ISSN: 2322 - 0902 (P)
ISSN: 2322 - 0910 (O)

International Journal of Ayurveda and Pharma Research

Research Article

EFFECT OF ETHYL ACETATE SOLUBLE PROANTHOCYANIDINS FROM *COCOS NUCIFERA* L. INFLORESCENCE ON PROGESTERONE AND OESTROGEN LEVELS IN FEMALE RATS

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Received on: 20/06/2015 Revised on: 16/07/2015 Accepted on: 22/07/2015

ABSTRACT

The immature inflorescence of *Cocos nucifera* L. variety aurantiaca is used by Ayurvedic and traditional medical practitioners for the treatment of menorrhagia in Sri Lanka. We have previously reported the extraction, purification and characterization of ethyl acetate soluble proanthocyanidins (EASPA) in the inflorescence of *Cocos nucifera* L. EASPA obtained from immature *Cocos nucifera* L. (var. aurantiaca) inflorescence was evaluated for its effect on the reproductive hormonal levels of female rats. EASPA (0.33 mg/day) dissolved in water was administered orally to female rats for 28 consecutive days. At the end of the study period, oestrogen and progesterone levels were measured and compared with the control group (water). Statistical analysis was performed with one-way ANOVA, followed by student T test using Minitab 17.0 software. The length of the reproductive cycle was 4.44 ± 0.15 days and 4.56 ± 0.15 days for the control and test group rats, respectively. No significant changes were noticed in the length of the cycle nor were there any difference in vaginal cytology in test and control group rats. There was no significant difference in the estrogen level between control and test group animals. However, there was a highly significant increase in progesterone levels of the test group as compared to the control ($P \leq 0.001$). This result suggests a possible mode of action to explain the use of coconut inflorescence in controlling menorrhagia in traditional medicine in Sri Lanka.

KEYWORDS: *Cocos nucifera* inflorescence, menorrhagia, proanthocyanidins, progesterone.

INTRODUCTION

Cocos nucifera L. is a member of the monocotyledonous family Arecaceae and is the only species of the genus. It mainly grows in tropical coastal areas and in Sri Lanka; it is also a plantation crop.

Ethnomedical usages and biological activities of different parts of the *Cocos nucifera* L. have been reported. Different extracts of husk fibre have been shown to possess antimicrobial[1], radical scavenging[2], analgesic[2], anti-inflammatory[3], anthelmintic activity[4] and antiproliferative activities[5]. Recent studies have revealed that the different extracts from mesocarp exhibit antimalarial[6], vasorelaxant[7] and antihypertensive activities[7]. Several studies found that virgin coconut oil has numerous medicinal properties, including cardioprotective[8], antithrombotic[9] and hypolipidemic activities[9]. Coconut water exhibit antioxidant[10] and hypolipidemic[11] activities. The results obtained from a recent study suggested that the coconut inflorescence has cytoprotective and antihyperglycemic properties[12]. Protective and curative effects of *Cocos nucifera* L. inflorescence on alloxan-induced pancreatic cytotoxicity in rats has also been reported[13].

Cocos nucifera L. is classified in to three varieties in Sri Lanka: Typica, Nana and Aurantiaca[14]. Of this, inflorescence of orange coloured variety aurantiaca is used by Ayurvedic and traditional medical practioners for the treatment of menorrhagia in Sri Lanka. We have previously reported the extraction, purification and characterization of the ethyl acetate soluble proanthocyanidins (EASPA) of the inflorescence of *Cocos nucifera* L.[15]. There is evidence also to suggest that proanthocyanidins may play a role in the treatment of menorrhagia[16].

In women, menorrhagia is menstruation at regular cycle intervals but with excessive flow and duration. Clinically it is defined as total blood loss exceeding 80 mL per cycle or menstruation lasting longer than 7 days. According to World Health Organization data, approximately 18 million women worldwide are affected by menorrhagia[17]. A number of conditions may cause menorrhagia, including endocrine disorders, uterine abnormalities, coagulation disorders and other pelvic diseases[18]. Eighty percent of women treated for menorrhagia have no anatomical pathology and over a third of the women undergoing

hysterectomies for menorrhagia have normal uteri removed[18,19]. Therefore, drug therapy, with the avoidance of unnecessary surgery, is a better alternative. A broad spectrum of medications is used to treat menorrhagia. According to a study conducted in UK, progestogens make up 55% in total of prescriptions for menorrhagia[20].

Given the predominance of proanthocyanidins in the inflorescence of *Cocos nucifera* L., we considered the possibility that they play a role in controlling menorrhagia by influencing the reproductive hormone levels. In this paper, we report the effect of ethyl acetate soluble proanthocyanidin (EASPA) fraction of *Cocos nucifera* L. inflorescence on reproductive hormonal levels of female rats.

MATERIALS AND METHODS

Materials

Light microscope (Meiji MT5000) with x10 and x40 objective lenses were used for vaginal cytology observations. Estrogen and progesterone levels were assessed using AxSYM Estradiol and AxSYM Progesterone test kits respectively.

Plant material

Inflorescences were collected from healthy adult *Cocos nucifera* L. (var. aurantiaca) palms situated in the University of Sri Jayewardenepura premises, Sri Lanka from May 2012 to April 2014. Immature inflorescence (the inflorescence which was situated just above the freshly opened inflorescence in the palm) was plucked and the spathe was removed. The inflorescence was botanically authenticated by Mr. I. U. Kariyawasam at Department Of Botany and voucher specimen (Assess. No. A3 S13, 001) was deposited in the herbarium of the Department Of Botany, Faculty of Applied Sciences, University of Sri Jayewardenepura, Sri Lanka.

Extraction and Purification

Extraction and purification of ethyl acetate soluble proanthocyanidin (EASPA) fraction in the immature inflorescence of *Cocos nucifera* L. has already been reported[15]. EASPA fraction of an acetone/water (7:3) extract of *Cocos nucifera* L. inflorescence was purified on Sephadex LH-20 to yield purified EASPA as an off white powder in 0.03% and was used for the study.

Experimental Animals

Female Wistar albino rats (origin- Wistar Institute of Biology, USA, *Rattus norvegicus*), approximately 14-16 weeks old, weighing 200-250 g exhibiting regular reproductive cycles obtained from Animal Centre, Medical Research Institute, Colombo 8, Sri Lanka, were used for the study. The animals were housed in standard cages, three per cage with sawdust as bedding. They were fed pelleted standard rat feed twice daily and watered *ad libitum*. Rats were exposed to a 12 hours light/dark cycle at room temperature. They were identified by colour markings on their body. This study was approved by the Ethics Review Committee, Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka. The rats were handled in accordance with the CPCSEA guidelines for the care and use of laboratory animals.

Test method

Animals were weighed and divided in to two groups with each containing eight rats. Group I animals were administered orally using a sondi needle with 0.33 mg of purified EASPA dissolved in 2 mL of tap water for 28 consecutive days. Available knowledge on usage of immature florescence of *Cocos nucifera* L. in Ayurveda was used to calculate the dose. Human dose in grams was extrapolated to rat dose according to the standard chart given in literature [21]. The resulting value was multiplied by the yield of purified EASPA to obtain the final dose. The group II animals received 2 mL of tap water in the same way for 28 days and served as control group. The animals were observed daily for behavioral activities. Body weight was recorded every day during the study period.

Vaginal cytology

Every afternoon during the 28-day study period, between 2.00 and 3.00 pm each animal cage was carried to the experimental room. The vaginal cell samples of the female rats were obtained by washing the vagina with normal saline. A blunt pipette with normal saline was gently inserted into the entrance of the vagina to a depth of 2-5 mm and the saline was flushed into the vagina and washings were collected into the pipette two or three times by gently squeezing and releasing the bulb of the pipette. A small amount of the cell suspension was then expelled onto a labeled glass slide and covered with a cover slip. This unstained cell suspension was examined under a light microscope (with x10 and x40 objective lenses) to determine the cytology of the vaginal epithelium in order to study the reproductive cycle. The phases of the reproductive cycle and its duration were determined as described in literature [22].

Serum oestrogen and progesterone levels

Blood samples were collected from rats after 28 days of study period at proestrous phase by tail vein bleeding under anesthetic ether anesthesia and estrous phase by cardiac puncture technique immediately after euthanasia. Rats were euthanized by overdosing of anesthetic ether inhalation. The blood was then allowed to stand for 40 minutes at room temperature to clot and centrifuged at 3000 r/min for 10 minutes. The supernatant (serum) was then tipped off in to a separate tube. Those of proestrous and estrous phases were subsequently subjected for the assessment of estrogen and progesterone levels, respectively.

Statistical analysis

The results are represented as the mean ± SEM. Every statistical analysis was performed with one-way ANOVA, followed by student T test using Minitab 17.0 software. Differences were accepted as statistically significant at $P \leq 0.05$ and $P \leq 0.001$.

RESULTS AND DISCUSSION

The reproductive cycle of female rats is characterized by proestrous, oestrus, metestrous and diestrous phases, which last for 12, 12, 21 and 57 hours, respectively[22]. Ovulation occurs from the beginning of the proestrous phase up to the end of the estrous phase. From the onset of sexual maturity up to the age of 12 months, the mean cycle length in a female rat is 4 days and this short cycle length makes the rat an ideal animal for investigation of changes occurring during the reproductive cycle. Female rats are characterized by a very distinct vaginal cytology depending on the phase of the reproductive cycle. Vaginal smear cytology was performed during the 28 day study period to determine the phase and the length of the estrous cycle. Three main cell types cornified, epithelial, and leucocytes were observed in the vaginal smears as expected. The difference in proportions among these cell types permitted the identification of the different phases of the estrous cycle between successive ovulations[22]. Depending on the time of smearing, transitional smears were also observed. Proestrous phase smears were characterized by rounded, usually nucleated, epithelial cells generally in low to moderate (occasionally high) numbers. Oestrous phase smears consisted entirely of cornified cells, in high numbers and usually forming clumps and sheets and that of metestrous phase consisted of large numbers of leucocytes and smaller numbers of large, non-granular and non-nucleated epithelial cells. Dioestrous phase smears predominantly consisted of leucocytes with variable numbers of epithelial and small-cornified cells. The cell numbers are low to moderate and the general appearance was similar to metestrous but usually with far fewer cells and without the tightly packed clumps. Dioestrous was the most difficult phase to recognize because of the variability in the numbers and ratios of the cells. Figure 1 shows the unstained vaginal smears from control group female rats at different phases.

Levels of the reproductive hormones oestrogen and progesterone, vary during the reproductive cycle. The highest levels of oestrogen are observed during the early proestrous phase while lowest levels are observed during oestrous and metestrous phases. With respect to progesterone, the highest levels are observed during late proestrous and early estrous phases with lower levels being observed during metestrous to diestrous phases[23]. The oestrogen and progesterone levels were measured in their peak phases, proestrous phase and oestrous phase, respectively.

Weight changes of test and control group rats during the study period are shown in Figure 2. These results indicate that both test and control group rats have gained weight during the study period. There is no significant difference in the weight gained of the female rats after 28 days of oral administration with EASPA when compared with respective controls ($P \leq 0.05$). There were no treatment related changes in the behavioral pattern of rats in the test group as compared to the control group. Rats belonging to both control and test groups appeared healthy and alert. This suggests that EASPA is safe to be used on female rats at the dose level employed. The length of the reproductive cycle was 4.44 ± 0.15 days and 4.56 ± 0.15 days for the control and test group rats, respectively. No significant changes were noticed in the length of the cycle nor was there any difference in vaginal cytology in test and control group rats. The levels of oestrogen and progesterone for the control group rats were within the normal range. There was no significant difference in the estrogen level between control and test group animals. However, there was a highly significant increase in progesterone levels of the test group compared to the control ($P \leq 0.001$). The results are given in Table 1.

Table 1: Effect of EASPA on length of oestrous cycle and reproductive hormone levels of group I and group II female rats

Parameter	Group I (EASPA administered)	Group II (Control)
Duration of estrous cycle (days)	4.56 ± 0.15	4.44 ± 0.15
Progesterone level (ng/mL)	77.82 ± 1.65*	32.79 ± 1.60
Oestrogen level (pg/mL)	55.75 ± 4.23	53.13 ± 4.06

*n = 8, all values are presented as Mean ± SEM. *significantly different from control value (P≤0.001)*

The fact that there was a significant difference in progesterone levels in the test group compared to the control group suggest that the administration of EASPA fraction may directly or indirectly lead to an increase of progesterone levels in female rats at the EASPA dose level employed. In western medicine, synthetic progestogens such as norethisterone are used to treat heavy menstrual bleeding in women (menorrhagia). They are also used in combination with oestradiol in the combined oral contraceptive pill, which in turn is also used to treat menorrhagia. When progestogens are used alone in the treatment of menorrhagia, the reduction of blood loss is marked when they are started prior to ovulation in the proliferative phase, when given for 21 days (from days 5 to 26 of the menstrual cycle)[18]. Progestogens act by

opposing the action of oestrogens by minimizing the effects of estrogen on target cells, thereby maintaining the endometrium in a state of down-regulation. The end result is suppression of endometrial glandular growth, stromal decidualization, leukocytic infiltration, glandular atrophy and stromal focal necrosis, thereby leading to a reduction in menstrual blood loss[24]. The same principle might be applied in Ayurvedic therapy to reduce the menstrual blood flow. Hence this could be the basis for the use of immature inflorescence of *cocos nucifera* L. in Ayurvedic medicine to treat menorrhagia.

CONCLUSION

The increase in progesterone levels in female rats administered with EASPA suggests a possible mode of action, which explains the use of coconut inflorescence in controlling menorrhagia in traditional medicine in Sri Lanka. To the best of our knowledge this is the first report of the effect of proanthocyanidins on reproductive hormone levels. It is significant that proanthocyanidins, are chemically different to progestogens used in modern medicine. Our findings open the possibility of clinical use of a new class of drug, which can be used in women diseases through its influence on progesterone levels.

ACKNOWLEDGEMENT

The research work was financially supported by the university research grant of Sri Jayewardenepura University, Sri Lanka: ASP/06/RE/SCI/2010/11.

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Cite this article as:
C. Padumadasa, D. Dharmadana, A. M. Abeysekera, M. G. Thammitiyagoda. Effect of Ethyl Acetate Soluble Proanthocyanidins from *Cocos nucifera* L. Inflorescence on Progesterone and Oestrogen Levels in Female Rats. International Journal of Ayurveda and Pharma Research. 2015;3(7):1-6.
Source of support: Nil, Conflict of interest: None Declared

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STUDY PHOTOGRAPHS

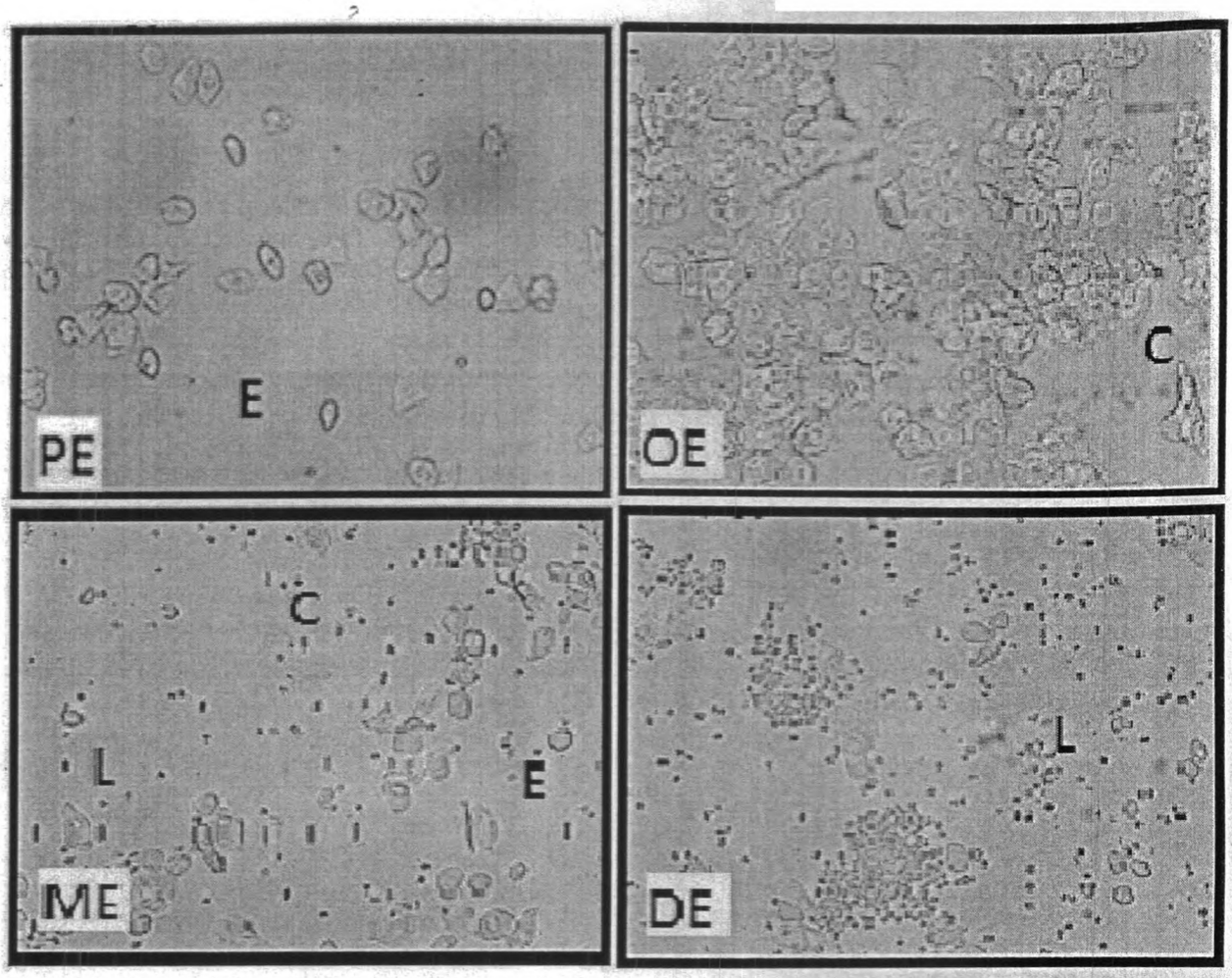

Figure 1: Unstained vaginal smears from control group female rats at proestrous (PE), oestrous (OE), metestrous (ME) and diestrous (DE) phases. Leukocytes (L), epithelial (E) and cornified (C) cells are indicated

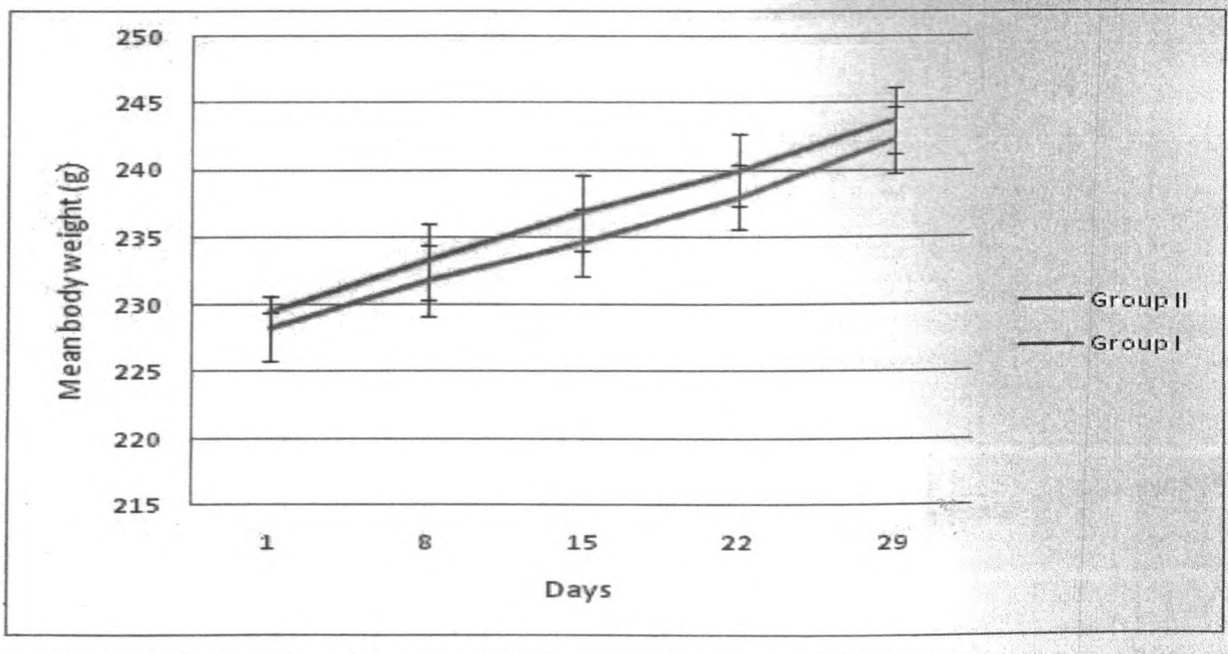

Figure 2: Variation of mean body weight during the study period of EASPA administered and control group female rats