ESTROGENIC ACTIVITY OF *Elaeis Guineensis Leaf*

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Summary

To assess the estrogenic activities of Oil Palm Fronds (*Elaeis guineensis*) extracts a vaginal cytology assay in normal and ovariectomized rats were used. The alcoholic extract of *Elaeis guineensis* (OPLME) was administered orally to adult normal cycling and ovareiectomized Sprague-Dawley rats. Bilaterally ovariectomized rats were divided into four groups (n = 8) receiving different treatments, consisting of vehicle, 150 and 300 mg/kg body weight OPLME and Premarin (conjugated estrogens) at a dose of 2.5 mg/kg body weight. Estrogenic activity was assessed by taking percentage vaginal cornification and uterine wet weight as parameters of assessment. The OPLME resulted in an irregular estrous cycle with lengthened estrus in dose dependent manner. Restoration of normal estrous cycles after withdrawal of treatment indicates the reversible effect of alcoholic extract in normal rats. OPLME administration produced statistically significant ($P < 0.001$), 2.54-fold increase in circulating 17 β -estradiol levels. OPLME showed a significant increase in percentage vaginal cornification, and uterine wet weight ($P < 0.001$), compared to the control in a dose dependent manner in ovariectomized rat. This estrogenic property of OPLME may be a possible explanation for the OPLME effects on the blood lipid profile. The estrogenic activity shown by OPLME can be attributed to the presence of flavonoids and phenolic compounds.

Keywords: Oil palm; Polyphenols; vaginal cytology assay; Ovariectomized rats

Introduction

Oil palm (Elaeis guineensis Jacq.) is a perennial monocot belonging to the family Palmae and tribe Cocoineae. It gives the highest oil yield per hectare of all the economic oil crops and the most important industrial crops in Malaysia (1). Oil palm leaflet or frond is a major waste by-product of the palm oil industry and contain several flavonoid compounds. Irine et al. (2003) reported that the total polyphenolic content (TPC) of oil palm frond methanolic extract (OPFME) is 24.3 mg gallic acid equivalent (GAE)/g dry weight, which is significantly higher than that of green tea (22.5 mg GAE/g dry weight)(2). The OPLME possessed better in-vitro antioxidant activities compared to green chili, lemongrass and papaya shoot extracts (3). OPLME had a dual effect on the growth of MCF-7 human mammary adenocarcinoma cells, stimulated by low doses and suppressed by high doses. The result was similar to that of estrogen and soybean products which contain phytoestrogens especially genistein, which shown in many studies could play a positive role in the growth of MCF-7 cells at the low concentration range.

In the present study, a preliminary experiment was run to find the effect of methanol extract of oil palm fronds (*Elaeis guineensis*) on the estrous cycle in normal rat. The final goal was to evaluate the estrogenic effect of oil palm fronds (*Elaeis guineensis*) extracts on ovariectomized Sprague-Dawley rats.

Materials and methods

Preparation of plant material

Fresh oil palm leaves from University Putra Malaysia plantation were weighed, chopped and oven-dried at 40°C overnight. The dried material was ground with a blender, and used for the first experiment. For the second experiment Oil palm leaf powder triple extracted (10 g per 100 ml) with methanol, acetone, chloroform, hexane, and petroleum ether. As methanol gave the highest yield, it was used. Pooled extracts were vacuum – dried at 40°C, freeze-dried, flushed with nitrogen and stored at -20°C until analyzed.

Animals

Adult female Sprague-Dawley rats, weighing 200-250 g, with regular estrous cycles (4-5 days) for three consecutive cycles before the study period were used in this study. They were obtained from Sapphire Enterprise, Malaysia, individually housed in wire cages, and were maintained under controlled standard animal house conditions with access to food and water ad libitum.
The experimental protocol was approved by the Animal Ethical Committee in accordance with the guide for the care and use of laboratory animals' prepared by University Putra Malaysia.

Experimental design

Effect of OPLME on the estrous cycle of normal cycling female rats

The above selected animals were randomly divided into three groups containing eight animals in each group: a vehicle control, a low dose (150 mg/kg BW), and a high dose (300 mg/kg BW) of OPLME. The OPLME was measured and applied in morning daily to a 2 g fresh apple wedge used as vehicle. There was a dual advantage to using an apple vehicle as Skibola (2005) mentioned: (1) it eliminated stress associated with gavage, (2) rats eagerly ate the apple and the Oil palm leaves in this manner, making it easy to monitor and ensure complete deliverance of the powder of oil palm leaf (4). Vaginal smears from each rat were monitored daily between 09:00 and 10:00h using staging criteria described by Everett (5) during treatment period and for two weeks after the termination of treatment.

To assure that all rats were cycling normally, the estrous cycle was monitored for 14 days prior to initiation of treatment. The vaginal epithelium cells observed under the microscope were classified into three types: leukocyte cells (L), nucleated cells (O) and cornified cells (Co). The representative cell type was determined by choosing the majority of cells. The results of examined vaginal smear cells from five rats in each treatment group were expressed as a mode value (the most frequently occurring cell type in five rats). After 6 week, all rats were removed from treatment.

For serum hormone studies 2 ml blood was collected via cardiac puncture during the morning of proestrous (determined by vaginal cytology). Immediately following the blood draw, rats were given low dose (150 mg/ kg) or high dose (*300mg/kg)* OPLME extract. At 2- and 4-wk intervals, blood was redrawn during the morning of proestrus. Blood samples were allowed to clot at room temperature and were centrifuged for 10 min at 2000 × g. Serums were aspirated and frozen at -20° C until further analysis. Blood serum 17 β -Estradiol was assayed by a radioimmunoassay COAT.A.COUNT (Siemens Medical Solutions Diagnostics).

Effect of OPLME on ovariectomized rats

Estrogenic activity of OPLME was assessed in bilaterally ovariectomized female Sprague-Dawley rats using a standardized method with few modifications, taking percentage vaginal cornification, and uterine wet weight as parameters of assessment. Adult animals were ovariectomized under ketamine (87 mg/ml) and xylazine (13 mg/ml) anesthesia at a dose of 0.1 ml/kg and allowed to recover for 21 days prior to use in the uterotrophic or vaginal cytology studies. The ovariectomized rats were divided into 4 groups each consisting of eight animals. In the first, rats were gavaged daily with 0.7 ml distilled water and kept as a negative control. In the second, rats were gavaged daily with 2.5 mg/kg BW Premarin (conjugated estrogens) in 0.7 ml of distilled water and kept as a positive control. In the third and fourth, rats were gavaged daily OPLME at doses of 150 and 300 mg/kg BW in 0.7 ml of distilled water, respectively. Generally, duration of experiment was 14 days and treatments were performed at 10:00-11:00 h, vaginal smears were checked daily between 0.9:00 and 10:00 AM in all rats throughout the experimental period. At the end of treatment period, rats were euthanized; the uteri were dissected and weighed, thereafter. The serum was analysed in an automatic blood chemical analyser for the levels of total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, and total cholesterol/HDL ratio.

Statistical Analysis

Analysis of variance (ANOVA) was used to determine the differences of means of estradiol levels. The observed significance was then confirmed using the least significant difference (LSD) test. One-way analysis of variance (ANOVA) was used to analyze the difference in uterine wet weight, and vaginal cornification between different groups of treatments. Significance level was set at $P < 0.05$. The SPSS, a statistical analysis program, Version 11.5, was used.

Results

Effect of OPLME on the estrous cycle of normal cycling rats

Based on distribution and density of cell types, each daily vaginal smear was assigned one of four estrous cycle stages: proestrus, oestrus, metoestrus, and diestrus. Untreated SD female rats displayed estrus once every 4 to 5 d. The effect of OPLME on rat estrous cycle is shown in Fig. 1 As shown, both dose of OPLME employed produced some irregularities in the estrous cycle 4 day after treatment, specifically, with frequent appearance of estrus. Thereafter, the cycling changed to PE within next 14 days, and the condition generally lasted till the termination. Daily doses of 300 mg/kg maintained rats in estrus for a longer period of time than a daily dose of 150 mg/kg. After the termination of the treatment the rats resumed their regulation 4-day cycles.

Effect of OPLME on the estradiol levels

Following the 150 mg/kg BW treatment for 2 wk, mean serum estradiol levels were increased from 20.80 ± 4.43 to 33.78 ± .2 pg/ml (P = 0.09) and after 4 wk at the same dose levels were significantly increased from baseline to 42.17± 6.37 pg/ml (P = 0.01). In high dose group (300 mg/kg BW) after 2 wk treatment mean serum estradiol levels were increased from 21.46 ± 2.88 to 35.42 ± 6.37 pg/ml (P = 0.05) and after 4 wk increased from baseline to 72.10 ± 8.87 pg/ml (P = 0.00), suggesting an effect of dosing over time.

Effect of OPLME on vaginal cytology of ovarectomomized rats

All rats had only L-type cells throughout the pre-treatment period after ovariectomy. It was confirmed by the fact that the ovaries were completely removed and no endogenous ovarian estrogens were produced. The administration of distilled water did not influence the vaginal epithelium, and only L-type cells were found. In contrast, synthetic estrogen induced a cornification of the vaginal epithelium as early as the third day of treatment. As shown in Fig.2 the occurrence of vaginal cornification in rats was dependent on doses of OPLME. The higher dose of OPLME showed an earlier response.

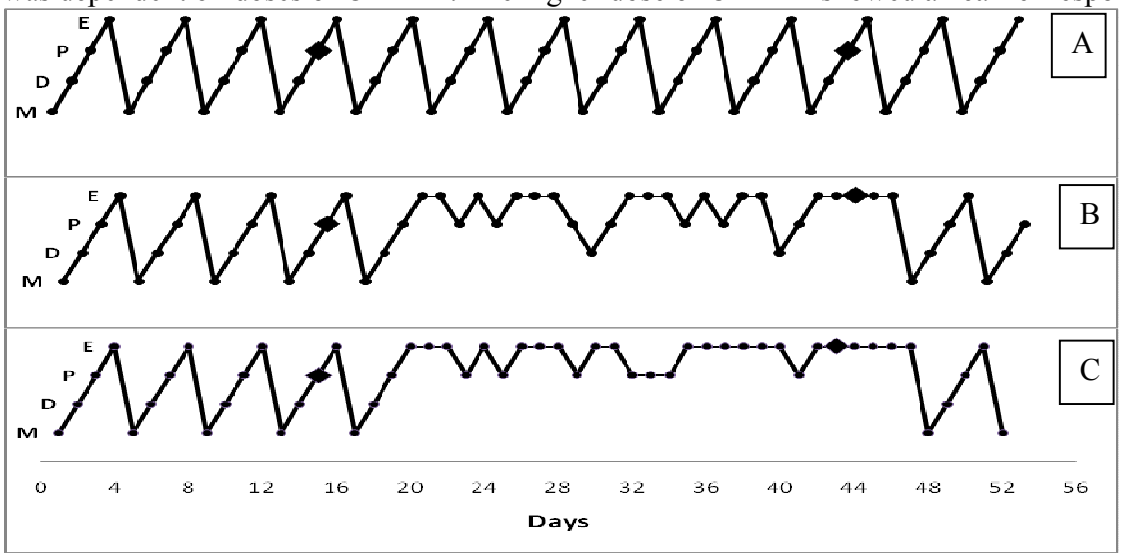

Fig.1. Effect of OPLME on rat estrous cycle.
A.control group receiving vehicle; B. experimental group receiving 150 mg/kg OPLME ; C. experimental group receiving 300 mg/kg OPLME.Abbreviations are: E, estrus; P, proestrus; D, diestrus; M, metestus; ⧫ , starting and termination of the treatment.

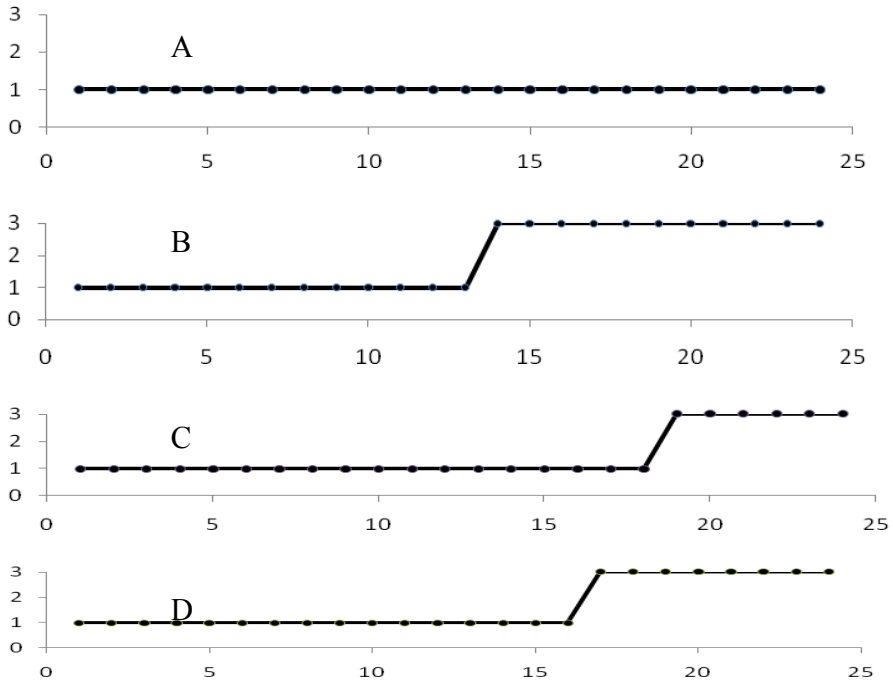

Fig.2 Comparison of the estrogenic activity of OPLME and Premarin, by vaginal cytology assay in ovariectomized rats. A. negative control group receiving vehicle; B. positive control group receiving 2.5 mg/kg BW Premarin; C. experimental group receiving 150 mg/kgBW OPLME ; D. experimental group receiving 300 mg/kgBW OPLME. 1, 2, and 3 indicate leukocyte cells, nucleated cells and cornified cells, respectively.

Effect of OPLME on Uterus weight of ovarectomomized rats

The increment of uterus weight at the end of treatment period in both OPLME groups of rats agreed with changes of vaginal epithelium, that is, the higher doses of OPLME showed heavier uterus weight (Table 1). For instance, the uterus weights of rats treated with 300 mg/kg BW of OPLME were heavier than those treated with 150 mg/kg BW.

Tab.1. Uterus weights at the end of treatment period in ovariectomized rats treated with distilled water, Premarin and two dose of OPLME.

Experimental Group	**Uterus weight (mg)**
Negative control (distilled water)	18±2.5
Positive control (Premarin)	73.75±6.7***
Low OPLME (150mg/kg)	23±3.2*
High OPLME (300mg/kg)	38±2.3**

* $P < 0.05$ compared to the mean of distilled water group.
** $P < 0.01$ compared to the mean of distilled water group.
*** $P < 0.001$ compared to the mean of distilled water group.

Effect of OPLME on lipid profile

Table 2. shown serum levels of total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, and total cholesterol/HDL ratio at the end of treatment period with distilled water (control), Premarin and two dose of OPLME in ovariectomized rats compare to sham group (un-ovariectomized rats). OPLME has a lowering effect on total cholesterol, and triglyceride ($P < 0.001$), this effect was similar to premarin. The effect of OPLME on lowering total cholesterol, and triglyceride was dose dependent. OPLME has an increasing effect on HDL profile, especially high dose of OPLME has more significant effect ($P < 0.001$) compare to low dose OPLME, and premarin ($P < 0.01$).

No significant differences in food consumption or body weights were observed between the control and treatment groups in OVX rat during the treatment period suggesting that the treatments had no side effects on the general health condition.

Table 2. serum levels of total cholesterol, triglyceride, HDL cholesterol, LDL cholesterol, and total cholesterol/HDL ratio at the end of treatment period with distilled water (control), Premarin and two dose of OPLME in ovariectomized rats compare to sham group (un-ovariectomized rats).

Group	Sham	Control	Premarin	Low OPLME	High OPLME
T.CH	1.65±0,26	1.53±0.29	1.61±0.24**	1.41±0.13**	1.3±0.1***
TRI.G	1.09±0.51	1.48±0.83	0.470±0.07**	0.463±0.15***	0.38±0.03***
HDL	0.53±0.07	0.40±0.01	0.48±0.05**	0.48±0.04**	0.57±0.1***
LDL	0.610±0.18	0.473±0.33	0.920±0.02	0.801±0.16**	0.725±0.1
RATIO	3.1±0.1	3.0±0.1	3.3±0.1*	2.9±0.1*	2.5±0.3**

* $P < 0.05$ compared to the mean of distilled water group.
** $P < 0.01$ compared to the mean of distilled water group.
*** $P < 0.001$ compared to the mean of distilled water group.

Body weights and food consumption

Discussion

The present results demonstrate that OPLME can induce various changes in the reproductive tract of normal cycling rats. Most notable changes were the disappearance of normal cyclicity and appearance of PE (persistent estrus) in vaginal cytology, the effects being time- and dose-dependent.

It is curious, however, that in intact, regularly cycling females, treatment with low doses of estrogenic compounds induces pseudopregnancy and not persistent or constant estrus (6). Thus, the smear of the intact, estrogen-treated female will initially exhibit a persistence of leukocytes because of the effect of estrogen on prolactin secretion, which in turn rescues and maintains functional corpora lutea. Apparently, the endogenous progesterone in such animals prevents the action of exogenous estrogens on the vaginal epithelium. After prolonged treatment, however, the smear will become cornified. This generally occurs when the ovaries have atrophied as a result of the low gonadotropin (LH and follicle-stimulating hormone) secretion attributable to the estrogen or estrogenic compounds effect on the brain and pituitary. Thus, when intact young-adult females are treated, the rapid development of persistent vaginal cornification is not the best indication that the compound is an estrogen. This was demonstrated by Laws *et al*. (1996), who reported that oral exposure to ethinyl estradiol and the putative environmental estrogen 4-tertoctylphenol induced an immediate pseudopregnancy as indicated by leukocytic smears and elevated serum progesterone on day 10 of treatment (7).

However, there do appear to be some conflicting observations in this regard, because Blake and Ashiru (1997) reported that when regularly cycling females were injected subcutaneously with 4-tert-octylphenol, a persistent estrus was observed beginning 3 days after the initiation of treatment (8).

In this study, persistent estrus was observed 22 days after treatment with OPLME, which is not consistent with sc exposure to 17β estradiol, which has been reported to initially cause an extended period of diestrus (6), suggesting that perhaps OPLME capable of altering estrous cyclicity via multiple mechanisms.

The OPLME administration produced statistically significant ($P < 0.001$), 2.54-fold increase in circulating 17 β -estradiol levels in day of 14 and 28 of treatment. We were unable to detect significant correlation between the specific days of the cycle with hormonal levels. Additional studies are needed especially when exposure occurs during critical periods. Normal cycling women who were administrated with GnRH antagonist reduced the pulse amplitude and frequency of LH. Serum levels of FSH, LH, estradiol, and ir-inhibin decrease during the menstrual cycle. These changes are consistent with an increase in length of the follicular phase and total menstrual cycle. Cassidy and colleagues observed that the inclusion in the diet of modest amounts of soy protein containing isoflavonoids extended the follicular phase of the menstrual cycle and attenuated the preovulatory LH and FSH surges (9).

Vaginal cytology assay which has been firstly used by Cook in 1933 is a reliable, sensitive, simple and inexpensive method. It is particularly used to determine the estrogenic activity of the synthetic estrogens, xenoestrogens and phytoestrogens (10, 11). If female rats are ovariectomized, the resultant lack of estrogen causes atrophy of the uterus and the reproductive tract; administration of estrogenic substances to ovariectomized rats leads to uterotrophic effects, vaginal cornification, increase in uterine glycogen content and proliferative changes in uterine endometrium (12). Estrogenic compounds are known to cause the keratinisation and cornification of the vaginal epithelium, causing the superficial cells to be shed into the lumen to form large squamous cells (13). This specific action of estrogens is due to the presence of specific receptors, ERα and ERβ (14). Both ERα and ERβ function in normal ovarian follicular development, vascular endothelia cells, myocardial cells, smooth muscle, and breast tissue (15). ERα is involved in bone maturation in both males and females, however, only ERβ plays a role in bone maintenance in females (15). ERα is more important in maintaining follicle stimulating and luteinizing hormone concentrations in blood, and ERβ is involved in frontal lobe mediated learning and memory (15). The dominant form of estrogen in the body is 17β estradiol, although any compound that induces receptor dimerization and subsequent binding to the ERE, can be considered an estrogen. Antagonistic effects can occur when a compound is able to bind to the receptor but dimer formation either does not occur or the correct configuration to activate the ERE is not attained. Some compounds act as estrogen agonists and antagonists and are referred to as Selective Estrogen Receptor Modulators (SERMs). As an example, the antiestrogen tamoxifen acts as an estrogen antagonist in breast tissue but as an agonist in the uterus, bone and vascular system (16). These agonist/antagonistic effects are believed to be responsible for the differential effects of phytoestrogens compared to estradiol.

All rats had only L-type cells throughout the pre-treatment period after ovariectomy. Synthetic estrogen induced a cornification of the vaginal epithelium as early as the third day of treatment, whereas the administration of distilled water did not influence the vaginal epithelium, and only L-type cells were found. The occurrence of vaginal cornification in experimental groups of rats was dependent on doses of OPLME. The higher dose of OPLME showed an earlier response.

Thus the dose dependent increase in percentage vaginal cornification shown by OPLME can be attributed to its estrogenic activity. Estrogenic potency and efficacy have traditionally been expressed in terms of uterotrophic effects in immature or ovariectomized female rats (17).

In OVX rats, treated for 3 months with estradiol (0.5 mg/day/animal, via food) vaginal cornification was regularly observed, whereas the sham-treated animals have an unestrous vaginal smear (11). There are many studies about the effects of phytoestrogens on genital tract of women and female animals. Miroestrol produced cornification of the vaginal epithelium in the immature female mice (18). Dietary supplementation with phytoestrogens led to increased vaginal cytological maturation in women (19). Six-month treatment of soy-rich diet to the asymptomatic post-menopausal women increased vaginal cornification of epithelium, karyopycnotic index (KI) and maturation value (MV), identical to those found in the hormonal replacement women (20).

The OPLME showed a dose dependent, statistically significant ($P < 0.001$) increase in uterine wet weight compared to control (Table 1).The standard drug Premarin produced statistically significant ($P < 0.001$), threefold increase in uterine wet weight. Literature review conducted on OPLME indicated the presence of flavonoids, and phenolic compounds. Flavonoids and phenolic compounds are known to possess estrogenic activity (21, 22). Thus the estrogenic activity shown by OPLME can be attributed to the presence of flavonoids and phenolic compounds.

In this study OPLME also had some hypocholesterolemic effects. Irine *et al* (2003) reported that palm leaf diets had hypocholestrolemic effects on hypercholesterolemic rabbits, via their fibre content (2).

The cardioprotective effect of low-dose estrogen therapy after menopause has been demonstrated repeatedly, but the mechanism of protection is uncertain. Two randomized crossover studies from Brigham and Women's Hospital in Boston indicate that one mechanism may be improvement in the lipid profile. In the first trial, 31 healthy postmenopausal women received conjugated estrogens in a daily dose of 0.625 mg or 1.25 mg for three months, and placebo for another three- month period. Relative to placebo, the low and high doses of estrogen decreased levels of LDL cholesterol by 15 percent and 19 percent, respectively, and increased HDL cholesterol levels by 16 percent and 18 percent. In the second trial, nine postmenopausal women received oral micronized estradiol (2 mg per day), transdermal estradiol (0.1 mg twice per week), or placebo during three different six-week periods. Compared with placebo, oral estradiol reduced LDL levels by 14 percent and increased HDL levels by 15 percent; transdermal estradiol had no effect on lipids. These trials provide strong evidence that estrogen therapy has a short-term beneficial effect on lipid profiles in postmenopausal women (23). The estrogenic activity shown by OPLME can be attributed to the presence of flavonoids and phenolic compounds. This estrogenic activity of OPLME may be a possible mechanism for effects of OPLME on lipid profile in this study.
Elucidation of the exact mechanism of action of the estrogenic principle of the OPLME demands isolation of active constituents, ER selectivity studies of such isolated compounds and performance of more extensive studies.

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