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# A STUDY OF THE EFFECT OF ETHYL ACETATE SOLUBLE PROANTHOCYANIDINS ON HISTOLOGICAL CHANGES OF FEMALE RAT ENDOMETRIUM

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**ABSTRACT**

Proanthocyanidins are secondary metabolites found in plants and they belong to a class of polyphenolic compounds called flavonoids. Proanthocyanidins have been reported to exhibit diverse biological activities. We have previously reported the extraction, purification, characterization and progestrogenic activity of ethyl acetate soluble proanthocyanidins (EASPA) in the inflorescence of *Cocos nucifera* L. EASPA was evaluated for its effect on histological changes of female rat endometrium during proestrous and oestrous phases of the reproductive cycle. 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, uteri were dissected and subjected to histological studies. There were no detectable histological changes of the endometrium of the test group rats compared to that of the respective control groups. This may be due to an inadequacy of the dose and time duration that EASPA was administered to female rats. This may also be due to the reproductive cycle of the rats being different to that of humans and the histological changes of the endometrium of female rats being independent of the increase in the progesterone levels after administration of EASPA.

**KEYWORDS**

Proanthocyanidins, Menorrhagia, Histology, Proestrous phase, Oestrous phase and Endometrium.

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## INTRODUCTION

*Cocos nucifera* L., the coconut palm is found widely distributed in the Asian continent and in parts of South America and Africa[1]. It is a member of the monocotyledonous family Arecaceae (Palmae) and is the only species of the genus. Coconut palm is a cultivated crop in Sri Lanka, which is a major producer of coconut in the world.

In Sri Lanka, the immature inflorescence of *Cocos nucifera* L. is used by Ayurvedic and traditional

medical practitioners for the treatment of menorrhargia. *Cocos nucifera* L. Is classified in to three varieties in Sri Lanka: Typica, Nana and Aurantiaca[2]. Of this, the orange coloured variety aurantiaca is used for this purpose. Our previous studies revealed that the inflorescence of *Cocos nucifera* L. contains high level of proanthocyanidins[3]. Proanthocyanidins have recently attracted a considerable amount of attention in the fields of medicine, health and nutrition. They have been reported to exhibit antioxidant[4], anti-inflammatory[4], bacterial anti-adhesion[5], anticancer[6], and cardioprotective[7] activities. There is evidence also to suggest that proanthocyanidins may play a role in the treatment of menorrhagia[8]. Proanthocyanidins are oligomers or polymers made up of flavan-3-ol monomeric units. The most common flavan-3-ol units are (+)-catechin, (-)-epicatechin, (+)-gallocatechin and (-)-epigallocatechin, while (+)-afzelechin and (-)-epiafzelechin have been reported to a lesser extent (Figure No. 1)[9].

Menorrhagia is clinically 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[10]. A number of conditions may cause menorrhagia, including endocrine disorders, uterine abnormalities, coagulation disorders and other pelvic diseases[11]. 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[11,12]. 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[13].

We have previously reported the extraction, purification and characterization of ethyl acetate soluble proanthocyanidins (EASPA) of the inflorescence of *Cocos nucifera* L.[3]. In addition, progestogenic activity of EASPA of *Cocos nucifera* L. inflorescence has also been reported in relation to its ethnomedical usage[14]. In allopathic medicine, synthetic progestogens are used to treat menorrhagia. It is significant that proanthocyanidins, are chemically different to progestogens used in allopathic medicine. Our finding suggested a possible mode of action, to explain the use of coconut inflorescence in controlling menorrhagia in Ayurveda and traditional medicine in Sri Lanka. Since in humans, the changes in progesterone levels are associated with histological changes in endometrium, it was interesting to find out whether the extracted EASPA fraction from the inflorescence of *Cocos nucifera* L. also causes histological changes in rat endometrium. Here, we report the effect of EASPA on the histological changes of female rat endometrium.

## MATERIALS AND METHODS

### Materials

Light microscope (Meiji MT5000) with x10 and x40 objective lenses were used for vaginal cytology observations. Histological assessment was done by Light microscopic (OLYMPUS CH-2) examination of the stained slides under scanning (x4), low power (x10) and high power (x40) objectives.

### 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 EASPA fraction in the immature inflorescence of *Cocos nucifera* L. has already been reported[3]. 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, *Rattusnorvegicus*), approximately 14-16 weeks old, weighing 200-250 g obtained from Animal Centre, Medical Research Institute, Colombo-8, Sri Lanka, were used for the study. The animals were housed in standard cages, total of 24 rats, 3 in each 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**

Rats were checked for regular reproductive cycles by observing vaginal cytology for two weeks prior to the study period. Rats exhibiting regular reproductive cycles were used for the study. Rats were weighed and divided in to four groups with each containing six rats. Group I and group II 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 the usage of the immature inflorescence 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. The resulting value was multiplied by the yield of purified EASPA to obtain the final dose. The group III and group IV animals received 2 mL of tap water in the same way for 28 days and served as control groups. The animals were observed daily for behavioral activities.

**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 as previously reported[14] and examined under a light microscope (with x10 and x40 objective lenses) to determine the cytology of the vaginal epithelium in order to identify the different phases of the reproductive cycle as described in literature[15].

**Histology of endometrium**

After 28 days of study period rats were euthanized at proestrous and oestrous phases and uterus was dissected out from each rat. Group I and group III animals were euthanized at proestrous phase, while group II and IV animals were euthanized at oestrous phase. Rats were euthanized by overdosing of anesthetic ether inhalation. Dissected uteri were fixed in 10% neutral buffered formalin solution. Following adequate fixation, tissue sections were taken from the endometrium. Histology sections were processed according to the standard histology techniques and embedded in paraffin wax. The paraffin blocks were sectioned at 4 µm thickness by the rotary microtome. The tissue sections were subjected to rehydration by exposing them to decreasing concentrations of alcohol, ranging from 100% to 10% and then stained with the routine haematoxylin and eosin stain and observed under light microscope[16]. Significant histopathological changes were documented and photographed for comparison with the control slides.

**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 $P \leq 0.05$.

**RESULTS AND DISCUSSION**

Rats have bicornuate uteri consisting of two lateral horns that join distally into a single body in contrast to the simple uterus with a single chamber found in humans. Uterus is suspended from the dorsal body wall by the mesometria, which are heavy broad ligaments carrying blood and lymphatic vessels as well as abundant nerves. The body of uterus consists of fundal (cranial) and cervical (caudal) segments. Female rat uterus is shown in Figure No. 2 and 3. The fundal segment is divided into two cavities by a median septum. The cervical segment consists of a

single cavity that protrudes into the vaginal opening[17]. The uterus of female rats consists of an inner epithelial lined mucosa named as the endometrium, which covers the myometrium or muscular layer of the uterus. In non-pregnant stage, the endometrium is arranged in elevated transverse folds supplied by vessels and nerves. The mucosal epithelium consists of simple columnar cells that extend into branched tubular glands within the endometrial stroma. The myometrium is composed of an inner circular layer and outer longitudinal layer of smooth muscle fibers separated by loose, highly vascular connective tissue termed stratum vasculosum. The outer serosa is continuous with the mesometrium[18]. The endometrium consists of a surface epithelium and an underlying delicate stroma. Surface epithelium consists of uniform columnar cells resting on a basement membrane. Endometrial stroma which lies immediately beneath the surface epithelium consists of endometrial glands and round to ovoid shaped endometrial stromal cells[18].

In female rats, the histology of the endometrium shows changes depending on the stage of the reproductive cycle[18]. The reproductive cycle of female rats is characterized by proestrous, oestrus, metestrous and diestrous phases[15]. First of all vaginal cytological studies were carried out to identify the different phases of the reproductive cycle as previously published[14]. Levels of the reproductive hormones, oestrogen and progesterone also vary during the reproductive cycle. The highest levels of oestrogen and progesterone have been reported from proestrous and oestrous phases of the reproductive cycle respectively[19].

Thus, the histological changes of the rat endometrium during proestrous and oestrous phases of the reproductive cycle were separately assessed and compared with the respective control groups. Endometrium of control group (Group III) rats during proestrous phase consisted of medium sized columnar epithelial cells lining the endometrial surface as well as forming endometrial glands. Mitotic activity was low. There were occasional neutrophils within the endometrial stroma. These results were in accordance with published reports for histological findings for rat endometrium at proestrous phase[20]. The histological findings of the endometrium of rats at proestrous phase of test group (Group I) were similar to that of the control group (Group III). Histological evidence of cell injury or any other histological alteration was absent. Endometrium of control group (Group IV) rats during oestrous phase of the cycle showed large, tall columnar cells lining the glands and surface of the endometrial cavity. Epithelial cells exhibited vacuolar degeneration. A few apoptotic bodies were noted. Endometrial stroma showed a significant number of neutrophils. These results were similar to that of published reports for histological findings of the rat endometrium at oestrous phase[20]. There were no detectable histological changes of rat endometrium at oestrous phase between the control group (Group IV) and the test group (Group II). Once again histological evidence of cell injury or any other histological alteration was absent. Histological observations for rat uteri of control groups, Group III and IV at proestrous and oestrous phases respectively are given in Figure No. 4. There was also no significant difference in uterus weight of test groups at proestrous and oestrous phases compared to that of respective control groups (Table No. 1).

The administration of EASPA into wistar rats at the dose of 0.33 mg/day for a period of 28 days did not result in histological changes of the endometrium compared to respective controls at the proestrus and oestrus phases of the reproductive cycle, although changes in serum progesterone level were observed at the dose level employed in our previous studies[14]. In humans, histological changes of the endometrium are associated with changes in reproductive hormone levels. These changes were not observed in our study. This may be due to an inadequacy of the dose or time duration that EASPA was administered or the dissimilarity of the oestrus cycle of the rats and the menstrual cycle of humans due to the fact that the female rats do not shed the endometrium. In addition, this may also be as a result of the histological changes of the

endometrium of female rats being independent of the increase in the progesterone levels after administration of EASPA.

**Table No. 1: Effect of EASPA on weight of rat uterus**

| S.No | Parameter | Group I | Group II | Group III | Group IV |
|---|---|---|---|---|---|
| 1 | Relative uterus weight | 0.42±0.03 | 0.40±0.02 | 0.40±0.01 | 0.41±0.04 |

*n = 6, all values are presented as Mean ±SEM. Uterus weights are expressed as relative to body weight [(uterus (g)/ final body weight (g)) × 100]*

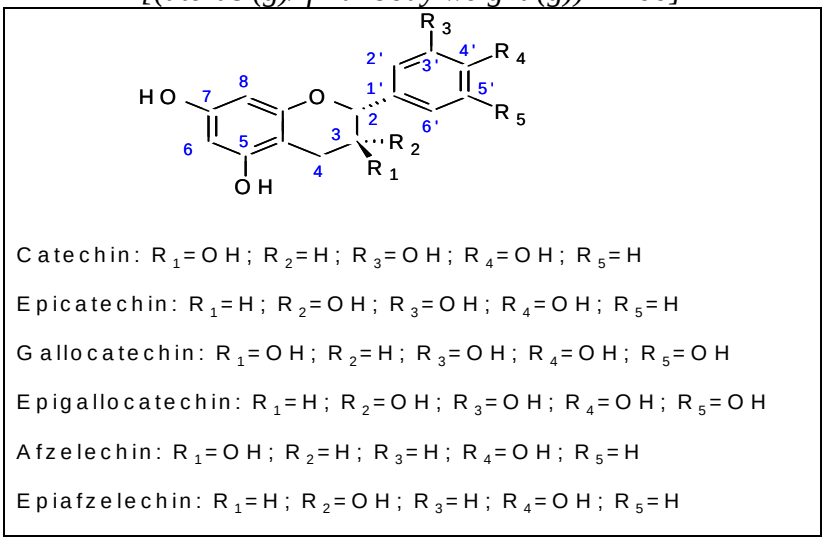



**Figure No. 1: Chemical structures of flavan-3-ol units found in proanthocyanidins**

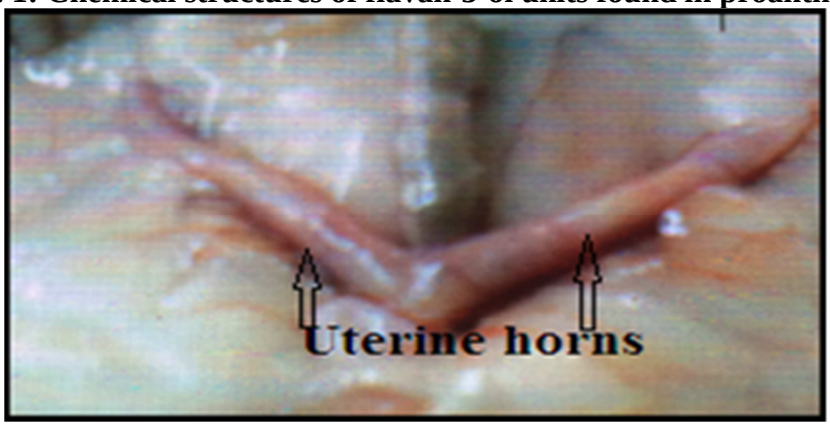



**Figure No. 2: Rat uterus *in situ***

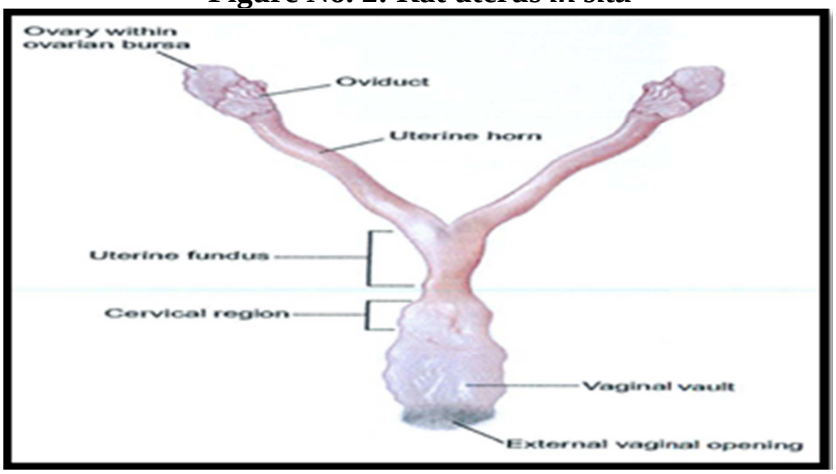
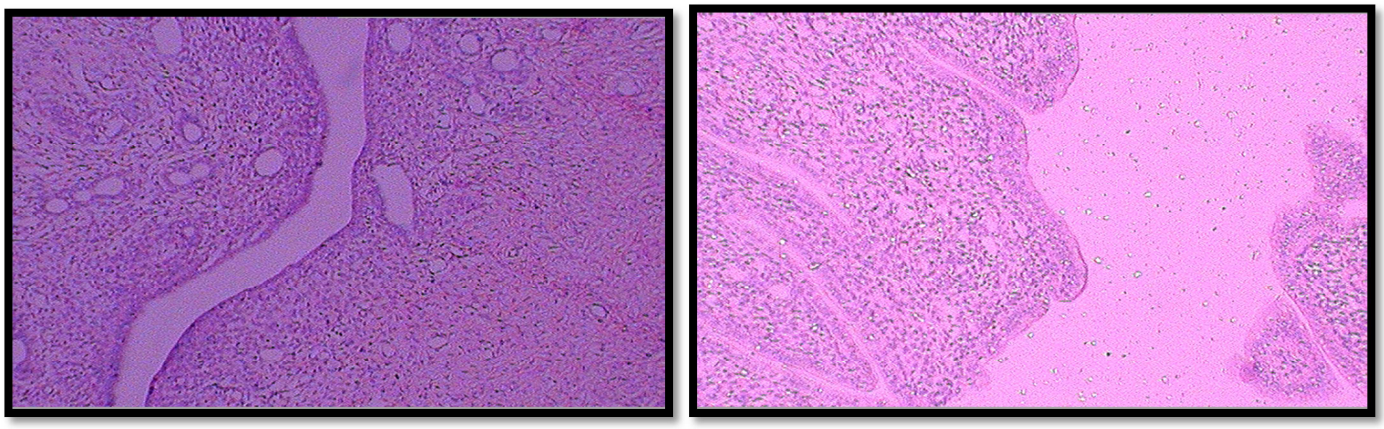



**Figure No. 3: Labeled diagram of reproductive tract of female rat**



(a) (b)

(a) Group III rat uterus at proestrous phase (H and E x4). Luminal and glandular epithelium were reduced in height; both were composed of low columnar cells.
(b) Group IV rat uterus at oestrous phase (H and Ex4). Endometrial epithelium was composed of tall columnar cells.

**Figure No. 4: Group III and Group IV rat endometrium at proestrous (A) and oestrous (B) phases respectively**

## CONCLUSION

The administration of EASPA into female wistar rats at the dose of 0.33 mg/day for a period of 28 days did not result in histological changes of endometrium compared with respective controls at proestrous and oestrous phases of the reproductive cycle, although changes in serum progesterone levels were shown by our previous studies.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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