Standardized quassinoid-rich *Eurycoma longifolia* extract improved spermatogenesis and fertility in male rats via the hypothalamic–pituitary–gonadal axis

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**Abstract**

*Ethnopharmacological relevance:* *Eurycoma longifolia* Jack, a small Simaroubaceae tree, known locally as 'Tongkat Ali' is popularly used as a sexual tonic in traditional medicine for aphrodisiac activity and improvement of fertility and male libido.

**Aim of the study:** To investigate the effects of the standardized bioactive fraction of *E. longifolia* and its chemical constituents on the male fertility and the mechanisms of action involved.

**Material and methods:** The powdered roots of *E. longifolia* were extracted separately with methanol and water. The organic extract upon further fractionation on HP 20 resin and elution with the methanol/water mixture afforded four fractions (F1–F4). These fractions, together with the crude aqueous (W) and organic extracts were standardized following their respective major quassinoid content and profile. The effects of the fractions on the rat spermatogenesis were compared with that of the aqueous extract (W) to determine the bioactive fraction. The effects of the bioactive fraction on the sperm count and quality, the histological morphometric changes on the spermatogenesis cycle, fertility and hormonal changes of plasma testosterone, luteinizing hormone (LH), follicle stimulating hormone (FSH) and estrogen in the animals upon oral administration were determined. The effects of the bioactive quassinoids on the testosterone release from the isolated testicular interstitial cells rich in Leydig cells, were also described.

**Results:** The male rats orally administered with 25 mg/kg of F2 and 250 mg/kg of W, significantly increased the sperm concentration when compared with that of the control animals (*P* < 0.05). High performance liquid chromatography analysis revealed that 25 mg/kg of F2 and 250 mg/kg of W were almost similar in concentration of eurycomanone, the major and most potent quassinoid. Microscopic morphometrical analysis of the rat testis following treatment with F2, showed significant increase in the number of spermatocytes and round spermatids at Stage VII of the spermatogenesis cycle when compared to that of the control (*P* < 0.05). The estimated spermatozoa production rate and the number of Leydig cells were also elevated (*P* < 0.001). The fertility index, fecundity index and the pup litter size delivered from the females after mating with the males treated with F2 were increased. The plasma testosterone level of the animals given 25 mg/kg of F2 orally was significantly different at day-26 (*P* < 0.05) and day-52 (*P* < 0.01) from those of control but was not different at day-104. The testicular testosterone also peaked in the animals treated with 25 mg/kg of F2 and was higher than that in the plasma. The plasma LH and FSH levels of the rats treated with 25 mg/kg of F2 were higher than those of the control (*P* < 0.001). In contrast, the plasma estrogen level was significantly lower than that of the untreated control. Amongst the isolated quassinoids of F2, eurycomanone and 13α(21)-dihydroeurycomaone significantly increased the testosterone level from the Leydig cells of the testicular interstitial cells cultured in vitro (*P* < 0.05).

**Conclusion:** The standardised extract F2 of *E. longifolia* and its major quassinoids especially eurycomanone improved the rat spermatogenesis by affecting the hypothalamic–pituitary–gonadal axis and the potential efficacy may be worthy of further investigation.

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1. Introduction

Infertility is a global problem that affects one in ten couples (Burns and Covington, 2006), where about 20% of them is due to the male partner (Raman et al., 2005). Over 90% of fertile males were diagnosed as oligoasthenozoospermia (Winston, 1986), due primarily to one or combination of factors that included lifestyle (frequent uses of electronic devices, wearing tight attire, horse riding, etc.), diet, working habits, (long hours of driving, stress, strenuous work, etc.) (Sharpe and Franks, 2002). Presently, infertile males have been treated by various medical procedures including in vitro fertilization (IVF) aided by semen intra-fallopian transfer, intra-cytoplasmic sperm injection (ICSI), surgical and hormonal therapy. These procedures are not only costly and uncomfortable, but have also causing socio-economic problems (Katz et al., 2002). As a result, alternative herbal remedies with a long history of usage and safety have resurfaced and are popularly sought (Adimoeja, 2000).

Eurycoma longifolia Jack, known as ‘Tongkat Ali’ in Malaysia and ‘Pasak Bumi’ in Indonesia, is traditionally used as a sexual tonic (Gimlette, 1971) besides the other remedies for boils, fever, bleeding gums and wound ulcer (Bhat and Karim, 2010). The quassinoids indigenously found in this Simaroubaceae family displayed anti-ulcer, antimalarial, antipyretic properties and cytotoxicity against cancerous cells (Tada et al., 1991; Chan et al., 1995, 2004; Kuo et al., 2004). Other studies have reported that the *E. longifolia* extracts improved the sexual performance in rats (Ang and Sim, 1998; Zanoli et al., 2009), increased the sperm quality (Noor et al., 2004), and reversed estrogen-induced infertility (Wahab et al., 2010) and *Andrographis paniculata*-induced oligosperma (Chan et al., 2009) in rats. In addition, clinical studies have shown that the sperm quality of idiopathic infertile males and the testosterone level of late-onset hypogonadism were improved when treated with the plant aqueous extract (Tambi and Imran, 2010; Tambi et al., 2011).

Despite the potential male reproductive pharmacology of *E. longifolia*, the chemical constituents contributing to the increase of sperm production and aphrodisiac activity have not been confirmed. Previous studies have suggested that a glycopeptide of 4.3 kDa in the aqueous extract of *E. longifolia* was the aphrodisiac marker (Asiah et al., 2007) and male fertility enhancer (Tambi et al., 2011). Hitherto, its absolute structural configuration is still not documented. Moreover, glycopeptides are also commonly found in other plants families and are not indigenously in the Simaroubaceae plants, especially *E. longifolia* (Asiah et al., 2007). Furthermore, the aqueous extract of *E. longifolia* also contained quassinoids, especially eurycomanone was reported in low concentration (Shuid et al., 2011; Tambi et al., 2011). In contrast, the crude methanol extract of *E. longifolia* contained amount of quassinoids > 2 % (w/w) including eurycomanone, but without any glycopeptides also displayed significant improvement in sperm concentration over the normal and oligospermic rats (Chan et al., 2009). Consequently, a standardized extract containing a higher quassinoid concentration of more than 10 fold of eurycomanone and its analogues, 13x(21)-epoxyeurycomanone, 13x,21-dihydroeurycomanone and eurycomanol was prepared following the method of Low et al. (2011). The detailed efficacy studies of the quassinoid-rich *E. longifolia* extract on the spermatogenesis cycle and fertility have not been investigated. Presently, more detailed studies were conducted to investigate the extract on the spermatozoa concentration and quality including the morphometrical changes in histology of the spermatogenesis cycle upon oral administration in male rats. The effects of the extract on the plasma levels of the follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone and estrogen, including studies of the quassinoids on the in vitro testostereone release from the testis interstitial cells rich in Leydig cells were also investigated to preliminary predict the mode of action.

2. Material and methods

2.1. Chemicals

The solvents for high performance liquid chromatography (HPLC) analysis were in analytical grade and were purchased from Merck (Darmstadt, Germany). All the chemicals were purchased from Sigma Chemicals (St Louis, USA). The external standard eurycomanone (EN; Purity > 96%), 13x(21)-epoxyeurycomanone (EP; Purity > 95%), 13,21-dihydroeurycomanone (ED; Purity > 95%) and eurycomanol (EL; Purity > 95%) were isolated and purified as described previously (Teh et al., 2011). The purity of the compounds was determined with Empower 2 workstation software (Waters, Milford, MA, USA) operated in a Waters Delta Prep HPLC system equipped with a Waters 2996 photodiode array detector.

2.2. Plant material and preparation of extract

*Eurycoma longifolia* roots were purchased from Biotropics Malaysia Berhad, Kuala Lumpur, Malaysia. A voucher specimen of the plant has been deposited at Penang Botanical Garden, Malaysia, with a reference no. of JT81189. The air-dried and powdered roots of *E. longifolia* (11.6 kg) were extracted with 6 x 4 L of 95% methanol for 6 days at 60 °C. The combined methanol extract upon evaporation to dryness yielded 485 g of dark brown residue which was next chromatographed on a Diaion HP 20 (Mitsubishi Chemical, Japan) packed-column. The residue was eluted with several gradient mixtures of H2O–MeOH at the ratio of 1:0 to 0:1 to obtain 4 fractions (F1–F4) (Low et al., 2011). For aqueous extract preparation, the powdered roots were extracted with distilled water at the ratio of 1:5 for 8 h per day at 45–50 °C for 5 days consecutively. The extract was then filtered and the filtrate was concentrated in vacuo at ambient temperature followed by freeze-drying to yield 6.8% (w/w) of crude aqueous extract (W).

2.3. Analysis of quassinoid in *E. longifolia* extract by HPLC

The high performance liquid chromatographic (HPLC) analysis of the bioactive extract quassinoids was carried out using an Agilent Technologies 1120 Compact LC system (Waldbronn, Germany) with TC-C18 column (Agilent Technologies, Santa Clara, CA, USA, 4.6 mm i.d. x 250 mm, 5 µm), pre-connected with a guard column (Zorbax SB-C18, 4.6 mm i.d. x 12.5 mm, 5 µm, Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisting of acetonitrile/distilled water (8:92, v/v), was delivered at a flow-rate of 0.9 mL/min at ambient room temperature. Ultraviolet detection was performed at a wavelength of 238 nm for quantification of EN, EP, ED and 210 nm for EL in the extracts. In order to make standard calibration curves, the stock solution (500 µg/mL) of each quassinoid was diluted with deionized water to give a range of 0.1–10.0 µg/mL prior to injection into the HPLC column. The quassinoid calibration curves were developed from the plot of peak height against the respective concentration. The fitting equations of the four quassinoids were as follows: EN, y = 27349x − 2233.5 (r² = 0.999); EP, y = 15081x − 208.02 (r² = 0.999); ED, y = 9882.9x − 89.07 (r² = 0.999) and EL, y = 6624.2x + 628.67 (r² = 0.999).

2.4. Animals

Sprague-Dawley male rats weighing 230–270 g obtained from the Animal Research and Service Centre, Universiti Sains Malaysia, Penang, Malaysia, were fed with a commercial pellet diet (Gold Coin, Penang, Malaysia) and the water was provided ad libitum. All the animals were kept under controlled ventilation and hygienic...
condition with 12:12 h light:dark schedule at ambient room temperature. All animal experimentation was humanely conducted and maintained in accordance with Guide for the Care and Use of Laboratory Animals 2010 Animal Research and Service Centre, Universiti Sains Malaysia and European legislation (EEC n. 86/609). The experimental designs and procedures were approved by the Animal Ethics Committee of the university.

2.5. Bioactive fraction determination

Male rats, weighing 250–280 g were divided into seven groups of six animals each. The control group was given distilled water only, whereas the other four groups were treated with 4 different fractions of *E. longifolia* (F1, F2, F3 and F4) at a dose of 25 mg/kg body weight. The remaining two groups of animals were treated with 25 mg/kg (W25) and 250 mg/kg (W250) of *E. longifolia* aqueous extract (W). All treatments were conducted orally with a metal oropharyngeal cannula (Popper and Sons Inc., UK) for 48 days consecutively. The animals were then sacrificed and determined for sperm concentration, sperm morphology and motility. The dose of 25 mg/kg chosen for the present study was derived from the preliminary dose-response studies of F2 at 5 to 50 mg/kg orally administered daily to the rats to investigate the spermatogenesis response (Supplementary Fig. 1). The results showed that a significant increase in spermatogenesis was observed from 10 to 25 mg/kg, and a maximum plateau response was seen at 25 and 50 mg/kg (Supplementary Fig. 1).

2.6. Spermatogenesis histological morphometric analysis

Male rats weighing 250–280 g were divided into two groups consisted of eight animals each. One group was given distilled water as control and the other group was orally administered with 25 mg/kg of F2 for 52 days consecutively. Testes from both control and treated animals were removed and the volume of each testis was measured by water displacement (Wing and Christensen, 1982). The testes were fixed in Bouin’s solution (Sigma Aldrich, St Louis, MO), dehydrated and subsequently embedded in paraffin before sectioning to a thickness of 3 μm. The sectioned tissues were stained with haematoxylin-eosin prior to the following spermatogenesis morphometrical analysis. The relative volume of the seminiferous tubules and the interstitial tissue was determined from the number of points over the respective structure divided by the total 25-point measuring over the section of the testicular tissue (Bourjadj et al., 1995; Wing and Christensen, 1982). The mean of the twenty measurements for each tissue represented the relative volume of the structure in each testis.

About one hundred seminiferous tubules for each testicular tissue were randomly sampled and the respective profile of each tubule was identified according to Russell et al. (1990). Ten stage VII seminiferous tubules for each testicular tissue were randomly selected and the crude counts of the Sertoli cells and the differentiated germ cells in the tubules were recorded. Subsequently, the true count was determined after the crude count values were corrected with the respective nuclear diameter of each cell type and the section thickness (Abercrombie, 1946; Russell et al., 1990).

The relationships between the volume, length and surface area of the seminiferous tubules were derived from the cylindrical tube model which considered the seminiferous tubule as a round tube (Wing and Christensen, 1982). The radius, diameter and the height for the germlinal epithelium of each seminiferous tubules (n=20 per testicular tissue) were measured using a graduated ocularmater. The cross-sectional area of seminiferous tubules (n=20 per testicular tissue) was then determined from the radius of the respective tubule. As the total volume of the seminiferous tubules could be calculated by multiplying the relative volume with the volume of the testis, therefore, the total length of the seminiferous tubules could be determined from the division of their total volumes by the cross-sectional area of the seminiferous tubules (Wing and Christensen, 1982).

The daily production of sperm per testis was estimated from round spermatids true count values divided by the respective duration of the seminiferous epithelium cycle preceded from stage I to stage X in rats (Wing and Christensen, 1982). The time necessary for the cycle to proceed from stage I to stage X is corresponding to 9.5 ± 0.2 days of the total complete duration 12.9 days in Sprague-Dawley rat, consistent with the earlier studies (Clermont and Harvey, 1965; Wing and Christensen, 1982). The number of Leydig cells per unit volume of the testis was determined as described previously (Chan et al., 2009).

2.7. Fertility study

Twenty males (300–320 g) and forty females (220–250 g) of Sprague-Dawley rats were selected for the study. Half of the males received distilled water alone as control, whereas the other half took 25 mg/kg of F2 daily for 48 days consecutively prior to mating. Two adult virgin females were housed together with one male. The females were examined daily for successive copulation by vaginal smear. Day-0 of gestation was identified when sperm were found in the smear. The numbers of pups given birth was counted and their gender determined. The mating, fertility, fecundity or pregnancy, gestation and viability indices were calculated by the following equations (Bailey et al., 2009):

\[
\text{Mating index} = \frac{\text{Number of copulated female}}{\text{Number of housed female}} \times 100\%
\]

\[
\text{Fertility index} = \frac{\text{Number of female given birth}}{\text{Number of housed female}} \times 100\%
\]

\[
\text{Fecundity index} = \frac{\text{Number of female given birth}}{\text{Number of copulated female}} \times 100\%
\]

\[
\text{Gestation index} = \frac{\text{Litter with live pups}}{\text{Number of pregnancy}} \times 100\%
\]

\[
\text{Viability index} = \frac{\text{Number of live pups}}{\text{Number of pups born alive}} \times 100\%
\]

2.8. Determination of testosterone, estrogen, luteinizing hormone and follicle stimulating hormone levels in the plasma and testosterone level in the testis homogenates

Male rats weighing 260–280 g were divided into four groups consisting of six animals each. One group was given distilled water as control and the other groups were treated with F2 at the doses of 10, 25 and 50 mg/kg for 48 days consecutively. All animals were sacrificed at the end of the study. The blood of the animals was collected by cardiac puncture and centrifuged at 3000 rpm for 15 min to collect the plasma prior to testosterone determination (Section 2.12). The luteinizing hormone (LH), follicle stimulating hormone (FSH) and estrogen levels in the plasma of the group orally administered with 25 and 50 mg/kg were determined (Section 2.12). The testes of the control and treated animals at the different doses were removed, kept in phosphate buffer saline (pH 7) and subsequently homogenized. An aliquot of the homogenized testes was centrifuged at 4000 rpm for 5 min and were subjected to testosterone level determination (Section 2.12).
2.9. Preparation of testicular interstitial cells

Adult male Sprague-Dawley rats weighing 300–350 g were euthanized by CO₂ asphyxiation and the testes were aseptically removed. The testicular interstitial cells was isolated following the collagenase dispersion method described by Browning et al. (1983) and Tsai et al. (1997) with slight modification. Briefly, six testes were decapsulated and were put into a 50 mL polypropylene tube containing culture medium (2 mL/testis) supplemented with 10 mg collagenase (bovine, Type II, Sigma Chemicals, St Louis, USA). The culture medium was made up of 0.1% bovine serum albumin (BSA, Fraction V, Sigma Chemicals, St Louis, USA) in tissue culture medium M199 (Sigma Chemicals, St Louis, USA), with N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES, 25 mM, Sigma Chemicals, St Louis, USA), sodium bicarbonate 0.22 g/L (Bendosen Laboratory Chemicals, Selangor, Malaysia), penicillin-G/streptomycin 100 IU/mL (Gibco®, Invitrogen™, Carlsbad, CA, USA), heparin 2550 units/L (Leo Pharma, Ballerup, Denmark) and aerated with 95% O₂ and 5% CO₂. The tubes were incubated at 34°C in a water bath shaken at 120 strokes/min. The digestion was stopped after 15 min of incubation by adding cold culture medium. The tube was allowed to stand for five min and was then filtered through two-layer nylon of 200 μm mesh size. The cell pellets collected after centrifugation at 300 g for 5 min, were washed with lysis buffer (0.5 mL/testis) to disrupt the red blood cells (RBCs) and were immediately replenished with 10 fold culture medium. The cell pellet was collected after centrifugation (300 g for 5 min at room temperature) and resuspended with culture medium (1 mL/testis). The viability and the concentration of the cells were determined by staining with trypan blue and haemocytometer measurement. Total cell proteins were determined (Bradford, 1976). The 3β-hydroxysteroid dehydrogenase (HSD) staining method was used to measure the abundance of Leydig cells in the preparation (Browning et al., 1983; Dirami et al., 1991). The preparation contained approximately 40% of Leydig cells with a viability of > 97%.

2.10. Effect of E. longifolia quassinoid-rich extract and quassinoid on testosterone production in testicular interstitial cells

Forty mL aliquot of cell suspensions (2.0 × 10⁵ cells/mL) pre-incubated with the culture medium at 34°C under a controlled atmosphere (95% O₂ and 5% CO₂) were prepared as stock and then 500 μL were dispensed into each microwell of the 24-well plate. A 100 μL solution of the E. longifolia quassinoid-rich extract (F2) at 0.02 and 0.2 mg/mL and EN, EP, ED and EL of 1.0 μM was next added to each well. The dose of 1 μM was derived from the dose-response studies of EN on the testosterone release from the testicular interstitial cells rich in Leydig cells and was 5-fold more than the ED₅₀ value of 0.2 μM for the quassinoid but was 10,000 fold less than highest cytotoxicity dose of 10 mM tested showing non cytotoxic with more than 95% viability (unpublished results). As EN, EP, ED and EL in the extract were quassinoid analogues with slight structural modifications, the dose selected from the ED₅₀ value of EN may therefore be appropriate to compare the quassinoid efficacies. EN alone at 0.1, 1.0 and 10.0 μM was also incubated with the isolated cells in the absence or presence of human chorionic gonadotropin (hCG, 0.05 IU/mL, Sigma Chemicals, St Louis, USA). After 2 h of incubation, 500 μL of ice-cold phosphate buffer saline with gelatin (PBSG, 0.1% gelatin (Sigma Chemicals, St Louis, USA) in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.5) were added to stop the incubation. The medium was then centrifuged at 4°C, 1000 g for 15 min and stored at −20°C prior to testosterone concentration determination (Section 2.12). Each experiment was repeated in three independence replicates and the testosterone level was measured in duplicate.

2.11. Sperm analysis

The cauda epidydimal semen fluid was collected by diffusion method with Dulbecco’s phosphate buffer saline as previously described (Remie, 2000). The sperm count was performed using Neubauer haemacytometer following WHO Laboratory Manual (1999) and the concentration was expressed as million/mL/g after correction with the testicular weight of the respective animals. The sperm motility was determined using the hanging drop preparation method as described by Akbarsha and Murugaiyan (2000). The percentage normality of spermatozoa morphology and motility were determined following WHO Laboratory Manual (1999).

2.12. Testosterone, estrogen and gonadotropins determination using enzyme-linked immunosorbent assay (ELISA) method

The testosterone and estrogen concentrations were measured using enzyme-linked immunosorbent assay kits purchased from Cusabio Biotech Co. Ltd. (Wuhan, China). The standard curves were constructed with the concentrations ranging from 0.1–25.6 ng/mL for testosterone and 40–1000 pg/mL for estrogen. The concentrations of the rats’ luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the rats’ plasma were determined using enzyme-linked immunosorbent assay kit purchased from Creative Diagnostics (New York, USA). The standard curves of LH and FSH were constructed with the concentrations ranging from 78–5000 μIU/mL and 1.25–80 mIU/mL, respectively.

2.13. Statistical analysis

The statistical comparisons of the results were conducted using parametric and non-parametric analysis. The data were presented as mean ± SEM (standard error mean). The significant levels of the sperm concentration, normality in morphology, motility and testicular morphometry parameters between groups were analyzed using Student’s t-test and one-way analysis of variance (ANOVA) with post-hoc multiple comparisons of Tukey’s HSD test. The significant difference of the means between groups of the hormone analysis was determined using Mann–Whitney U tests. Significant levels of the mating index, fecundity index and fertility index were calculated using Chi-square test. The confidence level at P < 0.05 was considered as statistically significant.

3. Results

The high performance liquid chromatography (HPLC) analysis revealed that E. longifolia fraction 2 (F2) contained 14.49 ± 0.26%, 7.39 ± 0.17%, 0.72 ± 0.06% and 9.54 ± 0.22% (w/w) of EN, EP, ED and EL, respectively (Fig. 1). The total amount of the four quassinoids in the extract was approximately 32.14% (w/w). Besides, fraction 3 (F3) contained 1.04 ± 0.11%, 0.31 ± 0.30%, 0.48 ± 0.13% and 2.90 ± 0.80% (w/w) of EN, EP, ED and EL, respectively. In contrast, the four quassinoid were not detected in fraction 1 (F1) and fraction 4 (F4). The E. longifolia aqueous extract contained 1.54 ± 0.06% (w/w) of EN and approximately 1.03% (w/w) of total EP, ED and EL.

The sperm concentration analysis of the animals treated with F1, F2, F3, F4 (at 25 mg/kg each) and the aqueous extract (W25 at 25 mg/kg and W250 at 250 mg/kg) and control are presented in Table 1. Amongst the four fractions, the animals treated with F2 at 25 mg/kg showed significantly higher sperm concentration than
those of the rest including the control \((P < 0.001)\). The sperm concentration of the animals treated with 250 mg/kg of \textit{E. longifolia} water extract (W250) was also significantly higher than control \((P < 0.001)\) and was comparable with that of F2. The morphology and motility of the sperm from the F2-treated animals were unchanged and similar to those of the untreated animals.

Upon oral administration of F2 (25 mg/kg), the mean testicular weight of the animals was higher than that of the control \((P < 0.01)\) (Table 2). The morphometrical analysis of the rat testicular tissues treated with 25 mg/kg F2 for 52 days showed that the height of the germ cells in the seminiferous tubules was significantly larger than the control \((P < 0.01)\). However, the diameter and the total length of the seminiferous tubules per testicular weight were not significantly different from those of the control. The corrected number of preleptotene spermatocytes, pachytene spermatocytes and round spermatids at Stage VII of spermatogenesis cycle was significantly higher than that of the control \((P < 0.05)\). From the round spermatid count, the estimated spermatozoa production rate corrected with the respective testicular

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**Fig. 1.** Representative chromatograms of \textit{Eurycoma longifolia} fractions, (a) Fraction 1, F1; (b) Fraction 2, F2; (c) Fraction 3, F3; (d) Fraction 4, F4 and (e) aqueous extract. Chromatograms were obtained at detection wavelength 210 nm, presented in black (top) and 238 nm in red (bottom). Eurycomanone (EN, 238 nm, RT: 25.30 min), 13\((21)\)-epoxyeurycomanone (EP, 238 nm, RT: 18.87 min), 13,21-dihydroeurycomanone (ED, 238 nm, RT: 26.66 min) and eurycomanol (EL, 210 nm, RT: 48.34 min). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).
Data are presented in mean ± SEM (n=6). Significant differences are marked with ***P < 0.001.

Table 2
Effect of quassinoid-rich *E. longifolia* F2 (25 mg/kg) on testicular morphometry parameters compared to the control.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>F2 (25 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Testicular weight (g)</td>
<td>2.57 ± 0.08</td>
<td>2.76 ± 0.10</td>
</tr>
<tr>
<td>Seminiferous tubules diameter (mm)</td>
<td>1.02 ± 0.02</td>
<td>1.05 ± 0.03</td>
</tr>
<tr>
<td>Seminiferous tubules diameter (mm)</td>
<td>0.56 ± 0.01</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>Germ height (mm)</td>
<td>2.58 ± 0.04</td>
<td>2.60 ± 0.05</td>
</tr>
<tr>
<td>Length of ST (m)</td>
<td>1.20 ± 0.03</td>
<td>1.24 ± 0.04</td>
</tr>
<tr>
<td>Corrected true count (Stage VII)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sertoli cells (Sc)</td>
<td>17.07 ± 0.56</td>
<td>19.00 ± 1.06</td>
</tr>
<tr>
<td>Preleptotene (Pl)</td>
<td>37.34 ± 1.38</td>
<td>48.39 ± 3.48</td>
</tr>
<tr>
<td>Pachytheme (Pc)</td>
<td>52.14 ± 2.96</td>
<td>62.24 ± 3.00</td>
</tr>
<tr>
<td>Round spermatid (Rs)</td>
<td>91.11 ± 3.29</td>
<td>127.40 ± 6.87</td>
</tr>
<tr>
<td>Production rate</td>
<td></td>
<td></td>
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<tr>
<td>(× 10⁶/days)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leydig cell</td>
<td>39.55 ± 10.44</td>
<td>52.96 ± 2.72</td>
</tr>
<tr>
<td>Fertility parameters</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of housed female</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>No. of housed male</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mating index (%)</td>
<td>60.00 ± 7.50</td>
<td>75.00 ± 10.00</td>
</tr>
<tr>
<td>Fecundity index (%)</td>
<td>75.00 ± 10.00</td>
<td>100.00*</td>
</tr>
<tr>
<td>Fertility index (%)</td>
<td>40.00 ± 7.50</td>
<td>75.00*</td>
</tr>
<tr>
<td>Viability index</td>
<td>98.15 ± 9.43</td>
<td>100.00*</td>
</tr>
<tr>
<td>Sex ratio index (M:F)</td>
<td>25.28 ± 8.89</td>
<td>86.89</td>
</tr>
<tr>
<td>Litter size</td>
<td>6.75 ± 0.7</td>
<td>11.67 ± 0.96*</td>
</tr>
</tbody>
</table>

Data are presented in mean ± SEM. Significant differences are marked with *P < 0.05, **P < 0.01.

The testosterone level in the testis homogenates of the animals treated with F2 at the dose of 25 mg/kg was significantly elevated compared to that of the control (P < 0.05) (Fig. 2). Moreover, the plasma testosterone level also increased significantly (P < 0.05) compared to lower dose of 10 mg/kg F2 and the control. The testosterone levels in the plasma and testis homogenates of the animals treated with 50 mg/kg F2 was not significantly different compared to that of the control (Fig. 2). The concentrations of the plasma luteinizing hormone (LH) and follicle stimulating hormone (FSH) of the F2-treated animals at 25 mg/kg were significantly higher (P < 0.001) than the control (Fig. 3a). However, at 50 mg/kg, the concentrations of LH and FSH were significantly reduced (P < 0.001) compared to that of 25 mg/kg. In contrast, the plasma estrogen level of the animals treated with 25 mg/kg was significantly reduced (P < 0.05), but at 50 mg/kg was not significantly elevated when compared to that of control (Fig. 3b).

The in vitro study of the F2 (0.02 mg/mL) and the four quassinoids EN, EP, ED and EL on the testicular interstitial cells containing about 20–40% of Leydig cells, revealed that the EN and ED significantly increased the testosterone secretion of the cells.
Po.05) compared to that of the control (Fig. 4). Furthermore, the testosterone elevation effect of the EN (0.1–10.0 μM) was dose-dependent (Fig. 5). The presence of positive control human chorionic gonadotropin (hCG, 0.05 IU/mL) significantly increased the testosterone level of the incubated cells (Po.05). The combination of EN at 10.0 μM and hCG (0.05 IU/mL) significantly increased the testosterone level (Po.05) compared to that of control and the hCG alone (Fig. 5).

4. Discussion

Earlier studies have revealed that the methanolic extract of E. longifolia improved the fertility of male rats by increasing the sperm concentration of the normal and oligospermic animals (Chan et al., 2009). To determine the bioactive chemical constituent of E. longifolia responsible for the improvement of spermatogenesis, the crude organic extract was further fractionated following bioactivity-guided separation of the chemical constituents, using the resin column chromatography to yield four fractions (F1–F4). The fraction F2 containing about 32.1% (w/w) of total quassinoids significantly increased the sperm concentration of rats at the dose of 25 mg/kg. Eurycomanone (EN) was the highest in concentration when compared to that of the other three quassinoids 13a(21)-epoxyeurycomanone (EP), 13,21-dihydroeurycomanone (ED) and eurycomanol (EL). Interestingly, the sperm concentration of the animals treated with 250 mg/kg of E. longifolia aqueous extract was similar in the sperm concentration of the F2 treated animals at 25 mg/kg. In contrast, the sperm concentration of the animals treated with 25 mg/kg of E. longifolia aqueous extract was not significantly different from that of the control. High performance liquid chromatography (HPLC) analysis revealed that the aqueous extract of E. longifolia contained 1.1%w/w of EN. As the content of EN was the highest in the E. longifolia bioactive extract, 250 mg/kg of the aqueous extract therefore contained 2.5 mg/kg of EN which was not far from the EN content (14.4%) of F2 at 25 mg/kg (containing 3.6 mg/kg of EN). Thus, EN and other quassinoids in F2 and the aqueous extract may play an important role in the spermatozoa production.

Along with the elevation in sperm concentration following the oral administration of F2 for 52 days consecutively, the increase of the male fertility index, fecundity index and litter size from the copulated females was observed. The increase in sperm concentration following the F2 treatment was further confirmed by the microscopic morphometry analysis of the testicular tissue showing that the populations of preleptotene spermatogonia, pachytene spermatocytes, round spermatids, spermatozoa production rate, the height of germinal epithelium and Leydig cells count were elevated. In the morphometrical studies, the Stage VII of the spermatogenesis cycle was chosen to evaluate the quantitatively changes in spermatogenesis because it contained variable differentiated germ cells such as...
as spermatagonia A, preleptotene, pachytene and round spermatids at Steps 18 and 19 (Singh and Singh, 2008). Thus, the increase of the sperm concentration upon treatment from *E. longifolia* was confirmed not only from the exogenous quantitative method of sperm counting using a cytometer chamber but also from the microscopic morphometrical studies providing more detailed spermatogenic cellular evidences than those provided from the earlier studies (Wahab et al., 2010).

The development and maintenance of the male reproductive system and the secondary sexual characteristics of males are mediated by testosterone. The production of testosterone by Leydig cells in the testes is influenced by the luteinizing hormone (LH) secreted from the anterior pituitary (Prakash, 2007). Beside LH, the follicle stimulating hormone (FSH) secreted from the anterior pituitary will bind to the Sertoli cells to facilitate spermatogenesis in seminiferous tubules (Prakash, 2007). The secretion of FSH and LH by pituitary gland is stimulated by the gonadotropin releasing hormone (GnRH) from the hypothalamus and back regulated mainly by the estrogen via the hypothalamic–pituitary–gonadal axis (Prakash, 2007). In the present study, the testosterone level of the animals treated with F2 for 52 days was augmented in agreement with the previous finding of (Zanoli et al., 2009) and Tambi et al. (2011). Beside the increase of the plasma testosterone level, the elevated levels of the two gonadotropins, LH and FSH, together with the reduction of estrogen level in the plasma as shown in the present study further substantiated the hypothesis that *E. longifolia* treatment may down-regulate the estrogen-mediated feedback effect for LH and FSH secretion in the hypothalamic–pituitary–gonadal axis. Thus, the absence or reduction of the feedback effect with the low plasma estrogen level allowed the pituitary gland to continuously release the two gonadotropins in the circulation system and finally increased the testosterone production. Testosterone together with FSH therefore enhanced the spermiogenesis processes in the seminiferous tubules as FSH and testosterone were the key modulators of these processes (O’Donnell et al., 1994; Ruwanpura et al., 2010). Interestingly, at the higher dose of 50 mg/kg F2, the testosterone level was not significantly increased when compared with that at 25 mg/kg dose. Furthermore, the LH and FSH levels were not significantly different when compared with those of the control, indicating that a compensatory physiological feedback mechanism may be triggered by testosterone and/or inhibit (Prakash, 2007) but not by estrogen as the plasma level plasma was lower and not significantly different from that of control.

Initially, the direct action of *E. longifolia* towards the hypothalamic–pituitary–gonadal axis was not well understood. The poor lipophilicity and bioavailability of the quassinoids (Low et al., 2011) further excluded the possible direct effect of the quassinoids on the gonadotropins secretion in hypothalamus. The detection of the quassinoid in the testes further confirmed the possible site of action (Chan et al., 2009). Among the four quassinoids in the extract F2, eurycomanone and 13,21-dihydroeurycomanone significantly increased the testosterone release from the Leydig cells. Furthermore, the agonistic effect of the eurycomanone on the testosterone elevation in the isolated cells was dose-dependent and the additive effect of the quassinoid was shown when combined with human chorionic gonadotropin (hCG). The significant high testosterone release from the Leydig cells upon concomitant treatment with hCG and eurycomanone may suggest that the quassinoid probably may act on the G-protein coupled receptor (GPCR) similar to that of LH/hCG as agonist to exert testosterone release (Simpson et al., 1987). This agonistic effect and the GPCR binding property requires further investigation and such experimental studies are currently on-going.

In general, the present study showed for the first time that the quassinoids *E. longifolia* especially the eurycomanone was involved in spermatogenesis process. The increase of the sperm concentration upon treatment with quassinoid-rich *E. longifolia* fraction treatment was further proven by the detailed microscopic morphometrical analyzing the germ cells followed by the estimated sperm production rate on the testicular tissue. The increase of the sperm concentration was also positively associated with the enhanced fertility index in the present study. The quassinoid-rich *E. longifolia* fraction induced the testosterone synthesis and elevated the LH, FSH, but reduced estrogen levels in the plasma provided evidences that the quassinoids of *E. longifolia* improved spermatogenesis via the hypothalamic–pituitary–gonadal axis.

The increase of the testosterone level following the administration of the plant extract may provide a warning on the safety of the extract. It was generally believed that the prostate cancer or benign prostatic hyperplasia originated from the elevated level of testosterone (Bosland, 2000). The oral administration of exogenous testosterone did not improve spermatogenesis in idiopathic male infertility (Sheckter et al., 1989) but may cause liver toxicity (Ishak and Zimmerman, 1987). Our recent findings with the single oral dose administration of F2 at 2000 mg/kg body weight (acute toxicity study), daily single dose of F2, 5–50 mg/kg for sub-chronic 90 days and 10–50 mg/kg for 180 days consecutively revealed no toxic effects on the male and female rodents studied (Low et al., submitted for publication). Moreover, the histologies on the male reproductive organs including testis, epididymis, seminal vesicles and prostate glands of the male animals followings F2 oral administration were normal when compared with those of the untreated animals. Therefore, these evidences may suggest that *E. longifolia* does not induce any prostate cancer or benign prostatic hyperplasia even though the plasma testosterone level was elevated. Besides, the over stimulated effect of testosterone production by *E. longifolia* probably may not be an issue as the peak testosterone level recorded at the first complete spermatogenesis process of 52 days was reduced back to the normal level even though the animals were given a higher dose (Supplementary Fig. 1) or prolonged treatment period (Supplementary Fig. 2), due probably to the effect of homeostasis. In fact, recent clinical and scientific evidences have shown that not all prostate cancer nor benign prostatic hyperplasia originated from high testosterone level (Morgentaler, 2006). Moreover, testosterone has also suggested been used to treat men with symptomatic prostate cancer (Morgentaler et al., 2011). Perhaps in the future, the quassinoids from *E. longifolia* may merit as medicaments for prostate cancer and testosterone deficient idiopathic infertile males but may not be suitable for sport due to its testosterone enhancement properties.

5. Conclusion

In conclusion, the significant increase in the spermatogenesis of the rats upon treatment with the quassinoid-rich *E. longifolia* extract on the hypothalamic–pituitary–gonadal axis may explain the fertility-enhancing property of the plant. As much attention was focused on the development of therapy against females’ infertility and lesser on male, *E. longifolia* may be worth further investigation for treating infertile male.

Conflict of interest

The authors declare no conflicts of interest.

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References


Supplementary data associated with this article can be found in Appendix A. Supporting information
