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Eurycomanone, the major quassinoid in *Eurycoma longifolia* root extract increases spermatogenesis by inhibiting the activity of phosphodiesterase and aromatase in steroidogenesis



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ABSTRACT

Ethnopharmacological relevance: *Eurycoma longifolia* Jack (Simaroubaceae family), known locally as 'Tongkat Ali' by the ethnic population, is popularly taken as a traditional remedy to improve the male libido, sexual prowess and fertility. Presently, many tea, coffee and carbonated beverages, pre-mixed with the root extract are available commercially for the improvement of general health and libido. Eurycomanone, the highest concentrated quassinoid in the root extract of *E. longifolia* improved fertility by increasing testosterone and spermatogenesis of rats through the hypothalamus–pituitary–gonadal axis, but the mechanisms underlying the effects are not totally clear.

Aim of the study: To provide evidences on the plant ethnopharmacological use and the involvement of eurycomanone, the major indigenous plant quassinoid in testosterone steroidogenesis and spermatogenesis increase.

Material and Methods: The rat testicular Leydig cell-rich interstitial cells were isolated and incubated in the culture medium M199. The viability of the cells was determined with trypan blue staining and the concentration of the viable cells was counted with a haemocytometer. The 3 β -hydroxysteroid dehydrogenase (HSD) staining method was used to measure the abundance of Leydig cells in the preparation. Eurycomanone and the standard steroidogenesis inhibitors were incubated with 1.0×10^5 cells, and after 2 h, the testosterone and the oestrogen concentrations were determined by the ELISA method. Computational molecular docking was performed to determine the binding affinity of the compound at the respective steroidogenesis enzymes.

Results: Eurycomanone (EN) significantly increased testosterone production dose-dependently at 0.1, 1.0 and 10.0 μ M ($P < 0.05$), but the two lower doses when combined with 3-isobutyl-1-methylxanthine (IBMX), the phosphodiesterase inhibitor were not significantly higher than EN or IBMX alone, except at a higher concentration. The molecular docking studies indicated EN and IBMX were binding at different sites of the enzyme. EN has no reversal of inhibition by aminoglutethimide, ketoconazole or nifedipine at the respective steroidogenesis enzyme. The quassinoid was also non-responsive to the inhibition of oestrogen receptor by tamoxifen, but displayed improved formestane inhibition of aromatase in reducing oestrogen production. The molecular docking studies further supported that EN and formestane bound to aromatase with similar orientations and free energy binding values.

Conclusion: Eurycomanone enhanced testosterone steroidogenesis at the Leydig cells by inhibiting aromatase conversion of testosterone to oestrogen, and at a high concentration may also involve phosphodiesterase inhibition. The quassinoid may be worthy for further development as a phytomedicine to treat testosterone-deficient idiopathic male infertility and sterility.

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1. Introduction

Infertility is generally defined as the inability of a couple to conceive after trying unprotected intercourse for a year (Prakash, 2007). In all cultures, infertility is recognised as a life crisis that can provoke the stability of an individual, the relationship with the

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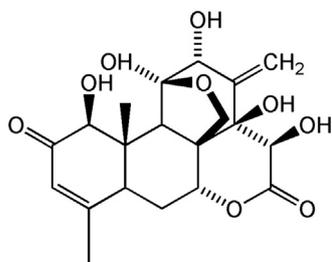


Fig. 1. The chemical structure of eurycomanone (EN).

spouse, the family members and the community (Burns and Covington, 2006). One in ten couples experienced infertility (Burns and Covington, 2006), affecting over 186 million globally (WHO, 2003). About 20% of the infertility problems originated from the male partner (Raman et al., 2005), and over 90% of the male problems were due to oligozoospermia, a clinical condition of low sperm count (< 15 million/mL, normal > 15 million/mL) and quality (Winston, 1986). Current approaches to overcome male infertility included *in vitro* fertilisation (IVF), intra-fallopian transfer, intra-cytoplasmic sperm injection (ICSI), surgery and hormonal therapy. These treatments were costly and also created socio-economic problems (Katz et al., 2002). Alternatively, the use of non-invasive traditional herbal remedies for the treatment of male infertility has been studied marginally and often lacks evidence. Therefore, plants traditionally indicated for aphrodisiac activity, improvement of libido and fertility may be suitable therapeutic candidates and should be thoroughly studied scientifically.

Eurycoma longifolia Jack, known as 'Tongkat Ali' in Malaysia and 'Pasak Bumi' in Indonesia and 'Cay ba binh' in Vietnam is traditionally used to improve the male libido, sexual prowess and fertility (Burkill and Hanif, 1930; Gimlette, 1971; Gimlette and Thomson, 1977). The root extract has been reported to induce aphrodisiac properties (Ang and Sim, 1998; Zanolli et al., 2009), increased the sperm quality (Noor et al., 2004), and reversed oestrogen-induced infertility (Wahab et al., 2010) and *Andrographis paniculata*-induced oligospermia (Chan et al., 2009) of rats. The extract also possessed beneficial treatment of androgen-deficient osteoporosis in rats (Razak et al., 2012). Presently, over 200 commercial products, containing the root extract pre-mixed in tea, coffee and carbonated drinks, are available in the domestic market recommended for the improvement of general health and libido (Cyranoski, 2005), but the scientific evidences based on the mechanism of action is still not documented. Our recent study on the oral administration of a quassinoid-rich *E. longifolia* extract in male rats has shown that the hypothalamic–pituitary–gonadal axis was triggered to decrease plasma oestrogen, increased testosterone, luteinizing hormone and follicle stimulating hormone levels (Low et al., 2013). Eurycomanone (Fig. 1) and the other major quassinoid analogues in the extract were responsible for the improved spermatogenesis and fertility. Eurycomanone, the quassinoid of the highest concentration in the spermatogenic-induced standardised *E. longifolia* extract, increased testosterone production in the isolated rat testicular Leydig cell-rich interstitial cells (Low et al., 2013). However, the mechanisms underlying the effects of the quassinoid on steroidogenesis were not clear. Presently, we examined the sites of eurycomanone activity on the steroidogenesis pathway related to testosterone and oestrogen production, using the rat testicular Leydig cell-rich interstitial cells. Computational molecular docking simulation was also performed to provide support to the experimental results and correlation of the quassinoid binding capabilities on the respective targeted protein sites.

2. Material and methods

2.1. Chemicals

All the chemicals were purchased from Sigma Chemicals (St Louis, MO, USA). The quassinoid, eurycomanone (EN; Fig. 1; purity $> 96\%$) was isolated and purified from the roots of *Eurycoma longifolia* Jack as described previously (Teh et al., 2011). The purity of the compounds was determined with the 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. Animals

Sprague–Dawley male rats, weighing 300–350 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 water was provided *ad libitum*. All animals were kept in a controlled ventilation room with 12/12-hour light/dark cycle at ambient room temperature. All animal experiments were humanely performed and maintained following the Institutional Guide for the Care and Use of Laboratory Animals 2010 from the Animal Research and Service Centre, Universiti Sains Malaysia. The Animal Ethics Committee of the university approved the experimental design and protocol.

2.3. Testicular interstitial cells preparation

Sprague–Dawley rats were euthanized by CO₂ asphyxiation and the testes were aseptically removed. The testicular interstitial cells were isolated following the collagenase dispersion method described by Browning et al. (1983) and Tsai et al. (1997) with slight modifications. Briefly, six testes were decapsulated and were put into a 50 mL polypropylene tube containing culture medium (2 mL/testis) supplemented with 10 mg bovine collagenase Type II. The culture medium was made up of 0.1% bovine serum albumin (Fraction V) in tissue culture medium M199 supplemented with N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES, 25 mM), sodium bicarbonate 0.22 g/L (Bendosen Laboratory Chemicals, Selangor, Malaysia), penicillin-G/streptomycin 100 IU/mL (Gibco[®], Invitrogen[™], Carlsbad, CA, USA) and heparin 2550 units/L (Leo Pharma, Ballerup, Denmark). The medium was aerated with 95% O₂ and 5% CO₂. The tubes were incubated at 34 °C in a water bath, and 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 5 min and then filtered through a 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 then immediately replenished with 10-fold culture medium and centrifuged. The final cell pellet collected after the second centrifugation, was resuspended with culture medium (1 mL/testis). The viability and the concentration of the cells were determined by staining with trypan blue and counted with a haemocytometer. The total cell proteins were determined following the method of Bradford (1976). The 3β-hydroxysteroid dehydrogenase (HSD) staining method was used to measure the abundance of Leydig cells (Browning et al., 1983; Dirami et al., 1991). The preparation contained approximately 20–40% of Leydig cells with a viability of $> 97\%$.

2.4. Experimental design

A 50 mL rat cell suspension (2.0×10^5 cells/mL) pre-incubated with the culture medium at 34 °C under controlled atmosphere of 95% O₂ and 5% CO₂, was prepared as stock. An aliquot of 500 μL

stock was dispensed into each microwell of the 24-well plate. A 100 μ L solution of **EN** at 0.1, 1.0 and 10.0 μ M was next added to each well incubated with the isolated cells in the absence and presence of 3-isobutyl-1-methylxanthine (1.0 mM), aminoglutethimide (25.0 mM), ketoconazole (20 μ M), nifedipine (1.0 μ M), tamoxifen (0.01 μ M–0.1 mM) and formestane (International Laboratory, USA) (1.0 nM–200 nM). The doses, 0.1–10.0 μ M of **EN** showing non-cytotoxicity, with more than 95% cell viability (unpublished results) were selected from the dose-response studies of **EN** on the testosterone release from the rat testicular Leydig cell-rich interstitial cells, and was at least 5-fold more than the ED₅₀ value of the quassinoid at 0.2 μ M, but was 10,000-fold less than highest cytotoxic dose at 10 mM. After 2 h of incubation, 500 μ L of ice-cold (4 °C) phosphate buffer saline with gelatin (0.1% gelatin in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.5) were added to stop the incubation (Tsai et al., 1997). The medium was then centrifuged at 4 °C, 1000 g for 15 min and stored at –20 °C prior to assays of testosterone and oestrogen concentrations. Each sample was performed in three independent replicates and the each hormone level was measured in duplicate.

2.5. Testosterone and oestrogen ELISA determinations

The testosterone and oestrogen concentrations were measured using the enzyme-linked immunosorbent assay (ELISA) kits purchased from Cusabio Biotech Co. Ltd. (Wuhan, China). The standard curves were constructed with concentrations ranging from 0.1–25.6 ng/mL for testosterone and 40–1000 pg/mL for oestrogen following the respective kit protocol.

2.6. Molecular docking simulation

The docking of the selected inhibitors to the protein structures was performed using the Autodock 3.0.5 (Morris et al., 1998). The X-ray structure of the respective proteins was taken from the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/home/home.do>). The inhibitor structure was extracted from PubChem database (<http://pubchem.ncbi.nlm.nih.gov/search>) and was subjected to a short energy minimisation to eliminate the steric clashes. All of the non-polar hydrogen from both proteins and inhibitors were merged. The map of 60 \times 60 \times 60 grid points at 0.375 Å spacing were generated using the Autogrid3 and centred on the potential binding site. Molecular docking simulation was carried out using the Lamarckian genetic algorithm with Autodock 3.0.5 (Morris et al., 1998). All docking simulations were realized with a total of 100 runs with an initial population of 250. The resulting docking orientations within 1.0 Å in the root-mean square tolerance were set as the docking input parameter. The lowest free energy for the binding of each conformation in the most populated cluster was selected.

2.7. Statistical analysis

The data were presented as mean \pm SD (standard deviation). The significant levels of the hormones between groups were determined using Kruskal–Wallis and Mann–Whitney *U* tests. All analyses were conducted using the statistical software SPSS[®] 9.0 (LEAD Technologies Inc., USA). A confidence level of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Inhibitory effects of eurycomanone on phosphodiesterase

The non-selective inhibitory effects on phosphodiesterase induced by 3-isobutyl-1-methylxanthine (IBMX) at concentration

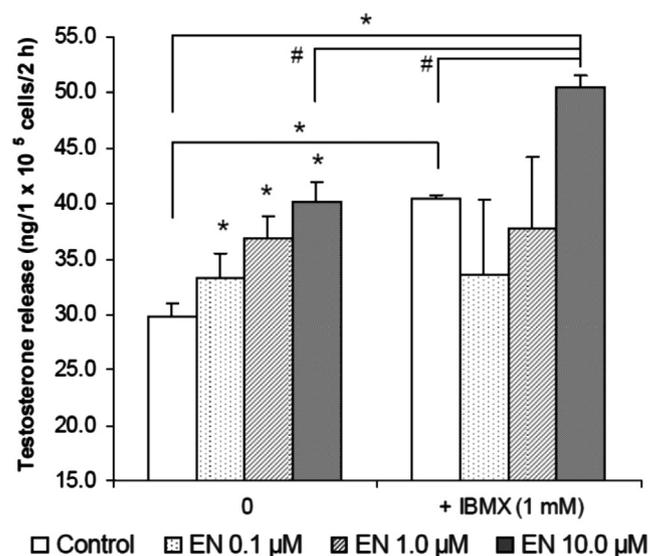


Fig. 2. Effects of the eurycomanone (**EN**, 0.1–10 μ M), 3-isobutyl-1-methylxanthine (IBMX, 1.0 mM) and the combination of **EN** (0.1–10 μ M) with 1.0 mM IBMX on the release of testosterone in the rat testicular Leydig cell-rich interstitial cells. Values are given in mean \pm SD ($n=3$ of three independent experiments). *Significant differences at $P < 0.05$ between the treatment groups and the non-treated control (first bar on left). #Significant differences at $P < 0.05$ between the treatment groups.

of 1.0 mM gave a significant increase in testosterone release ($P < 0.05$) over the non-treated rat testicular Leydig cell-rich interstitial cells (Fig. 2). Eurycomanone (**EN**) at 0.1–10 μ M significantly increased the testosterone level following a dose-dependent manner over that of the non-treated control (Low et al., 2013). However, similar increasing concentrations of **EN** at 0.1 and 1.0 μ M in the presence of 1.0 mM IBMX (Fig. 2) produced no significant increase in respective testosterone levels when compared to that of **EN** at 0.1 and 1.0 μ M alone, IBMX (1.0 mM) alone or the non-treated control ($P < 0.05$), except at the highest concentration of 10.0 μ M.

The docking studies showed that **EN** and IBMX bound to different sites of phosphodiesterase 4B (PDE4B, PDB ID: 1TB5) catalytic pocket (Fig. 3) with free energy binding (FEB) values of –9.53 and –7.73 kcal/mol, respectively. The xanthine ring of IBMX stacking against the phenolic ring of Phe446 formed a strong π – π bonding at 3.82 Å. The nitrogen atom in the xanthine ring of IBMX interacted with Asn395 via hydrogen bonding (1.71 Å). In contrast, the structure of **EN** fitted well into the binding pocket of PDE4B and may thus prevent the access of the substrate to the catalytic site of PDE4B (Fig. 3). The hydroxyl groups of **EN** at position C-1 and C-11 formed hydrogen bonding with Glu304 and Asp346 at distance of 1.95 Å and 1.74 Å, respectively. The N₈H1 and N₈H2 atoms of Asn283 approached the oxygen atoms of the C-11 and C-12 hydroxyl groups of **EN** at the distances of 2.42 Å and 2.10 Å, respectively (Fig. 2). In addition, the PDE4B–**EN** complex was further stabilized by another hydrogen bonding at 2.44 Å between the oxygen atom at the oxymethylene bridge of **EN** and the backbone amide hydrogen atom of Asn283.

3.2. Effects of eurycomanone on steroidogenesis enzymes and calcium ion channel

The administration of aminoglutethimide (AGTM, 0.5 mM), a cytochrome P450 side-chain-cleavage enzyme inhibitor and ketoconazole (KTZ, 20.0 μ M), a 17 β -hydroxysteroid dehydrogenase/17,20-lyase inhibitor separately to the Leydig cells, significantly decreased the respective testosterone levels ($P < 0.05$) when compared to that of non-treated control (Fig. 4). **EN** increased the testosterone level in a dose-dependent manner (0.1, 1.0 and

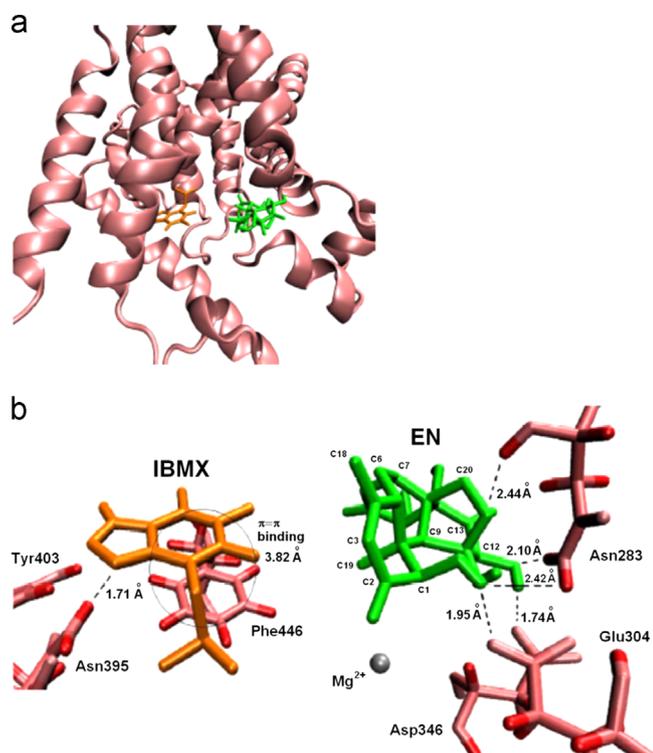


Fig. 3. (a) Binding mode of eurycomanone (EN) and 3-isobutyl-1-methylxanthine (IBMX) inside the catalytic site of phosphodiesterase 4B (PDE4B; PDB ID: 1TB5). (b) The docking studies showed that EN (green) and IBMX (orange) bound to different sites of PDE4B catalytic pocket with free energy binding (FEB) values of -9.53 and -7.73 kcal/mol, respectively. The Zn ion of PDE4B was not shown and Mg was coloured in grey. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

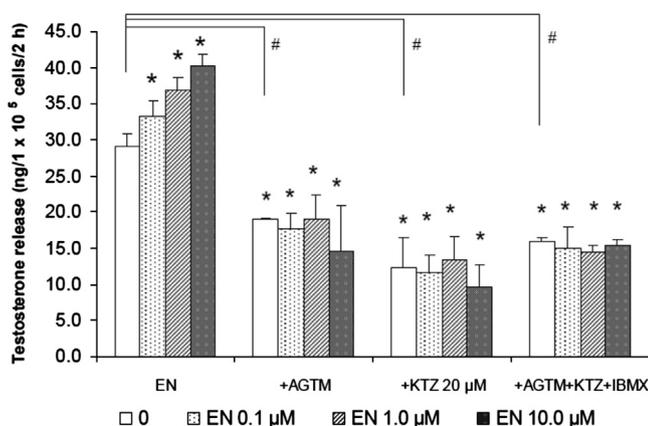


Fig. 4. Effects of the eurycomanone (EN, 0.1–10 μ M) on the release of testosterone following the incubation of rat testicular Leydig cell-rich interstitial cells with the aminoglutethimide (AGTM, 0.5 mM), ketoconazole (KTZ, 20 μ M) and the combination of AGTM (0.5 mM), KTZ (20 μ M) and 3-isobutyl-1-methylxanthine (IBMX, 1.0 mM). Values are given in mean \pm SD ($n=3$ of three independent experiments). *Significant differences at $P < 0.05$ between the treated groups and non-treated control (first bar on the left). #Significant differences at $P < 0.05$ between AGTM alone, KTZ alone, AGTM+KTZ+IBMX treated groups versus non-treated control (first bar on the left).

10.0 μ M). However, EN, at the three increasing doses when each combined with AGTM or KTZ, or with both inhibitors and IBMX displayed no significant difference in testosterone levels when compared to those of the respective inhibitor alone or the three combined inhibitors only (Fig. 4). EN has therefore no effect on the steroidogenesis enzymes responsible for producing testosterone. Moreover, the inhibitory effects of AGTM and KTZ on testosterone

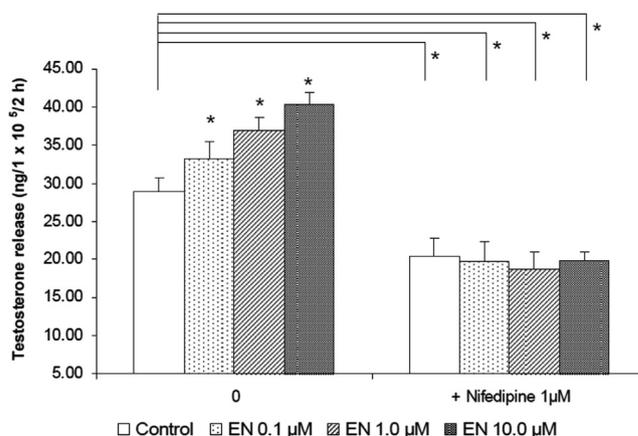


Fig. 5. Effects of the eurycomanone (EN, 0.1–10 μ M) on the release of testosterone following the incubation of rat testicular Leydig cell-rich interstitial cells with the nifedipine (1.0 μ M). Values are given in mean \pm SD ($n=3$ of three independent experiments). *Significant differences at $P < 0.05$ between the EN (0.1–10 μ M), nifedipine treated versus non-treated groups (first bar on left).

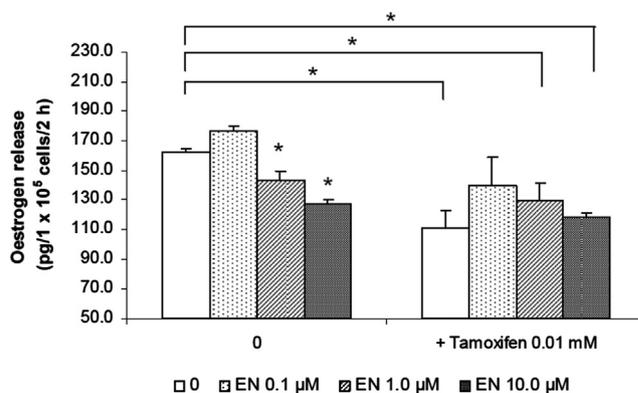


Fig. 6. Effects of the eurycomanone (EN, 0.1–10 μ M) on the release of oestrogen following the incubation of rat testicular Leydig cell-rich interstitial cells with the tamoxifen (0.01 mM). Values are given in mean \pm SD ($n=3$ of three independent experiments). *Significant differences at $P < 0.05$ between the treated groups versus the non-treated control (first bar on left).

steroidogenesis were not reversed by the highest concentration of EN at 10 μ M in combination with 1.0 mM IBMX (Fig. 4), suggesting that the quassinoid may not affect the described steroidogenesis pathway. Similarly, the treatment of nifedipine, a calcium ion channel blocker at 1.0 μ M, significantly decreased the testosterone level ($P < 0.05$) when compared with that of the non-treated control (Fig. 5). The testosterone increasing properties of EN at 0.1, 1.0 and 10.0 μ M were all significantly reduced in the presence of nifedipine ($P < 0.05$). However, the testosterone levels of nifedipine in combination with three doses of EN (0.1, 1.0 and 10.0 μ M) were not significantly different from that of nifedipine alone (Fig. 5).

3.3. Inhibitory effects of eurycomanone on oestrogen receptors

Fig. 6 shows that tamoxifen (0.01 mM) alone and EN (1 and 10 μ M) alone significantly decreased the oestrogen levels from the rat testicular Leydig cell-rich interstitial cells when compared to that of the non-treated control ($P < 0.05$). The different doses of EN with each in combination with 0.01 mM of tamoxifen similarly reduced significantly the oestrogen level compared to that of the control ($P < 0.05$) but were not significantly different from that of EN alone and tamoxifen alone at a similar dose (Fig. 6). The dose-response curves of tamoxifen alone and EN alone and in combination were analysed by non-linear regression analysis, the curves

were not parallel to one another suggesting that the two compounds lowered oestrogen following different mechanisms. The calculated EC_{50} values of tamoxifen and **EN** for the oestrogen levels released from the testicular interstitial cells were 1.93×10^{-7} M and 6.38×10^{-7} M, respectively. The EC_{50} values of **EN** at 0.01, 1.0 and 100 μ M with 0.01 mM of tamoxifen remained at 3.40×10^{-7} M, 5.47×10^{-7} M and 5.37×10^{-7} M, respectively within those of **EN** alone and tamoxifen alone, without shifting significantly lower, suggesting that **EN** may not be binding on the oestrogen receptor to reduce the oestrogen production.

Furthermore, the molecular docking studies revealed that **EN** was also found like hydroxytamoxifen (HO-TMX), the active metabolite of tamoxifen, inside the oestrogen receptor alpha ($ER\alpha$; PDB ID: 3ERT) and oestrogen receptor beta ($ER\beta$; PDB ID: 1QKM) (Supplementary Figs. 1 and 2). Unlike HO-TMX, no hydrogen bonding was formed between **EN** and the amino acid residues in $ER\alpha$ and $ER\beta$. **EN** was less stable showing a higher FEB value of -8.90 kcal/mol when compared with HO-TMX displaying -11.21 kcal/mol on $ER\alpha$. The FEB value of **EN** at -4.36 kcal/mol was similarly higher than that of HO-TMX (-7.23 kcal/mol), suggesting that HO-TMX was more stable than **EN** in binding to $ER\beta$. Hence, **EN** may lack the inhibitory effects of HO-TMX on both oestrogen receptor proteins.

3.4. Inhibitory effects of eurycomanone on aromatase

The present study showed that the administration of formestane (50.0 and 200.0 nM), the aromatase inhibitor, significantly decreased ($P < 0.05$) the oestrogen level of the non-treated testicular Leydig cell-rich interstitial cells (Fig. 7). The combination of **EN** (1.0 μ M) and formestane at 1.0, 10.0 and 200.0 nM showed significant oestrogen reductions ($P < 0.05$) when compared to that of formestane alone treated groups or the non-treated control.

The molecular docking studies showed that **EN** and formestane (FMS), bound to the same catalytic pocket of the enzyme aromatase (PDB ID: 3EQM) with similar orientations and similar FEB values of -13.53 kcal/mol and -13.20 kcal/mol, respectively (Fig. 8). FMS formed a single hydrogen bond at its hydroxyl group with Asp309 at 2.09 Å. In addition, two hydrogen bonds were formed between the carbonyl oxygen and N_H of Arg115 (2.86 Å) and the backbone amide hydrogen of Met374 at 1.81 Å. On the other hand, the oxygen atom of the carbonyl group at the C-2 of **EN** was found interacting directly with the heme Fe of the enzyme

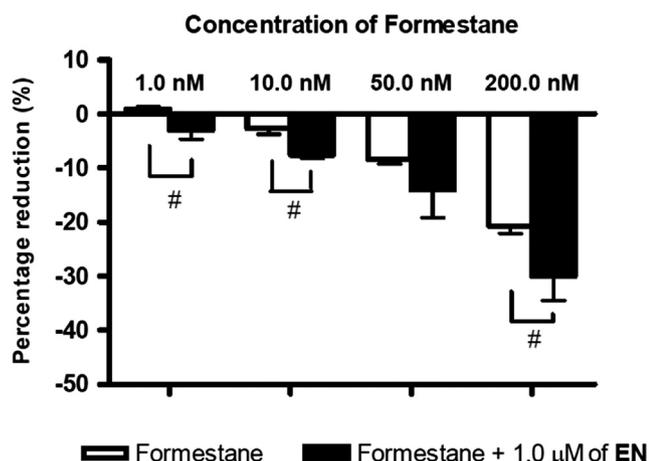


Fig. 7. Percentage reductions of the oestrogen levels compared to that of the non-treated control following the incubation of rat testicular interstitial cells with the formestane (1.0–200.0 nM) and the absence and presence of eurycomanone (**EN**) at 1.0 μ M. Values are given in mean \pm SD ($n=3$ of three independent experiments). #Significant differences at $P < 0.05$ between the presence of **EN** with formestane compared with formestane alone.

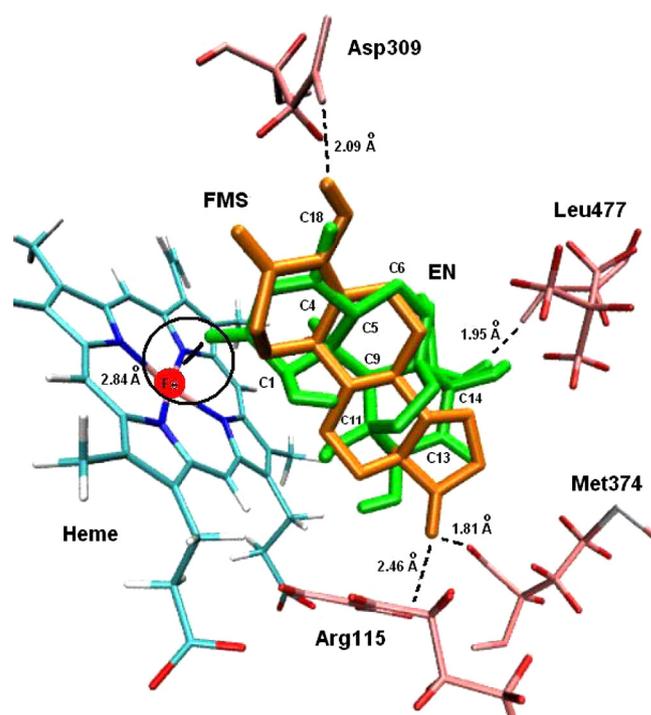


Fig. 8. Closer view of molecular interaction of eurycomanone (**EN**) superimposed with formestane (FMS) and the active amino acid residues inside the catalytic site of aromatase with Fe ion of heme coloured in red. The molecular docking studies showed that **EN** and FMS bound to the same catalytic pocket of aromatase (PDB ID: 3EQM). FMS formed hydrogen bonds with Asp309, Arg115 and Met374. The oxygen atom of the carbonyl group at C-2 of **EN** interacted directly with heme Fe of the enzyme and was further stabilized by hydrogen bonding with the backbone amide hydrogen of Leu477. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

at 2.84 Å. Furthermore, the aromatase-**EN** complex was further stabilized by hydrogen bonding between the oxygen atom at the C-15 hydroxyl moiety of **EN** and the backbone amide hydrogen atom of Leu477 at 1.95 Å (Fig. 8).

4. Discussion

Previous *in vitro* and *in vivo* studies have shown that the quassinoid, eurycomanone (**EN**) indigenously found in the plant, increased the testosterone level (Low et al., 2013). The present *in vitro* studies have shown that **EN** at the highest concentration in combination with IBMX showed significant increase in testosterone level over that of IBMX or **EN** alone. Furthermore, the computational docking results showed that IBMX and **EN** bound inside the phosphodiesterase catalytic pocket at different sites. IBMX showed more stable binding through π - π bonding and hydrogen bonding with the respective proteins at the sub-catalytic pocket of PDE as previously reported (Huai et al., 2004) whereas, **EN** showing a higher FEB value than IBMX, was binding to the catalytic pocket by weaker hydrogen bonding. Therefore, the testosterone increasing effect of **EN** may not be contributed strongly by PDE inhibition but may be from other inhibition route instead. In the presence of IBMX or **EN**, cyclic AMP was not able to degrade by PDE to AMP and the amount was therefore increased. The crystalline structure of the PDE4B applied in the present molecular docking study was highly homologous to the PDE8A that actively modulated the testosterone production in the mouse Leydig cells (Vasta et al., 2006). However, the crystalline structure of PDE8A was not available for studies and the recent study on the

inhibition of IBMX on PDE4 was more effective than PDE8A (Tsai and Beavo, 2011).

An earlier study has reported that *E. longifolia* activated the CYP17a enzymes to enhance the metabolism of pregnenolone and 17-OH-pregnenolone to yield more dehydroepiandrosterone (DHEA), 4-androstenedione, and eventually to produce testosterone (Tambi, 2009). However, the present studies showed that the presence of the specific inhibitors for the cytochrome P450_{SCC} (CYP11a), 17,20-lyase, 17 β -hydroxysteroid dehydrogenase (CYP17a) and calcium ion channel such as aminoglutethimide, ketoconazole and the calcium ion channel blocker, nifedipine, significantly affected the testosterone levels induced EN. In other words, the enzymes involved in the steroidogenesis such as CYP11a, 17,20-lyase, CYP17a and the calcium transportation system were not affected by the presence of EN. In fact, these pathways were vital for the eurycomanone-induced testosterone production. The results therefore suggested that EN at a high concentration may increase the steroidogenesis of testosterone by inhibiting phosphodiesterase to accumulate cyclic AMP. Consequently, the accumulated cyclic AMP may in turn activate the protein kinases and steroidal acute regulatory (StAR) protein to transport the cholesterol into inner mitochondria to produce pregnenolone, a precursor for testosterone biosynthesis (Prakash, 2007). According to Manna et al. (1999), the expression of the StAR genes was augmented with the elevated hCG level influenced by presence of calcium ion. The inhibition of nifedipine on the testosterone production was not reversed by EN indicating that calcium ion is essential for the EN-induced testosterone steroidogenesis.

The present study revealed that EN reduced the oestrogen release of the testicular interstitial cells in a dose-dependent manner. The reduction of the oestrogen level by EN has given two possible hypotheses regarding to the mechanism action of EN. Firstly, the quassinoid may compete with oestrogen as an antagonist at the oestrogen receptor (ER) and eventually reduced the oestrogen production. Secondly, the quassinoid may directly inhibit the aromatase enzyme that converts the testosterone to oestrogen. In order to determine the binding property of EN on the ER, a potent ER inhibitor, tamoxifen was used in the current study. Interestingly, our present study showed that tamoxifen reduced the oestrogen release of the testicular interstitial cells and its anti-oestrogenic effect was not affected by the presence of EN, indicating that EN may not react on the ER similar to that of tamoxifen. Furthermore, the molecular docking studies also revealed that tamoxifen or its active metabolite hydroxytamoxifen, a potent ER inhibitor, formed prominent hydrophobic van der Waals interactions with the non-polar residues at the binding pocket of the ER α (Shiau et al., 1998). In contrast, EN, a polar quassinoid with five hydroxyl groups, one carbonyl group and a lactone in the structure, has poor hydrophobic interaction with the active binding pocket, and may therefore not act on ER (Pike et al., 1999).

Interestingly, EN at different concentrations with formestane, the aromatase inhibitor gave additive oestrogen reduction, suggesting that the quassinoid may share the same mechanism of the aromatase inhibition with formestane. The computational docking of EN and formestane on the aromatase enzyme also indicated that EN and formestane bound at the same catalytic pocket of the enzyme. According to Osborne and Tripathy (2005), aromatase inhibitors can be subdivided into two categories: steroidal (type I) and non-steroidal (type II). Steroidal aromatase inhibitors such as formestane and exemestane competitively bind to the substrate-binding site of the enzyme, forming very tight, irreversible bonds that resulted in permanent enzyme inactivation. In contrast, the non-steroidal aromatase inhibitors reversibly interact with the haem moiety of the enzyme, consequently inhibit the steroidal aromatisation by excluding the substrate and oxygen for the heme.

The present computational molecular docking of EN showed that the quassinoid bound directly inside the catalytic site of the enzyme forming hydrogen bonds with the amino acid residues and interacted with haem of the aromatase. As aromatase inhibitors have potential to treat male infertility (Schlegel, 2012), therefore, it may be postulated that EN behaved like a non-steroidal aromatase inhibitor. As a result, the low plasma oestrogen level following the administration of the quassinoid-rich F2 may be due to the inhibition of aromatase but not ER by the quassinoid EN. Thus, the effect of EN on the testosterone and oestrogen release may explain the *in vivo* fertility improvement, testosterone elevation and anti-oestrogenic properties of *E. longifolia* as previously reported (Abdulghani et al., 2012; Chan et al., 2009; Zanolli et al., 2009).

The increase of the testosterone level following the administration of the plant extract, however, has provoked a warning on the safety of chronic consumption of the plant (Bosland, 2000). The recent report that the high concentration of *E. longifolia* extract at 2000 μ g/mL possessed harmful effect on human spermatozoa *in vitro*. However, the concentration for study was not a physiological concentration but was about 800-fold higher than the reported therapeutic dose (Erasmus et al., 2012). Our latest findings of using a single oral dose administration of eurycomanone-rich *E. longifolia* extract (F2) at 2000 mg/kg body weight for acute toxicity study, daily single dose of F2, at 5–50 mg/kg for sub-chronic 90-day and chronic 10–50 mg/kg for 180-day studies consecutively revealed no toxicity on the male and female rodents (unpublished observations). Further evidences have shown that not all prostate cancer and/or benign prostatic hyperplasia originated from high testosterone level (Morgentaler, 2006). In fact, testosterone has also been suggested to treat men with symptomatic prostate cancer (Morgentaler et al., 2011). Hence, eurycomanone may be worthy for further development as a phytomedicine to treat testosterone-deficient idiopathic male infertility and sterility. According to Schlegel (2012) aromatase inhibitors have potential to improve the male infertility.

5. Conclusions

The present studies explained for the first time that eurycomanone acted on testosterone and oestrogen production in the rat testicular Leydig cell-rich interstitial cells. The quassinoid inhibited aromatase to block the conversion of testosterone to oestrogen. The increase of testosterone may therefore improve spermatogenesis and male fertility.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2013.06.023>.

References

- Abdulghani, M., Hussin, A.H., Sulaiman, S.A., Chan, K.L., 2012. The ameliorate effects of *Eurycoma longifolia* Jack on testosterone-induced reproductive disorders in female rats. *Reproductive Biology* 12, 247–255.

- Ang, H.H., Sim, M.K., 1998. *Eurycoma longifolia* Jack and orientation activities in sexually experienced male rats. *Biological and Pharmaceutical Bulletin* 21, 153–155.
- Bosland, M.C., 2000. The role of steroid hormones in prostate carcinogenesis. *Journal of the National Cancer Institute* 27, 67–73.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, 248–254.
- Browning, J.Y., Heindel, J.J., Grotjan, H.E., 1983. Primary culture of purified Leydig cells isolated from adult rat testes. *Endocrinology* 112, 543–549.
- Burkill, I.H., Hanif, M., 1930. Malay village medicine, prescriptions collected. *Gardens Bulletin Strait Settlements* 6, 176–177.
- Burns, L.H., Covington, S.N., 2006. Psychology of infertility. In: Covington, S.N., Burns, L.H. (Eds.), *Infertility Counseling a Comprehensive Counseling Handbook for Clinician*, 2nd ed. Cambridge University Press, Cambridge, pp. 1–2.
- Chan, K.-L., Low, B.-S., Teh, C.-H., Das, P.K., 2009. The effect of *Eurycoma longifolia* on sperm quality of male rats. *Natural Product Communications* 4, 1331–1336.
- Cyranoski, D., 2005. Malaysian researchers bet big on home-grown viagra. *Nature Medicine* 11, 912.
- Dirami, G., Poulter, L.W., Cooke, B.A., 1991. Separation and characterization of Leydig cells and macrophage from rat testes. *Journal of Endocrinology* 130, 357–365.
- Erasmus, N., Solomon, M.C., Fortuin, K.A., Henkel, R.R., 2012. Effect of *Eurycoma longifolia* Jack (Tongkat ali) extract on human spermatozoa *in vitro*. *Andrologia* 44, 308–314.
- Gimlette, J.D., 1971. In: Thomson, H.W., Watson, M. (Eds.), *A Dictionary of Malayan Medicine*. Oxford University Press, Kuala Lumpur, p. 242.
- Gimlette, J.D., Thomson, H.W. (Eds.), 1977. *A Dictionary of Malayan Medicine*. Oxford University Press, Kuala Lumpur.
- Huai, Q., Liu, Y., Francis, S.H., Corbin, J.D., Ke, H., 2004. Crystal structures of phosphodiesterases 4 and 5 in complex with inhibitor 3-isobutyl-1-methylxanthine suggest a conformation determinant of inhibitor selectivity. *Journal of Biological Chemistry* 279, 13095–13101.
- Katz, P., Nachtigall, R., Showstack, J., 2002. The economic impact of the assisted reproductive technologies. *Nature Medicine* 8, S29–S32.
- Low, B.-S., Das, P.K., Chan, K.-L., 2013. Standardized quassinoid-rich *Eurycoma longifolia* extract improved spermatogenesis and fertility in male rats via the hypothalamic-pituitary-gonadal axis. *Journal of Ethnopharmacology* 145, 706–714.
- Manna, P.R., Pakarinen, P., El-Hefnawy, T., Huhtaniemi, I.T., 1999. Functional assessment of the calcium messenger system in cultured mouse Leydig tumor cells: regulation of human chorionic gonadotropin-induced expression of the steroidogenic acute regulatory protein. *Endocrinology* 140, 1739–1751.
- Morgentaler, A., Lipshultz, L.I., Bennett, R., Sweeney, M., Avila Jr., D., Khera, M., 2011. Testosterone therapy in men with untreated prostate cancer. *Journal of Urology* 185, 1256–1261.
- Morgentaler, A., 2006. Testosterone and prostate cancer: an historical perspective on modern myth. *European Urology* 50, 935–939.
- Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., Olson, A.J., 1998. Automated docking using a Lamarckian genetic algorithm and empirical binding free energy function. *Journal of Computational Chemistry* 19, 1639–1662.
- Noor, M.M., Shaari, M.N.A.H., Lukman, C.H., 2004. The effect of *Eurycoma longifolia* Jack (Tongkat Ali) on sexual behaviour and sperm quality in rats. *Malaysian Journal of Pharmaceutical Sciences* 2, 53–60.
- Osborne, C., Tripathy, D., 2005. Aromatase inhibitors: rationale and use in breast cancer. *Annual Review Medicine* 56, 103–116.
- Pike, A.C.W., Brzozowski, A.M., Hubbard, R.E., Bonn, T., Thorsell, A.-G., Engström, O., Ljunggren, J., Gustafsson, J.-A., Carlquist, M., 1999. Structure of the ligand-binding domain of estrogen receptor beta in the presence of a partial agonist and full antagonist. *EMBO Journal* 18, 4608–4618.
- Prakash, G., 2007. *Reproductive Biology*. Alpha Science International, Oxford, pp. 12.8–12.10.
- Raman, J.D., Nobert, C.F., Goldstein, M., 2005. Increased incidence of testicular cancer in men presenting with infertility and abnormal semen analysis. *Journal of Urology* 174, 1819–1822.
- Razak, H.S.A., Shiud, A.N., Mohamed, I.N., 2012. Combined effects of *Eurycoma longifolia* and testosterone on androgen-deficient osteoporosis in a male rat model. *Evidence-based Complementary and Alternative Medicine*, 872406, <http://dx.doi.org/10.1155/2012/872406>.
- Schlegel, P.N., 2012. Aromatase inhibitors for male infertility. *Fertility and Sterility* 98, 1359–1362.
- Shiau, A.K., Barstad, D., Loria, P.M., Cheng, L., Kushner, P.J., Agard, D.A., Greene, G.L., 1998. The structure basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95, 927–937.
- Tambi, M.I.B.M., 2009. Nutrients and botanicals for optimizing men's health. Examining the evidence for *Eurycoma longifolia* Jack, the Malaysian ginseng in men's health. *Asian Journal of Andrology* 11, 37–38.
- Teh, C.H., Abdulghani, M., Morita, H., Shiro, M., Hussin, A.H., Chan, K.L., 2011. Comparative X-ray and conformational analysis of a new crystal of 13 α ,21-dihydroeurycomanone with eurycomanone from *Eurycoma longifolia* and their anti-estrogenic activity using the uterotrophic assay. *Planta Medica* 77, 128–132.
- Tsai, S.-C., Chen, J.-J., Chiao, Y.-C., Lu, C.-C., Lin, H., Yeh, J.-Y., Lo, M.-J., Kau, M.-M., Wang, S.-W., Wang, P.S., Wang, S.-W., Wang, P.S., 1997. The role of cyclic AMP production, calcium channel activation and enzyme activities in the inhibition of testosterone secretion by amphetamine. *British Journal of Pharmacology* 122, 949–955.
- Vasta, V., Shimizu-Albergine, M., Beavo, J.A., 2006. Modulation of Leydig cell function by cyclic nucleotide phosphodiesterase 8 A. *Proceedings of National Academic of Science* 103, 19925–19930.
- Wahab, N.A., Mokhtar, N.M., Halim, W.N.H.A., Das, S., 2010. The effect of *Eurycoma longifolia* Jack on spermatogenesis in estrogen-treated rats. *Clinics* 65, 93–98.
- WHO, World Health Organization, 2003. *Progress in reproductive health research no. 63*. (<http://www.who.int>) (accessed 20.09.2010.).
- Winston, R.M.L., 1986. *Infertility a Sympathetic Approach*. P. G. Publishing Pte. Ltd, Singapore p. 134.
- Zanoli, P., Zavatti, M., Montanari, C., Baraldi, M., 2009. Influence of *Eurycoma longifolia* on the copulatory activity of sexual sluggish and impotent male rats. *Journal of Ethnopharmacology* 126, 308–313.