Eurycoma longifolia aqueous extract increases sexual activities in male and female rats

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Introduction

Eurycoma longifolia Jack (Simaroubaceae), identified by its local name as Tongkat Ali is commonly found along the hilly jungle slopes of Malaysia. Pharmacological evaluations on this plant showed that it possessed antimalarial (Chan et al., 1986, 1989; Kardono et al., 1991; Ang et al., 1995), cytotoxic (Kardono et al., 1991; Morita et al., 1990, 1993; Itokawa et al., 1991), antiulcer (Tada et al., 1991) and antipyretic (Chan et al., 1995) properties. However, in Malaysia, it has gained notoriety as a male aphrodisiac since it is reputed to increase male prowess (Gimlette and Thompson 1977) and it is usually taken as a water decoction of roots. However, this is largely based on subjective rather than scientific verification. In vivo physiological studies employing sexually naive male rats treated with this extract exhibit increased sexual motivation; pendiculation, stimulated erectogenic effects and copulatory behaviour and improved sperm quality. However, the biochemical mechanism underlying its aphrodisiac properties especially with regard to its erectogenic effect is yet to be comprehended. In order to elucidate the mechanism of action of E. longifolia aqueous extract which has been reported to evince aphrodisiac properties, in vivo studies using male and female rats were carried out in this present study. Male and female Spraque Dawley rats were given various concentrations of aqueous extract of E. longifolia (0, 30, 60, 90, 150 mg/kg) orally for 7 days.

Materials and Methods

Animals and treatments

Male and female Spraque Dawley rats (3-4 months old) were given various concentrations of aqueous extract of E. longifolia (30, 60, 90, 150 mg/kg) orally everyday for 7 days. The control rats were given distilled water. They were kept under conditions of controlled temperature and relative humidity with water and standard laboratory food available. Each group consisted of 5 animals.

Sexual attractiveness test

This test was performed at the beginning of the dark phase of the light-dark cycle, under subdued light, in a quiet room with adequate ventilation. They were tested after dosing the sexually experienced male and female rats with the respective doses of E. longifolia aqueous extract from day 1 to day 7. Orientation activities or the sexual behaviour (mounting, licking, anogenital sniffing) of the male rats towards the receptive female rats and female rats towards male rats were tested according to the method previously described by Hull et al. (1984).

Surgical procedures

The day after each treatment, male and female rats were killed using chloroform and their blood were collected from the vein using 10ml syringe. Then, blood were centrifuged at 3000 rpm for 5 minutes to get the serum. Penis and brain were rapidly excised and snap - frozen in liquid nitrogen. Both organs were stored at –80°C until further experimentation.

Hormone assay

The mechanism of action of E. longifolia was studied by examining the hormonal profile which compared testosterone and dihidrotestosterone in male rats while in female rats estrogen (estradiol and estrone) and progesterone levels. Enzyme-linked immunoassay (EIA) kit from DRG International, Inc. USA was employed.
Time taken for the male rats to copulate the estrus female rats (seconds) on a graph showing the time taken for male rats treated with different concentrations of *E. longifolia* aqueous extract (0, 30, 60, 90, 150 mg/kg BW) to respond sexually towards the estrus female rats.

**Extraction of nitric oxide synthase brain and penis of rats**

Rats brain and penis from −80°C were left at room temperature and pooled with 10ml extraction buffer (0.05M Tris-HCl, 0.5M EDTA, 0.5M phenylmethylsulfonylfluoride, 2µg/ml leupeptin, 2% Triton X-100). Each sample were homogenized and the homogenates were allowed in the ice for 30 minutes. Homogenates were then centrifuged for 1 hour at 15 000 rpm at 4°C. The supernatant was collected and protein concentration determined according to Bradford (Bradford, 1976).

**Extraction of phosphodiesterase (PDE) enzyme in rats’ penises**

Penises from −80°C were allowed at room temperature and homogenized in extraction buffer (20Mm Tris-HCl, 2mM magnesium acetate, 5mM EDTA, 0.5M dithiothreitol, 0.1M phenylmethylsulfonylfluoride, 2000 U/ml aprotinin, 10mg/ml leupeptin). Homogenates were allowed in the ice for 30 minutes and then centrifuged at 17 000 rpm for 1 hour at 4°C. The supernatant was collected and protein concentration determined according to Bradford (Bradford, 1976).

**SDS – PAGE and western blot analysis**

Protein extracts were subjected to slab gel electrophoresis using 10% polyacrylamide separating gel and 5% polyacrylamide stacking gel. 30µg of each protein extract were separated on. Perfect protein markers (Sigma Chemical Company) were co-electrophoresed. After electrotransfer onto PVDF membrane (PerkinElmer Life Science) according to the method previously described by Towbin *et al.* (1979); nNOS, eNOS, PDE3, PDE4 and PDE5 subunits were immunodetected using specific affinity-purified antibodies (Transduction Laboratories) at dilutions 1:2500 for nNOS and eNOS, 1:2000 for PDE3, PDE4 and PDE5. The secondary antibody was an anti-mouse IgG linked to HRP (1:30000). Reacted proteins were revealed by enhanced chemiluminescence system (ECL-NEN Life Science Products). Blots were exposed to Kodak BioMax Light films (Eastman Kodak, Rochester, NY).

**Results**

**Effects of *E. longifolia* aqueous extract on sexual behaviour of male rats**

Sexual attractiveness test showed that *E. longifolia* increased the sexual behaviour with regard to the lesser time taken for the male to copulate the estrus female using four dosages of 30, 60, 90 and 150mg/kg BW (Figure 1). At the dosage of 30mg/kg BW, time taken for treated male rats to copulate the estrus female was only 70.0 ± 8.0 seconds compared to control 108.0 ± 11.0 seconds on day 1. Time taken for treated male rats to respond sexually towards the estrus female significantly decreased until day 7 (P<0.05) which only took 25.0 ± 9.0 seconds. At dosages of 60, 90 and 150mg/kg BW, sexual activities of treated male rats vigorously increased from day 1 to day 7 by the lesser time taken to copulate the estrus female (16.0 ± 4.0 seconds). It was showed that *E. longifolia* aqueous extract increased the sexual activities in a dose and time dependent manner.

**Effects of *E. longifolia* aqueous extract on sexual behaviour of female rats**

In order to determine the effects of aqueous extract of *E. longifolia* on the sexual behaviour of female rats, we conducted the sexual attraction test for female rats. As shown in Figure 2, time taken for the female rats to copulate towards the male rats were decreased with increasing dosages of the extract. This means that, the aqueous extract of *E. Longifolia* also increased sexual responsiveness of female rats.
Time taken for the female rats to copulate to the male rats (seconds)

FIGURE 2 Time taken (seconds) for female rats treated with different concentrations of *E. longifolia* aqueous extract (0, 30, 60, 90, 150 mg/kg BW) to respond sexually towards the male rats.

Testosterone levels (ng/ml)

FIGURE 3 Effect of different concentrations of aqueous extract of *E. longifolia* (0, 30, 60, 90, 150 mg/kg BW) on testosterone levels of male rats.

It was observed that on day 1, sexual responsiveness increased from 46% (160.0 ± 15.0 seconds) to 93.57% (25.0 ± 4.0 seconds) on day 7 at the highest dosage of 150 mg/kg BW of *E. longifolia* aqueous extract.

**Effect of *E. longifolia* aqueous extract on androgen levels of male rats**

Results showed that testosterone levels in treated male rats increased with treatment duration (Figure 3). A 100% (0.8 ± 0.1 ng/ml) increase of testosterone level was observed compared to the control on day 1 (0.4 ± 0.02 ng/ml) onwards until day 6 whereby testosterone levels reached almost at 663.2% (2.9 ± 0.04 ng/ml). However, on day 7, testosterone levels ‘slogged off’ to 500%, possibly because of the effect of DHT.

Testosterone levels were significantly enhanced in a time and dose dependent manner. Nevertheless, a negative feedback regulation occur at higher dosage (150 mg/kg BW). It was found from this study that *E. longifolia* aqueous extract gives the highest increase in testosterone levels at the optimum dosage of 90 mg/kg BW.

However, aqueous extract of *E. longifolia* increased DHT levels gradually from day 3 onwards at 30 mg/kg BW which is 250.0 ± 8.0 pg/ml and increased to 1380.0 ± 15.0 pg/ml on day 7 compared to control 49.0 ± 1.5 pg/ml (Figure 4). However, no significant increase of DHT were observed till day 7 for dosages 60 and 90 mg/kg BW.

For the treatment with 150 mg/kg BW, DHT levels increased from day 6 onwards. DHT does not play an active role in enhancing sexual activities. Nevertheless, its presence is essential for the maintenance of testosterone promoted sexual stimulations and activities.

Treatment with 60 mg/kg BW of aqueous extract of *E. longifolia* increased testosterone levels more than 30 mg/kg BW for each day of treatment. The testosterone levels on day 7 increased to 675% (3.1 ± 0.2 ng/ml). At 90 mg/kg BW, increased testosterone levels on day 1 by 400% (2.0 ± 0.05 ng/ml) and was augmented to 900% (4.0 ± 1.6 ng/ml) on day 7. Whereas, at the highest concentration (150 mg/kg BW) used in this experiment, testosterone levels subsided.

FIGURE 4 Effect of different concentrations of aqueous extract of *E. longifolia* (0, 30, 60, 90, 150 mg/kg BW) on Dihidrotestosterone (DHT) levels of male rats.
Effect of *E. longifolia* aqueous extract on sex hormones of female rats

Sexual activities always correlate with sex hormones. So, in order to determine the effect of aqueous extract of *E. longifolia* on female sexual behaviour, we also evaluated the effect of the extract on testosterone, DHT, progesterone, estrone and estradiol levels in female rats. Figure 5 shows that testosterone levels also increased in female rats but ‘slogged off’ with days of treatment. At 30mg/kg BW, testosterone levels increased by 118.8% (0.7 ± 0.02 ng/ml) compared to control group (0.32 ± 0.01 ng/ml) On day 2 to day 5 the levels of testosterone were decreased but not altered on days 6 and 7.

Increasing concentration of aqueous extract of *E. longifolia* at 60 and 90 mg/kg BW increased the testosterone levels but decreased at 150mg/kg BW. This is possibly because testosterone was converted to DHT. As shown in Figure 6, DHT levels increased on days 6 and 7 in a concentration dependent manner.

To evaluate the effect of the aqueous extract of *E. longifolia* on estrogen, we assessed the levels of estradiol (Figure 7) and estrone (Figure 8) in treated female rats. Results showed that at 30mg/kg BW, estradiol levels increased to 614.29% (50.0 ± 1.0 pg/ml) on day 1 but decreased with duration of treatment to 81.82% (10.0 ± 2.8 pg/ml) on day 4 and was unaltered until day 7. The profile of estradiol levels were similar for all concentrations used in this experiment. The aqueous extract of *E. longifolia* enhanced the levels of estradiol in the early days of treatment. Then, the estradiol levels ‘slogged off’ with treatment duration. On days 6 and 7, estradiol levels equalled to that of control.

On the contrary, estrone levels were not altered at all concentrations of *E. longifolia* aqueous extract until day 4. On day 5, estrone levels increased to 100% (20.0 ± 2.0 pg/ml) at 150mg/kg BW compared to the controls (10.0
 Estrone slightly increased on days 6 and 7. Estrone levels increased in a concentration dependent manner. At 30mg/kg BW, estrone levels increased to 50% (15.0 ± 1.7 pg/ml) on day 6 and 100% (20.0 ± 1.0 pg/ml) on day 7. At 60mg/kg BW, estrone levels increased to 100% (20.0 ± 2.0 pg/ml) and 150% (25.0 ± 1.5 pg/ml) on days 6 and 7 respectively. Estrone levels increased to 200% (30.0 ± 15.0 pg/ml) at 90mg/kg BW on day 7 but at 150mg/kg BW, the levels of estrone were unaltered. Decreased of estradiol levels and increased of estrone levels on days 6 and 7 showed that estradiol possibly change to estrone. Reciprocal effects on estrone and estradiol indicate a possible conversion of estradiol to estrone.

The significant increased of progesterone levels (P<0.05) in treated female rats were seen on day 4 for each dosage of the extract (Figure 9). However, at 30mg/kg BW, no significant (P>0.05) increases of progesterone levels from day 1 to day 7 were found. At 60mg/kg BW, progesterone levels increased significantly on day 5 (116.2%) to day 7 (140%). At 90mg/kg BW, progesterone levels were enhanced until day 5 (163.5%). Likewise, at 150mg/kg BW, progesterone levels increased with treatment duration. On day 1, progesterone increased to 37.5% (11.0 ± 2.0 ng/ml) and on day 7, the progesterone levels reached 166% (20.0 ± 1.0 ng/ml).

**Effect of E. longifolia aqueous extract on NOS and eNOS of male rats**

In order to determine the mechanism of action of E. longifolia aqueous extract, the effect of the extract on nitric oxide (NO) was monitored. NO which is a neurotransmitter in penile erection was assessed in this studies by measuring the rate limiting enzyme, nitric oxide synthase (NOS). The two isoforms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) were observed.

Results are presented in Figure 10 shows the aqueous extract of E. longifolia increased nNOS in brain with duration of treatment. At 30mg/kg BW, on the day 1, nNOS was slowly increased compared to control. This nNOS concentration was went up to 411.1% (OD = 46.0 ± 1.53) on day 7. A similar pattern was observed for dosages 60 and 90 mg/kg BW. However, by day 7, 150mg/kg BW extract augmented nNOS levels to almost 700% but lacked statistical significant (P>0.05). Initially, there was a decrease in nNOS levels
at 150mg/kg BW compared to 90mg/kg BW till day 6. This finding suggested that nNOS in the brain of male rats were increased concomitantly by the dosage 90mg/kg BW of aqueous extract of *E. longifolia*.

![Western blot analysis of nNOS in rat penises treated with different concentrations of aqueous extract of *E. longifolia* (30, 60, 90, 150 mg/kg BW). C= control. (b)Densitometric analysis for expression of nNOS in rat penises.](image)

(Apparently, penile nNOS and eNOS levels were also increased with increasing concentrations of aqueous extract of *E. longifolia* and treatment durations. Figure 11 demonstrates that levels of penile nNOS enhanced on day 3 until day 6 at dosages 30 and 60 mg/kg BW. On day 7, nNOS levels appears to have decreased but not significantly (P>0.05). On the contrary, at 90mg/kg BW, nNOS increased significantly until day 7 (P<0.05). The highest level of penile nNOS which is 83.3% (OD = 42.0 ± 2.08) was achieved at the dosage of 150mg/kg BW on day 7.

Since eNOS is present in the penile corpora cavernosa, most likely in the endothelium of sinusoids and blood vessels, it is important to determine whether the aqueous extract of *E. longifolia* also affects its expression. Figure 12(a) presents the effects of the extract on a single 140kDa eNOS polypeptide in penises. From Figure 12(b), it is evident that eNOS was enhanced from day 3 onwards at 30mg/kg BW. As for 60, 90 and 150 mg/kg BW, eNOS levels increased from day 1 onwards. eNOS increased with increasing concentration of aqueous extract of *E. longifolia* and time duration. The highest percentage increase of eNOS expression was 680% (OD = 39.0 ± 1.53) on day 7 at 150mg/kg BW compared to control (OD = 5.0 ± 1.0).

![Western blot analysis of eNOS in rat penises treated with different concentrations of aqueous extract of *E. longifolia* (30, 60, 90, 150 mg/kg BW). C= control. (b)Densitometric analysis for expression of eNOS in rat penises.](image)

**Effect of *E. longifolia* aqueous extract on brain NOS of female rats**

To gain further insight into the mechanism of action of aqueous extract of *E. longifolia*, we also assessed the effect of the extract on brain nNOS of female rats. Results presented in Figure 13 showed that nNOS brain increased with concentrations of *E. longifolia* and treatment duration. However, the expression of brain nNOS of female rats is lower than male rats. The highest level of brain nNOS of treated female rats is 640% on day 7, at 150mg/kg BW. (OD = 55.5 ± 1.528) when compared to the control group (OD = 7.5 ± 1.361).
Effect of E. longifolia aqueous extract on Phophodiesterase (PDE) levels of male rats

PDE3, PDE4 and PDE5 which occur in penis are also responsible for the erection process. The effect of the aqueous extract of E. longifolia on these PDEs were assessed to see whether this extract inhibited these enzymes or not. The present study found that this extract inhibited all three isoforms of PDE with increasing concentration and duration of treatment.

Figure 14 depicts the effect of various concentrations of E. longifolia aqueous extract on the expression of PDE3 in penis of male rats. PDE3 (130kDa) was gradually inhibited for all dosages (30, 60, 90 and 150 mg/kg mg/kg BW) till day 7 of treatment. The expression of PDE3 from day 1 at 30mg/kg BW (OD = 51.0 ± 2.52) was reduced by 83.3% by day 7 (OD = 8.5 ± 1.53). Likewise, PDE4 (140kDa) expression reduced by was 5.96% from day 1 (OD = 35.5 ± 3.01) to 78.57% (OD = 7.5 ± 1.53) by day 7 (Figure 15). A similar observation was noted with PDE5 (155kDa), whereby a marked reduction in PDE5 expression with dosage and treatment duration was apparent (P<0.05) (Figure 16).

(a) C 30 60 90 150 Day

(b) 0 10 20 30 40 50 60 70 Time (Day) Mean Density control 30mg/kg 60mg/kg 90mg/kg 150mg/kg

FIGURE 13(a) Western blot analysis of nNOS in female rats brain treated with different concentration of aqueous extract of E. longifolia (30, 60, 90, 150 mg/kg BW). C= control. (b) Densitometric analysis for expression of nNOS in brain of female rats.

FIGURE 14(a) Western blot analysis of PDE3 in rat penises treated with different concentrations of aqueous extract of E. longifolia (30, 60, 90, 150 mg/kg BW). C= control. (b) Densitometric analysis for expression of PDE3 in rat penises.

FIGURE 15(a) Western blot analysis of PDE4 in rat penises treated with different concentrations of aqueous extract of E. longifolia (30, 60, 90, 150 mg/kg BW). C= control. (b) Densitometric analysis for expression of PDE4 in rat penises.
FIGURE 16 (a) Western blot analysis of PDE5 in rat penises treated with different concentrations of aqueous extract of *E. longifolia* (30, 60, 90, 150 mg/kg BW). C= control. (b) Densitometric analysis for expression of PDE5 in rat penises.

**Discussion**

These present study provides evidence that the aqueous extract of *E. longifolia* acts specifically on sex hormones, neurotransmitter NO in penile erection and also PDE in an effort to enhance sexual performance and increase libido. The extract increased the levels of testosterone in congruence with DHT in male rats. The highest level of testosterone was observed when the extract at 90mg/kg BW was used. At the highest concentration used in this experiment (150mg/kg BW) testosterone levels ‘slogged off’. In medication, this feature is very useful because elevated levels of testosterone may lead to prostate cancer (Azimahtol, 1998).

In treated female rats, aqueous extract of *E. longifolia* also regulates the testosterone and DHT levels including estrogen and progesterone which are important hormones in female reproductive systems. Sexual behaviour of female rats increased with testosterone and DHT. It is interesting that DHT prevents the erectile failure. A notable feature of this extract which is down-regulation of estrogen dominance by up-regulation of progesterone is important in the treatment of cancer especially breast cancer and endometrial cancer. Estrogen at high levels is a cause for cancer onset. Increases in progesterone will suppress the dominant effects of estrogen. Hence, the aqueous extract of *E. longifolia* has the ability to regulate these hormones in a manner that increases sexual activities but is not detrimental.

From this studies, *E. longifolia* was also found to maintain penile erection. This extract acts via NO and PDE. Aqueous extract of *E. longifolia* increased NOS expression to form NO and also inhibited PDE3, PDE4 and PDE5. NO is known to be involved in a myriad of biochemical processes in the human body including smooth muscle relaxation (Nitric oxide 1999). Thippeswamy & Marris reported that NO produced by nNOS induced synthesis of cGMP. Penile erection is a NO-mediated process that has been shown to be androgen dependent. In penile erection, NO locates its target molecule of soluble guanylyl cyclase on the surface of corpus cavernosum smooth muscle cell and causes a conformational change in the enzyme that leads to an increase in the production of the second messenger guanosine 3’-5’-cyclic monophosphate (cGMP) from guanosine triphosphate (GTP). cGMP induces smooth muscle relaxation in the corpus cavernosum and allows blood to flow into the penis where it becomes trapped. The degradation and subsequent disappearance of cGMP from the smooth muscle tissue results in contraction and normal blood flow into and out of the corpus cavernosum. PDE5 which is dominant in penis, breaks down cGMP to GMP by catalyzing a reaction that breaks the phosphodiester bond (Garret *et al.*, 1999). *E. longifolia* helps maintain high levels of cGMP in the corpus cavernosum by suppressing PDE5 from breaking down cGMP. Inhibition of PDE5 increases the length of time that cGMP remains in the smooth muscle tissue and therefore increase chances of erection. *E. longifolia* also inhibits PDE3 and PDE4 which catalyzes breakdown of adenosine 3’,5’-cyclic monophosphate (cAMP). cAMP activates protein kinase A (PKA) which then leads to dephosphorylation of the light chain of myosin and initiate relaxation of smooth muscle. Therefore, cGMP and cAMP are the final products of several steps needed to initiate and maintain a penile erection. Since *E. longifolia* enhances cAMP levels which play a significant role in glycogen degradation
and energy metabolism, it can also be regarded as an ‘energy booster’.

In conclusion, *E. longifolia* has the potential to be a pharmacotherapeutic agent which is relatively safe to treat the often complex problem of impotence. Hence, this findings may explain the profound erectogenic effects of aqueous extract of *E. longifolia* in *in vivo* models.

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**References**


