



Regulation of androgens and steroidogenic enzymes gene expression in Sprague-Dawley rats treated with *Eurycoma longifolia* extract.

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Introduction

Eurycoma longifolia (EL) or also popularly known in Malaysia as 'Tongkat Ali' belongs to the Simaroubaceae family that is traditionally used to treat a variety of illnesses including fever, hypertension and tuberculosis. It is also claimed that consuming this plant could elevate energy level, increase endurance and stamina, enhance physical and mental performance, enhance immune system and also improve skin and muscle tone.¹ However, the extract is majorly consumed for its aphrodisiac property by men to elevate their manliness during sexual activities.² Recently, there are about 65 compounds that have been isolated from EL.³ This plant is rich in bioactive compounds which the major portion consists of quassinoids and alkaloids, eurycomaoside, eurylactone, eurycomalactone and eurycomanone.⁴

The peptides found in water extract of EL roots was claimed to enhance the biosynthesis of various androgens especially production of testosterone.⁵ Testosterone is important in development of male sexual characteristics and sexual function as well as stimulates anabolism⁶. There are many mechanisms involved in the elevation of serum testosterone level by EL. Quassinoids found in roots of EL have been claimed for its aphrodisiac activity⁷ which induce testosterone synthesis and increase luteinizing hormone and follicle-stimulating hormone levels.⁸ Eurycomanone also increases testosterone production by Leydig cell through the inhibition of phosphodiesterase and aromatase.⁸ Thus, this plant has been used as a natural alternative for testosterone replacement therapy but without its side effects.^{3, 9} Elevation of testosterone level, sperm count and motility in animal samples have also been linked to the presence of biological active peptide compound of 4.3 kDa.¹⁰

Although there are studies that showed that EL extract increases testosterone level, no studies have reported the regulation of dihydrotestosterone (DHT) which is another important androgen that has 3-10 fold higher potency than testosterone.¹¹ Additionally, to the best of our knowledge, no studies have reported the effect of EL on two major androgen precursors, i.e. dehydroepiandrosterone (DHEA) and androstenedione (ASD). Thus, the present study aimed to elucidate the effect of EL on the regulation of DHT, DHEA and ASD and the enzymes [i.e. cytochrome 17A1 type 3 (CYP17A1), 17 β hydroxysteroid dehydrogenase (17 β HSD) and 5 α reductase (type 2)] involved in the synthesis of these androgenic steroids (Figure 1).

Methods

Animal & Treatment

Sixteen healthy male Sprague-Dawley (SD) rats weighing between 150 and 250 grams were kept in individual ventilated cages (four animals per cage). The animals were acclimatized for 3 days prior to treatment. The rats were maintained in 12-h light and 12-h dark and were provided with food (Gold Coin Feedmills, Malaysia) and water supply *ad libitum*. The rats were divided into two groups, control group that received distilled water and treatment group that received 800 mg/kg body weight of EL aqueous extract (LJack Sdn. Bhd, Malaysia). The EL powdered extract was dissolved in distilled water and force-fed to animals daily for 14 days. Body weights of the rats were recorded daily and the health of the animals were monitored throughout the treatment duration. On day 15, animals were anaesthetized with 0.1 g/kg of Zoletil 100 followed by blood collection via

cardiac puncture. The blood sample were left overnight at 4°C and then centrifuged at 3000 rpm for 15 minutes to separate serum. The testes, epididymis, seminal vesicles and prostate gland were excised immediately weighed. Testis was frozen in liquid nitrogen and kept in -80° C until used for gene expression quantitation. Ethic approval for the present study was obtained from UiTM Care (Committee on Animal Research and Ethics: #198/2017).

Hormone assay

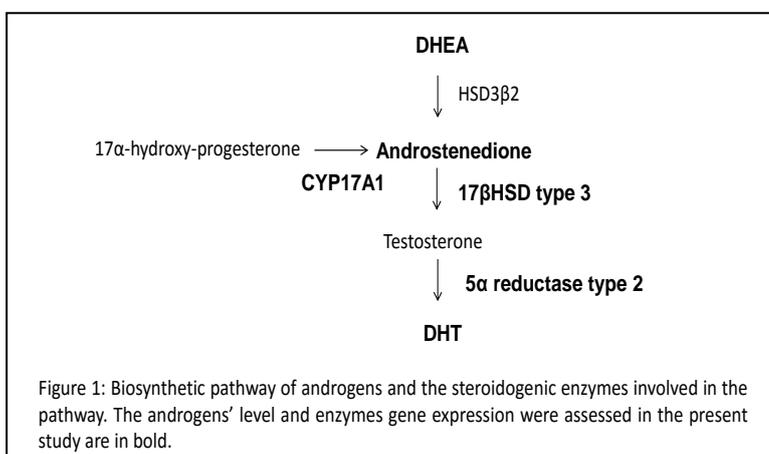
The DHT, DHEA and ASD serum level were measured using enzyme immunoassay kits according to the manufacturers' protocol. The DHT and ASD kits were purchased from Elabscience Biotechnology, China and DHEA were purchased from Shanghai Qayee Biotechnology, China.

Real-time polymerase chain reaction (RT PCR)

RNA extraction from tissue sample of the rat testis was conducted by using the RNeasy Plus extraction kit from Qiagen, Germany according to the manufacturer's protocol. Thirty mg of the testis tissue were used for the RNA extraction. RNA concentration was determined using the Microvolume Spectrophotometer (NanoDrop ND-1000). The complementary DNA was synthesized by reverse transcription using QuantiNova™ Reverse Transcription Kit. The RT PCR was conducted using QuantiNova SYBR® Green PCR Kit from Qiagen. The table below shows the primer sequence of the target genes (CYP17A1, 5 α reductase type 2, 17 β HSD type 3) and the house keeping genes (Gadph, ADH1 and Actb). Gene expression was calculated using relative quantitation method ($2^{-\Delta\Delta Ct}$).

Table 1: Primer sequence of target androgens and house keeping genes.

Gene name		Sequence (5' – 3')
Cyp17a1	Forward	ACGGTGGGAGACATCTT TGG
	Reverse	TGGTCAATCTCCTT TTGGATCTTC
5 α reductase type 2	Forward	ACATCCACAGTGACTACACCCTGC
	Reverse	TCC ATT CAA TAA TCT CGC CCAG
17 β HSD type 3	Forward	TTC TGCAAGGCTTTACCAGG
	Reverse	ACAAACTCATCGGGGCTCTT
Gadph	Forward	ACCACAGTCCATGCCATCAC
	Reverse	TCCACCACCCTGTTGCTGTA
ADH1	Forward	TGCTCCGTGCTGGAAAGATATCC
	Reverse	TAAGGTTGTGATGTGGCTGGCG
Actb	Forward	TTGCTGATCCACATCTGCTG
	Reverse	GACAGGATGCAGAAGGAGAT



Statistical Analysis

Descriptive statistics including the mean and standard deviation (SD) were performed by SPSS software (version 23.0). Significant difference between control and EL treatment group was determined by using independent T-Test (parametric data) and Mann-Whitney U Test (non-parametric data). Pearson's correlation coefficient was used for correlation of body weight changes during the 14 days of treatment. A P-value of < 0.05 was considered significant.

Results

Body and organ weights

Table 2 . Body and organ weight of control rats and EL treated group

Weight (g) (mean \pm SD)	Control	EL
Mean body	232.6 \pm 2.8	221.2 \pm 2.7*
Right testis	1.293 \pm 0.092	1.504 \pm 0.075
Left testis	1.300 \pm 0.099	1.526 \pm 0.083
Right epididymis	0.495 \pm 0.029	0.591 \pm 0.036
Left epididymis	0.490 \pm 0.027	0.528 \pm 0.032
Seminal vesicles	0.501 \pm 0.081	0.451 \pm 0.160
Prostate	0.885 \pm 0.038	1.144 \pm 0.023

** P<0.05 when tested against control group

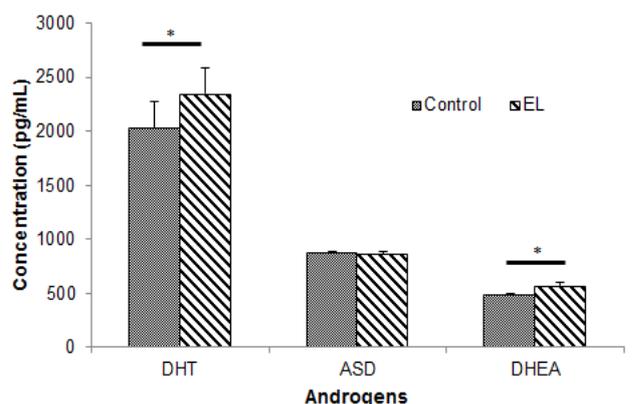


Figure 2: Serum concentration of androgens in control and EL treated animals. * P<0.05 when compared against control.

The results of mean daily body weight showed significant decrease for EL group compared to control ($P=0.004$) (Table 2). However, there were no significant changes in weight of reproductive organs compared to control (Table 2)

Androgen level

Figure 1 shows the androgens and the enzymes assessed in the present study. There was a significant increase in serum DHT ($P = 0.045$) and DHEA ($P = 0.027$) level of EL group when compared to control (Figure 2). However no change was seen in the ASD level in the EL treatment group compared to the control (Figure 2).

Gene expression

The 17β HSD type 3 expression showed a significant up-regulation ($p<0.05$) with fold change of 1.13 for EL treated rats. Meanwhile, gene expression of CYP17A1 and 5α reductase type 2 showed an up-regulation of 5 and 0.86 fold, respectively for EL group compared to control but was not significant.

Discussion

The present study assessed the effects of an aphrodisiac plant, EL on the regulation of androgens and the gene expression of the enzymes that is involve in its synthesis. The changes of body and reproductive organs weight were also evaluated. From the results of total body weight obtained, it shows that there were a significant decrease by 4.89% in weight between control and EL group ($P = 0.004$). There was also a moderate correlation of the weight changes during the 14 days of treatment within each of the groups (Control, $r=0.487$, $P=0.00$; EL treatment, $r=0.511$; $P=0.00$). Our findings concurred with study by Solomon et al. (2014) which has reported that there were also a decrease in body weight after treatment with EL but at much lower dose than this study. The authors postulated that the decrease in body weight is due to increase in testosterone and decrease in fat mass due to decrease in omentum fat. A study using drug aphrodisiac, Tadalafil however shows no significant changes in body weight after treatment at same length of time.¹²

Interestingly, we found that there was a significant increase in concentration of DHT of the rats treated with EL 800 mg/kg ($P=0.027$). This finding is novel as no previous study that has reported the effect of EL on the DHT level. Previous studies have reported the increase of serum testosterone level in a group of patients suffering from late-onset with hypogonadism with 200 mg per day of EL for 1 month.¹ The present study found that an up-regulation of 17β HSD type 3 ($P=0.043$) which is expressed exclusively in the testicular Leydig cells that convert ASD to testosterone. This supports the previous studies that reported increase in the testosterone level that is due to increase in the 17β HSD type 3.¹³ Circulating testosterone acts as a pro-hormone at target tissues to produce DHT by the irreversible 5α -reductase type 2 enzyme reaction.¹⁴ We found an up-regulation of 5α -reductase type 2 gene expression but it was not significant. DHT is also produced through the 'backdoor pathway' bypassing DHEA, ASD and testosterone¹⁵. It is possible that the increase of DHT was contributed by the 'backdoor pathway'. This is further supported with the levels of androgen precursor, ASD that did not change between the treated and control group ($p>0.05$). Although there was an increase in the CYP17A1 (converts 17α -hydroxy-progesterone to ASD) but it was not significant. The 3β HSD type 2 enzyme converts ASD to DHEA in the gonad and the adrenal.¹⁶ Although we did not find any changes in the ASD level but the DHEA was significantly increase in the EL treatment group compared to the control. Future study should assess the expression of 3β HSD type 2.

Conclusion

The present study shows that EL aqueous extract fed to SD male rats at 800 mg/body weight resulted in an increase in the androgenic steroids (DHT and DHEA) levels and the expression of enzymes involves in their synthesis. Here we have shown that the EL extract affect the androgenic steroids pathway.

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