan kepuasan keseluruhan (33.33%). Penyelidikan ini menunjukkan peningkatan fungsi seksual setelah selesai suplemen *E. longifolia*, memberikan bukti bahawa penggunaan *E. longifolia* sebagai afrodisiak.

Kesan *E. longifolia* berikutan suplementasi 8 minggu tidak menunjukkan peningkatan kekuatan isokinetik dan daya anaerobik. Oleh itu, suplemen *E. longifolia* sahaja tidak menunjukkan kesan ergogenik di kalangan orang dewasa muda, yang mungkin disebabkan oleh kekurangan perubahan tahap testosteron selepas supplementasi *E. longifolia* selama 8 minggu.

Sebagai kesimpulan, ujian sitotoksisitas menunjukkan bahawa *E. longifolia* mempunyai aktiviti dehidrogenase laktat yang lebih rendah di SMC, menunjukkan tahap ketoksikan yang rendah. Walaupun hasil semasa menunjukkan peningkatan tahap testosteron setelah suplemen jangka pendek, hasil penemuan juga memberikan bukti bahawa *E. longifolia* tidak mempengaruhi paksi HPG. Tambahan pula, *E. longifolia* meningkatkan keadaan mood dan seksual, memberikan bukti bahawa penggunaan *E. longifolia* sebagai adaptogen dan afrodisiak. Hasil kajian juga menunjukkan suplementasi 600 mg *E. longifolia* sahaja adalah dos tertinggi pada masa ini tidak mempunyai kesan ergogenik terhadap kekuatan otot dan daya anaerobik.

Kata kunci: *Eurycoma longifolia*, testosteron, mood, fungsi seksual, kekuatan

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A-549	:	Human lung cell line
ALT	:	Alanine transaminase
ALP	:	Alkaline phosphatase
AST	:	Aspartate transaminase
BRUMS	:	The Brunel Mood Scale
BSFI	:	Brief Sexual Function Inventory
Caov-3	:	Human ovarian cell line
cm	:	Centimeters
C_2C_{12}	:	Murine skeletal muscle cell
CCD11114sk	:	Normal skin cell
CI	:	Confidence interval
CAM	:	Complementary and alternative medicine
CYP17	:	17α-hyroxylase/17, 20 lyase
CO_2	:	Carbon dioxide
CON	:	Control
DM	:	Differentiation medium
DHEASO4	:	Dehydroepiandrosterone
DMEM	:	Dulbecco's modified eagles medium
dH_2O	:	Distilled water
EL	:	*Eurycoma longifolia* group
E	:	Erection
EJ	:	Ejaculation
ED50	:	Effective dose
EC50	:	Effective concentration
FBS	:	Foetal bovine serum

FSH	:	Follicle-stimulating hormone
GI50	:	Growth inhibition
GM	:	Growth media
GnRH	:	Gonadotropin-releasing hormone
HPG	:	Hypothalamic gonadal
HS	:	Heat-inactivated horse serum
HeLa	:	Cervical cell line
Hep G2	:	Human liver cell line
HM3KO	:	Melanoma cells
HT1080	:	Fibrosarcoma cell line
IC50	:	Half-maximal inhibitory concentration
IGF-1	:	Insulin-like growth factor-1
kDa	:	Kilodaltons
KB	:	KERATIN-forming tumour cell line HeLa
KB-V1	:	Human cervix carcinoma
K-562	:	Leukaemia cell line
KCl	:	Potassium Chloride
LDH	:	Lactate dehydrogenase
LH	:	Luteinizing hormone
LNCap	:	Human prostate cells
LOH	:	Hypogonadism
Ml	:	Millilitre
Mg	:	Milligram
MTT	:	3-(4,5-dimethylthiazol-2-yl)-2–5-diphenyltetrazolium bromide)
MCF-7	:	Breast cell line

MCF10A	:	Non-cancerous breast cell
MDBK	:	Bovine kidney
MC3T3-E1	:	Osteoblastic cell line
MTS	:	3-(4,5-dimethylthiazol-2-**yl**)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NBCS	:	Heat-inactivated newborn calf serum
NaCl	:	Sodium Chloride
OS	:	Overall satisfaction
P-388	:	Murine lymphocytic leukemia
PBMC	:	Peripheral blood mononuclear cells
PS	:	Penicillin-streptomycin
PBS	:	Phosphate buffered saline
P13	:	Passage 13
P26	:	Passage 26
PLA	:	Placebo
POMS	:	Profile of mood states
PA	:	Problem assessment
RWPE-1	:	Normal prostate
S	:	Seconds
SHBG	:	Sexual hormone binding globulin
SRB	:	Sulforhodamine B
SQ40	:	Standardised quassinoids 40%
SD	:	Sexual desire
SMC	:	Skeletal muscle cells
WRL-68	:	Liver cell lines
WHO	:	World Health Organization

°/s	:	Degree per second
%	:	Percent
µl	:	Microliter

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

According to the World Health Organisation (WHO), herbal medicine includes herbs, herbal preparations, herbal materials, and all finished herbal products that contain parts of plants, or other plant materials or combinations, as active ingredients. To date, approximately 80 percent of people worldwide use herbal medicine as one of the alternatives for their health care needs due to the naturally occurring substances known as 'phytochemicals'. The phystochemicals could exert medicinal or therapeutic properties and enhance general health status, along with preventing or treat illnesses (WHO, 2002; Firenzuoli & Gori, 2007; Gunnels & Bloomer, 2014). Herbal medicines are credited with many health benefits and are widely used. However, many of the herbs remain untested, and their usage has not been monitored (WHO, 2002).

Eurycoma longifolia Jack (*E. longifolia*), commonly known in Malaysia as 'Tongkat Ali', often prescribed in complementary or alternative medicine or herbal therapy, is well-known in Asia and Western countries (Rehman, Choe & Yoo, 2016). *E. longifolia* has gained its reputation due to its ability to alleviate various illnesses, such as aches, intermittent fever, sexual insufficiency, dysentery, glandular swelling and fatigue, based on a limited number of positive scientific findings (Ang & Sim, 1998; Ang & Cheang, 2001; Ang, Lee & Kiyoshi, 2004; Tambi & Imran, 2010; Zanoli, Zavatti, Montanari & Baraldi, 2009; Wahab, Mokhtar, Halim & Das, 2010; Bhat & Karim, 2010; Low, Choi, Wahab, Das, & Chan, 2013a; Li *et al*., 2013), which have led to a higher demand for *E. longifolia* globally.

E. longifolia is one of the four species of the plant commonly known as 'Tongkat Ali'. The other three species are known as *Eurycoma apiculata*, *Polyathia bullata*, and *Goniothalamus* sp. (Aziz, Sarmidi, Kumaresan, Taher, & Foo, 2003; Athimulam, Kumaresan, Foo, Sarmidi & Aziz, 2006; Hassan *et al*., 2012). *E. longifolia* is a tall, slender, shrubby tree that grows as tall as 15 to 18 metres in sandy soil and grows up 2 to 3 cm long green fruit when mature (Figure 1.1). Almost all the parts of the plant, including the roots, stems, and bark are used as remedies to improve health and wellness.

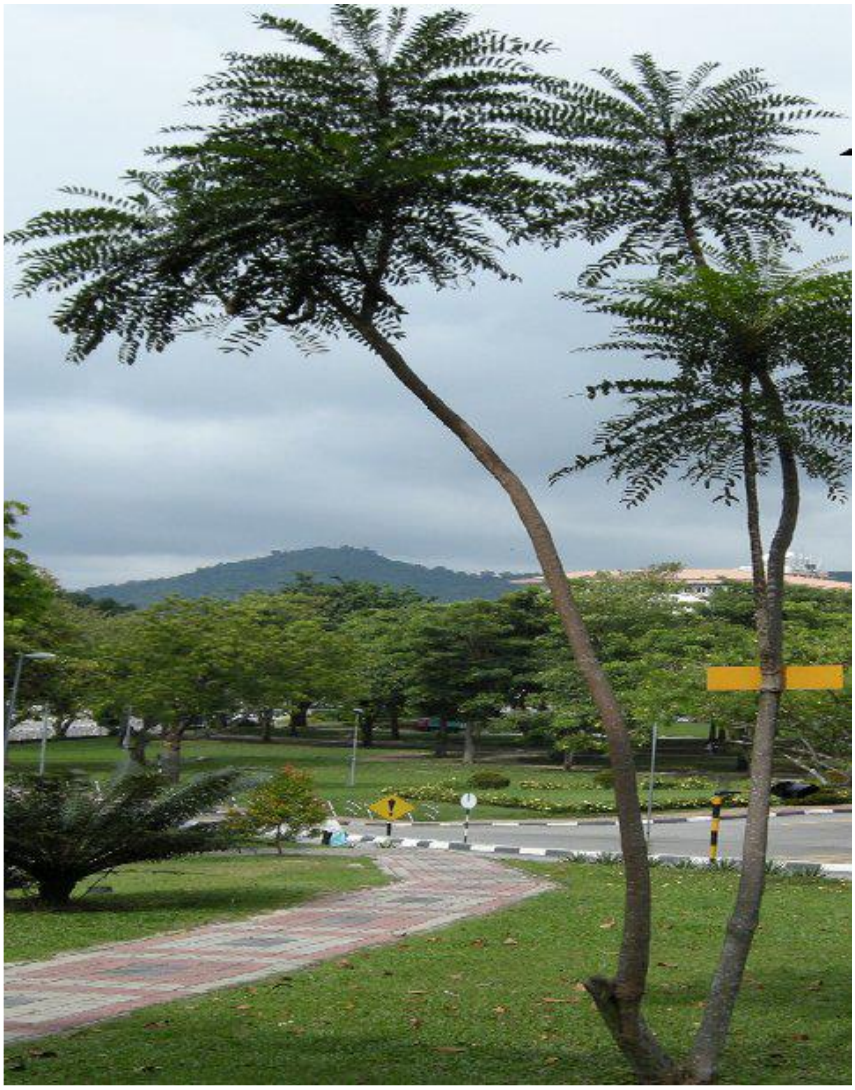


Figure 1.1 *Eurycoma longifolia* (Source: Bhat & Karim, 2010)

Apart from herbal supplements, such as ginseng, *Tribulus terrestris*, fenugreek and many more, the root extract of *E. longifolia* is also available in the market (Bhat & Karim, 2010). The extract is used commonly to treat sexual dysfunction, malaria, cancer, diabetes, anxiety, aches, constipation, fever, leukaemia, osteoporosis, stress, syphilis, and glandular swelling. However, most of these claims remain to be verified via randomized controlled trial. It is also used to improve exercise recovery and increase energy and strength (Darise, Kohda, Mizutani & Tanaka, 1982; Chan, Lee, Sam & Han, 1989; Grieco and Morre, 1998; Kuo, Damu, Lee & Wu, 2004; Hussein, Ibrahim & Kiong, 2006; Miyake, Tezukam, Awale, Li & Kadota, 2009; Fiaschetti, Grotzer, Shalaby, Castelletti & Arcaro, 2011).

E. longifolia may offer an attractive alternative to more mainstream products. Its wide-ranging properties are deemed useful in increasing energy, inducing weight loss, promoting muscle growth or inducing other physiological or metabolic responses that may be conducive to enhancing exercise performance (William, 2006). An earlier study suggests that *E. longifolia* improves muscle strength post-supplementation (Hamzah & Yusof, 2003); however, the underlying mechanisms were not well explained. Following Hamzah and Yusof (2003), many have shown that the increased strength and body composition in older adults and young participants supplemented with *E. longifolia* are associated with increased testosterone levels. According to Talbott, Talbott, George, and Pugh (2013), *E. longifolia* is considered an extract that can restore low testosterone levels to normal circulating level in human and do not enhance testosterone beyond the normal circulating level. While the improvement in older individuals could be due the result of *E. longifolia* restoring low testosterone levels, the improvement in young individuals with normal testosterone level could be due to the combination of *E. longifolia*'s ability to restore or maintain circulating testosterone levels with strength training (Hamzah & Yusof, 2003). Strength training had the most considerable influence on the secretion of

testosterone and, thus, increased strength (Ahtiainen, Pakarinen, Kraemer & Hakkinen, 2003; Ahtianen, Pakarinen, Alen, Kraemer & Hakkinen, 2005; Linnamo, Pakarinen, Komi, Kraemer & Hakkinen, 2005; McCaulley *et al*. 2009). Additional intake of *E. longifolia* may potentially lead to an increase in muscle mass and strength (Hamzah & Yusof, 2003; Chen *et al*., 2019). However, further examination in this respect is necessary to determine whether the muscle strength improvement is due to the strength training programme, a combination of strength training and *E. longifolia*, or *E. longifolia* alone.

Apart from being used to enhance physical attributes, *E. longifolia* is used as a folk medicine for anxiety; however, there has been limited research to prove this claim. There are three related studies; one conducted on animals while two were conducted on humans. These showed a significant decrease and improvement in tension, anger, confusion (Talbott *et al*., 2013), and increased vigour (George, Udani, Zainal Abidin & Yusof, 2018). *E. longifolia* may be a practical approach to shielding the body from the detrimental effects of day-to-day chronic stress, such as stress in planning a daily dietary intake to lose weight, deprivation of sleep, and exercise training (Talbott *et al*., 2013). Nevertheless, more studies are needed to substantiate the findings of previous studies.

Numerous studies have been conducted on animals and humans to investigate the therapeutic effectiveness of *E. longifolia* on male sexual dysfunction. The efficacy of *E. longifolia* in managing male sexual health is due to the presence of the 4.3 kDa peptide (Sambandan, Rha, Kadir, Aminudim & Saad, 2004). The bioactive peptide (4.3 kDa) (patented: PI 20003988, MAL: 10/362697, USA; 01920972.5, EUROPE and 2002-522919, JAPAN) was isolated from *E. longifolia* is a phytoandrogen, which was reported to increase testosterone level in rat Leydig cells. Efforts have been put into identifying the mechanism involved in enhancing testosterone and how testosterone regulation via

the negative feedback loop is affected. Hayes, DeCruz, Seminara, Boepple, and Crowley (2001) proposed that luteinising hormone (LH) and follicle-stimulating hormone (FSH) of the pituitary gland regulate the activity of the Leydig cells which produce testosterone. Also, the aromatase enzyme acts by catalysing the irreversible conversion of testosterone to oestrogen. In an animal study by Low, Choi, Abdul Wahab, Das, and Chan (2013a), *E. longifolia* was shown to interfere with the HPG axis by inhibiting the aromatase enzyme and, thus, prevent the conversion of testosterone to oestrogen. Eurycomanone, a bioactive compound extracted from *E. longifolia,* is responsible for the inhibition of aromatase and it increases the level of testosterone circulation (Low *et al*., 2013a). This mechanism allows more testosterone to remain in the system, and the inhibition of aromatase leads to a lower negative feedback signal to the hypothalamus. The decline in negative feedback causes a stimulation of the anterior pituitary to increase LH and FSH production. LH and FSH's increased secretion stimulates the Leydig and Sertoli cells to produce more testosterone and spermatogenesis (Bhatnagar, Müller, Schenkel, Trunet, Beh & Schieweck, 1992). There is a lack of supporting data in humans concerning the mechanism involved. The study by Low *et al*. (2013a) for the first time demonstrated that *E. longifolia* could increase the availability of testosterone via interference in the negative feedback loop mechanisms by inhibiting aromatase and thus prevent the conversion of testosterone to oestrogen. Hence, the increase in testosterone was speculated to enhance muscular strength, mood state and sexual functions in the male population.

The various benefits demonstrated by *E. longifolia* has also prompted extensive research into its cytotoxicity and anti-proliferative effects on human cell lines. Various compounds isolated from *E. longifolia* show cytotoxic and anti-proliferative effects (Rehman, Choe & Yoo, 2016) when tested on various human cell lines, as well as on

various solid tumours, including lung, breast, and cervical cell lines (Jiwanjinda, Santisopasri & Murakami, 2002; Kuo, Damu, Lee & Wu, 2004). This compound has demonstrated a cytotoxic response against many cancer cell types. It is said that the approach of killing that is induced by most anticancer agents is by apoptotic cell death. The anti-proliferative activity and cytotoxicity of eurycomanone have been investigated in cancerous cell lines (Caov-3, HeLa, Hep G2, HM3KO, and MCF-7) by triggering apoptotic cell death. The antiproliferative activity demonstrated by eurycomanone is due to the induction of apoptosis. These were determined using Tdt-mediated dUTP nick end labelling (TUNEL) assay with the apoptotic detection kit and further confirmed using the Nuclear staining assay to evaluate the morphological changes of apoptotic cells, chromatin condensation and formation of apoptotic bodies (Mahfudh & Azimahtol, 2006). However, eurycomanone is relatively non-toxic on non-cancerous cell lines, and this was evaluated using the cell proliferation assay (Rubinstein *et al*., 1990; Kardano *et al*. 1991; Kuo *et al*., 2003; Tee & Azimahtol, 2005; Mahfudh & Azimahtol, 2006; Park *et al*., 2014; Tong *et al*., 2015). Hajjouli *et al*. (2014) have shown that the major quassinoid, eurycomanone and its derivative, eurycomanol are regulators of signalling pathways involved in proliferation, cell death, and inflammation without affecting healthy cells.

1.2 Problem Statement

Eurycoma longifolia is generally marketed as a testosterone booster and is growing in popularity amongst the male population, based on its acclaimed benefits in alleviating illness, and increasing strength, physical performance, sexual function and mood state. Despite its extensive use in South East Asian countries and the infiltration of western countries as a supplement to improve health and treat and prevent diseases, the ergogenic properties affecting exercise performance and wellbeing among male adults and their

underlying mechanisms are not well explained. Due to its claim of being a putative replacement of testosterone, there is a risk of intramuscular injection of the extract. No preliminary study is examining the cytotoxicity effect of *E. longifolia* on skeletal muscle cells.

Cytotoxicity studies are commonly used as the initial step in determining potential toxicity of a test substance, including plant extracts or bioactive compounds isolated from plants. In relation to *in vitro* cell culture study, a substance is considered cytotoxic when it interferes with cells' attachment, alters morphology, affects rate of cell growth or even causes cells death (McGaw *et al*., 2014). At present, extensive *in vitro* studies examining the cytotoxicity and anti-proliferation effect of various bioactive compounds of *E. longifolia* on numerous cancer cell lines have been conducted, and many have demonstrated cell death and diminished cell growth (Thu, Hussain, Mohamed & Shuid, 2018). Two studies show no cytotoxicity and anti-proliferation in normal cells (Nurhanan, Azimahtol, Mohd Ilham & Mohd Shukri, 2005; Mahfudh and Pihie, 2008; Tong *et al*., 2015). *E. longifolia* is believed to be an alternative to testosterone replacement therapy but yet to be verified (George & Henkel, 2014). It may have the same functionality as testosterone administration, which promotes hypertrophy in myoblasts (Deane *et al*., 2013). However, no study has investigated the cytotoxicity effect of *E. longifolia* on the skeletal muscle cell line to date. Thus far, only oral supplementation of *E. longifolia* shows enhanced testosterone levels and improved muscular strength in young and old individual with or without low circulating testosterone levels, but these changes have not been investigated at the cellular level, particularly in myoblasts.

On the other hand, two animal studies have attempted to explain the mechanism behind the increase production of testosterone following supplementation of *E. longifolia*. It is

suggested that the increase in testosterone is due to the inhibition of the aromatase enzyme, which converts testosterone to oestrogen (Low *et al*., 2013a; Low, Das & Chan, 2013b). In humans, *E. longifolia* supplementation has been shown to improve testosterone production in older participants with low testosterone levels (Tambi *et al*., 2012; Talbott *et al*., 2013) and young, healthy men with normal testosterone levels (Ismail *et al*., 2012; Chen *et al*., 2014; Chen *et al*., 2019). However, the regulation of all the hormones (testosterone, free testosterone, LH, FSH, sexual hormone-binding globulin, and oestrogen) involved in the negative feedback has not been examined. Though a plausible mechanism has been shown in rats (Low *et al*., 2013a and 2013b), no studies have demonstrated this in humans.

In their pilot study, Hamzah and Yusof (2003) showed an increase in muscular strength after a combination of a period of strength training and *E. longifolia* supplementation. However, no study has been conducted to examine young men's muscular strength using only *E. longifolia* supplementation. Whether supplementation of *E. longifolia* alone, without strength training, augment muscle mass and strength in men with normal circulating testosterone levels is still unknown. Similarly, the effects of *E. longifolia* on psychological mood state and sexual function have been studied using animal models, but research on the effectiveness of *E. longifolia* in humans is still scarce (Talbott *et al*., 2013; George *et al*., 2018).

The present thesis examines the hormonal changes in humans to understand the mechanism involved in testosterone regulation and how the interference of the feedback loop will improve the said benefits. The findings will also allow a better understanding of the reputed ergogenic properties of *E. longifolia*, especially in terms of muscular strength and power.

1.3 Research Aim and Objectives

The research's primary aim was to examine the effect of *E. longifolia* in skeletal muscle cell growth, wellbeing and exercise performance in adult males.

The primary aim would be realised by addressing the following objectives in the four study chapters:

(i) to examine the cytotoxicity of *E. longifolia* on the murine skeletal muscle cells (SMC).

(ii) to elucidate the changes in selected reproductive hormone due to *E. longifolia* supplementation.

(iii) to compare the short-term versus long-term effects of *E. longifolia* supplementation on psychological mood state and sexual function among healthy young adults.

(iv) to determine the ergogenic properties of *E. longifolia* with regard to physical measures (muscular strength and power) following supplementation among healthy young adults.

1.4 Research hypothesis

1.4.1 Murine skeletal muscle (C_2C_{12}) cells treated with *E. longifolia* significantly increase cytotoxicity compared to untreated C_2C_{12} (control).

1.4.2 Supplementation of 600 mg/day of *E.longifolia* for 2 and 8 weeks would significantly increase the hormonal concentrations such as testosterone, free testosterone, luteinising hormone, follicle-stimulating hormone, and decreased oestrogen concentration compared to placebo (maltodextrin) consumption.

1.4.3 Supplementation of 600 mg/day of *E. longifolia* for 2 and 8 weeks improve participant's psychological mood such as anger, confusion, depression, tension, fatigue, and vigour compared to placebo (maltodextrin) consumption.

1.4.4 Supplementation of 600 mg/day of *E. longifolia* for 2 and 8 weeks would significantly improve participant's sexual function, such as sexual drive, erection, ejaculation, problem assessment, and overall satisfaction compared to placebo (maltodextrin) consumption.

1.4.5 Supplementation of 600 mg/day of *E. longifolia* for 8 weeks would significantly increase muscular strength and anaerobic capacity compared to placebo (maltodextrin) consumption.

1.5 Significance of the Study

This present study will add considerably to the body of literature pertinent to the usage of *E. longifolia* among male individuals globally. These studies' findings can facilitate professionals or other health care practitioners to conduct consistent, safe, and effective *E. longifolia* supplementation to all individuals in Malaysia, and even to individuals from the west. Once the efficacy of *E. longifolia* is established, the mode of action, potential

adverse reactions, contraindications and interactions with existing pharmaceutical drugs can be determined in future studies to promote safe and rational use of the extract. Since *E. longifoli*a is said to be an alternative treatment to exogenous testosterone, it can be used as a natural supplement to slow down age-related muscle loss. The present findings will provide preliminary information regarding whether *E. longifolia* intake will alter the endogenous steroid profile similar to exogenous testosterone consumption.

A cytotoxicity test is performed to predict potential toxicity by using cultured cells. These tests involve short-term exposure of cultured cells to test substances to detect how cultured cells may be affected by *E. longifolia* extract. Thus, the cytotoxicity test in the present study can determine the potential toxicity of *E. longifolia* towards SMCs prior to performing efficacy and safety studies in humans.

The fundamental data from this study may indicate future research directions. It can stimulate multiple research ideas, especially molecular physiology and interventional studies of populations with or without disease or populations involved in sport. This study may also be relevant for the public, the authorities, researchers, health care practitioners, care service providers, coaches, athletes, and family members in garnering a shared effort for the wellbeing of an individual with health issues and individuals that want to improve their sporting performance and health benefits.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction to literature

Eurycoma longifolia is native to Southeast Asia countries such as Malaysia, Indonesia, Thailand, Philipines and Vietnam. *E. longifolia* has known by many for its medicinal properties since ancient times, particularly in South East Asia. *E. longifolia* is popular for its aphrodisiac properties, a substance that increases sexual desire, sexual attraction, sexual pleasure or sexual behaviour. Also, every single part of the plant is used for the preparation of traditional remedies to treat various illnesses such as aches, intermittent fever, sexual insufficiency, dysentery, glandular swelling and fatigue (Ang & Sim, 1998; Ang & Cheang, 2001; Ang, Lee & Kiyoshi, 2004; Zanoli, Zavatti, Montanari & Baraldi, 2009; Tambi & Imran, 2010; Wahab, Mokhtar, Halim & Das, 2010; Bhat & Karim, 2010; Low, Choi, Wahab, Das, & Chan, 2013a; Li *et al*., 2013). An aphrodisiac is a substance that can increase sexual desire, sexual attraction, or sexual pleasure. Despite being known for the extensive variety of traditional uses, *E. longifolia* is most well-known for its aphrodisiac properties and is highly sought after in the western world for its potential in enhancing sexual performance (Norhidayah *et al*., 2015). Hence, *E. longifolia* with its many bioactive compounds capable of improving general wellbeing and most importantly, enhancing testosterone production, was chosen in this study.

A study by Ab Rahman *et al*. (2011) found that 40 percent of the 1331 male participants aged 40 years and above used *E. longifolia* as part of their treatment of sexual problems because they believed that its testosterone-enhancing abilities contributed to the aphrodisiac properties. Approximately 8,550 out of 20,211 *E. longifolia* products from local manufacturers in Malaysia are produced locally. Any imported products must be

registered with the Drug Control Authority. These products are sold in the market in various forms, such as capsule, polls, liquid formulation, pre-mixed coffee and canned processed drinks (Ang & Lee, 2007; Ang *et al*., 2004; Effendy *et al*., 2012) and can be found and purchased easily from any pharmacies. Due to its ubiquity, extensive animal and human clinical evaluations have been performed (Tambi, 2009); however, findings related to cytotoxicity on SMCs, how *E. longifolia* works and its efficacy concerning reproductive hormones, mood, sexual function and muscular strength in humans are still scarce.

2.2 Bioactive phytochemicals of *E. longifolia*

The major bioactive compounds isolated from parts of *E. longifolia* are presented in Table 2.1. All the bioactive compounds are compiled from studies spanning more than three decades and provided findings related cancer cell lines' effectiveness. The compounds are categorised into: quassinoids, alkaloids, squalene derivatives, triterpene type tirucallane, and many more (Mahfudh & Pihie, 2008; Ang, Cheang & Yusof, 2000; Kuo *et al*., 2004; Miyake *et al*., 2009; Tran *et al*., 2014).

According to Rehman *et al*. (2016) and Fiaschetti, Grotzer, Shalaby, Castelletti, and Arcaro (2011), quassinoids are the main bioactive compounds of *E. longifolia*, found especially in the root of the plant. Quassinoids are a group of nortriterpenoids that have numerous pharmacological properties. Quassinoids such as eurycomanone, eurycomalactone, trihydoxyklaineanone, laurycolactones, eurycomalactone, eurycomalides, longilactone, and eurycolactone derivatives have mostly been isolated from the roots and leaves of the *E. longifolia* plant (Morita, Kishi, Takeya & Itokawa, 1992; Mitsunaga *et al*., 1994; Jiwajinda *et al*., 2002). While quassinoids are mostly

isolated from the roots, alkaloids, squalene derivatives and tirucallane-type triterpenes are mainly found in the herbal plant's bark, stem, and roots. The successful isolation and characterisation of over 150 quassinoids have prompted numerous *in vitro* and *in vivo* studies using bioactive compounds (Fiaschetti *et al*., 2011).

2.2.1 Bioavailability, pharmacokinetics and pharmacodynamics of *E. longifolia*

While there are few animal studies examining the bioavailability, pharmacokinetics and pharmacodynamics of *E. longifolia*, none were conducted on humans (Han *et al*., 2015; Low *et al*., 2005; Pan *et al*., 2014). Bioavailability was conducted to measure the transport of an active ingredient to its site of action to cause the predicted effects (Ahmad *et al*., 2018). Previous studies conducted by Low *et al*. (2005), Ahmad *et al.* (2018) and Ma *et al*. (2015) demonstrated poor absorption after oral administration and low bioavailability. However, the authors also demonstrate that the plasma volume distribution of *E. longifolia* is high, which suggests that they are distributed in various tissues, slow elimination rate and does not show inhibitory effects on cytochrome P450 isoenzymes (Thu *et al*., 2018). *In vitro* assessment of the effect of eurycomanone on cytochrome P450 (CYP) isoforms CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2E1 and CYP3A4 demonstrated eurycomanone did not inhibit any of the investigated isoforms (Pan *et al*., 2014; Han *et al*., 2015). Hence, the purported mechanism suggested by Low *et al*. (2013) that *E. longifolia* inhibit aromatase and prevent the conversion of testosterone to oestrogen may not be true.

E. longifolia's exhibit various pharmacodynamic response against various animal and human diseases such as male fertility enhancement (Low *et al*., 2013a, 2013b), antimalarial (Chan *et al*., 1986; Kardono *et al*., 1991; Ang *et al*., 1995; Kuo *et al*., 2004;

Wernsdorfer *et al*., 2009; Low *et al*., 2011), cytotoxic (Kuo *et al*., 2004; Kardono *et al*., 1991; Rehman et al., 2016), antiproliferative (Wong *et al*., 2012; Hajjouli *et al*., 2014), and antiulcer (Tada et al., 1991), anti-osteoporotic (Shuid *et al*., 2011; Saadiah *et al*., 2012; Tambi & Henkel, 2012; Mirza & Canalis, 2015), sexual function (Tambi *et al*., 2012; Ismail *et al*., 2012; Udani *et al*., 2014; George & Henkel, 2014; Henkel *et al*., 2014) effects, are generally accredited to the quassinoids, specifically eurycomanone. Studies haven shown that eurycomanone is the compound responsible for the reported activities using *in vitro* and *in vivo* studies on rats and cell lines. To date, there are *in vivo* and human clinical studies which have explored the effectiveness of *E. longifolia* in the management of male sexual dysfunction, but mostly on older, hypogonadic, infertile individuals (Tambi *et al*., 2012l; Udani *et al*., 2014; George & Henkel, *2014*; Henkel *et al*., 2014).

2.3 Cytotoxicity effects of *E. longifolia* extract

Many extensive investigations using animal and human cell lines have been performed related to the cytotoxicity effect of *E longifolia* (Table 2.2. and Table 2.3). While most studies used methanol to extract the *E. longifolia*, later it was partitioned using chloroform and butanol before compounds were isolated through various chromatographic resin and isolation techniques (Park *et al*., 2014; Kuo *et al*., 2003; Mahfudh & Azimahtol, 2008). Results from water extract *E. longifolia* to examine the cytotoxicity effect on cell lines are limited (Table 2.3). *E. longifolia*'s active phytochemicals, such as quassinoids, alkaloids, squalene derivatives, and tirucallane-type triterpenes, show a broad range of cytotoxicity and anti-proliferative activity (Thu *et al*., 2018).

Cytotoxicity and anti-proliferative test are commonly used in *in vitro* toxicology studies to detect cytotoxicity or cell viability and the inhibition of cell growth following exposure to toxic substances (Fotakis & Timbrell, 2006). The cytotoxicity test is also known as a means of assessing the loss of membrane integrity, membrane metabolic activity, the loss of monolayer adherence, and the arrest of cells in various stages of the cell cycle.

The most common cytotoxicity and anti-proliferation test used to test *E.longifolia* are the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and Sulforhodamine B (SRB) assay. Apart from these assays, the Lactate dehydrogenase (LDH) assay is also known for its reliability, speed and simple evaluation characteristics (Decker & Lohmann-Matthes, 1988). However, the LDH assay has never been used in examining the cytotoxicity effect of *E. longifolia* on cell lines. This assay is considered more reliable compared to the MTT assay, aquabluer, and neutral red assays. It has also been suggested that the LDH assay can be useful in screening herbal medicine (Specian *et al*., 2016). The cytotoxicity effect of *E. longifolia* extract on various cell lines is presented in Table 2.3.

Table 2.1: Bioactive compound isolated from *E. longifolia* from its various plant parts

Chemical compounds	Parts	References
Quassinoids		
Eurycomonanone derivatives	Roots	Kuo *et al*. (2004); Darise *et al*. (1982); Tran *et al*. (2014); Park *et al*. (2014); Chan *et al*. (1998); Udani *et al*. (2014); Chan *et al*. (1986); Kardono *et al*. (1991); Low *et al*. (2011); Wernsdorfer *et al*. (2009); Ang *et al*. (1995a); Low *et al*. (2005); Ang *et al*. (1995b); Darise *et al*. (1983); Wong *et al*. (2012)
Eurycomanol derivatives	Roots	Kuo *et al*. (2004); Darise *et al*. (1982); Chan *et al*. (1991); Meng *et al*. (2014); Chan *et al*. (1998); Udani *et al*. (2014); Chan *et al*. (1986); Ang *et al*. (1995a); Ang *et al*. (1995b); Darise *et al*. (1983)
Trihydroxyklaineanone derivatives	Leaves, Roots	Jiwajinda *et al*. (2001); Kuo *et al*. (2004); Tran *et al*. (2014); Chan *et al*. (1998); Park *et al*. (2014); Chan *et al*. (1998); Itokawa *et al*. (1993)
Laurycolactones A and B	Roots	Miyake *et al*. (2009); Itokawa *et al*. (1993)
Eurycomalactone	Roots	Kuo *et al*. (2004); Tran *et al*. (2014); Chan *et al*. (1992); Park *et al*. (2014); Chan *et al*. (1998); Udani *et al*. (2014); Kardono *et al*. (1991); Itokawa *et al*. (1993); Ang and Lee (2002)
Eurycomalides derivatives	Roots	Kuo *et al*. (2004); Miyake *et al*. (2009); Tran *et al*. (2014)
Longilactone derivatives	Leaves, Roots	Kuo *et al*. (2004); Miyake *et al*. (2009); Tran *et al*. (2014); Chan *et al*. (1998); Itokawa *et al*. (1993); Morita *et al*. (1993); Jiwajinda *et al*. (2002)
Eurycolactone derivatives	Roots	Miyake *et al*. (2009); Ang *et al*. (2000); Tran *et al*. (2014); Park *et al*. (2014); Ang *et al*. (2002); Kuo *et al*. (2004); Tada *et al*. (1991)

Table 2.1: Bioactive compound isolated from *E. longifolia* from its various plant parts (Continued)

Chemical compounds	Parts	References
Alkaloids		
Alkaloids derivatives	Bark, Stem, Roots	Kuo *et al*. (2004); Tran *et al*. (2014); Low *et al*. (2011); Itokawa *et al*. 1993); Kuo *et al*. (2003); Miyake *et al*. (2010); Lin *et al*. (2001); Souza-Almeida *et al*. (2011); Donkwe *et al*. (2012); Jiang and Zhou (2008); Mitsunaga *et al*. (1994); Kardono *et al*. (1991); Varghese *et al*. (2013); Kuo *et al*. (2003); Siregar *et al*. (2009); Siregar *et al*. (2004); Maziah and Rosli (2009)
Squalene		
Squalene derivatives	Stem	Morita *et al*. (1993); Morimoto *et al*. (1998); Hioki *et al*. (2004)
Tirucallane-type triterpenes		
Niloticin, dihydronilocticin, piscidinol A, bourjotinolone A, 3-episapelin A, melianone, hispidone	Stem	Itokawa *et al*. (1993)

Table 2.2: Summary of investigation implementing isolated bioactive compounds on numerous cell lines.

Model	Dose	Toxicity	Outcome variable	References
MCF-7, colon, HT1080, A-549, melanoma, KB and P-388	7 - 5000 ng/ml	ED50 : 2.0 - 12.0 µg/mL	Cytotoxic against all cell lines except for P-388	Kardono *et al*. (1991)
A-549 cell line MCF-7 cell line	NA	ED50: < 2.5 µg/mL	Significant cytotoxicity against cell lines	Kuo *et al*. (2004)
A-549 cell line MCF-7	NA	ED50: < 2.5 µg/mL -4.2 µg/mL	Significant cytotoxicity against cell lines	Kuo *et al*. (2003)
MCF-7 cell line	2.5 µg/ml	EC50: 2.2 ± 0.18 µg/ml	Anti-proliferative against cell line	Chuen and Pihie (2004)
A-549 cell line	5 - 20 µg/ml	GI50: 5.1 µg/ml	Inhibited proliferation in a dose dependent manner	Wong *et al*. (2012)
Caov-3, HeLa, HepG2, HM3KO, MCF-7	5 µM	IC50 : 2.13 ± 0.09 - 4.21 ± 0.2 µg/ml	Cytotoxicity effect on cell lines	Mahfudh and Pihie (2008)
K-562 cell line	25 ± µg/ml 6 µg/ml	IC50: 19 ± 3 µg/ml IC50: 6 ± 1 µg/ml	Cytotoxicity effect against cell line Strong anti-proliferative against a cell line	Al-Salahi *et al*. (2014)
A-549, MCF-7 HeLa cell lines	1 ,10 and 100 µM	21.01 ± 2.46 to 66.9 ± 6.67µM 21.01% ±2.46% to 66.9% ± 6.67	Cytotoxicity effect against cell lines	Park *et al*. (2014)
K562 cell line Jurkat cell line	100 µM	IC50: 5.7 µM IC50: 6.2 µM	Inhibited cell lines viability and proliferation	Hajjouli *et al*. (2014)
LNCaP,RWPE-1, WRL-68 cell lines	2.5 - 100 µg/ml	IC50: 5.97 µg/ml	Selective cytotoxicity	Tong *et al*. (2015)
KB, DU-145, RD, MCF-7, CaOV-3 cell lines	0.1 - 100 µg/ml	IC50: 3.2µg/mL	Cytotoxicity effect against cell lines	Nurhanan *et al*. (2005)
HepG2	5.0 µg/ml	IC50: 3.8 ± 0.12 µg/ml - 45 ± 0.15 µg/ml	Toxicity and inhibitory effect towards cell line	Zakaria *et al*. (2009)
MC3T3-E1 cell line	25 µg/ml	-	Proliferation or growth of MC3T3-E1	Thu *et al*. (2017)

2.3.1 The cytotoxicity effect due to various dosage of *E. longifolia*

Cytotoxicity and anti-proliferation tests have been conducted on numerous cell lines and found to be toxic to the cell and prevent cell growth when exposed to *E. longifolia*. However, a small number of studies have shown reduced levels of toxicity and no cytotoxicity effect in healthy human cell lines. Most isolated bioactive compounds of *E. longifolia* have been used extensively to examine cytotoxicity and the anti-proliferation of malignant neoplasm (Rehman *et al*. 2016) by using various assay options, such as MTT and SRB (Table 2.3). A malignant neoplasm is a group of diseases involving unregulated cells, and these cells divide and grow uncontrollably to form a malignant tumour and then spread to nearby parts of the human body. Isolated alkaloids, such as 9-methoxycanthin-6-one, 9-methoxycanthin-6-one N-oxide, 9-hydroxycanthin-6-one, and 9-hydroxycanthin-6-one-N-oxide, has cytotoxic effects against breast, colon, fibrosarcoma, lung, melanoma, KB, KB-V1, and murine lymphocytic leukaemia cell lines (Kardono *et al*., 1991). Since then, Kuo *et al*. (2004) have isolated sixty-five compounds from *E. longifolia*, with some of the alkaloids showing similarities to the compounds isolated by Kardono *et al*. (1991) and Kuo *et al*. (2003), namely 9-methoxycanthin-6-one, canthin-6-one and canthin-6-one-9-O-β-glucopyranoside, which also showed potent cytotoxicity on the human lung (A-549) cell line and human breast (MCF-7) cell lines with an effective dose (ED50) and inhibitory concentration (IC50) ranging from 2.0 - 12.0 μg/ml. While isolated bioactive compound of *E. longifolia* was tested on various cell lines, it has not been tested on muscle cells.

The studies discussed above confirm the cytotoxicity effect of *E. longifolia* in cancer cell lines. However, studies tested on healthy cell lines showed the methanolic extract of *E. longifolia* and eurycomanone were 95 and 30 times less toxic than imatinib, a medication used to treat cancer (Al-Salahi *et al*., 2014). Also, the studies have shown that healthy cells, such as peripheral blood mononuclear cells (Hajjouli *et al*. 2014), non-cancerous breast cells (MCF10A) (Chuen & Pihie, 2004), normal prostate (RWPE-1), liver (WRL-68) cell lines (Tong *et al*., 2015), MDBK (bovine kidney) and Vero (African green monkey kidney) (Mahfudh and Azimahtol, 2008), treated with eurycomanone did not demonstrate high cytotoxicity levels and did not inhibit proliferation. In addition, no cytotoxicity was detected post 9-methoxycanthin-6-one administration on normal bovine kidney (MDBK) cell lines (Nurhanan *et al*., 2005).

One study reported that crude water extract of *E. longifolia* had no toxicity effect on non-malignant cells, such as Chang's liver and human normal skin cells (CCD11114sk) (Zakaria *et al*., 2009). Interestingly, a recent study by Thu *et al*. (2017) has shown that *E. longifolia* water extract promotes proliferation and osteogenic differentiation of osteoblastic cell lines (MC3T3-E1). A low concentration of the water extract of *E. longifolia* (5 to 50 μg/ml) that contains proteins, eurypeptide, glycosaponins, and eurycomanone treated on MC3T3-E1 demonstrate significant proliferation at day three and day six of treatment. However, with a higher concentration of 50 - 2500 μg/ml, a progressive decrease in proliferation consistently over time, due to the cell undergoing apoptosis at a higher dose, was observed (Thu *et al*., 2017).

Most compounds of *E. longifolia* isolated using the methanol extraction method demonstrated cytotoxicity towards human cancer cell lines. However, water extract *E. longifolia* showed lower cytotoxicity levels and even induced proliferation with a low

dose of *E. longifolia*. Thus, administration of *E. longifolia* in low doses may have the potential to be an alternative approach to testosterone replacement therapy, such as osteoporosis management. Based on these findings, there is an indication that a specific bioactive compound administered in low doses was not toxic or harmful to normal cells. Thus, there is a possibility that low doses of *E. longifolia* may enhance proliferation and reduce cytotoxicity in SMCs. However, no studies have indicated the dose needed to reduce or increase proliferation and cytotoxicity on SMCs; thus, more investigation is warranted.

2.3.2 Cell Death

Cell death is a critical and active process that maintains tissue homeostasis and eliminates potentially harmful cells (Green and Llambi, 2015). Apoptosis and necrosis are two types of cell death (Galluzi *et al*., 2007). Apoptosis is the process of programmed cell death and often characterized by cell shrinkage, membrane blebbing, chromatin condensation, nuclear fragmentation, and chromosal DNA fragmentation (Kerr *et al*., 1972; Green and Llambi, 2015). Apoptosis can be triggered through the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. Is it said that drugs used for chemotherapy resulted in DNA damage in cells through a p53 dependent apoptotic pathway (Elmore, 2007; Green and Llambi, 2015). On the other hand, necrosis is characterized by cell swelling and plasma membrane rupture, and a loss of organellar structure without chromatin condensation. Also, necrosis is referred to as the degradation processes that occur after cell death. This form of cell death can result from active signalling pathways that depend on the activity of the protein kinase RIP3 (Elmore, 2007; Green and Llambi, 2015).

Cytotoxicity happens due to apoptosis by up-regulating p53 (apoptotic trigger) and down regulating Bcl-2 (the apoptosis suppressor gene) (Zakaria *et al*., 2009). Eurycomanone, the main quassinoid, was used to examine the mechanism of cytotoxicity or the mode of cell death. Previous studies found that eurycomanone induced apoptosis through the up-regulation of p53 and Bax protein and down-regulation of Bcl-2 protein in the liver cell line (Mahfudh & Azimahtol, 2008; Cheun & Pihie, 2004; Zakaria *et al*., 2009).

2.3.2.1 Effect of E. longifolia on liver function toxicity

Based on animal studies, the oral lethal dose 50 (LD50) of the alcohol extract of *E. longifolia* is between 1500 - 2000 mg/kg, while the aqueous extract is between 3000 - 5000 mg/kg (Satayavivad *et al*. 1998; Shuid *et al*. 2011). Effendy *et al.* (2012) stated that to extrapolate an animal dosage to humans, the normalisation of body surface area is used, and it shows that LD50 for *E. longifolia* is extrapolated to be 810 mg/kg in an adult man. Clinical data has demonstrated safe consumption of the water-soluble *E. longifolia* extract to be between 200 and 600 mg (Tambi, 2005). In the study by Tambi (2005), a high dose of *E. longifolia* supplementation (600 mg/day) over two months taken by twenty healthy males, aged from 38 to 58 years old, showed no influence on the blood profile (haemoglobin, red blood cell, white blood cell, platelet count) or any deleterious effects on measures of liver or renal functions. The quality of *E. longifolia* is based on its level of eurycomanone, which is the main active ingredient found in *E. longifolia* (Norhidayah *et al*., 2015). The Department of Standards Malaysia functions as the National Standards Body and the National Accreditation Body, which provides credible accreditation services to laboratories, inspections and certification bodies for greater market access and global recognition for technical competence and quality assurance.

Table 2.3: Cytotoxicity and anti-proliferative responses of various bioactive compound isolated from *E. longifolia*

Bioactive compound	Method	Toxicity	Cytotoxicity and anti-proliferative outcome	References
Methanol extract alkaloids 9-Methoxycanthin-6-one, 9-Hydroxycanthin-6-one, 9-Methodoxycanthin-6-one-N-oxide, 9-Hydroxycanthin-6-one-N-oxide, canthin-6-one 9-O-β-glucopyranoside	MTT assay SRB assay	ED50: 2.0 µg/mL - 12.0 µg/mL IC50: 3.2 µg/mL	Cytotoxicity effect on the breast (MCF-7), colon, fibrosarcoma, lung (A-549), melanoma, KB, and murine lymphocytic leukaemia (P-388), epidermoid carcinoma (KB), prostate cancer (DU-145), rhabdosarcoma (RD), Ovarian cancer (CaOV-3). No significant cytotoxic effect detected on bovine normal kidney (MDBK) cell line	Kardono *et al*. (1991); Kuo *et al*. (2004); Kuo *et al*. (2003); Nurhanan *et al*. (2005)
Methanol extract quassinoids eurycomalactone, 6dehydroxylongilactione, longilactone, 14, 15β-dihydroxyklaineanone, pasakbumin B, C, eurycomanone, semi-purified eurycomanone TAF273 13,21-dihydroeurycomanone 13α(21)-epoxyeurycomanone Eurycomanol epoxyeurycomanone	MTT assay Tunnel assay CellTiter assay SRB assay	ED50: < 2.5µg/mL EC50:2.2 ± 0.18 µg/ml GI50: 5.1 µg/ml IC50: 3.8 µg/ml - 59.26 µg/ml	Cytotoxicity toward A-549, MCF-7, Caov-3, HeLa, Human liver (HepG2), HM3KO, MCF-7 cell lines. Inhibited A549, HepG2, K-562, Jurkat cell line proliferation in a dose-dependent manner Selectively cytotoxic to LNCaP prostate cancer cells, RWPE-1 and WRL-68 cell line No cytotoxicity effect on MDBK and Vero, Chan's liver, and WLR-68 cell line No cytotoxic effect on normal prostate cells.	Kuo *et al*. (2004) Chuen and Pihie (2004) Wong *et al*. (2012) Mahfudh and Azimahtol (2008) Zakaria *et al*. (2009) Al-Salahi *et al*. (2014) Park *et al*. (2014) Hajjouli *et al*. (2014) Tong *et al*. (2015)
Water Extract *E. longifolia* extract	MTT assay	IC50: 45 ± 0.15 µg/ml	Higher toxicity to HepG2. Non-malignant cells (Chang's liver and CCD11114sk) was not affected Promoting proliferation and osteogenic differentiation of osteoblastic cell line (MC3T3-E1)	Zakaria *et al*. (2009) Thu *et al*. (2017)

Note: MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; SRB: Sulforhodamine B assay; ED50: median effective dose; IC50: Half maximal inhibitory concentration

The Malaysian Standard (2011), issued by the Department of Standards Malaysia, states that the level of eurycomanone should be from 0.8 to 1.5 w/v (%). The *E. longifolia* used in the present study reported a 1.21 w/v (%) and complied with the Malaysian Standard (2011). Thus, a dose of 600 mg/kg body weight (Tambi, 2005) is safe for consumption.

2.4 Steroid hormones production pathways

Steroid hormones are grouped into three classes by function, which is glucocorticoids (i.e. cortisol), mineralocorticoids (i.e. aldosterone) and sex hormones (i.e. androgen, oestrogen). These are synthesized in the adrenal glands. In addition, sex hormones can also be synthesized by the gonads and placenta (Arlt *et al*., 2002; Schiffer *et al*., 2019). Mineralcorticoids hormones are synthesized in the adrenal cortex known as zona glomerulosa to regulate the concentration of electrolytes circulating in the blood. On the other hand, glucocorticoids are synthesized in the zona fasciculate, which regulate the process of cortisol, and lastly, the sex hormones (i.e. testosterone, oestrogen) are synthesized in the adrenal cortex known as zona reticularis, which promote sexual characteristics and maturation of reproductive organs (Shier *et al*., 2010; Connell & Davies, 2005; Schiffer *et al*., 2019). Steroid hormones are synthesized in the adrenal glands from cholesterol, a substance for the biosynthesis of pregnenolone. Pregnenolone is the precursor steroid in the biosynthesis of all the steroid hormones to form aldosterone, cortisol and androstenodione (Figure 2.1). Steroid hormones are hydrophobic, and the hormones are transported in the blood by albumin, corticosteroid-binding globulin and sex hormone-binding globulin (Schiffer *et al*., 2019). While the glucocorticoids and mineralcorticoids are beyond this review's scope, the review will focus on the androgen hormone synthesizing into androstenedione, a precursor to testosterone which is produced by the adrenal gland and gonads. Subsequently, the precursor is converted to testosterone,

which then aromatized to oestrogen (King *et al*., 1999). Testosterone hormones are regulated via the HPG axis.

2.4.1 Feedback systems involved in testosterone regulation

The production of testosterone by the Leydig cells of the testes is regulated by the HPG axis, forming a homeostatic testosterone negative feedback loop. The hypothalamus secretes the gonadotrophin-releasing hormone (GnRH) and, subsequently, stimulates the anterior pituitary to secrete LH and FSH, further stimulating the Leydig and Sertoli cells to increase the production of testosterone and spermatogenesis. However, the increase in testosterone level will then feeds back to the HPG to reduce the LH secretion, and this mechanism is called the negative feedback loop (Tilbrook & Clarke, 2001) (Figure 2.2).

The development of the negative feedback loop is essential for the homeostasis of circulating testosterone concentration in males (O'Hara *et al*., 2015). On the other hand, testosterone produced can be metabolised in the peripheral and neuroendocrine tissue by either 5α-reductase to the androgen 5α-dihydrotestosterone (DHT) or by aromatase to oestrogens (Bhatnagar *et al*., 1992). Aromatase is an enzyme responsible for the conversion of testosterone to estrogen (de Ronde & de Jong, 2011), but it cannot convert DHT to estrogen (Bhatnagar *et al*., 1992). Thus, testosterone and DHT are aromatisable and non-aromatisable androgens, respectively. The existence of 5α-reductase (Martini, 1983) and aromatase (Naftolin *et al*., 1975) in the neuroendocrine system influences the hypothalamic-pituitary unit to maintain homeostasis has been well recognised. Thus in the hypothalamic-pituitary unit, testosterone can apply its regulatory influence on gonadotrophin secretion by three probable mechanisms which are either directly, after conversion to the non-aromatisable DHT or after aromatisation to estrogens. The first two mechanisms which cause the secretion of gonadotrophin by the anterior pituitary through

the direct influence of testosterone or after conversion to DHT mechanisms are not affected by aromatase inhibition. However, the third mechanism responds to aromatase inhibition by detecting a decline in the negative feedback signal due to a reduction of oestrogen, thus effecting increased gonadotrophin production. The increase in gonadotrophin stimulates the anterior pituitary to increase LH and FSH secretion and, consequently, cause an increase in testosterone production from the testes. The negative feedback exerted by testosterone on gonadotropin secretion is dependent on the aromatisation of testosterone to oestrogen (Bhatnagar *et al*., 1992).

While there is research on *E. longifolia* in animal and human models, the effect of *E. longifolia* on increasing testosterone levels in humans remains inconclusive. Further work is required to explore the possible mechanisms concerning the wide-ranging benefits claimed, especially in the changes in sex hormones due to *E. longifolia* supplementation.

2.4.2 Effect of testosterone on skeletal muscle growth

Sex hormones play an important role in the maintenance and growth of muscles. A gradual decrease in sex hormones such as testosterone, oestrogens and progesterone will cause the loss of muscular function and mass (Kim *et al*., 2016). Muscle hypertrophy or muscle growth is said to be regulated by a mechanical, hormonal and nutritional signal. Sex hormones responsible for muscle growth are testosterone and insulin-like growth factor-1 which are known to be potent anabolic agents. To enable the interaction between testosterone and skeletal muscle cells to enhance muscle mass, the androgen hormone needs to bind to androgen receptors (AR) (Hulmi *et al*., 2008). In addition, endogenous

and exogenous testosterone also enhance muscle protein synthesis (Griggs *et al*., 1989; Urban *et al*., 1995; Brodsky *et al*., 1996; Arny et al., *1998*).

While testosterone demonstrates a marked increased in muscle protein synthesis, muscle protein breakdown was not examined in the current study. Whether there is any testosterone effect on the net protein synthesis, it still remains unclear. However, according to Arny *et al*., (1998) and other studies, a continuous increase in muscle protein synthesis would eventually increase lean body mass, provided that protein synthesis was to be greater than protein breakdown over time (Bhasin *et al*., 1996; Brodsky *et al*., 1996).

Androgen is shown to elevate the mRNA levels of insulin-like growth factor-I (IGF-I), which subsequently plays a role in androgen-mediated muscle growth via the IGF-I pathway (Urban *et al*., 1995; Xu *et al*., 2004). It is said that IGF-IEc also known as the mechano growth factor (MGF) exist as one of the splice variants of IGF-I mediates satellite cell activation. Study shows an increased proliferation in C_2C_{12} myoblast upon MGF administration (Yang & Goldspink, 2002; Matheny *et al*., 2010). However, it is unknown whether that MGF is the actual effector towards the activation of satellite cells (Matheny et al., 2010). In addition, the circulating level of testosterone and androgen receptor in the blood are affected by exercise, especially performing resistance exercise. A single resistance exercise session (Bamman *et al*., 2001) and long-term strength training (Hameed *et al*., 2004) can affect the insulin-like growth factor-I (IGF-I) and MGF or IGF-IEa. There is a significant correlation between AR and IGF-IEa (MGF) responses to resistance exercise such as the anabolic effects of androgens in the muscle (Lewis *et al*., 2002; Hulmi *et al*., 2008).

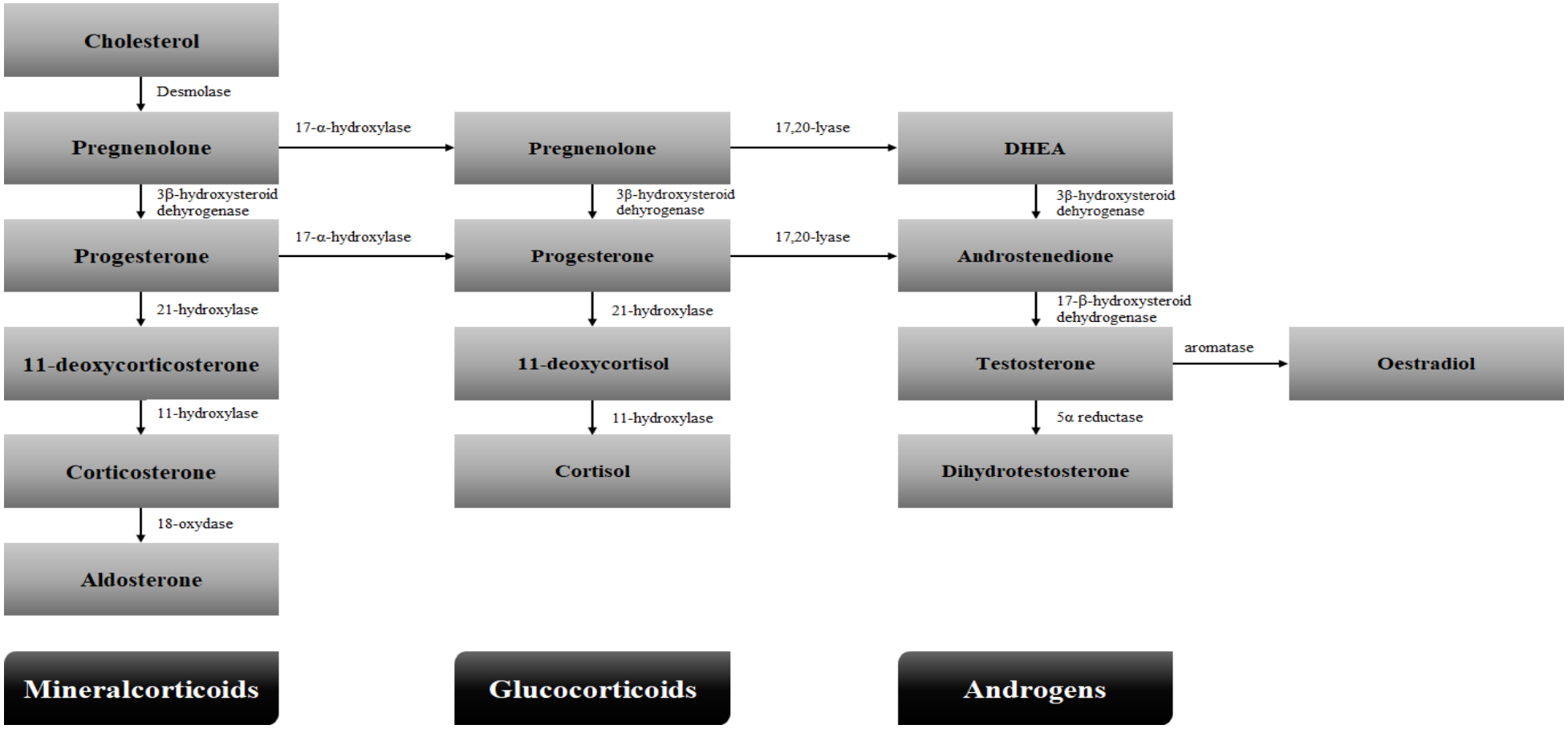


Figure 2.1 Steroid hormones biosynthesis pathway

2.5 Effect of *E. longifolia* on reproductive hormones

Studies have shown that *E. longifolia* supplementation increases testosterone production and strength in humans and animals (Tambi, 2009; George & Henkel, 2014) (Table 2.4). Animal studies are available to show the aphrodisiac and testosterone-enhancing effects of *E. longifolia* extract (Ang & Sim, 1998). While in humans, a study that examined 76 patients with late-onset hypogonadism (LOH) (below 6 nmol testosterone per litre) supplemented with 200 mg of a standardised water-soluble extract of *E. longifolia* for one month showed an increase in testosterone concentration (5.66 ±1.52 to 8.31 ± 2.47 nM) (Tambi *et al*., 2012).

To further substantiate the findings of Tambi *et al*. (2012), a small number of studies have shown an increase in testosterone concentration following *E. longifolia* consumption (Henkel *et al*., 2014; Talbott *et al*., 2013; Udani *et al*., 2014). Talbott *et al*. (2013) and Henkel *et al*. (2014) demonstrated a significant increase in testosterone after consuming 200 mg/day and 400 mg/day of *E. longifolia* for four weeks and five weeks, respectively. Interestingly, testosterone concentration in female participants also recorded an increase in total (48.6%) and free testosterone (122%) (Henkel *et al*., 2014), which, they suggested but did not further explain, was due to a significant decrease in the sex hormone-binding globulin (SHBG). However, a study conducted by Henkel *et al*. (2014) comparing the effect of *E. longifolia* between male and female cyclist with no control group. Hence, the study is not able to establish a cause-and-effect relationship of *E. longifolia* and the outcomes could have been confounded by external variables.

The liver produces and secretes SHBG into the bloodstream, where it will then bind the androgens, such as testosterone and oestrogen with high affinity, regulating their bioavailability (Simó *et al*., 2015). SHBG drives free testosterone and bioavailable testosterone levels, and it is said that the hepatic production of SHBG is regulated positively by estradiol and negatively by testosterone. Thus, an increase in testosterone levels would increase free testosterone levels directly, and indirectly lower the SHBG levels (Brand & van der Schouw, 2010).

Another study administered a combination of *E. longifolia* (300 mg) and *Polygonum minus* to examine sexual performance and wellbeing in twelve men aged between 40 - 65 years for 12 weeks. *Polygonum minus* is a herbal plant commonly used as a food additive, flavouring agent and traditionally used to treat stomach and body aches. This herbal plant was reported to have high antioxidant content (Christapher *et al*., 2015). Total testosterone levels increased significantly from 359.2 ng/dL to 396.4 ng/dL ($p < 0.005$) with an increase of 10.36% in total testosterone in the treatment group, and a significant decrease in free testosterone was recorded for the treatment group (10.73 ± 1.12 to 8.55 ± 1.07 ng/dL). The increased levels in total testosterone and decreased levels in free testosterone poses numerous questions regarding testosterone metabolism. *E. longifolia* might increase the production of SHBG, leading to increased metabolism and the breakdown of free testosterone, and a decrease in free testosterone (Udani *et al*., 2014). The increase in total testosterone in the previous studies may be due to the herb being most effective in participants with lower baseline levels of testosterone (Tambi *et al*., 2012; Tambi *et al*., 2010).

While the studies reviewed reported a significant improvement in testosterone concentration, a study by Ismail *et al*. (2012) reported otherwise. Ismail *et al*. (2012)

examined the effect of *E. longifolia* on the improvement of quality of life and sexual wellbeing in males aged between aged 30 to 55 years and found no significant changes in testosterone, IGF-1, SHBG and dehydroepiandrosterone sulfate (DHEA-SO4). Even with a longer supplementation period (12 weeks), administration of 300 mg/day of *E. longifolia* did not induce any testosterone concentration change (Ismail *et al*., 2012). Another double-blind, placebo-controlled, cross-over study, examining 13 healthy male recreational athletes supplemented with 400 mg of *E. longifolia* for six weeks, also did not demonstrate a significant increase in testosterone: epitestosterone ratio (Chen *et al*., 2014).

At present, only Low *et al*. (2013a & 2013b) have suggested that the eurycomanone, the bioactive compound in *E. longifolia*, may enhance the testosterone in the Leydig cells by inhibiting aromatase conversion of testosterone to oestrogen, thus affecting the HPG axis. However, this postulated mechanism of action behind the reputed effects of *E. longifolia* has only been tested on an animal model. Thus further investigation of the mechanism of *E. longifolia* is needed to rule out a possible effect of certain steroid-like compounds found in *E. longifolia*, such as eurycomanone, that are mediated by receptor or feedback loop regulation rather than bioconversion into steroids (Bucci, 2000).

The quality of *E. longifolia* was based on the level of eurycomanone (Norhidayah *et al*., 2015), and The Malaysian Standard (2011) stated that the level of eurycomanone should be from 0.8-1.5 w/v (%). Although it was suggested that eurycomanone, is responsible for the enhanced production of testosterone, studies have not reported the content of eurycomanon prescribed in those studies (Table 2.3). However, a few studies have reported that the supplement contained 22% eurypeptide which showed improvement in testosterone and muscular strength (Ali & Saad, 1993; Sareena & Yusof,

2003; Tambi, 2005). Efficacy report by Tambi (2005) showed that dosage of 200, 400 and 600 mg of *E. longifolia* (22% Bioactive Eurypeptide) is not toxic to liver function, renal function, haematological profile, lipid profile, body electrolytes and body immune system as well as cancer markers such as Prostate Specific Antigen. Thus far, the efficacy is only based on the assumption that all *E. longifolia* do consist of eurycomanone and eurypeptide.

2.5.1 *Eurycoma longifolia* effects of reproductive hormones regulation

Eurycoma longifolia contains a broad mixture of bioactive compounds (Morita *et al*., 1993; Ang *et al*., 2002; Bedir *et al*., 2003) and eurypeptides with a molecular weight of 4.3 kDa known to have effects in improving energy status and sex drive in animal studies (Asiah *et al*., 2007; Zanoli *et al*., 2009; Ang *et al*., 2001). In addition, eurypeptide is a glycopeptide and has about 36 amino acids. These eurypeptides can activate the CYP17 (17α-hyroxylase/17, 20 lyase) enzyme to enhance the metabolism of pregnenolone and progesterone to produce more dehydroepiandrosterone (DHEA), androstenedione and testosterone, respectively (Sambandan *et al.*, 2006; Tambi, 2009; George and Henkel, 2014).

Low *et al*. (2013a) demonstrated that *E. longifolia* could increase testosterone levels, LH, FSH, and decrease oestrogen through the stimulation of the rats' HPG axis. The HPG axis regulates the circulating concentration of testosterone (Borst & Mulligan, 2007) via the endocrine feedback loop, with LH and FSH as the main hormonal signals (Amory & Bremner, 2003) (Figure 2.2).

Table 2.4: Studies conducted to examine the effectiveness of *E. longifolia* on testosterone production

Study	Dose & duration	Participants/Specimen	Outcome	Reference
Eurycomanone, the major quassinoid in *Eurycoma longifolia* root extract increases spermatogenesis by inhibiting the activity of phosphodiesterase and aromatase in steroidogenesis	100 µL solution of Eurycomanone at 0.1, 1.0 and 10 µM	Testicular interstitial cells	Significantly increased testosterone production dose-dependently at 0.1, 1.0, and 10.0 µM ($p < 0.05$) at the Leydig cells by inhibiting aromatase conversion of testosterone to oestrogen.	Low *et al*. (2013a)
Standardised quassinoid-rich *Eurycoma longifolia* extract improved spermatogenesis and fertility in male rats via the hypothalamic-pituitary-gonadal axis.	Four different fractions of *E. longifolia* at a dose of 25 mg/kg body weight	Male rats	*E. longifolia* fraction (F2) and eurycomanone significantly increased the testosterone level from the Leydig cells, and the plasma LH and FSH levels were significantly higher than the control group.	Low *et al*. (2013b)
Standardized water-soluble extract of *Eurycoma longifolia*, *Tongkat Ali*, as testosterone booster for managing men with late-onset hypogonadism?	200 mg for 1 month	76 of 320 LOH patients 28 – 70 years	Prior to treatment, 35.5% (n = 27) had normal testosterone (5.66 ± 1.51 nm) level. Post treatment, 90.8% (n = 69) showed normal (8.31 ± 2.47 nm) values.	Tambi *et al*. (2012)
Tongkat Ali as a potential herbal supplement for physically active male and female seniors - a pilot study	400 mg *E. longifolia* 5-weeks	13 physically active male and 12 female 57 - 72 years	Significant increase in total testosterone (+15.1%) and free testosterone concentrations (+61.1%) ($p<0.05$)	Henkel *et al*. (2014)
Effect of *Tongkat Ali* on stress hormones and psychological mood state in moderately stressed subjects.	200 mg daily for 4 weeks	63 male aged 45 - 65 years moderately psychological stressed male	Significant increase in testosterone levels in all sub-fertile men.	Talbott *et al*. (2013)

Table 2.4: Studies conducted to examine the effectiveness of *E. longifolia* on testosterone production (continued)

Study	Dose & duration	Participants/specimens	Outcome	Reference
Effect of a proprietary freeze-dried water extract of *Eurycoma longifolia* and *Polygonum minus* on sexual performance and well-being in men: A randomised, double-blind, placebo-controlled study	300 mg combination of *E. longifolia* and *Plygonum minus* 12 weeks	30 healthy males aged 40 - 65 years	Significant increase in total testosterone from 359.2 ng/dL to 396.4 ng/dL ($p < 0.05$)	Udani *et al*., (2014)
Randomised clinical trial on the use of Physta freeze-dried water extract of Eurycoma longifolia for the improvement of quality of life and sexual well-being in men	300 mg/day 12 weeks	109 males 30 - 55 years	No significant changes in testosterone, IGF-1, SHBG, and DHEASO4among the *E. longifolia* group or differences to placebo	Ismail *et al*. (2012)
Supplementation of Eurycoma longifolia Jack Extract for 6 weeks does not affect urinary testosterone: epitestosterone ratio, liver and renal functions in male recreational athletes	400 mg/day 6 weeks	13 healthy male recreational athletes 29.0 ± 5.5 years	T: E ratio was not significantly different following 6 weeks supplementation of either E. longifolia or placebo group compared with respective baseline values.	Chen *et al*. (2014)
Effect of Eurycoma longifolia extract on anabolic balance during endurance exercise	100 mg/day 24 hours	15 male cyclist	Significantly decreased cortisol levels and increased testosterone levels	Talbott et al. (2006)

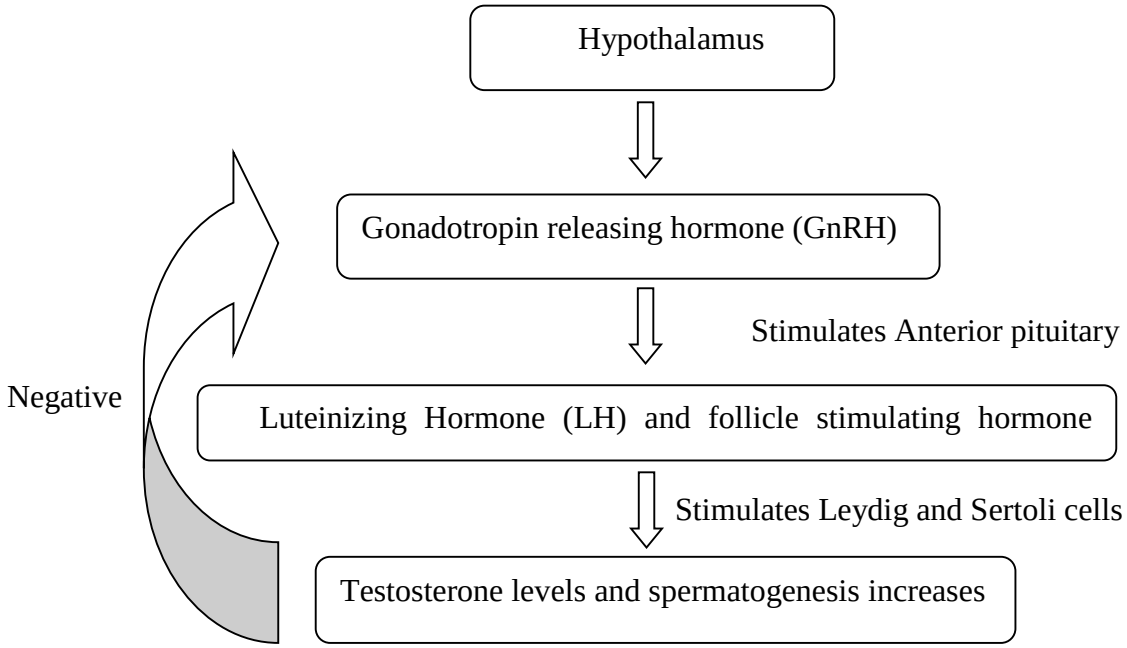


Figure 2.2: Hypothalamic-pituitary-gonadal axis regulates the circulating concentration of testosterone.

Inhibition of aromatase occurs when *E. longifolia* is supplemented (Low *et al*., 2013a). Rat testicular Leydig cells showed an increase in testosterone production dose-dependently at 0.1, 1.0 and 10.0 μM compared to the non-treated group, and dosing eurycomanone alone on rat testicular Leydig cell-rich interstitial cells significantly decreased the oestrogen levels. The reduction of oestrogen levels by eurycomanone could be due to competition between the quassinoid and oestrogen at the oestrogen receptor, eventually culminating in reduced oestrogen production, or quassinoid may directly inhibit the aromatase enzyme that converts testosterone to oestrogen. To test the hypothesis, a combination of formestane (1.0, 10.0, 200 nM), an aromatase inhibitor with eurycomanone (1.0 μM) was administered; it showed a significant reduction in oestrogen ($p < 0.05$) compared to the group administrated with formestane alone or the control group. These findings indicate that eurycomanone may not react on the oestrogen receptor; however, the quassinoid may share the exact aromatase inhibition mechanism as with formestane. It is conclusive that eurycomanone acts on oestrogen production in the rat testicular Leydig cell by inhibiting aromatase from blocking testosterone's

conversion to oestrogen and, subsequently, explains the elevation of testosterone levels (Figure 2.3).

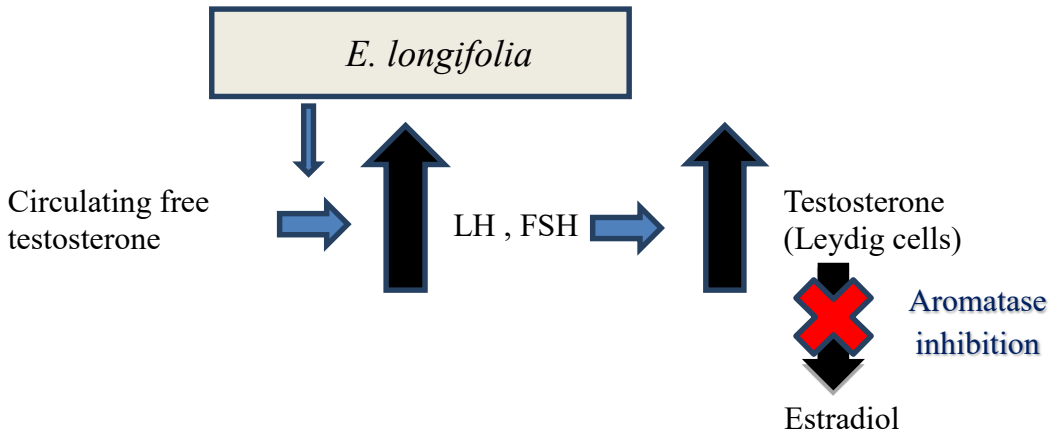


Figure 2.3: Inhibition of aromatase to prevent the conversion of testosterone to oestradiol

Findings by Low *et al*. (2013a) were further substantiated by another study conducted in the same year (Low *et al*., 2013b), where male rats were treated with bioactive fractions of *E. longifolia* at doses of 10, 25, 50 mg/kg for 48 days. There was an increase in plasma testosterone levels and gonadotropins, LH and FSH. The study also reported a reduction in plasma oestrogen levels. The authors concluded that *E. longifolia* treatment did indeed down-regulate the oestrogen-mediated feedback effect for LH and FSH secretions in the HPG axis. The reduction or absence of the oestrogen-mediated feedback effect and the low level of oestrogen allowed the pituitary gland to continuously release LH and FSH (Figure 2.3) and increase testosterone production.

2.6 Effect of *E. longifolia* on sexual function and psychological mood state

The ability of *E. longifolia* to increase and maintain testosterone levels provides numerous benefits, such as increased muscle mass and reduced body fat, improved psychological vigour, such as mental or physical strength, and general wellbeing (Miller, 2009; Grossmann, 2011). The roots of *E. longifolia* are typically used as an adaptogen to help individuals that have reduced mood levels and libido due to ageing (Adimoelja, 2000; Cyranoski, 2005; Joseph *et al*., 2005; Wan Hassan, 2007; Zhari *et al*. 1999). Adaptogen is defined as a substance used in herbal medicine to increase the body's ability to resist the harmful effects of stress and support or restore normal physiological functioning (Panossian & Wagner, 2005). Tambi and Kadir (2005) found that *E. longifolia* improved haematological and lipid profile and sexual function. Testosterone levels start to decrease by approximately 1 to 2% annually after the age of 30 years (Gatti & De Palo, 2011), thus affecting the psychological mood state and sexual wellbeing of individuals. *E. longifolia* could, therefore, be used as a supplement for testosterone replacement to improve psychological parameters such as mood, sense of wellbeing and improved sexual function in those with hypogonadism (Talbott *et al*., 2013; Ang & Cheang, 1999).

2.6.1 Effect of *E. longifolia* on sexual functions

In three studies, the rats were obstructed by an electric grid (0.10 mA) in the electrical copulation cage to investigate how much an aversive stimulus (crossing the electric grid) the male rats were prepared to overcome to reach the oestrous receptive female rats in the other side of the cage. Interestingly, there was a decrease in the hesitation time in reaching the oestrous receptive female in the goal cage after eight weeks of *E. longifolia*

supplementation. Thus, three studies' authors suggested that the extract improves sexual qualities in male rats (Ang *et al*., 2003a; Ang & Lee, 2002; Ang & Ngai, 2001).

Earlier, Ang *et al*. (2000) investigated the effects of *E. longifolia* on the initiation of the sexual performance of castrated male rats. Each male rat was supplemented with *E. longifolia* at various dosages for 50 to 80 days, and sexual performance tests were performed in the copulation cage after ten days of treatment. In the test, the male rats were introduced to the female rats. The rats were monitored for any mounting without intromission or vaginal intromissions. If either of these two copulatory behaviours ended with ejaculation, the performance test was terminated. Thus, they demonstrated a dose-dependent increase in sexual performance of *E. longifolia* treated rats by eliciting an increase in mounting, intromission, and ejaculation.

Ang and Lee (2002) further substantiates this phenomenon by showing increases in orientation activities in male rats, which have been fed with *E. longifolia*, toward the receptive females (anogenital sniffing, licking and mounting), an increase in genital grooming of themselves and a preference for restricted movements within a particular area of the cage with the female rats, with decreased interest in the external environment (climbing, rearing, exploring).

Most studies examining the impact of *E. longifolia* in managing sexual dysfunction is via *in vivo* animal models (Ang & Ngai, 2001; Ang & Lee, 2002; Ang *et al*., 2003a; Ang *et al*., 2003b; Ang & Lee, 2004), with limited research conducted in human trials (Thu *et al*., 2017; Tambi, 2009; Tambi *et al*., 2012; Ismail *et al*., 2012; Muhammad *et al*., 2010; Udani *et al*., 2014; Tambi & Imran, 2010). Although human studies have provided some significant findings, the effectiveness of *E. longifolia* on sexual function in human

has only been demonstrated in three studies, and the mechanisms behind the changes are still unknown. The three studies (Tambi *et al*., 2012; Ismail *et al*., 2012; Udani *et al*., 2014) recruited similar age groups ranging from 30 to 65 years (Table 2.4). While Tambi *et al*. (2012) recruited participants diagnosed with hypogonadism, Ismail *et al*. (2012) and Udani *et al*. (2014) recruited healthy participants, along with some participants with chronic medical illnesses such as controlled diabetes mellitus and hypertension.

Tambi *et al*. (2012) in their clinical study, showed improvement in ageing male symptoms (AMS) among participants with hypogonadism. The AMS was used to assess ageing symptoms under different health conditions, evaluate the severity of symptoms, and measure changes post androgen replacement therapy (Heinemann *et al*., 2003; Badia & Herdman, 2001). The AMS was categorised into no complaints, a few complaints, mild complaints and severe complaints. Prior to the treatment, 10.5% of the patients did not show any complaints, but this increased to 71.1% after being treated with *E. longifolia* for four weeks. Increased testosterone concentration was observed as well (Tambi *et al*., 2012). However, in Ismail *et al*. (2012) administration of 300 mg of *E. longifolia* to 109 healthy men found significant improvements in overall erectile function, sexual libido and overall sexual satisfaction scores in the *E. longifolia* group ($p < 0.001$). Also, in a double-blind and placebo-controlled study on 30 healthy males, improvements in the frequency of sexual performance, penile erection and hardness, and overall sexual wellbeing were observed when treated with *E. longifolia* (Udani *et al*., 2014). Thus, the three studies' findings demonstrated the effectiveness of *E. longifolia* not only on elderly participants diagnosed with LOH but also on healthy elderly participants in improving quality of life and sexual wellbeing.

Despite *E. longifolia*'s promising efficacy of improving sexual function demonstrated in numerous animal model studies and limited human studies (Table 2.5), more data related to *E. longifolia* and male sexual function are required to determine whether age or health play a role in determining the effectiveness of *E. longifolia*.

2.6.2 Psychological mood state following *E. longifolia* consumption

Consumption of *E. longifolia* can reduce anxiety levels in animal models (Ang & Cheang, 1999). Ang and Cheang (1999) demonstrated an anti-anxiety effect in various behavioural tests, such as emotional state anxiolytic and anxiogenic drug effects in rodents following *E. longifolia* feeding. In this study as well, the *E. longifolia* anxiolytic effect was reported to be similar to that of the positive control diazepam, a controlled drug substance used to treat anxiety (Table 2.5). Thus the effects are thought to be due to a restoration of normal testosterone levels post *E. longifolia* supplementation.

Table 2.5: Studies demonstrating the potential *of E. longifolia* in improving male sexual function

Test	Subjects	Dosing regimen and duration	Findings	Reference
Sexual Intercourse Attempt diary, Erection Hardness Scale, Sexual Health Inventory of Men, and Aging Male Symptom	30 healthy male aged 40 - 65 years	300 mg daily 6 months	Significant improvements in all test	Udani *et al.* (2014)
The Ageing Males' Symptoms (AMS) and the serum testosterone concentration	76 patient with LOH aged 30 - 64 years	200 mg daily 1 month	Improved AMS score, serum testosterone concentration. Improvement in sexual performance	Tambi *et al.* (2012)
International Index of Erectile Function (IIEF) and Sexual Health Questionnaires (SHQ); Seminal Fluid Analysis (SFA)	109 healthy men aged 30 - 55 years	300 mg daily 3 months	Improved in the domain Physical Functioning, Erectile Function domain, sexual libido	Ismail *et al.* (2012)
Electrical copulation cage	Sexually sluggish old adult male rats	0.5 g/kg *E. longifolia*	Decreased the hesitation time of noncopulator male rats Transient increase in the percentage of the male rats responding to the right choice	Ang and Ngai (2001)
Monitoring orientation activities towards the receptive females	Adult middle-aged 9 months old and retired breeders	200, 400 and 800 mg/kg of *E. longifolia* fraction	Increased orientation activities towards the receptive females (anogenital sniffing, licking and mounting), increased genital grooming towards themselves	Ang *et al.* (2000)
Electrical copulation cage during the dark phase of the light-dark cycle and in subdued light.	Adult male rats, 9 months old and retired breeders	0.5 g/kg of *E. longifolia* control group : 3 ml/kg of normal saline daily for 12 weeks	Enhanced sexual qualities by decreasing their hesitation time to controls.	Ang *et al.* (2003a)

The survey conducted by Talbott *et al*. (2013) investigated *E. longifolia* on stress hormones and the psychological mood state in a moderately stressed human subject. The survey assessed mood states, such as vigour, depression, anger, confusion, fatigue and anxiety. Significant improvements were reported in *E. longifolia* groups for tension (-11%), anger (-21%), and confusion (-15%) compared to placebo. The hormone profile (salivary cortisol and testosterone) was also tested, and findings showed significant improvement post supplementation, with reduced cortisol level (- 16%) and an increase in testosterone (+37%), with an overall improvement in cortisol: testosterone ratio by -36% (Table 2.6).

In a more recent randomised, double-blind and placebo-controlled study (George *et al*., 2018), 93 participants aged 25 to 65 years were supplemented with 50 mg of *E. longifolia* water extract plus multivitamins for 12 weeks, and they found an increasing trend in the profile of mood states scale (POMS) at week 12 and a reduction of 15% in physical stress. However, when the participants were subdivided according to age, those aged 46 to 65 showed significant improvement in the POMS-vigour (+14.1%) domain compared to the younger age group. The older group's improvement was due to a reduction in muscle strength and endurance with age, changes in body fat percentages, and poor nutrition. Thus, *E. longifolia* supplementation improved muscles and strength acted in an anti-ageing capacity and enhanced vigour (George *et al*., 2018). According to the findings to date, it is suggested that this plant could be beneficial for individuals affected by daily chronic stress, dieting, sleep deprivation and exercise training stress.

Table 2.6: Selected studies on the effect of *E. longifolia* on psychological mood state.

Study	Test	Dosage	Outcome	Reference
Studies on the anxiolytic activity of *E. longifolia* Jack roots in mice.	Inbred adult albino mice Supplementation twice daily for 5 days. Open-field test, elevated plus-maze, and anti-fighting tests.	*E.longifolia* fractions: Chloroform (0.3 g/kg) Methanol (0.3 g/kg) Water (0.3 g/kg) Butanol (0.3 g/kg) Control (3 ml/kg) Diazepam (1 mg/kg)	*E. longifolia* attenuated anxiety parameters in open-field and plus-maze test and inhibited footshock-induced fighting.	Ang and Cheang (1999)
Effect of Tongkat Ali on stress hormones and psychological mood state in moderately stressed subjects.	POMS survey in 63 moderately stress men and women. Supplemented for 4 weeks	200 mg / day	Significant improvement for Tension, Anger, and Confusion Cortisol and testosterone significantly improve, reduced cortisol exposure and increased testosterone	Talbott *et al*. (2013)
Efficacy and safety of *Eurycoma longifolia* (Physta) water extract plus multivitamins on quality of life, mood, and stress	POMS survey in 93 subjects. *E. longifolia* plus multivitamins 12 weeks	50 mg /day	Increased trend in POMS vigour domain Significant improvement on POMS vigour for age 46-65 men	George *et al*. (2018)

2.7 Effects of *E. longifolia* on physical strength

Eurycoma longifolia offers the potential of improving strength and power for those engaged in sport and exercise (Table 2.7). There are preliminary data that support the claims made on *E. longifolia* concerning improvements in strength and body composition (Hamzah & Yusof, 2003; George *et al*., 2013). At present, only Hamzah and Yusof (2003) and Chen *et al*. (2019) have conducted studies on young men, while other studies recruited participants aged between 30 to 72 to examine the effectiveness of *E. longifolia* in muscular strength. In their pilot study, Hamzah and Yusof (2003), investigated the ergogenic effects of *E. longifolia* supplementation on fourteen healthy men by ingesting 100 mg per day of *E. longifolia* water extract and performing an intense strength training programme for five weeks. The combination of supplementation and strength training-induced an increase in muscle strength by 6.8% ($p < 0.05$). Also, the pilot study showed a significant increase in lean body mass, reduction in the percentage of body fat, and increased mean arm circumference in the *E. longifolia* treated group. In a more recent study, 40 young males were assigned into four groups: control group, *E. longifolia* group, *E. longifolia* combined with resistance training and resistance training. Participants in the *E. longifolia* and *E. longifolia* combined with training, were dosed with 200 mg *E. longifolia* for eight weeks (Chen *et al*., 2019). This study showed that the combination of *E. longifolia* administration and resistance training, and even supplementation alone, improved isokinetic power and anaerobic power. Although there was no significant improvement in strength, there was a trend towards increased strength when *E. longifolia* was taken while resistance training was performed.

Table 2.7: Effect of *E. longifolia* supplementation in improving muscular strength

Methodology	Participant	Study duration and dose	Findings	Reference
Performed intense strength training with supplementation of *E. longifolia* water-soluble extract.	14 healthy men	5 weeks 100mg/ day	1RM test showed a significant increase in the treatment group compared to the placebo group. *E. longifolia* 's strength (6.78%) increase larger than placebo (2.77%)	Hamzah and Yusof (2003)
A total of four interval visits and required to performed physical testing at each visit and blood sample collect to analyse Testosterone:Epitestosterone ratio	40 Malaysian men, 30 - 55 years	12 weeks 300 mg/day	T: E ratio did not change during the 12 weeks supplementation period. Muscular strength improved significantly in the back and leg	George *et al*. (2013)
Supplementation of *E. longifolia* for 5 weeks. A resting, fasting blood sample were collected before treatment and during week 3 and 5. Muscular strength assess using the handgrip dynamometer	13 males and 12 physically female 57 - 72 years	5 weeks 400 mg/day	Significant increase in total and free testosterone and muscular force in men and women	Henkel *et al*. (2014)
Resistance training with *E. longifolia* supplementation. Strength examined using handgrip strength. Muscle circumference determines using an ultrasound method.	31 middle-aged women, 45 - 59 years	12 weeks 100 mg/day	Increased handgrip strength and bigger quadriceps muscles as determined by cross-section of the rectus femoris muscle compared to placebo.	Sarina *et al*. (2009)

Previous studies examining the effectiveness of *E. longifolia* on muscular strength mainly recruited older participants. Ooi *et al*. (2015) are one of the most recent studies to examine the combined effects of *E. longifolia* and a circuit training programme on muscular strength and power in men aged around 46 years old. In this study, eight weeks of 400 mg of *E. longifolia* and circuit training programme was found to have improved muscular strength and power compared to the group that performed circuit training alone. However, Ooi *et al.* (2015) did not report any possible confounding factors such as dietary intakes, daily physical activities, or whether the training involved a specific target heart rate zone in standardizing the intensity.

In a later study conducted by Sarina *et al*. (2009), for the first time on women aged 45 to 59 years, 100 mg per day of *E. longifolia* was supplemented for 12 weeks, and strength training was performed twice weekly (Sarina *et al*., 2009). The treatment period demonstrated an increase in muscle strength and a more significant increase in quadriceps muscle size. There followed a study by Henkel *et al*. (2014), which saw 12 active female and 13 active male recruits aged between 57 to 72 years supplemented with 400 mg *E. longifolia* daily for five weeks and demonstrating increased muscular strength while performing the handgrip test. Although females reported increased in strength, the muscular strength remained significantly lower than in the male participants. The findings further affirm the ergogenic benefits of *E. longifolia* through enhanced muscle strength not just in men but also in females. However, this study (Henkel *et al*., 2014) compared between male and female cyclist with no control group. In order to determine whether there is an effect to a treatment, a control group is needed to provide a baseline data. Furthermore, testosterone is not measured in females to indicate a possible relationship between strength and the testosterone-enhancing *E. longifolia*. Hence, the increase in strength could be due to the resistance training itself and not entirely due to *E. longifolia*.

At present, only one randomised, double-blind, and placebo-controlled trial with 40 male non-athletes aged between 30 to 55 years supplemented with 300 mg/day of *E. longifolia* for 14 weeks with no strength training has been carried out by George *et al*. (2013). There was a significant increase in back and leg strength in the *E. longifolia* supplemented group; however, other fitness tests did not show any changes. It speculated that a higher dose and longer duration of supplementation in young and older participants might produce better improvement in physical strength. However, the effectiveness of *E. longifolia* alone in improving strength is still debatable, especially in young males. Previous studies have shown that supplementation of *E. longifolia* demonstrates an increase in testosterone. However, no study has been conducted to date to determine whether supplementation of *E. longifolia* alone will affect muscular strength in young, healthy males with normal testosterone concentration.

2.8 Summary of literature review

Cytotoxicity tests are important steps to determine the cytotoxicity and inhibition of cell growth following exposure to a toxic substance. Numerous bioactive compounds isolated from *E. longifolia* have shown positive cytotoxic and anti-proliferation responses towards various cell lines, especially cancer cells. However, a small number of examinations have shown that *E. longifolia* is not cytotoxic on normal cell lines but improves normal healthy cells' proliferation. *E. longifolia* has been described as a testosterone replacement due to its androgen characteristics, and this claim can increase in the usage of *E. longifolia* in the near future. Thus, whether the administration of *E. longifolia* on SMCs will promote hypertrophy or promote cell death are still not being investigated or answered.

Previous studies have shown that *E. longifolia* can induce aromatase inhibition and, thus, prevent the conversion of testosterone to oestrogen, allowing more testosterone concentration production. This inhibition of aromatase will then cause the hypothalamus to sense a decline in the negative feedback signal. This inhibition will prompt the anterior pituitary to secrete more LH and FSH and, subsequently, produce more testosterone by the Leydig cells. The authors have demonstrated the mechanism of *E. longifolia* in their animal studies and suggested that similar mechanisms may happen in humans to increase testosterone. However, it is still unknown whether the mechanism demonstrated in their study (Low *et al*. 2013a) will be the same in humans.

The testosterone level in humans has been shown to increase in fat-free mass, muscle size, and strength in young, healthy men using *E. longifolia* supplementation with a combination of strength training. Physically active older men and older men with LOH have also demonstrated an increase in muscular strength. However, the increase in muscular strength may be due to the high-intensity strength training and low testosterone concentration due to the ageing process, and the herbal supplement may have an anabolic effect. Apart from muscular strength improvement through supplementation of *E. longifolia*, studies have shown that the plant extract effectively improves the psychological mood state and sexual function wellbeing in animal studies. Although a small number of human studies have been conducted, it is still too early to conclude its efficacy in improving mood state and sexual function in various age groups.

In conclusion, studies have been conducted to elucidate the mechanism of *E. longifolia* via the changes in reproductive hormones to determine the effect of the plant on muscular strength, mood and sexual function. Numerous cytotoxicity tests have been

performed on numerous cell lines, but more extensive studies in human and skeletal muscles cells are needed to substantiate the data found from these previous studies.

CHAPTER 3: THE CYTOTOXICITY OF *E. longifolia* ON C_2C_{12} MURINE SKELETAL MUSCLE CELLS

3.1 Introduction

Various parts of the plant demonstrate anti-cancer, anti-ulcer, anti-tumour, anti-pyretic, anti-schistosomal and anti-malarial properties (Ismail *et al*., 1999; Harun *et al*., 2015). Numerous bioactive compounds, such as quassinoid, alkaloids, triterpenes, and steroidal compounds, have been isolated. However, not all of the bioactive compounds have been screened for their cytotoxic effects. Quassinoids and alkaloids exhibit a cytotoxic effect on certain cells lines: for example, epidermoid carcinoma (KB) and murine lymphocytic leukaemia (P-388) cell lines (Morita *et al*., 1990; Kardono *et al*., 1991; Morita *et al*., 1993; Kuo *et al*., 2004; Chan *et al*., 2004).

Many studies have been conducted to isolate the bioactive compound of *E. longifolia*, which has then been used to determine the cytotoxicity effect on various cell lines, and a preliminary *in vitro* cell culture study was performed to examine any potential cytotoxicity of *E. longifolia* on SMCs, which exhibits the characteristics of normal myoblastic cells. In the present study, C_2C_{12} cells from the thigh muscle of 2-month-old normal mice were used to examine the response of SMCs to exposure to *E. longifolia* during differentiation. C_2C_{12} cells are derived from murine SMCs, and there is a well-established model to study muscle regeneration and differentiation. An extract is considered cytotoxic if it interferes with the attachment of SMCs, significantly changing the morphology, affecting the rate of cell growth, and causing the cells to die (Horvath, 1980). A cytotoxicity study is the first step in examining the toxicity of *E. longifolia*, and this is the first time it is being tested on SMCs.

3.2 Literature Review

Several studies have shown the androgen-like or testosterone-enhancing effects of *E.longifolia. E. longifolia* has been found to increase the copulatory activity, leavator ani muscle activity, the initiation of sexual performance, and spermatogenesis in animal studies (Ang *et al*., 2000; Ang & Cheang, 2001; Ang & Lee, 2002; Zanoli *et al*., 2009; Tambi & Imran, 2010; Wahab *et al*., 2010; Erasmus *et al*., 2012). In human studies, individuals with LOH, is a condition in older men that has low testosterone levels have shown improvements when treated with *E. longifolia* (Tambi *et al*., 2012). These animal and human studies collectively show that *E. longifolia* has the potential to be an alternative to testosterone therapy. However, *E. longifolia* also exhibits cytotoxicity effects on cancer cell lines (Thu *et al*., 2017).

3.2.1 Cytotoxicity Effect of *E. longifolia* on Cell Lines

Table 2.2 in Chapter 2 summarise the findings of studies related to the cytotoxicity effect of *E. longifolia* on various cell lines at a different dosage of bioactive compounds isolated from it. The studies demonstrate cytotoxicity and anti-proliferative effects mainly from *E. longifolia* methanol and chloroform extracts with doses ranging from 2.5 to 100 µg/ml of *E. longifolia* on A-549 lung cancer cell line, MCF-7 breast cancer cell line, HT1080 fibrosarcoma cancer cell line, HeLa cervical cancer cell line, CaoV-3 human ovarian cancer cell line, HepG2 human liver cancer cell line, HM3KO melanoma cells, LNCap human prostate cancer cells, and murine lymphocytic leukaemia (P-388) (Table 3.1). (Kardono *et al*., 1991; Kuo *et al*., 2003, 2004; Chuen & Pihie, 2004; Nurhanan *et al*., 2005; Mahfudh & Pihie, 2008; Zakaria *et al*., 2009; Wong *et al*., 2012; Al-Salahi *et al*., 2014; Hajjouli *et al*., 2014; Park *et al*., 2014; Tong *et al*., 2015). Their findings

indicate the termination of cell growth or cell death post-*E. longifolia* administration in cancer cell lines.

While many demonstrate cytotoxicity in cancer cells, little is known about *E. longifolia* water extract's effect on normal cell lines. Although limited, one study has shown no cytotoxic effect when *E. longifolia* is administered to bovine kidney cells (MDBK) (Nurhanan *et al*., 2005). Also, concentrations ranging from 5 - 2500 μg/ml of *E. longifolia* were administered to osteoblast MC3T3-E1 cell line, but only the concentration of 1 - 25 μg/ml *E.longifolia* showed a significant increase in the proliferation in MC3T3-E1 (Thu *et al*., 2017). Both studies suggest that *E. longifolia* extract is not toxic or harmful to normal cells and can prevent osteoporosis by increasing proliferation rate in a selective, time and dose-dependent manner. However, no cytotoxicity test has been conducted on SMCs, and it is still unknown whether it can produce the same results found in Nurhanan *et al*. (2015) and Thu *et al*. (2017).

3.2.2 Cytotoxicity Assay

Various tests to measure the bioactive compounds' cytotoxicity effect, e.g. MTT, SRB, and MTS assay. The MTT assay is used to measure cellular activity to determines cell viability. Among all the cytotoxicity tests, the MTT assay is the most commonly used method to test the cell culture's cell growth rate and toxicity. Although the MTT assay is accurate compared to other detection methods, there are problems in the application. For example, the test results are not the same when using different doses of extract: at high concentrations, the particles accumulate in the bottom of the culture wells and hinder the measurement of optical density; it is also a cumbersome procedure (Allen *et al*., 1994; Li *et al*., 2015).

The Lactate dehydrogenase (LDH) assay has been developed as a standard test and is used regularly in human medicine as a diagnostic test for myocardial injury and neoplasia (Allen *et al*., 1994). The levels of LDH activity in samples of cell lysate are measured using an LDH assay kit. The enzyme is released into the culture medium only if the plasma membrane is damaged, and the release of LDH from cells has been used extensively as an accurate marker for cell death.

In the present study, the LDH assay is used as a cytotoxicity detection method. It has high reliability and is suitable for the screening and characterising of plant extracts as phytotherapeutic compounds (Specian *et al*., 2016). Furthermore, according to the LDH assay's basis shown in Figure 3.1, LDH levels can be detected accurately to determine whether there is any cell death happening due to the plant extract. Since plant extract users have increased tremendously over the years for various purposes, such as improving strength and muscle mass, it is vital to examine *E.longifolia* crude extract on SMC to identify potential cytotoxicity at an early stage. Hence, the present study is aimed at examining the cytotoxicity of *E. longifolia* on the murine SMCs.

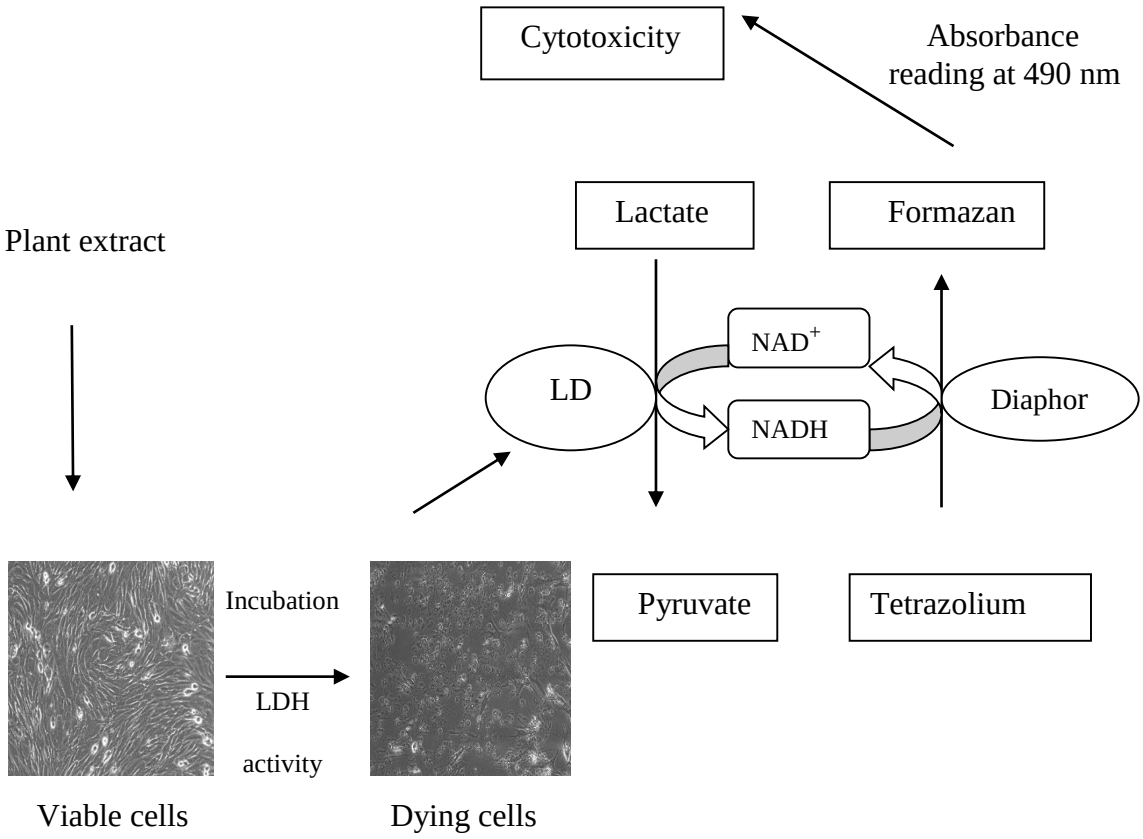


Figure 3.1: Lactose dehydrogenase (LDH) assay principles

3.3 Methods

3.3.1 General Cell Culture for Skeletal Muscle

Cell culture was conducted under the cell culture hood by Kojair Biowizard Silverline class II hood (Kojair. Vippula, Finland), and all cells cultured were incubated in the HERA cell 150i Incubator (ThermoScientificInc. Cheshire, UK) at 5% CO_2 and 37 ºC. Commercially available C_2C_{12} cells population were purchased from ATCC and cultured on T75 cm^2 (Nunc. Roskilde, Denmark) culture flasks. Experiments were also conducted on cell monolayers seeded on sterile six-well plates (Nunc. Roskilde, Denmark). All culture flasks and the six-well plates were coated with 2 ml/l porcine gelatin solution (~90-110 g Bloom; Sigma-Aldrich Company Ltd. Dorset, UK) to enable cell adhesion. Waste media, supernatant, phosphate-buffered saline (PBS) wash, and any other liquid were discarded using an extraction pump (Charles Austen Pump Ltd, Surrey, UK). As for the solution's preparation, dH_2O from a MilliQ water purification system (Merck KGaA. Darmstadt, Germany) was used.

Skeletal muscle cell culture was performed prior to the experimental study. All T75 flask was gelatinised with 8 ml of gelatin (0.2% gelatin solution) using the 10 ml pipette in a sterile condition, and was T75 was left under the flow hoods for 10 minutes at room temperature. Following that, T75 was relocated to the incubator at 37ºC for 10 minutes. At the same time, the growth media were warmed up in the small oven at 37 ºC. Whilst the culture flasks are warming in the incubator, one vial of cells was taken out from the liquid nitrogen storage. The vial is then placed in the water bath and leave the cells in the vial to thaw for approximately two to three minutes in the water bath. When the vial of cells is thawed, the oven's growth was place under the hood. Next, the T75 was removed from the incubator, and the excess gelatin was aspirated off, and 14 ml of growth media

was added to the T75 using a 25 ml pipette. Pipette 1 ml from the vial on top of the media into the T75 flask. The T75 flask was gently moved from side to side six times in each direction to ensure the cells are evenly distributed and are not clumped together. Following that, the T75 flask was clean with a paper towel with 70 % ethanol before placing the flask into the incubator at 37 ºC (5 % CO_2). The T75 was incubated for 72 hours to allow the cells to attach and proliferate to confluence. A similar procedure was performed on the six-well plates.

3.3.2 Reagents, Media, and Cell Culture Preparation

Reagents consisted of Dulbecco's Modified Eagles Medium (DMEM), purchased from Sigma-Aldrich (Sigma-Aldrich Co. Dorset, UK). Heat-inactivated horse serum 2% (HS), 10 % heat-inactivated foetal bovine serum (FBS), 10% heat-inactivated newborn calf serum (NBCS) and 1% penicillin-streptomycin (PS) were purchased from Gibco (Life Technologies. California, US). Phosphate buffered saline (PBS) for cell monolayer washes was purchased in tablet form from Sigma-Aldrich and reconstituted to give a working concentration of 0.01 M phosphate buffer, 0.0027 M KCl, and 0.137 M NaCl, pH 7.4 in dH_2O.

Primary culture myoblast growth media (GM) for murine C_2C_{12} cells consisted of DMEM, 10% FBS (50 ml), 10% NBCS (50 ml), 1% penicillin-streptomycin (50 units penicillin / 50 µg streptomycin) and 2 mM L-Glutamine (5 ml). Differentiation media (DM) preparation for C_2C_{12} growth consisted of DMEM, 2% heat-inactivated horse serum (10 ml) and 1% penicillin-streptomycin (5 ml) and 2 mM L-Glutamine (5 ml). Both penicillin-streptomycin and L-Glutamine were filtered through a 0.445 µl filter before adding into DMEM.

Before using the cell culture hood and all equipment placed under the hood were sprayed with 70% ethanol for sterilisation. Next, the T75 flask was gelatinised with approximately 5 to 8 ml of 0.2 % gelatine solution using a large pipettor and 10 ml pipettes in sterile conditions. Gelatine is a translucent and colourless substance extracted from an animal, which acts as an extracellular matrix (ECM) protein so that C_2C_{12} attaches to the base of the flask via integrin binding. Gelatinised flask was left under the hood for 10 minutes at room temperature and subsequently transferred into the incubator 37 ºC for another 10 minutes.

According to Pronsato *et al*. (2013), skeletal muscle cells, C_2C_{12} with passage numbers less than 20 and passage numbers more than 60, are considered low and high passage numbers. High passage numbers exhibit alteration in cell morphology by demonstrating reduced proliferation or differentiation. Thus a low (passage 13) and mid (passage 26) passage number were chosen for the present study. Both passage numbers can grow confluent better than other passage numbers below passage 30 in the present laboratory.

Vials of C_2C_{12} cells from passages 13 and 26 were thawed for 2 to 3 minutes. Next, 1 ml of C_2C_{12} cells was seeded onto the gelatinised T75 at 1 x 10^6 cells/ml in a growth medium pre-heated to 37 ºC. The cells were then distributed evenly by moving the T75 flask side to side gently six times in each direction. Immediately the T75 was cleaned with 70 % ethanol just before placing it in the incubator at 37 ºC (5% CO_2). The cells were incubated for 72 hours to allow the cells to attach and proliferate to confluence. The cells were checked for any contamination, and growth media was changed at 24 and 48 hours during the incubation period.

3.3.3 *Eurycoma longifolia* Stock Solution Preparation

Eurycoma longifolia was sponsored for this study (ERP Two One Technologies & Innovations Sdn Bhd). The quality of *E. longifolia* was based on the level of eurycomanone, which is the main compound found in *E. longifolia* (Norhidayah *et al*., 2015). The Malaysian Standard (2011) stated that the level should be from 0.8-1.5 w/v (%). The *E. longifolia* used in the present study complied with the standard since eurycomanone content was 1.21 w/v (%) (Appendix B).

The stock solution of *E. longifolia* was prepared prior to addition to the media. To prepare a 200 mg/ml stock solution, mix 1000mg of *E. longifolia* with 5 ml of GM. Following that, the stock was filtered with a 0.2 µl filter. Since no previous studies have been conducted on C_2C_{12}, various doses were tested at 2.0, 1.0, 0.5, 0.25, 0.125 mg/ml of *E. longifolia,* and differentiation media (DM) as a control. A dilution of 1:100 of the *E. longifolia* stock (200 mg/ml) produced 2.0 mg/ml starting volume. A 0.1 ml of *E. longifolia* from the stock volume was added to 9.9 ml of DM to make 10.0 ml of 2.0 mg/ml *E. longifolia*. Double dilution was performed to prepare the 1.0, 0.5, 0.25, 0.125 mg/ml of *E. longifolia*. Differentiation media was made and act as a control treatment (CON)**.** The five doses of *E. longifolia* and CON were seeded onto a 6-well plate seeded with C_2C_{12}.

3.3.4 Microscopy

All cell imaging and photography were performed on a Leica DMI 6000B inverted research microscope (Leica Biosystems GmbH. Nussloch, Germany). Images were captured with a 10X objective and 0.5 magnification c-mount lens fitted to a camera.

3.3.5 Dose-response and Cytotoxicity Lactate Dehydrogenase Assay

After 72 hours of incubation, C_2C_{12} cells were examined under the microscope. Once 80% confluence was attained, cells were trypsinised. Existing growth media (GM) in the T75 flask was aspirated and subsequently rinsed twice with PBS. Next, 1 ml of trypsin was injected into the T75 flask slowly, and the dish was tapped to make sure the trypsin solution covered the entire flask. The flask was then incubated at 37 ºC for 5 minutes. After 5 minutes, the cells were examined under the microscope to ensure non-adherence to the flask. Following that, 4 ml of fresh GM was added into the T75 to neutralise the trypsin. It was then mixed by collecting up and down approximately 5 to 10 times in a 10 ml pipette tube and transferred into a 50 ml tube.

Neubauer haemocytometer was used for cell counting. The cells were prepared as a 1:1 suspension in 0.4% trypan blue stain and loaded into the haemocytometer. A 50 µl of cell suspension was collected and mixed with 50 µl of Trypan blue stain (1:1 ratio). The mixture was then pipetted onto the haemocytometer and was placed under the microscope. Cells were counted under the microscope at 10X magnification. Only viable cells were counted (small, round, and visible). The cells were then seeded at 100,000 cells/ml on gelatinised six-well plates (Nunc, Roskilde, Denmark). The cells were allowed to proliferate to 80% confluence for 72 hours of incubation using similar cell culture procedures mentioned above.

3.3.6 Phase 1: *E. longifolia* treatment on C_2C_{12} passage 13 (P13) and passage 26 (P26).

Once 80% confluence, C_2C_{12} cells were treated with 2.0, 1.0, 0.5, 0.25, 0.125 mg/ml of *E. longifolia* and CON. The treated C_2C_{12} cells were then incubated. The image under the microscope was photographed at 0, 24, and 48 hours. Cells treated with the various doses of *E. longifolia* and CON were observed to determine any detachment of cells from the flask.

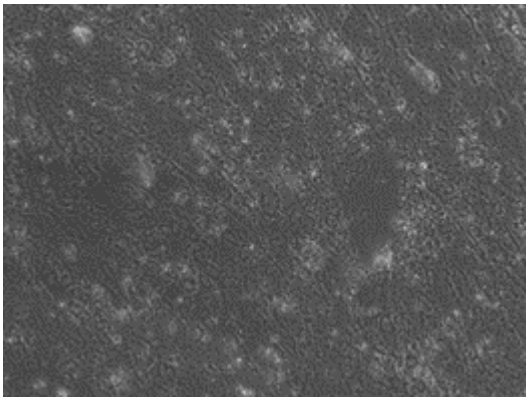


Figure 3.2 Example of cell death through imaging

3.3.7 Phase 2: Lactate Dehydrogenase (LDH) Cytotoxicity

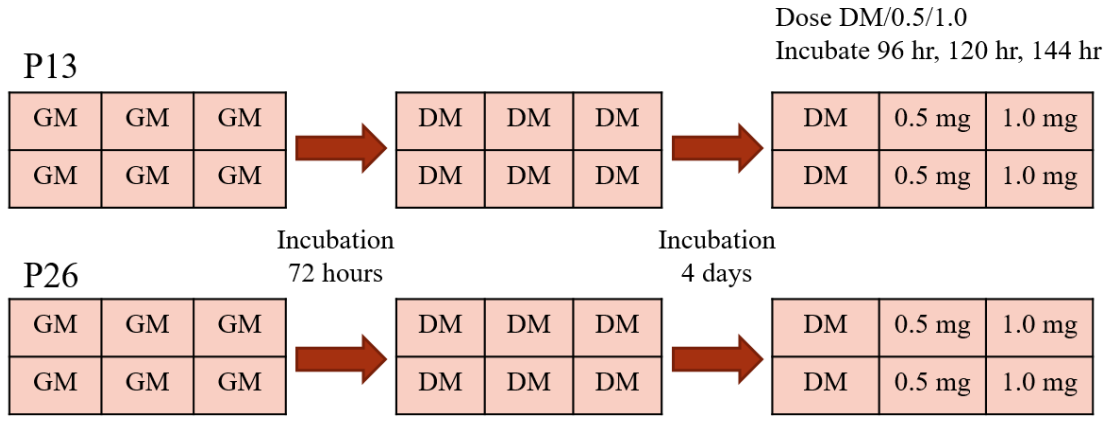
A similar procedure explained in section 3.3.4 was conducted in phase 2. Once C_2C_{12} passage 13 (P13) and passage 26 (P26) achieved 80% confluent, the GM was aspirated from the 6-well plate and washed with PBS. Before *E. longifolia* treatment, fresh DM was then added to the 6-well plate to allow the C_2C_{12} P13 and P26 cells to fused and differentiate. The 6-well plate was incubated for 72 hours. The C_2C_{12} P13 and P26 cells were examined under the microscope to make sure the cells had fused and differentiated. Immediately, the fused cells were washed with PBS and then treated with 1.0 and 0.5 mg/ml of *E. longifolia* and CON. Images were photographed using the microscope, and

supernatants were collected at 96, 120, and 144 hours from differentiated cells, and the cytotoxicity LDH test was conducted.

Cytotoxicity LDH assay was used to quantitate cytotoxicity based on the measurement of LDH activity released from C_2C_{12} cells, which have gone through population doubling and administration of *E. longifolia* extract. Thus, the cytotoxicity detection kit was used to determine cell death after being dosed with *E. longifolia* at two different doses (1.0 and 0.5 mg/ml of *E. longifolia*) and CON.

At each time point (96, 120 and 144 hours), 200 µl of supernatant was harvested from each well and stored at -20 °C until further analysis. The supernatant was thawed, and 100 µl supernatant was transferred into the sterile 96-well tissue culture plate. A total of 50 µl was utilised for the LDH (Roche, Germany) test and was prepared before the cytotoxicity test according to the manufacturer's protocol. According to the manufacturer's instruction, the reaction mix was transferred onto the 96-well plate using a multichannel pipette and was then immediately transferred into a cabinet to avoid bright light. It was kept at room temperature for 20 minutes. An increase in the number of dead or plasma membrane-damaged cells was indicative of an increase in the total LDH enzymatic activity in the cell culture supernatant. This increase in LDH activity directly correlates with the amount of formazan product formed. The red formazan product is water-soluble and has a broad absorbance maximum of around 500 nm. According to the manufacturer's protocol, the value of spectral absorption of the resulting formazan at 490 nm is directly proportional to the number of damaged cells. Thus the samples were analysed via spectrophotometry at 490 nm absorbance using an ELISA plate reader (Clariostar, BMG Labtech, Offenburg, Germany) to quantified LDH activity. Cell

viability was then determined by dividing the treatment mean value divide with the mean control treatment and multiply with 100%.



Note: Photographs at each time points (96, 120 and 144 hours
Samples were analysed using spectrophotometry at 490nm absorbance and ELISA plate reader (Clariostar, BMG Labtech, Offenburg, Germany) was used to quantified LDH activity

Figure 3.3 Skeletal muscle cell culture process prior to LDH assay analysis

3.3.8 Statistical Analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) (v.20, IBM Corporation. New York, US). For the comparison of group means, a *t*-test was used, and where a comparison of multiple time points of means was required, a repeated measure analysis ANCOVA was used. Normal distribution of data sets was first checked for any violation of the assumption of normality using the Shapiro-Wilk test. Data violated the assumption of Mauchly's test of sphericity, Greenhouse-Geiser, or Huyn-Feldt correction factors were used. Significance was assumed when α reached < 0.05. All data are presented as mean ± standard deviation (SD).

3.4 Results

3.4.1 Dose-response on Murine Skeletal Muscle Cell

Dosages used for the phase 1 study were 2.0, 1.0, 0.5, 0.25, and 0.125 mg/ml of *E. longifolia*, while differentiation media (DM) was used as the control treatment (CON). Figure 3.4 shows the image of C_2C_{12} P13 photographed at 0, 24, and 48 hours. Figure 3.4 (A) showed the image of C_2C_{12} P13 before the treatment of *E. longifolia* and CON. Figures 3.4 (B - K) showed the images of C_2C_{12} P13 dosed with 2.0, 1.0, 0.5, 0.25 and 0.125 mg/ml of *E. longifolia* following incubation for 24 hours and 48 hours, respectively. During the incubation period, no cell death was observed, and the cell remains adherent to the flask for C_2C_{12} P13 dosed with 1.0 mg/ml (Figure 3.4 D and E) and 0.5 mg/ml (Figure 3.4 F and G) of *E. longifolia*. However, in phase 1, cell death was only monitored through imaging and cell death was observed in a low dose of 0.25, 0.125 mg/ml, and a high dose of 2.0 mg/ml of *E. longifolia* (Figure 3.4 B, C, H, I, J, K).

Murine skeletal muscle C_2C_{12} P26 cells were also photographed at 0, 24, and 48 hours, as shown in Figure 3.5. Figure 3.5 (A) shows the pre-treatment of *E. longifolia* and CON. C_2C_{12} P26 cells treated with 2.0, 1.0 0.5, 0.25, and 0.125 mg/ml of *E. longifolia* and incubated for 24 and 48 hours are shown in Figure 3.5 B - K respectively, and imaging shows no cell death was observed after the treatment duration, and cells are still adherent to the plate for cell treated with 1.0 and 0.5 mg/ml of *E. longifolia*. However, general observation through imaging shows cell death was observed in C_2C_{12} P26 cells at 48 hours with 2.0 mg/ml (Figure 3.5 C), 0.25 mg/ml (Figure 3.5 I) and 0.125 mg/ml (Figure 3.5 K) treatment.

3.4.2 Cytotoxicity lactate dehydrogenase effect of *E. longifolia* on murine skeletal muscle C_2C_{12}

Through imaging, general observation shows that cells treated with E. longifolia (0.5 mg/ml and 1.0 mg/ml) are still adherent to the flask and did not observed any cell detached. However, images showed larger myotube (Figure 3.6 D, E) growth when compared to P13 dosed with 1.0 mg/ml (Figure 3.6 B, C) of *E. longifolia*. Also, skeletal muscle C_2C_{12} P26 cells dosed with 0.5 mg/ml also showed larger myotube at 144 hours (Figure 3.7 E) when compared to P26 dosed with 1.0 mg/ml of *E. longifolia* at 120 and 144 hours (Figure 3.7 B, C) and CON at 96 hours (Figure 3.7 A). Similar to P13, C_2C_{12} P13 and P26 treated with E. longifolia remained adherent to the flask and did not observe any cell death.

Meanwhile, Figure 3.8 shows the cytotoxicity activity at 96, 120, and 144 hours post *E. longifolia* administration on P13 and P26 C_2C_{12}. There was no significant main interaction between group and time for LDH activity ($F_{1.066,\ 12.791} = 0.060$, $p = 0.825$). However, there is significant main effect of time ($F_{1.066,\ 12.791} = 17.419$, $p = 0.001$) (Figure 3.8 A, B, C).

ANCOVA repeated measurement showed significant increase in LDH activity in P13 C_2C_{12} treated with CON from 96 hours [Mean(M) = 0.138, 95% CI = 0.116, 0.160] to 120 hours (M = 0.438, 95% CI = 0.224, 0.653) and 144 hours (M = 0.612, 95% CI = 0.188, 1.035). A significant increase in LDH activity in P13 C_2C_{12} treated with 0.5 mg/ml *E. longifolia* from 96 hours (M = 0.138, 95% CI = 0.116, 0.160) to 120 hours (M = 0.425, 95% CI = 0.237, 0.614) and 144 hours (M = 0.526, 95% CI = 0.230, 0.822).

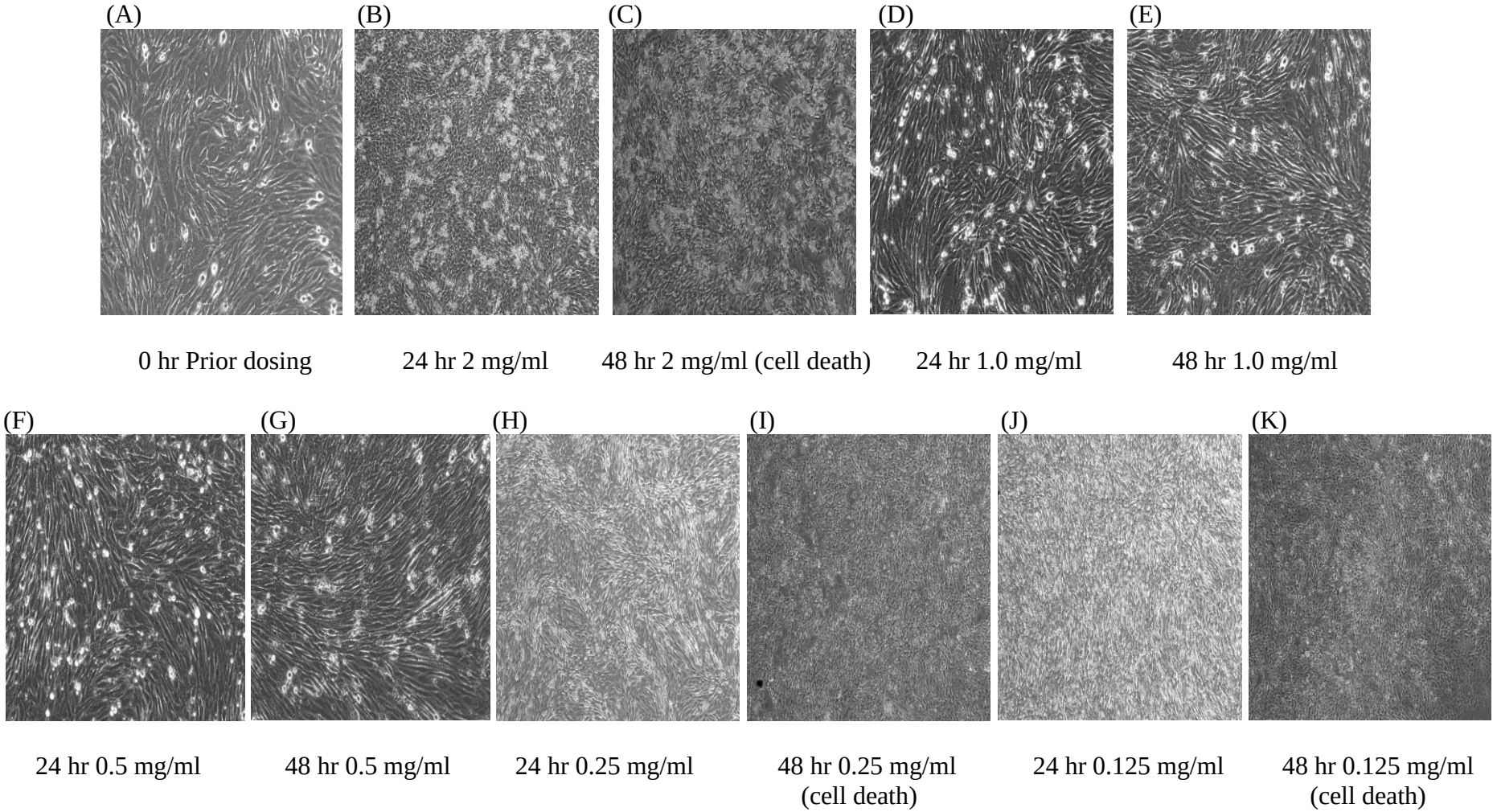


Figure 3.4: ***E. longifolia*** **treatment at different dosage on passage 13 C_2C_{12} murine skeletal muscle cell**

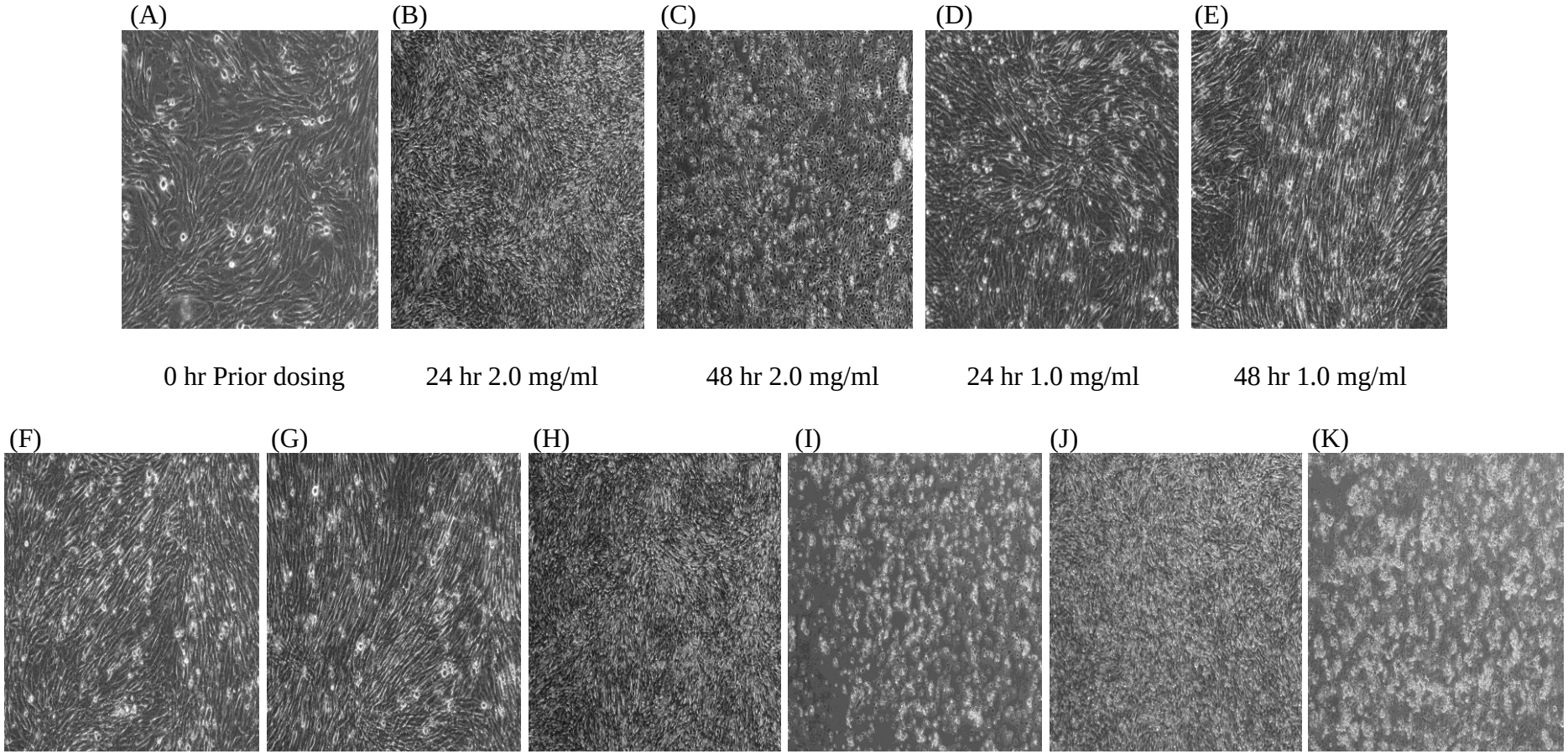
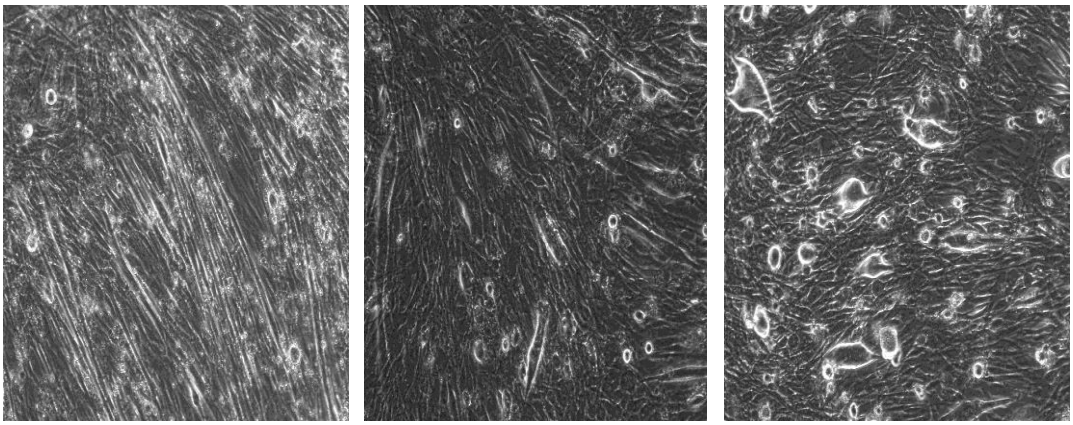


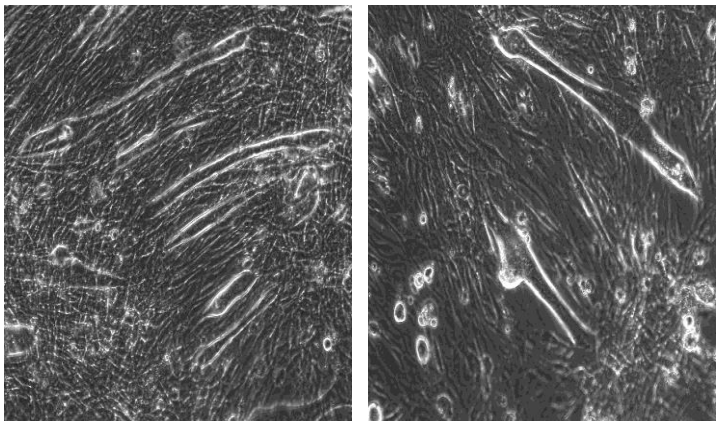
Figure 3.5: Pre-treatment and post *E. longifolia* treatment on passage 26 C_2C_{12} murine skeletal muscle cell

(A) (B) (C)



P13 DM 96 hr Prior dosing P13 1.0 mg/ml 120 hr P13 1.0 mg/ml 144 hr

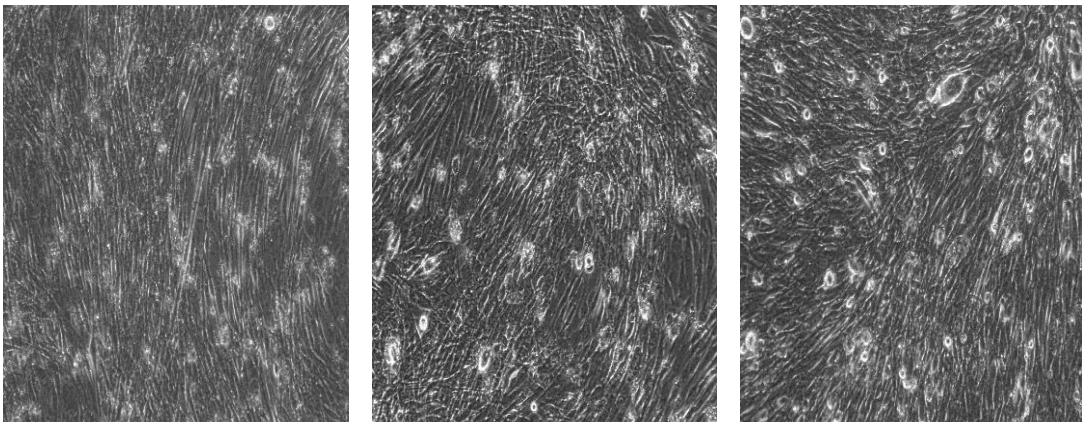
(D) (E)



P13 0.5 mg/ml 120hr P13 0.5 mg/ml 144hr

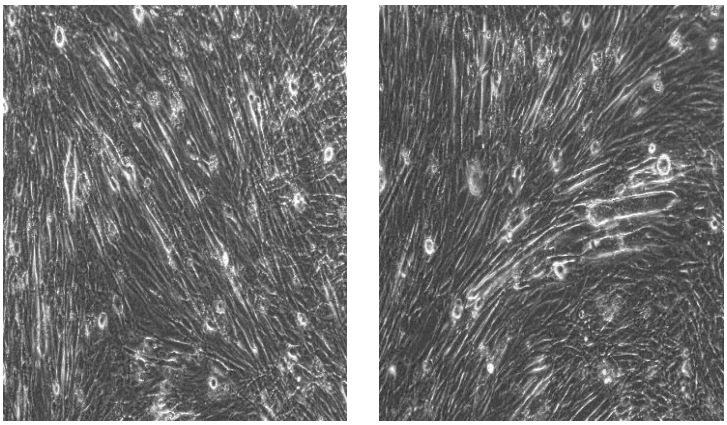
Figure 3.6: Pre treatment and post *E. longifolia* treatment at two different dosages (0.5 and 1.0 mg/ml of *E. longifolia*) dosed after cells are fused on passage 13 C_2C_{12} murine skeletal muscle cell

(A) (B) (C)



P26 DM 96 hr Prior dosing P26 1.0 mg/ml 120 hr P26 1.0 mg/ml 144 hr

(D) (E)



P26 0.5 mg/ml 120 hr P26 0.5 mg/ml 144 hr

Figure 3.7: Pre-treatment and post *E. longifolia* treatment at two different dosages (0.5 and 1.0 mg/ml of *E. longifolia*) dosed after cells are fused on passage 26 C_2C_{12} murine skeletal muscle cell

Similarly, significant increase in C_2C_{12} treated with 1.0 mg/ml *E. longifolia* from 96 hours (M = 0.138, 95% CI = 0.116, 0.160) to 120 hours (M = 0.345, 95% CI = 0.215, 0.476) and 144 hours (M = 0.448, 95% CI = 0.260, 0.635). While P13 treated with CON shows 3.27 fold increase from 96 hours to 120 hours, P13 treated with 0.5 mg/ml and 1.0 mg/ml demonstrate only 3.2 and 2.58 fold increase respectively in LDH activity from 96 hours to 120 hours. Finding also found that P13 treated with CON has a higher cell viability of 327 ± 189 % compare to P13 treated with 0.5 mg/ml (320 ± 169 %) and 1.0 mg/ml (258 ± 120 %) of *E. longifolia from* 96 hours to 120 hours. Also, LDH activity from 96 to 144 hours also shows P13 treated with CON has higher fold increase (4.61) in LDH activity compared to 3.92 and 3.35 fold increase in P13 treated with 0.5 mg/ml and 1.0 mg/ml, respectively. Similarly, CON has a non significant higher cell viability of 461 ± 362 % compared to P13 treated with 0.5 mg/ml (392 ± 257 %) and 1.0 mg/ml (335 ± 169 %) of *E. longifolia* from 96 hours to 144 hours. A lower LDH activity in P13 dosed with *E. longifolia* (CON > 0.5 mg/ml > 1.0 mg/ml) was observed.

Similar findings were mostly observed in P26 cells treated with DM and *E. longifolia* extract. ANCOVA repeated measurement showed no significant increase in LDH activity in P26 treated with DM from 96 hours (M = 0.124, 95% CI = 0.115, 0.133) to 120 hours (M = 0.384, 95% CI = 0.100, 0.668) but an increase from 96 hours to 144 hours (M = 0.544, 95% CI = 0.123, 0.965) was observed.

A significant increase in LDH activity in P26 C_2C_{12} treated with 0.5 mg/ml *E. longifolia* from 96 hours (M = 0.124, 95% CI = 0.115, 0.133) to 120 hours (M = 0.330, 95% CI = 0.177, 0.484) and 144 hours (M = 0.413, 95% CI = 0.182, 0.644). Similarly, significant increase in C_2C_{12} treated with 1.0 mg/ml *E. longifolia* from 96 hours (M = 0.124, 95% CI = 0.115, 0.133) to 120 hours (M = 0.327, 95% CI = 0.138, 0.517) and 144

hours (M = 0.486, 95% CI = 0.176, 0.795). Similarly, P26 treated with CON shows a 3.02 fold increase in LDH activity from 96 hours to 120 hours, and P26 treated with 0.5 mg/ml and 1.0 mg/ml demonstrate a lower increase in LDH activity of 2.61 and 2.59 fold increase respectively from 96 hours to 120 hours. P26 treated with CON has a non-significant higher cell viability of 302 ± 231 % compare to P13 treated with 0.5 mg/ml (261 ± 122 %) and 1.0 mg/ml (259 ± 150 %) of *E. longifolia from* 96 hours to 120 hours. Also, LDH activity from 96 to 144 hours shows P26 treated with CON has a higher fold increase (4.28) in LDH activity compared to 3.27 and 3.84 fold increase in P26 treated with 0.5 mg/ml and 1.0 mg/ml, respectively. Cell treated with CON demonstrated higher cytotoxicity compared to the two *E. longifolia* doses. The lower dose (0.5 mg/ml) of *E. longifolia* demonstrated lower cytotoxicity compared to the higher *E. longifolia* dose (1.0 mg/ml after 48 hours of treatment in P26 C_2C_{12}. In addition, CON has a higher cell viability of 428 ± 345 % compared to P26 treated with 0.5 mg/ml (327 ± 188 %) and 1.0 mg/ml (384 ± 250 %) of *E. longifolia* from 96 hours to 144 hours.

A repeated study (Figure 3.9) with similar cell culture procedure and LDH assay test were conducted to validate the previous findings. A paired-samples t-test was conducted to compare three different treatments treated in P13 and P26 C_2C_{12}. Whilst a significant difference in the scores for P26 treated with CON (M = 0.344, SD = 0.043) and P26 treated with 0.5mg/ml *E. longifolia* (M = 0.291, SD = 0.032) conditions; t(5) = 5.072, p = 0.004, there are no significant difference in P26 treated with CON with P26 treated with 1.0 mg/ml *E. longifolia* (M = 0.320, SD = 0.042); t(5) = 2.219, p = 0.077. Also, there is a significant difference between P26 treated with 0.5 mg/ml and 1.0 mg/ml of *E.longifolia* (t_5 = -3.817, p = 0.012). These results suggest that C_2C_{12} cell treated with 0.5 mg/ml *E. longifolia* has lower LDH activity compared to 1.0 mg/ml *E. longifolia* and CON.

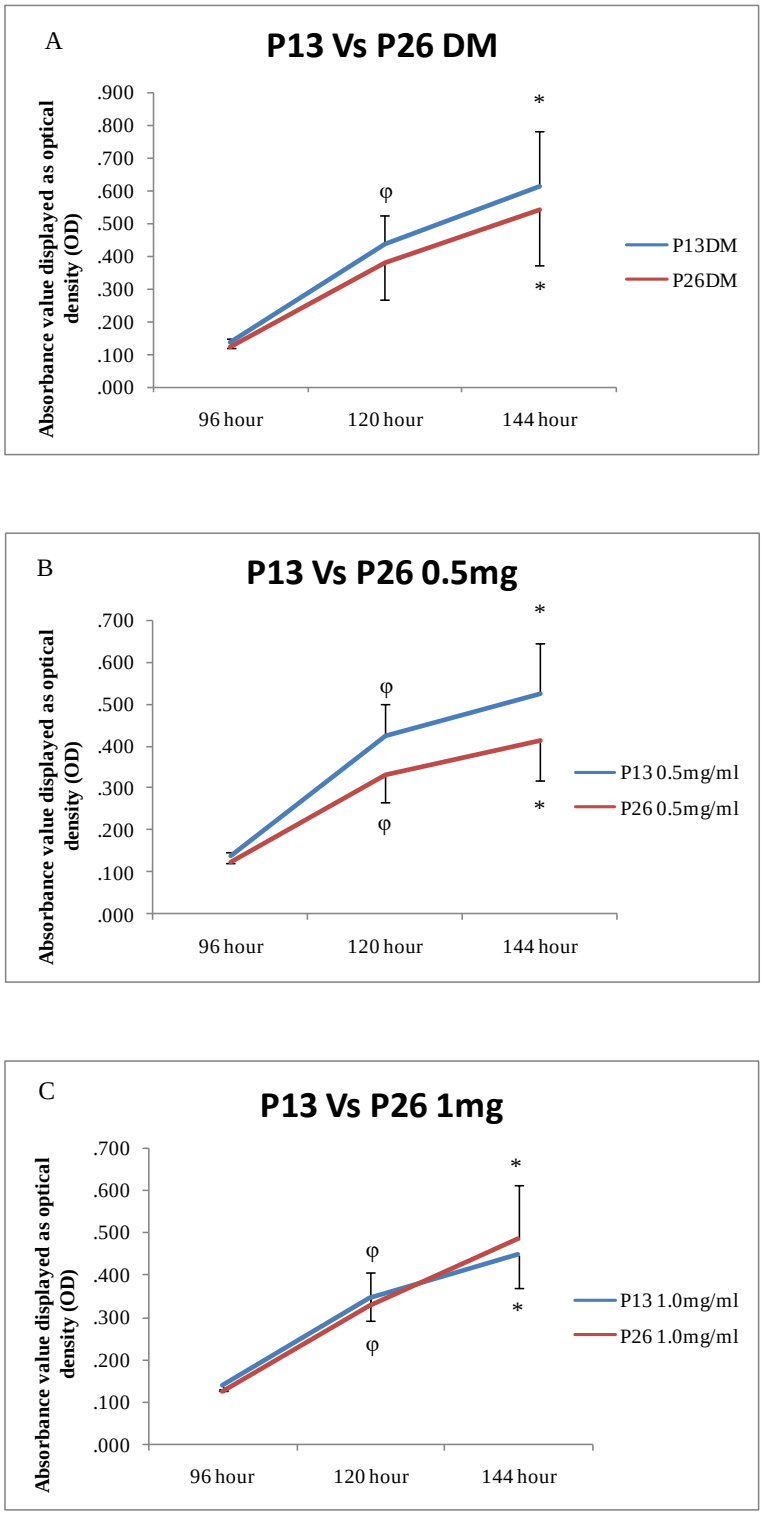


Figure 3.8: Effect of *E. longifolia* (0.5 and 1.0 mg/ml) and DM (control) treated on C_2C_{12} (P13 and P26) on the lactate dehydrogenase (LDH) activity over time

φ, *Denotes indicate there is a significant change over time; φ Denotes indicate a significant increase from 96-120 hour; * Denotes indicate a significant increase from 96 -144 hours.

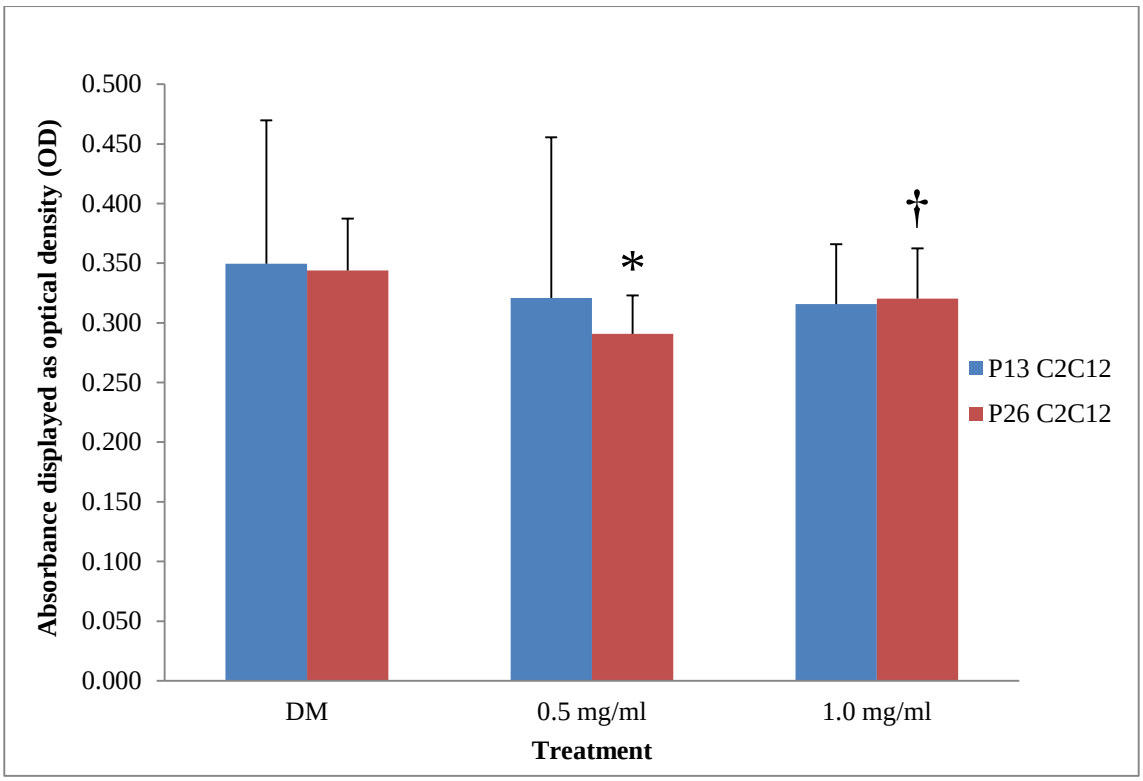


Figure 3.9: Lactate dehydrogenase activity differences within and between P13 and P26 treated with three different treatment doses at 144 hours.
*† Denotes indicate significant differences within P13 and P26 C_2C_{12} cells; *Denote indicate a significant lower LDH activity between DM and 0.5 mg/ml; †Denotes indicate significant differences between 0.5 mg/ml and 1.0 mg/ml

However, P13 C_2C_{12} treated with the three treatment did not show any significant differences. No significant differences in the scores for P13 treated with CON (M = 0.350, SD = 0.120) and P13 treated with 0.5mg/ml *E. longifolia* (M = 0.321, SD = 0.135) conditions; t (5) = 1.903, p = 0.115. Also, no significant difference in P13 treated with CON with P13 treated with 1.0 mg/ml *E. longifolia* (M = 0.316, SD = 0.050); t (5) = 0.577, p = 0.589. Lastly, no significant difference between P13 treated with 0.5 mg/ml and 1.0 mg/ml of *E. longifolia* [(t (5) = 0.080, p = 0.939]. Although no significant differences were observed, *E. longifolia* treated cells demonstrated lower LDH activity compared to the control treatment CON.

Whilst findings show some differences within the two cells, there are no differences between P13 and P26 when the cells are treated with CON, and the two *E. longifolia* doses. There was no significant difference in the LDH activity between P13 and P26 treated with CON [t (10) = 0.105, p = 0.918], 0.5 mg/ml *E. longifolia* [t (10) = 0.531, p = 607], and 1.0 mg/ml *E. longifolia* [t (10) = -0.174, p = 0.865].

3.5 Discussion

The present study aims to examine *E. longifolia* water extract's cytotoxicity on murine SMC C_2C_{12} cell line for the first time. Various concentrations of *E. longifolia* were used to determine the appropriate dosage, and the chosen dosage (0.5 mg and 1.0 mg/ml) was then used to examine the cytotoxicity effect on C_2C_{12} murine SMCs. The cells treated with 0.5 mg/ml and 1.0 mg/ml demonstrated lower cytotoxicity level compared to cells treated with DM as a control treatment. Also, an increase in cell viability in C_2C_{12} P13 and P26 treated with *E. longifolia*, indicating potential cell growth.

General observation through imaging found that *E. longifolia* concentrations of 2.0, 0.25, and 0.125 mg/ml administered to C_2C_{12} resulted in cell detaching from the flask, but this was not the case for 0.5 mg/ml and 1.0 mg/ml concentrations. Hence this concentration was used to examine the cytotoxicity effect. Differentiated C_2C_{12} (P13 and P26) cell lines were dosed with 0.5 and 1.0 mg/ml and incubated for 120 and 144 hours. During this incubation period, general observation showed the myotube increased in size compared to cells treated with DM. Since this is the first study of its kind, the reason for the increase in myotube size is still not apparent.

Whilst LDH activity was used to examine cytotoxicity and cell viability upon *E. longifolia* treatment, the pathway causing cell death (apoptosis or necrosis) due to

cytotoxicity was not determined in the current study. Lactate dehydrogenase (LDH) is a cytosolic enzyme available in numerous different cells types, e.g. SMCs. As soon as the plasma membrane is damaged, LDH is released into the cell culture media. Cell membrane damage leads to the release of cytoplasmic enzymes, and the measurement of LDH release was used to estimate cell membrane integrity and to quantify cell cytotoxicity (Mosmann, 1983; Korzeniewski & Callewaert, 1983; Roehn *et al*., 1991; Legrand *et al*., 1992; Arechabala *et al*., 1999; Choi *et al*., 2009). Once cells are impaired by stress, injury, chemicals or intercellular signals, LDH is rapidly released from the cell membrane. The amount of released LDH into the surrounding extracellular space after the cell membrane integrity is compromised can be used as a cell death marker (Kumar *et al*., 2018).

The LDH cytotoxicity test demonstrated a non-significant increase in LDH level over time in P13 and P26 dosed with 0.5 mg/ml and 1.0 mg/ml of *E. longifolia.* Interestingly the LDH activity level was lower than the CON, indicating a possible reduction in cytotoxicity or cell death rate. While there is no study conducted on C_2C_{12} prior to the current study, Thu *et al*. (2017) found *E. longifolia* is less toxic than the control treatment, and the extract increased proliferation. The fold increase of cytotoxicity level in *E. longifolia* treated cells was lower when compared to the cells treated with CON. While lower cytotoxicity was observed in C_2C_{12} cell, the previous study has shown that higher concentrations and longer durations of *E. longifolia* treatment with cancer cells inhibit cell growth and cell death occurs (Hajjouli *et al*., 2014; Tong *et al*., 2015). In addition, cell viability also demonstrated that C_2C_{12} treated with *E. longifolia* has no significant difference with CON, indicating the murine cells survived after being treated with *E. longifolia*. Also, 1.0 mg/ml of *E. longifolia* has shown higher cell viability compared to 0.5mg/ml for P26. It is suggested that the high concentration of superoxide dismutase (SOD) found in *E. longifolia* (Tambi & Imran, 2010) accelerate the bone formation and

reduce the rate of resorption (Effendy *et al*., 2012). Hence, the high concentration of SOD may potentially reduce harmful oxygen molecules in cells to prevent damage to tissues and subsequently increase the proliferation, decreased apoptosis and growth (Hollander *et al*., 1999; Che *et al*., 2016), which demonstrate why C_2C_{12} treated with *E. longifolia* has lower cytotoxic activity compared to the control treatment and reported higher cell viability in C_2C_{12} especially in P26. Thus indicating that there is a dose response towards older murine cell culture. Hence, the present study concurs with previous studies that *E. longifolia* treatment of healthy cells is non-toxic towards healthy cells (Hajjouli *et al*., 2014; Thu *et al*., 2017).

Another possible reason behind these findings could be due to the availability of *E. longifolia*'s bioactive compound, which comprised of 1% eurycomanone, 22% protein, 30% polysaccharides, and 35% glycosaponin (Lin *et al*., 2001; Tambi & Kadir, 2005; Athimulam *et al*., 2006). The protein component (a type of protein not mentioned in any published article) in *E. longifolia* may contribute to the growth in hypertrophy. A review of muscle protein synthesis in response to nutrition by Atherton and Smith (2012) found that the anabolic effects of a meal were attributable to essential amino acids (Smith *et al*., 1992). Atherthon and Smith (2012) further suggest that the anabolic response is transient, and if the adaptive increase in muscle protein breakdown is neglected, hypertrophy can be achieved by just eating protein. However, more studies to examine the effect of the *E. longifolia* component, especially the protein, in increasing hypertrophy are needed.

The administration of *E. longifolia* to SMCs demonstrate a different response when compared to cancer cells, where inhibition in growth and cell death is not observed. One possible reason that *E. longifolia* provokes lower LDH activity than the control treatment may be due to the other compounds belonging to the alkaloid class and not just eurycomanone. Alkaloids and triterpene act as antioxidants and have been reported to

reduce bone loss and maintain the bone formation rate (Effendy *et al*., 2012). Although not proven in the present study, triterpenes in *E. longifolia* may increase murine skeletal muscle. Kunkel *et al*. (2011) and Kunkel *et al*. (2012) found that ursolic acid, a natural triterpene found in various herbs, can induce skeletal muscle hypertrophy by increasing the protein kinase Akt in murine skeletal muscle and cultured C_2C_{12} skeletal myotube. Hence a further study looking into this compound in inducing SMC hypertrophy is warranted.

The current study on the cytotoxicity effect of *E. longifolia* on C_2C_{12} has demonstrated low toxicity level and increased cell viability in both P13 and P26. Whilst finding demonstrate *E. longifolia* is not toxic to C_2C_{12} cell line and even increased cell viability, the possible factors contributed to the study outcome such as the concentration of SOD (Tambi & Imran, 2010), protein contents (Lin *et al*., 2001; Tambi & Kadir, 2005; Athimulam *et al*., 2006), triterpene (Kunkel *et al*., 2012) was not examined. Also, whether *E. longifolia* prevent cell growth or promote growth, investigating the signalling pathway of apoptosis and necrosis is warranted. While the C_2C_{12} cell line derived from murine SMCs is a useful cellular model for investigating muscle regeneration and differentiation (Schöneich *et al*., 2014), the data presented here served as a preliminary study investigating for the first time its effect on C_2C_{12} cell line and may not precisely mirror human.

In this preliminary study, *E. longifolia* treatment on C_2C_{12} resulted in lower toxicity and increased cell viability indicating an increase in growth. However, more studies, such as an evaluation of the cell death pathway, myogenic potential (myogenic differentiation (MyoD) and myogenin) of *E. longifolia*, the pharmacological effect of superoxide dismutase in *E. longifolia*, and the protein content in *E. longifolia* are warranted to strengthen the outcome of the current study.

3.5.1 Conclusion

Eurycoma longifolia was examined for the first time for its dose-response and cytotoxicity effect on the murine C_2C_{12}. The present study has shown that there was a dose-dependent effect toward C_2C_{12} murine SMCs. A dose of 0.5 and 1.0 mg/ml of *E. longifolia* did not induce cell death, but there was an increase in cell viability, indicating possible myotube growth. Interestingly cytotoxicity levels increased in the control treatment over time, but C_2C_{12} cells treated with *E. longifolia* showed lower toxicity. Also, *E. longifolia* showed a favoured response in reducing toxicity in ageing cells. Thus, *E. longifolia* could be beneficial in slowing down age-related muscle loss.

CHAPTER 4: EFFECT OF SHORT AND LONG-TERM CONSUMPTION OF *E. Longifolia* ON REPRODUCTIVE HORMONE REGULATION AMONG ADULT MALES

4.1 Introduction

In recent years, *E. longifolia* has gained popularity due to its ability to increase the production of reproductive hormones such as testosterone, which is important in the development of secondary male characteristics such as voice deepening, increase in penis size, and growth of facial and body hair. Although studies have examined the effectiveness of *E. longifolia* in enhancing testosterone levels, clinical trials are scarce, and the findings based on the limited studies are inconclusive: some show an increase (Tambi *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2013; Udani *et al*., 2014) and others a decrease (Ismail *et al*., 2012; Chen *et al*., 2014). Also, the underlying mechanisms on how *E. longifolia* regulates testosterone levels in humans have not been examined.

The regulation of testosterone production is controlled by the hypothalamic gonadal (HPG) axis to ensure normal blood concentrations are maintained (Palaparthi, 2017). Testosterone is the primary regulator of gonadotrophin-releasing hormone (GnRH) secretion, which stimulates LH and FSH production from the anterior pituitary, which in turn stimulates the production of testosterone and sperm by the Leydig cells and Sertoli cells (Bhatnagar *et al*., 1992). A study examining the effect of *E. longifolia* supplementation in rats has shown similar testosterone regulation in humans, with an increase in testosterone level, LH and FSH. (Low *et al*., 2013b). Indeed, based on the findings of Low *et al*. (2013), it is proposed that the *E. longifolia* treatment down-regulates the negative feedback, stimulating the pituitary gland to release LH and FSH,

which subsequently increases testosterone production (Tambi *et al*., 2012; Low *et al*., 2013b).

Most related studies have been carried out on older participants, particularly those diagnosed with late-onset of hypogonadism (LOH). The optimal duration of treatment is debatable, where short-term supplementation (2 - 5 weeks) (Qureshi *et al*., 2014) shows an increase in testosterone, while longer-term (> 5 weeks) (Quinn *et al*., 2010) does not show an increase. Moreover, *E. longifolia* supplementation for 4 to 12 weeks (long-term) does not demonstrate any testosterone boosting effect. Tachyphylaxis and drug tolerance have been used to describe the phenomena causing reduced drug effectiveness. Tachyphylaxis is known to develop quickly when drugs are used repeatedly over a short period, with no response occurring when the dosage is increased. However, the effect can be restored by stopping the administration for a short time (Binder, 2012). On the other hand, tolerance is known as the prolonged administration of a drug at a set dose, producing either a reduced effect or no further change in response (Branch, 1993). The drugs' effect can be improved by increasing the dosage given over a short time interval, but efficacy is not restored if the treatment is halted temporarily (Binder, 2012). Based on this, it is speculated that the effect of *E. longifolia* may have achieved tolerance due to dosage (Ismail *et al*., 2012; Chen *et al*., 2014; George *et al*., 2018; Chen *et al*., 2019).

Apart from older adults, there are a substantial number of reports showing that the younger population are affected by psychological stress (Vo *et al*., 2008; Sifferlin, 2013; Pascoe *et al*., 2019); however, to date, there is no study investigating the effect of *E longifolia* on this population. The transition from childhood to adulthood involves the attainment of sexual maturity and the attainment of a variety of psychological and social factors (Sisk and Zehr, 2005; Lürzel *et al*., 2010). Many have perceived that testosterone

treatment improves or alleviates psychological outcomes such as mood, stress and wellbeing in men with low testosterone levels and have even suggested that testosterone levels are reduced in response to stress (Choi *et al*., 2012). Many stressors, such as psychological stress and physical stress, decrease LH by inhibiting its release by the anterior pituitary, leading to decreased testosterone production (Rivest & Rivier, 1995; Chichinadze & Chichinadze, 2008).

It is uncertain at present whether short and long-term administration with a higher dose of *E. longifolia* supplementation will have any impact on reproductive hormones in the younger population due to its testosterone boosting effect. Hence, an investigation is needed to determine the changes in testosterone, free testosterone, luteinizing hormone (LH), follicle-stimulating hormone (FSH), oestrogen and sexual hormone-binding globulin (SHBG) concentration levels to understand the mode of action of *E. longifolia*. In order to demonstrate a more significant improvement, appropriate dosage and duration are needed to prevent any possible tolerance. In this study, the dosage, duration (short and long-term) of supplementation, age and health of participants were taken into consideration to enable a thorough investigation into the efficacy of *E. longifolia* on the changes in reproductive hormones.

Therefore, this study aims to examine the effect of *E. longifolia* on male reproductive hormone regulation in healthy young adults and assess the toxicity effect of *E. longifolia* on liver function after two and eight weeks of supplementation.

4.2 Literature Review

4.2.1 The effects of *Eurycoma longifolia* on testosterone production

To date, nine studies have investigated the effect of *E. longifolia* on testosterone levels in humans and animals (Tables 4.1 and 4.2). In humans, most studies show an increase in testosterone levels following *E. longifolia* supplementation. Data from many of them were extracted from participants diagnosed with a history of LOH (Tambi *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2013; Udani *et al.,* 2014), with ages ranging from 30 to 72 years. However, there are three studies in which testosterone levels did not change when supplemented with *E. longifolia* (Ismail *et al*., 2012; Chen *et al*., 2014; George *et al*., 2018). The lack of change could be attributed to the participants' characteristics, including health status and physical activity level. It is also worth noting that greater changes in testosterone levels were seen when initial levels were low (LOH and moderately stressed individuals) compared to a healthy individual with normal testosterone levels. *E. longifolia* supplementation appeared to restore the testosterone concentration to the optimal level among LOH and moderately stressed participants (Talbott *et al*., 2013). However, as time passed, *E. longifolia* demonstrated a diminishing effect in increasing testosterone levels. Hence, studies examining a higher dose of E. longifolia supplement on healthy young men level at different duration to determine its efficacy in increasing testosterone production in a population that has normal testosterone level is needed.

Table 4.1: Studies conducted to examine the effect of *E. longifolia* on testosterone levels in human

Dose (Duration)	Outcome	Participants	References
200 mg (4 weeks)	Increased T but maintained at normal values	LOH patients (30 - 64 years)	Tambi *et al*. (2012)
300 mg (12 weeks)	No significant changes in T, IGF-1, SHBG, and DHEASO4	Healthy males (30 - 55 years)	Ismail *et al*. (2012)
200 mg (4 weeks)	Increase T	Sub-fertile and moderately psychological stressed male (45 - 65 years)	Talbott *et al*. (2013)
400 mg (5 weeks)	Increase T and FT	Physically active male and female (57 - 72 years)	Henkel *et al*. (2014)
300 mg *EL* and *PM* (12 weeks)	Increased T	Healthy males (40 - 65 years)	Udani *et al*. (2014)
400 mg (6 weeks)	No difference in the T and EP ratio	Recreational athletes (29.0 ± 5.5 years)	Chen *et al*. (2014)
50 mg EL and MV (24 weeks)	No changes in T, increased in FT	Moderately stressed male (25 - 65 years)	George *et al*. (2018)
200 mg EL 8 weeks	No significant changes in T/E ratio	Young male (19 – 25 years)	Chen *et al*. (2019)

Note: Late-onset of hypogonadism (LOH); *E. longifolia* (EL); *Polygonum minus* (PM); Testosterone (T); Epitestosterone (EP); Free testosterone (FT) Insulin-like growth factor-1 (IGF-1); Sexual hormone-binding globulin (SHBG); Dehydroepiandrosterone sulfate (DHEASO4)

4.2.2 Factors Affecting the Regulation of Testosterone Due to *Eurycoma longifolia* Consumption.

According to the findings shown in Table 4.1, several inter-related factors, such as dose and duration of supplementation and participants' age and health status, may affect the regulation of testosterone due to *E. longifolia* consumption. Supplementation of 200 and 400 mg of *E. longifolia* in older participants with LOH or moderately stressed participants demonstrated a significant increase in testosterone level (Tambi *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2013); however, this was not observed by Chen *et al*. (2019) which they supplemented *E. longifolia* on young healthy adults with no history of

low testosterone level. While LOH is a syndrome associated with advancing age and characterised by symptoms and a deficiency in serum testosterone levels (Huhtaniemi, 2014; Wang *et al*., 2009), evidence suggests that there is also a reduction in testosterone levels in response to stress (Afrisham *et al*., 2016). Studies have also shown that low testosterone levels were reported in individuals facing psychological stress, physical stress, actual stress, anxiety and irritability (Schulz *et al*., 1996; Sternbach,1998; Choi *et al*., 2012). Rivest and Rivier (1995) indicated that stressors, such as psychological stress, decrease LH and, consequently, testosterone level by inhibiting the secretion of LH from the pituitary gland. Thus the potential mechanism of *E. longifolia* in increasing testosterone levels may be its ability to stimulate the secretion of LH to produce more testosterone in the HPG axis and, subsequently, increase the level of testosterone in this group of individuals. However, the level of LH during stress remains conflicting and not clearly described. Thus examining the gonadotropin-releasing hormone responsible for the release of LH and FSH from the anterior pituitary may be vital in understanding whether *E. longifolia* stimulated LH and FSH in the HPG axis.

After the age of 30, there could be a 1% decline in testosterone level per year due to ageing (Belanger *et al*., 1994; Litman *et al*., 2006). Participants from previous studies (Table 4.1) were mainly between the ages of 30 to 72 years and diagnosed with LOH symptoms and stress that may contribute to a lower testosterone level. At present, the dose administered in humans ranges from 100 mg to 400 mg and is based on limited studies: a low dose might be considered below 300 mg, while above 300 mg might be considered a high dose. Studies by Ismail *et al*. (2012) and Chen *et al*. (2019) show that the administration of a low dose of *E. longifolia* shows no increase in testosterone level in a healthy male with normal testosterone levels (19 to 55 years). These indicate that this dose of *E. longifolia* has no effect on physically active young and older healthy

participants with testosterone levels within the normal concentration range. Hence a higher dose may be warranted to demonstrate a significant effect.

Studies using a combination of 300 mg of *E. longifolia* with *Polygonum minus* and 50 mg of *E. longifolia* with multivitamins for 12 weeks and 24 weeks respectively showed an increase in testosterone and free testosterone (Ismail *et al*., 2012; Udani *et al*., 2014). *Polygonum minus* is a herbal plant that contains antioxidants, flavonoids and phenolic compounds (Qader *et al*., 2012). Apart from quassinoids, which are often said to be the compound that induces testosterone elevation, flavonoids in *Polygonum minus* may also contribute to the increase in testosterone (Zohre *et al*., 2015). Hence, with the combination of *E. longifolia* and *Polygonum minus*, there is an increase in the amount of bioactive compound especially in quassinoids and flavonoid responsible for the increase in testosterone and may not solely from *E. longifolia*. In addition, a dosage above 300 mg of *E. longifolia* has demonstrated an increase in testosterone levels among healthy participants (Henkel *et al*., 2014). A recent study by Chen *et al*. (2019) has shown that 200 mg of *E. longifolia*, supplemented daily for eight weeks, did not increase testosterone levels, and it is suggested that the dose is insufficient to elicit any effects in young individuals.–Studies are needed to investigate the effects of flavonoids in increasing testosterone levels and at a different dosage.

The duration of supplementation may be another potential factor that may determine the effectiveness of *E. longifolia*. In general, two to five weeks of dietary supplementation is considered short-term (Qureshi *et al*., 2014), while over five weeks might be considered long-term (Quinn *et al.,* 2010). Apart from *E. longifolia*, a multitude of herbal supplements have the potential to increase testosterone after a period of supplementation: for example, *astragalus membranaceus* (Jiang *et al*., 2006; Kim *et al*.,

2012), *fenugreek* (Bushey *et al*., 2009; Steels *et al*., 2011), *barrenwort* (Zhang *et al*., 2011), *ginseng* (Fahim *et al*., 1982), *passiflora incarnate* (Dhawan & Sharma, 2002 & 2003), *mucuna pruriens* (Shukla *et al*., 2009; Shukla *et al*., 2010; Muthu & Krishnamoorthy, 2013), *ginkgo biloba* (Markowitz *et al*., 2005; Ahmed *et al*., 2009), *Tribulus Terrestris* (Neychev & Mitev, 2005; Rogerson *et al*., 2007; Wankhede *et al*., 2014; Maheshwari *et al*., 2017) and *ashwagandha* (Sengupta *et al*., 2018; Lopresti *et al*., 2019). These herbal supplements have been supplemented between 2 to 12 weeks in both animals and humans. Supplementation for longer than four weeks in humans did not show any increase in testosterone levels. The shortest duration of *E. longifolia* supplementation is four weeks and the longest 12 weeks. Previous studies shown in Table 4.1 have demonstrated that short-term supplementation might increase testosterone levels (Tambi *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2014), but long-term supplementation has demonstrated otherwise (Ismail *et al*., 2012; Chen *et al*., 2014; George *et al*., 2018). Supplementation of *E longifolia* lasting between 6 and 12 weeks did not demonstrate any increase in testosterone levels. However, the testosterone level remains in the healthy range (Ismail *et al*., 2012; Chen *et al*., 2014; George *et al*., 2018).

Tolerance is often said to happen when the effect of a specific drug dose decreases over some time, and to further improve on the efficacy, an increase in the dosage is required (Branch, 1993; Siegel, 2005; Campbell & Cohall, 2017). However, tachyphylaxis is another term used to demonstrate the effect of repeatedly used drugs over a short period with no response occurring when the dosage is increased (Binder, 2012). Repeated exposure to *E. longifolia* longer than six weeks may cause gradual diminishing effects of the herbal extract, and it is speculated that *E. longifolia* has achieved tolerance and not tachyphylaxis since few studies have demonstrated an increase in testosterone level with a high dose of *E. longifolia* (Ismail *et al*., 2012; Chen *et al*., 2014; George *et*

al., 2018; Chen *et al*., 2019). However, *E. longifolia* has not been used to investigate the aspect of tolerance and tachyphylaxis thoroughly. The effect of *E. longifolia* on testosterone regulation needs to be examined further through continuous monitoring of different dosages and duration of supplementation.

4.2.3 The Postulated Mechanism of Action on *E. longifolia* Supplementation.

The hypothalamus and pituitary gland regulate the circulating concentration of testosterone and testosterone production. Upon detecting low circulating testosterone levels, the gonadotrophin-releasing hormone (GnRH) is secreted by the hypothalamus to the anterior pituitary, where it stimulates the production of LH and FSH. Following that, LH and FSH then stimulate the Leydig cells and Sertoli cells in the testes to produce testosterone and stimulate spermatogenesis. An elevated testosterone concentration in men causes negative feedback inhibition of the HPG axis by decreasing GnRH, LH and FSH from the hypothalamus and pituitary gland. Also, testosterone is converted into oestrogen by the enzyme aromatase, thus increasing the circulating concentration of oestrogen, which subsequently down-regulates the HPG axis, resulting in a further reduction in GnRH, LH and FSH production. As a result, testosterone concentration is decreased and maintained in its optimal range (Ullah *et al*., 2014; Majzoub & Saganegh, 2016) (Figure 4.1).

At present, the mechanisms involved in raising testosterone levels have been shown in animal studies conducted by Low *et al*. (2013a & 2013b). Both studies have demonstrated that 2 hours and 48 hours of *E. longifolia* supplementation increases testosterone production, LH and FSH secretion but reduces oestrogen levels in rats. Initially, the mechanism behind the effects of *E. longifolia* on testosterone was postulated by Low *et*

al. (2013a) (Table 4.2), where steroidogenesis and spermatogenesis were explored by incubating the extract with rat Leydig cells. Testosterone production increased when treated with 0.1, 1.0, and 10.0 μM ($p < 0.05$) of the plant extract, with a concomitant reduction in oestrogen. The authors speculated that these observations could be due to aromatase inhibition, thus preventing the conversion of testosterone to oestrogen. In a subsequent study by Low *et al*. (2013b) (Table 4.2), 25 mg/kg of *E. longifolia* reported an increase in plasma testosterone, LH and FSH levels, while oestrogen levels were significantly suppressed. These findings provide a preliminary understanding of the effects of *E. longifolia*, which may, potentially, down-regulate the oestrogen-mediated feedback effect on LH and FSH secretion in the HPG axis.

Low *et al*. (2013a; 2013b) concluded that *E. longifolia* affects hormonal regulation, namely testosterone, free testosterone, oestrogen, LH, FSH, and SHBG, activating the HPG axes. Although these preliminary findings demonstrate the possible mechanism of *E. longifolia* on the HPG axis, both studies were conducted in cell cultures and rat models (Table 4.2). Rats are often used in biomedical and comparative medicine research due to their many similarities to human in terms of anatomy and physiology (Bryda, 2013). However, it is to early to speculate that what works in rats will in humans. To date, the mechanism behind the reputed effect of *E. longifolia* in terms of testosterone regulation in humans has not been examined and, thus, remains unknown. Hence, additional studies are needed to fully understand the underlying mechanism of *E. longifolia* supplementation in increasing testosterone production.

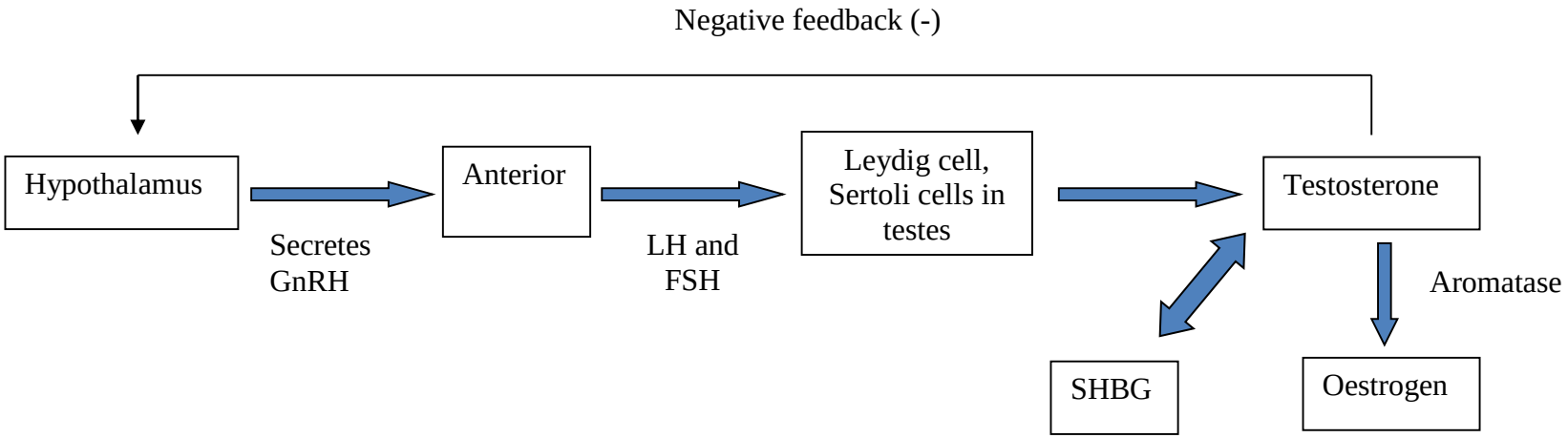


Figure 4.1 The testosterone negative feedback loop. The hypothalamus secretes GnRH and stimulates the anterior pituitary to secrete LH and FSH. Leydig cells and Sertoli cells in testes then produce testosterone. Increased testosterone production causes negative feedback, and the hypothalamus stops GnRH production. A concomitant reduction in LH and FSH secretion results in reduced testosterone production.
Note: Gonadotrophin-releasing hormone (GnRH), luteinising hormone (LH), follicle-stimulating hormone (FSH), sexual hormone binding globulin (SHBG)

Table 4.2: Studies conducted to examine the effect of *E. longifolia* on testosterone production in animals

Dose	Outcome	Participants	Mechanism	References
100 µL solution Eurycomanone at 0.1, 1.0 and 10µM (2 hours incubation)	T increased	Rat testicular cells	inhibiting aromatase conversion of T to oestrogen	Low *et al*. (2013a)
25 mg/kg body weight (bioactive fraction of EL (48 days)	EL fraction (F2) and eurycomanone increased T	Male rats	LH and FSH levels were significant higher	Low *et al*. (2013b)

Note: *E. longifolia* (EL); Luteinizing hormone (LH); Follicle-stimulating hormone (FSH); Testosterone (T)

Animal and human studies have revealed the potential of *E. longifolia* in increasing testosterone levels following the administration of *E. longifolia* extract. Evidence suggests that the increase in testosterone happens largely in older participants with LOH and those who are moderately stressed. Current health status, *E. longifolia* dose, and age appear to play a role in determining the effects of *E. longifolia* in increasing testosterone levels. Based on previous studies, long-term supplementation did not demonstrate a significant increase in testosterone level, and it is suggested that higher dosages may be needed to continue to observe improvement, especially in healthy individuals.

4.3 General methods

4.3.1 Participants

A total of 16 males for short-term supplementation and 21 males for long-term supplementation were recruited for study Chapters 4, 5 and 6. They were between 18 to 30 years old, and a summary of participants' characteristics are listed in Table 4.3. The participants recruited were sedentary male adults, who did not participate in any high-performance sport. Due to the scarcity of study examining *E. longifolia* toward young adult males, the sample size was determined based on previous studies that recruited young adult males ranging from 13 to 30 participants (Talbott *et al.*, 2006; Chen *et al.*, 2014). The study was approved by the Liverpool John Moores University Ethics Committee (14/SPS/013), and all participants provided their informed consent. Study Chapter 4 was conducted concurrently with Chapter 5, which examined the psychology mood state and sexual function and Chapter 6, which examined the muscular strength, anaerobic power and body composition. All participants were free from any musculoskeletal injury and did not exhibit any health problems such as diabetes mellitus, heart disease, kidney disease, liver disease, and sleep apnoea. In addition, those who smoked or used other recreational or prescribed drugs were excluded. Also, the study participants do not participate in any physical activity or compete in any high-level competition throughout the supplementation period. Elite athletes were also excluded due to the active phytochemicals content of the supplement. Competitive athletes who were subject to anti-doping regulations and drug tests were excluded from the study to mitigate a positive drug test risk.

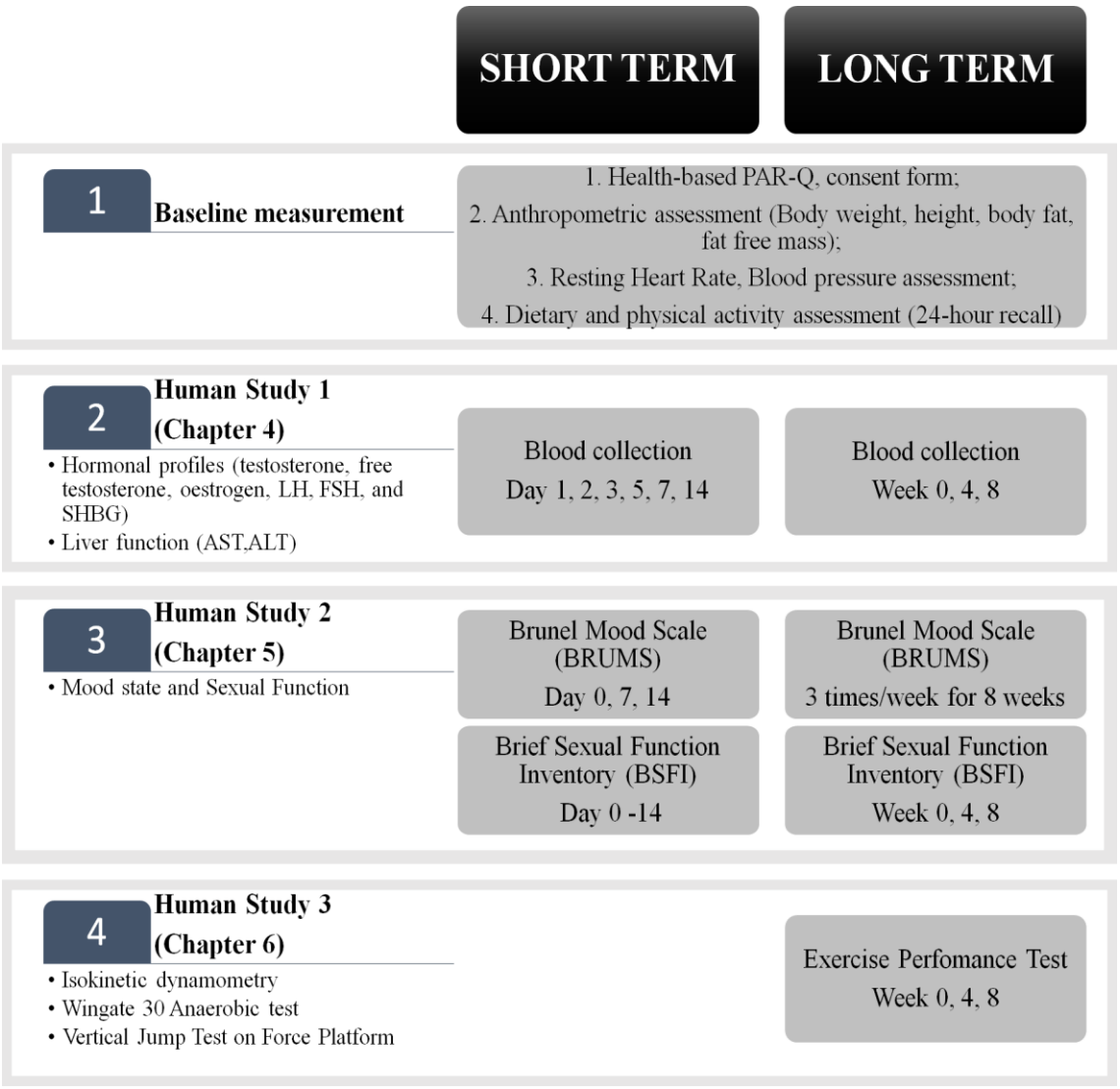


Figure 4.2 General method for study chapter 4, 5 and 6.

Table 4.3 Demographic data for participants enlisted in the short-term and prolonged supplementation phases

	Short-term			Long-term		
	EL(n = 8) M ± SD /Med (IqR)	**PLA(n = 8) M ± SD /Med (IqR)**	***p***	**EL(n=11) M ± SD /Med (IqR)**	**PLA(n=10) M ± SD /Med (IqR)**	***p***
Age (years)	25.3 ± 5.0	23.5 ± 4.5	0.470†	22.6 ± 4.1	22.8 ± 3.4	0.905†
Body Weight (kg)	74.5 ± 8.7	72.9 ± 8.6	0.709†	78.7 ± 8.3	76.9 ± 5.7	0.579†
Height (cm)	172.5 ± 7.5	176.21 ± 5.6	0.282†	180.0 ± 9.3	180.0 ± 7.5	0.960†
Body Fat (%)†††	18.95 (5.2)	13.55 (2.6)	0.052††	14.78 ± 4.7	14.22 ± 3.6	0.764†
Lean Body Mass (kg)†††	60.30 (6.4)	57.9 (11.5)	0.834††	66.92 ± 6.5	66.08 ± 5.6	0.757†

Notes: Mean (M); Standard Deviation (SD); *E. longifolia* (EL); Placebo (PLA)
†denotes *p* value based on parametric test (independent *t*-test)
††denotes *p* value based on non-parametric test (Mann-Whitney test)
†††denotes that body fat % and Lean Body Mass are reported in Median (Med), Interquartile range(IqR)

4.3.2 Experimental Design

The study was a randomized double-blind, placebo-controlled, body weight matched-paired design to investigate the effect of *E. longifolia* on hormonal changes among adult males. The study consisted of two phases; the short-term consumption phase (2 weeks) and the long-term consumption phase (8 weeks). Participants in both phases were assigned to the placebo (PLA) and *E. longifolia* treatment group (EL) using a matched-paired design according to their body weight. The participants are grouped into pairs, based on their body weight. Then, within each pair, the participants are randomly assigned to E. longifolia or Placebo treatment. The participants and the researcher in the studies did not know who is received the placebo or EL treatment. The *E. longifolia* and placebo were assigned by the researcher's supervisor. This procedure was conducted to prevent any bias in the research outcomes.

4.3.2.1 Short-term supplementation phase

Participants were assigned to either a PLA (n = 8) or EL (n = 8) group. A total of six laboratory visits were scheduled: Visit 1 - Baseline anthropometric assessment and blood collection (Day 1); Visits 2 to 4 - Blood collections (Days 2, 3, 5); Visit 5 - Interim anthropometric assessment and blood collection (Day 7); and Visit 6 - Post-supplementation anthropometric assessment and blood collection (Day 14). All blood collection was collected in the morning from 8 a.m to 9 a.m.

4.3.2.2 Long-term supplementation phase

Participants recruited to the prolonged supplementation phase were assigned to either a PLA (n = 10) or EL (n = 11) group. A total of three laboratory visits were scheduled: Visit 1 - Baseline anthropometric assessment and blood collection (Week 0); Visit 2 - Interim anthropometric assessment and blood collection (Week 4); and Visit 3 - Post-supplementation anthropometric assessment and blood collection (Week 8).

4.3.3 Experimental Protocol

Pre-baseline sessions consisted of a health screen that incorporated measurement of resting heart rate and blood pressure and the implementation of a health-based physical activity readiness questionnaire (PAR-Q) to ensure that each participant fulfils the study criteria. Thorough instruction on supplementation and prior testing criteria was given to ensure valid and reliable data collection.

Each subsequent laboratory visits for the short (Days 1, 2, 3, 5, 7, 14) and prolonged supplementation phase (Week 0, 4, 8) involved health-related assessments of each participant, including anthropometric profiling (weight, height, body fat percentage, fat-free mass); cardiovascular measures (resting heart rate and blood pressure and blood lipid profiles); hormonal profiles (testosterone, free testosterone, oestrogen, LH, FSH, and SHBG); and dietary and physical activity assessment (24-hour recall). The dietary and physical activity recording was needed to identify whether participants were consuming any other performance-enhancing supplements and involved in any high-intensity exercise and to enable replication of diet and physical activity before subsequent visits. Participants administered 600 mg of *E. longifolia* or placebo daily throughout the short (2 weeks) and prolonged (8 weeks) phase. Prior to each visit, all participants were asked to complete an overnight fast and refrain from alcohol and caffeine consumption, strenuous activity, and sexual activity eight hours before testing commenced. Participants' adherence to the protocol was assessed using the dietary recall diary, where the time they took the supplement and how they felt at different times during the day were recorded. Additionally, reminders were sent out via text messages to ensure compliance.

4.3.3.1 Anthropometric assessment

Height and body weight were determined using a stadiometer and weighing scales (SECA, Hamburg). Body compositions were determined using Bioelectrical Impedance Analysis (BIA; Bodystat 1500, Douglas, Isle of Man, UK). Body fat percentage, lean body mass, total weight, and body water was recorded. Prior to the testing, participants were asked to remove their shoes and socks, accessories such as watches or bracelet on the right side to reduce the possibility of impeding the electrodes' correct placement. Participants were assessed in a supine position. Before the commencement of the test,

participants were asked to rest for 5 minutes. The legs and arms were spread out, so they couldn't make contact with other part of their body. Alcohol wipes were used to clean the area of the skin where the electrodes are to be attached. The self-adhesive disposable electrodes were attached to the right hand and right foot before the leads were connected.

4.3.3.2 Blood pressure assessment

Prior to any performance test, resting heart rate and blood pressure were tested using an automated sphygmomanometer (Critikon, Tampa, Florida, USA). Participants were asked to rest in a supine position for 10 minutes before the blood pressure measurement.

4.3.3.3 Dietary supplementation

A dose of 600 mg per day of *E. longifolia* or 600 mg placebo (maltodextrin) in a double-blind manner was administered. The participants were required to consume supplements each day between 8 a.m to 9 a.m and asked not to change their dietary or physical activity patterns significantly throughout this period. Participants completed the 24-hour diet recall and physical activity questionnaire on the first visit and were asked to replicate the same diet and physical activity before the second visit. However, dietary and physical activity was not accessed. Participants' adherence to the protocol was assessed using the dietary recall diary, where the time they took the supplement and how they felt at different times during the day were recorded. Additionally, reminders were sent out via text messages to ensure compliance. For the short supplementation phase, participants were given two weeks supply of *E. longifolia* or placebo in a capsule that contained 600 mg/day of EL (efficacy reported in Tambi, 2005). Participants for the prolonged phase were given two month's supply of *E. longifolia* or placebo in capsule form. The quality

of EL was based on the level of eurycomanone, which is the main compound found in EL (Norhidayah *et al*., 2015). The Malaysian Standard (2011) stated that the level should be from 0.8-1.5 w/v (%). The *E. longifolia* used in the present study complied with the standard since eurycomanone content was 1.21 w/v (%). A capsule of 600 mg of *E. longifolia* consists of 7.26 mg of eurycomanone based on the 1.21% w/v.

4.3.3.4 Blood collection and analysis

The collection of venous blood (15 ml) was done by a qualified phlebotomist from an antecubital vein using serum separation tubes (BD Vacutainer, New Jersey, USA) in the morning between 8 a.m to 9 a.m. After one hour, at room temperature (20°C), blood was centrifuged at 4000 rpm (Sigma 3-16KL, Germany), and serum was separated and transferred to micro-tubes and stored at -80 °C until analysis.

a) Liver damage tests (AST and ALT) were assessed using enzymatic assays and automated benchtop spectrophotometry (RX Daytona) supplied by Randox Laboratories Limited (Crumlin, Co. Antrim, UK). ALT is an enzyme within the aminotransferase group and also the most sensitive liver enzymes. The normal concentration levels of ALT in the blood are low. However, when the liver is damaged, the level of ALT increases. Aspartate aminotransferase is measured together with ALT to examine liver damage. Aspartate aminotransferase is an enzyme found in the body, and an elevated level in the blood is directly correlated to the severity of the tissue damage.
b) Hormone profiles (testosterone, free testosterone, sexual hormone-binding globulin (SHBG), oestrogen, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) were assessed by enzyme-listed immunosorbent assay (ELISA;

IBL International, Hamburg, Germany) coupled to a spectrophotometer at 490 nm absorbance for detection (Clariostar, BMG Labtech, Offenburg, Germany). The ELISA Kit is a solid phase enzyme-linked immunosorbent assay based on the principle of competitive binding. Prior to the analysis, all reagents, samples, control and standards were prepared according to the manufacture procedures. Next, the samples, standards and control were added to the 96-well plate pre-coated with antibodies. HRP-Conjugate was added to the 96-well plate, and the plate was covered with aluminium foil. The wells were allowed to incubate at 37º for 1 hour. Following incubation and washing, 100 µL TMB substrate solution was added into all wells and incubate for another 15 minutes at room temperature in the dark. Lastly, add 100 µL stop solution into all wells and shake the microplate gently. Measure the absorbance of the sample at 490 nm.

4.3.4 Statistical analysis

All statistical analysis for study Chapter 4, 5 and 6 were conducted using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (SPSS Inc., Chicago, USA). Baseline data were analysed using the parametric (independent *t*-test) and non-parametric test (Mann Whitney test). All data in text and tables are presented as means ± SD (parametric) and median (interquartile range) with p values < 0.05 indicating statistical significance. Assumptions of normality, homogeneity of variances, compound symmetry and homogeneity of regression were checked and fulfilled using the Shapiro-Wilk test before the repeated measurement analysis of covariance (ANCOVA) was performed to determine the effect of the *E. longifolia* supplementation over the entire period of studies (2 weeks and 8 weeks). Analysis of covariance for repeated measures (ANCOVA) was performed to determine the group's treatment effects and between-

group differences. In consideration of participants having individual differences at baseline and this differences might have an impact on the outcome of the treatment, the repeated measures ANCOVA was used to correct the baseline variables and were considered as covariates in each of the studies in order to prevent bias (de Boer *et al*., 2015)

4.4 Results

The demographic characteristics of participants for short and long term studies in EL and PLA groups are presented in Table 4.1. There was no significant difference ($p > 0.05$) in all parameters between the two groups. Similarly, no significant difference ($p > 0.05$) between the EL and PLA were identified in the prolonged supplementation phase.

4.4.1 Liver function test

Baseline results indicated no significant difference in the AST between EL (20.3 ± 11.95 U/L) and PLA (20.8 ± 12.96 U/L); t (14) = -0.070, $p = 0.945$ and ALT level between EL (16.9 ± 4.1 U/L) and PLA (31.3 ± 48.8 U/L); t(14) = -0.830, $p = 0.420$ in the two weeks supplementation phase. Figure 4.2 shows findings of liver function after two weeks of *E. longifolia* supplementation. No significant main effect of time $F_{1.274,\ 16.565} = 2.666$, $p = 0.115$) and also no significant main interaction between group and time for AST ($F_{1.274,\ 16.565} = 0.933$, $p = 0.372$). ANCOVA repeated measurement with the baseline measure as covariate showed no significant changes within EL and PLA after two weeks of supplementation. Findings shows no significant difference between Day 1 (20.3 ± 11.95 U/L) and Day 14 (20.94 ± 9.1) [Mean difference (MD) = -0.625 U/L, 95% CI = -7.120, 5.870, $p = 1.000$] in EL. Placebo also showed no significant difference between

day 1 (20.8 ± 12.96 U/L) and day 14 (33.5 ± 35.2 U/L) (MD = -12.75 U/L, 95% CI = -75.046, 49.546, p = 1.000). AST level in EL is highest at Day 3 but reduces in the following days and PLA has the highest AST level at Day 14. Also, no significant difference between EL and PLA at all time point. Upon completion of the study at day 14, no significant difference in AST level was observe between EL (M = 20.94 ± 9.1 U/L) and PLA (33.5 ± 35.2 U/L), t (14) = - 0.978, p = 0.345.

In Figure 4.2(B), there is a significant main effect of time ($F_{1.816,\ 23.609}$ = 6.616, p = 0.006). However, there was no significant main interaction between group and time for ALT ($F_{1.816,\ 23.609}$ = 1.066, p = 0.354). ANCOVA repeated measurement showed no significant changes within EL and PLA after two weeks of supplementation. Findings shows no significant difference between Day 1 (16.9 ± 4.1 U/L) and Day 14 (17.4 ± 4.7) [Mean difference (MD) = -0.563 U/L, 95% CI = -6.77, 5.65, p = 1.000] in EL. PLA also showed no significant difference between day 1 (31.3 ± 48.8 U/L) and day 14 (28.3 ± 25.0 U/L) (MD = 3.00 U/L, 95% CI = -27.71, 33.71, p = 1.000). However, in PLA, there are significant drop in ALT level from Day 1 to Day 5 (MD = 6.88 U/L, 95% CI = 2.22, 11.53, p = 0.007) and also from Day 1 to Day 7 (MD = 8.125, 95% CI = 3.63, 12.62, p = 0.002). ALT level in EL is highest at Day 3 and reduces in the following days. PLA has the lowest ALT level at Day 7 before the level increases back on Day 14. Similary, independent sample t-test shows no significant difference between EL and PLA at all time point. At day 14, no significant difference in ALT level between EL (M = 17.4 ± 4.7 U/L) and PLA (28.3 ± 25.0 U/L), t (14) = - 1.205, p = 0.248.

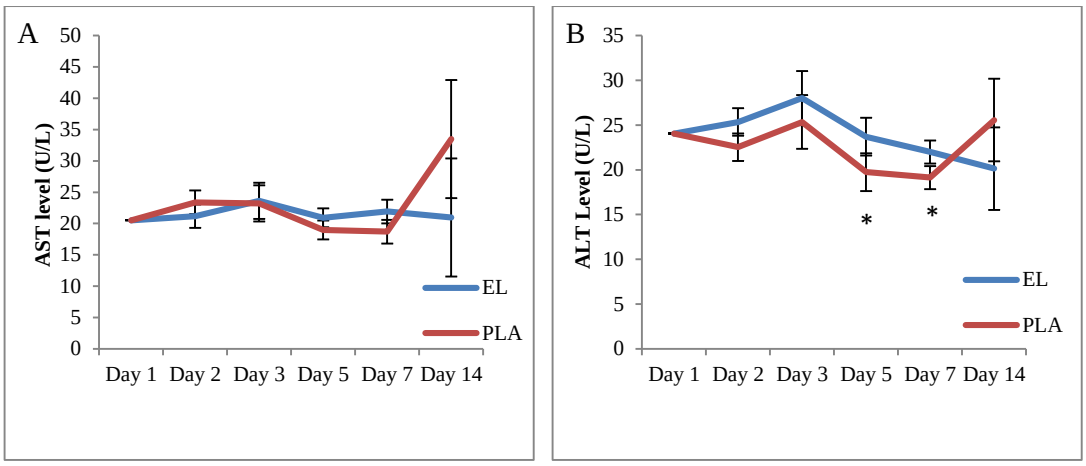


Figure 4.3 Effect of two weeks *E. longifolia* supplementation on liver function
Note: Aspartate Aminotransferase (AST); Alanine Aminotransferase(ALT)
Repeated measures ANCOVA between-group analysis with regard to time was applied. Numerical covariates (baseline – D0) were controlled by using repeated measurement ANCOVA. Assumption of normality, homogeneity of variances, compound symmetry, and homogeneity of regression were checked and were fulfilled.
* denotes indicate significant differences within *E.longifolia* (EL) group and Placebo (PLA) group compared to baseline with ANCOVA repeated measurement applied.
*denotes level of significance was set at 0.05 (two-tailed)

Following the two weeks supplementation period, the eight week *E. longifolia* supplementation was performed. Baseline results indicated no significant difference in the AST between EL (18.64 ± 6.4 U/L) and PLA (22.05 ± 7.1 U/L); t (19) = -1.158, p = 0.261 and ALT level between EL (19.32 ± 9.3 U/L) and PLA (21.65 ± 5.6 U/L); t (19) = -0.688, p = 0.500 in the eight weeks supplementation phase.

Figure 4.4 shows findings of liver function after eight weeks of *E. longifolia* supplementation. No significant main effect of time ($F_{2, 36}$ = 0.756, p = 0.100) and no significant main interaction between group and time for AST ($F_{2, 36}$ = 0.788 p = 0.463) (Figure 4.4A). ANCOVA repeated measurement showed no significant changes within EL and PLA after eight weeks of supplementation. Findings shows no significant difference between baseline (18.64 ± 6.4 U/L) and post-test (20.30 ± 7.7 U/L) (MD = -1.664 U/L, 95% CI = -8.115, 4.788, p = 1.000] in EL. Placebo also showed no significant difference between baseline (22.05 ± 7.1 U/L) and post-test (19.24 ± 4.8 U/L) (MD =

2.81 U/L, 95% CI = -0.830, 6.450, p = 0.145). AST level in EL increase at interim but reduces at post test and placebo demonstrate a decreasing trend from baseline to post test.

As for ALT level, there was no significant main effect of time ($F_{2,36}$ = 1.325, p = 0.221) (Figure 4.4B) and no significant main interaction between group and time for ALT ($F_{2,36}$ = 1.314, p = 0.281). However, ANCOVA repeated measurement showed no significant changes within EL and PLA after eight weeks of supplementation. Findings shows no significant difference between baseline (19.32 ± 9.3U/L) and post-test (23.23 ± 10.0 U/L) (MD = -3.91 U/L, 95% CI = -12.352, 4.533, p = 0.622) in EL. PLA also showed no significant difference between baseline (21.65 ± 5.6 U/L) and post-test (19.95 ± 8.9 U/L) (MD = 1.70 U/L, 95% CI = -6.770, 10.170, p = 1.000).

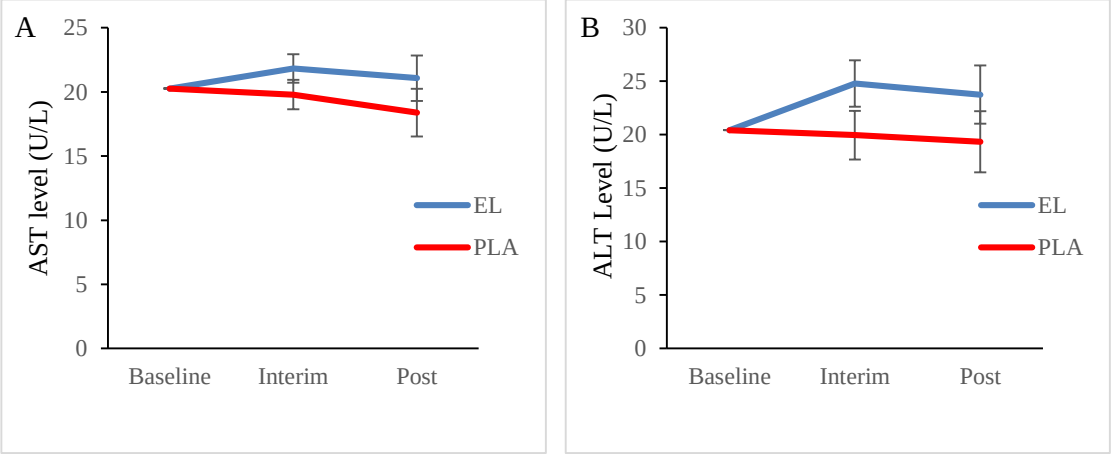


Figure 4.4 Effect of eight weeks *E. longifolia* supplementation on liver function
Note: Aspartate Aminotransferase (AST); Alanine Aminotransferase(ALT)
Repeated measures ANCOVA between-group analysis with regard to time was applied. Numerical covariates (baseline – D0) were controlled by using repeated measurement ANCOVA. Assumption of normality, homogeneity of variances, compound symmetry, and homogeneity of regression were checked and were fulfilled.

Similar to AST level, ALT level in EL also increases at interim but reduces at post test and PLA also demonstrates a decreasing trend from baseline to post test. Independent sample t-test show no significant difference in ALT level between EL and PLA (MD = 3.277, t (19) = 0.792, p = 0.438) at post-test.

4.4.2 Effects of *E. longifolia* supplementation on reproductive hormones

Table 4.4 shows no significant difference between EL and PLA for all reproductive hormones on Day 1. The findings were measured using the independent sample t-test for testosterone, oestradiol, FSH and SHBG. Free testosterone and LH were analysed using the Mann-Whitney U test and reported in the median and Interquartile range.

Similar to the short-term phase, the long-term phase also examined a similar hormonal profile, including testosterone, free testosterone, oestradiol, LH, FSH, and SHBG after 8 weeks *E. longifolia* supplementation. The baseline result (Table 4.5) showed no significant difference between EL and PLA for all parameters.

Table 4.4: Baseline measurement on the effect of two weeks *E. longifolia* supplementation on selected reproductive hormones

	EUR (n = 8)	**PLA (n = 8)**	***t* (14) / *U***	***p***
	M ± SD/ Med (IqR)	**M ± SD/ Med (IqR)**		
Testosterone(ng/ml)	8.71 ± 2.0	8.63 ± 1.5	-0.037	0.971†
Free Testosterone(pg/ml)†††	7.66 (3.7)	6.30 (6.8)	26.00	0.529††
Oestradiol(pg/ml)	44.1 ± 18.1	63.65 ± 21.0	-1.994	0.066†
LH(mIU/ml)†††	8.18 (5.4)	8.13 (3.9)	31.00	0.916††
FSH(mIU/ml)	4.75 ± 1.3	5.40 ± 1.9	-0.794	0.44†
SHBG(nmol/L)	47.77 ± 11.0	36.07 ± 27.7	1.112	0.285†

Notes: Mean (M); Standard deviation (SD); Median (Med); Interquartile range (IqR); luteinizing hormone (LH); follicle-stimulating hormone (FSH); sexual hormone-binding globulin (SHBG)
†denotes *p* value based on parametric test (independent t-test).
††denotes *p* value based on non-parametric test (Mann-Whitney *U* test).
†††denotes that Free Testosterone and LH are reported in Median, Interquartile range

Table 4.5: Baseline measurement on sex-related hormonal profile prior to 8 weeks supplementation.

	EUR (n = 11)	PLA(n = 10)	t (19) / *U*	*p*
	M ± SD/Med (IqR)	M ± SD/Med (IqR)		
Testosterone(ng/ml)	6.85 ± 2.4	7.25 ± 1.7	0.436	0.668†
Free Testosterone(pg/ml)	6.77 ± 1.9	7.61 ± 2.2	-0.939	0.359†
Oestradiol(pg/ml)†††	31.74 (17.6)	32.49 (65)	52.00	0.863††
LH(mIU/ml)†††	4.12 (2.1)	2.67 (2.6)	31.00	0.099††
FSH(mIU/ml)†††	4.73 (5.2)	2.89 (4.1)	52.00	0.863††
SHBG(nmol/l)†††	15.86 (3.5)	16.85 (4.2)	48.00	0.654††

Note: Mean(M); Median (Med); standard deviation (SD), Interquartile range (IqR); luteinizing hormone (LH), follicle-stimulating hormone (FSH); sexual hormone-binding globulin (SHBG)
† Denotes *p* value based on parametric test (independent t-test)
†† Denotes *p* value based on non-parametric test (Mann-Whitney *U* test)
††† Denotes that Oestradiol, LH, FSH, and SHBG are reported in Median, Interquartile range

4.4.2.1 Effects of two weeks and eight weeks of E. longifolia supplementation on selected reproductive hormones

Two weeks of *E. longifolia* supplementation

Significant main effect of time ($F_{2.40, 31.17} = 5.416$, $p = 0.007$ was observed and there is no significant main interaction between group and time for testosterone level ($F_{2.40, 31.17} = 4.221$, $p = 0.118$). EL shows significant increase (11.1%) in testosterone level from day 1 (8.71 ± 2.0 ng/ml) to day 14 (9.68 ± 0.7 ng/ml), (MD = -0.97 ng/ml, 95% CI = -1.908, -0.029, $p = 0.043$) and PLA did not demonstrate any significant increase but a decrease (-8.4%) in testosterone level from day 1 (8.63 ± 1.5 ng/ml) to day 14 (7.90 ± 1.5 ng/ml) (MD = 0.73 ng/ml, 95% CI = -1.977, 3.432, $p = 1.000$). Significant higher testosterone level in EL compared with PLA was observed at day 14 [t (14) = 3.037, $p = 0.009$) (Figure 4.5A).

No significant main effect of time ($F_{1.07, 13.95} = 0.204$, $p = 0.676$) and no significant main interaction between group and time ($F_{1.07, 13.95} = 0.631$, $p = 0.451$) was observed in free testosterone level. No significant differences within and between EL and PLA after two weeks of supplementation (Figure 4.5B).

Following testosterone and free testosterone level, no significant main effect of time ($F_{5, 65} = 2.446$, $p = 0.443$) was observed in oestradiol level. Oestradiol level showed no significant main interaction between group and time ($F_{5, 65} = 0.474$, $p = 0.794$). No significant changes within EL and PLA throughout the supplementation period. Only at day 3, PLA (66.23 ± 12.5 pg/ml) has significant higher oestradiol level compared to EL (45.94 ± 22.7 pg/ml), t (14) = -2.216, $p = 0.004$ (Figure 4.5C).

Next, data shows no significant main effect of time ($F_{1.99, 25.90} = 2.014$, $p = 0.154$) and no significant main interaction between group and time ($F_{1.99, 25.90} = 1.389$, $p = 0.267$) for LH level. As for FSH level, there is a significant main effect of time ($F_{5, 65} = 2.583$, $p = 0.034$) but no significant main interaction between group and time ($F_{5, 65} = 0.617$, $p = 0.687$) was observed. Both LH and FSH level shows no significant differences within and between EL and PLA after two weeks of supplementation (Figure 4.5D and 4.5E).

Lastly, no significant main effect of time ($F_{5, 65} = 1.918$, $p = 0.103$) but there is a significant main interaction between group and time ($F_{5, 65} = 4.268$, $p = 0.002$) in SHBG level. Within group analysis shows no significant changes within EL and PLA. There is a significant higher SHBG level in EL (53.06 ± 15.4 nmol/l) compared to PLA (30.55 ± 17.7 nmol/l) at day 7, t (14) = 2.718, $p = 0.017$ (Figure 4.5F).

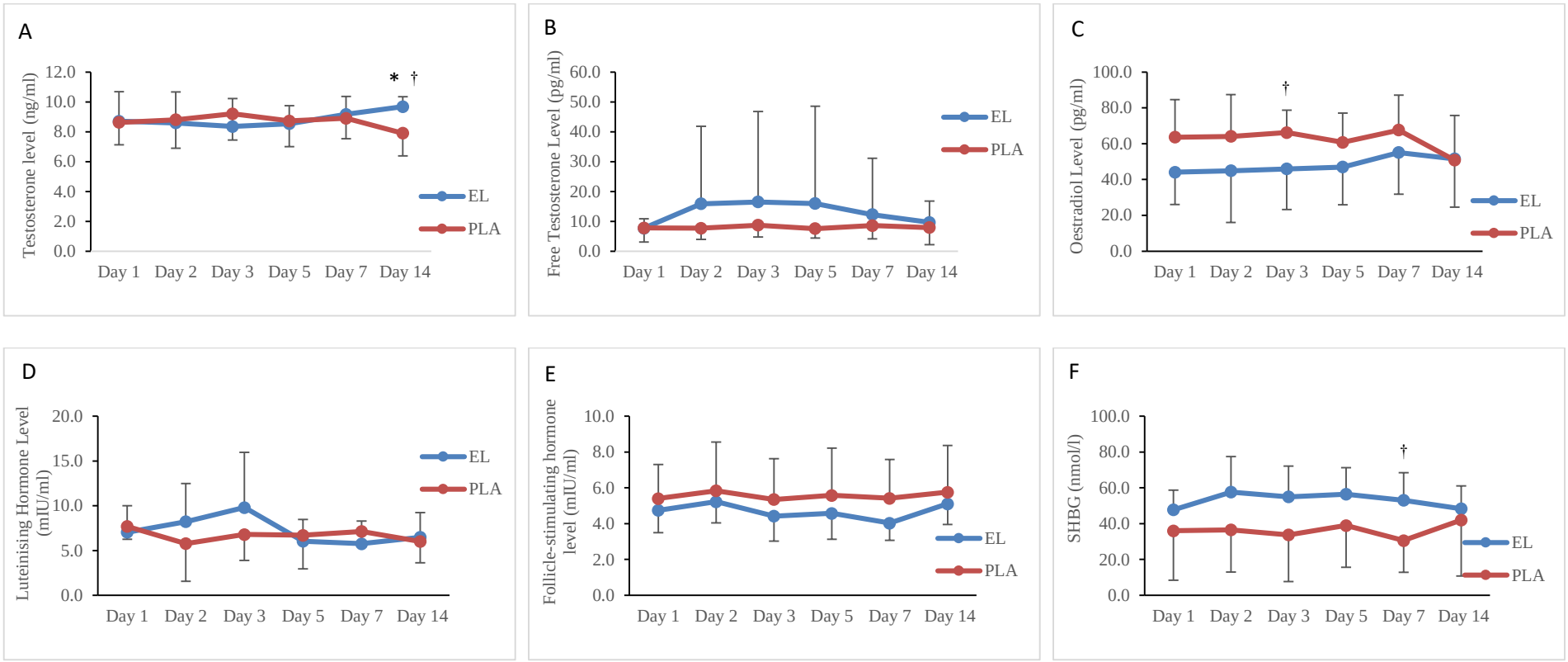


Figure 4.5 Effect of 2 weeks of *E. longifolia* supplementation on testosterone (A), free testosterone (B), oestradiol (C), LH (D), FSH (E) and SHBG (F)

Note: Repeated measurement ANCOVA between-group analysis with regard to time was applied. Numerical covariates (baseline – D0) were controlled by using repeated measurement ANCOVA. Assumption of normality, homogeneity of variances, compound symmetry, and homogeneity of regression were checked and were fulfilled.
* denotes indicate that there is a significant increase compare to day 1; †denotes indicate significant differences between *E. Longifolia* group and Placebo group.
*,†denotes level of significance was set at 0.05 (two-tailed).

Table 4.6 shows the mean differences and the percentage of changes after two weeks of *E. longifolia* and placebo supplementation on all hormones. All hormones increase in EL except for the LH level. On the other hand, PLA shows decreased in testosterone, oestradiol, and LH and a minimal increase in free testosterone, FSH, and SHBG.

Table 4.6: Effect (Week 0 vs. Week 2) of *E. longifolia* and placebo supplementation on hormone after 14 days of supplementation.

Variable	EL(n = 8)			PLA(n = 8)		
	MD [95% CI]	***p***	**%**	**MD [95% CI]**	***p***	**%**
Testosterone (ng/ml)	0.97 [-1.91,-0.03]	0.04*	11.1	-0.73 [-1.98,3.43]	1.00	-8.4
Free testosterone(pg/ml)	1.94 [-8.76, 4.88]	1.00	24.7	0.12 [-5.06, 4.81]	1.00	1.6
Estradiol (pg/ml)	7.46 [-55.60, 40.69]	1.00	17.0	-12.83 [-27.11, 52.78]	1.00	-20.0
LH (mIU/ml)	-0.54 [-2.31, 3.40]	1.00	-7.81	-1.69 [-1.95, 5.33]	1.00	-22.0
FSH (mIU/ml)	0.35 [-2.11, 1.4]	1.00	7.6	0.37 [-3.90, 3.16]	1.00	6.8
SHBG (nmol/L)	0.60 [-16.38, 15.17]	1.00	1.3	5.94 [-25.76, 13.88]	1.00	16.5

Note: Day 0 (D0); Day 14 (D14); MD=Mean difference; CI=Confidence interval; LH=luteinising hormone; FSH=follicle-stimulating hormone; SHBG=sexual hormone binding globulin; Numerical covariate (baseline) was controlled by using ANCOVA repeated measurement.
*Level of significance was set at 0.05 (two-tailed); % increased or decreased (-)

Eight weeks of *E. Longifolia* supplementation

No significant main effect of time ($F_{2, 36} = 5.287$, $p = 0.110$) and no significant main interaction between group and time for testosterone level ($F_{2, 36} = 0.233$, $p = 0.794$). EL and PLA show no significant changes in testosterone level within and between groups from baseline to post test (Figure 4.6A).

There is a significant main effect of time ($F_{2, 36}$ = 4.142, p = 0.024) was observed in free testosterone level. Findings shows no significant main interaction between group and time ($F_{2, 36}$ = 1.398, p = 0.260) in free testosterone level. However, EL shows significant decrease in free testosterone level from baseline (6.77 ± 1.9 pg/ml) to interim (5.67 ± 0.8 pg/ml), (MD = 1.09 pg/ml, 95% CI = 0.38, 1.81, p = 0.005) before a slight increase at post test (MD = -0.51 pg/ml, 95% CI = -2.52, 1.50, p = 1.000). PLA did not demonstrate any significant changes from baseline to post test. Between group analysis shows PLA (7.47 ± 2.8 pg/ml) had significant higher free testosterone at interim compared to EL (5.67 ± 0.8 pg/ml), t (19) = -2.151, p = 0.045 (Figure 4.6B).

Next, a significant main effect of time ($F_{2, 36}$ = 7.603, p = 0.002) was found in oestradiol level there but no significant main interaction between group and time ($F_{2, 36}$ = 0.235, p = 0.791). The pairwise comparison shows no significant changes within and between EL and PLA throughout the three time point Figure 4.6(C).

No significant main effect of time ($F_{2, 36}$ = 2.747, p = 0.078) for LH level. The data also shows no significant main interaction between group and time ($F_{2, 36}$ = 0.825, p = 0.446). As for FSH level, no significant main effect of time ($F_{1.46, 26.31}$ = 0.649, p = 0.484) and no significant main interaction between group and time ($F_{1.46, 26.31}$ = 0.575, p = 0.687) was observed. Similar to the hormone level found in the short term, both LH and FSH level also shows no significant differences within and between EL and PLA after eight weeks of supplementation (Figures 4.6D and 4.6E).

Lastly, no significant main effect of time ($F_{2, 36}$ = 2.345, p = 0.11) in SHBG level but there is a significant main interaction between group and time ($F_{2, 36}$ = 5.388, p = 0.009). Within group analysis shows significant increase from baseline (17.33 ± 5.3 nmol/l) to

post test (22.96 ± 13.8 nmol/l) in EL (MD = -5.64 nmol/l, 95% CI = -10.46, - 0.818, p = 0.022 but no significant changes in PLA. Also, analysis also shows no significant difference between EL and PLA at each time point (Figure 4.6F).

Table 4.7 shows the mean differences and the percentage of changes after eight weeks of *E. longifolia* and placebo supplementation on all hormones. In EL, all hormones reduce except for FSH and SHBG. PLA, on the other hand, changes after eight weeks is minimal.

Table 4.7: Effect (Baseline vs. Post) of *E. longifolia* and placebo supplementation on endocrine hormone after 8-weeks of supplementation.

	EL (n = 11)			**PLA (n = 10)**		
Variable	**Mean difference (95% CI)**	***p***	**%**	**Mean difference (95% CI)**	***p***	**%**
Testosterone	-0.142 [-1.99, 2.27]	1.00	-2.00	0.054 [-1.67, 1.56]	1.00	0.80
Free Testosterone	-0.582 [-1.35, 2.51]	1.00	-8.60	0.169 [-3.01, 2.68]	1.00	2.20
Oestradiol	-10.105 [-7.16, 27.37]	0.36	-26.52	-0.037 [-18.62, 18.70]	1.00	-0.09
LH	-1.003 [-0.78, 2.78]	0.40	-24.63	0.063 [-1.77, 1.65]	1.00	2.06
FSH	0.546 [-2.37, 1.28]	1.00	12.56	-0.332 [-2.51, 3.18]	1.00	-7.84
SHBG	5.64 [-10.45, -0.82]	0.022*	32.49	-1.655 [-1.15, 4.46]	0.34	-9.19

Note: MD=Mean difference; CI=Confidence interval; LH=luteinising hormone; FSH=follicle-stimulating hormone; SHBG=sexual hormone-binding globulin; EL= *Eurycoma longifolia.* Numerical covariate (baseline) was controlled by using ANCOVA repeated measurement.
*Level of significance was set at 0.05 (two-tailed); % increased or decreased (-)

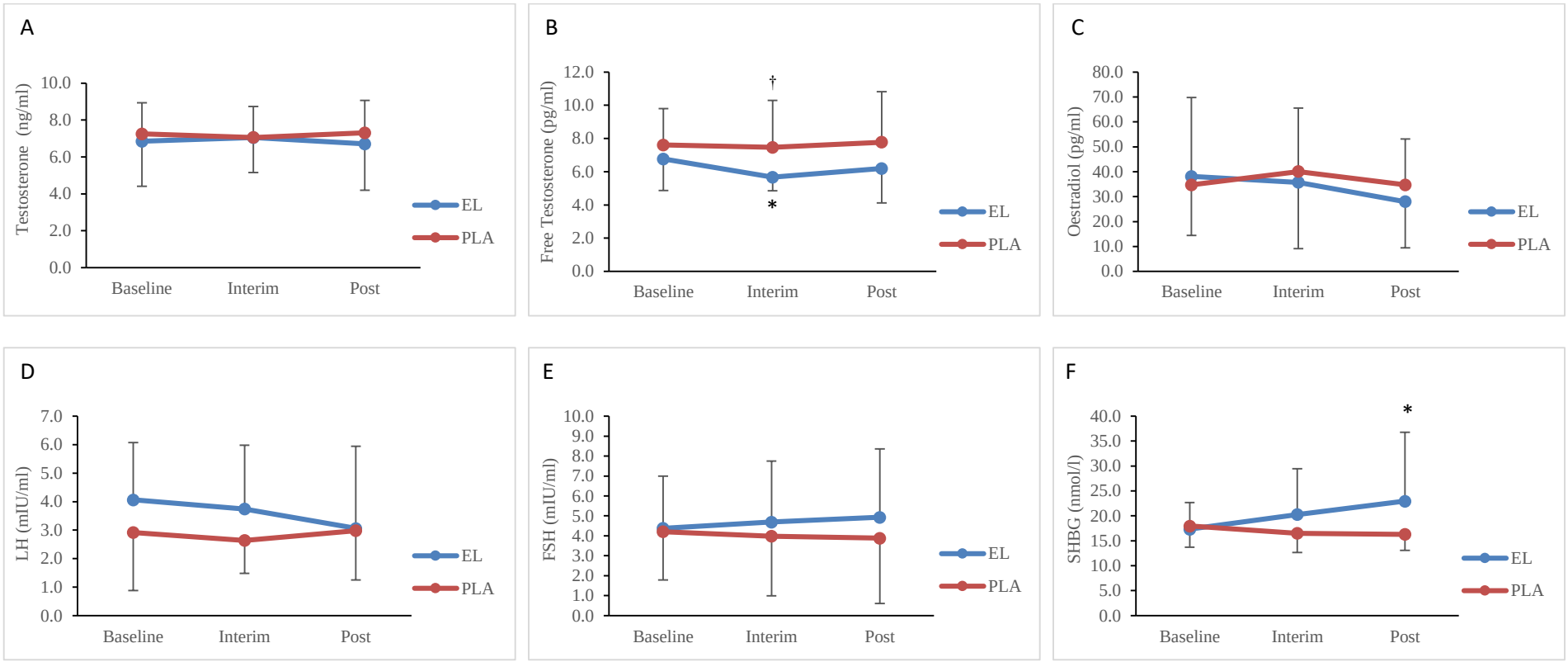


Figure 4.6 Effect of eight weeks of *E. longifolia* supplementation on testosterone (A), free testosterone (B), oestradiol (C), LH (D), FSH (E) and SHBG (F)

Note: Repeated measurement ANCOVA between-group analysis with regard to time was applied. Numerical covariates (baseline – D0) were controlled by using repeated measurement ANCOVA. Assumption of normality, homogeneity of variances, compound symmetry, and homogeneity of regression were checked and were fulfilled.
* denotes indicate that there are significant changes within groups; †denotes indicate significant differences between *E. Longifolia* group and Placebo group. *,†denotes level of significance was set at 0.05 (two-tailed).

4.5 Discussion

This chapter aims to determine the efficacy of *E. longifolia* on reproductive hormones. Data have demonstrated that the administration of *E. longifolia* for two weeks produces significantly higher total testosterone (11.1%) and a non-significant increase in free testosterone (24.7%) levels compared to PLA. PLA recorded a decrease in testosterone (8.4%; $p > 0.05$) and an increase of 1.6% ($p > 0.05$) in free testosterone level from D0 to D14. However, a decrease in testosterone (-2.0%) and free testosterone levels (-8.6%) in EL and a minimal increase in PLA; testosterone (0.8%) and free testosterone (2.2%) after eight weeks of supplementation. The findings for the long-term supplementation shows no significant changes. Although supplementation of 2 weeks of *E. longifolia* increased the total testosterone levels among young participants, supplementation of 8 weeks of *E. longifolia* demonstrates otherwise. Findings also show that *E. longifolia* may not affect the LH and oestrogen in the HPG axis.

The current study findings demonstrated similarity with previous studies which reported that short-term supplementation of *E. longifolia* increased total testosterone (Tambi et al., 2012; Talbott et al., 2013; Henkel *et al*., 2013) and long-term supplementation studies (Ismail *et al*., 2012; Chen *et al*., 2014; Chen *et al*., 2019) showed no changes. A low dose (200 to 400 mg) of *E. longifolia* did not elicit any significant increase in testosterone levels among young, healthy males with normal testosterone levels (Chen *et al*., 2014; Chen *et al*., 2019). However, present findings demonstrated that two weeks of a higher dose of 600 mg of *E. longifolia* supplementation increased testosterone levels significantly compared to placebo.

It is postulated that the increase in total testosterone is said to be due to a bioactive peptide of 4.3 kDa (Tambi *et al*., 2012; Talbott *et al*., 2013; George *et al*., 2018) and eurycomanone, identified and isolated in *E. longifolia*, which restores and increases testosterone (Low *et al*., 2013a and 2013b). While data demonstrated an increase in testosterone and free testosterone in just two weeks of *E. longifolia* supplementation, the increase may also be affected by other confounding variables such as participants performing resistance training, food and supplements. Although participants are monitored through dietary and physical activity recording, there is also a possibility that participant may not comply with the study criteria, which may contribute to the outcome of the study.

An increase in free testosterone ($p > 0.05$) was observed after two weeks of supplementation. However, individual data were examined and found that two participants' data reported a higher increase from day 1 to day 14 than the other six participants. In addition to the variability of the individual's data, a larger decrease in PLA testosterone level migh have contributed to the significant difference between the two groups (Figure 4.5A). In addition, no significant difference between the lean body mass in both short-term and long-term study. However, the EL group had a higher lean body mass compared to PLA in the short term. Generally, men with high total testosterone levels had more lean body mass (Mouser *et al*., 2016). Hence, EL play a possible role in producing higher testosterone in the short term study and might promote an increase in lean body mass.

Data relating to the use of *E. longifolia* for two weeks from the human clinical trial are still scarce. Thus, further investigation is needed to validate whether *E. longifolia* (600 mg) on healthy young adults is able to increases testosterone in a short period (2 weeks).

However, in the long-term study, no changes were observed throughout the study period, indicating that *E. longifolia* supplementation alone may not inhibit any increase in testosterone level in healthy young adults. Hence, the present study shows that the current dose of 600 mg may not be sufficient if the duration is extended for more than two weeks.

In relation to the effect of *E. longifolia* on the HPG axis, the present study reported a non-significant decrease in the LH level in EL after two weeks and eight weeks of supplementation. The HPG axis regulates testosterone production, and when testosterone levels are low, GnRH is secreted by the hypothalamus, which results in LH production from the anterior pituitary. The LH then stimulates the Leydig cells in the testes to produce testosterone. In contrast, when testosterone levels are high, the negative feedback will inhibit the HPG axis by decreasing GnRH and LH production and, subsequently, reduce testosterone production. Total testosterone and free testosterone regulate the negative feedback loop, and one of its roles is to suppress the secretion of LH (Veldhuis *et al*., 1984; Mulligan *et al*., 1997; Veldhuis, 1999; Friedl, 2005; Stanković*et al*., 2013). Even though there is an increase in testosterone level in the short-term supplementation, findings suggest that *E. longifolia* did not stimulate LH production, which subsequently increases testosterone levels.

The current study shows that the oestradiol concentration increased from day 1 to day 14 in EL. Also, a decrease was observed after supplementation for eight weeks in EL. However, the outcome did show any significant changes in the oestradiol levels. Hence there is no indication that *E. longifolia* inhibits aromatase from preventing testosterone conversion to oestradiol to increase the testosterone level. It would seem that the higher testosterone in the short-term study has prompted a higher conversion to oestrogen and, thus, increased the circulating concentration of oestrogen, which, subsequently, down-

regulates the HPG axis, resulting in further decreased production of LH (Ullah *et al*., 2014; Majzoub & Saganegh, 2016). However, the total testosterone level still increased at day 14, suggesting that *E. longifolia* does not directly affect the HPG axis activity in increasing LH secretion. According to Figure 4.5C, oestrogen level does not significantly differ between EL and PLA, and the increase in oestrogen in EL may be due to inter-individual variance. Therefore, the data are not in agreement with the study conducted by Low *et al*. (2013a and 2013b), which reported that *E. longifolia* enhanced testosterone production in rats by inhibiting the aromatase conversion of testosterone to oestrogen. Hence, further investigation is required to elucidate this mechanism suggested by Low *et al*. (2013a and 2013b) before a conclusion can be made since the present study did not measure the aromatase enzyme.

Two weeks of *E.longifolia* supplementation have shown a low concentration level in SHBG in EL (1.3%) compared to PLA (16.5%), demonstrating higher circulating total testosterone (11.1%) and free testosterone (24.7%) levels unbound to SHBG in EL compared to the total circulating testosterone (-8.4%) and free testosterone (1.6%) in PLA. In the long term supplementation, a significant increase in SHBG in EL demonstrates decreased testosterone and free testosterone. Testosterone is bound with SHBG, and only a small fraction of about 2 to 5% circulates as free testosterone in the body. As circulating SHBG increases with age, the total testosterone concentration level will decrease gradually. An increase in SHBG and a decrease in testosterone result in a greater decline in free testosterone (Rosner *et al*., 2007; Wu *et al*., 2008; Hammond *et al*., 2012; Antonio *et al*., 2016). The precise mechanism by which *E. longifolia* restores normal testosterone levels are still not well explained, but it has been suggested by Tambi (2003) that *E. longifolia* influences the release rate of free testosterone from SHBG.

However, the current study's findings did not demonstrate *E. longifolia* influencing the testosterone and free testosterone from SHBG.

While the current study did not show E. longifolia affect the HPG axis, an earlier study on endurance cyclists showed that the cortisol levels were 32% lower and testosterone levels were 16% higher in the *E. longifolia*-supplemented cyclist compared to placebo (Talbott *et al*., 2006). Another study also reported a reduction in cortisol levels and increased testosterone levels (Talbott *et al*., 2013). Cortisol produced from the HPA axis may have inhibited testosterone by reducing HPG activity and blocking androgen receptors (Sherman *et al*., 2016; Viau, 2002; Chen *et al*., 1997). Thus, the increase in testosterone in EL may be due to *E. longifolia* affecting the HPA axis, resulting in a possible reduction in the stress hormone cortisol and causes an increased in testosterone (George *et al*., 2018; Sherman *et al*., 2016).

Findings from the long-term study have indicated that 600 mg of *E. longifolia* did not elicit the same effect found in the short-term study due to the human body trying to maintain homeostasis in individuals with a normal circulating level. Repeated exposure to *E. longifolia* for eight weeks did not demonstrate any diminishing effect of the *E. longifolia* extract. Thus, the current study is not able to determine whether there is any dose-dependent effect on the tolerance level because the dosage used in the current study may not be sufficient in affecting changes in the hormones. A higher dose may be needed to produce or enhance the effect of *E. longifolia* in the production of testosterone in young, healthy male individuals. However, a high dose may not be needed for older individuals since previous studies (Table 4.1) have shown a low dosage (200 to 400 mg) of *E. longifolia* supplemented for 4 to 12 weeks can increase the testosterone level. Hence,

age, health and dosage appear to play a significant role in determining the efficacy of *E. longifolia*.

Although there is a significant increase in the testosterone level in the short-term supplementation, there is a possible confounding factor affecting the outcome or the study. It is said that the ELISA kit may need to be validated analytically, physiologically or biologically (Gholib *et al*., 2020) to enable more reliable measurement of testosterone, free testosterone, LH, FSH, oestrogen and SHBG. However, the current study did not validate the reliability of the kit. Nevertheless, current studies' findings provide valuable information regarding *E. longifolia* and its mechanism in affecting testosterone levels via the HPG axis. However, the current study also acknowledges that the high inter-individual variation may obscure the outcome of the findings due to the measurement's reliability.

The *E. longifolia* used in the current study has a eurycomanone content of 1.21 w/v, equivalent of 7.26 mg of eurycomanone in a capsule of 600 mg *E. longifolia*. The euryomanone content used complied with the standard recommendation (0.8 – 1.5 w/v %). The amount of active ingredient available in the dosage supplemented to the participants may not be sufficient. Also, previous studies have not reported the appropriate amount of active ingredient available in the dosage they used in their supplementation studies. Hence, a study investigating different doses of the active ingredient may be warranted.

The current study has demonstrated that the supplementation of 600 mg of *E.longifolia,* which is considered the highest dose supplemented in humans at the moment, did not induce toxicity to the liver among the studies' participants, based on the

measurement of AST and ALT. Although lower AST and ALT levels were recorded in short-term treatment and higher AST and ALT in long-term treatment than PLA, the AST (8 - 48 U/L) and ALT (7 - 55 U/L) levels were still at the normal concentration levels. Thus, this supports the study by Tambi (2005), who demonstrated that 600 mg of *E. longifolia* is not toxic to healthy males' liver function. This suggests that supplementation of 600 mg of *E. longifolia* is safe for prolonged supplementation in humans.

4.6 Conclusion

The present study has investigated, for the first time, the potential relationship between *E. longifolia* and the hormonal changes in humans, specifically young, healthy males. Although there are differences in total testosterone and free testosterone levels between short and long-term studies, our findings are in agreement with several previous studies, which have demonstrated an increase in testosterone levels. The findings show a beneficial effect in short-term supplementation by increasing testosterone and free testosterone but not in the long-term supplementation of *E. longifolia* in young individuals. However, possible confounding factors such as exercising during the supplementation period and a larger decrease in testosterone in PLA may affect the study's outcome. The findings also demonstrate that *E. longifolia* did not affect the HPG axis. Hence the claim that *E. Longifolia* acts as a testosterone booster might not be accurate in a population with normal circulating testosterone levels, even with a high dose and extended supplementation period.

CHAPTER 5: THE EFFECTS OF *Eurycoma longifolia* ON MOOD AND SEXUAL FUNCTION FOLLOWING SHORT AND LONG-TERM CONSUMPTION

5.1 Introduction

Herbal medicines have been used to treat various health challenges (WHO, 2004), such as those concerned with psychological mood and sexual function (Litwin *et al*., 1998; Shabsigh *et al*., 1998; Laumann *et al*., 1999; Althof, 2002). It is known that a significant development in hormonal changes among adolescents in the endocrine system may be due to the activation of the HPG axis, which enhances the secretion of sex hormones to induce adaptations in physical (i.e., sexual health) and psychological characteristics (i.e., mood) (Naughton *et al*., 2000).

Most testosterone therapies are prescribed to middle-aged and older men with age-related declines in testosterone (US Food and Drug Administration, 2014). While the effects of exogenous testosterone on physical functions are well documented (Anderson & Wu, 1996; Bhasin *et al*., 1996; Bhasin *et al*., 2001), much remains to be learned about its effects on mood and sexual function. The results concerning the use of testosterone therapy in men and data from previous studies examining the effects of testosterone supplementation in men with low testosterone level on mood and sexual health are limited and conflicting, with some suggesting improvements (Steidle *et al.*, 2003; Wang *et al.*, 2004; Cavallini *et al.*, 2004; Daniell *et al.*, 2006; Wang *et al.*, 2011), while some suggest otherwise (Seidman *et al.*, 2001; Shores *et al.*, 2009; Pope *et al.*, 2010). Thus, alternative and complementary medicines, such as herbal supplements, are considered replacements to conventional therapeutics to improve mood and sexual function.

Eurycoma longifolia has a wide range of therapeutic properties as general health remedies, which include enhancement in physical and mental energy levels and aspects of overall quality of life, such as mental health, physical functioning, role limitations due to physical health, role limitations due to emotional health, energy/fatigue ratio (vitality), emotional wellbeing, social functioning, pain and general health (Ali & Saad, 1993; Bhat & Karim, 2010). Most importantly, *E. longifolia* can increase testosterone production in humans (Tambi *et al*., 2012; Henkel *et al*., 2014), which may have a beneficial effect in improving mood, physical status (i.e., strength, body composition), and sexual function (Miller, 2009; Grossmann, 2011).

Although limited, studies involving the consumption of *E. longifolia* have demonstrated exciting findings that show a higher testosterone level is associated with an improved psychological mood state (Talbott *et al*., 2013; George *et al*., 2018). Also, in recent years, *E. longifolia* has been shown to improve sexual function (Goreja, 2004; Bhat & Karim, 2010; Talbott *et al*., 2013; Wizneh & Asmawi, 2014) through the elevation of reproductive hormones, in particular, testosterone (Talbott *et al*., 2006; Tambi *et al*., 2012; Henkel *et al*., 2013; Udani *et al*., 2014). Earlier studies have identified that eurypeptides, a group of bioactive peptide fractions (4300-dalton glycopeptide with 36 amino acids) found in *E. longifolia*, are able to increase muscle size and strength (Hamzah and Yusof, 2003; Sarine *et al*., 2009), improve overall sexual wellbeing (Ismail *et al*., 2012; Udani *et al*., 2014), accelerate recovery from exercise (Talbott *et al*., 2006), reduce stress and improve mood (Talbott *et al*., 2013), and reduce symptoms of fatigue (Tambi, 2003; Tambi, 2005; Tambi, 2007) by elevating testosterone (Ang *et al*., 2001; Asiah *et al*., 2007; Zanoli *et al*., 2009; Tambi *et al*., 2012). Recent studies then reported how eurycomanone, a quassinoid isolated from *E. longifolia*, demonstrated an ability to

increase the production of testosterone in isolated rat testicular Leydig cells (Low *et al*., 2013a, 2013b)

Daily stress and aspects of lifestyle, such as diet, exercise and sleep patterns, among the youth may hasten the lowering of testosterone levels, affecting their psychological mood state and sexual function. Therefore, maintaining a youthful testosterone level should enable the human body to maintain or improve its psychological mood and general wellbeing, including sexual function (Miller, 2009; Grossmann, 2011). Though there are a couple of studies that have reported on an older age population (George *et al*., 2013; Talbott *et al*., 2013), there are no studies that have reported on the effects of *E. longifolia* on psychological and sexual function among a younger cohort of people who are going through ageing, daily stress, and changes in lifestyle, which are subsequently affecting their mood and sexual function.

The present study aims to address the following:

(i) to investigate the short and long-term effects of *E. longifolia* supplementation on psychological mood state among collegiate males; and

(ii) to investigate the short and long-term effects of *E. longifolia* supplementation on sexual function among collegiate males.

5.2 Literature Review

5.2.1 Testosterone Levels on Psychological Wellbeing and Sexual Function

Testosterone levels decrease gradually by 40% between the ages of 40 to 70 years (Davidson *et al*., 1983; Seidman, 2007; Bhasin *et al*., 2010). Hypogonadism is described as involving one or more symptoms, including low libido, erectile dysfunction and

osteoporosis. It is also defined as involving two or more symptoms, such as sleep disturbance, depressed mood, lethargy or low physical performance, or a total testosterone level below 300 ng/dL (Araujo *et al*., 2007; Johnson *et al*., 2013). Studies have shown that testosterone replacement therapy significantly improves sexual function (sexual desire, enjoyment without a partner, enjoyment with a partner, percentage of full erection, self-assessment of satisfaction with erection), positive mood scores (alert, full of energy, friendly, feeling well/good) and combats negative mood scores (angry, irritable, sad or blue, tired and nervous (Bhasin *et al*., 2010; Bhasin & Basaria, 2011). However, studies also suggest no correlation between lower testosterone and depressive disorders, and further study is warranted (Araujo *et al*., 1998; Barrett-Connor *et al*.,1999; Booth *et al*., 1999; Johnson *et al*., 2013). The potential adverse effects associated with exogenous testosterone administration are still limited and unknown (Grech *et al*., 2014; Ryan and Nathan., 2017).

5.2.2 Effect of *E. longifolia* consumption on psychological mood

Complementary and alternative medicine (CAM) has become popular among adults to alleviate stress and avoid the possible adverse effects of pharmaceutical drugs (George *et al*., 2018). However, few studies have sought the perspective of young adults on how CAM is used, a population said to be facing high levels of stress and poor mental health (Patel *et al*., 2007; Blanco *et al*., 2008; Storrie *et al*., 2010; Sifferlin, 2013; Eisenberg *et al*., 2013). Perceived stress and poor mental health are prevalent among young adults in the range of 16 to 24 years old (Storrie *et al*., 2010; Eisenberg *et al*., 2013), and often this condition is related to anti-social behaviour and poor social relations (Johnson, 1991; Sandi & Haller, 2015). This stress condition, if left untreated, will significantly increase the risk of developing an array of symptoms, such as anxiety, depression, and fatigue

(Lazarus, 1999; George *et al*., 2018). Pharmaceutical drugs are commonly used to treat stress and anxiety disorder. While there is a concern that someone taking anti-depressant drugs may become addicted and dependent on them (Fernandes *et al*., 2018), data are still limited.

At present, only two studies have examined the effect of *E. longifolia* on psychological mood state in humans using the POMS questionnaire (Table 5.1). Both studies recruited similar age groups (46 to 65 years), and the participants were moderately stressed. Talbott *et al*. (2013) revealed that 200 mg/day of *E. longifolia* showed improvement in the tension (-11%), anger (-12%), and confusion (-15%) domains. On the other hand, George *et al.* (2018) found the combination of *E. longifolia* (50 mg) and multivitamins showed improvement in the vigour domain (14.1%) and decreased negative mood. The authors suggest that a positive mood state, which indicates positive mental health or desirable emotional health status among the older population, is achievable by administrating *E. longifolia*. It is postulated that the improvement may be due to the eurypeptides in *E. longifolia* which improve energy status and sex drive by raising testosterone in rodent's studies.

Also, eurypeptides are said to influence free testosterone release of from SHBG (Chaing *et al*., 1994; Tambi, 2003). The stress hormone cortisol increases under stressed states and, as a result, the opposite effect is that the testosterone levels decrease (Sherman *et al*., 2016). While Talbott *et al*. (2013) reported a rise in testosterone levels and a reduction in cortisol levels, George *et al*. (2018) only managed to report an increase in testosterone without measuring the cortisol level. Cortisol, a primary stress hormone, is produced via the HPA axis. However, no studies examined how *E. longifolia* affect the glucocorticoid biosynthesis pathway in producing cortisol. Thus, the precise mechanism

of restoring testosterone levels via HPG and reducing the cortisol levels via HPA is still unknown and, at the very least, requires further study.

A positive mood state (i.e., an increase in vigour and a reduction in tension, anger, depression and fatigue) can be achieved when testosterone levels are restored to their normal range (Anderson *et al*., 1999; Wang *et al*., 2000; O'Connor *et al*., 2001). Testosterone has long been associated with mood, and often it is reported in case reports involving exogenous testosterone administration (Talih *et al*., 2007; Johnson *et al*., 2013). Talbott *et al*. (2013) and George *et al*. (2018) have suggested that the positive mood state improvement is likely due to the association with the restoration of normal sexual function through the consumption of *E. longifolia*. However, only a small number of studies have provided information on the ability of *E. longifolia* to improve mood by restoring or maintaining healthy testosterone levels (Ang & Cheang, 1999; Talbott *et al*., 2013; Johnson *et al*., 2013).

Table 5.1: Studies on the effect of *E. longifolia* on psychological mood state.

Study	Test	Dosage	Outcome	Reference
The anxiolytic activity of *E. longifolia* Jack roots	Adult mice Twice daily for 5 days. Open-field test, elevated plus-maze, and anti-fighting tests.	*E.longifolia* fractions: Chloroform (0.3 g/kg) Methanol (0.3 g/kg) Water (0.3 g/kg) Butanol (0.3 g/kg) Control (3 ml/kg) Diazepam (1 mg/kg)	*E. longifolia* attenuated anxiety parameters in open-field and plus-maze test and inhibited footshock-induced fighting.	Ang and Cheang (1999)
Effect of *E. longifolia* on stress hormones and psychological mood state	POMS in 63 moderately stress men and women. Supplemented for 4 weeks	200 mg / day	Significant improvement for tension, anger, and confusion Cortisol and testosterone significantly improve, reduced cortisol exposure and increased testosterone	Talbott *et al*. (2013)
Efficacy and safety of *E. longifolia* (Physta) water extract plus multivitamins on quality of life, mood, and stress	POMS in 93 moderately stress subjects. *E. longifolia* plus multivitamins 12 weeks	50 mg /day	Increased trend in POMS vigour domain Significant improvement on POMS vigour for age 46-65 men	George *et al*. (2018)

5.2.3 The effect of *E. longifolia* supplementation on sexual function

Eurycoma longifolia root is a popular traditional supplement to enhance sexuality and fertility (Ang and Sim, 1998; Tambi and Imran, 2010). The water extract contains various bioactive compounds with pro-fertility and aphrodisiac properties, as seen in animal and human studies (Sambandan *et al*., 2006; Asiah *et al*., 2007; Tambi and Imran, 2010).

E. longifolia improves erectile dysfunction in hypogonadal men by enhancing testosterone levels (Hussein *et al*., 2007; Bhat & Kharim, 2010; Hassali *et al*., 2009). Hence demonstrating the aphrodisiac effects of *E. longifolia* in improving libido and countering erectile dysfunction (Ang and Thai, 2001; Ang and Lee, 2002). Bioactive compounds, such as phenolic compounds, polypeptides, diterpenoids, alkaloids and quassinoids, found in *E. longifolia* have been reported to be the factors behind the aphrodisiac effect (Ang and Thai, 2001; Ang and Lee, 2002). Various fractions of *E. longifolia* have been used to investigate the effect on sexual function in rats (Ang and Lee, 2003; and Ang *et al*., 2003) and have highlighted that eurypeptides exhibited aphrodisiac effects by increasing testosterone levels (Ang and Lee, 2003; and Ang *et al*., 2003). While studies examining eurypeptide influencing testosterone increase are scarce, recent studies have shown eurycomanone, a quassinoid in *E. longifolia*, also exhibiting the ability to increase testosterone levels in rats by affecting the HPG axis (Low *et al*., 2013a & 2013b).

The impact of *E. longifolia* in handling male sexual dysfunction has primarily been studied using *in vivo* animal models (Ang and Thai, 2001; Ang and Lee, 2002; Ang *et al*., 2003; Ang *et al*., 2004) and some human models (Tambi, 2009; Tambi *et al*., 2012; Ismail *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2014; George and Henkel, 2014; Udani *et*

al., 2014) (Table 5.2). In a study conducted by Udani *et al*. (2014), a 12 week randomised, double-blind, placebo-controlled, and parallel-group study demonstrated that the combination of 300 mg of *E. longifolia* and *Polygonum minus* (an antioxidant) improves sexual intercourse performance, the concentration of male sex hormone, sex libido and overall sexual wellbeing in men between 40 to 65 years old.

Also, two earlier studies, by Ismail *et al*. (2012) and Tambi *et al*. (2012), have shown similar findings. The effect of 300 mg of *E. longifolia* daily in healthy men between 30 to 55 years old have shown improvements in sexual libido, sexual activities and sexual satisfaction, and positive effects on seminal parameters, which include the volume and concentration of sperm and the proportion of sperm having normal motility (Ismail *et al*., 2012). A lower dose of 200 mg per day in hypogonadic men between 30 to 64 years old found an improvement in sexual performance, including erectile function, sexual intercourse performance, and penile hardness (Tambi *et al.*, 2012). All three studies demonstrated an improvement in testosterone levels.

Several other studies have been performed to investigate the effect of *E. longifolia* on male sexual libido. Talbott *et al*. (2013) performed a clinical placebo-controlled trial in 63 subjects by supplementing 200 mg of *E. longifolia* for four weeks and found significant improvement in testosterone levels, with concurrent improvement in sexual activities. Another study by Henkel *et al*. (2014) examining the effects of 400mg of *E. longifolia* on age-related sexual impotence among 13 physically active males aged between 57 to 72 years for five weeks found an improved sexual function with an improvement in the total and free testosterone concentrations. Similarly, the significance of *E. longifolia* in improving male sexual libido and testosterone levels have been shown in a study conducted by George and Henkel (2014). They reported the supplementation of *E.*

longifolia had successfully restored serum testosterone levels compared to the conventional testosterone replacement therapy and demonstrated that *E. longifolia* could improve male sex libido in elderly patients with 40 to 50 % lower testosterone levels compared to young patients. The increase in testosterone levels, reported in the above published clinical studies, may contribute to improving sexual performance in male adults, especially in adults between 30 to 65 years old who have lower baseline testosterone levels.

Considering the adaptogenic nature of *E. longifolia*, the plant could be more effective in adult participants with lower baseline levels of testosterone and not in those with optimum health and wellbeing (Tambi *et al*., 2012; Udani *et al*., 2014). While there are reports of high numbers of sexual problems and rates of sexual dysfunction in adulthood, few recent survey studies have found that there is such a high prevalence of problems in sexual functioning among adolescents and young adults (Akre, Berchtold, Gmel & Suris, 2015; Mitchell *et al*., 2016; O'Sullivan *et al*., 2016). Based on the clinical evidence from previous studies demonstrating the benefits of *E. longifolia* in men age 30 to 64 years in improving sexual function, it would be misleading to make any definitive claims that the effects of *E. longifolia* concerning sexual function will be the same on young, healthy participants below the age of 30 years, with normal circulating testosterone levels. At the very least, this requires further study.

Table 5.2: Studies demonstrating promising potential in improving male sexual function

Test	Subjects	Dosing regimen and duration	Findings	Reference
Sexual Intercourse Attempt diary, Erection Hardness Scale, Sexual Health Inventory of Men, and Aging Male Symptom	30 healthy male aged 40 - 65 years	300 mg daily 6 months	Significant improvements in all test	Udani *et al*. (2014)
The Ageing Males' Symptoms (AMS) and the serum testosterone concentration	76 patient with LOH aged 30 - 64 years	200 mg daily 1 month	Improved AMS score, serum testosterone concentration. Improvement in sexual performance	Tambi *et al*. (2012)
International Index of Erectile Function (IIEF) and Sexual Health Questionnaires (SHQ); Seminal Fluid Analysis (SFA)	109 healthy men aged 30 - 55 years	300 mg daily 3 months	Improved in the domain Physical Functioning, Erectile Function domain, sexual libido	Ismail *et al*. (2012)
Electrical copulation cage	Sexually sluggish old adult male rats	0.5 g/kg *E. longifolia*	Decreased the hesitation time of noncopulator male rats Transient increase in the percentage of the male rats responding to the right choice	Ang and Ngai (2001)
Monitoring orientation activities towards the receptive females	Adult middle-aged 9 months old and retired breeders	200, 400 and 800 mg/kg of *E. longifolia* fraction	Increased orientation activities towards the receptive females (anogenital sniffing, licking and mounting), increased genital grooming towards themselves	Ang *et al*. (2000)
Electrical copulation cage during the dark phase of the light-dark cycle and in subdued light.	Adult male rats, 9 months old and retired Breeders	0.5 g/kg of *E. longifolia* control group : 3 ml/kg of normal saline daily for 12 weeks	Enhanced sexual qualities by decreasing their hesitation time to controls.	Ang *et al*. (2003)

5.3 Methods

5.3.1 Participants

The participant information and participant's anthropometric is presented in Chapter 3, Table 4.3. The study was approved by the Liverpool John Moores University Ethics Committee (14/SPS/013), and all participants provided their informed consent before embarking on the study.

5.3.1.1 Experimental Design

The study was a double-blind, placebo-controlled matched-paired design and was conducted concurrently with the study of the reproductive hormone presented in Chapter 4. The duration of the short and long term phase was 2 weeks and 8 weeks, respectively. Participants for the short term phase were asked to complete the Brunel of Mood Scale (BRUMS) questionnaire every day and the Brief Male Sexual Function Inventory (BSFI) questionnaire on days 0, 7, and 14. As for the long-term phase, participants were asked to complete the BRUMS questionnaire three times each week for 8 weeks and the BSFI at baseline (Day 0), interim (week 4), and post supplementation (week 8).

5.3.2 Experimental Protocol

5.3.2.1 Mood state measurement

The participant's mood was assessed using the BRUMS (Terry *et al*., 2003). The BRUMS questionnaire consists of six subscales including anger, confusion, depression, tension, fatigue and vigour assessed using 24 self-report items with a five-point Likert scale (0 = not at all, 1 = a little, 2 = moderately, 3 = quite a lot, 4 = extremely). Each

subscale consists of four items. Tension consists of items such as "worried" and "anxious", anger includes "furious" and "bad-tempered", fatigue include "worn out" and "exhausted", vigour includes "lively and "energetic", confusion include "mixed-up" and "uncertain" and lastly depression includes item such as "miserable" and "downhearted". Mood states are presented as standard scores illustrated in Table 5.3. To identify the standard score for each domain, the items were added accordingly. Items 1, 13, 14, and 18 for tension score, items 5, 6, 12 and 16 for depression score, items 7, 11, 19 and 22 for anger score, items 2, 15, 20 and 23 for vigour score and items 3, 9, 17 and 24 for confusion score. BRUMS was developed by Terry *et al*. (1999) and has undergone rigorous validity testing (Terry *et al*., 2003). BRUMS reported a high internal consistency, with Cronbach coefficient alphas ranging from 0.74 to 0.90 for each of the subscales (Terry *et al*., 1999). The test-retest reliability demonstrates a coefficient ranging from 0.26 to 0.53 over a one-week period, which is suitable for measuring transient feeling states.

The raw score obtained was converted into the standard score (Table 5.3). The standard score on the BRUMS was converted into a standard T-score format (Mean = 50.0 and SD =10.0) corresponding to the raw score for each subscale (Karageorghis, 2017). The standard score has a range of 1 to 100 and can be interpreted as a percentage score. The standard score is identified by reading across from the raw score (i.e., a raw score of 4 has a standard score of 57 for tension, a raw score of 7 for depression has a standard score of 67). High vigour score (standard score more than 50) and low tension, depression, anger, fatigue, and confusion (standard score below 50) indicate a positive mood.

Table 5.3: BRUMS standard score for adults.

Raw Score	Tension	Depression	Anger	Vigour	Fatigue	Confusion
0	42	43	44	34	38	42
1	46	47	48	36	40	45
2	49	50	51	39	42	48
3	53	54	54	42	45	51
4	57	57	58	45	47	55
5	61	60	61	47	49	58
6	65	64	64	50	51	61
7	69	67	67	53	54	64
8	72	71	71	55	56	68
9	76	74	74	58	58	71
10	80	77	77	61	61	74
11	84	81	81	64	63	77
12	88	84	84	66	65	81
13	92	88	87	69	67	84
14	96	91	90	72	70	87
15	99	94	94	75	72	91
16	103	98	97	77	74	94

Source: Karageorghis (2017)

5.3.2.2 Sexual function measurement

Sexual function was assessed using the Brief Male Sexual Function Inventory (BSFI; O'Leary *et al.*, 1995). The questionnaire includes 11 questions divided into five domains: sexual drive (two items), erection (three items), ejaculation (two items), problem assessment (three items) and overall satisfaction (one item), of which the responses to each are based on a Likert scale from 0 to 4. The first ten-item covers the functional aspects of male sexuality, and the last item in overall satisfaction is not considered a functional question (MyKletun *et al.*, 2005). Participants were required to answer

questions related to the five domains, such as "During the past 30 days, on how many days have you felt sexual drive?', "Over the past 30 days, how often have you had partial or full sexual erections when you were sexually stimulated in any way?", "In the past 30 days, how much difficulty have you had ejaculating when you have been sexually stimulated?", "In the past 30 days, to what extent have you considered a lack of sexual drive to be a problem?", "Overall, during the past 30 days, how satisfied have you been with your sex life?". The findings are presented according to the average score added for each item in respective domains. BSFI reported an internal consistency, with Cronbach coefficient alphas ranging from 0.62 to 0.95 for each domain. Test-retest reliability for a 1-week interval showed an intra-class correlation coefficient of 0.70 to 0.90.

5.3.3 Statistical Analysis

All statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (SPSS Inc., Chicago, USA). Within-between group differences to examine the time-treatment interaction within-group differences to examine time effect and between-group differences to examine treatment effect was analysed using the repeated measurement ANCOVA. Assumption of normality, homogeneity of variances, compound symmetry, and homogeneity of regression was checked and fulfilled using the Shapiro-Wilk test. Baseline data were also analysed using the parametric (independent *t*-test). All data in text, figures, and tables were presented as means ± SD with P values ≤ 0.05 indicating statistical significance.

5.4 Results

5.4.1 Effect of *E. longifolia* on mood state based on the Brunel Mood Scale (BRUMS) assessment.

Prior to the *E. longifolia* and placebo intervention for the short and long-term supplementation period, baseline measurements were recorded (Table 5.4). Baseline measurements showed that the participants in both short and long-term supplementations matched the inclusion criteria and did not exhibit any mood state and sexual function abnormality. There were no differences at baseline between EL and PLA in both the short and long-term supplementation studies for the BRUMS domains, i.e., tension, anxiety, depression, vigour, fatigue, and confusion. Statistically, there is no significant ($p > 0.05$) difference between the EL and PLA on mood state profile throughout both short and long term supplementation periods, respectively.

Figure 5.1A and B shows the mood state profile of both short and long term supplementation periods at baseline. A positive mood state demonstrates high vigour activity above the average standard score of 50.0 and low or below the average standard score of 50.0 in the negative mood domains such as anger, confusion, depression, fatigue, and tension (Morgan, 1980). The baseline findings showed that all the mood domains well above the average standard score (Figure 5.1A and 1B). In the short-term supplementation study shown in Figure 5.1A, vigour recorded a higher score compared to depression, anger, fatigue, and confusion but lower than the tension score. A similar trend is also found in the long term supplementation period (Figure 5.1B), and all domains are above the average standard score, with tension scoring higher than vigour.

Table 5.4: Baseline measurement on mood states in short and long term *E. longifolia* or placebo supplementation

	Short term			Long term		
	EL *(n = 8)*	**PLA (n = 8)**	***p***	**EL (n = 11)**	**PLA (n = 10)**	***p***
	M ± SD	**M ± SD**		**M ± SD**	**M ± SD**	
Tension	67.13 ± 11.61	65.13 ± 7.95	0.694	64.46 ± 6.50	70.20 ± 9.92	0.130
Anger	61.13 ± 5.64	59.88 ± 3.18	0.594	61.36 ± 5.50	62.00 ± 4.90	0.783
Depression	61.75 ± 7.11	60.00 ± 6.16	0.607	59.18 ± 4.49	63.40 ± 6.65	0.102
Vigour	63.63 ± 7.01	61.25 ± 6.69	0.500	62.00 ± 8.31	60.40 ± 9.11	0.678
Fatigue	58.63 ± 9.47	58.13 ± 7.16	0.907	56.64 ± 7.84	58.10 ± 6.61	0.651
Confusion	60.50 ± 6.55	58.75 ± 3.50	0.516	58.46 ± 6.59	59.10 ± 5.47	0.811

p value based on parametric test (independent *t*-test); EL: *E. longifolia* treated group; PLA: Placebo

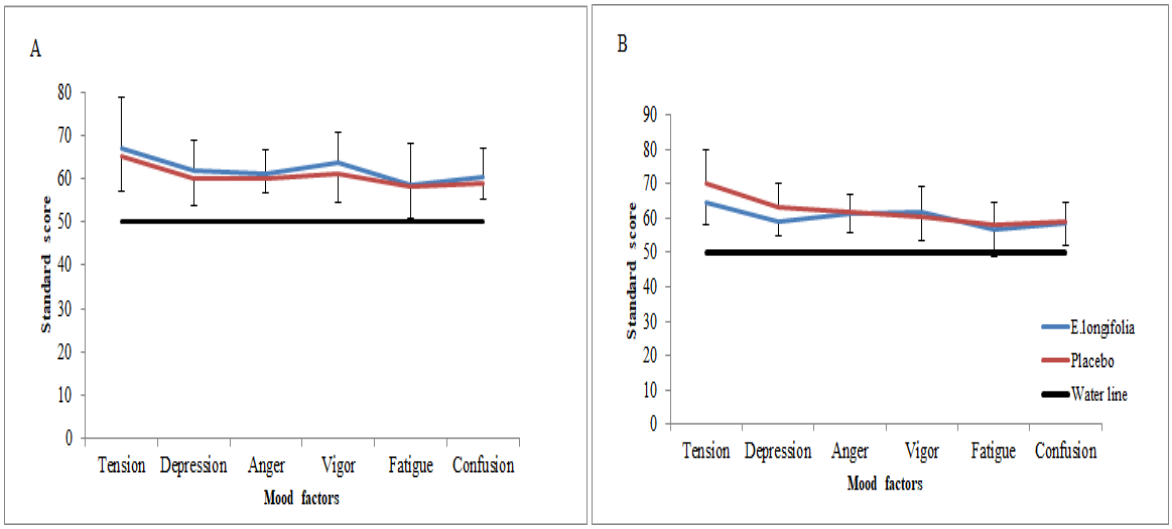


Figure 5.1: Baseline measurement of mood state profiling for (A) short term and (B) long-term supplementation period.

5.4.1.1 Short term E. longifolia supplementation

Throughout the short term supplementation study, ANCOVA repeated measurement was used to analyse the findings. No significant main effect of time for tension ($F_{2, 26}$ = 5.941, p = 0.088) was observed throughout the short-term period. There is no significant main interaction between group and time for tension score ($F_{2, 26}$ = 0.299, p = 0.744). Pairwise comparison within EL from baseline (67.13 ± 11.61%) to post (61.75 ± 9.48%) (MD = 5.38%, 95% CI = - 4.438, 15.188, p = 0.365) supplementation and PLA from baseline (65.13 ± 7.95%) to post tests (63.75 ± 9.30%) (MD = 1.38%, 95% CI = - 9.90, 12.652, p = 1.000) shows no significant changes in tension. No significant differences between EL and PLA in tension score at post test, t (14) = - 0.426, p = 0.677 (Figure 5.2A).

In the anger domain, there was a significant main effect of time ($F_{1.293, 16.813}$ = 8.961, p = 0.005) observed in the anger domain. There is no significant interaction between group and time ($F_{1.293, 16.813}$ = 1.442, p = 0.255). After two weeks of *E. longifolia* supplementation, a significant decrease in anger score in EL from baseline (61.13 ± 5.64%) to post test (58.00 ± 0.00%) (MD = -3.13%, 95% CI = 3.125, 3.125, p < 0.001) compared with PLA (MD = 0.50%, 95% CI = -3.99, 2.99, p = 1.000). Independent t-test shows no significant changes in anger score between EL and PLA at post-test, t (14) = - 1.199, p = 0.251) (Figure 5.2B).

In the depression domain, no significant main effect of time ($F_{2, 26}$ = 8.697, p = 0.100) and no significant interaction between group and time ($F_{2, 26}$ = 0.355, p = 0.705) was reported. Pairwise comparison shows no significant changes within EL from baseline (61.75 ± 7.11%) to post-test (59.13 ± 4.91%) (MD = 2.63%, 95% CI = - 2.812, 8.062, p

= 0.491) and also in PLA from baseline (60.00 ± 6.16%) to post-test (60.38 ± 4.96%) (MD = -0.38%, 95% CI = - 3.822, 3.072, p = 1.000). Also no significant differences between EL and PLA observed throughout the two-week supplementation, t (14) = -0.136, p = 0.620 (Figure 5.2C).

No significant main effect of time ($F_{2, 26}$ = 1.377, p = 0.270) and no significant main interaction between group and time ($F_{2, 26}$ = 0.829, p = 0.448) for the vigour domain. Pairwise comparison within group did not report any significant changes in EL from baseline (63.63 ± 7.00%) to post-test (64.00 ± 9.10%) (MD = - 0.37%, 95% CI = - 9.721, 8.971, p = 1.000) and also in PLA from baseline (61.25 ± 6.69%) to post-test (59.88 ± 10.64%) (MD = 1.38%, 95% CI = - 11.980, 14.730, p = 1.000). No significant difference between EL and PLA was observed at post test, t (14) = 0.833, p = 0.419 (Figure 5.2D).

In the fatigue domain, there was a significant main effect of time ($F_{1.405, 18.265}$ = 6.288, p = 0.014) but no significant main interaction between group and time ($F_{1.405, 18.265}$ = 0.172, p = 0.766). Pairwise comparison shows a significant decrease in fatigue score in EL from baseline (58.63 ± 9.47%) to post test (52.13 ± 5.08%) (MD = 6.50%, 95% CI = 0.385, 12.615, p = 0.039) and also in PLA from baseline (58.13 ± 7.16%) to post test (51.50 ± 4.50%) (MD = 6.63%, 95% CI = 1.537, 11.713, p = 0.016) (Table 5.5). There was no significant difference between EL and PLA at post-test, t (14) = 0.260, p = 0.798 (Figure 5.2E).

In the confusion domain, a significant main effect of time ($F_{2, 26}$ = 2.398, p = 0.011) and no significant main interaction between group and time ($F_{2, 26}$ = 1.524, p = 0.237) was observed. Pairwise comparison showed a significant decrease in confusion score in EL from baseline (60.50 ± 6.55%) to post test (55.75 ± 2.12%) (MD = 4.75%, 95% CI =

2.722, 6.778, $p = 0.001$) but no significant changes in PLA from baseline (58.75 ± 3.50%) to post test (58.50 ± 5.95%) (MD = 0.25%, 95% CI = -4.587, 5.087, $p = 1.000$) was observed (Table 5.5). There is no significant difference between EL and PLA at post-test, t (14) = - 1.231, $p = 0.239$ (Figure 5.2F).

Table 5.5: Treatment effect of *Eurycoma longifolia* and placebo after two week supplementation and the percentage changes from baseline to post-test.

Variable	EL (n = 8)			PLA (n = 8)		
	MD (95% CI)	*p*	%	MD (95% CI)	*p*	%
Tension	-5.375[-4.438, 15.19]	0.365	-8.01	-1.375[-9.90, 12.65]	1.000	-2.12
Anger	-3.125[3.125, 3.125]	0.000*	-5.12	0.500 [-3.99, 2.99]	1.000	0.84
Depression	-2.625 [-2.81, 8.06]	0.491	-4.24	0.375 [-3.82, 3.07]	1.000	0.63
Vigour	0.375 [-9.72, 8.97]	1.000	0.58	-1.375 [-11.98, 14.73]	1.000	-2.24
Fatigue	-6.50 [0.39, 12.62]	0.039*	-11.09	-6.63 [1.54, 11.71]	0.016*	-11.41
Confusion	-4.750 [2.72, 6.778]	0.001*	-7.85	-0.250 [-4.59, 5.09]	1.000	-0.43

Note: Day 0 (Baseline); Day 14 (Post); MD = Mean difference; CI = Confidence interval; EL = *E. longifolia* group; PLA = Placebo.
Numerical covariate (baseline) was controlled by using ANCOVA repeated measurement.
*Level of significance was set at 0.05 (two-tailed); % increased or decreased (-)

5.4.1.2 Long-term E. longifolia supplementation

There was a significant main effect of time ($F_{2, 36} = 1.041$, $p = 0.044$) and no significant main interaction between group and time for tension ($F_{2, 36} = 0.584$, $p = 0.563$) was observed. After 8 weeks of *E. longifolia* supplementation, pairwise comparison showed a significant decrease in tension score in EL from baseline (64.46 ± 6.50%) to post test (59.18 ± 3.74%) (MD = -5.27%, 95% CI = 1.901, 8.645, $p = 0.004$) compare with PLA from baseline (70.20 ± 9.92%) to post-test (69.40 ± 16.58%) (MD = 0.80%, 95% CI = -13.024, 14.624, $p = 1.000$) (Table 5.6). Between group analysis shows no significant difference between EL and PLA at post test, t (19) = -1.994, $p = 0.061$ (Figure 5.3A).

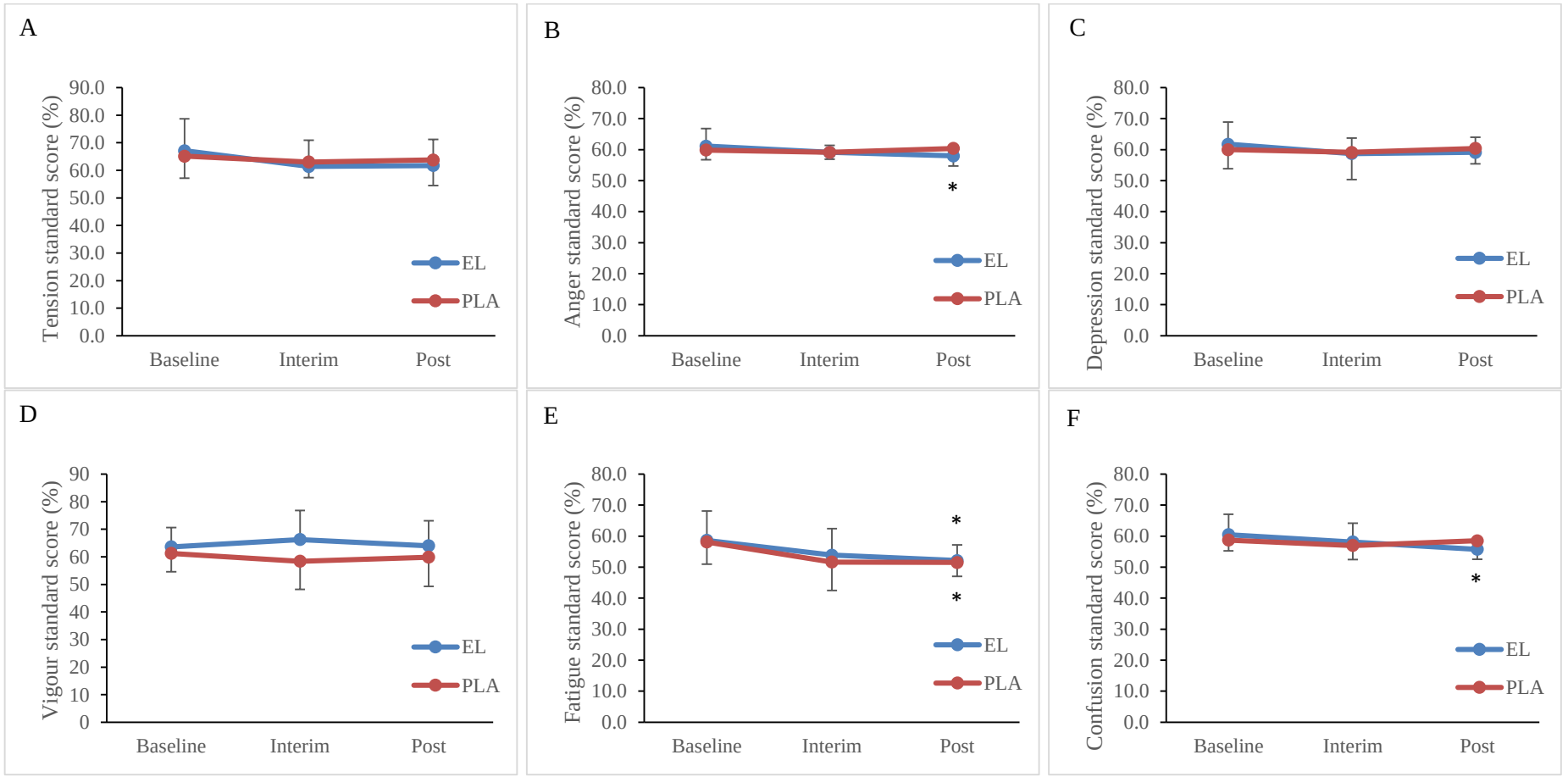


Figure 5.2: Effect of *E. longifolia* on tension (A), anger (B), depression (C), vigour (D), fatigue (E), and confusion (F) based on BRUMS assessment in the short-term phase to examine the time effect within-group and between-group (2 weeks)

*indicates significant differences within group with ANCOVA repeated measurement applied (Baseline vs. Post).

* Denotes level of significance was set at 0.05 (two-tailed)

There was significant main effect of time ($F_{1.233, 22.188} = 6.399$, $p = 0.014$) and there was no significant interaction between group and time ($F_{1.233, 22.188} = 0.352$, $p = 0.604$) for anger domain. Pairwise comparison show a significant decrease in anger in EL from baseline (61.36 ± 5.50%) to post test (58.82 ± 1.94%) (MD = -2.55%, 95% CI = 0.782, 4.309, $p = 0.007$) but PLA did not show significant changes from baseline (62.00 ± 4.90%) to post-test (60.50 ± 4.22%) (MD = 1.50%, 95% CI = -2.50, 5.503, $p = 0.874$ (Table 5.6). Independent t-test shows no significant differences in anger score between EL and PLA at post-test, t (19) = -1.192, $p = 0.248$ (Figure 5.3B).

No significant main effect of time ($F_{1.389, 25.003} = 1.402$, $p = 0.259$) and no significant interaction between group and time ($F_{1.389, 25.003} = 0.218$, $p = 0.723$) was reported in the depression domain. Pairwise comparison shows no significant changes in depression score in EL from baseline (59.18 ± 4.49%) to post test (58.55 ± 4.23%) (MD = 0.64%, 95% CI = - 3.268, 4.541, $p = 1.000$) and also in PLA from baseline (63.40 ± 6.65%) to post-test (62.40 ± 8.73%) (MD = 1.00%, 95% CI = - 5.68, 7.68, $p = 1.000$) (Table 5.6). There is no significant difference between EL and PLA was observed post eight week supplementation, t (19) = - 1.307, $p = 0.207$ (Figure 5.3C).

In vigour no significant main effect of time ($F_{2, 36} = 2.634$, $p = 0.086$) and no significant main interaction between group and time ($F_{2,36} = 0.178$, $p = 0.838$). Pairwise comparison shows no significant changes in depression score in EL from baseline (62.00 ± 8.31%) to post test (61.64 ± 9.77%) (MD = 0.36%, 95% CI = -8.341, 9.068, $p = 1.000$) and also in PLA from baseline (60.40 ± 9.11%) to post-test (63.90 ± 11.03%) (MD = -3.50%, 95% CI = - 13.93, 6.93, $p = 1.000$) (Table 5.6). There are no significant differences between EL and PLA at post-test, t (19) = - 0.499, $p = 0.624$. (Figure 5.3D).

In the fatigue domain, no significant main effect of time ($F_{2, 36} = 4.429$, $p = 0.119$) but no significant main interaction between group and time ($F_{2, 36} = 0.674$, $p = 0.516$). However, in the pairwise comparison shows no significant changes in fatigue score in EL from baseline (56.64 ± 7.84%) to post test (54.27 ± 6.41%) (MD = 2.36%, 95% CI = -3.014, 7.742, $p = 0.688$) and also in PLA from baseline (58.10 ± 6.61%) to post-test (54.60 ± 8.823%) (MD = 3.50%, 95% CI = -4.474, 11.474, $p = 0.667$) (Table 5.6). Also there is no significant differences between EL and PLA after 8 weeks of supplementation, t (19) = -0.098, $p = 0.923$ (Figure 5.3E).

In the confusion domain, no significant main effect of time ($F_{2, 36} = 0.611$, $p = 0.548$) and no significant main interaction between group and time ($F_{2, 36} = 1.743$, $p = 0.189$) was observed. Pairwise comparison showed no significant changes in EL from baseline (58.46 ± 6.59%) to post test (56.36 ± 2.46%) (MD = 2.09%, 95% CI = -0.19, 4.37, $p = 0.075$) but a significant increase in PLA from baseline (59.10 ± 5.47%) to post test (63.00 ± 11.81%) (MD = -3.90%, 95% CI = -7.64, -0.16, $p = 0.041$ (Table 5.6). There are no significant differences observed between EL and PLA at post-test, t (19) = -1.825, $p = 0.084$. (Figure 5.3F).

Table 5.6: Treatment effect of *E. longifolia* and placebo after 8-week supplementation and the percentage changes from baseline to post-test.

Variable	EL (n = 11)			PLA (n = 10)		
	Mean difference (95% CI)	*p*	%	Mean difference (95% CI)	*p*	%
Tension	5.273 [1.90, 8.65]	0.004*	-8.19	0.800 [-13.02, 14.62]	1.000	1.14
Anger	2.545 [0.78, 4.31]	0.007*	-4.14	1.500 [-2.50, 5.50]	0.874	-2.42
Depression	0.636 [-3.27, 4.54]	1.000	-1.07	1.000[-5.68, 7.68]	1.000	-1.58
Vigour	0.364 [-8.34, 9.07]	1.000	-0.58	-3.50 [-13.93, 6.93]	1.000	5.80
Fatigue	2.364 [-3.01, 7.74]	0.688	-4.18	3.500 [-4.47, 11.47]	0.667	-6.02
Confusion	2.091 [-0.19, 4.37]	0.075	-3.59	-3.900 [-7.64, -0.163]	0.041*	6.60

Note: CI=Confidence interval; EL = *E. longifolia*; PLA = placebo
Numerical covariate (baseline) was controlled by using ANCOVA repeated measurement.
*Level of significance was set at 0.05 (two-tailed); % increased or decreased (-)

5.4.2 Effect of *E. longifolia* on sexual function based on The Brief Sexual Function Inventory (BSFI) assessment

Baseline measurements were recorded prior to the supplementation of *E. longifolia* and placebo (Table 5.7). Baseline measurements showed that the participants in both short and long-term supplementations matched the inclusion criteria and did not exhibit sexual function abnormality. There were no differences at baseline between EL and PLA in both the short and long-term supplementation studies for the BSFI domains, i.e., sexual desire, erection, ejaculation, problem assessment, overall satisfaction.

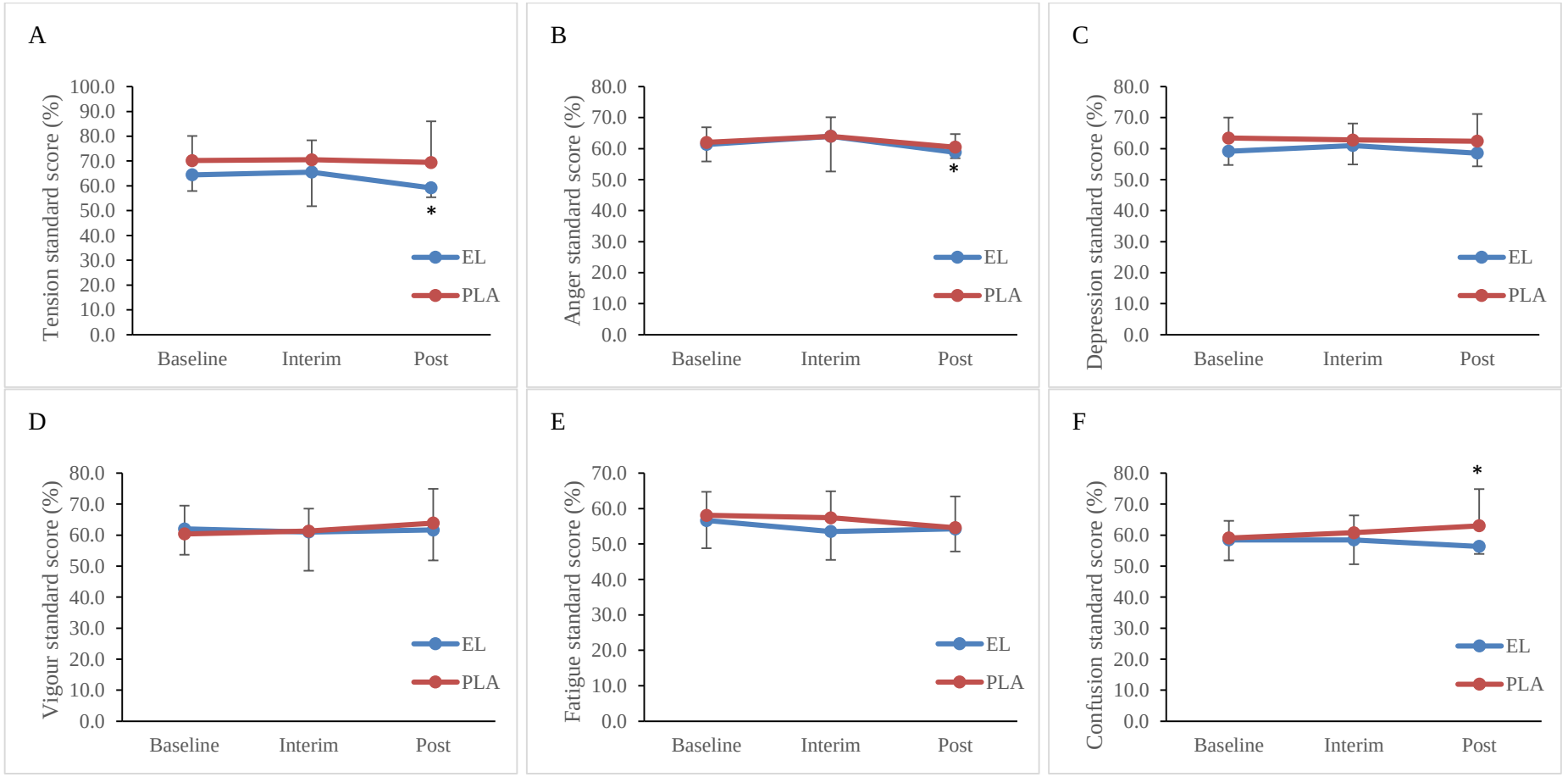


Figure 5.3: Effect of *E. longifolia* on tension (A), anger (B), depression (C), vigour (D), fatigue (E) and confusion (F) based on BRUMS assessment in the long term phase to examine the time effect within-group and between-group (8 weeks)

*denotes indicate significant differences within group with ANCOVA repeated measurement applied (Baseline vs. Post).

* Denotes level of significance was set at 0.05 (two-tailed)

Table 5.7: Baseline measurement on sexual function prior to *E. longifolia* and placebo supplementation

	Short			Long		
	EL (n = 8) M ± SD	**PLA (n = 8) M ± SD**	***p***	**EL (n = 11) M ± SD**	**PLA (n = 10) M ± SD**	***p***
Sexual desire	3.19 ± 0.70	2.38 ± 0.92	0.067	2.40 ± 0.80	2.35 ± 0.78	0.866
Erection	3.71 ± 0.38	3.34 ± 0.69	0.200	2.82 ± 1.14	3.04 ± 0.87	0.624
Ejaculation	3.75 ± 0.38	4.00 ± 0.00	0.104	3.32 ± 1.27	3.85 ± 0.24	0.201
Problem assessment	4.00 ± 0.00	3.71 ± 0.38	0.069	3.52 ± 0.41	3.54 ± 0.67	0.929
Overall satisfaction	3.00 ± 0.76	2.63 ± 0.52	0.266	2.46 ± 1.13	2.40 ± 0.70	0.897

† Denotes *p* value based on parametric test (independent t-test)
Note: E. longifolia (EL); placebo (PLA), mean (M); standard deviation (SD)

5.4.2.1 Short-term supplementation phase

There was no significant main effect of time ($F_{2, 26}$ = 1.653, p = 0.211) and no significant main interaction between group and time for sexual desire domain ($F_{2, 26}$ = 5.663, p = 0.089) was observed. Pairwise comparison showed no significant changes in sexual desire score in EL from baseline (3.19 ± 0.70) to post test (3.00 ± 0.71) (MD = 0.188, 95% CI = -0.13, 0.51, p = 0.307) and also in PLA from baseline (2.38 ± 0.92) to post-test (2.50 ± 0.93) (MD = -0.13, 95% CI = -0.56, 0.31, p = 1.000) (Table 5.8). Between group analysis also showed no significant difference between EL and PLA at post test, t (14) = 1.214, p = 0.245 (Figure 5.5A).

In the erection domain, no significant main effect of time ($F_{1.361, 17.693}$ = 1.244, p = 0.296) and no significant interaction between group and time ($F_{1.361, 17.693}$ = 3.915, p = 0.053). Pairwise comparison showed no significant changes in erection score in EL from

baseline (3.71 ± 0.38) to post test (3.71 ± 0.29) (MD = 0.00, 95% CI = -0.143, 0.143, p = 1.000) and also in PLA from baseline (3.34 ± 0.69) to post-test (3.50 ± 0.72) (MD = -0.163, 95% CI = -0.48, 0.16, p = 0.439) (Table 5.8). No significant changes in erection domain score between EL and PLA at post-test, t (14) = 0.779, p = 0.449 (Figure 5.5B).

A significant main effect of time ($F_{2, 26}$ = 8.449, p = 0.001) was reported in the ejaculation domain but no significant interaction between group and time ($F_{2,26}$ = 0.478, p = 0.626). In the pairwise comparison, there was a significant increase in EL from baseline (3.75 ± 0.38) to post-test (3.94 ± 0.18) [MD = -0.19, 95% CI = -0.32, -0.06, p = 0.011) (Table 5.8) but no significant was found in PLA from baseline (4.00 ± 0.00) to post-test (3.94 ± 0.18) [MD = 0.06, 95% CI = -0.13, 0.26, p = 1.000). No significant differences between EL and PLA after two weeks supplementation, t (14) = 0.000, p = 1.000 (Figure 5.5C).

The problem assessment domain showed a significant main effect of time ($F_{2, 26}$ = 41.016, p < 0.001) but no significant main interaction between group and time ($F_{2,26}$ = 0.206, p = 0.815). Pairwise comparison showed no significant changes in EL from baseline (4.00 ± 0.00) to post test (3.96 ± 0.11) [MD = 0.04, 95% CI = -0.08, 0.16, p = 1.000) but PLA showed a significant increase from baseline (3.71 ± 0.38) to post-test (3.89 ± 0.16) [MD = -0.18, 95% CI = -0.29, -0.07, p = 0.006) (Table 5.8). No significant differences between EL and PLA, t (14) = 1.128, p = 0.278. (Figure 5.5D).

In overall satisfaction domain, no significant main effect of time ($F_{2, 26}$ = 1.337, p = 0.280) and no significant main interaction between group and time ($F_{2, 26}$ = 2.706, p = 0.086) was observed (Table 5.8). Pairwise comparison showed no significant changes in overall satisfaction score in EL from baseline (3.00 ± 0.76) to post test (3.38 ± 0.74) (MD

= -0.38, 95% CI = -0.98, 0.23, p = 0.263) and also in PLA from baseline (2.63 ± 0.52) to post-test (2.75 ± 0.71) (MD = -0.13, 95% CI = -0.55, 0.30, p = 1.000) (Table 5.8). No significant differences between EL and PLA after 2 weeks of supplementation, t (14) = 1.722, p = 0.107 (Figure 5.5E).

Table 5.8: Effect (Baseline vs Post-test) of *E. longifolia* and placebo supplementation on sexual function after 14 days of supplementation.

Variable	EL (n = 8)			PLA (n = 8)		
	Mean difference (95% CI)	*p*	%	Mean difference (95% CI)	*p*	%
Sexual desire	0.19 [-0.13, 0.51]	0.307	-5.96	0.13 [-0.56, 0.31]	1.000	5.46
Erection	0.00 [-0.14, 0.14]	1.000	0.00	0.16 [-0.48, 0.16]	0.439	4.80
Ejaculation	0.23 [-0.32, -0.06]	0.011*	6.13	0.11 [-0.13, 0.26]	1.000	-2.75
Problem assessment	0.04 [-0.08, 0.16]	1.000	-1.00	0.18 [-0.29, -0.07]	0.006*	4.90
Overall satisfaction	0.38 [-0.98, 0.23]	0.263	12.67	0.13 [-0.55, 0.30]	1.000	4.94

Note: CI = Confidence interval; EL = *E. longifolia*; PLA = placebo
Numerical covariate (baseline) was controlled by using ANCOVA repeated measurement.
*Level of significance was set at 0.05 (two-tailed); % increased or decreased (-)

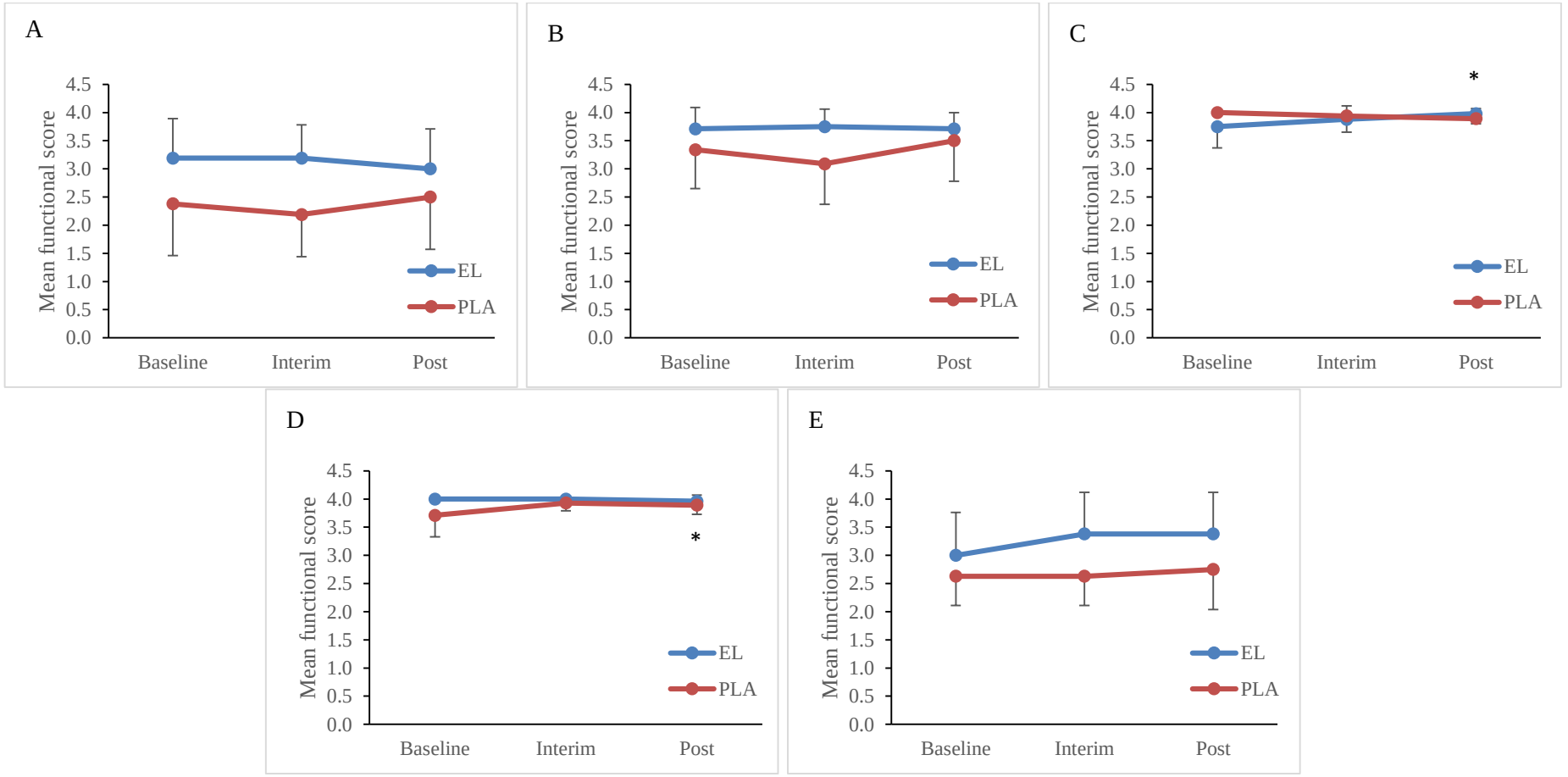


Figure 5.4: Effect of *E. longifolia* on sexual desire (A), erection (B), ejaculation (C), problem assessment (D), and overall satisfaction(E) domains based on BSFI assessment between EL and PLA group to examine the time effect within-group and between-group (2 weeks)

*indicates significant changes in overtime within-group (Baseline vs Post-test)with ANCOVA repeated measurement applied.

*Denotes level of significance was set at 0.05 (two-tailed)

5.4.2.2 Long-term supplementation period

There was no significant main effect of time ($F_{2, 36}$ = 0.677, p = 0.503) and no significant main interaction between group and time for the sexual desire domain ($F_{2, 36}$ = 0.377, p = 0.688) observed. Although not significant, pairwise comparison showed an increase in sexual desire domain score within EL from baseline (2.40 ± 0.80) to post-test (2.96 ± 0.85) [MD = -0.55, 95% CI = -1.12, 0.03, p = 0.063) and no significant change in PLA from baseline (2.35 ± 0.78) to post-test (2.70 ± 0.89) [MD = -0.35, 95% CI = -1.171, 0.471, p = 0.703) (Table 5.9) as well. Between group analysis also showed no significant difference between EL and PLA at post-test, t (19) = 0.671, p = 0.510 (Figure 5.6A).

In erection domain, there was a significant main effect of time ($F_{2, 36}$ = 25.274, p < 0.001) but no significant interaction between group and time ($F_{2, 36}$ = 0.683, p = 0.512). Pairwise comparison showed a significant increase from baseline (2.82 ± 1.14) to interim (3.42 ± 0.69) [MD = -0.60, 95% CI = -1.08, -0.12, p = 0.016) and to post-test (3.55 ± 0.50) [MD = -0.763, 95% CI = -0.91, -0.54, p < 0.001) but no significant change found in PLA from baseline (3.04 ± 0.87) to post test (3.40 ± 0.80) [MD =-0.36, 95% CI = -1.02, 0.30, p = 0.407) (Table 5.9). However, no significant differences in erection score between EL and PLA at post-test, t (19) = 0.506, p = 0.619 (Figure 5.6B).

There was significant main effect of time ($F_{2, 36}$ = 192.495, p < 0.001) in the ejaculation domain but no significant interaction between group and time ($F_{2,36}$ = 0.628, p = 0.540). In the pairwise comparison a significant increase from baseline scores (3.32 ± 1.27) to interim (3.82 ± 0.25) [MD = -0.50, 95% CI = -0.72, -0.28, p < 0.001) and to post-test (3.82 ± 0.34) [MD = -0.50, 95% CI = -0.73, -0.27, p < 0.001) was observed, but no significant change found in PLA from baseline (3.85 ± 0.24) to post-test (3.95 ± 0.16)

[MD =-0.100, 95% CI = -0.24, 0.04, p = 0.180) (Table 5.9). However, no significant changes between EL and PLA after eight weeks supplementation, t (19) = -1.127, p = 0.274 (Figure 5.6C).

The problem assessment domain showed no significant main effect of time was observed ($F_{1.277,\ 22.994}$ = 0.314, p = 0.114) but no significant main interaction between group and time ($F_{1.277,\ 22.994}$ = 0.155, p = 0.758). Pairwise comparison showed no significant changes in EL from baseline scores (3.52 ± 0.41) to post-test (3.66 ± 0.49) [MD = -0.145, 95% CI = -0.573, 0.282, p = 1.000) and also in PLA from baseline (3.54 ± 0.67) to post test (3.60 ± 0.77) [MD =-0.06, 95% CI = -0.69, 0.57, p = 1.000) (Table 5.9). Also, there was no significant difference between EL and PLA at post-test, t (19) = 0.227, p = 0.823. (Figure 5.6D).

There was a significant main effect of time ($F_{2,\ 36}$ = 15.328, p < 0.001) but no significant main interaction between group and time ($F_{2,\ 36}$ = 1.479, p = 0.241) observed in overall satisfaction domain. In the pairwise comparison there was a significant increase from baseline scores (2.46 ± 1.13) to post-test (3.27 ± 0.79) [MD = -0.82, 95% CI = -1.44, -0.19, p = 0.012) in EL and significant changes was also found in PLA from baseline (2.40 ± 0.70) to post test (3.00 ± 0.67) [MD =-0.60, 95% CI = -1.19, -0.01, p = 0.047) (Table 5.9). There were no significant differences between EL and PLA after 8 weeks of supplementation, t (19) = 0.853, p = 0.404 (Figure 5.6E).

Table 5.9: Effect (Baseline vs. Post-test) of *E. longifolia* and placebo supplementation on sexual function after 8-weeks of supplementation.

Variable	EL (n = 11)			PLA (n = 10)		
	Mean difference (95% CI)	*p*	%	Mean difference (95% CI)	*p*	%
Sexual desire	0.55 [-1.12, 0.03]	0.063	22.92	0.35 [-1.17, 0.47]	0.703	14.89
Erection	0.73 [-0.91, -0.54]	0.000*	25.89	0.36 [-1.01, 0.29]	0.407	11.84
Ejaculation	0.50 [-0.73, -0.27]	0.000*	15.06	0.10 [-0.24, 0.04]	0.18	2.60
Problem assessment	0.15 [-0.57, 0.28]	1.000	4.26	0.06 [-0.69, 0.57]	1.000	1.70
Overall satisfaction	0.82 [-1.44, -0.19]	0.012*	33.33	0.60 [-1.19, -0.007]	0.047*	25.00

Note: CI = Confidence interval; EL = *Eurycoma longifolia* group; PLA = placebo
Numerical covariate (baseline) was controlled by using ANCOVA repeated measurement.
*Level of significance was set at 0.05 (two-tailed); % increased or decreased (-)

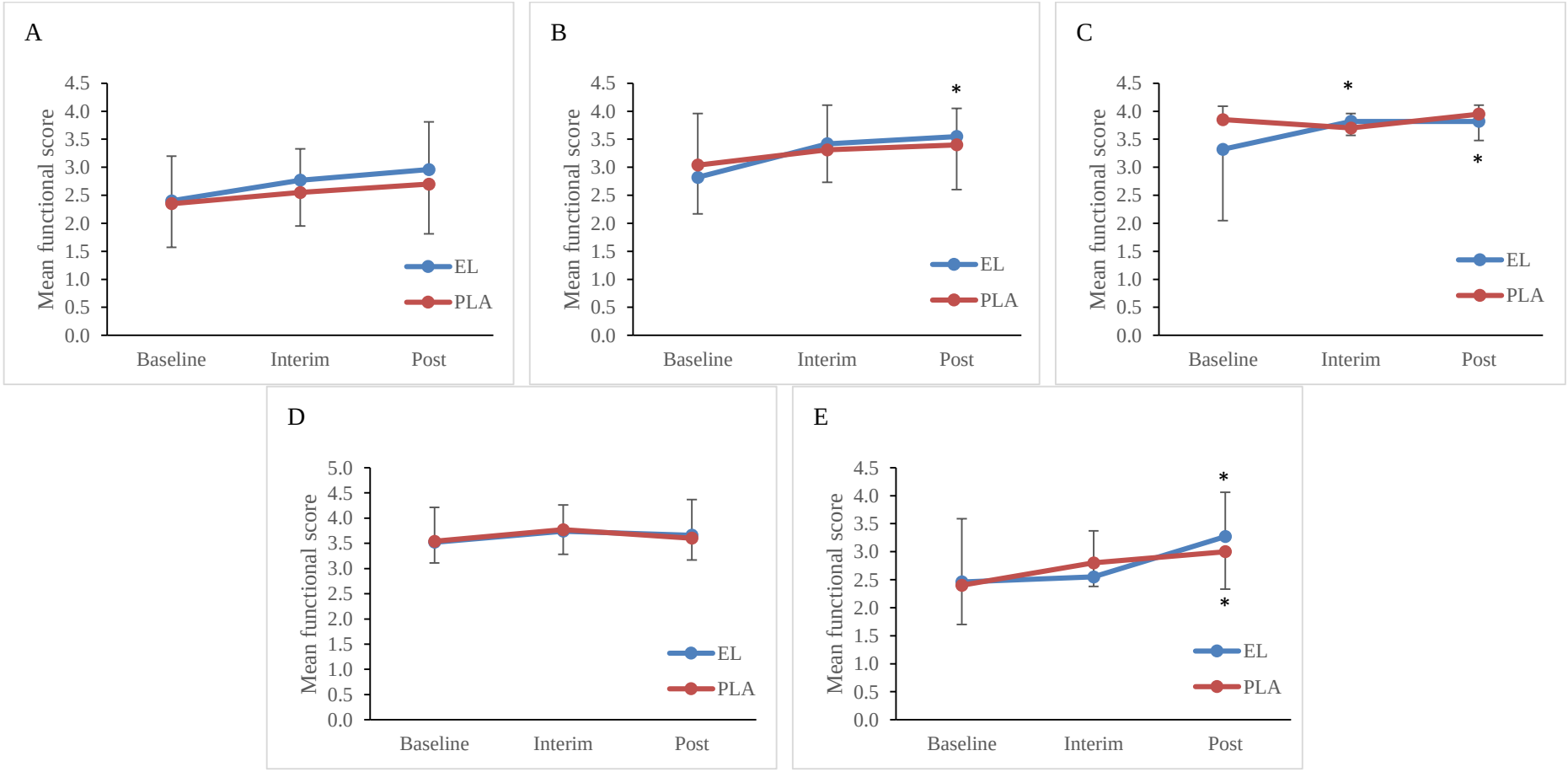


Figure 5.5: Effect of 8 week *E. longifolia* on sexual drive (A), erection (B), ejaculation (C), problem assessment (D) and overall satisfaction (E) domains based on BSFI assessment between EL and PLA group to examine the time effect within-group and between-group (8 weeks)

* indicates significant changes in overtime within-group (Baseline vs Interim, Baseline vs Post-test)with ANCOVA repeated measurement applied.

*Denotes level of significance was set at 0.05 (two-tailed)

5.5 Discussion

The present short and long-term supplementation of *E. longifolia* has demonstrated improved psychological mood state and sexual function among young male adults. Significant improvement in anger, fatigue, and confusion has been observed after short-term *E. longifolia* supplementation. Although not significant, improvement in tension, depression, and vigour has also been demonstrated. On the other hand, long-term supplementation has shown improvement in all negative mood domains (tension, anger, depression, fatigue, confusion), with significant improvement observed in tension and anger domains only. However, a minimal decrease in vigour domain post-long-term supplementation has been observed. In contrast, long-term supplementation of *E. longifolia* has also shown improvement in sexual function, with three domains demonstrating significant improvement compared to short-term supplementation, with only one significant improvement.

The increase (short term) and no change (long-term) in the secretion of sex hormones (i.e., testosterone) may provide some form of adaptation in physical and psychological characteristics. While testosterone concentration could benefit the psychological mood state and sexual function, current findings found that *E. longifolia* did not improve all the domains but only selected mood and sexual function domains in both mood state and sexual function. Findings demonstrate some benefits in sexual function assessment and mood state, which concur with previous studies demonstrating the aphrodisiac and adaptogen effects of *E. longifolia.* However, other confounding factors such as stress, anxiety, depression, poor sleep, interaction with people, poor nutrition, to name a few, may have contributed to the findings. Hence, a further investigation involving the

monitoring of possible confounding factors affecting mood state and sexual function is needed.

5.5.1 Psychological Mood State

A positive mood state profile indicates positive mental health (Morgan, 1980) and should demonstrate more vigour (positive domain) and less anger, tension, depression, fatigue and confusion (negative domains), which was demonstrated in Morgan (1980). Unfortunately, the participants supplemented with *E. longifolia* after two weeks and eight weeks of supplementation in the present study did not demonstrate an optimal peak in vigour above 50% of the standard score and recorded a low negative mood state (below 50% of the standard score). Hence, the present short and long-term supplementation period study did not demonstrate a positive mood state profile among young men. Although the present findings did not show a positive mood state profile, there were still changes within the negative mood domains and positive mood domains. Indeed, findings are in agreement with previous studies that *E. longifolia* does improve certain psychological mood state parameters (Talbott *et al*., 2013; George *et al*., 2018) but does not improve the mood state profile.

The measurement for all mood state domains was reported to be above the average standard score of 50 before supplementation. Hence, *E. longifolia* supplementation may not be able to improve a positive mood state profile. However, the current short-term and long-term supplementation period found that daily supplementation with *E. longifolia* (600 mg/day) improves all individual negative mood state domains (tension, depression, anger, fatigue and confusion). The anger, fatigue and confusion domains in the short-term, and the tension and anger domains in the long-term supplementation period, showed

significant improvement. These findings are in agreement with several supplementation trials performed with humans, suggesting *E. longifolia* may be an effective extract to reduce the negative effects of chronic stress (Talbott *et al*., 2006; Talbott *et al*., 2013, George *et al*., 2018). While Talbott *et al*. (2013) reported improvement in tension, anger, and confusion, the present short and long-term supplementation period not only reported improvement in the same three domains, it also saw an improvement in reducing depression and fatigue. Another study by George *et al*. (2018) highlighted a significant improvement in vigour and improvement in the negative mood domains. However, findings showed no significant difference between PLA, indicating that the EL group's improvement may not be entirely due to *E. longifolia*.

Although it is perceived by many that testosterone administration improves mood in men with low testosterone levels, the association between testosterone and psychological mood state outcomes remains poorly understood. Observational studies have shown a weak association of low testosterone with depressed mood, fatigue and reduced vigour (Barrett-Connor *et al*., 1999; Araujo *et al*., 2007). Also, few studies have found differences between findings, such as no significant differences in mood between testosterone and placebo, inconsistent effects of testosterone, or improvements in depression (Seidman *et al*., 2001; Steidle *et al*., 2003; Cavallini *et al*., 2004; Shores *et al*., 2009; Pope *et al*., 2010). However, open-label, uncontrolled studies have found supplementation of testosterone in men with low testosterone levels improves mood and wellbeing (Wang *et al*., 2004; Daniell *et al*., 2006; Wang *et al*., 2011).

The present study examining the psychological mood state was performed concurrently with reproductive hormones. The participants demonstrated an increase in testosterone in the short-term supplementation period, while testosterone remained in a

normal concentration range without observing any changes during long-term supplementation. It has been suggested that the ability of *E. longifolia* to increase testosterone levels in humans (Tambi, 2007) may contribute to the improvement in mood state, especially in the vigour domain, among older participants facing a gradual decline in testosterone levels (Talbott *et al*., 2013; George *et al*., 2016; George *et al*., 2018). However, this is not the case in participants aged between 18 to 45 years with normal circulating testosterone levels, as was demonstrated in a study conducted by George *et al*. (2018) and repeated in the current study. Hence, the testosterone and free testosterone finding demonstrate any association with the improvement of the psychological mood domains.

Also, the vigour domain did not see any changes after two and eight weeks of *E. longifolia* supplementation. A possible factor affecting the study's outcome may be due to participants' testosterone remain unchanged presented in Chapter 4 and participants are not actively involved in physical activities. Furthermore, the current study did not have any resistance training intervention. Resistance training has been shown to improve testosterone level and overall psychological profile (Zanuso *et al*., 2012). Studies examining a group of cyclist and a group of people participating in a weight loss programme which involve exercise intervention have shown a reduction in cortisol level after supplementing with *E. longifolia*, indicating a shift to an anabolic hormone state and the subsequent improvement in certain psychological mood states (Talbott *et al*., 2006; Talbot *et al*., 2013). Although there are a strong validity and reliability reported by Terry *et al*. (1999) and it is suitable to be used for adolescent and adults (Terry *et al*., 1999; Terry *et al*., 2003), a pilot test to check on the reliability was not performed in the current study. The BRUMS questionnaire was generated from data collected using the response time frame, "How do you feel right now?" may not be relevant to data collected using

other response timeframes (Terry *et al*., 1999). The current study did not personally meet the participants to monitor the process of filling up the BRUMS and was asked to fill up the forms independently. Hence, they could have been affected by situational differences, such as various stress or lifestyle factors, such as diet, exercise or sleep patterns while participating in the studies. These factors could affect the study's outcome for the whole duration of the study. The current study did not project a positive mood profile suggested by Morgan (1980), and the improvement may not be entirely due to the supplementation of *E. longifolia*.

The current study utilised young men who have normal testosterone concentration levels and may not be suffering any decline in testosterone levels. They reported a higher baseline score in all the negative mood domains. Hence, the effect on young participants was less evident in the current short and long-term supplementation period study and may require longer supplementation duration beyond eight weeks. Nevertheless, any improvement in the psychological domains in both the short and long-term supplementation period that did take place do not seem to have any possible association between *E. longifolia*'s testosterone-enhancing ability with psychological mood state among young, healthy male participants due to the lack of changes in the testosterone presented in Chapter 4. Hence, the improvement may be due to various confounding factors such as participating in physical activity, dietary and other environmental factors. However, based on the present findings, *E. longifolia* demonstrate adaptogen effects and could be an alternative to improving psychological mood, but better control towards the confounding factor may provide a better outcome finding.

5.5.2 Sexual function

Eurycoma longifolia is commonly used for the treatment of sexual dysfunction and the improvement of sexual functions. Numerous studies have been carried out using *in vivo* animal models (Ang & Ngai, 2001; Ang & Lee, 2002; Ang *et al.,* 2003a; Ang *et al*., 2003b; Ang *et al*., 2004). Only a limited number of human clinical trials have examined the impact of *E. longifolia* in managing sexual dysfunction (Tambi, 2009; Tambi and Imran, 2010; Muhammad *et al*., 2010; Tambi *et al*., 2012; Tambi *et al*., 2012; Udani *et al*., 2014). Supplementation of *E. longifolia* exhibited improvement within EL in the ejaculation domain during the short-term period and improved ejaculation, erection, and overall satisfaction domains in the long-term supplementation period. Current findings concur with the view that *E. longifolia* does have a beneficial effect on male sexual function, as has been shown in previous human clinical trials examining sexual health and sexual disorder (Ang *et al*., 2000; Tambi, 2009; Bhat & Karim, 2010; Tambi *et al*., 2012). However, data reported no significant difference between EL and PLA, demonstrating that the improvement in certain sexual function domains may not entirely due to the supplementation of *E. longifolia.*

Evidence from the current study using the BSFI for the first time to examine *E. longifolia* supplementation for two and eight weeks found significant improvement in certain sexual function domains, i.e., ejaculation, erection and overall satisfaction. In addition, the *E. longifolia* treated group scored better but not significantly in most sexual function domains compared to PLA, in either interim or post-supplementation. Based on the data, the improvement in the domains of the sexual functions observed in the current study may indicate that *E. longifolia* can exhibit aphrodisiac effects in humans (Sambandan *et al*., 2006; Asiah *et al*., 2007; Tambi and Imran, 2010). However, a closer

evaluation of Figure 5.4 and 5.5 shows that the difference between EL and PLA is minimal. The improvement in EL may not be entirely due to supplementation but may be affected by other confounding factors that are not measured in the current study, i.e. resistance exercise and cortisol. The improvement may also be due to the improvement in certain mood state domains shown in Figure 5.2 and 5.3. Although the precise mechanism is postulated based on animal studies, some human clinical trials were performed to examine the effects of *E. longifolia* on sexual functions. One study demonstrated the efficacy of *E. longifolia* on men's sexual health by its ability to reduce the stress hormone profile, which is correlated with male sexual performance (Tambi, 2006).

Eurypeptides in *E. longifolia* have been shown to exert and enhance an effect on the biosynthesis of androgens. Eurypeptides activate the CYP17 (17 α - hydroxylase/17, 20 lyase) enzyme to enhance the metabolism of progesterone and 17 - OH - progesterone to androstenedione and testosterone (Ali and Saad, 1993). The increase in testosterone levels was thought to be a factor in the improvement of sexual function. The dosage of 600 mg *E. longifolia* used in the current study was the highest compared to all previous studies (Ismail *et al*. 2012; Tambi *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2014; Udani *et al*., 2014; Chen *et al*., 2014; George *et al*., 2018; Chen *et al*., 2019) but did not demonstrate changes in testosterone level. The dosage between 200 to 400 mg of *E. longifolia* improved testosterone levels in older participant age between 30 to 72 years old (Tambi *et al*., 2012; Ismail *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2014; Udani *et al*., 2014; George et al., 2018). However, the same dosage did not increase testosterone levels in young participants (Chen *et al*., 2014; Chen *et al*., 2019).

Previous studies reported improved sexual activity and wellbeing (Talbott *et al*., 2013), sexual performance, sexual libido (Tambi *et al*., 2012; Udani *et al*.,2014; George & Henkel, 2014; Henkel *et al*., 2014), sexual intercourse performance, the concentration of male sex hormone, overall sexual wellbeing (Udani *et al*.,2014), erectile function, sexual intercourse performance and penile hardness (Tambi *et al*., 2012), with an improvement in testosterone level. However, the investigation was performed mostly on elderly individuals with a history of erectile dysfunction, LOH and idiopathic infertility. George and Henkel (2014) suggested that the possibility of *E. longifolia* improving male sex libido is higher in 60-year old patients having 40 to 50% lower testosterone levels than young individuals. Testosterone levels remained in the normal range level among healthy young adults presented in Chapter 4. It is speculated that *E. longifolia* acts as a maintainer of normal testosterone levels (Talbott *et al*. 2013), and because of this, the present findings did not present vast differences in the sexual function domain between the young adults in EL and PLA. While data showed improvement in some sexual functions, the finding does not demonstrate any possible association between *E. longifolia* supplementation with sexual function. The current findings can only be compared with previous studies that mainly recruited elderly participants diagnosed with LOH and sexual dysfunction. Hence, explaining the positive findings of significant efficacy in young adults is challenging.

The Brief Sexual Function Inventory (BSFI) was designed to be brief, self-administered, and clinically useful in assessing current sexual functioning. MyKletun *et al*. (2005) examined male sexual function using the BSFI and found that mean levels of sexual drive, erection, ejaculation, problem assessment, and overall satisfaction decreases from age 20 to 29 years to 40 to 49 years, followed by a more substantial decline among participants aged 70 to 79 years. This investigation may be the first study investigating

the extract's effect on young participants who did not suffer from LOH. However, being healthy, young, and having a sexual partner (MyKletun *et al*., 2005) may affect the higher scores of the BSFI domains reported in the current study. Also, the low participation rate due to this study's nature, which required parrticipants to disclose information such as testosterone levels and sexual ability, may affect the study's outcome. Despite this, the findings in the current study demonstrate *E. longifolia* having some influence in managing male sexual function among young males but may not be the main contributor to the improved sexual function in this study.

5.5.3 Conclusion

At present, the improvement in mood and sexual function is claimed to be associated with *E. longifolia's* ability to increase the testosterone level in the human body and reduce the stress hormone known as cortisol. The consumption of *E. longifolia* for two and eight weeks has exhibited significant improvement in specific mood states, and sexual function domains following short and long-term supplementation corroborate *E. longifolia*'s adaptogen and aphrodisiac properties. However, considering that the participants recruited are healthy, young, and possibly having a sexual partner may confound the outcome, not necessarily due to *E. longifolia* alone. The precise mechanism behind the increase in testosterone is still unknown in humans, and evidence of the association between *E. longifolia* with mood and sexual function is still limited and not shown in the current study. Nevertheless, the current study has highlighted the role of *E. longifolia* as a potential herbal supplement against various negative mood states, such as anxiety, depression, anger and fatigue, and male sexual health disorders, such as sexual desire, erection, ejaculation and problem assessment functioning. However, further clinical investigations into stress hormones, male infertility (i.e., quality and quantity of

sperm) among young males, and considering possible confounding factors are needed to determine the impact and association of *E. longifolia* on mood and sexual function.

CHAPTER 6: THE EFFECTS OF *EURYCOMA LONGIFOLIA* ON BODY COMPOSITION, MUSCLE STRENGTH AND ANAEROBIC POWER

6.1 Introduction

Individuals involved in sports and exercise are constantly looking for effective ways to enhance their performance. Many athletes and non-athletes believe that herbal supplements can improve sport and exercise by increasing energy levels, inducing weight loss, promoting muscle growth and strength or even inducing other physiological or metabolic responses. (Herbold *et al*., 2004; Williams, 2006; Sellami *et al*., 2018).

To study such effects, many scientific investigations with controlled clinical trials have been carried out. Various herbal supplements, including, but not limited to, *E. longifolia*, are believed to have medicinal and ergogenic effects (William, 2006; Rogerson *et al*., 2007; Poole *et al*., 2010). Although some have shown evidence and putative effects in improving health and wellbeing, the investigation into quality and safety remains limited. Furthermore, the mechanisms concerning how the supplements enhance physical performance are not clearly understood (Yavuz & Őzkum, 2014).

In the context of exercise performance, an increase in muscle mass and strength is associated with *E longifolia* supplementation (Hamzah & Yusof, 2003; Henkel *et al*., 2013; Chen *et al*., 2019). It has been observed in physically active individuals (Hamzah *et al*.,2003; Henkel *et al*., 2013; Chen *et al*., 2019) and healthy young (Sarina *et al*., 2009; George *et al*., 2013) and older adult populations.

E. longifolia is said to increase testosterone levels (Tambi *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2013; Udani *et al*., 2013). It is expected to act similarly to exogenous testosterone in increasing strength. Indeed, improving physical performance, such as muscular strength and anaerobic power, is important for athletes and those involved in bodybuilding wanting to enhance muscle mass and strength (Bhat & Karim, 2010; George *et al*., 2013). However, the potential ergogenic effects of *E.longifolia* remain inconclusive, with some demonstrating an increase in testosterone (Tambi *et al*., 2012; Talbott *et al*., 2013; Henkel *et al*., 2013; Udani *et al*., 2014) and others not (Ismail *et al*., 2012; Chen *et al*., 2014).

Furthermore, exercise intervention in most studies carried out along with *E longifolia* supplementation in young men may have contributed significantly to the strength improvement. Several studies have shown that a combination of *E. longifolia* with exercise improves muscular strength. So, it is unknown whether *E longifolia* alone can improve strength in young, healthy adults.

6.2 Literature Review

The ergogenic effects of *E. longifolia* supplementation have been studied in both athletic endurance and muscular strength performance. Preliminary evidence suggests a lack of effect of *E. longifolia* on endurance in humans (Ooi *et al*., 2001; Ooi *et al*., 2003; Muhamad *et al*., 2010) but positive findings in muscular strength (Hamzah & Yusof, 2003; Sarina *et al*., 2009; Henkel *et al*., 2013; George *et al*., 2013). The increase in testosterone levels following *E. longifolia* supplementation with or without exercise, reported in several studies (Tambi *et al*., 2012; Talbott *et al*., 2013; Low *et al*., 2013a & 2013b; Henkel *et al*., 2013; Udani *et al.*, 2014) is often linked to the increase in muscle

mass and strength. However, only one study (Henkel *et al*., 2013) reported that the administration of *E. longifolia* increased testosterone levels and, subsequently, improved muscular strength. At the same time, other studies have demonstrated an improvement in strength post-*E. longifolia* administration, these studies did not measure testosterone levels (Hamzah & Yusof, 2003; Sarina *et al*., 2009; Ooi *et al*., 2015). Hence, published clinical research associating testosterone and muscular strength when supplemented with *E. longifolia* remains inconclusive.

6.2.1 Effect of *E. longifolia* on Muscular Strength

Six studies shown in Table 6.1 have investigated the effect of *E. longifolia* supplementation on muscular strength. All reported an increase in muscular strength and muscle size after a period of *E. longifolia* supplementation (5 to 12 weeks). The combination of *E. longifolia* with resistance training may have contributed to muscular strength improvement (Hamzah & Yusof, 2003; Sarina *et al*., 2009; Henkel *et al*., 2013; Ooi *et al*., 2015; Chen *et al*., 2019). From the six studies, four investigated the effect of *E. longifolia* by combining *E. longifolia* supplementation (100 mg) with resistance training for 5 to 12 weeks (Hamzah & Yusof, 2003; Sarina *et al*., 2009; Ooi *et al*., 2015; Chen *et al*., 2019). The participants were male in Hamzah and Yusof (2003), Ooi *et al*. (2015), and Chen *et al*. (2019), while in Sarina *et al*. (2009), the participants were middle-aged women. All the studies demonstrated increases in muscle size and strength. However, only abstracts were published for studies by Hamzah & Yusof, 2003 and Sarina *et al*., 2009 and, thus, the information they yielded was limited.

The testosterone to cortisol ratio is measured to determine the anabolic or catabolic status of a muscle during exercise (Hakkinen, 1989). According to Adlercreutz *et al*.

(1986), whenever an individual exercise, there is a change in the hormonal status from a catabolic state toward an anabolic state. The testosterone to cortisol ratio diminishes with increased training loads (Hoogeveen & Zonderland, 1996; Mujika *et al*., 1996), which indicates overtraining and fatigue (Adlercreutz *et al*., 1986; Hakkinen & Pakarinen, 1991). Henkel *et al*. (2013) reported higher testosterone values to cortisol ratio in men after *E. longifolia* administration, indicating *E. longifolia* increases the body's anabolic status (Henkel *et al*., 2013). Sedentary males and physically active males and females were recruited in George *et al*. (2013), and Henkel *et al*. (2013) and an increase in muscular strength was found, even though strength training was not performed. Henkel *et al*. (2013) reported an increase in muscle force production, and this finding was associated with a significant increase in total and free testosterone concentrations due to *E. longifolia*. Hence, it is suggested that *E. longifolia*, with an ability to enhance testosterone with or without resistance training, might increase muscle size and strength in males.

Administration of exogenous testosterone is associated with increases in muscle mass and strength (Blazevich & Giorgi, 2001). Also, the use of exogenous testosterone in conjunction with heavy resistance training has shown strength gains (Bhasin *et al*., 1996). Heavy resistance training increases muscle pennation and muscle size, and the administration of supraphysiological doses of testosterone may have hastened the gain of muscle mass and strength compared to individuals not performing any resistance training (Giorgi *et al*., 1999; Blazevich & Giorgi, 2001). While testosterone was not measured in Hamzah and Yusof (2003) and Ooi *et al*. (2015), Chen *et al*. (2019) reported no changes in the testosterone to epitestosterone ratio. These three studies demonstrated that the combination of *E. longifolia* with resistance training increases muscle size and strength compared to control groups. On the other hand, in studies without performing any resistance training and involving just the consumption of *E. longifolia,* an improvement

in muscle mass and strength has been identified (Sarina *et al*., 2009; Henkel *et al*., 2013; George *et al*. 2013), indicating the increase in circulating testosterone may have contributed to the improvement.

However, George *et al*. (2013), in their randomised, double-blind, placebo-controlled trial, examined 40 men aged between 30 to 55 years supplemented with *E. longifolia* for 12 weeks. Although testosterone is known to affect muscles (Herbst & Bhasin, 2004), *E. longifolia* may not be restricted to enhancing testosterone. The study showed no increase in testosterone levels but, surprisingly, reported an increase in muscular strength, indicating the increase in strength may not be related to the testosterone hormone. The increase in strength may be due to *E. longifolia* being a natural adaptogenic energiser in healthy ageing in men with suboptimal testosterone levels (George *et al*., 2013). As men age, they encounter a decrease in muscle mass and subsequent decrease in strength and physical function due to lower testosterone production (Harman *et al*., 2001; Feldman *et al*., 2002; Standworth & Jones, 2008). It is suggested that the supplementation of *E. longifolia* may not generally increase testosterone levels but modulate it to optimal levels based on the individual's present hormonal level status (Henkel *et al*., 2013; George *et al*., 2013).

Table 6.1: Studies on the effect of *E. longifolia* in exercise and sports.

Studies	Participants	Dosage & Duration	Test	Findings
Hamzah & Yusof (2003)	14 healthy men (age)	100 mg 5 weeks	Body composition, arm circumference, 1RM, (sEMG) Strength training intervention	Increased FFM, reduced BF, and increased muscle strength and size. T not measured
Sarina *et al*. (2009)	31 women 45 - 59 years.	100 mg 12 weeks	Handgrip test, muscle circumference measured	Increased handgrip strength and bigger quadriceps muscle.
Henkel *et al*. (2013)	13 male and 12 physically active female 57 - 72 years	400 mg 5 weeks	Handgrip test Standard hematological parameters	Increased in T and FT Enhanced muscle force.
George *et al*. (2013)	40 men 30 - 55 years	300 mg 12 weeks	sit and reach, handgrip, back, and leg strength, sit up, push up, and body composition. The ratio of T:E was analyzed	Ratio T:E did not change Muscular strength improved significantly in the back and leg.
Ooi *et al*. (2015)	40 male 35 to 55 years	400 mg 8 weeks	Isokinetic strength and power Circuit training	Strength and power improvement T not measured
Chen *et al*. (2019)	40 males 19 to 25 years	200 mg 8 weeks	Isokinetic muscular strength and power, anaerobic power Resistance training	Muscular strength and peak power increased T:E ratio no change

Note: Fat free mass (FMM); One repetition maximum (1RM) ; surface electromyography (sEMG); Testosterone (T); Epitestosterone (E); Free Testosterone (FT); Body Fat (BF)

Another reason for the increase in strength may be due to *E. longifolia* extract comprising of more than 22 percent protein and peptides, which could add to the ergogenic benefit, even without a significant increase of testosterone (Sambandan *et al*., 2006). It is believed that protein may boost protein synthesis to assist in the repair and regeneration of skeletal muscle that has been damaged during resistance training, which may have an implication for the improvement of both muscle size and strength (Hoffman *et al*., 2006; Tarnopolsky *et al*., 1995; Roy *et al*., 2000; Ratamess *et al*., 2003; Kraemer *et al*., 2006). In addition, protein may influence the anabolic hormones involved in muscle regeneration and, subsequently, improve strength (Lemon *et al*., 1992; Chandler *et al*., 1994; Volek *et al*., 1997). Future studies are necessary to examine the effects of proteins and peptides found in *E. longifolia* on muscles' contractile properties.

Short or long-term consumption of *E. longifolia* has demonstrated its ability to promote strength gains (Table 6.1). Four studies recruited participants aged between 30 to 72 years, and the remaining two studies recruited young participants between 19 to 25 years. According to Travison *et al*. (2017), testosterone's normal range in a healthy non-obese population age between 19 to 39 years is between 264 to 916 ng/dL. Due to ageing, the older participants in Henkel *et al*. (2014) and George *et al*. (2013) reported suboptimal testosterone levels (384 ng/dL and 457.92 ng/dl) before the test. Owing to the putative effects of *E. longifolia* in increasing testosterone levels (442. Ng/dL and 483.29 ng/dL), the two studies demonstrated an increase in strength from week 5 to week 12 (Henkel *et al*., 2013; George *et al*., 2013). A dosage of 300 mg may be sufficient to induce strength gain, and the duration of supplementation to see improvement can be observed as early as week 5 in the older population.

On the other hand, strength also improved in just five weeks of *E. longifolia* supplementation among young participants dosed with 100 mg of *E. longifolia* (Hamzah and Yusof, 2003). Chen *et al*. (2019) demonstrated an increase in strength when the participants were supplemented with 200 mg of *E. longifolia* for eight weeks. However, both studies included resistance training with the supplementation, and this combination may have amplified the improvement in strength. According to a study conducted by Ooi *et al.* (2015), their findings show an enhanced synergistic effect of *E. longifolia* supplementation combined with resistance training on isokinetic muscular strength and power compared to supplementation alone (Ooi *et al*., 2015). Thus, a combination of resistance training with supplementation of just 100 mg to 200 mg for five to eight weeks appears to be sufficient to demonstrate strength improvement. At present, a dosage ranging between 100 to 400 mg of *E. longifolia* shows improvement in strength within 5 to 12 weeks among participants aged 30 to 72 years. The improvement in strength among the young participants may be due to the resistance training and not entirely due to the *E. longifolia* supplementation. Hence, the appropriate dosage and duration of *E. longifolia* supplementation alone in young participants to see any improvement in strength is still unknown.

Based on this, the present study aimed to assess the following:

To distinguish the potential ergogenic properties in relation to exercise performance (isokinetic strength and anaerobic power) following *E. longifolia* supplementation among college students.

6.3 Methods

6.3.1 Participants

A total of 21 males were recruited for the study aged between 18 to 30 years old, and their characteristics are tabulated in Table 4.3 and inclusion criteria is stated in Chapter 4, page 93. The study was approved by the University Ethics Committee (14/SPS/013), and all participants provided informed consent.

6.3.2 Experimental Design

The study was a randomized, double-blind, placebo-controlled matched-paired design. Participants were assigned to *E. longifolia* (EL) (n=11) or placebo (PLA) (n=10) group using a matched-pairs design according to their body weight. The study was performed concurrently with the effect of long term consumption of *E. longifolia* on reproductive hormones regulation among collegiate men presented in Chapter 4. A total of three laboratory visits were scheduled: 1. Baseline performance, physiological, health, and well-being tests (week 0); 2. Interim performance, physiological, health, and well-being tests (week 4); and 3. Post-supplementation performance, physiological, health, and well-being tests (week 8). Before baseline measures, participants visited the laboratory on two separate occasions for familiarisation with tests and equipment.

6.3.3 Experimental Protocol

Familiarisation sessions consisted of a health screen that incorporated measurements of resting heart rate and blood pressure and implementing a health-based PAR-Q to ensure that each participant fulfils the study criteria. Familiarisation also occurred to ensure valid

and reliable data collection, particularly concerning the performance tests that required some degree of participant training.

Each subsequent laboratory visits (visits 1 to 3) throughout the eight weeks testing period involved exercise performance tests in assessing quadriceps and hamstring muscle force production (Isokinetic dynamometry), anaerobic power (Wingate test and Vertical Jump test); health-related assessments, including anthropometric profiling (weight, height, body fat percentage, fat-free mass); cardiovascular measures (resting heart rate and blood pressure). Before the muscle strength and anaerobic power assessment, participants completed a warm-up exercise. The warm-up consisted of 5 minutes of dynamic exercise using a Monark 894E cycle ergometer (Monark Exercise AB, Vansbro, Sweden) maintained at 50 Watt, followed by stretching all major muscle groups.

A 24-hour recall concerning diet and physical activity was performed by each participant on each visit to the laboratory. The 24-hour recall questionnaire was used to identify whether the participants were consuming any other performance-enhancing supplements and involved in any high-intensity exercise and to enable replication of diet and physical activity before each visit. According to the group they were assigned to, participants were administered *E. longifolia* or placebo daily throughout the eight weeks period. Prior to each visit, all participants were asked to complete an overnight fast and refrain from alcohol and caffeine, strenuous activity, and sexual activity eight hours before testing commenced.

6.3.3.1 Anthropometric Measurements, Cardiovascular Measurements and Dietary Supplementation

As mentioned in Chapter 4 general method, the participants recruited are the same. Hence the anthropometric and cardiovascular measurement is reported in Chapter 4, page 93 and page 94. The supplementation of *E. longifolia* is also presented on page 94.

6.3.3.2 Muscle Force Production Assessment

Force production of the quadriceps and hamstring muscles was assessed using an isokinetic dynamometer (Biodex Medical Systems, New York, USA) on the dominant leg at two angular velocities (60 and 240°/sec). Participants were placed in a seated position with arms folded and secured according to the standard Biodex procedure using fixation girdle on the shin, thigh, pelvis, and upper torso. The axis of rotation of the dynamometer was aligned with the knee joint axis, defined as the most prominent point on the femur's lateral epicondyle. The dynamometer's shin brace was placed 2 cm above the medial malleolus while being tightly secured to the dynamometer lever arm, where a pad with 2 mm of high-density foam was secured tightly to the tibia to avoid any discomfort. The range of motion was fixed with participant's maximum extension, and flexion movements are comfortable and without an excessive stretch of their hamstrings. Based on the individual, the most extended position was set to anatomical zero. Also, the gravity correction for limb weight was applied using the manufacturer protocol. Clear instructions and standardized verbal motivation were given to the participants to produce maximum muscular effort throughout the range of motion for both knee extensors and knee flexors, at a constant angular velocity of 60°/s and 240°/s. The test was preceded by a warm-up involving six repetitions at 50 and 75% effort. Three to five submaximal

repetition was performed to familiarise participants with the test prior to the six maximal effort trial. Each test involved maximum concentric extension and flexion of the knee, and there was one-minute rest between each maximum concentric extension and flexion of the knee test. Test-retest reliability of the Biodex isokinetic mode for knee extension peak torque at 60/sec was r = 0.97, and at 240/sec was r = 0.95 (Feiring *et al*., 1990).

6.3.3.3 Anaerobic Power Measurement

Anaerobic power was assessed via the Wingate test (Ayalon *et al*., 1974) and the vertical jump test. Monark 894E cycle ergometer (Monark, Stockholm, Sweden) was used to perform the Wingate test with a suggested resistance of 0.075 kg per kg body mass applied to elicit the highest possible power output (Ayalon *et al*., 1974). The Wingate test duration was set at 30 seconds, and peak power, average peak power, relative peak power, and fatigue index were recorded. According to Bar-Or (1987), the correlation coefficients for Wingate tests performed under standardized environment condictions reported a ranged between 0.89 to 0.98

The vertical jump involves participants performing a countermovement jump on a force platform (Kisler Instrument Ltd, Winterthur, Switzerland). The participant was asked to keep their hands on their hips during the test, which involved push-off, flight and landing and ground reaction force was recorded. In a countermovement jump, the participant started from an upright standing position, bend downward by flexing at the knees and hips, and then immediately and vigorously extended the knees and hips again to jump vertically up off the ground. The platform recorded the force applied to it by the participants is called the ground reaction force. The vertical component of the jumper's ground reaction force was sampled at 960 Hz and recorded by a computer. Data

acquisition and analysis of the jump were performed using a custom program that was written using Excel. The participant performed three countermovements jump forcefully upon instruction from the researcher, and max force and max power were recorded.

6.3.4 Statistical Analysis

All statistical analysis was conducted using the Statistical Package for the Social Sciences (SPSS) for Windows version 22.0 (SPSS Inc., Chicago, USA). Within-group differences to examine the time effect was analyzed using the repeated measurement ANCOVA and the baseline was used as a covariate. Assumption of normality, homogeneity of variances, compound symmetry, and homogeneity of regression was checked and fulfilled using the Shapiro Wilk test. Baseline data were also analyzed using the parametric (independent t-test) and non-parametric test (Mann Whitney test). All data in text, figures, and tables were presented as means ± SD or median (interquartile range) with *P* values < 0.05 indicating statistical significance.

6.4 Results

A total of 22 participants with a mean age of 22.7 ± 3.7 years completed the experiment. This group of participants did not participate in the short term supplementation study. The mean body weight for EL (78.66 ± 8.30 kg) was statistically similar to PLA (76.89 ± 5.69 kg), (t_{19} = 0.565, p = 0.579). Both groups also were similar for body fat percentage (t_{19} = 0.304, p = 0.764) and lean body mass (t_{19} = 0.314, p = 0.757). Anthropometry measurement between groups and within 8 weeks of supplementation is shown in Figure 6.1. No significant main effect of time ($F_{1.330, 23.947}$ = 2.982, p = 0.087) and no significant main interaction between group and time ($F_{1.330, 23.947}$

= 0.457, p = 0.560) observed in body weight (Figure 6.1A). In body fat assessment, no significant main effect of time ($F_{1.630, 29.336}$ = 1.115, p = 0.331) and no significant main interaction between group and time ($F_{1.630, 29.336}$ = 0.123, p = 0.845) (Figure 6.1B). Also in lean body mass, no significant main effect of time ($F_{1.332, 23.970}$ = 1.304, p = 0.277) and no significant main interaction between group and time ($F_{1.332, 23.970}$ = 1.068, p = 0.333) (Figure 6.1C). Pairwise comparison and independent t-test shows no significant differences within and between EL and PLA in body weight, body fat and lean body mass.

6.4.1 Quadriceps peak torque and average quadriceps peak torque 60°/sec

In quadriceps peak torque, no significant main effect of time ($F_{1.427, 25.680}$ = 0.456, p = 0.574) and no significant main interaction between group and time ($F_{1.427, 25.680}$ = 0.015, p = 0.957) observed. In the pairwise comparison within groups, no significant changes but a decrease from baseline (206.26 ± 20.3Nm) to post-test (201.36 ± 37.7Nm) [MD = 4.900Nm, 95% CI = -22.960, 32.760, p = 1.000] in EL and also in PLA from baseline (200.03 ± 23.8Nm) to post test (195.28 ± 22.0Nm) [MD = 4.75Nm, 95% CI = -3.390, 12.890, p = 0.349). There are no significant differences between EL and PLA after 8 weeks of supplementation at post-test (t_{19} = 0.661, 0.661).

Also in average quadriceps peak torque, no significant main effect of time ($F_{1.486, 26.746}$ = 1.084, p = 0.335) and no significant main interaction between group and time ($F_{1.486, 26.746}$ = 0.022, p = 0.950). Similarly, no significant within and between EL and PLA (p > 0.05) (Figure 6.2A).

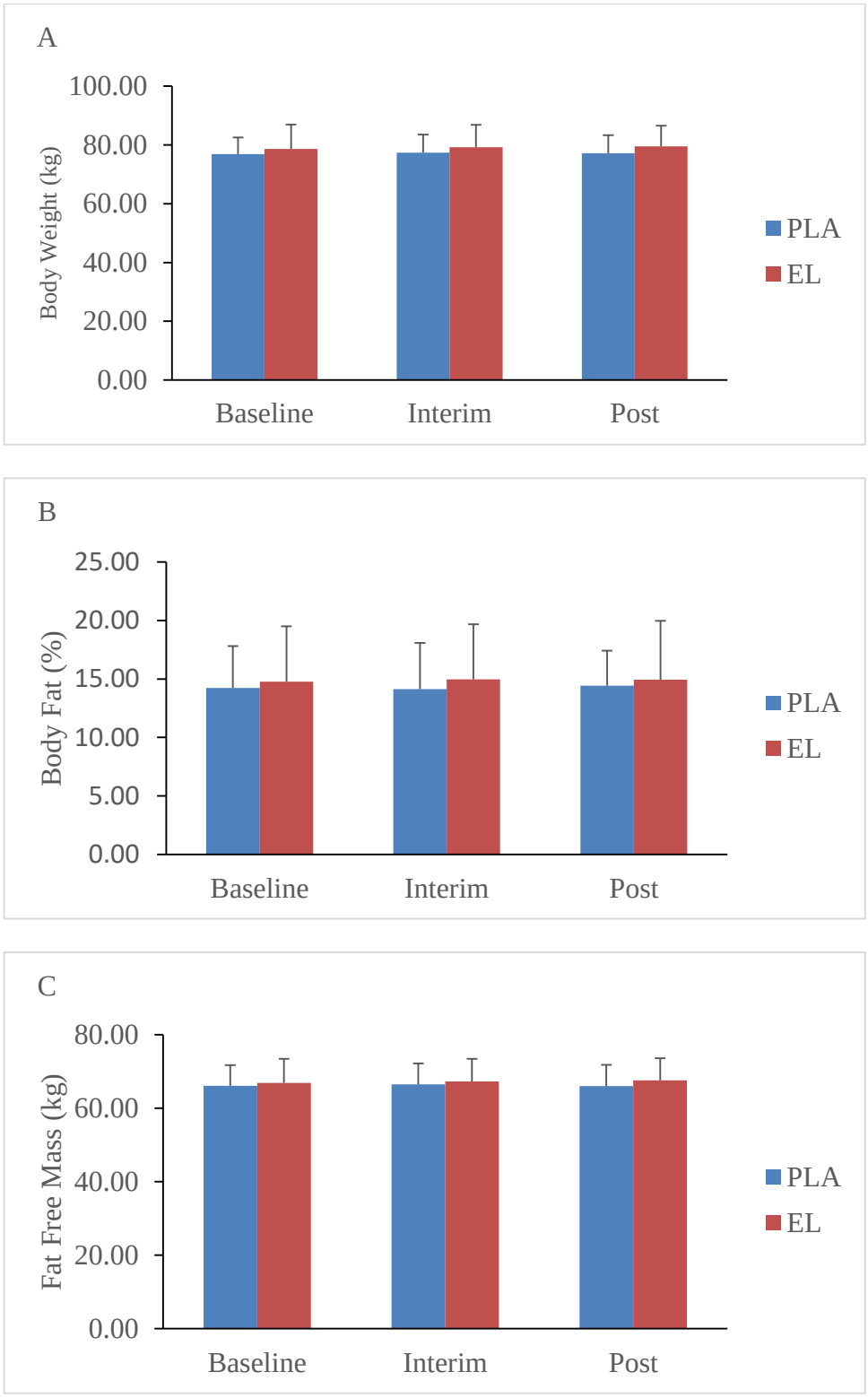


Figure 6.1: Anthropometric measurement between and within EL and PLA groups at baseline (Week 0), interim (Week 4) and Post (Week 8)
Note: body weight (A); body fat (B); fat free mass (C)

6.4.2 Hamstring peak torque and average hamstring peak torque 60°/sec

In hamstring peak torque, a significant main effect of time ($F_{2, 36} = 10.719$, $p < 0.001$) but no significant main interaction between group and time ($F_{2, 36} = 0.711$, $p = 0.498$). In the pairwise comparison within groups, there is no significant changes but a decrease from baseline (126.22 ± 24.4Nm) to post-test (120.27 ± 11.0Nm) [MD = 5.95Nm, 95% CI =-3.583, 15.473, $p = 0.301$] in EL and also in PLA from baseline (129.15 ± 23.3Nm) to post test (122.76 ± 17.6Nm) [MD = 6.39Nm, 95% CI = -0.312, 13.092, $p = 0.062$). There are no significant differences between EL and PLA after 8 weeks of supplementation at post test ($t_{19} = -0.392$, p = 0.699) (Figure 6.2B).

In average hamstring peak torque, a significant main effect of time ($F_{2, 36} = 4.697$, $p = 0.015$) but no significant main interaction between group and time ($F_{2, 36} = 0.658$, $p = 0.524$). Pairwise comparison analysis shows no significant changes from baseline (114.69 ± 119.3Nm) to post test (109.6 ± 13.4Nm) in EL [MD = 5.10Nm, 95% CI = -4.905, 15.105, $p = 0.507$] but a significant decrease in PLA from baseline (119.31 ± 24.1Nm) to post test (112.14 ± 14.5Nm) [MD = 7.17Nm, 95% CI = 1.467, 12.873, $p = 0.016$] (Figure 6.2B).

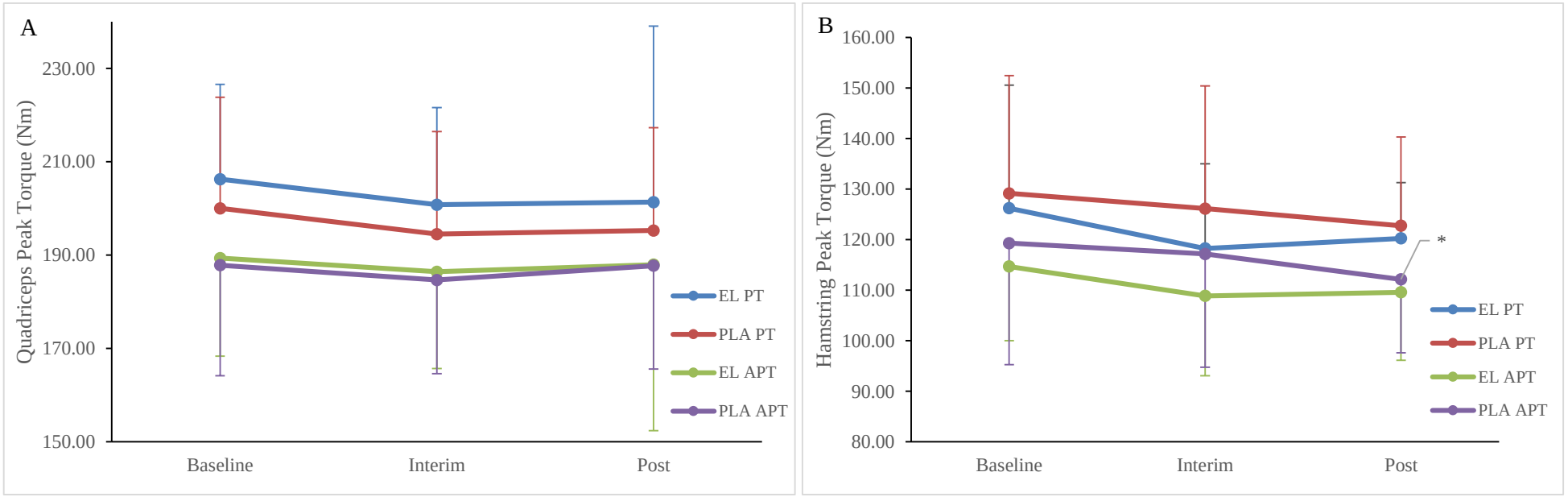


Figure 6.2: Quadriceps (A) and hamstring (B) peak torque and average peak torque at 60°/s in *E. longifolia* and Placebo group to examine the time effect within-group and between-groups at baseline (Week 0), interim (Week 4) and Post (Week 8)

Note: EL: *E. Longifolia* group; PLA: placebo group; Peak torque (PT); Average peak torque (APT)

* indicates significant changes within group compare with baseline;* Denotes level of significance was set at 0.05 (two-tailed)

6.4.3 Quadriceps peak torque and average quadriceps peak torque 240°/sec

In quadriceps peak torque, a significant main effect of time ($F_{2, 36}$ = 7.347, p = 0.002) observed but no significant main interaction between group and time ($F_{2,36}$ = 0.136, p = 0.873). Pairwise comparison within groups shows no significant changes but a minimal increase from baseline (136.60 ± 23.1Nm) to post-test (139.18 ± 15.0Nm) [MD = -2.58Nm, 95% CI = -11.331, 6.168, p = 1.000] in EL and also in PLA from baseline (127.46 ± 19.1Nm) to post test (133.04 ± 18.6Nm) [MD = -5.58Nm, 95% CI = -12.942, 1.782, p = 0.155). There are no significant differences between EL and PLA after 8 weeks of supplementation at post test (t_{19} = 0.835, p = 0.414). Also in average quadriceps peak torque, no significant main interaction between group and time ($F_{2, 36}$ = 2.475, p = 0.098) but significant main effect of time ($F_{2,36}$ = 4.125, p = 0.024). Pairwise comparison show no significant change throughout the 8 weeks supplementation. Between group analysis saw a significant higher average peak torque in EL (131.42 ± 23.2Nm) compare to PLA (114.11 ± 7.2Nm) at interim (t_{19} = 2.259, p = 0.036) but no significant differences at post-test (Figure 6.3A).

6.4.4 Hamstring peak torque and average hamstring peak torque 240°/sec

Hamstring peak torque, a significant main effect of time ($F_{2, 36}$ = 8.158, p = 0.001) but no significant main interaction between group and time ($F_{2,36}$ = 0.084, p = 0.919) observed. Pairwise comparison within groups shows no significant changes but a minimal increase from baseline (86.39 ± 11.7Nm) to post-test (89.92 ± 9.9Nm) [MD = -3.53Nm, 95% CI = -12.354, 5.299, p = 0.814] in EL and also in PLA from baseline (90.82 ± 11.8Nm) to post test (91.85 ± 11.2Nm) [MD = -1.03Nm, 95% CI = -10.406, 8.346, p = 1.000). There are no significant differences between EL and PLA after 8 weeks of

supplementation at post test, (t_{19} = -0.420, p = 0.679). Also in average hamstring peak torque, significant main effect of time ($F_{2,36}$ = 13.411, p < 0.001) but no significant main interaction between group and time ($F_{2, 36}$ = 0.528, p = 0.595). Within group and between group analysis also did not show any significant differences (p > 0.05) throught the supplementation period (Figure 6.3B).

6.4.5 Wingate 30 seconds anaerobic test (WAnT)

In WanT peak power assessment, no significant main effect of time ($F_{2, 36}$ = 0.187, p = 0.830) observed and no significant main interaction between group and time ($F_{2,36}$ = 1.701, p = 0.197) and. Pairwise comparison within groups shows no significant changes but an increase from baseline (826.22 ± 144.87W) to post-test (840.11 ± 141.66W) [MD = -13.89W, 95% CI = -66.098, 38.318, p = 1.000] in EL and but PLA show a significant decrease from baseline (755.67 ± 68.22W) to interim (715.27 ± 46.49W) [MD = 40.40W, 95% CI = 7.843, 72.949, p = 0.017) and increases back at post test (p > 0.05). Between group analysis saw a significant higher peak power in EL (844.08 ± 158.16W) compared to PLA (715.27 ± 46.49W), (t_{19} = 2.581, p = 0.024) at interim. However, no significant differences between EL and PLA at post-test (p > 0.05).

Average peak power findings found significant main effect of time ($F_{2,36}$ = 9.584, p < 0.001) and significant main interaction between group and time ($F_{2, 36}$ = 3.389, p = 0.045) was observed. Within-group analysis within EL and PLA did not find any significant changes over the 8 weeks supplementation. Between-group analysis shows significant higher average peak power (580.04 ± 63.06W) in EL compared to PLA (516.62 ± 41.3W), (t_{19} = 2.695, p = 0.014) after 4 weeks supplementation but no significant difference at post test (p > 0.05) (Figure 6.4).

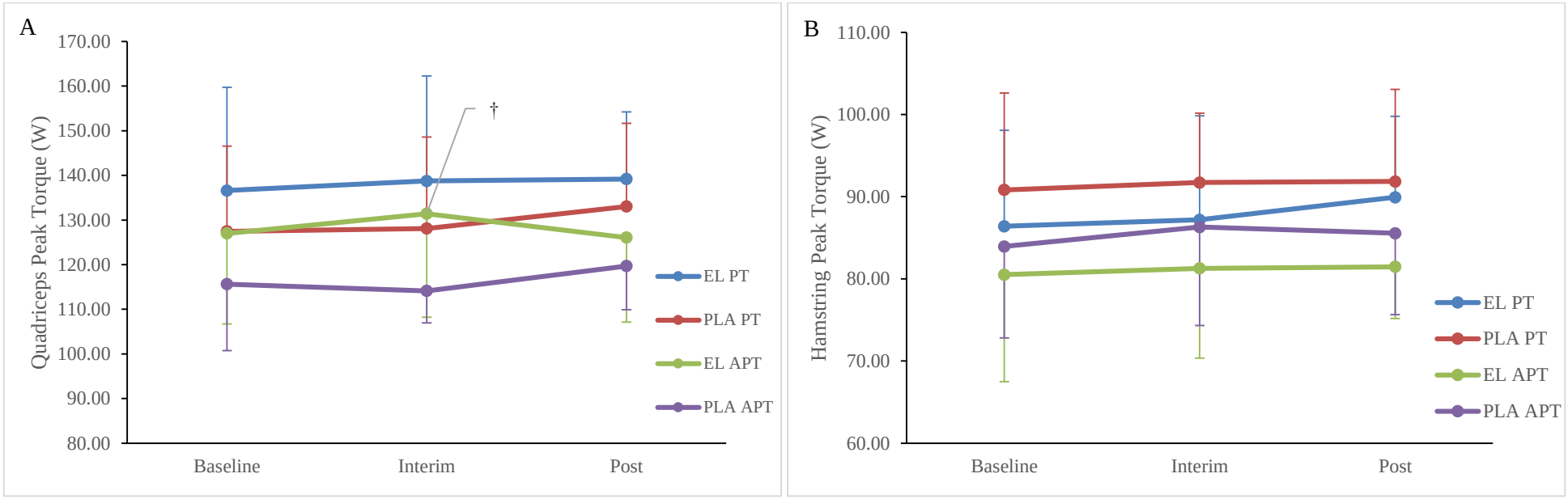


Figure 6.3: Quadriceps (A) and hamstring (B) peak torque and average peak torque at 240°/s in *E. longifolia* and Placebo group to examine the time effect within-group and between-groups at baseline (Week 0), interim (Week 4) and Post (Week 8)

Note: *E. Longifolia* group (EL); placebo (PLA); Peak torque (PT); Average peak torque (APT)

† indicates a significant difference in average peak torque between EL and PLA group; †Denotes level of significance was set at 0.05 (two-tailed)

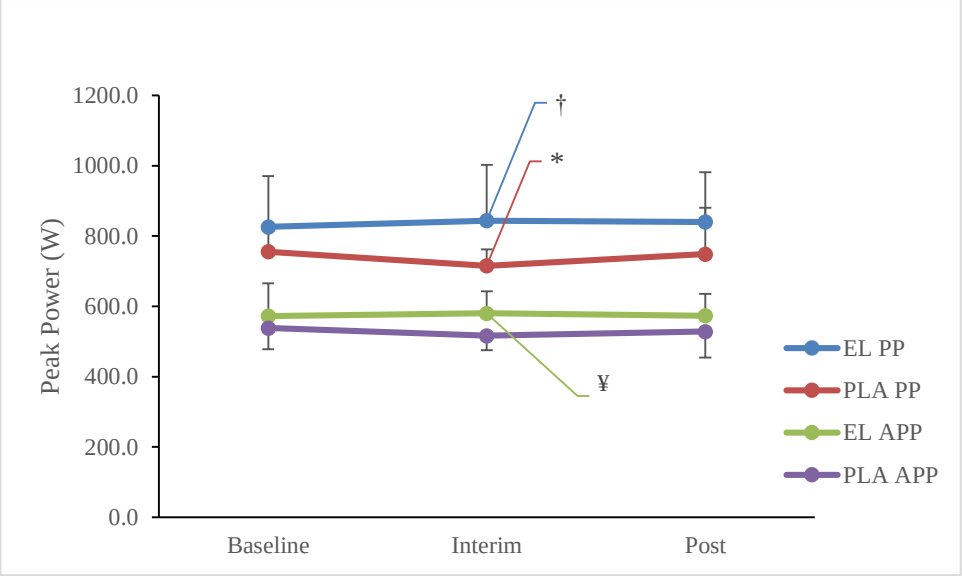


Figure 6.4: Peak power and average peak power (30s Anaerobic Wingate Test) on *E. longifolia* and placebo group to examine the time effect within-group and between-groups at baseline (Week 0), interim (Week 4) and Post (Week 8)
Note: *E. Longifolia* (EL); placebo (PLA); Peak power (PP); Average (A)
* indicates that there is significant changes compare to baseline; † Denotes indicate significant differences between EL and PLA Peak Power; ¥ Denotes indicate significant difference between EL and PLA Average Peak Power.
*,† Denotes level of significance was set at 0.05 (two-tailed)

There is a significant main effect of time ($F_{2, 36} = 8.979$, $p = 0.001$) but no significant main interaction between group and time ($F_{2,36} = 2.817$, $p = 0.073$) in relative peak power. Pairwise comparison within groups shows no significant increased in relative peak power from baseline (10.85 ± 1.80 W/kg) to interim (10.70 ± 1.54 W/kg) [MD = 0.147 W/kg, 95% CI = -1.14, 1.44, $p = 1.000$] and no significant increase from baseline to post-test (10.60 ± 1.28 W/kg) [MD = 0.250 W/kg, 95% CI = -0.772, 1.27, $p = 1.000$] in EL. PLA show significant decrease from baseline (9.87 ± 0.72 W/kg) to interim (9.31 ± 0.57 W/kg) [MD = 0.564 W/kg, 95% CI = 0.120, 1.00, $p = 0.015$) but no singnificant changes from baseline to to post-test (9.40 ± 0.89 W/kg) [MD = 0.477, 95% CI = -0.420, 1.37, $p = 0.442$). Between group analysis saw a significant higher relative peak power in EL compared to PLA at interim ($t_{19} = 2.688$, $p = 0.015$) and in post-test ($t_{19} = 2.477$, $p = 0.023$) (Figure 6.5)

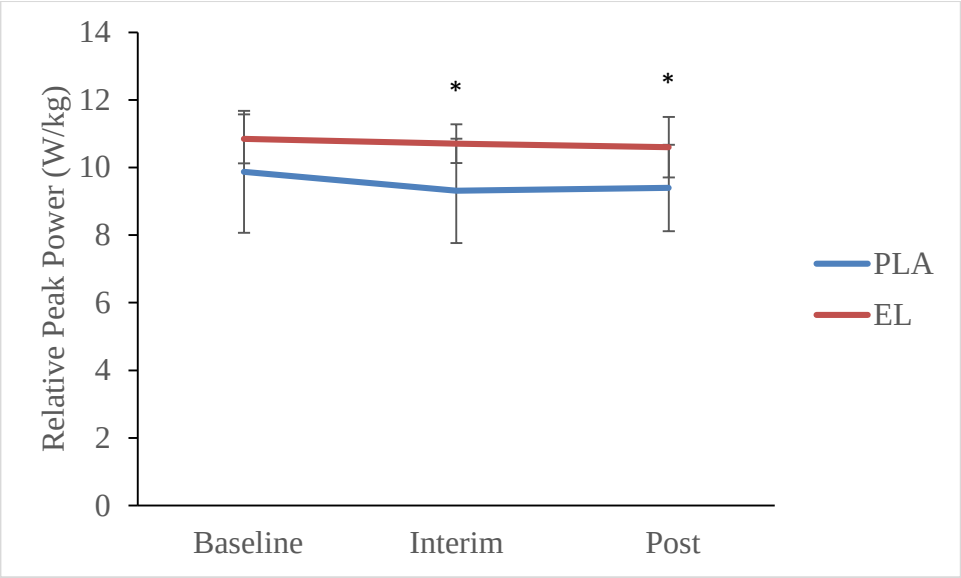


Figure 6.5: Relative peak power (30s Anaerobic Wingate Test) on *E. longifolia* and placebo group to examine the time effect within-group and between-groups at baseline (Week 0), interim (Week 4) and Post (Week 8)
Note: *E. Longifolia* (EL); placebo (PLA)
* indicates significant differences between EL and PLA
*Denotes level of significance was set at 0.05 (two-tailed)

In WanT, fatigue index was determined as well. There is a significant main effect of time ($F_{2, 36} = 45.50$, $p = 0.000$) and significant main interaction between group and time ($F_{2,36} = 3.991$, $p = 0.027$). Pairwise comparison within groups shows significant increased in fatigue index from baseline (59.93 ± 14.67 %) to interim (66.14 ± 5.63 %) [MD = -6.216 %, 95% CI = -11.42, -1.05, $p = 0.002$] but no significant increase from baseline to post-test (62.55 ± 6.34 %) in EL. PLA show no significant changes from baseline (58.0 ± 12.9 %) to interim (58.1 ± 3.6 %) [MD = -0.063 %, 95% CI = -3.64, 3.51, $p = 1.000$) and also to post-test (57.6 ± 9.2 %) [MD = 0.445 %, 95% CI = -7.76, 8.65, $p = 1.000$) Between group analysis saw no significant fatigue index in EL (844.08 ± 158.16W) compared to PLA ($p > 0.05$).

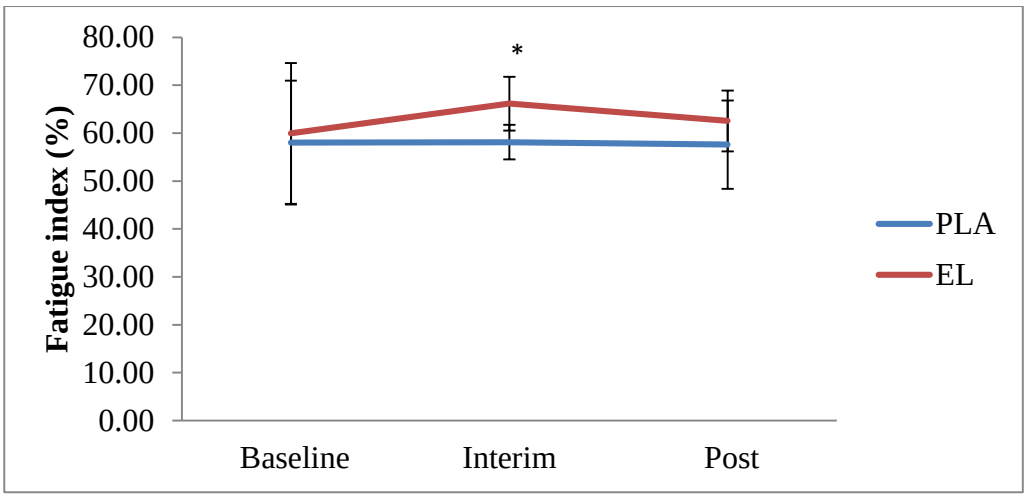


Figure 6.6: Fatigue index (30s Anaerobic Wingate Test) on *E. longifolia* and placebo group at baseline (Week 0) and Post (Week 8)
Note: *E. Longifolia* (EL); placebo (PLA)
* indicates significant differences within EL
*Denotes level of significance was set at 0.05 (two-tailed)

6.4.6 Force generation via vertical jump test

For baseline measurement, max force scores of EL (Median = 2075N) were not different than PLA (Median = 1892N). A Mann-Whitney test indicated that no statistically significant, U (EL = 11, PLA = 10) = 36.50, z = -1.303, p = 0.197. However, in max power, independent t-test shows significant difference between EL (4103.18 ± 509.67N) and PLA (3555.9 ± 383.52N) in max power, (t_{19} = 2.757, p = 0.013).

In max force, ANCOVA repeated measurement with baseline at the covariate shows no significant main effect of time ($F_{1.356,\ 24.410}$ = 2.892, p = 0.091) and no significant main interaction between group and time ($F_{1.356,\ 24.410}$ = 0.012, p = 0.959) was observed. Pairwise comparison within groups shows no significant changes but an increase from baseline (2141 ± 385N) to post-test (2154 ± 415N) [MD = -13.00N, 95% CI = -290.386, 264.386, p = 1.000] in EL and also similar in PLA from baseline (1862 ± 189N) to post

(1936 ± 213N) [MD = -73.70, 95% CI = -234.124, 86.724, p = 0.610). Between group analysis shows no significant difference in max force between EL (2154 ± 415N) and PLA (1936 ± 213N) (t_{19} = 1.535, p = 0.145) at post-test (Figure 6.5A).

In max power, ANCOVA repeated measurement shows no significant main effect of time ($F_{2, 36}$ = 0.272, p = 0.764) but significant main interaction between group and time ($F_{2, 36}$ = 3.650, p = 0.036) was observed. Pairwise comparison within groups shows no significant changes from baseline (4103 ± 510W) to post-test (4076 ± 585W) [MD = 27.455, 95% CI = -128.955, 183.864, p = 1.000] in EL but there is significant increase in max power in PLA from baseline (3556 ± 384W) to post (3736.6 ± 421W) [MD = -180.70W, 95% CI = -301.033, -60.367, p = 0.006). Between group analysis shows no significant difference in max power between EL (4076 ± 585W) and PLA (3736.6 ± 421W), (t_{19} = 1.510, p = 0.148) at post test (Figure 6.5B).

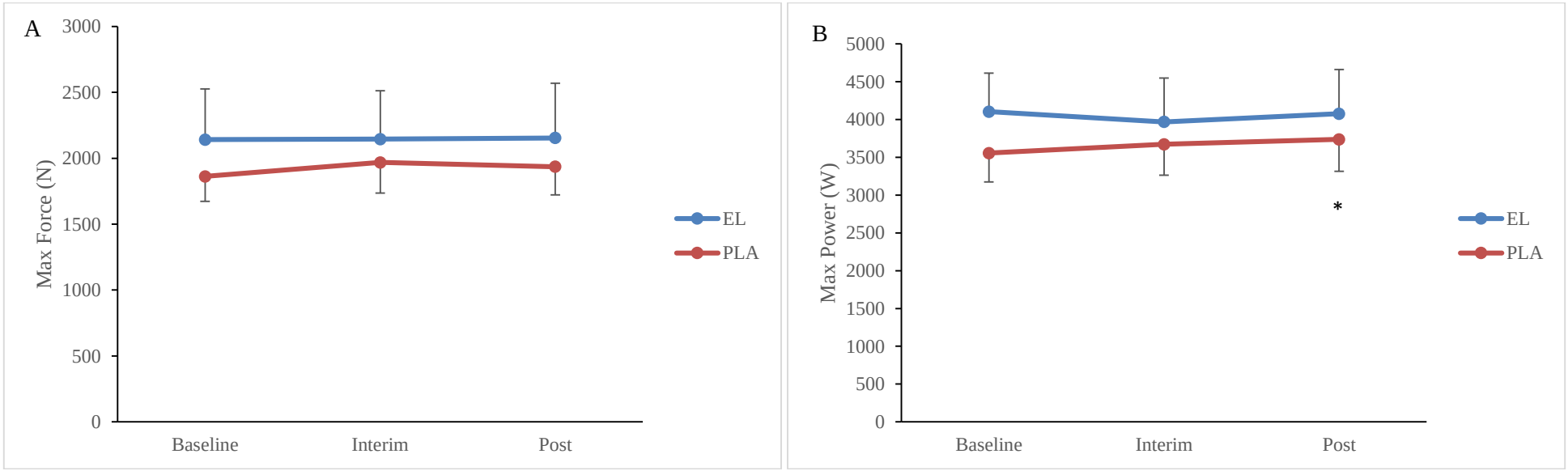


Figure 6.7: Max force (A) and max power(B) (vertical jump on force platform) in *E. longifolia* and Placebo group to examine the time effect within-group and between-groups at baseline (Week 0), interim (Week 4) and Post (Week 8)

Note: *E. Longifolia* (EL) and placebo (PLA)

*indicates that there is significant changes compare to baseline.

* denotes level of significance was set at 0.05 (two-tailed)

6.5 Discussion

The present results indicate that high dose supplementation (600 mg) alone does not significantly improve strength, anaerobic power or body composition in healthy young men. An increase in testosterone was associated with increased skeletal muscle mass, strength and power (Storer *et al*., 2008). The testosterone level presented in Chapter 4 did not indicate any significant increase after *E. longifolia* supplementation. Hence, the lack of strength, power and body composition improvement was observed.

While findings in the current study did not improve in strength or power after *E. longifolia* supplementation, several previous studies have shown improvements. Previous studies were performed on participants aged between 30 to 72 years old with a possibility of an age-related reduction in circulating testosterone levels (George *et al*., 2013; Henkel *et al*., 2014). Both studies demonstrated an increase in muscle strength after being supplemented with 100 mg and 400 mg of *E. longifolia*, respectively. However, only Henkel *et al*. (2014) found an increase in strength with testosterone levels elevated. It has been suggested that the improvement is due to the peptides found in *E. longifolia* which add additional ergogenic benefits (Sambandan *et al*., 2006). The peptides enhance protein synthesis to assist in the repair and regeneration of skeletal muscle fibres, which promote improvements in both muscle size and strength in the older population (Tarnopolsky *et al*., 1995; Roy *et al*., 2000; Ratamess *et al*., 2003; Hoffman *et al*., 2006; Kraemer *et al*., 2006). However, the current long-term supplementation of 600 mg of *E. longifolia* did not improve strength and power. While *E. longifolia* exhibits greater changes in testosterone levels when initial levels are low (Talbott *et al*., 2013), baseline testosterone levels in the current study (Chapter 4) remain at the normal circulating levels. As a

consequence, the strength and power within the *E. longifolia* and placebo treated group remain unchanged.

The present study recruited adult males that do not participate in any physical activities. In order to determine whether supplementation of *E. longifolia* alone increases testosterone level and subsequently improves strength and power, resistance training intervention was not included in the study. However, without resistance training intervention, it may have a lesser effect in enhancing strength and power. The combination can elicit a greater effect in enhancing muscle strength and power than supplementation alone, considering exercise increases testosterone and results in higher testosterone to cortisol ratio values, which subsequently increase the individual's anabolic status. It is suggested that the supplementation of *E. longifolia* during this anabolic state enhances muscle size and strength (Henkel *et al*., 2013; Adlercreutz *et al*., 1986). A combination of 400 mg per day of *E. longifolia* and circuit training for eight weeks has demonstrated better improvement than *E. longifolia* alone or exercise alone in increasing muscular strength and power (Ooi *et al*., 2015). An earlier pilot study found an intensive 8-week strength training programme supplemented with 100 mg per day of *E. longifolia* exhibited a significant increase (6.78%) in strength compared to the placebo group (Hamzah & Yusof, 2003). Another study using 100 mg of *E. longifolia,* combined with strength training, found increased muscle strength and larger quadriceps muscles (Sarina *et al*., 2009). A more recent study, utilizing 40 males aged between 19 to 25 years, administered 200 mg per day of *E. longifolia* and, combined with resistance training, found significant muscular strength and power improvement. The improvement in muscular strength may be attributed to the resistance training acting as a stimulus to initiate muscular growth, and this growth, in turn, triggers protein signalling to activate and stimulate the synthesis of muscle protein, leading to increased muscle strength (Chen

et al., 2019). It is proven that strength can increase through performing resistance training (Brown and Wilmore, 1974; Anderson and Kearney, 1982; Morganti *et al*., 1995; Starkey *et al*., 1996; Chilibeck *et al*., 1998; Faigenbaum et al., 1999; Hagerman *et al*., 2000). Previous studies have shown that the combination of *E. longifolia* and resistance training demonstrates a higher synergistic effect than *E. longifolia* alone in increasing muscular strength and power. Hence, the supplementation of *E. longifolia* alone in this study reported no improvement in strength.

Also, a high dose of *E. longifolia* (600 mg) supplemented in adult men did not elicit any increase in strength and anaerobic power. Although there was a significantly higher relative peak power in EL than PLA, there were no significant changes within the EL. The significant difference could be due to the decrease of relative peak power in PLA, and most importantly, baseline relative peak power was higher in EL. Based on the fatigue index data, there is a higher decline in power output during the test in EL. According to Tambi (2005), 600 mg of *E. longifolia* is currently the highest dose tested on humans and is non-toxic. Thus, the current findings suggest that even a high dose of *E. Longifolia* will not produce a more beneficial effect on muscular strength among healthy young males; a combination of *E. longifolia* with resistance training may be needed. Although the participants were asked to perform two times of familiarization prior to the isokinetic dynamometer, Wingate and vertical jump test, there is a possibility that the muscular strength and power assessment may not obtain accurate and reliable measurements. According to Dias *et al*. (2005) and Vrbik *et al*. (2016), the familiarization of two to three familiarization sessions with previous experience of performing the testing activities is needed to measure strength and power accurately. It is also suggested that one familiarization trial is sufficient to demonstrate excellent reproducibility for peak moment, average power and total work parameters. Also, one familiarization is

recommended prior to testing to reduce the effect of practice-based improvement (Nugent *et al*., 2015). Hence, a better assessment of familiarization is needed prior to testing future studies to obtain reliable measurements.

The present study did not significantly improve body weight, body fat percentage, and fat-free mass. Hamzah and Yusof (2003) found that eight weeks of resistance training combined with *E. longifolia* exhibited a five percent increase in lean mass compared to the placebo group. The positive findings in lean mass improvement may have been due to the combination of intensive strength training and the supplementation of *E. longifolia*, which provides further evidence that a combination of *E. longifolia* and resistance training may significantly improve lean body mass. Thus, suggesting that this traditional remedy may be a practical approach to gaining lean body mass and, subsequently, losing body fat. However, the current study does not demonstrate this effect, and further research is warranted with a proper evaluation of diet intake and physical activities.

6.6 Conclusion

Administrating a high dose of *E. longifolia* for eight weeks did not elicit any effects in increasing isokinetic strength, anaerobic power or body composition among young men who had no previous health issues, including low testosterone levels. There are limitations in the current study, including the lack of exercise training in the experimental protocol, and participants might not have familiarized themselves with the exercise testing. Also, participants might have performed a certain amount of exercise throughout the trial period, which may confound the current findings. Biochemical markers, such as cortisol, growth hormone, dehydroepiandrosterone sulfate, total insulin-like growth factor-1, creatine kinase, can be measured to determine the anabolic or catabolic status after

consuming *E. longifolia*. Future studies involving muscle biopsies to assess the changes directly in the skeletal muscle tissues rather than indirectly through measures in the blood may provide substantial information such as skeletal muscle fiber hyperthrophy. The present study shows that the supplementation of *E. longifolia* alone, especially in young, healthy men, did not exhibit beneficial effects, despite long-term supplementation of high doses (600 mg). While it is suggested that a combination of resistance training and *E. longifolia* may augment muscular strength, anaerobic power or body composition, further studies are needed to confirm this.

CHAPTER 7: SUMMARY AND CONCLUSION

7.1 Summary

This thesis has examined the efficacy of *E. longifolia* supplementation on exercise performance and wellbeing among male adults. This chapter provides a synthesis of the primary outcomes of the investigations presented in the thesis. Limitations and the future direction of research will also be discussed.

The first objective is addressed in Chapter 3. The cytotoxicity effect of *E. longifolia* on C_2C_{12} murine SMCs is assessed using the LDH assay. A concentration of 0.5 mg/ml and 1.0 mg/ml *E. longifolia* was administered to differentiated C_2C_{12} cell lines (P13 and P26) throughout this cytotoxicity study. The findings reveal that P13 has a greater increase in myotube size compared to P26, with no cell death observed during the test period. While there is no cell death, the LDH cytotoxicity test demonstrated that the cytotoxicity level in the *E. longifolia* treated cells is lower than the cells treated with the differentiation medium (CON). The reduction in LDH activity indicates that *E. longifolia* reduces the rate of cytotoxicity or cell death. Also, there is increased cell viability in P13 and P26 cells treated with 0.5 and 1.0 mg/ml of *E. longifolia* compared to the non treated control cells. However, the cell viability is lower than the P13 and P26 C2C12 cells treated with DM, demonstrating that there are more living cells in both P13 and P26 treated with *E. longifolia*. A higher dose is needed in P26 to induce higher cell viability. Findings suggest that *E. longifolia*, with its bioactive compounds, may have the ability to stimulate the myotube's proliferation and differentiation. However, further studies are needed to identify which bioactive compounds are responsible for the current study outcome.

The second objective is addressed in Chapter 4. Two weeks of 600 mg of *E. longifolia* supplementation increased testosterone (11.1%), but this effect diminishes when the supplementation is extended to eight weeks. The increase in testosterone and free testosterone is thought to be due to an active ingredient known as eurycomanone. However, the prolonged administration of *E. longifolia* did not demonstrate any change in the reproductive hormones. While the current study shows that a high dose (600 mg) can increase short-term testosterone levels, the outcome may have been due to participants participating in exercise without the researcher's knowledge. Also, other possible factors affecting the outcome such as dietary and supplementation intake, were not examined. Future investigations considering all the possible confounding factors are needed to allow a more reliable and accurate outcome.

The current findings demonstrate that *E. longifolia* supplementation does not stimulate LH production nor reduce oestrogen levels. Instead, two weeks of *E. longifolia* supplementation shows a lower level in SHBG (1.3%) compared to placebo (16.5%). However, supplementation of *E. longifolia* for eight weeks demonstrated a significant increase in SHBG, and a reduction in free testosterone is observed. While a lower SHBG value was reported in EL than PLA in the short-term supplementation period, there are no significant changes from day 1 to day 14. Thus, demonstrating that E. longfolia did not influence SHBG to release free testosterone to circulate around the body. The current study shows that *E. longifolia* did not boost testosterone in a healthy individual beyond the normal circulating levels and did not affect the LH, FSH, oestrogen and SHBG in the HPG axis.

Chapter 5 addresses objective number three. The current study shows that participants supplemented with 600 mg of *E. longifolia* for either two or eight weeks do not demonstrate an optimal peak in vigour and low negative mood state above or below the

50% standard score, respectively. Hence, the present short and long-term supplementations of *E. longifolia* do not demonstrate a positive mood state profile among adult men. There is a significant reduction in the anger and confusion domains in the two-week supplementation period, and all the negative domains demonstrate improvement after the long-term supplementation period, with tension and anger showing a significant decrease. Not all the psychological mood states are significantly increased in the current study; thus, the hypothesis is only partially accepted. Further research in this area, especially to examine the precise mechanism of *E. longifolia* in enhancing testosterone levels and reducing the cortisol level, is required. Although an improvement was observed in certain domains, the improvements within and between EL and PLA are too minimal to conclude the effectiveness of *E. longifolia* causes this outcome.

Concerning sexual function, the *E. longifolia* supplemented group shows a significant increase only in the ejaculation domain and a non-significant increase in overall satisfaction after two weeks of supplementation. Alternatively, long-term supplementation demonstrates a significant increase in three sexual domains (erection, ejaculation, and overall satisfaction) and a higher increase in sexual desire and problem assessment than placebo. Although there is no indication that *E. longifolia* supplementation for two weeks contributes to sexual function improvement, there are improved sexual function in all domains over time compared to placebo. Similar to mood state, the improved sexual function may not be due to *E. longifolia* as testosterone level did not change significantly. However, the outcome may be affected due to confounding factors such as the participants recruited being healthy, young, and may have a sexual partner, and not directly related to *E. longifolia.*

The final objective is addressed in Chapter 6. *E. longifolia* supplementation (600 mg) alone does not demonstrate any significant improvement in strength, anaerobic power and

body composition in healthy young men. In order to improve muscular strength, anaerobic power and body composition, resistance training is needed. It has been shown in previous studies that the combination of *E. longifolia* and resistance training demonstrates a synergistic effect in increasing muscular strength and power compared to those that only participate in resistance training (Hamzah & Yusof, 2003; Sarina *et al*., 2009; Henkel *et al*., 2013; George *et al*., 2013; Ooi *et al*., 2015; Chen *et al*. 2019). In addition, supplementation of *E. longifolia* with a dosage of 600 mg per day for eight weeks does not increase the testosterone levels in young adult participants with normal circulating levels. It should be noted that the human body constantly regulates testosterone levels to maintain homeostasis (O'Hara *et al*., 2015), and for this reason, 600 mg of *E. longifolia* administered alone for a prolonged period may not be effective in increasing testosterone. Testosterone is perceived as a determining factor in enhancing strength. Hence, the lack of significant improvement in muscular strength, anaerobic power and body composition may be due to the lack of significant changes in testosterone level and the absence of resistance training. Further studies examining the synergistic effect of combined *E. longifolia* supplementation with resistance training on adult participants, and investigation into the possible perturbation of homeostasis due to the supplementation of *E. longifolia*, are warranted.

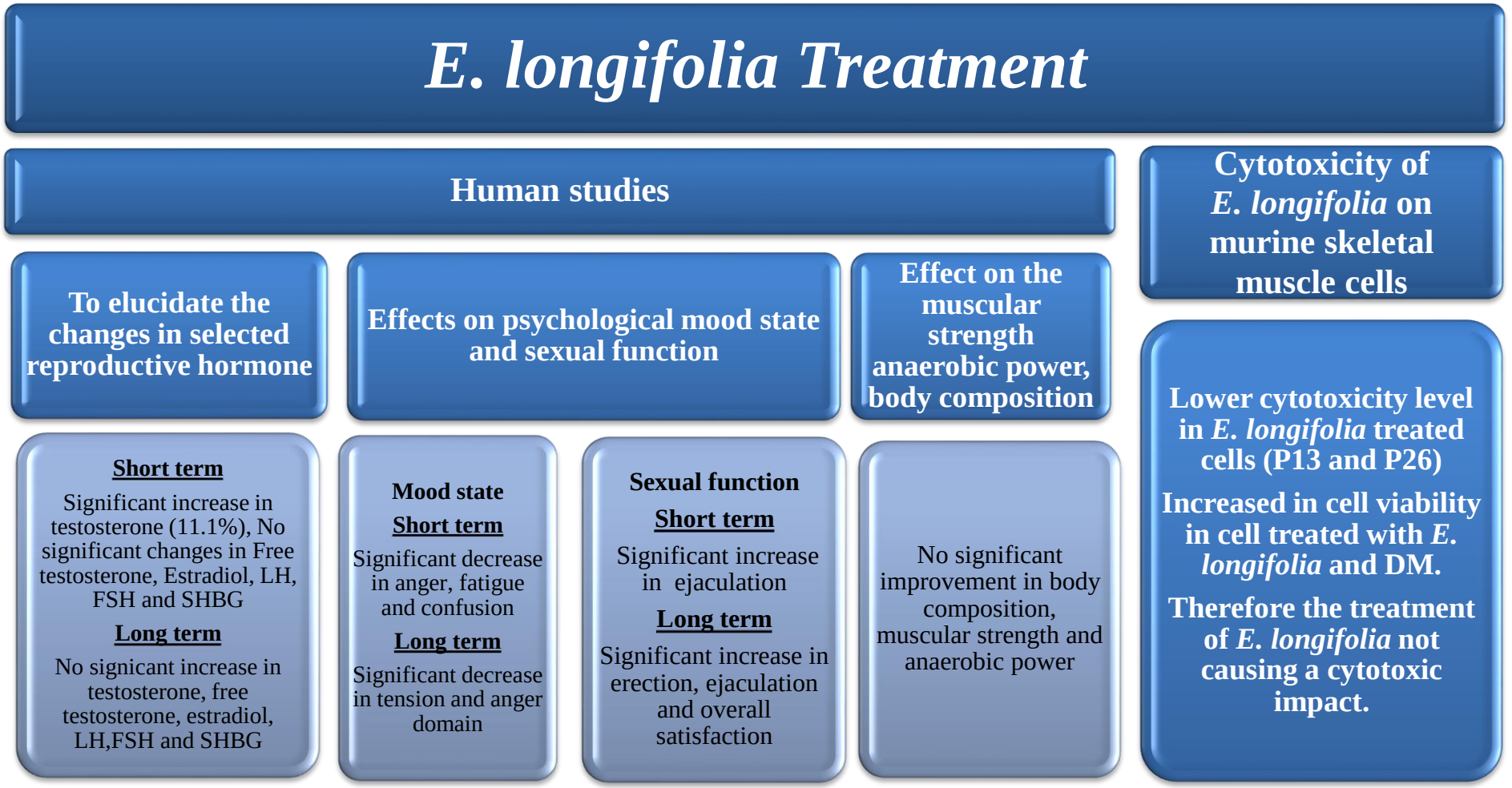


Figure 7.1 Summary of studies

7.2 Limitations of the Study

Previous studies have mostly examined the cytotoxicity of *E. longifolia* in various cancer cell lines using various bioactive compounds isolated from *E. longifolia*. In this study, the cytotoxicity test on SMCs is examined for the first time. Thus, there are no specific guidelines on the appropriate dose of *E. longifolia* to be used. The pilot test was conducted using various doses that were tested repeatedly on SMCs with different passage numbers to determine the most suitable dose and the cell passage number. While the current study shows lower cytotoxicity levels in murine SMCs compared to the control treatment, the reason behind the myotube growth observed after being treated with *E. longifolia* is unknown. This study is limited to cytotoxicity tests within cell culture models, and myotube size and gene expression were not performed to examine the muscle growth, hypertrophy post-*E. longifolia* treatment.

Following the *in vivo* study, humans studies were conducted to examine the efficacy of *E. longifolia,* which is presented in Chapter 4 through to Chapter 6. The main obstacle faced during this *in vitro* study is the recruitment process. The participants' recruitment is done in the United Kingdom, where *E. longifolia* is not widely used and was unfamiliar as a herbal supplement. Potential participants may not have been comfortable consuming the extract, thus hampering wider recruitment. In addition, the cost to purchase instruments (i.e. butterfly needles for phlebotomy, blood collection tube, assay kits for hormone analysis) also contribute to the smaller number of participants.

The study examining the possible mechanisms involved in *E. longifolia* supplementation in a human is performed for the first time. The current study is limited to analysing of the reproductive hormones, such as testosterone, free testosterone, the

luteinising hormones, FSH, oestrogen, and sexual hormone-binding globulin to determine whether *E. longifolia* influenced the HPG axis.

Chapter 4 demonstrate an increase and no change in testosterone levels after two and eight weeks of the supplementation period, respectively, with oestrogen levels remaining elevated. These findings suggest that aromatase is not inhibited after the *E. longifolia* supplementation period. However, we cannot draw a well-founded conclusion that *E. longifolia* indeed does not inhibit aromatase since the aromatase activity is not examined. Therefore, the effect of *E. longifolia* on the aromatase activity warrants more investigation.

Testosterone levels in the body are regulated predominantly via the HPG axis and, to a lesser extent, the HPA axis. However, the current study can only speculate that the lack of a significant increase in testosterone levels in Chapter 4 may be due to a higher level of circulating cortisol hormones. The cortisol hormones play an important role in regulating testosterone in both the HPG and HPA axes. That their levels are not measured indicates another main limitation in the thesis.

A combination of strength training and exogenous testosterone improves muscular strength significantly compared to participants who performed strength training alone. While a synergistic effect arising from the combined *E. longifolia* and resistance training regime on muscular strength and power, the exclusion of resistance training in the experimental protocol in Chapter 6 demonstrates another limitation in this study, and further research is needed to confirm the synergistic effects of the combination of *E. longifolia* supplementation and resistance training among adults males.

7.3 Future Directions

The present cytotoxicity study shows lower cytotoxicity levels in murine SMCs than the control treatment, and myotube growth is observed. The *E. longifolia* effects on skeletal muscle differentiation and hypertrophy are still unknown. Thus, future work examining the gene expression of MyoD, muscle regulatory factor 4 (MRF4), myogenic factor 5 (Myf5), and myogenin may provide a better understanding of skeletal muscle differentiation and hypertrophy post-administration of *E. longifolia*. Hence the adoption of muscle cells versus primary human culture through muscle biopsies to address cell behaviour via gene expression may facilitate a better understanding of skeletal muscle differentiation and hypertrophy. The ELISA kit used for human hormone measurements may need to be validated analytically (specificity, sensitivity, accuracy, precision, and parallelism of the dose-response relationship for the standard of the assay) and biologically (comparing changes in hormone levels) to ensure the accurate and reliable outcome. Also, studies investigating the claim ability in regulating signaling patways involved in proliferation, cell death and inflammation.

At present, the effect of *E. longifolia* in increasing testosterone levels is based solely on the findings by Low *et al.* (2013a & 2013b), which demonstrated *E. longifolia* interfered with the HPG axis through the inhibition of aromatase enzymes and, thus, prevented the conversion of the testosterone to oestrogen. This mechanism allows more testosterone to stay in the system, and the inhibition of aromatase leads to a lower negative feedback signal to the hypothalamus. However, Chapter 4 suggests otherwise; hence, future work examining the aromatase activity in humans may provide a better mechanistic understanding of whether *E. longifolia* affects aromatase inhibition via humans' HPG axis.

E. longifolia at a 600 mg/day dosage was well-tolerated and considered safe for consumption by participants. However, this dosing regimen did not increase testosterone levels following eight weeks of supplementation, the levels remaining within the normal biological range. The present study did not measure cortisol levels, something which may provide valuable information about and understanding of the efficacy of *E. longifolia* in various stress domains and sexual function parameters. Previous studies have shown a reduction in cortisol levels after supplementing with *E. longifolia*, indicating a shift to an anabolic hormone state. Apart from the measurement of testosterone hormones, other biochemical markers, such as cortisol, growth hormones, dehydroepiandrosterone sulfate, total insulin-like growth factor-1 and creatine kinase, should be considered in future studies to determine the anabolic or catabolic status. Muscle biopsies could be an important future study direction to evaluate the skeletal muscle tissues' modifications rather than using indirect measures in the blood. This may provide significant information regarding the effects of *E. longifolia* on muscle strength and hypertrophy. Also, studies investigating male fertility (semen volume, spermatozoa count, and motility) in human may provide better information regarding the aphrodisiac properties of *E. longifolia*.

The current study does not demonstrate any muscular strength, anaerobic power or body composition improvement, even after participants had been supplemented with *E. longifolia*. *E. longifolia* supplementation with exercise training may need to be conducted concurrently to demonstrate a possible synergistic effect on muscular strength and anaerobic power. Hence, future studies involving the combination of an exercise intervention with *E. longifolia* supplementation in young men with normal testosterone levels are warranted.

7.4 Conclusion

The LDH cytotoxicity test demonstrates that *E. longifolia* treatment displays low LDH activity in SMCs compared with the cells treated with differentiation medium, indicating *E. longifolia* treatment does not exhibit toxicity. Also, SMCs treated with *E. longifolia* demonstrate an increase in cell viability. This thesis also demonstrates that *E. longifolia* did not demonstrate efficacy in increasing the reproductive hormones, mood state, sexual function and exercise performance in the human study. While the current findings show that *E. longifolia* does not interfere with the HPG axis to stimulate more testosterone production by affecting the LH and oestradiol, more work is needed to examine the HPG and HPA axes concurrently. This thesis also was the first to explore the reproductive hormones associated with the HPG axis. It is acknowledged that many confounding factors were unavoidable, and caution was needed in interpreting the findings to eliminate any bias toward *E. longifolia* supplementation. Ultimately, the study's outcome provides a new perspective on how *E. longifolia* could be conducted on murine skeletal muscle cells and a better understanding of the claim ability of *E. longifolia* in affecting the HPG axis via the reproductive hormones, mood state, sexual function and exercise performance.

REFERENCES

Adan, A., Kiraz, Y., and Baran, Y. (2016). Cell proliferation and cytotoxicity assays. *Current Pharmaceutical Biotechnology, 17*(14), 1213 - 1221.

Adimoelja, A. (2000). Phytochemicals and the breakthrough of traditional herbs in the management of sexual dysfunctions. *International Journal Andrology, 23*(Suppl. 2), 82–84.

Adlercreutz, H., Harkonen, K., Kuoppasalmi, K., Näveri, H., Huhtaniemi, I., Tikkanen, H., Remes, K., Dessypris, A., and Karvonen, J.(1986). Effects of training on plasma anabolic and catabolic steroid hormones and their responses during physical exercise. *International Journal of Sports Medicine, 7*(Suppl 1), 27–28.

Afrisham, R., Sadegh-Nejadi, S., SoliemaniFar, O., Kooti, W., Ashtary-Larky, D., Fatima, A., Aberomand, M., Najjar-Asl, S., and Khaneh-Keshi, A. (2016). Salivary testosterone levels under psychological stress and its relationship with rumination and five personality traits in medical students. *Psychiatry Investigation, 13*(6), 637-643.

Ahmad, N., Samiulla, D.S., Teh, B.P., Zainol, M., Zolkifli, N.A., Muhammad, A., Matom, E., Zulkapli, A., Abdullah, N.R., Ismail, Z., and Mohamed, A.F.S. (2018). Bioavailability of Eurycomanone in its pure form and in a standardized Eurycoma longifolia water extract. *Pharmaceutics, 10*(90), 1 – 16.

Ali, J.M. and Saad, J.M. (1993). Biochemical effect of *Eurycoma longifolia* jack on the sexual behavior, fertility, sex hormone, and glycolysis. *Department of Biochemistry, University of Malaya, PhD Dissertation*.

Ang, H.H., Chan, K.L., and Mak, J.W. (1995a). Effect of 7-day daily replacement of culture medium containing *Eurycoma longifolia* Jack constituents on the Malaysian Plasmodium falciparum isolates. *Journal of Ethnopharmacology, 49*(3), 171–175.

Ang, H.H., Chan, K.L., and Mak, J.W. (1995b). *In vitro* antimalarial activity of quassinoids from *Eurycoma longifolia* against Malaysian chloroquine-resistant Plasmodium falciparum isolates. *Planta Medica, 61*(2), 177–177.

Ang, H. H., and Cheang, H. S. (1999). Studies on the anxiolytic activity of *Eurycoma longifolia* jack Roots in mice. *The Japanese Journal of Pharmacology, 79*(4), 497-500.

Ang, H.H., and Cheang, H.S. (2001). Effects of *Eurycoma longifolia* Jack on laevator ani muscle in both uncastrated and testosterone-stimulated intact male rats. *Archives of Pharmacal Research, 24*(5), 437 – 440.

Ang, H., and Lee, K. (2002). Effect of *Eurycoma longifolia* Jack on orientation activities in middle-aged male rats. *Fundamental and Clinical Pharmacology, 16*(6), 479–483.

Ang, H.H., and Ngai, T.H. (2001), Aphrodisiac evaluation in non-copulator male rats after chronic administration of *Eurycoma longifolia* Jack. *Fundamental and Clinical Pharmacology*, *15*(4), 265–268.

Ang, H. H., Ngai, T. H., and Tan, T. H. (2003a). Effects of *Eurycoma longifolia* Jack on sexual qualities in middle aged male rats. *Phytomedicine, 10*(6-7), 590-593.

Ang, H.H., Lee, K.L., and Kiyoshi, M. (2003b). *Eurycoma longifolia* Jack enhances sexual motivation in middle-aged male mice. *Journal of Basic and Clinical Physiology and Pharmacology*, *14*(3), 301–308.

Ang, H.H., Lee, K.L., and Kiyoshi, M. (2004). Sexual arousal in sexually sluggish old male rats after oral administration of *Eurycoma longifolia* Jack. *Journal of Basic and Clinical Physiology and Pharmacology*, *15*(3-4), 303–309.

Ang, H.H., Cheang, H.S., and Yusof, A.P. (2000). Effects of *Eurycoma longifolia* Jack (Tongkat Ali) on the initiation of sexual performance of inexperienced castrated male rats. *Experimental Animals*, *49* (1), 35-38.

Ahn, H. S., Park, C. M., and Lee, S. W. (2002). The clinical relevance of sex hormone levels and sexual activity in the ageing male. *BJU international, 89*(6), 526-530.

Ang, H.H., Ikeda, S., and Gan, E.K. (2001). Evaluation of the potency activity of aphrodisiac in *Eurycoma longifolia* Jack. *Phythotherapy Research, 15*(5), 435 – 436.

Ang, H.H., and Sim, M.K. (1998). *Eurycoma longifolia* increases sexual motivation in sexually naive male rats. *Archives of Pharmacal Research, 21*, 779–81.

Athimulam, A., Kumaresan, S., Foo, D.C.Y., Sarmidi, M.R., and Aziz, R.A. (2006). Modelling and optimisation of *Eurycoma longifolia* water extract production. *Food Bioproduct Process, 84,*139–149.

Ahmed, H.H., Abdel-Rahman, M., Ali, R.S., and Moneim, A.E.A. (2009). Protective effect of ginkgo biloba extract and pumpkin seed oil against neurotoxicity of rotenone in adult male rats. *Journal of Applied Sciences Research, 5*(6), 622-635.

Ahtiainen, J.P., Pakarinen, A., Kraemer, J., and Hakkinen, K. (2003). Acute hormonal and neuromuscular responses and recovery to force vs. maximum repetitions multiple resistance exercises. *International Journal of Sports Medicine, 24,* 410–418.

Ahtiainen, J.P., Pakarinen, A., Alen, M., Kraemer, W.J., and Hakkinen, K. (2005). Short versus long rest periods between the sets in hypertrophic resistance training: influence on muscle strength, size, and hormonal adaptations in trained men. *Journal of Strength Conditioning Research, 19,* 572–582.

Akre, C., Berchtold, A., Gmel, G., and Suris, J. (2015). The evolution of sexual dysfunction in young men aged 18–25 years. *Journal of Adolescent Health*, *55*(6), 736–743.

Alexander, G.M., Swerdloff, R.S., Wang, C., Davidson, T., McDonald, V., Steiner, B., and Hines, M. (1998). Androgen–behaviour correlations in hypogonadal men and eugonadal men. *Hormones and Behavior, 33* (2), 85 – 94.

Althof, S.E. (2002). Quality of life and erectile dysfunction. *Urology, 59*(6), 803–810.

Al-Salahi, O.S.A., Ji, D., Majid, A.M.S.A., Kit-Lam, C., Abdullah, W.Z., Zaki, A., Din, S.K.K.J., Yusoff, N.M., and Majid. A.S.A. (2014). Anti-tumor activity of *Eurycoma longifolia* root extracts against K-562 cell line: *In vitro* and *in vivo* study. *PLoS ONE, 9*(1), 1-13.

Allen, M., Millett, P., Dawes, E., and Rushton, Neil. (1994). Lactate dehydrogenase activity as a rapid and sensitive test for the quantification of cell numbers *in vitro*. *Clinical Materials, 16*(1994), 189-194.

Amory, J.L., and Bremner, W.J. (2003). Regulation of testicular function in men: Implications for male hormonal contraceptive development. *The Journal of Steroid Biochemistry and Molecular Biology*, *85*(2-5), 357-361.

Anderson, R.A., and Wu, F.C.W. (1996). Comparison between testosterone enanthate induced azoospermia and oligozoospermia in a male contraceptive study. II. Pharmacokinetics and pharmacodynamics of once weekly administration of testosterone enanthate. *Journal Clinical Endocrinology Metabolism, 81*, 896–901.

Anderson, R.A., Martin, C.W., Kung, A.W.C., Everington, D., Pun, T.C., Tan, K.C.B., Bancroft,J., Sundaram, K., Moo-Joung, A.J., and Baird, D.T. (1999). 7α-Methyl-19-nor-testosterone maintains sexual behavior and mood in hypogonadal men. *Journal of Clinical Endocrinology Metabolism, 84*, 3556-3562.

Antonio, L. Wu, F.C., O'Neill, T.W., Pye. S.R., Ahern,T.B., Laurent, M.R., Huhyaniemi, I.T., Lean, M.E., Keevil, B.G., Rastrelli, G., Forti, G., Bartfai, G., Casanueva, F.F., Kula, K., Punab, M., Giwecman, A., Claessens, F., Decallonne, B., and Vanderschueren, D. (2016). Low free testosterone is associated with hypogonadal sign and symptopms in men with normal total testosterone. *Journal of Clinical Endocrinology and Metabolism, 101*(7), 2647-2657.

Araujo, A.B., Durante, R., Feldman, H.A., Goldstein, I., and McKinlay, J. (1998). The relationship between depressive symptoms and male erectile dysfunction: Cross-sectional results from the Massachusetts male aging study. *Journal of Biobehavioral Medicine, 60*(4), 458 – 465.

Araujo, A.B., Esche, G.R., Kupelian, V., O'Donnell, A.B., Travison, T.G., William, R.E., Clark, R.V., and McKinlay, J.B. (2007). Prevalence of symptomatic androgen deficiency in men. *The Journal of Clinical Endocrinology and Metabolism, 92*(11), 4241 – 4247.

Araujo, A.B., and Wittert, G.A. (2011). Endocrinology of the aging male. *Best Practice & Research Clinical Endocrinology & Metabolism, 25*(2), 303–319.

Arechabala, D., Coiffard, C., Rivalland, P., Coiffard, L.J., and de Roeck-Holtzhauer, Y. (1999). Comparison of cytotoxicity of various surfactants tested on normal human

fibroblast cultures using the neutral red test, MTT assay and LDH release. *Journal of Applied Toxicology, 19*(3), 163-165.

Arlt, W., Martens, J.W.M., Song, M., Wang, J.T., Auchus, R.J., and Miller, W.L. (2002). Molecular evolution of adrenarche: structural and functional analysis of P450c17 from Four Primate Species. *Endocrinology, 143*(12), 4665 – 4672.

Asiah, O., Nurhanan, M.Y., Ilham, M.A. (2007). Determination of bioactive peptide (4.3kDa) as an aphrodisiac marker in six Malaysia plants. *Journal of Tropical Forest Science, 19*(1), 61 – 63.

Azarneoshan, F., KhatamsazSaz, S., and Sadeghi. H. (2009). The effects of hydro alcoholic extract of doremaaucheri on blood concentration of gonadotropin and androgen hormones in adult rats. *Armaghane Danesh, 14*(3), 65-70. [In Persian]

Aziz, R.A., Sarmidi, M.R., Kumaresan, S., Taher, Z.M. and Foo, D.C.Y. (2003). Phytochemical processing: the next emerging field in chemical engineering—aspects and opportunities. *Jurnal Kejuruteraan Kimia Malaysia, 3,* 45–60.

Badia, X., and Herdman, M. (2001). The importance of health-related quality-of-life data in determining the value of drug therapy. *Clinical Therapeutics, 23*(1), 168–75.

Bamman, M.M., Shipp, J.R., Jiang, J., Gower, B.A., Hunter, G.R., Goodman, A., McLafferty Jr. C.L., Urban, R.J. (2001). Mechanical load increases muscle IGF-I and androgen receptor mRNA concentrations in humans. *American Journal of Physiology Endocrinology and Metabolism, 280*(3), E383–E390.

Bar-Or, O. (1987). The Wingate anaerobic test. An update on methodology, reliability and validity. *Sports Medicine, 4*(6), 381-394.

Barret-Connor, E., Von Műhlen, D.G., and Kritz-Sliverstein, D. (1999). Bioavailable testosterone and depressed mood in older men: the Rancho Bernardo Study. *Journal of Clinical Endocrinology and Metabolism, 84*(2), 573 – 577.

Bedir, E., Abou-Gazar, H., Ngwendson, J. N., and Khan, I. A. (2003). Eurycomaoside: a new quassinoid-type glycoside from the roots of *Eurycoma longifolia*. *Chemical & Pharmaceutical Bulletin, 51*(11), 1301-1303.

Belanger, A., Candas, B., Dupont, A., Cusan, L., Diamond, P., Gomez, J.L., and Labrie, F. (1994). Changes in serum concentrations of conjugated and unconjugated steroids in 40- to 80- year old men. *The Journal of Clinical Endocrinology & Metabolism, 79*(4), 1086-1090.

Bhasin, S., Cunningham, G.R., Hayes, F., Matsumoto, A.M., Snyder, P.J., Swerdloff, R.S., and Montori, V.M. (2010). Testosterone therapy in men with androgen deficiency syndromes : An endocrine society clinical practice guideline. *The Journal of Clinical Endocrinology and Metabolism, 95*(6), 2536 – 2559.

Bhasin, S., and Basaria, S. (2011). Diagnosis and treatment of hypogonadism in men. *Best Practice Research Clinical Endocrinology Metabolism, 25*(2), 251 – 270.

Bhasin, S., Storer, T.W., Berman, N., Callegari, C., Clevenger, B., Phillips, J., Bunnell, T.J., Tricker, R., Shirazi, A., and Casaburi, R. (1996). The effects of supraphysiologic doses of testosterone on muscle size and strength in normal men. *The New England Journal of Medicine, 335*(1), 1-7.

Bhasin, S., Storer, T.W., Berman, N., Yarasheski, K.E., Clevenger, B., Philips, J., Lee, W.P., Bunnell, T.J., and Casaburi, R. (1997). Testosterone replacement increases fat-free mass and muscle size in hypogonadal men. *Journal Clinical Endocrinology and Metabolism, 82*(2), 407–413.

Bhasin, S., Woodhouse, L., Casburi, R., Singh, A.B., Bhasin, D., Berman, N., Chen, X., Yarasheski, K.E., Magliano, L., Dzekov, C., Dzekov, J., Bross, R., Phillips, J., Sinha-Hokim, I., Shen, R., and Storer, T.W. (2001). Testosterone dose-response relationships in healthy young men. *American Journal of Physiology Endocrinology and Metabolism, 281,* E1172– E1181.

Bhat, R., and Karim, A.A. (2010). Tongkat Ali (*Eurycoma longifolia* Jack): a review on its ethnobotany and pharmacological importance. *Fitoterapia, 81*(7), 669-679.

Bhatnagar, A.S., Müller, P.H., Schenkel, L., Trunet, P.F., Beh, I., and Schieweck, K. (1992). Inhibition of estrogen biosynthesis and its consequences on gonadotrophin secretion in the male. *The Journal of Steroid Biochemistry and Molecular Biology, 41*(3 - 8), 437 - 443.

Blanco, C., Okuda, M., Wright, C., Hasin, D.S., Grant, B.F., Liu, S.M., and Olfson, M. (2008). Mental health of college students and their non–college-attending peers: results from the national epidemiologic study on alcohol and related conditions. *Archives General Psychiatry, 5*(12), 1429–1437.

Blazevich, A.J., and Giorgi. A. (2001). Effect of testosterone administration and weight training on muscle architecture. *Medicine and Science in Sports and Exercise, 33*(10), 1688–1693.

Branch, M.N. (1993). Behavioral factors in drug tolerance. In F. van Haaren (Ed.), Methods in behavioral pharmacology, *Amsterdam, Elsevier,* 329-347.

Brand, J.S., and van der Schouw., Y.T. (2010). Testosterone, SHBG and cardiovascular health in postmenopausal women. *International Journal of Impotence Research, 22*, 91 – 104.

Braun, H., Koehler, K., Geyer, H., Kleinert, J., Mester, J., and Schänzer, W. (2009). Dietary supplement use among elite young German athletes. *International Journal of Sport Nutrition & Exercise Metabolism, 19*(1), 97-109.

Brodsky, I.G., Balagopal, P., and Nair, K.S. (1996). Effects of testosterone replacement on muscle mass and muscle protein synthesis in hypogonadal men — a clinical research center study. *Journal of Clinical Endocrinology and Metabolism, 81*(10), 3469 - 3475.

Brown, G.A., Vukovich, M.D., Reifenrath, T.A., Parsons, K.A., Sharp, R.L., and King, D.S. (2000). Effects of anabolic precursors on serum testosterone concentration and

adaptations to resistance training in young men. *International Journal of Sport Nutrition Exercise Metabolism, 10,* 340-359.

Borst, S.E., and Mulligan, T. (2007). Testosterone replacement therapy for older men. *Clinical Interventions in Aging, 2*(4), 561-566.

Bryda, E. (2013). The Mighty Mouse: The impact of rodents on advances in biomedical research. *Missouri Medicine, 110*(3), 207 – 211.

Bucci, L.R. (2000). Selected herbals and human exercise performance. *The American Journal of Clinical Nutrition, 72*(2), 624S-636S.

Bushey, B., Taylor, L.W., Wilborn, C.W., Poole, C., Foster, C.A., Campbell, B., Kreider, R.B., and Willoughby, D.S. (2009). Fenugreek extract supplementation has no effect on the hormonal profile of resistance-trained males. *International Journal of Exercise Science, 2*(1).

Calfee, R., and Fadale, P. (2006). Popular ergogenic drugs and supplements in young athletes. *Pediatrics, 117*(3), 577-589.

Campbell, J.E., and Cohall, D. (2017). Pharmacodynamic- A pharmacognosy Perspective. *Pharmacognosy, 2017,* 513-525.

Cavallini, G., Caracciolo, S., Vitali, G., Modenini, F., and Biagiotti, G. (2004). Carnitine versus androgen administration in the treatment of sexual dysfunction, depressed mood, and fatigue associated with male aging. *Urology, 63*(4), 641– 646.

Chan, K.L., O'Neill, M.J., Phillipson, J.D., and Warhurst, D.C. (1986). Plants as sources of antimalarial drugs. Part 31 *Eurycoma longifolia*. *Planta Medica, 52*(2), 105–107.

Chan, K., Lee, S., Sam, T., and Han, B. (1989). A quassinoid glycoside from the roots of *Eurycoma longifolia*. *Phytochemistry, 28*, 2857–2859.

Chan, K., Lee, S., Sam, T., Tan, S., Noguchi, H., and Sankawa, U. (1991). 13β,18-dihydroeurycomanol, a quassinoid from *Eurycoma longifolia*. *Phytochemistry, 30* (9), 3138–3141.

Chan, K.L., Choo, C.Y., Morita, H., and Itokawa, H. (1998). High performance liquid chromatography in phytochemical analysis of *Eurycoma longifolia*. *Planta Medica. 64*,741–745.

Chan, K.L., Choo, C. Y., Abdullah, N.R., and Ismail, Z. (2004). Antiplasmodial studies of *Eurycoma longifolia* Jack using the lactate dehydrogenase assay of *Plasmodium falciparum*. *Journal of Ethnopharmacology, 92*(2-3), 223-227.

Chandler, R.M., Byrne, K., Patterson, J.G., and Ivy, J.L. (1994). Dietary supplements affect the anabolic hormones after weight-training. *Journal of Applied Physiology, 76*, 839–845.

Chaing, H.S., Merino-Chavez, G., Yang, L.L., Wang, F.N., and Hafez, E.S. (1994). Medicinal plants: conception/contraception. *Advances in Contraceptive Delivery System, 10*(3–4), 355–363.

Che, M., Wang, R., Li, X., Wang, H. Y., & Zheng, X. (2016). Expanding roles of superoxide dismutases in cell regulation and cancer. *Drug Discovery Today, 21*(1), 143–149.

Chen, S., Wang, J., Yu, G., Liu, W., and Pearce, D. (1997). Androgen and glucocorticoid receptor heterodimer formation. A possible mechanism for mutual inhibition of transcriptional activity. *The Journal of Biological Chemistry, 272*, 14087–14092.

Chen, C. K., Muhamad, A. S., and Ooi, F. K. (2012). Herbs in exercise and sports. *Journal of physiological anthropology, 31*(4).

Chen, C. K., Wan Mohamad, W.M.Z., Ooi, F. K., Ismail, S.B., Abdullah, M.R., and George, A. (2014). Supplementation of *Eurycoma longifolia* Jack extract for 6 weeks does not affect urinary testosterone: epitestosterone ratio, liver and renal functions in male recreational athletes. *International Journal of Preventive Medicine, 5*(6), 728 – 733.

Chen, C. K., Ooi, F. K., Abu Kasim, N. A., and Asari, M. A. (2019). Effects of *Eurycoma longifolia* Jack supplementation combined with resistance training on isokinetic muscular strength and power, anaerobic power, and urinary testosterone: epitestosterone ratio in young males. *International Journal of Preventive Medicine, 10*(118), 1 – 8.

Chhatre, S., Nesari, T., Somani, G., Kanchan, D., and Sathaye, S. (2014). Phytopharmacological overview of *Tribulus terrestris*. *Pharmacognosy Reviews, 8*(15), 45-51.

Chichinadze, K., and Chichinadze, N. (2008). Stress-induced increase of testosterone: Contributions of social status and sympathetic reactivity. *Physiology and Behavior, 94*, 595 – 603.

Christapher, P.V., Parasuraman, S., Christina, J.M.A., Azmawi, M.Z., and Vikneswaran, M. (2015). Review on *Polygonum minus*. Huds, a commonly used food additive in Southeast Asia. *Pharmacognosy Research, 7*(1), 1 – 6.

Chuen, C.S., and Pihie, A.H.L. (2004). Eurycomanone exerts antiproliferative activity via apoptosis upon MCF-7 cells. *The 4th Annual Seminar of National Science Fellowship.*

Choi, J.Y., Lee, S.H., Na, H.Y., An, K., Hyeon, T., and Seo, T.S. (2010). In vitro cytotoxicity screening of water-dispersible metal oxide nanoparticles in human cell lines. *Bioprocess and Biosystems Engineering, 33*(1), 21-30.

Choi, J.C., Chung, M., and Lee, Y. (2012). Modulation of pain sensation by stress-related testosterone and cortisol. *Anaesthesia, 67*(10), 1146–1151.

Connell, J. M. C., and Davies, E. (2005). The new biology of aldosterone. *Journal of Endocrinology, 186*(1), 1-20.

Corona, G., and Maggi, M. (2010). The role of testosterone in erectile dysfunction. *Nature Review Urology, 7*(1), 46–56.

Cuthbertson, D., Smith, K., Babraj, J., Leese, G., Waddell, T., Atherton, P., Wackerhage, H., Taylor, P.M. and Rennie, M.J. (2005). Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. *FASEB Journal, 19*(3), 422–424.

Cyranoski, D. (2005). Malaysian researchers bet big on homegrown Viagra. *Natural Medicine, 11*, 912.

Daniell, H.W., Lentz, R., and Mazer, N.A. (2006). Open-label pilot study of testosterone patch therapy in men with opioid-induced androgen deficiency. *The Journal of Pain, 7*, 200–210.

Danneskiold-Samsoe, B., Kofod, V., Munter, J., Grimby, G., Schnohr, P., and Jensen, G. (1984). Muscle strength and functional capacity in 78-81-year-old men and women. *European Journal Applied Physiology, 52*, 310-314.

Darise, M., Kohda, H., Mizutani, K., and Tanaka, O. (1982). Eurycomanone and eurycomanol, quassinoids from the roots of *Eurycoma longifolia*. *Phytochemistry, 21*, 2091–2093.

Darise, M., Kohda, H., Mizutani, K., and Tanaka O. (1983). Revision of configuration of the 12-hydroxyl group of eurycomanone and eurycomanol, quassinoids from *Eurycoma longifolia*. *Phytochemistry, 22*(6), 1514.

Davidson, J.M., Chen, J.J., Crapo, L., Gray, G.D., Greenleaf, W.J., and Catania, J.A. (1983). Hormonal changes and sexual function in aging men. *Journal of Clinical of Endocrinology and Metabolism, 57*(1), 71–77.

Deane, C.S., Hughes, D.C., Sculthorpe, N., Lewis, M.P., Stewart, C.E., and Sharples, A.P. (2013). Impaired hyperthrophy in myoblasts is improved with testosterone administration. *Journal of Steroid Biochemistry and Molecular Biology, 138*(2013), 152 - 161.

Decker, T., and Lohmann-Matthes, M.L. (1988). A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *Journal of Immunological Methods*, *115*(1), 61-69.

de Ronde, W., and de Jong, F.H. (2011). Aromatase inhibitors in men: effects and therapeutic options. *Reproductive Biology and Endocrinology, 9*(93), 1-7.

Donkwe, S.M.M., Happi, E.N., Wansi, J.D., Lenta, B.N., Devkota, K.P., Neumann, B., Stammler, H.-G., and Sewald, N. (2012). Oxidative burst inhibitory and cytotoxic activity of constituents of the fruits of *Odyendyea gabonensis*. *Planta Medica, 78*(18), 1949–1956.

Dhawan, K., and Sharma, A. (2002). Antitussive activity of the methanol extract of Passiflora incarnate leaves. *Fitoterapia*, *73*(5), 397-399.

Dias, Raphael Mendes Ritti, Cyrino, Edilson Serpeloni, Salvador, Emanuel Péricles, Caldeira, Lúcio Flávio Soares, Nakamura, Fábio Yuzo, Papst, Rafael Raul, Bruna, Nelson, & Gurjão, André Luiz Demantova. (2005). Influence of familiarization

process on muscular strength assessment in 1-RM tests. *Revista Brasileira de Medicina do Esporte, 11*(1), 34-38.

Edwards, D., Heufelder, A., and Zimmermann, A. (2012). Therapeutic effects and safety of Rhodiola rosea extract WS® 1375 in subjects with life-stress symptoms – results of an open-label study. *Phytotherapy Research, 26*(8), 1220 – 1225.

Effendy, M.N., Mohamed, N., Muhammad, N., Mohamad, I.N., Shuid, A.N. (2012). *Eurycoma longifolia*: Medicinal plant in the prevention and treatment of male osteoporosis due to androgen deficiency. *Evidence Based Complementary Alternative Medicine, 2012* (123761), 1 – 9.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology, 35*(4), 495 – 516.

Erasmus, N., Solomon, M., Fortuin, K., and Henkel R. (2012). Effect of *Eurycoma longifolia* Jack (Tongkat Ali) extract on human spermatozoa *in vitro*. *Andrologia, 44*(5), 308–314.

Eisenberg, D., Hunt, J., and Speer, N. (2013). Mental health in American colleges and universities: variation across student subgroups and across campuses. *Journal of Nervous and Mental Disease, 201*(1), 60–67.

Fahim, M.S., Fahim, Z., Harman, J.M., Clevenger, T.E., Mullins, W., and Hafez, E.S. (1982). Effect of Panax ginseng on testosterone level and prostate in male rates. *Archives of Andrology, 8*(4), 261-263.

Feiring, D.C., Ellenbecker, T.S., Derscheid, G.L. (1990). Test-retest reliability of the biodex isokinetic dynamometer. *Journal Orthopaedic Sports Physical Therapy, 11*(7), 298-300.

Feldman, H.A., Longcope, C., Derby, C.A., Johannes, C.B., Araujo, A.B., Coviello, A.D., Bremner, W.J., and McKinlay, J.B. (2002). Age trends in the level of serum testosterone and other hormones in middle-aged men: longitudinal results from the Massachusetts male aging study. *Journal of Clincal Endocrinology and Metabolism, 87*(2), 589–598.

Ferrando, A.A., Tipton, K.D., Doyle, D., Philips, S.M., Cortiella, J., and Wolfe, R.R. (1998). Testosterone injection stimulates net protein synthesis but not tissue amino acid transport. *American Journal of Phyiology, 275*(5), e864 – e871.

Fernandes, A.C., Chandran, D., Khondoker, M., Dewey, M., Shetty, H., Dutta, R., and Stewart, R. (2018). Demographic and clinical factors associated with different antidepressant treatments: a retrospective cohort study design in a UK psychiatric healthcare setting. *BMJ Open, 8*(9), 1 – 12.

Fiaschetti, G., Grotzer, M., Shalaby, T., Castelletti, D., and Arcaro, A. (2011). Quassinoids: From traditional drugs to new cancer therapeutics. *Current Medicinal Chemistry, 18*(3), 316–328.

Firenzuoli, F., and Gori, L. (2007). Herbal medicine today: Clinical and research issues. *Evidence Based Complement Alternative Medicine, 4*(Suppl 1), 37 – 40.

Forbes, G.B., Porta, C.R., Herr, B.E., Griggs, R.C. (1992). Sequence of changes in body composition induced by testosterone and reversal of changes after drug is stopped. *Journal of the American Medical Association,* 267(3), 397–399.

Foss, B., and Dyrstad, S.M. (2011) Stress in obesity: cause or consequence? *Medical Hypotheses, 77*(1), 7–10.

Fotakis, G., and Timbrell, J.A. (2006). In vitro cytotoxicity assays: Comparison of LDH, neurtal red, MTT, and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicology letters, 160*(2), 171 – 177.

Friedl, K.E., Dettori, J.R., Hannan, C.J. Jr., Patience, T.H., and Plymate, S.R. (1991). Comparison of the effects of high dose testosterone and 19-nortestosterone to a replacement dose of testosterone on strength and body composition in normal men. *Journal of Steroid Biochemistry and Molecular Biology, 40*(4–6), 607–612.

Friedl, K. E. (2005). Effects of testosterone and related androgens on athletic performance in men. In Kraemer, W. J., and Rogol, A. D. (Eds.), the endocrine system in sports and exercise (pp. 525–543). Oxford, UK: Blackwell.Gatti, R., and de Palo, E.F. (2011). An update: salivary hormones and physical exercise. *Scandinavian Journal of Medicine & Science in Sports, 21*, 157-169.

Frontera, W. R., Hughes, V. A., Lutz, K. J., and Evans, W. J. (1991). A cross-sectional study of muscle strength and mass in 45- to 78-yr-old men and women. *Journal of Applied Physiology, 71,* 644-650.

Gatti, R., and De Palo, E. F. (2011). An update: salivary hormones and physical exercise. *Scandinavian Journal of Medicine & Science in Sports, 21*(2), 157–169.

Gautaman, K., and Ganesan, A.P. (2008). The hormonal effects of *Tribulus terrestris* and its role in the management of erectile dysfunction: An evaluation using primates, rabbits and rat. *Phytomedicine, 15*, 44–54.

George, A., and Henkel, R. (2014). Phytoandrogenic properties of *Eurycoma longifolia* as natural alternative to testosterone replacement therapy. *Andrologia, 46*(7), 708 - 721.

George, A., Liske, E., Chen, C.K., and Ismail, S.B. (2013). The *Eurycoma longifolia* freeze dried water extract-Physta does not change normal ratios of testosterone to epitestosterone in healthy males. *Journal of Sports Medicine & Doping Studies, 3*(2), 1 - 6.

George, A., Suzuki, N., Abas, A., Mohri, K., Utsuyama, M., Hirokawa, K., and Takara, T. (2016). Immunomodulation in middle-aged humans via the ingestion of Physta ® standardized root water extract of *Eurycoma longifolia* Jack – A randomized, double-blind, placebo-controlled, parallel study. *Phytotherapy Research, 30*(4), 627 – 635.

George, A., Udani, J., Zainal Abidin, N., and Yusof, A. (2018). Efficacy and safety of *Eurycoma longifolia* (Physta® water extract plus multivitamins on quality of life, mood and stress: a randomized placebo-controlled and parallel study. *Food and Nutrition Research, 62*(2018), 1 – 16.

Giorgi, A., Weatherby, R.P., and Murphy, P.W. (1999). Muscular strength, body composition and health responses to the use of testosterone enanthate: a double blind study. *Journal of Science and Medicine in Sport, 2*(4), 341–355.

Grech, A., Breck, J., and Heidelbaugh, J. (2014). Adverse effects of testosterone replacement therapy: an update on the evidence and controversy. *Therapeutic Advances in Drug Safety, 5*(5), 190 – 200.

Green, G.A., Catlin, D.H., and Starcevic, B. (2001). Analysis of over the counter dietary supplements. *Clinical of Journal of Sports Medicine, 11*(4), 254– 259.

Green, D.G., and Llambi, F. (2015). Cell death signalling. *Cold Spring Harbor Perspectives in Biology, 7*, 1 – 25.

Grieco, P.A., and Morre, D.M. (1998). Mode of action of the anticancer quassinoids - Inhibition of the plasma membrane NADH oxidase. *Life Sciences, 63*(7), 595–604.

Griggs, R. C., Kingston, W., Jozefowicz, R. F., Herr, B. E., Forbes, G., and Halliday, D. (1989). Effect of testosterone on muscle mass and muscle protein synthesis. Journal of Applied Physiology, 66, 498–503.

Grossmann, M. (2011). Low testosterone in men with ypype 2 diabetes: Significance and treatment. *Journal of Clinical Endocrinology Metabolism, 96*(8), 2341-2353.

Gunnels, T., and Bloomer, R. (2014). Increasing circulating testosterone: Impact of herbal dietary supplements. *Journal of Plant Biochemistry & Physiology, 2*(130), 1-9.

Goldman, R.D., Kaplan, N.O., and Hall, T.C. (1964). Lactic dehydrogenase in human neoplastic tissues. *Cancer Research, 24*(3), 389–399.

Goreja, W.G. (2004). Tongkat Ali: The tree that cures a hundred diseases. New York: *Amazing Herb Press, TNC International Inc, 2*, 10–11.

Hajjouli, S., Chateauvieux, S., Teiten, M.H., Orlikova, B., Schumacher, M., Dicato, M., Chee, Y.C., and Diederich, M. (2014). Eurycomanone and Eurycomanol from *Eurycoma longifolia* Jack as regulators of signaling pathways involved in proliferation, cell death and inflammation. *Molecules, 19*(9), 14649-14666.

Hakkinen, K., Keskinen, K.L., Alen, M., Komi, P.V., and Kauhanen, H. (1989). Serum hormone concentrations during prolonged training in elite endurance-trained and strength-trained athletes. *European Journal of Applied Physiology and Occupational Physiology, 59*(3), 233 – 238.

Hakkinen, K., and Pakarinen, A. (1991). Serum hormones in male strength athletes during intensive short term strength training. *European Journal of Applied Physiology and Occupational Physiology, 63*(3 – 4), 194 – 199.

Hameed, M., Lange, K.H.W., Andersen, J.L., Schjerling, P., Kjaer, M., Harridge, S.D., and Goldspink, G. The effect of recombinant human growth hormone and resistance training on IGF-I mRNA expression in the muscles of elderly men. *Journal of Physiology, 555*(Pt 1), 231–240.

Hammon, G.L. (2011). Diverse roles for sex hormone-binding globulin in reproduction. *Biology of Reproduction, 85*(3), 431 – 441.

Hammond, G.L., Wu, T.S., Simard, M. (2012). Evolving utility of sex hormone-binding globulin measurements in clinical medicine. *Current Opinion in Endocrinology and Diabetes and Obesity, 19*(3), 183-189.

Hamzah, S., and Yusof, A. (2003). The ergogenic effects of Tongkat Ali (*Eurycoma longifolia*): A Pilot study. *British Journal of Sports Medicine, 37*(5), 464-447.

Harman, S.M., Metter, E.J., Tobin, J.D., Pearson, J., and Blackman, M.R. (2001). Longitudinal effects of aging on serum total and free testosterone levels in healthy men. Baltimore Longitudinal Study of Aging. *Journal of Clinical Endocrinology and Metabolism, 86*(2). 724–731.

Harun, N.H., Abdul-Aziz, A., and Aziz, R. (2015). Effect of number of steps on the quality of *Eurycoma longifolia* extract and cost efficiency of the extraction process. *Transactions on Science and Technology, 2*(2), 36 - 47.

Hassali, M.A., Khan, T.M., Shafie, A.A., and Nazir, M. (2009). Public knowledge about herbal beverages in Penang, Malaysia. *Australasian Medical Journal, 1*(6), 1-12.

Hassan, N.H., Abdullah, R., Kiong, L.S., Ahmad, A.R., and Abdullah, N., *et al*. (2012). Micropropagation and production of eurycomanone, 9-methoxycanthin-6-one and canthin-6-one in roots of *Eurycoma longifolia* plantlets. *African Journal of Biotechnology, 11*(26), 6818-6825.

Hayes, F.J., DeCruz, S., Seminara, S.B., Boepple, P.A., and Crowley, W.E. (2001). Differential regulation of gonadotropin secretion by testosterone in the human male: absence of a negative feedback effect of testosterone on follicle-stimulating hormone secretion. *Journal of Clinical Endocrinology Metabolism, 86*(1), 53 - 58.

Herbold, N.H., Visconti, B.K., Frates, S., and Bandini, L. (2004). Traditional and non traditional supplement use by collegiate female varsity athletes. *International Journal of Sport Nutrition, 14* (5), 586–93.

Heinemann, L.A.J., Saad, F., Zimmermann, T., Novak, A., Myon, E., Badia, X., Potthoff, P., T'Sjoen, G., Pollanen, P., Goncharow, N.P., Kim, S., and Giroudet, C. (2003). The Aging Males' Symptoms (AMS) scale: Update and complication of international versions. *Health and Quality of Life Outcome, 1*(15), 1 – 5.

Henning, P.C., Park, B.S., and Kim, J.S. (2011). Physiological decrements during sustained military operational stress. *Military Medicine, 176*(9), 991– 997.

Hussein, S., Ibrahim, R., and Kiong, L.P. (2007). A summary of reported chemical constituents and medicinal uses of *Eurycoma longifolia*. *Journal of Tropical Medicinal Plants, 8*(1), 103-10.

Huhtaniemi, I. (2014). Late-onset hypogonadism: current concepts and controversies of pathogenesis, diagnosis and treatment. *Asian Journal of Andrology, 16*(2), 192-202.

Hulmi, J.J., Ahtiainen, J.P., Selänne, H., Volek, J.S., Häkkinen, K., Kovanen, V., and Mero, A.A. (2008). Androgen receptors and testosterone in men – Effects of protein ingestion, resistance exercise and fiber type. *Journal of Steroid Biochemistry and Molecular Biology, 110,* 130 – 137.

Heikkinen, A., Alaranta, A., Helenius, I., and Vasankari, T. (2011). Use of dietary supplements in Olympic athletes is decreasing: a follow-up study between 2002 and 2009. *Journal of the International Society of Sports Nutrition, 8*(1), 1-8.

Henkel, R.R., Wang, R.X., Bassett, S.H., Chen, T., Liu, N., Zhu, Y., and Tambi, M.I. (2014). Tongkat Ali as a potential herbal supplement for physically active male and female seniors-a pilot study. *Phytotherapy Research*, *28*(4), 544–550.

Hioki, H., Yoshio, S., Motosue, M., Oshita, Y., Nakamura, Y., Mishima, D., Fukuyama, Y., Kodama, M., Ueda, K., and Katsu, T. (2004). Enantioselective total synthesis of Eurylene, 14-Deacetyl Eurylene, and their 11-Epimers: The relation between Ionophoric nature and cytotoxicity. *Organic Letters, 6*(6), 961–964.

Hoffman, J.R., Ratamess, N.A., Kang, J., Falvo, M.J., and Faigenbaum, A.D. (2006). Effect of protein intake on strength, body composition and endocrine changes in strength/power athletes. *Journal of the International Society of Sports Nutrition, 3*(2), 12 – 18.

Hoogeveen, A.R., and Zonderland, M.L. (1996). Relationships between testosterone, cortisol and performance in professional cyclist. *International Journal of Sports Medicine, 17*(6), 423 – 428.

Hollander, J., Fiebig, R., Gore, M., Bejma, J., Ookawara, T., Ohno, H., and Ji, L.L. (1999). Superoxide dismutase gene expression in skeletal muscle: fiber-specific adaptation to endurance training. *Regulatory and Integrative Physiology, 277*(3), R856 – R862.

Horvath, S. (1980). Cytotoxicity of drugs and diverse chemical agents to cell cultures. *Toxicology, 16*(1), 59-66.

Husen, R., Pihie, A. H. L., and Nallappan, M. (2004). Screening for antihyperglycaemic activity in several local herbs of Malaysia. *Journal of Ethnopharmacology, 95*(2-3), 205-208.

Hussein, S., Ibrahim, R., and Kiong, A.L.P. (2006). Adventitious shoots regeneration from root and stem explants of Eurycoma longifolia Jack-an important tropical medicinal plants. *International Journal of Agricultural Research, 1,* 183–193.

Ismail, Z., Ismail, N., and Lassa, J. (1999). Malaysian herbal monograph. *Malaysian Monograph Committee, Kuala Lumpur.*

Ismail, S.B., Wan Mohammad, W.M., George, A., Nik Hussain, N.H., Musthapa Kamal, Z.M., Liske, E. (2012). Randomized clinical trial on the use of PHYSTA freeze-dried water extract of *Eurycoma longifolia* for the improvement of quality of life and sexual well-being in men. *Evidence Based Complement Alternative Medicine, 2012*(429268), 1 - 10.

Itokawa, H., Qin, X.-R., and Morita, H., Takeya K. (1993). C18 and C19 quassinoids from *Eurycoma longifolia*. *Journal of Natural Product, 56*(10), 1766–1771.

Jameson, J.L. and Finlayson, C. (2010). Endocrinology of sexual maturation and puberty. Jameson, J.L. and DeGroot, L.J. (Eds.), *Endocrinology (6th ed.), Saunders Elsevier, Philadelphia, PA*, 2229-2238.

Jiang, M.X., and Zhou, Y.J. (2008). Canthin-6-one alkaloids from *Picrasma quassioides* and their cytotoxic activity. *Journal of Asian Natural Product Research, 10*(11-12), 1009–1012.

Johnson, T.P. (1991). Mental health, social relations, and social selection: A longitudinal analysis. *Journal of Health and Social Behavior, 32*(4), 408–23.

Johnson, J.M., Nachtigall, L.B., and Stern, T.A. (2013). The effect of testosterone levels on mood in men: A review. *Psychosomatics, 54*(6), 509-514.

Jiwajinda, S., Santisopasri, V., and Murakami, A. (2002). In vitro anti-tumor promoting and anti-parasitic activities of the quassinoids from *Eurycoma longifolia*, a medical plant in Southeast Asia. *Journal of Ethnopharmacology. 82*(1), 55 - 58.

Joseph, S., Sugumaran, M., Kate, L., Lee, W. (2005). Herbs of Malaysia. An Introduction to the medicinal, culinary, aromatic and cosmetic use of herbs. *Federal Publications Sdn Berhad, Kuala Lumpur, Malaysia.*

Kallman, D.A., Plato, C.C., and Tobin, J.D. (1990). The role of muscle loss in the age-related decline of grip strength: cross-sectional and longitudinal perspectives. *Journal of Gerontology, 45*(3), M82 – 88.

Karageorghis, C.I. (2017). Applying music in exercise and sport. *Champaign, Illinois: Human Kinetics*.

Kardono, L.B., Angerhofer, C.K., Tsauri, S., Padmawinata, K., Padmawinata, K., Pezzuto, J.M., and Kinghorn, A.D. (1991). Cytotoxic and antimalarial constituents of the roots of *Eurycoma longifolia*. *Journal Natural Products, 54*(5), 1360-1367.

Katznelson, L., Finkelstein, J.S., Schonenfeld, D.A., Rosenthal, D.I., Anderson, E.J., Klibanski, A. (1996). Increase in bone density and lean body mass during testosterone administration in men with acquired hypogonadism. *Journal Clinical Endocrinology and Metabolism, 81*(12), 4358–4365.

Kendig, D.M., and Tarloff, J.B. (2007). Inactivation of lactate dehydrogenase by several chemicals: Implications for in vitro toxicology studies. *Toxicology In Vitro, 21*(1), 125-132.

Khera, M. (2009). Androgens and erectile function: a case for early androgen use in postprostatectomy hypogonadal men. *Journal of Sexual Medicine, 6*(S3), 234–238.

Kim, W., Kim, S.H., Park, S.K., and Chang, M.S. (2012). *Astragalus membranaceus* Ameliorates Reproductive Toxicity Induced by Cyclophosphamide in Male Mice. *Phytotherapy Research, 26*, 1418-1421.

Kim, Y.J., Tamadon, A., Park, H.T., Kim, H., and Ku, S.Y. (2016). The role of sex steroid hormones in the pathophysiology and treatment of sarcopenia. *Osteoporosis and Sarcopenia, 2*(3), 140 – 155.

Kraemer, W.J., and Ratamess, N.A. (2005). Hormonal responses and adaptations to resistance exercise and training. *Sports Medicine, 35*(4), 339–361.

Kumar, P., Nagarajan, A., and Uchil, P.D. (2018). Analysis of cell viability by the lactate dehydrogenase assay. *Cold Spring Harbor Protocol, 2018*(6)

Kuo, P.C., Damu, A.G., Lee, K.H., and Wu, T.S. (2004). Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*. *Bioorganic & Medicinal Chemistry, 12*(3), 537–544.

Kuo, P.C., Shi, L.S., Damu, A.G., Su, C.R., Huang, C.H., Ke, C.H., Wu, J.B., Lin, A.j., Bastow, K.F., and Lee, K.H. (2003). Cytotoxic and antimalarial β-carboline alkaloids from the roots of *Eurycoma longifolia*. *Journal of Natural Products, 66*(10), 1324-1327.

Kunkel, S.D., Suneja, M., Ebert, S.M., Bongers, K.S., Fox, D.K., Malmberg, S.E., Alipour, F., Shields, R.K., and Adams, C.M. (2011). mRNA Expression Signatures of Human Skeletal Muscle Atrophy Identify a Natural Compound that Increases Muscle Mass. *Cell Metabolism, 13*(6), 627 – 638.

Kunkel, S.D., Elmore, C.J., Bonger, K.S., Ebert, S.M., Fox, D.K., Dyle, M.C., Bullard, S.A., and Adams, C.M. (2012). Ursolic acid increases skeletal muscle and brown fat and decreases diet-induced obesity, glucose intolerance and fatty liver disease. *PLoS ONE, 7*(6), 1 – 8.

Korzeniewski, C., and Callewaert D.M. (1983). An enzyme-release assay for natural cytotoxicity. *Journal of Immunological Methods, 64*(3), 313-320.

Kraemer, W.J., Ratamess, N.A., and Volek, J.S. (2006). The effects of amino acid supplementation on hormonal responses to overreaching. *Metabolism, 55*,282–291.

Larsson, L., Grimby, G., and Karlsson, J. (1979). Muscle strength and speed of movement in relation to age and muscle morphology. *Journal of Applied Physiology, 46*(3), 451 – 456.

Laumann, E.O., Paik, A., and Rosen, R.C. (1999). Sexual dysfunction in the United States. Prevalence and predictors. *Journal of the American Medical Association, 281*(6), 537–544.

Lazarus, R.S. (1999). Stress and emotion. A new synthesis. New York: Springer Publishing Company, Incorporated

Leifke, E., Korner, H.C., Link, T.M., Behre, H.M., Peters, P.E., and Nieschlag, E. (1998). Effects of testosterone replacement therapy on cortical and trabecular bone mineral density, vertebral body area and paraspinal muscle area in hypogonadal men. *European Journal of Endocrinology, 138*(1), 51–58.

Lemon, P.W.R., Tarnopolsky, M.A., MacDougal, J.D., Atkinson, S.A. (1992). Protein requirements and muscle mass/strength changes during intensive training in novice bodybuilders. *Journal of Applied Physiology, 73,* 767–775.

Lewis, M.I., Horvitz, G.D., Clemmons, D.R., and Fournier, M. (2002). Role of IGF-I and IGF-binding proteins within diaphragm muscle in modulating the effects of nandrolone. *American Journal Physiology Endocrinology and Metabolism, 282,* E483–E490.

Li, C. H., Liao, J. W., Liao, P. L., Huang, W. K., Tse, L. S., Lin, C. H., Kang, J.H., and Cheng, Y. W. (2013). Evaluation of acute 13-week subchronic toxicity and genotoxicity of the powdererd root of Tongkat Ali (*Eurycoma longifolia* Jack). *Evidence-Based Complementary and Alternative Medicine, 2013*(2013), 1-11.

Li, W., Zhou, J., and Xu, Y. (2015). Study of the *in vitro* cytotoxicity testing of medical devices. *Biomedical reports, 3*(5), 617-620.

Liu, C.C., Lee, Y.C., Tsai, V.F.S., Cheng, K.H., Wu, W.J., Bao, B.Y., Huang, C.N., Yeh, H.C., Tsai, C.C., Wang, C.J., and Huang, S.P. (2015). The interaction of serum testosterone levels and androgen receptor CAG repeat polymorphism on the risk of erectile dysfunction in aging Taiwanese men. *Andrology, 3*, 902 – 908.

Litman, H.J., Bhasin, S., Link, C.L., Araujo, A.B., and McKinlay, J.B. (2006). Serum androgen levels in black, Hispanic, and white men. *The Journal of Clinical Endocrinology & Metabolism, 91*(11), 4326-4334.

Lin, L.C., Peng, C.Y., Wang, H.S., Lee, K.W., and Wang, P.S. (2001). Reinvestigation of the chemical constituents of *Eurycoma longifolia*. *The Chinese Pharmaceutical Journal, 53*(2), 97–106.

Linnamo, V., Pakarinen, A., Komi, P.V., Kraemer, W.J., and Hakkinen, K. (2005). Acute hormonal response to submaximal and maximal heavy resistance and explosive exercise in men and women. *Journal of Strength and Conditioning Research, 19*(3), 566–571.

Litwin, M.S., Nied, R.J., and Dhanani, N. (1998). Health-related quality of life in men with erectile dysfunction. *Journal of General Internal Medicine, 13*(3), 159–166.

Liverman, C.T., and Blazer, D.G. (2004). Testosterone and health outcomes. In Liverman, C.T., and Blazer, D.G. (Eds), *Testosterone and Aging: Clinical Research Directions.*Washington, DC: National Academies Press (US).

Lopresti, A.L., Smith, S.J., Malvi, H., and Kodgule, R. (2019). An investigation into the stress-relieving and pharmacological actions of an ashwagandha (Withania somnifera) extract: A randomized, double-blind, placebo-controlled study. *Medicine, 98*(37).

Low, B.S., Ng, B.H., Choy, W.P., Yuen, K.H., and Chan K.L. (2005). Bioavailability and pharmacokinetic studies of eurycomanone from *Eurycoma longifolia*. *Planta Medica, 71*(9), 803–807.

Low, B.S., Teh, C.H., Yuen, K.H., and Chan, K.L. (2011). Physico-chemical effects of the major quassinoids in a standardized *Eurycoma longifolia* extract (Fr 2) on the bioavailability and pharmacokinetic properties, and their implications for oral antimalarial activity. *Natural Product Communications, 6*(3), 337–341.

Low, B. S., Choi, S. B., Abdul Wahab, H., Das, P. K., and Chan, K. L. (2013a). Eurycomanone, the major quassinoid in *Eurycoma longifolia* root extract increases spermatogenesis by inhibiting the activity of phosphodiesterase and aromatase in steroidogenesis. *Journal of Ethnopharmacology, 149*(1), 201-207.

Low, B.S., Das, P.K., and Chan, K.L. (2013b). Standardized quassinoid-rich Eurycoma longifolia extract improved spermatogenesis and fertility in male rats via the hypothalamic-pituitary-gonadal axis. *Journal of Ethnopharmacology, 145*(3), 706-714.

Ma, H.Q., Ebrahimi, F., Khan, N.A.K., and Chan, K.L. (2015). Investigating the double-peak phenomenon in oral pharmacokinetics of *Eurycoma longifolia* quassinoids in lipid-based solid dispersion. *Journal of Bioequivalence Studies, 1*, 1 – 10.

Majzoub, A., and Sabanegh, E. (2016). Testosterone replacement in the infertile man. *Translational Andrology and Urology, 5*(6), 859-865.

Mahfudh, N., and Azimahtol, H.L.P. (2006). Eurycomanone exerts antiproliferative activity via apoptosis in HeLa cells. *International Conference on Mathematics and Natural Sciences.*

Mahfudh, N., and Pihie, A.H.L. (2008). Eurycomanone induces apoptosis through the up-regulation of p53 in human cervical carcinoma cells. *Journal of Cancer Molecules, 4*(4), 109 - 115.

Malaysian Standard. (2011). Phytopharmaceutical aspects of freeze dried water extract from Tongkat Ali roots-specification. *Jabatan Standard Malaysia.* 12-13.

Martini, L. (1983). Androgen metabolism in the brain and in the anterior pituitary: role in the feedback control of gonadotropin secretion. In A.S. Bhatnagar (Ed), *The Anterior Pituitary Gland*. (pp. 73 – 82). Raven Press, New York.

Matheny, R.W. Jr., Nindl, B.C., and Adamo, M.L. (2010). Minireview: Mechano-Growth Factor: A Putative Product of IGF-I Gene Expression Involved in Tissue Repair and Regeneration. *Endocrinology, 151*(3), 865–875.

Maughan, R. J., Depiesse, F., and Geyer, H. (2007). The use of dietary supplements by athletes. *Journal of Sports Sciences, 25*(Suppl 1), S103-S113.

Maziah, M., and Rosli, N. (2009). The Production of 9-methoxycanthin-6-one from callus cultures of (*Eurycoma longifolia* Jack) Tongkat Ali. In: (Methods in Molecular Biology) Saxena P.K., Jain S.M., editors. Protocols for in vitro cultures and secondary metabolite analysis of aromatic and medicinal plants. Volume 547. *Humana Press; New York, NY, USA, 547*, 359–369.

McArdle, W.D., Katch, F.I., and Katch, V.L. (2007). Exercise physiology: Energy, nutrition, and human performance. 6th ed., *Lippincott William & Wilkins, USA*.

McCaulley, G.O., McBridge, J.M., Cormie, P., Hudson, M.B., Nuzzo, J.L., Quindry, N.T., and Toiplett, N.T. (2009). Acute hormonal and neuromuscular responses to hypertrophy, strength and power type resistance exercise. *European Journal of Applied Physiology*, *105*(5), 695–704.

McGaw, L.J., Elgorashi, E.E., and Elof, J.N. (2014). Cytotoxicity of African Medicinal Plants Against Normal Animal and Human Cells. *Toxicological Survey of African Medicinal Plants*, 181 – 233.

McMillan, J.H. (2007). Randomized field trials and internal validity: not so fast my friend. *Practical, Assessment and Evaluation, 12*(15), 1- 6.

Meng, D., Li, X., Han, L., Zhang, L., An, W., and Li, X. (2014). Four new quassinoids from the roots of *Eurycoma longifolia* Jack. *Fitoterapia, 92*, 105–110.

Milanović, Z., Pantelić, S., Trajković, N., Sporiš, G., Kostić, R., and James, N. (2013). Age-related decrease in physical activity and functional fitness among elderly men and women. *Clinical Interventions in Aging, 8*, 549 – 556.

Miller, K.K. (2009). Androgen deficiency: effects on body composition. *Pituitary, 12*(2), 116–24.

Miloski, B., Marcelo, A., Camila, G.D.F., Ademir, F.S.D.A., Helena, S.D.M., Gustavo, D., Thiago, O.B., and Alexandre, M. (2015). Does testosterone modulate mood states and physical performance in young basketball players? *The Journal of Strength and Conditioning Research, 29*(9), 2474 – 2481.

Mitchell, K.R., Geary, R., Graham, C., Clifton, S., Mercer, C. H., Lewis, R., Macdowall, W., Datta, J., Johnson, A.M., and Wellings, K. (2016). Sexual function in 16 to 21-year-olds in Britain. *Journal of Adolescent Health*, *59*(4), 422–428.

Mitsunaga, K., Koike, K., Tanaka, T., Ohkawa, Y., Kobayashi, Y., Sawaguchi, T., and Ohmoto, T. (1994). Canthin-6-one alkaloids from *Eurycoma longifolia*. *Phytochemistry, 35*(3), 799 - 802.

Miyake, K., Tezuka, Y., Awale, S., Li, F. and Kadota, S. (2009). Quassinoids from *Eurycoma longifolia*. *Journal of Natural Product, 72*, 2135–2140.

Miyake, K., Tezuka, Y., Awale, S., Li, F., and Kadota, S. (2010). Canthin-6-one alkaloids and a tirucallanoid from *Eurycoma longifolia* and their cytotoxic activity against a human HT-1080 fibrosarcoma cell line. *Natural Product Communications, 5*(1), 17–22.

Morgan, W.P. (1980). The trait psychology controversy. *Research Quarterly for Exercise Sport, 51*(1), 50–76

Morita, H., Kishi, E., Takeya, K., Itokawa, H., and Tanaka, O. (1990). New quassinoids from the roots of *Eurycoma longifolia*. *Chemistry Letters, 19*(5). 749–752.

Morita, H., Kishi, E., Takeya, K., and Itokawa, H. (1992). Biphenylneolignans from wood of *Eurycoma longifolia*. *Phytochemistry, 31*(11), 3993 - 3995.

Morita, H., Kishi, E., Takeya, K., Itokawa, H., and Iitaka, Y. (1993). Highly oxygenated quassinoids from *Eurycoma longifolia*. *Phytochemistry, 33*(3), 691–696.

Morimoto, Y., Iwai, T., Yoshimura, T., and Kinoshita, T. (1998). Diastereoselective two-directional synthesis and cation transport ability of the central tristetrahydrofuranyl unit of meso polyether glabrescol as naturally occurring podand. *Bioorganic & Medicinal Chemistry Letters, 8*(15), 2005–2010.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*(1-2), 55-63.

Mouser, J.G., Loprinzi, P.D., and Loenneke, J.P. (2016). The association between physiologic testosterone levels, lean mass, and fat mass in a nationally representative sample of men in the United State. *Steroids, 115*, 62 – 66.

Moore, D.R., Robinson, M.J., Fry, J.L., Tang, J.E., Glover, E.I., Wilkinson, S.B., Prior, T., Tarnopolsky, M.A., and Phillips, S.M. (2009). Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *American Journal of Clinical Nutrition*, *89*(1), 161–168.

Muhamad, A.S., Chen, C.K., Ooi, F.K., Abdullah, M.R., and Lam, C.T. (2010). Effects of *Eurycoma longifolia* Jack supplementation on recreational athletes' endurance running capacity and physiological responses in the heat. *International Journal of Aeronautical and Space Sciences*, *22*(2), 1–19.

Mulligan, T., Iranmanesh, A.m Johnson, M.L., Staume, M., and Veldhuis, J.D. (1997). Aging alters feed-forward and feedback linkages between LH and testosterone in healthy men. *Regulatory and Integrative Physiology, 273*(4), R1407-R1413.

Mujika, I., Chatard, J.C., and Geyssant, A. (1996). Effects of training and taper on blood leucocyte populations in competitive swimmers: relationships with cortisol and performance. *International Journal of Sports Medicine, 19*, 213-217.

Mykletun, A., Dahl, A.A., O'Leary, M.P., and Fossa, S.D. (2005). Assessment of male sexual function by the Brief Sexual Function Inventory. *BJU International, 97*, 316 – 323.

Naftolin, F., Ryan, K. J., Davies, I. J., Reddy, V. V., Flores, F., Petro, Z., and Kuhn, M. (1975). The formation of estrogens by central neuroendocrine tissue. *Recent Progress in Hormone Research, 31*, 295-319.

Naughton, G., Farpour-Lambert, N.J., Carlson, J., Bradney, M., and Praagh, E.V. (2000). Physiological issues surrounding the performance of adolescent athletes. *Sports Medicine. 30*, 309-325.

Nugent, E.P., Snodgrass, S.J., and Callister, R. (2015). The effect of velocity and familiarization on the reproducibility of isokinetic dynamometer. *Isokinetics and Exercise Science, 23*(2015), 205 – 214.

Nurhanan, M.Y., Azimahtol, H.L.P., Mohd Ilham, A., and Mohd Shukri, M.A. (2005). Cytotoxic effects of the root extract of *Eurycoma longifolia* Jack, *Phytotherapy Research, 19*(11), 994 - 996.

Norhidayah, A., Vejayan, J., and Yusoff, M. (2015). Detection and quantification of eurycomanone levels in Tongkat Ali herbal products. *Journal of Applied Sciences, 15*(7)**,** 999-1005.

O'Connor, D.B., Archer, J., Hair, W.H., and Wu, F.C.W. (2001). Exogenous testosterone, aggression, and mood in eugonadal and hypogonadal men. *Physical Behavior, 75*,557-566.

O'Connor, D.B., Archer, J., and Wu, F.C. (2004). Effects of testosterone on mood, aggression, and sexual behaviour in young men: a double-blind, placebo-controlled, cross-over study. *Journal of Clinical Endocrinology and Metabolism, 89*(6), 2837 – 2845.

O'Hara, L., Curley, M., Ferreira, M.T., Cruickshanks, L., Milne, L., and Smith, L.B. (2015). Pituitary androgen receptor signalling regulates prolactin but not gonadotrophins in the male mouse. *PLoS ONE, 10*(3), 1-18.

O'Leary, M.P., Fowler, F.J., Lenderking, W.R., Barber, B., Sagnier, P.P., Guess, H.A., and Barry, M.J. (1995). A brief male sexual function inventory for urology. *Urology, 46*(5), 697 – 706.

Ooi, F.K., Singh, R., Sirisinghe, R.G., Suen, A.B., Jamalullail, S.M.S. (2001). Effects of a herbal ergogenic drink on cycling performance in young cyclists. *Malaysia Journal Nutrition, 7*(1&2), 33-40.

Ooi, F.K., Singh, R., Sirisinghe, R.G., Suen, A.B., and Jamalullail, S.M.S. (2003). Effects of a herbal drink on cycling endurance performance. *The Malaysian Journal of Medicine Sciences, 10*(1), 78 – 85.

Ooi, F.K., Afifah, M.H., Chen, C.K., and Asnizam, A.M. (2015). Combined effects of *Eurycoma longifolia* Jack supplementation and a circuit training programme on bone metabolism markers, muscular strength and power and immune functions in adult men. *International Journal of Engineering Research and Sports Science, 2*(3), 1 - 10.

O'Sullivan,L. F., Byers, E. S., Brotto, L. A., Majerovich, J. A., and Fletcher, J. (2016). A longitudinal study of problems in sexual functioning and related sexual distress among middle to late adolescents. *Journal of Adolescent Health, 59*(3), 318–324.

Page, S.T., Amory, J.K., Bowman, F.D., Anawlt, B.T., Matsumoto, A.M., Bremner, W.J., and Tenover, J.L. (2005). Exogenous testosterone (T) alone or with finasteride increases physical performance, grip strength, and lean body mass in older men with low serum T. *Journal of Clinical Endocrinology and Metabolism, 90*(3),1502–1510.

Palaparthi, S. (2017). Role of Homeostasis in Human Physiology: A review. *Journal of Medical Physiology & Therapeutics, 1*(2), 1-5.

Panossian, A.G., and Wagner, H. (2005). Stimulating effect of adaptogens: An overview with particular reference to their efficacy following single dose administration. *Phytotherapy Research, 19*(10), 819 – 838.

Park, S., Nhiem, N.X., Van Kiem, P., Van Minh, C., Tai, B.H., Kim, N., Yoo, H.H. Song, J.H., Ko, H.J., and Kim, S.H. (2014). Five new quassinoids and cytotoxic constituents from the roots of *Eurycoma longifolia*. *Bioorganic & medicinal chemistry letters, 24*(16), 3835-3840.

Pascoe, M.C., Hetrick, S.E., and Parker, A.G. (2019). The impact of stress on students in secondary school and higher education. *International Journal of Adolescence and Youth*, 1-9

Patel, V., Flisher, A.J., Hetrick, S., McGorry, P. (2007). Mental health of young people: a global public-health challenge. *Lancet. 369*(9569):1302–13.

Pronsato, L., La Colla, A., Ronda, A.C., Milanesi, L., Boland, R., and Vasconsuelo, A. (2013). High passage numbers induce resistance to apoptosis in C2C12 muscle cell. *Biocell, 37*(1), 1-9.

Poole, C., Bushey, B., Foster, C., Campbell, B., Willoughby, D., Kreider, R., Taylor, L., Wilborn, C. (2010). The effects of a commercially available botanical supplements on strength, body composition, power output, and hormonal profiles in resistance-trained males. *Journal of the International Society of Sports Nutrition, 7*(34), 1-9.

Pope, H.G.Jr., Amiaz, R., Brennan, B.P., Orr, G., Weiser, M., Kelly, J.F., Kanayama, G., Siegel, A., Hudson, J.I., and Seidman, S.N. (2010). Parallel-group placebo-controlled trial of testosterone gel in men with major depressive disorder displaying an incomplete response to standard antidepressant treatment. *Journal of Clinical Psychopharmacology, 30*(2), 126 –134

Qader, S.W., Abdulla, M.A., Chua, L.S., and Hamdan, S. (2012). Potential bioactive property of *Polygonum minus* Huds (kesum) review. *Scientific Research and Essays, 7*(2), 90 – 93.

Qureshi, A., Naughton, D.P., and Petroczi, A. (2014). A systematic review on the herbal extract *Tribulus terrestris* and the roots of its putative aphrodisiac and performance enhancing effect. *Journal of Dietary Supplements, 11*(1), 64-79.

Quinn, M., Drummond, R.J., Ross, F., Murray, J., and Macdonald, A. (2010). Short course pre-operative ferrous sulphate supplementation—is it worthwhile in patients with colorectal cancer? *Annals of the Royal College of Surgeons of England, 92*(7), 569-572.

Razak, M.F.A., and Aidoo, K.E. (2011). Toxicity studies of *Eurycoma longifolia* (Jack)-Based remedial products. *Asian Journal of Pharmaceutical and Clinical Research, 4*(3), 23 - 27.

Ratamess, N.A., Kraemer, W.J., and Volek, J.S. (2003). The effects of amino acid supplementation on muscular performance during resistance training overreaching. *Journal of Strength and Conditioning Research, 17*, 250–258.

Rehman, S.U., Choe, K., and Yoo, H.H. (2016). Review on a traditional herbal medicine, *Eurycoma longifolia* Jack (Tongkat Ali): Its traditional uses, chemistry, evidence-based pharmacology and toxicology. *Molecules, 21*(3), 1 – 31.

Rehman, S.U., Choi, M.S., Han, Y.M., Kim, S.H., Piao, X.L., and Yoo, H.H. (2017). Determination of eurycomanone in rat plasma using hydrophilic interaction liquid chromatography-tandem mass spectrometry for pharmacokinetic study. *Biomedical Chromatography, 31*(4), 1 – 8.

Rivest, S., and Rivier, C. (1995). The role of corticotropin-releasing factor and interleukin-1 in the regulation of neurons controlling reproductive functions. *Endocrine Review, 16*(2), 177–99.

Rockafellow, B.D., and Saules, K.K. (2006). Substance use by college students: the role of intrinsic versus extrinsic motivation for athletic involvement. *Psychology of Addictive Behaviors, 20*(3), 279 – 287.

Roy, B.D., Fowles, J.R., Hill, R., and Tarnopolsky, M.A. (2000). Macronutrient intake and whole body protein metabolism following resistance exercise. *Medicine Science in Sports and Exercise, 32*(8), 1412–1418.

Rubinstein, L., Shoemaker, R., Paull, K., Simon, R., Tosini, S., Skehan, P., Scudiero, D., Monks, A., and Boyd, M. (1990). Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *Journal of the National Cancer Institute, 82*(13), 1113-1118.

Roehm, N.W., Rodgers, G.H., Hatfield, S.M., and Glasebrook, A.L. (1991). An improved colorimeter assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *Journal of Immunological Methods, 142*(2), 257-265.

Rogerson, S., Riches, C.J., Jennings, C., Weatherby, R.P., Meir, R.A., Marshall-Gradisnik, S.M. (2007). The effect of five weeks of *Tribulus terrestris* supplementation on muscle strength and body composition during preseason training in elite rugby league players. *Journal of Strength and Conditioning Research, 21*(2), 348-353.

Rosner, W., Auchus, R.J., Azziz, R., Sluss, P.M., and Raff, H. (2007). Position statement: utility, limitations, and pitfalls in measuring testosterone: an Endocrine Society position statement. *Journal of Clinical Endocrinology and Metabolism, 92(2), 4*05-413.

Ryan, C. P., and Nathan, A. B. (2017). Testosterone therapy: Review of clinical applications. *American Family Physician, 96*(7), 441 – 449.

Sambandan, T.G., Rha, C., Kadir, A.A., Aminudim, N., and Saad, J.M. (2004). Bioactive fraction of *Eurycoma longifolia*, US Patent Application Publication, US 2004/0087493 A1.

Sambandan, T.G., Rha, C., Aminudim, N., Saad, J.M., and Kadir, A.A. (2006). Bioactive fraction of *Eurycoma longifolia*. Patent number: WO0217946; Other reference

numbers: JP2004521075, US2004087493, EP1313491, DK1313491, DE60125955.

Sarina, M.Y, Zaiton, Z., Aminudin, A.H.K., Nor, A.K. and Azizol, A.K. (2009). Effects of resistance training and *Eurycoma longifolia* on muscle strength, lipid profile, blood glucose, and hormone level in middle-aged women. In 4th Asia-Pacific Conference on Exercise and Sport Science & 8th International Sports Science Conference. *Academy of Medicine of Malaysia*.

Sandi, C., and Haller, J. (2015). Stress and the social brain: behavioural effects and neurobiological mechanisms. *Nature Reviews Neuroscience, 16*(5), 290–304.

Satayavivad, J., Noppamas, S., Aimon, S., and Yodhathai T. (1998). Toxicological and antimalaria activity of *Eurycoma longifolia* Jack extracts in mice. *Thailand Journal of Phytopharmacology, 5*, 14–27.

Schiffer, L., Barnard, L., Baranowski, E.S., Gilligan, L.C., Taylor, A.E., Arlt, W., Shackleton, C.H.L., and Storbeck, K.H. (2019). Human steroid biosynthesis, metabolism and excretion are differentially reflected by serum and urine steroid metabolomes: A comprehensive review. *Journal of Steroid Biochemistry and Molecular Biology, 194*(105439), 1 – 26.

Schrader, E. (2000). Equivalence of St John's wort extract (Ze 117) and fluoxetine: a randomized, controlled study in mild-moderate depression. *International Clinical Psychopharmacology, 15*(2), 61– 68.

Schöneich, C., Dremina, E., Galeva, N., and Sharov, V. (2014). Apoptosis in differentiating C2C12 muscle cells selectively targets Bcl-2-deficient myotubes. *Apoptosis, 19*(1), 42–57.

Scofield, D.E., and Unruh, S. (2006). Dietary supplement use among adolescent athletes in central Nebraska and their sources of information. *Journal of Strength and Conditioning Research, 20*(2), 452-455.

Seidman, S.N., Spatz, E., Rizzo, C., and Roose, S.P. (2001). Testosterone replacement therapy for hypogonadal men with major depressive disorder: A randomized, placebo-controlled clinical trial. *The Journal of Clinical Psychiatry, 62*(6), 406 – 412.

Seidman, S.N. (2007). Androgens and the aging male. *Psychopharmacology Bulletin, 40*(4), 205–218.

Sellami, M., Slimeni, O., Pokrywka, A., Kuvačić, G., Hayes, L.D., Milic, M., and Padulo, J. (2018). Herbal medicine for sports: a review. *Journal of the International Society of Sports Nutrition, 15*(14), 1 – 14.

Shabsigh, R., Klein, L.T., Seidman, S., Kaplan, S.A., Lehrhoff, B.J., and Ritter, J.S. (1998). Increased incidence of depressive symptoms in men with erectile dysfunction. *Urology, 52*(5), 848– 852.

Sharples, A.P., Al-Shanti, N., Lewis, M.P., and Stewart, C.E. (2011). Reduction of myoblast differentiation following multiple population doublings in mouse C2 C12

cells: a model to investigate ageing? *Journal of Cellular Biochemistry, 112*(12), 3773-3785.

Sher, L., Grunebaum, M.F., Sullivan, G.M., Burke, A.K., Cooper, T.B., Mann, J.J., and Oquendo, M.A. (2012). Testosterone levels in suicide attempters with bipolar disorder. *Journal of Psychiatric Research, 46*(10), 1267 – 1271.

Sherman, G. D., Lerner, J. S., Josephs, R. A., Renshon, J., and Gross, J. J. (2016). The interaction of testosterone and cortisol is associated with attained status in male executives. *Journal of Personality and Social Psychology, 110*(6), 921–929.

Shier, D., Butler, J., Lewis, R. (2012). "Adrenal Glands." Hole's Human Anatomy & Physiology. 12th ed. *New York: McGraw-Hill*, 504-508

Shuid, A. N., Abu Bakar, M. F., Abdul Shukor, T. A., Muhammad, N., Mohamed, N., and Soelaiman, I. N. (2011). The anti-osteoporotic effect of *Eurycoma Longifolia* in aged orchidectomised rat model. *The Aging Male, 14*(3), 150-154.

Shukla, K.K., Mahdi, A.A., Ahmad, M.K., Shankhwar, S.N., Rajender, S., and Jaiswar, S.P. (2009). Mucuna pruriens improves male fertility by its action on the hypothalamus-pituitary-gonadal axis. *Fertility and Sterility, 92*(6), 1934-1940.

Shores, M.M., Kivlahan, D.R., Sadak, T.I., Li, E.J., (2009). Matsumoto, A.M. A randomized, double-blind, placebo-controlled study of testosterone treatment in hypogonadal older men with subthreshold depression (dysthymia or minor depression). *The Journal of Clinical Psychiatry, 70*(7), 1009–1016.

Siegel, S. (2005). Drug tolerance, drug addiction, and drug anticipation. *Current Directions in Psychological Science, 14*(6).

Sifferlin, A. (2013). The most stressed out generation. Time Magazine. Retrieved from http://healthland.time.com/2013/02/07/the-most-stressed-out-generation-youngadults.

Sinha-Hikim, I., Artaza, J., Woodhouse, L., Gonzalez-Cadavid, N., Singh, A.B., Lee, M.I., Storer, T.W., Casaburi, R., Shen, R., and Bhasin, S. (2002). Testosterone-induced increase in muscle size in healthy young men is associated with muscle fiber hypertrophy. *American Journal of Physiology, Endocrinology & Metabolism, 283*(1), E154–E164.

Siregar L.A.M., Peng-Lim, B., and Lai-Keng, C. (2004). Effect of cell source and pH of culture medium on the production of canthin-6-one alkaloids from the cell cultures of Tongkat Ali (*Eurycoma longifolia* Jack). *Journal of Plant Biotechnology, 6*(2), 125–130.

Siregar, L.A.M., Keng, C.L., and Lim, B.P. (2009). Effects of medium constituents on growth and canthinone accumulation in cell suspension cultures of *Eurycoma longifolia* Jack. *HAYATI Journal of Biosciences, 16*(2), 69–77.

Simó, R., Sáez-López, C., Barbosa-Desongles, A., Hernández, C., and Selva, D.M. (2015). Novel insights in SHBG regulation and clinical implications. *Trends in Endocrinology and Metabolism, 26*(7), 1 – 8.

Storer, T.W., Woodhouse, L., Magliano, L., Singh, A.B., Dzekov, C., Dzekov, J., and Bhasin, S. (2008). Changes in muscle mass, muscle strength, and power but not physical function are related to testosterone dose in healthy older men. *Journal of the American Geriatrics Society, 56*(11), 1991 – 1999.

Sobal, J., and Marquart, L. F. (1994). Vitamin/mineral supplement use among athletes: a review of the literature. *International Journal of Sport Nutrition, 4*(4), 320-334.

Souza-Almeida, E.S., Niero, R., Clasen, B.K., Balogun, S.O., and Oliveira-Martins, D.T. (2011). Pharmacological mechanisms underlying the anti-ulcer activity of methanol extract and canthin-6-one of *Simaba ferruginea* A. St-Hil. in animal models. *Journal of Ethnopharmacology, 134*(3), 630–636.

Specian, A.F.L., Serpeloni, J.M., Tuttis, K., Ribeiro, D.L., Ciliao, H.L., Varanda, E.A., Sannomiya, M., Martinez-Lopez, Vilegas, W., Vilegas, W., and Colus, M.S. (2016). LDH, proliferation curves and cell cycle analysis are the most suitable assays to identify and characterized new phytoterapeutic compound. *Cytotechnology, 68*, 2729-2744.

Stanković, A., Đorđević-Nikić, M., Kukić, F., Petrović, M., Cvijanović, N., and Todorović, N. (2013). The effect of strength training on the testosterone level in men. *Fizička Kultura, 67*(2), 157-166.

Stanworth, R. D., and Jones, T. H. (2008). Testosterone for the aging male; current evidence and recommended practice. *Clinical Interventions in Aging, 3*(1), 25-44.

Steidle, C., Schwartz, S., Jacoby, K., Sebree, T., Smith, T., Bachand, R. (2003). AA2500 testosterone gel normalizes androgen levels in aging males with improvements in body composition and sexual function. *The Journal of Clinical Endocrinology and Metabolism, 88*(6), 2673 – 2681.

Steels, E., Rao, A., and Vitetta, L. (2011). Physiological aspects of male libido enhanced by standardized Trigonella foenum-graucum extract and mineral formulation. *Phytotherapy Research, 25*(9), 1294-1300.

Sternbach, H. (1998). Age-associated testosterone decline in men: clinical issues for psychiatry. *The American Journal of Psychiatry, 155*(10), 1310–1318.

Storrie, K., Ahern, K., and Tuckett, A. (2010). A systematic review: students with mental health problems—a growing problem. *International Journal of Nursing Practice, 16*(1), 1–6.

Schulz, P., Walker, J.P., Peyrin, L., Soulier, V., Curtin, F., and Steimer, T. (1996). Lower sex hormones in men during anticipatory stress. *Neuroreport, 7*, 3101–3104.

Smith, K., Barua, J.M., Watt, P.W., Scrimgeour, C.M., and Rennie, M.J. (1992). Flooding with L-[1-13C] leucine stimulates human muscle protein incorporation of continuously infused L-[1-13 C]valine. *American Journal Physiology Endocrinology and Metabolism, 262*, E372–E376.

Snyder, P.J., Peachey, H., Berlin, J.A., Hannoush, P., Haddad, G., Dlewati, A., Santanna, J., Loh, L., Lenrow, D.A., Holmes, J.H., Kapoor, S.C., Atkinson, L.E., and Strom, B.L. (2000). Effects of testosterone replacement in hypogonadal men. *Journal Clinical Endocrinology Metabolism*, 85(8), 2670–2677.

Tada, H., Yasuda, F., Otani, K., Doteuchi, M., Ishihara, Y., Shiro, M. (1991). New antiulcer quassinoids from *Eurycoma longifolia*. *European Journal of Medicinal Chemistry, 26*(3), 345–349.

Talbott, S., Talbott, J., Negrete, J., Jones, M., Nichols, M., and Roza, J. (2006). Effect of *eurycoma longifolia* extract on anabolic balance during endurance exercise [abstract]. *Journal of International Society Sports Nutrition, 3*(1), S32.

Talbott, S.M., Talbott, J.A., George, A., and Pugh, M. (2013). Effect of Tongkat Ali on stress hormones and psychological mood state in moderately stressed subjects. *Journal of the International Society of Sports Nutrition, 10*(28), 1-7.

Talih, F., Fattal, O., and Malone, D. Jr. (2007). Anabolic steroid abuse: psychiatric and physical costs. *Cleveland Clinic Journal of Medicine, 74*(5), 341–344, 346, 349–352.

Tambi, M.I. (2003). Glycoprotein water-soluble extract of *Eurycoma longifolia* Jack as a health supplement in management of healthy aging in aged men. *The Aging Male*, (1), 41–70.

Tambi, M.I. (2007). Standardized water soluble extract of *Eurycoma longifolia* maintains healthy aging in man. *The Aging Male. 10*(2), 77–87.

Tambi, M.I.M. (2005). Standardised water-soluble extract of *Eurycoma longifolia* (LJ100) on men's health. *International Journal of Andrology, 28*(Suppl. 1), 27.

Tambi, M.I. (2009). Nutrients and botanicals for optimizing men's health. Examining the evidence for *Eurycoma longifolia* Jack, the Malaysian Ginseng in men. *Asian Journal of Andrology, 11*(Suppl. 5), 37–38.

Tambi, M.I., Imran, M.K., and Henkel, R.R. (2012). Standardised water-soluble extract of *Eurycoma longifolia*, Tongkat Ali, as testosterone booster for managing men with late-onset hypogonadism? *Andrologia, 44*(S1), 226–230.

Tambi, M.I., and Imran, M.K. (2010). *Eurycoma longifolia* Jack in managing idiopathic male infertility. *Asian Journal of Andrology, 12*(3), 376 – 380.

Tambi, M.I., and Kadir, A.A. (2005). Human toxicology and clinical observations of *Eurycoma longifolia* on men's health. *International Journal of Andrology, 28*(Suppl 1), 37-38.

Tarnopolsky, M.A., Atkinson, S.A., and MacDougal, J.D. (1992). Evaluation of protein requirements for trained strength athletes. *Journal of Applied Physiology, 73*, 1986–1995.

Tee, T.T., and Azimahtol, H.L.P. (2005). Induction of apoptosis by *Eurycoma longifolia* Jack extracts. *Anticancer research, 25*(3B), 2205-2213.

Terry, P.C., Lane, A.M., Lane, H.J., and Keohane, L. (1999). Development and validation of a mood measure for adolescents. *Journal of Sports Sciences, 17,* 861-872.

Terry, P.C., Lane, A.M., and Fogarty, G.J. (2003). Construct validity of the Profile of Mood States – Adolescents for use with adults. *Psychology of Sport and Exercise, 4*(2), 125 – 139.

Thu, H.E., Mohamed, I.N., Hussain, Z., and Shuid, A.N. (2017). *Eurycoma longifolia* as a potential alternative to testosterone for the treatment of osteoporosis: Exploring time-mannered proliferative, differentiative and morphogenic modulation in osteoblasts. *Journal of Ethnopharmacology, 195*(2017), 143-158.

Thu, H.E., Hussain, Z., Mohamed, I.N., and Shuid, A.N. (2018). Exploring dynamic biomedical algorithm of *Eurycoma longifolia* Jack and its bioactive phytochemicals: A review of pharmacokinetic and pharmacodynamic implication and future prospects. *Asian Pacific Journal of Tropical Medicine, 11*(2), 89 - 97.

Tilbrook, A.J., and Clarke, I.J. (2001). Negative feedback regulation of the secretion and actions of gonadotropin-releasing hormone in males. *Biology of Reproduction, 64*(3), 735-742.

Tong, K.T., Chan, K.L., AbuBakar, S., Low, B.S., Ma, H.Q., and Wong, P.F. (2015). The in vitro and in vivo anti-cancer activities of a standardized quassinoids composition from *Eurycoma longifolia* on LNCaP human prostate cancer cells. *PLoS ONE, 10*(3), 1-21.

Tran, T.V.A., Malainer, C., Schwaiger, S., Atanasov, A.G., Heiss, E.H., Dirsch, V.M., and Stuppner, H. (2014). NF-κB inhibitors from *Eurycoma longifolia*. *Journal of Natural Products, 77*(3), 483-488.

Traish, A.M., Goldstein, I. and Kim, N.N. (2007). Testosterone and erectile function: from basic research to a new clinical paradigm for managing men with androgen insufficiency and erectile dysfunction. *European Urology, 52*(1), 54–70.

Traish, A.M., Miner, M.M., Morgentaler, A., and Zitzmann, M. (2011). Testosterone deficiency. *The American Journal of Medicine, 124*(7), 578–87.

Tyagi, V., Scordo, M., Yoon, R.S., Liporace, F.A., and Greene, L.W. (2017). Revisiting the role of testosterone: Are we missing something? *Reviews in Urology, 19*(1), 16-24.

Udani, J.K., George, A.A., Musthapa, M., Pakdaman, M.N., and Abas, A. (2014). Effects of a proprietary freeze-dried water extract of *Eurycoma longifolia* (Physta) and *Polygonum minus* on sexual performance and well-being in men: A randomized, double-blind, placebo-controlled study. *Evidence-Based Complementary and Alternative Medicine, 2014*, 1 - 10.

Ullah, M.I., Riche, D.M., and Koch, C.A. (2014). Transdermal testosterone replacement therapy in men. *Drug Design, Development and Therapy, 8*, 101-112.

Urban, R.J., Bodenburg, Y.H., Gilkison, C., Foxworth, J., Coggan, A.R., Wolfe, R.R., and Ferrando A. (1995). Testosterone administration to elderly men increases skeletal muscle strength and protein synthesis. *American Journal of Physiology, 269*(5 Pt 1), E820–E826.

U.S. Food and Drug Administration. Joint meeting for Bone, Reproductive and Urologic Drugs Advisory Committee (BRUDAC) and the Drug Safety and Risk Management Advisory Committee (DSARM AC). September 17, 2014.

Vajrabhaya, L.O., and Korsuwannawong, S. (2018). Cytotoxicity evaluation of a Thai herb using tetrazolium (MTT) and sulforhodamine B (SRB) assays. *Journal of Analytical Science and Technology, 9*(15), 1 - 6.

Varghese, C., Ambrose, C., Jin, S., Lim, Y., and Keisaban, T. (2013). Antioxidant and anti-inflammatory activity of *Eurycoma longifolia* Jack. A traditional medicinal plant in Malaysia. *International Journal of Pharmaceutical Sciences and Nanotechnology, 5*(4), 1875–1878.

Veldhuis, J.D., Rogol, A.D., Samojlik, E., and Ertel, N.H. (1984). Role of endogenous opiates in the expression of negative feedback actions of androgen and estrogen on pulsatile properties of luteinizing hormone secretion in man. *The Journal of Clinical Investigation, 74*(1), 47-55.

Viau, V. (2002). Functional cross-talk between the hypothalamic-pituitary gonadal and adrenal axes. *Journal of Neuroendocrinology, 14,* 506 – 513.

Vo, D.V., and Park, M.J. (2008). Stress and stress management among youth and young men. *American Journal of Men's Health, 2*(4), 353-366.

Volek, J.S., Kraemer, W.J., and Bush, J.A. (1997). Testosterone and cortisol in relationship to dietary nutrients and resistance exercise. *Journal of Applied Physiology, 82*, 49–54.

Vrbik, I., Sporis, G., Stefan, L., Madic, D., Trajkovic, N., Valantine, I., and Milanovic, Z. (2016). The influence of familiarization on physical fitness test results in primary school-aged children. *Pediatric Exercise Science, 29*(2), 1 – 19.

Wan Hassan, W.E. (2007). Healing herbs of Malaysia. *Federal Land Development Authority (FELDA).*

Wang, C., Alexander, G., Berman, N., Salehian, B., Davidson, T., McDonald, V., Steiner, B., Hull, L., Callegari, C., and Swerdloff, R.S. (1996). Testosterone replacement therapy improves mood in hypogonadal men–a clinical research center study. *Journal Clinical Endocrinology and Metabolism, 81*(10), 3578–3583.

Wang, C., Swerdloff, R.S., Iranmanesh, A., Dobs, A., Snyder, P.J., Cunningham, G., Matsumoto, A.M., Weber, T., and Berman, N. (2000). Transdermal testosterone gel improves sexual function, mood, muscle strength, and body composition parameter in hypogonadal men. *Journal of Clinical Endocrinology Metabolism, 85*, 2839-2853.

Wang, C., Cunningham, G., Dobs, A., Iranmanesh, A., Matsumoto, A.M., Snyder, P.J., Weber, T., Berman, N., Hull, L., and Swerdloff, R.S. (2004). Long-term testosterone gel (androgel) treatment maintains beneficial effects on sexual function and mood, lean and fat mass, and bone mineral density in hypogonadal men. *Journal of Clinical Endocrinology Metabolism, 89*(5), 2085–2098.

Wang, C., Nieschlag, E., Swerdloff, R., Behre, H.M., Hellstrom, W.J., Gooren, L.J., Kaufman, J.M., Legros, J.J., Lunenfeld, B., Morales, A., Morley, J.E., Schulman, C., Thompson, I.M., Weidner, W., and Wu, F.C. (2009). Investigation, treatment, and monitoring of late-onset hypogonadism in males: ISA, ISSAM, EAU, EAA, and ASA recommendations. *European Urology, 55*(1), 121 – 130.

Wang, C., Ilani, N., Arver, S., McLachlan, R.I., Soulis, T., and Watkinson, A. (2011). Efficacy and safety of the 2% formulation of testosterone topical solution applied to the axillae in androgen-deficient men. *Clinical Endocrinology (Oxf),75*(6), 836–843.

Wankhede, S., Mohan, V., and Thakurdesai, P. (2016). Beneficial effects of fenugreek glycoside supplementation in male subjects during resistance training: A randomized controlled pilot study. *Journal of Sport and Health Science, 5*(2), 176-182.

Wahab, N.A., Mokhtar, N.M., Halim, W.N.H.A., and Das, S. (2010). The effect of *Eurycoma longifolia* Jack on spermatogenesis in estrogen treated rats. *Clinics, 65*(1), 93 - 98.

Wernsdorfer W.H., Ismail S., Chan K.L., Congpuong K., Wernsdorfer G. Activity of *Eurycoma longifolia* root extract against Plasmodium falciparum *in vitro*. *Wiener Klinische Wochenschrift, 121* (Suppl 3), 23–26.

World Health Organization. (1999). WHO Monographs on Selected Medicinal Plants. Geneva: *World Health Organization*.

World Health Organization. (2002). WHO Monographs on Selected Medicinal Plants. Geneva, Switzerland: *World Health Organization, 2*.

Williams, M. (2006). Dietary Supplements and Sports Performance: Herbals. *Journal of the International Society of Sports Nutrition, 3*(1), 1-6.

Wizneh, F.M., and Asmawi, M.Z. (2014). *Eurycoma longifolia* Jack (Simarubaceae); Advances in Its Medicinal Potentials. *Pharmacognosy Journal, 6*(4), 1 – 9.

Woodhouse, L.J., Reisz-Porszasz, S., Javanbakht, M., Storer, T.W., Lee, M., Zerounian, H., and Bhasin, S. (2003). Development of models to predict anabolic response to testosterone administration in healthy young men. *American Journal of Physiology, Endocrinology and Metabolism, 284*(5), E1009–E1017.

Wong, P.F., Cheong, W.F., Shu, M.H., Teh, C.H., Chan, K.L., and Abu Bakar, S. (2012). Eurycomanone suppresses expression of lung cancer cell tumor markers, prohibitin, annexin 1 and endoplasmic reticulum protein 28. *Phytomedicine, 19*(2), 138–144.

Wu, F.C., Tajar, A., Pye, S.R., Silman, A.J., Finn, J.D., O'Neill, T.W., Bartfai, G., Casaneuva, F., Forti, G., Giwercman, A., Huhtaniemi, I.T., Kula, K., Punab, M., Boonen, S., and Vanderschueren, D. (2008). Hypothalamic-Pituitary-Testicular Axis Disruption in Older Men are Differentially Linked to Age and Modifiable Risk Factors: The European Male Aging Study. *The Journal of Clinical Endocrinology and Metabolism, 93*(7), 2737-2745.

Xu, T., Shen, Y., Pink, H., Triantafillou, J., Stimpson, S.A., Turnbull, P., and Han, B. (2004). Phosphorylation of p70s6 kinase is implicated in androgen-induced levator ani muscle anabolism in castrated rats. *Journal of Steroid Biochemistry Molecular Biology, 92*(5), 447–454.

Yang, S.Y., and Goldspink, G. (2002). Different roles of the IGF-I Ec peptide (MGF) and mature IGF-I in myoblast proliferation and differentiation. *FEBS Letter, 522*(1-3), 156–160.

Yavuz, H.U., and Őzkum, D. (2014). Herbs potentially enhancing sports performance. *Current Topics in Nutraceutical Research, 12*(1), 25 – 34.

Young, N.R., Baker, H.W., Liu, G., Seeman, E. (1993). Body composition and muscle strength in healthy men receiving testosterone enanthate for contraception. *Journal of Clinical Endocrinology and Metabolism, 77*(4):1028–1032.

Zanuso, S., Sieverdes, J.C., Smith, N., Carraro, A., and Bergamin, M. (2012). The effect of a strength training program on affect, mood, anxiety, and strength performance in older individuals. *International Journal of Sport Psychology, 43*, 53 – 66.

Zanoli, P., Zavatti, M., Montanari, C., and Baraldi, M. (2009). "Infuence of *Eurycoma longifolia* on the copulatory activity of sexually sluggish and impotent male rats," *Journal of Ethnopharmacology, 26*(2), 308–313.

Zakaria, Y., Rahmat, A., Pihie, A.H.L., Abdullah, N.R., and Houghton, P.J. (2009). Eurycomanone induce apoptosis in HepG2 cells via up-regulation of p53. *Cancer Cell international, 9*(16), 1-21.

Zhang, Y.J., Dang, H.S., Wang, Y., Li, X.W., and Li, J.Q. (2011). A taxonomic revision of unifoliolate Chinese *Epimedium L.* (Berberidaceae). *Kew Bulletin, 66*(2).

Zhari, I., Ismail, N., and Lassa, J. (1999). Malaysian herbal monograph. Radix Eurycoma, Eurycoma root. *Malaysian Monograph Committee, Kuala Lumpur*, 29–32.

APPENDIX

Appendix A: Ethical approval for the research project (14/SPS/013)

Ethical Approval

Williams, Mandy

Wed 4/23/2014 3:56 PM

To Chan, Kai Quin <K.Q.Chan@2013.ljmu.ac.uk>;

Cc Chester, Neil <N.Chester@ljmu.ac.uk>;

Dear Kai

With reference to your application for Ethical approval.

14/SPS/013, Kai Quin Chan, PGR, The effects of 12 Weeks Eurycoma longifolia (Tongkat Ali) ingestion on exercise performance, health and wellbeing (Dr Neil Chester).

Liverpool John Moores University Research Ethics Committee (REC) has reviewed the above application and I am pleased to inform you that ethical approval has been granted and the study can now commence.

Approval is given on the understanding that:

- any adverse reactions/events which take place during the course of the project are reported to the Committee immediately;
- any unforeseen ethical issues arising during the course of the project will be reported to the Committee immediately;
- the LJMU logo is used for all documentation relating to participant recruitment and participation eg poster, information sheets, consent forms, questionnaires. The LJMU logo can be accessed at http://www.ljmu.ac.uk/corporatecommunications/60486.htm

Where any substantive amendments are proposed to the protocol or study procedures further ethical approval must be sought.

Applicants should note that where relevant appropriate gatekeeper / management permission must be obtained prior to the study commencing at the study site concerned.

For details on how to report adverse events or request ethical approval of major amendments please refer to the information provided at http://www.ljmu.ac.uk/RGSO/93205.htm

Please note that ethical approval is given for a period of five years from the date granted and therefore the expiry date for this project will be April 2019. An application for extension of approval must be submitted if the project continues after this date.

LIVERPOOL JOHN MOORES UNIVERSITY

Mandy Williams, Research Support Officer
Graduate School, Research and Innovation Services
Kingsway House, Hatton Garden, Liverpool L3 2AJ
t: 01519046467 e: a.f.williams@ljmu.ac.uk

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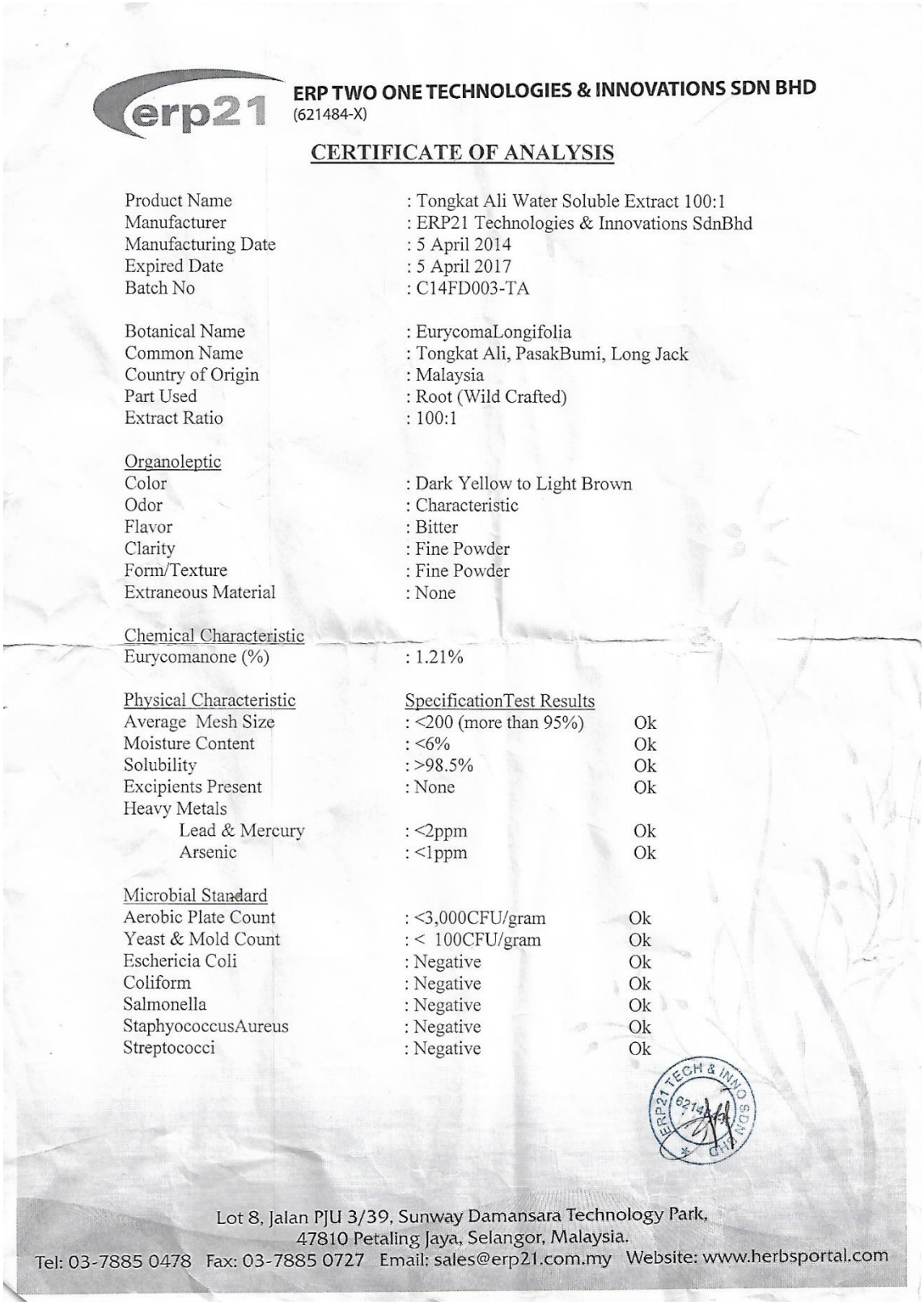
Appendix B: *E. longifolia* certificate of analysis



ERP TWO ONE TECHNOLOGIES & INNOVATIONS SDN BHD
(621484-X)

CERTIFICATE OF ANALYSIS

Product Name	: Tongkat Ali Water Soluble Extract 100:1
Manufacturer	: ERP21 Technologies & Innovations SdnBhd
Manufacturing Date	: 5 April 2014
Expired Date	: 5 April 2017
Batch No	: C14FD003-TA
Botanical Name	: EurycomaLongifolia
Common Name	: Tongkat Ali, PasakBumi, Long Jack
Country of Origin	: Malaysia
Part Used	: Root (Wild Crafted)
Extract Ratio	: 100:1

Organoleptic	
Color	: Dark Yellow to Light Brown
Odor	: Characteristic
Flavor	: Bitter
Clarity	: Fine Powder
Form/Texture	: Fine Powder
Extraneous Material	: None

Chemical Characteristic	
Eurycomanone (%)	: 1.21%

Physical Characteristic	Specification	Test Results
Average Mesh Size	: <200 (more than 95%)	Ok
Moisture Content	: <6%	Ok
Solubility	: >98.5%	Ok
Excipients Present	: None	Ok
Heavy Metals		
Lead & Mercury	: <2ppm	Ok
Arsenic	: <1ppm	Ok

Microbial Standard		
Aerobic Plate Count	: <3,000CFU/gram	Ok
Yeast & Mold Count	: < 100CFU/gram	Ok
Eschericia Coli	: Negative	Ok
Coliform	: Negative	Ok
Salmonella	: Negative	Ok
StaphyococcusAureus	: Negative	Ok
Streptococci	: Negative	Ok

ERP21 TECH & INNO SDN BHD 621484-X

Lot 8, Jalan PJU 3/39, Sunway Damansara Technology Park,
47810 Petaling Jaya, Selangor, Malaysia.
Tel: 03-7885 0478 Fax: 03-7885 0727 Email: sales@erp21.com.my Website: www.herbsportal.com

Appendix C: Certificate of analysis for *E. longifolia* by LGC laboratory accredidated by United Kingdom Accreditation Service (UKAS)

UKAS Testing Laboratory No: 1187

LGC
Newmarket Road
Fordham
Cambridgeshire
CB7 5WW
UK

Tel: +44 (0)1638 720500
Fax: +44(0)1638 724200
Email: info@lgcgroup.com
www.lgcgroup.com

Neil Chester
Liverpool John Moores University
School of Sport and Exercise Sciences
Tom Reilly Building
Byrom Street
Liverpool
L3 3AF
UK

Date Issued: 15 September, 2014

CERTIFICATE OF ANALYSIS: 92718

LGC Supplement Screen

Consignment Number:	Post
Delivery Date:	23 July, 2014
Date Analysis Commenced:	14 August, 2014
Purchase Order Number:	N/A

Product:	Tongkat Ali		
Flavour:		**Programme Category:**	Routine
Batch No:	C14FD003-TA	**Pack Size:**	
Batch Expiry:	5/4/2017	**LGC Reference:**	701601

The sample was analysed using documented LGC screening methods for the compounds specified within the Service Level Agreement: Nutritional Supplements V2.0.

GCMS:
Screening tests indicated the presence of 1,4-androstadiene-3,17-dione.

All other compounds: None were found.

LCMS:
Sample unsuitable for the analysis of nikethamide.

All other compounds: None were found.

Signed

Sam Kay
Team Leader

Test results apply to the portion of product taken.
* or isomers of - as specified within the service level agreement.

R-2616

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Appendix D: Participation information sheet

LIVERPOOL JOHN MOORES UNIVERSITY
PARTICIPANT INFORMATION SHEET

Title of Project: The effects of 12 Weeks *Eurycoma longifolia* (Tongkat Ali) ingestion on exercise performance, health and wellbeing

Name of Researcher and School/Faculty:
Kai Quin Chan
School of Sport and Exercise Sciences, Faculty of Science

Academic Supervisor:
Dr Neil Chester
School of Sport and Exercise Sciences, Faculty of Science

"You are invited to take part in a sports science research project to examine the effects of a natural Asian herb, *Eurycoma Longifolia* (EL) on exercise performance, health and well-being"
I am looking for males aged 18 to 30 years old and 55 to 70 years old, free from musculoskeletal injury and are in good health. You are not eligible to take part in this research project if you have diabetes mellitus, heart disease, liver disease and sleep apnoea. Also, if you smoke or take other recreational or prescription drug and have artificial pacemaker, you will not be eligible to participate in the research.

The herb contains an ingredient, boldione, which is prohibited by the World Anti-Doping Agency and therefore those who may be subject to anti-doping tests (i.e. competitive athletes) are excluded from participating in the study. Whilst boldione is present in Tongkat Ali, the concentration/amount is extremely low and the dose administered as part of the study is within the range of 18 to 30 ng per day which is deemed to be innocuous from a health and safety perspective.

Please, take the time to read the details of the project on this participant information sheet. Feel free to ask any questions you may have about the research and the processes involved. When you are ready you can decide whether you would like to take part in the research process or not. Thank you."

1. What is the purpose of the study?

The purpose of this study is to assess the effects of the oral supplementation of a herb, *Eurycoma longifolia* (600mg/day) relative to placebo (maltodextrin) for 2 weeks, on health (heart rate and blood pressure; body composition; blood lipid profile i.e. cholesterol) and well-being (mood state and libido). The herb is considered to be a natural testosterone booster and therefore is reputed to increase testosterone levels in the human body.

Testosterone is the predominant male sex hormone known for their androgenic (masculinizing) and anabolic (muscle-building) actions which create possible advantages to those engaged in sport. *Eurycoma longifolia* (EL) supplementation could increase testosterone levels and subsequently increase sports performance and effect health and wellbeing.

2. Do I have to take part?

Your participation in this process is completely voluntary. If you decide to take part in this study you will be asked to sign a volunteer consent form and will be given this information sheet. If at

any time during the study you would like to withdraw from the study you are free to do so without your rights, future treatment or service you receive being affected. Please ensure you read this and ask any question no matter how small a detail it may seem.

3. What will happen to me if I take part?

The duration of this study is 2 weeks and you will need to consume *Eurycoma longifolia* or placebo (maltodextrin) in capsule form throughout the duration (i.e. 600 mg per day). You will need to attend six (6) laboratory visits for blood sampling. Prior to each visit you will be asked to complete an overnight fast (8 hours), refrain from alcohol and caffeine, strenuous activity and sexual activity 24 hours prior to the blood collection day.

The laboratory testing includes:

1) Body composition will be determined by assessment of your height, and weight. In addition a technique known as 'bioelectrical impedance analysis' will be used to assess body composition. This technique involves the passage of a very low (unnoticeable) electrical current through the body to determine electrical resistance. This measurement corresponds with body composition.
2) Heart rate and blood pressure will be assessed using a method involving an inflatable cuff around the upper arm.
3) Measurement of haematological parameters and steroid and lipid profiles (i.e. cholesterol) will involve venepucture of a vein in the arm and the collection of a small sample of blood (15 ml divided into two separate tubes).
4) Mood state will be assessed using a simple questionnaire involving the rating of your mood according to six states (i.e. vigour, depression, anger, confusion, fatigue and anxiety)
5) Sexual function will be assessed by a simple questionnaire (11 questions) involving the rating of your sex drive.

4. Are there any risks / benefits involved?

Benefits

Increases in testosterone level will provide potential benefits including: improvements in exercise performance (muscle strength and anaerobic power); health (heart rate and blood pressure; body composition; blood lipid profile i.e. cholesterol); and wellbeing (mood state and libido).

Risks

There are limited reported side effects following the supplementation with *Eurycoma longifolia,* however possible side effects include insomnia, anxiety, restlessness, and reduced blood glucose. Symptoms of low blood glucose are trembling or shakiness, fast heartbeat, sweating, anxiety, dizziness, feeling hungry, tiredness, blurred vision, confusion and difficulty concentrating. Subjects who experience such side-effects should report this to the researcher and may terminate their involvement in the study immediately. Men with breast cancer or prostate cancer, diabetes mellitus, heart disease, kidney disease, liver disease, sleep apnoea should avoid supplementation and will be excluded from the study via the pre-investigation health screen. The assessment of sexual function involves sensitive information and may cause distress/embarrassment to participants. Any distress will be alleviated through the completion of the questionnaire on-line, in a private room adjacent to the laboratory. The use of an on-line questionnaire will ensure the anonymity of the data since it will only be identifiable from a specific code number. The data will be encrypted and the computer will be password protected to ensure the data is not retrievable by a third party other than the researcher and supervisor.

The Tongkat Ali administered contains a prohormone, boldione. Prohormones have the potential to adversely affect cholesterol levels if taken in high quantities. Research suggests that there are no effects in quantities of 100 mg per day and therefore quantities in this study of within the range of 18 to 30 ng per day (greater than a million times less) is deemed to be innocuous from a health and safety perspective. Also, as boldione is currently prohibited by the World Anti-Doping Agency (WADA) there is a risk of testing positive if an individual is a competitive athlete and is subject to anti-doping drug testing. For this reason competitive athletes are excluded from participating in this study. Also, those recruited to take part in this **should not** take part in any competitive athletic events that are subject to WADA regulations.

5. Will my taking part in the study be kept confidential?

Should you choose to participate, your confidentiality will be assured in a number of ways. These include data encryption, secure file storage, and destruction of data after the valid period of use.

This study has received ethical approval from LJMU's Research Ethics Committee *(14/SPS/013 – 23/4/2013)*

If you have any concerns regarding the research please contact the research or research supervisor:

Contact Details of Researcher
Kai Quin Chan
k.q.chan@2013.ljmu.ac.uk

Contact Details of Academic Supervisor
Dr Neil Chester
N.Chester@ljmu.ac.uk

Appendix E: Consent form

LIVERPOOL JOHN MOORES UNIVERSITY
CONSENT FORM

Project title: The effects of 12 Weeks *Eurycoma longifolia* (Tongkat Ali) ingestion on exercise performance, health and wellbeing
Investigator : Kai Quin CHAN
Supervisor : Dr Neil Chester
School of Sport and Exercise Sciences, Faculty of Science

Please initial the appropriate response for each of the following statements before signing the form:

		Yes	***No***
1.	I confirm that I have read and understand the information provided for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily	☐	☐
2.	I understand that my participation is voluntary and that I am free to withdraw at any time, without my medical care and my legal rights being affected.	☐	☐
3.	I understand that any personal information collected during the study will be anonymised and remain confidential	☐	☐
4.	I agree to take part in the above study *(Ingestion of Eurycoma longifolia supplement or placebo and perform strength training programme)* i.	☐	☐
5.	I understand that my results will be coded, stored and will not be possible to trace them back to me by individuals not associated with the project	☐	☐
6.	I consent to blood samples being collected and stored for biochemical analysis	☐	☐
7.	I understand that the blood samples are to be considered a gift as such, once the samples are analysed they will be destroyed.	☐	☐

I, ... agree to take part in the above named project/procedure, the details of which have been fully explained to me and described in writing.

Signed ..... Date..... (Participant)

I, ... certify that the details of this project/procedure have been fully explained and described in writing to the subject named above and have been understood by him/her.

Signed ..... Date..... (Investigator)

I, ... certify that the details of this project/procedure have been fully explained and described in writing to the subject named above and have been understood by him/her.
Signed ..... Date..... (Witness)

Appendix F: Physical activity readiness questionnaire (PAR-Q)

Physical Activity Readiness Questionnaire (PAR-Q)

Title of Project: The effects of 12 Weeks *Eurycoma longifolia* (Tongkat Ali) ingestion on exercise performance, health and wellbeing.

Name of Researcher (Student): Kai Quin Chan
Academic Supervisors: Dr Neil Chester and Professor Claire Stewart
School: Sport and Exercise Sciences, Faculty of Science
Contact details : k.q.chan@2013.ljmu.ac.uk

Many exercise performance assessment are associated with physical activity and completion of Physical Activity Readiness Questionnaire (PAR-Q) is important before participating in a physical activity laboratory testing. For most people physical activity should not pose any problem or hazard and the PAR-Q is designed to identify the small number of adults for whom physical activity might be inappropriate or those who should seek further advice from a doctor before participating in any exercise performance laboratory testing.

Please read carefully and answer each one honestly. You are required to check YES or NO.		**YES**	**NO**
1.	Has your doctor ever said that you have a heart condition OR high/low blood pressure		
2.	Do you feel pain in your chest at rest, during your daily activities of living, OR when you do physical activity?		
3.	Do you lose balance because of dizziness OR have you lost consciousness in the last 12 months? Please answer NO if your dizziness was associated with over-breathing (including during vigorous exercise).		
4.	Do you have an artificial pacemaker implanted under your skin of your chest (below your collar bone) to help you heart muscle to pump blood regularly?		
5.	Is there any history of Coronary Heart Disease in your family?		
6.	Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)?		
7	Do you have Diabetes Mellitus or any other metabolic disease (liver disease, kidney diseases)?		
8.	Has your doctor ever said you have raised cholesterol (serum level above 6.2mmol/L)?		
9.	Do you have sleep apnoea? (A type of sleep disorder characterized by pauses in breathing or instances of shallow or infrequent breathing during sleep).		
10.	Have you been on medication for low testosterone (hypogonadism)?		
11.	Are you currently taking prescribed drugs or medications for a chronic medical condition?		
12.	Do you have a bone or joint problem that could be made worse by becoming more physically active? Please answer NO if you had a joint problem in the past, but it does not limit your current ability to be physically active. For example, knee, back, ankle, or hip.		
13.	Has your doctor ever said that you should only do medically supervised physical activity?		

14.	Have you ever suffered from unusual shortness of breath at rest or with mild exertion?		
15.	Do you currently drink more than the recommended safe limit of alcohol per week (21 units; 1 unit = ½ pint of beer/cider/lager or 1 small glass of wine).		
16.	Do you currently smoke?		
17	Do you know of any other reason why you should not participate in a programme of physical activity? (If yes, please specify :…….)		

If you answered:

YES to one or more questions:
You should consult with your doctor to clarify that it is safe for you to participate in this research project at this current time and in in your current state of health. Inform your doctor what questions you answered YES to on PAR-Q or present your PAR-Q copy.

No to all questions:
If you answered PAR-Q accurately, you have reasonable assurance of your present suitability for the research project.

I have read, understood and accurately completed this questionnaire. I confirm that I am voluntarily engaging in an acceptable level of physical activity. Any questions I had were answered to my full satisfaction.

NAME: _____
SIGNATURE: _____
DATE: _____

NAME OF WITNESS: _____
SIGNATURE: _____
DATE: _____

Appendix G: The control of substances hazardous to health (COSHH) risk assessment form – Routine cell culture

LIVERPOOL JOHN MOORES UNIVERSITY

Life Sciences Building – COSHH Risk Assessment Form

Protocol/Procedure: Routine cell culture

Name of person carrying out procedure: Kai Quin Chan (654076)	
Job Title: PhD Student	Tel no. & e-mail: 07870825516 K.Q.Chan@2013.ljmu.ac.uk
Faculty: Science	School: Sports and Exercise Science
Name of supervisor: Professor Claire Stewart Tel no. & e-mail: C.E.Stewart@ljmu.ac.uk Signature of supervisor:	
Location of procedure to be carried out: 1.24, 1.25, 1.26, 1.27, 1.28, 2.08 Life sciences building, Byrom street	
Has the person been trained in this protocol/procedure? Yes Is training/supervision required? See above If yes, please give details:	
Date of assessment: 05.01.2015 Signature of person carrying out procedure: Kai Quin Chan	

Description of Activity/Procedure/Process (include standard operating procedures (SOPs) as a Control): -Aspirate off existing growth media -Wash with PBS -Add 1ml of Trypsin -Add new growth media -Collect cells -Homogenise cells with syringe -Add 20-50ul of Trypan blue to same amount of cell suspension -Count using haemocytometer The dose response for Tongkat Ali will be between 0 and 20 mM will be determined in preliminary studies. The concentration is several folds lower than the approved dose of 6g used for daily human ingestion and it is considered safe for consumption. The non-human waste will be autoclaved.
Who is at risk? (staff/students/others): All users of the Cell culture lab including; Post-Doc's, lecturers, PhD and MSc students, BSc Students.
What is the duration of exposure? Minimum 30 mins, Maximum 8 Hours
What is the frequency of exposure? Frequent (often daily)
What is the maximum number of people in the room/lab: 5
Are there any ethical issues Y/N? YES (when using human cells) If yes, has consent been obtained? N/A – only commercially available rodent cell samples being utilised.
Is health surveillance required? No Health Surveillance is required if the procedure involves substances which are respiratory sensitisers or skin sensitisers (risk phrases R42, R43 or R42/43). If other substances with potential health effects are used and if any health effects are observed that is believed to have resulted from its use then Occupational Health should be contacted. Consideration should be made of the existing health status of the user of hazardous substances. Are special arrangements required? E.g. for types of PPE (ref:SCP9).
Will the individual be working outside of normal working hours, overnight or at weekends? No (if the need arises, a separate risk assessment in line with this form will be undertaken. If yes, has the appropriate form (SCP11 – Out of hours working) been completed and authorisation given? Will the experiment be left to run unattended out of hours? No

If yes, has the appropriate form (SCP22 – Unattended experiments) been completed and authorisation given? N/A If out of hours work is required in future, relevant forms will be completed.

Starting substances:	Rodent Skeletal Muscle Cells. DMEM (Dulbeccos Modified Eagle Medium) Foetal Bovine Serum/ Newborn calf serum Phosphate Buffered Saline Trypsin EDTA Solution Trypan Blue
End products:	Same as above.
Hazard categories:	H319 Causes serious eye irritation. H315 Causes skin irritation. H302 harmful if swallowed H350 May cause cancer. H340 May cause genetic defects. H361 Suspected of damaging fertility or the unborn child.
What are the potential risks?	Risk to Eyes, skin, if ingested, inhaled. Use of trypan blue: Risk of mutagenesis particularly of blood and reproductive system. Risk of needle stick injury when homogenising cells. Cut from glass pipette aspirator
How will these risks be controlled?	Avoid contact with skin, by wearing lab coats and nitrile gloves. Always work with samples in a ventilated safety hood with the glass screen lowered according to manufacturer's specifications. Avoid inhalation or ingestion via avoiding working in a confined space/ poorly ventilated space. Use Trypan blue in a ventilated safety hood behind a glass screen or inside class II cabinet Keep mouth closed when using trypan blue. Discard trypan blue tips/ependorfs in yellow sharp bin yellow cardboard biobin and eppendofs, close lid and put in yellow clinical waste bag. When homogenising cells using a syringe and needle, do-not re-sheath needles, and place in a plastic yellow sharps bin after use. Carry out homogenisation within safety hood.
What personal protective equipment (PPE) is required? (ref:SCP9)	Nitrile gloves and howie lab coat For Trypan Blue only dispense inside the class II cabinet. Once the coverslip is on the haemocytometer, remove from cabinet to view under the microscope, therefore not risking eye exposure

Chemical storage procedure:

Store all these reagents in the fridge at 1-4 degrees. Foetal bovine serum/ newborn calf serum and trypsin can be stored in minus15-20 degrees until needed. Throw away once use by date expires place into yellow clinical waste bags which when full are tied shut, placed in large yellow bin in room 1.29 for collection and disposal by licenced contractor. If Trypan blue reaches its expiry date it must be collected in a suitable container, labeled with waste, substance name, concentration (if applicable), supplier name and catalogue code. Bottles must be stored in suitable locations, i.e. flammable liquids inside fume hood or white flammables cabinet. Until transfer to chemical stores in the James Parsons tower building for collection and disposal by a licensed company. Keep containers tightly closed.

Waste disposal procedure:

Reagents can be disposed of in the chemical/clinical waste bags then into clinical waste bin for incineration.
sharps (needles, blades etc go into yellow, contaminated sharps bins which when full are closed and stored in room 1.29 for collection and removal by licenced company
For human samples: Pipette tips/stripettes are placed in yellow bio-bins which when full are sealed shut, placed in large yellow bin in room 1.29 for collection and disposal by licenced contractor. For non-human samples, autoclave bags are used, which when full are sealed shut with autoclave tape and autoclaved prior to routine disposal
Plastic plates etc are placed into yellow clinical waste bags which when full are tied shut, placed in large yellow bin in room 1.29 for collection and disposal by licenced contractor.

Hazardous liquid waste is stored in suitable containers labelled with waste, substance name, concentration if required, supplier name and catalogue code and is stored in suitable location such as fume hood, white flammables cabinet (if flammable) or grey chemical cabinet until transfer to chemical stores in the James Parsons tower building until collection and disposal by a licenced company.
Non-hazardous liquids which may contain cells are aspirated into a sealed container with Virkon and left for at least 24hours before disposal down the drain.

Spillage/leaks procedure:

Small spillages: While wearing gloves and lab coats then absorb using blue roll and place in clinical waste disposal bags, and clean surface with Virkon solution, then water.

Emergency action procedures – action required if a substance is,

Ingested:
Seek medical aid immediately. Do not induce vomiting unless directed to do so by medical personnel. Never give anything by mouth to an unconscious person. If vomiting occurs naturally, have individual lean forward.

Inhaled:
If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen if available. Get medical aid immediately.

Contact with skin:
In case of contact, immediately flush skin with plenty of water for at least 15 minutes while removing contaminated clothing and shoes. Get medical aid/ contact physician immediately. Wash clothing before reuse. When homogenising cells using a syringe and needle, do-not re-sheath needles in, and place in a plastic yellow sharps bin after use.

Contact with eye:
In case of contact, immediately flush eyes with plenty of water for at least 15 minutes. Get medical aid, contact physician. For Trypan Blue wear safety goggles.

Injected:

In case of an emergency please contact:

Supervisor: Professor Claire Stewart- 01519046234
Health and Safety Officer - Monica Barclay ext 6211 (0151 904 6211)
First aider - Monica Barclay ext 6211 (0151 904 6211)
First aider - Louise Coyne ext 6283 (0151 904 6283)
First aider - Ellen Dawson ext 6264 (0151 904 6264)

For a serious incident, call security on ext 2222 (0151 904 2222) and they contact emergency services.

In case of business interruption (e.g. power shutdown, flood), what are the Contingency Procedures for work and waste?
Leave items in the hoods, with lids on, vacate area and return when told it is safe to do so, ensure the CO_2 sensor is working before returning.

In case of fire or explosion: Raise alarm, immediately evacuate area, call security on ext 2222 (0151 231 2222 from an external line) and inform them of the location and source of the fire, they will contact Merseyside Fire and Rescue Service. Inform fire warden on site of the location and source of the fire.

Based on the COSHH information below, the overall risk of the procedure/protocol is low.

COSHH material safety data – This needs to be completed for each chemical used in the procedure that is classed as harmful, toxic, corrosive, an irritant or poses any other risk to human health.

Hazardous substance: Trypan Blue
What are the hazards and hazard codes?

H341 - Suspected of causing genetic defects H350 - May cause cancer H361 - Suspected of damaging fertility or the unborn child	
Is it solid, liquid or gas? Liquid	Quantity used: Maximum of 50ul per sample tested
What is the route of entry into the body? Eyes, skin, ingestion and inhalation	What are the target organs? No data available
What is the working limit exposure (WEL)? No data available	
What control measures, handling precautions and PPE are in place? Nitrile gloves and howie lab coat, wear safety goggles. Work in safety hood when dispensing trypan blue. If this is not possible, wear safety goggles outside the hood.	
What is the disposal procedure (if different from page 2): Prevent product from entering drains.	
What is the spillage procedure (if different from page 2): Soak up with inert absorbent material.	
What are the emergency procedures (if different from page 2): • Swallowed: Never give anything by mouth to an unconscious person. Consult a physician. • Inhaled: Move to fresh air. Consult a physician. • Injected: Gently squeeze wound (DO NOT SUCK AREA), Wash affected area with soap & water (DO NOT SCRUB) Rinse mucous membranes with warm water (DO NOT SWALLOW WATER) Cover broken skin with waterproof dressing e.g. elastoplast, if not allergic • Contact with skin: Wash off immediately with soap and plenty of water removing all contaminated clothes and shoes. If symptoms persist, call a physician. • Contact with eyes: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. If eye irritation persists, consult a specialist.	

For further information contact Health and Safety Unit (ext. 8167), refer to supplier's data sheet, contact supervisor.

Appendix H: COSHH risk assessment form – Storage and removal of samples from the liquid nitrogen storage waste

LIVERPOOL JOHN MOORES UNIVERSITY

Life Sciences Building – COSHH Risk Assessment Form

Protocol/Procedure: Storage and removal of samples from the Liquid Nitrogen storage vessel.

Name of person carrying out procedure: Kai Quin Chan (654076)	
Job Title: PhD Student	Tel no. & e-mail: 07870825516 K.Q.Chan@2013.ljmu.ac.uk
Faculty: Science	School:
Name of supervisor: Professor Claire Stewart Tel no. & e-mail: C.E.Stewart@ljmu.ac.uk Signature of supervisor:	
Location of procedure to be carried out: 1.24, 1.25, 1.26, 1.27, 1.28, 2.08 Life sciences building, Byrom street	
Has the person been trained in this protocol/procedure? Yes Is training/supervision required? See above If yes, please give details:	
Date of assessment: 05/01/2015 Signature of person carrying out procedure: Kai Quin Chan	

Description of Activity/Procedure/Process (include standard operating procedures (SOPs) as a Control):

1. Determine which rack/box you will be storing/removing your sample in/from.
2. Put on all required PPE.
3. Press the level alarm button to disable the alarm whilst using the vessel.
4. Remove the lid.
5. Raise the chosen rack holding it above the liquid nitrogen level in the dewer until all the liquid nitrogen has drained and then proceed to remove the entire rack from the vessel and onto a flat surface.
6. Replace the lid on the vessel to prevent unwanted liquid nitrogen evaporation.
7. Remove the safety bar to allow the chosen box to be removed.
8. Store/remove sample from chosen box and replace into the rack, reinserting the safety bar.
9. Remove the lid and replace the rack into vessel slowly.
10. Replace the lid and press the level alarm button to re-enable the alarm.

Who is at risk? (staff/students/others): Users of cell culture lab and are working with cells (Post – Docs, PhD, MSc, BSc students).

What is the duration of exposure? >10 minutes

What is the frequency of exposure? Fairly Frequent (Every time new cell culture experiment begins – varies)

What is the maximum number of people in the room/lab: 2

Are there any ethical issues: YES (when using human cells) If yes, has consent been obtained? N/A – Only using commercially available rodent cells.

Is health surveillance required? No
Health Surveillance is required if the procedure involves substances which are respiratory sensitisers or skin sensitisers (risk phrases R42, R43 or R42/43). If other substances with potential health effects are used and if any health effects are observed that is believed to have resulted from its use then Occupational Health should be contacted. Consideration should be made of the existing health status of the user of hazardous substances. Are special arrangements required? E.g. for types of PPE.

Will the individual be working outside of normal working hours, overnight or at weekends? No (Yes – if required, however, relevant risk assessments and documents will be obtained).
If yes, has the appropriate form (SCP11 – Out of hours working) been completed and authorisation given? If required in the future then relevant forms will be completed.
Will the experiment be left to run unattended out of hours? No
If yes, has the appropriate form (SCP22 – Unattended experiments) been completed and authorisation given? N/A

Starting substances:	Cells – Rodent skeletal muscle cells, liquid nitrogen
End products:	Cells – Rodent skeletal muscle cells, liquid nitrogen
Hazard categories:	H281 - refrigerated gas may cause cryogenic burns or injury
What are the potential risks?	Cryogenic burns or injury, asphyxiation in high concentrations of liquid nitrogen.
How will these risks be controlled?	PPE is worn to protect areas exposed such as skin and eyes. There is an oxygen monitor in the room to alert users if oxygen levels reduce.

What personal protective equipment (PPE) is required? (ref:SCP9)	Cryo gloves, Howie Lab coat, Face shield or safety glasses. Oxygen monitor is located in the room.

Chemical storage procedure: Liquid nitrogen is stored in cryogenic vessels with safety mechanisms to prevent pressure build up in a designated room with oxygen alarms which will sound if oxygen levels fall.

Waste disposal procedure: Unwanted liquid nitrogen should be left to evaporate in a well-ventilated area.

Spillage/leaks procedure: If liquid nitrogen is spilled, leave area for liquid nitrogen to evaporate naturally. All personnel should vacate the area until the nitrogen is fully evaporated and oxygen levels are safe.

Emergency action procedures – action required if a substance is,

Ingested: Ingestion is not considered a potential route of exposure.

Inhaled: Move to fresh air. If not breathing, give artificial respiration.

Contact with skin: In case of frostbite spray with water for at least 15 minutes. Apply a sterile dressing. Obtain medical assistance.

Contact with eye: Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Seek Medical Advice.

Injected: Injection is not considered a potential route of exposure.

In case of an emergency please contact:

In case of business interruption (e.g. power shutdown, flood), what are the Contingency Procedures for work and waste? In the event of a power cut the Cryo storage vessel should be left with the lid firmly in place until work can resume. The room should be vacated until oxygen level alarms are operational.

In case of fire or explosion: Raise alarm, immediately evacuate area, call security on ext 2222 (0151 231 2222 from an external line) and inform them of the location and source of the fire, they will contact the fire brigade. Inform fire marshal on site of the location and source of the fire.
Based on the COSHH information below, is the overall risk of your procedure/protocol high, medium or low?
LOW

COSHH material safety data – This needs to be completed for each chemical used in the procedure that is classed as harmful, toxic, corrosive, an irritant or poses any other risk to human health.

Hazardous substance: Liquid nitrogen	
What are the hazards and hazard codes? H281 - refrigerated gas may cause cryogenic burns or injury	
Is it solid, liquid or gas? Liquid	Quantity used: 50L
What is the route of entry into the body? Skin, eyes, inhalational	What are the target organs? No known toxicological effects from this product
What is the working limit exposure (WEL)? N/A	
What control measures, handling precautions and PPE are in place? Cryo-gloves, Howie laboratory coat, safety glasses/face shield	
What is the disposal procedure (if different from page 2):	
What is the spillage procedure (if different from page 2):	
What are the emergency procedures (if different from page 2) if • Swallowed: • Inhaled: • Injected: • Contact with skin: • Contact with eyes:	

Appendix I: COSHH risk assessment form – Freezing cells

LIVERPOOL JOHN MOORES UNIVERSITY

Life Sciences Building – COSHH Risk Assessment Form

Protocol/Procedure: Freezing Cells

Name of person carrying out procedure: Kai Quin Chan (654076)	
Job Title: PhD Student	Tel no. & e-mail: 07870825516 K.Q.Chan@2013.ljmu.ac.uk
Faculty: Science	School: Sports and Exercise Science
Name of supervisor: Professor Claire Stewart Tel no. & e-mail: C.E.Stewart@ljmu.ac.uk Signature of supervisor:	
Location of procedure to be carried out: 1.24, 1.25, 1.26, 1.27, 1.28, 2.08 Life sciences building, Byrom street	
Has the person been trained in this protocol/procedure? Yes Is training/supervision required? Yes If yes, please give details:	
Date of assessment: 26/01/2015 Signature of person carrying out procedure: Kai Quin Chan	

Description of Activity/Procedure/Process (include standard operating procedures (SOPs) as a Control): - Add 1ml DMSO (Dimethyl Sulphoxide) per 10ml of cell suspension - Aliquot solution into cryotubes. - Place in -80, in a Mr Frosty containing isopropanol for 24hours before moving into liquid nitrogen
Who is at risk? (staff/students/others): All users of the Cell culture lab including; Post-Doc's, lecturers, PhD and MSc students, BSc Students.
What is the duration of exposure? <30mins
What is the frequency of exposure? Variable: up to once per week
What is the maximum number of people in the room/lab: 5 for 1.28 and 8 for 1.25
Are there any ethical issues Y/N? Yes (when human cells) If yes, has consent been obtained? N/A – Only using commercially available rodent cells.
Is health surveillance required? No Health Surveillance is required if the procedure involves substances which are respiratory sensitisers or skin sensitisers (risk phrases R42, R43 or R42/43). If other substances with potential health effects are used and if any health effects are observed that is believed to have resulted from its use then Occupational Health should be contacted. Consideration should be made of the existing health status of the user of hazardous substances. Are special arrangements required? E.g. for types of PPE (ref:SCP9).
Will the individual be working outside of normal working hours, overnight or at weekends? No If yes, has the appropriate form (SCP11 – Out of hours working) been completed and authorisation given?If required in the future then relevant forms will be completed. Will the experiment be left to run unattended out of hours? No If yes, has the appropriate form (SCP22 – Unattended experiments) been completed and authorisation given? N/A

Starting substances:	Cells – Rodent skeletal muscle cells, liquid nitrogen Cells in DMEM (Dulbeccos Modified Eagle Medium) Foetal Calf Serum/Newborn calf serum DMSO (Dimethyl Sulphoxide) Isopropanol
End products:	Cells – Rodent skeletal muscle cells, liquid nitrogen Cells in DMEM (Dulbeccos Modified Eagle Medium) Foetal Calf Serum/Newborn calf serum DMSO (Dimethyl Sulphoxide) Isopropanol
Hazard categories:	H302 Harmful if swallowed.

	H315 Causes skin irritation. H335 May cause respiratory irritation. H319 Causes serious eye irritation.
What are the potential risks?	Risk to Eyes, skin, if ingested, inhaled. Dissolved chemicals may be carried into the body via skin contact
How will these risks be controlled?	Limit the amount of time that the DMSO vessel is open to a minimum. Avoid contact with skin through use of PPE detailed below
What personal protective equipment (PPE) is required? (ref:SCP9)	Nitrile gloves and howie lab coat. This procedure is carried out in a Class II cabinet, therefore safety glasses are not deemed necessary.

Chemical storage procedure:

Store Feotal bovine serum/ new born calf serum and DMEM in the fridge at 1-4 degrees and store Isopropanol and DMSO in grey, chemical cabinet. Foetal bovine serum can be stored in minus15-20 degrees until needed. Keep containers tightly closed.

Waste disposal procedure:

Pipette tips/stripettes are placed in yellow bio-bins which when full are sealed shut, placed in large yellow bin in room 1.29 for collection and disposal by licenced contractor.
Plastic plates etc are placed into yellow clinical waste bags which when full are tied shut, placed in large yellow bin in room 1.29 for collection and disposal by licenced contractor.

Non-hazardous liquids that may contain cells are aspirated into a sealed container with Virkon and left for at least 24hours before disposal down the drain.

Hazardous liquid waste is stored in suitable containers labelled with waste, substance name, concentration if required, supplier name and catalogue code and is stored in suitable location such as fume hood, white flammables cabinet (if flammable) or grey chemical cabinet until transfer to chemical stores in the James Parsons tower building until collection and disposal by a licenced company.

Spillage/leaks procedure:

Small spillages: While wear gloves and lab coats then absorb using blue roll and place in yellow clinical waste disposal bags, and clean surface with Virkon solution if the substance is non hazardous substance (DMEM, foetal bovine serum and newborn calf serum), then water.

It is not likely that a large spillage will occur, as the maximum volumes handled would be 500 ml. Further, all work is carried out in a class II hood, therefore spills would be treated as above, or sash on the class II hood would be closed while guidance sought from Health and Safety officer. Contact Monica Barclay (Health and Safety Officer) – 0151 904 6211 (ext. 6211) initially, if a very serious incident, call security on 0151 904 2222 (ext. 2222) and they contact emergency servicesfor assistance if unsure of procedures.

Emergency action procedures – action required if a substance is,

Ingested:

Do not induce vomiting. Obtain medical attention.

Inhaled:

Move to fresh air. If breathing is difficult, give oxygen. Get medical attention immediately if symptoms occur.

Contact with skin:

Wash off immediately with plenty of water for at least 15 minutes. Get medical attention immediately if symptoms occur.

Contact with eye:

Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Obtain medical attention.

Injected:

Injection is not considered a potential route of exposure

In case of an emergency please contact:

Health and Safety Officer - Monica Barclay ext 6211 (0151 904 6211)
First aider - Monica Barclay ext 6211 (0151 904 6211)
First aider - Louise Coyne ext 6283 (0151 904 6283)
First aider - Ellen Dawson ext 6264 (0151 904 6264)
For a serious incident, call security on ext 2222 (0151 904 2222)and they contact emergency services.

In case of business interruption (e.g. power shutdown, flood), what are the Contingency Procedures for work and waste?
Leave items in the hoods, with lids on, vacate area and return when told it is safe to do so, ensure the CO_2 sensor is working before returning.

In case of fire or explosion: Raise alarm, immediately evacuate area, call security on ext 2222 (0151 231 2222 from an external line) and inform them of the location and source of the fire, they will contact Merseyside Fire and Rescue Service. Inform fire warden on site of the location and source of the fire.

Based on the COSHH information below, the overall risk of the procedure/protocol is low

COSHH material safety data – This needs to be completed for each chemical used in the procedure that is classed as harmful, toxic, corrosive, an irritant or poses any other risk to human health.

Hazardous substance: DMSO (Dimethyl Sulphoxide)	
What are the hazards and hazard codes? H319 Causes serious eye irritation H335 May cause respiratory irritation H315 Causes Skin irritation H240- Heating may cause an explosion H313- Harmful in contact with skin (readily penetrates skin and may carry other dissolved chemicals into the body)	
Is it solid, liquid or gas? Liquid	Quantity used: 1ml per 10 ml of cell suspension (min 1ml, max 10mls)
What is the route of entry into the body? Eyes, skin, ingestion and inhalation	What are the target organs? Skin, Liver, Kidney, Spleen
What is the working limit exposure (WEL)? This product does not contain any hazardous materials with occupational exposure limits established by the region specific regulatory bodies.	
What control measures, handling precautions and PPE are in place? Nitrile gloves and howie lab coat, protective eyeglasses or chemical safety goggles	
What is the disposal procedure (if different from page 2): Same as page 2	
What is the spillage procedure (if different from page 2): Same as page 2	
What are the emergency procedures (if different from page 2) if; All same as page 2 •	

For further information contact Health and Safety Unit (ext. 8167), refer to supplier's data sheet, contact supervisor.

Hazardous substance: Isopropanol (2-Propanol)	
What are the hazards and hazard codes? H225 Highly flammable liquid and vapour. H319 Causes serious eye irritation. H336 May cause drowsiness or dizziness.	
Is it solid, liquid or gas? Liquid	Quantity used: 1ml max 100ml
What is the route of entry into the body? Eyes, skin, ingestion and inhalation	What are the target organs?
What is the working limit exposure (WEL)? STEL- 500 ppm, 1,250 mg/m3 TWE- 400 ppm, 999 mg/m3	
What control measures, handling precautions and PPE are in place? Nitrile gloves and howie lab coat. Decanting is carried out in a Class II safety hood, with the glass sash at appropriate working height, therefore face shields are not needed for this procedure. Face shield and safety glasses are recommended.	
What is the disposal procedure (if different from page 2): Same as page 2	
What is the spillage procedure (if different from page 2): Same as page 2	
What are the emergency procedures (if different from page 2) Same as page 2 •	

Appendix J: The Brunel Mood Scale

The Brunel Mood Scale (BRUMS: Terry et al., 1999, 2003)

Subject Name:
Subject Code No:
Date:
Birth date:

Directions: Describe ***HOW YOU FEEL RIGHT NOW*** by circling the most appropriate number after each of the words listed below:

No	FEELING	Not at all	A little	Moderate	Quite a bit	Extremely
1	Panicky	1	2	3	4	5
2	Lively	1	2	3	4	5
3	Confused	1	2	3	4	5
4	Worn out	1	2	3	4	5
5	Depressed	1	2	3	4	5
6	Downhearted	1	2	3	4	5
7	Annoyed	1	2	3	4	5
8	Exhausted	1	2	3	4	5
9	Mixed up	1	2	3	4	5
10	Sleepy	1	2	3	4	5
11	Bitter	1	2	3	4	5
12	Unhappy	1	2	3	4	5
13	Anxious	1	2	3	4	5
14	Worried	1	2	3	4	5
15	Energetic	1	2	3	4	5
16	Miserable	1	2	3	4	5
17	Muddled	1	2	3	4	5
18	Nervous	1	2	3	4	5
19	Angry	1	2	3	4	5
20	Active	1	2	3	4	5
21	Tired	1	2	3	4	5
22	Bad tempered	1	2	3	4	5
23	Alert	1	2	3	4	5
24	Uncertain	1	2	3	4	5

Terry, P. C., Lane, A. M., Lane, H. J., & Keohane, L. (1999). Development and validation of a mood measure for adolescents. *Journal Of Sports Sciences, 17*(11), 861-872.
Terry, P. C., Lane, A. M., & Fogarty, G. J. (2003). Construct validity of the Profile of Mood States-Adolescents for use with adults. Psychology of Sport and Exercise, 4(2), 125-139. doi: 10.1016/S1469-0292(01)00035-8

The Brief Sexual Function Index (BSFI: O'Leary et al., 1995)

Subject Code No:
Date:

"Let's define sexual drive as a feeling that may include to have a sexual experience (masturbation or intercourse), thinking about having sex, or feeling frustrated due to lack of sex".

Sexual drive						
1	During the past 30 days, on how many days have you felt sexual drive?	None	Only a few	Some	Most	Almost every day
		0	1	2	3	4
2	During the past 30 days, how would you rate your level of sexual drive?	None at all	Low	Medium	Medium-high	High
		0	1	2	3	4

Erection						
3	Over the past 30 days, how often have you had partial or full sexual erections when you were sexually stimulated in any way?	Not at all	A few times	Fairly often	Usually	Always
		0	1	2	3	4
4	Over the past 30 days, when you had erections, how often were they firm enough to have sexual intercourse?	Not at all	A few times	Fairly often	Usually	Always
		0	1	2	3	4
5	How much difficulty did you have getting an erection during the past 30 days?	No erections	A lot of difficulty	Some difficulty	Little difficulty	No difficulty
		0	1	2	3	4

Ejaculation						
6	In the past 30 days, how much difficulty have you had ejaculating when you have been sexually stimulated?	No sexual stimulation	A lot of difficulty	Some difficulty	Little difficulty	No difficulty
		0	1	2	3	4
7	In the past 30 days, how much did you consider the amount of semen you ejaculate to be a problem for you?	Did not climax	Big problem	Medium problem	Small problem	No problem
		0	1	2	3	4

Problem assessment		Big	Medium	Small	Very small	No problem
8	In the past 30 days, to what extent have you considered a lack of sexual drive to be a problem?	0	1	2	3	4
9	In the past 30 days, to what extent have you considered your ability to get and keep erection to be a problem?	0	1	2	3	4
10	In the past 30 days, to what extend have you considered your ejaculation to be a problem?	0	1	2	3	4

Overall satisfaction		Very dissatisfied	Mostly dissatisfied	Neutral or mixed	Mostly satisfied	Very satisfied
11	Overall, during the past 30 days, how satisfied have you been with your sex life?	0	1	2	3	4

O'Leary, M. P., Fowler, F. J., Lenderking, W. R., Barber, B., Sagnier, P. P., Guess, H. A., & Barry, M. J. (1995). A brief male sexual function inventory for urology. Urology, 46(5), 697-706

Appendix L: Presentation and abstracts

Chan, K.Q., Stewart, C.S., Yusof, A., Hanim, S., and Chester, N. (2015). Effects of *Eurycoma longifolia*, a natural testosterone booster, on muscular force production and anaerobic power. Day 1. Posters – Physiology and Nutrition, *Journal of Sports Sciences*, *33*(Sup 1), s33 – 34.

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Day 1. Posters – Physiology and Nutrition

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To link to this article: https://doi.org/10.1080/02640414.2015.1110321

Published online: 25 Nov 2015.

D1.P20. Effects of *Eurycoma longifolia*, a natural testosterone booster, on muscular force production and anaerobic power

KAI QUIN CHAN[1*], CLAIRE ELISABETH STEWART[1], ASHRIL YUSOF[2], SAREENA HANIM[2], AND NEIL CHESTER[1]

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Eurycoma longifolia (EL) is a well-known herbal sup-plement in South East Asia, believed to improve sporting performance and strength in humans (George et al., 2013, Journal of Sports Medicine & Doping Studies, 3, 2; Hamzah and Yusof, 2003, British Journal of Sports Medicine, 37, 464–470). Eurycomanone, the active ingredient, is also claimed to boost testosterone levels in animal models (Low et al., 2013, Journal of Ethnopharmacology, 149(1), 201–207). The aim of this study was to assess the ergogenic properties of EL in relation to muscular force production and anaerobic power. The study was a matched, double-blind, placebo-controlled design; participants were supplemented with 600 mg · day^{-1} EL or placebo (maltodextrin) for 8 weeks. Following institutional ethical approval, 22 males (mean age = 22.8 years, s = 3.7; stature = 1.79 m, s = 0.08; body mass = 77.3 kg, s = 7.3)(mean ± SD) were recruited, matched according to body mass and assigned to treatment (n = 11) and placebo (n = 11) groups. Three laboratory visits were scheduled throughout the 8-week protocol. Exercise performance tests to assess peak muscle force pro-duction (Isokinetic dynamometry at 60° · s^{-1} and 240° · s^{-1}), anaerobic power (Wingate test) and body composition were performed. Supplementation of EL resulted in no significant changes ($P > 0.05$) in peak muscle force production for quadriceps (EL: 211.5 ± 27.8 N · m vs. Placebo: 209.7 ± 34.5 N · m) and hamstrings (EL: 126.2 ± 24.4 N · m vs. Placebo: 135.1 ± 22.3 N · m) at 60° · s^{-1}. Also, no differences ($P > 0.05$) in peak muscle force production were evident at 240° · s^{-1} for quadriceps (EL: 136.6 ± 23.1 N · m vs. Placebo: 130.9 ± 19.7 N · m) and hamstrings (EL: 86.4 ± 11.7 N · m vs. Placebo: 98.5 ± 15.7 N · m). There were no differences ($P > 0.05$) in anaerobic power (EL: 902.4 ± 254.4 W vs. Placebo: 841.0 ± 259.7 W), lean mass (EL: 66.9 ± 6.5 kg vs. Placebo: 67.4 ± 5.3 kg) or percentage body fat (EL: 10.2 ± 3.8% vs. Placebo: 10.1 ± 4.5%). The results suggest that EL supple-mentation, in the current dosing regimen, is insuffi-cient to impact on muscular force production, anaerobic power and body composition in young men, assumed to be eugonadal. Investigation of alternative EL dosing regimens on sports perfor-mance in hypogonadal participants is warranted. Studies to challenge the reputed mechanisms of EL supplementation are also necessary.

Chan, K.Q., Stewart, C.E., Yusof, A., Hamzah, S.H., and Chester, N. (2017). Eurycoma longifolia; a herbal supplement that interferes with testosterone negative feedback loop. In International Conference on Food Science and Nutrition. *Universiti Malaysia Sabah*.

ORAL PRESENTATION

Eurycoma Longifolia; a Herbal Supplement that Interferes with Testosterone Negative Feedback Loop

K.Q. Chan [1, 2, 3, *], C.E. Stewart [2], A. Yusof [3], S.H. Hamzah [3], and N. Chester [2]

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[3]*Department of Exercise Science, Sport Centre, University Malaya, 50603 Kuala Lumpur, Malaysia, ashril@um.edu.my; sareena@um.edu.my; kaiquin@siswa.um.edu.my*

Abstract

Eurycoma longifolia (EL) or "Tongkat Ali" is a supplement believed to increase testosterone levels and to improve sporting performance. In animal studies, EL is postulated to boost testosterone by interfering with its negative feedback loop. This study therefore aimed to assess the impact of EL supplementation in young healthy men. Following ethical approval and informed consent, sixteen males (24.4±4.7years; 1.74±0.07m; 73.7±8.4kg) in a double blind matched-paired study (n=8/group), received 600mg/day EL or placebo for 2 weeks. Blood samples were collected on days (D) 1, 2, 3, 5, 7 and 14 for analysis of androgens and liver functions. EL resulted in a significant increase (0.97 ng/ml) in testosterone ($p=0.043$) at D14 vs D1. Significant increases from D0 vs D14 (*$p<0.05$) in EL vs placebo were observed in free testosterone (27.6%*), Estradiol (17.0%*) and Luteinising-Hormone (LH;9.9%*). Changes in Follicular-Stimulating-Hormone (FSH; 7.6%), Sex-Hormone-Binding-Globulin (1.3%), Aspartate-Aminotransferase (3.1%) and Alanine-Aminotransferase (3.3%) were not different between groups. EL supplementation for 2 weeks in young healthy men significantly increased testosterone levels, however, the secretion of LH and FSH, did not decrease. These preliminary data, therefore suggest an interference of the testosterone negative feedback loop in the presence of EL. Importantly, the increase in testosterone is within the normal healthy range for humans and the liver function tests suggest the dosage is safe for human consumptions. In conclusion, consuming 600mg/day EL for 14 days, could have positive outcomes on testosterone and other anabolic hormone levels, which may hasten recovery, improve sporting performance and reduce the impact of decreasing testosterone with age.

Keywords

Herbal supplement, testosterone booster, hormones, health, performance

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"Eurycoma Longifolia; a Herbal Supplement that Interferes with Testosterone Negative Feedback Loop"

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Chairman
ICFSN 2017

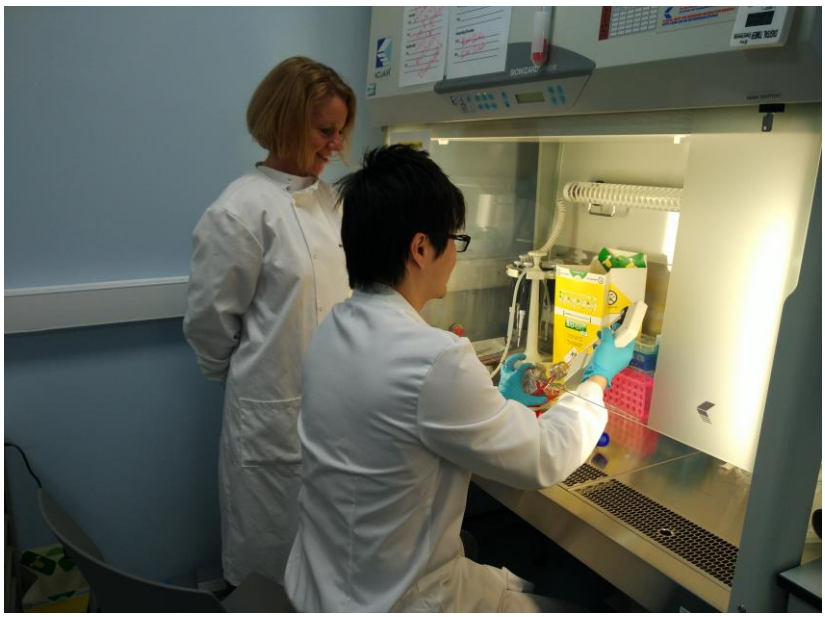
Professor Datuk Dr. D Kamarudin D Mudir
Vice Chancellor
UNIVERSITI MALAYSIA SABAH

Appendix M: Project photos

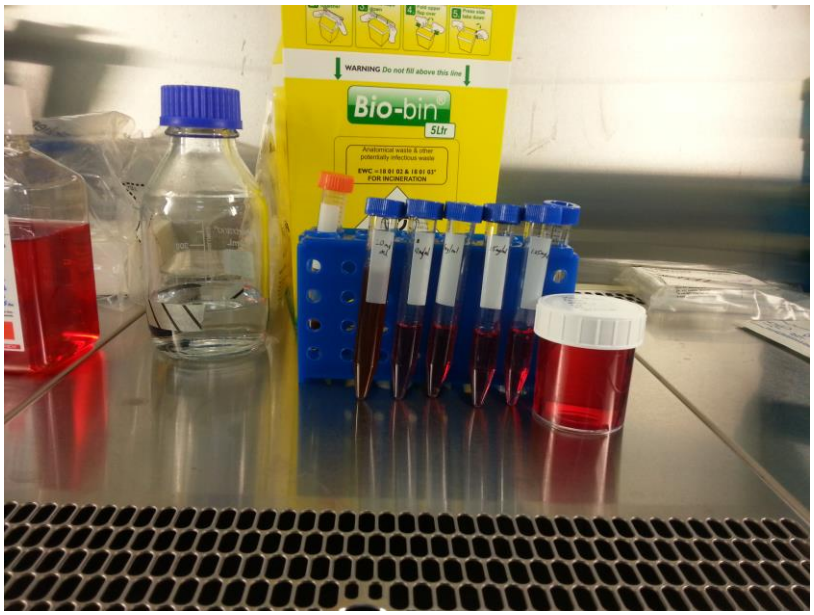
30 seconds Wingate Anaerobic test

Isokinetic muscular strength test

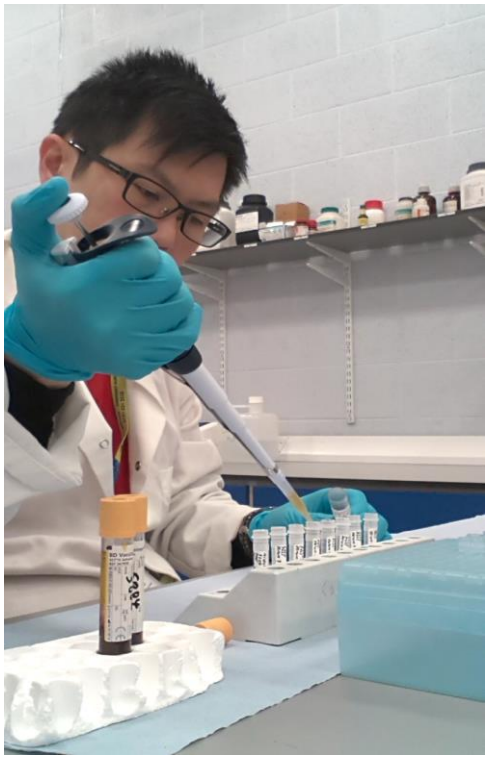
Vertical jump on the force platform



Cell culture project



Preparation of *E. longifolia* at different concentration.



Pipetting blood serum for reproductive hormone analysis

Performing a venipuncture using a butterfly needle to collect blood sample