Sudarshan Publication

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THE PHARMA RESEARCH

An International Journal of Pharmacy Research

ESTROGENIC ACTIVITY OF HYDRO-ALCOHOLIC EXTRACT OF *ARTOCARPUS LAKOOCHA* FRUIT ON FEMALE SPRAGUE-DAWLEY RATS

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ARTICLE INFO

Published on: 15-6-2015
ISSN: 0975-8216

Keywords:
Artocarpus lakoocha; estrogenic activity; uterotropic assay; vaginal cytology; vaginal opening

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ABSTRACT

Aim: Hormone replacement therapy was considered a safe treatment for short-term therapy. But, because of adverse outcomes such as increased risk of endometrial and breast cancer, stroke and pulmonary thrombo-embolism after its long-term use, women turned to use natural health remedies. Thus, the estrogenic activity of 70% hydro-alcoholic extract of *Artocarpus lakoocha* fruit (HEAL) was evaluated.

Materials and Methods: HEAL was subjected to phytochemical analysis as per standard protocol. Estrogenic potential of HEAL was performed by uterotropic assay, vaginal cytology and measurement of vaginal opening at different doses of 200 mg/kg, 400 mg/kg and 600 mg/kg bw *po* in Sprague-Dawley (SD) rats and compared with standard drug *β*-estradiol (2 mg/kg bw, *po*). Ovariectomized immature and mature female SD rats were administered with HEAL for 7 days.

Results: It showed the presence of tannins, saponins, triterpenes, flavonoids, steroids and alkaloids as major secondary metabolites in HEAL. Dose of 600 mg/kg administered in ovariectomized immature and mature female SD rats resulted in significant increase in the uterine wet weight of 219.60±8.71 mg and 906.19±26.91 mg respectively when compared with that of control rats of 110.55±1.16 mg and 505.66±27.38 mg respectively. Only cornified epithelial cells were seen in HEAL-treated rats at the dose of 600 mg/kg indicating the presence of estrogen. Significant vaginal opening was also seen in HEAL-treated rats at the dose of 600 mg/kg.

Conclusion: It was concluded that HEAL possess promising estrogenic activity.

INTRODUCTION

Estrogen plays an extremely essential role in growth regulation and function in numerous female target organs such as vagina, uterus, skeletal and cardiovascular systems[1,2]. Estrogen deficiency results in cessation of ovarian production which is associated with many complaints experienced by menopausal

women[3,4,5]. For decades, the hormone replacement therapy (HRT) had been used as an alternative for managing menopause-induced complaints[6,7]. It is presumably a safe treatment for short-term therapy. But, it was implicated in adverse outcomes such as increased risk of endometrial and breast cancer, stroke and pulmonary thrombo-embolism after long-term use[8,9]. Due to this, women turned to use natural health remedies. Thus, the interest in plant-derived phyto-estrogens raised significantly in the last decades.

The genus *Artocarpus* comprises approximately 50 species of deciduous and evergreen trees. It is economically important as a source of edible fruit and yields good timber. It is widely used in traditional folk medicines in South-East Asia and India for the treatment of several diseases like abscess, ulcer, inflammation, diarrhoea, diabetes, malaria and tapeworm infection[10,11,12]. *Artocarpus lakoocha* fruits are eaten fresh. Its edible fruit pulp act as a liver tonic. Its male flower spikes and raw fruits are used in pickles and chutney. Its brown powder named as Puag-Haad in Thailand is used as a traditional anthelmintic drug for the treatment of tapeworm infection[13,14]. Its bark containing tannin is chewed like betel nuts and is also used to treat skin diseases[15]. This plant has antiviral and anthelmintic activities and has also been used as skin-whitening agent in cosmetics[16,17,18]. *Artocarpus lakoocha* plant contains various phytoconstituents like *β*-sitosterol, cycloartenol, cycloartenone, α-amyrin acetate and lupeal acetate. It also contains artocarpin, norartocarpin, cycloartocarpin, resorcinol, oxyresveratrol, pentosans, lignin, tannin, holocellulose and α-cellulose[19]. The aim of study was designed for exploration purpose to evaluate the estrogenic activity of HEAL in SD rats by uterotropic assay, vaginal cytology and measurement of vaginal opening.

MATERIALS AND METHODS

COLLECTION AND AUTHENTICATION OF PLANT SPECIMEN

The fruits of *Artocarpus lakoocha* used in the present study were collected from local market of Lucknow, India and and authenticated by National Botanical Research Institute (NBRI), Lucknow (authentication reference number NBRI/CIF/260/2011).

PREPARATION OF HYDRO-ALCOHOLIC EXTRACT

Fruits were cut into small pieces and shade dried. The dried material was then powdered into coarse powder by a mechanical grinder. The dried coarse powder was evenly packed in a Soxhlet extractor for extraction. It was defatted with petroleum ether (60-80°). The temperature was maintained on an electric heating mantle with thermostat control. The defatted marc was further extracted with 70% ethanol for 24 hr. Extract was filtered and then concentrated to dryness in a rotavapor under reduced pressure and controlled temperature. It was stored in refrigerator and kept in desiccator few hours before use[20].

MATERIALS AND REAGENTS

β-Estradiol (National chemicals, Vadodara, Gujrat), methanol (Ranbaxy, India), normal saline (NaCl 0.9%), methylene blue, ketamine hydrochloride injection (Neon Laboratories Limited), chloroform (RFCL Ltd, New Delhi).

PRELIMINARY PHYTOCHEMICAL SCREENING

Phytochemical screening of HEAL was carried out for alkaloids (Mayer test), saponins (Foam test), flavonoids (Shinoda test), carbohydrates (Benedict test), tannins (Ferric chloride test) steroids and triterpenes (Liberman-Burchard test) according to standard methods.[21]

ANIMALS

Immature and mature female SD rats weighing between (60-70±10, 150-200±20 gm respectively) procured from the Central Drug Research Institute Lucknow, Uttar Pradesh were housed in polypropylene cages (22.5×37.5 cm^2) and maintained under standard laboratory environmental conditions viz., temperature 25±2°C, 12 h light:12 h dark cycle and 55±10% relative humidity with free access to standard pellet diet and water, *ad libitum*. The experimental protocol was approved by the Institutional Animal Ethics Committee, which follow the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and conform to the international norms of the Indian National Science Academy. Ethical norms were strictly followed during all experimental procedures [Hygia/M.Pharm./05/2011-12].

PREPARATION OF TEST SAMPLES AND DOSING

The ovariectomized rats were divided into 5 groups each consisting of 5 animals. Group 1 (control) received 0.6% (w/v) sodium CMC suspension (10 ml/kg, *po*), Group 2 (standard) aqueous suspension of *β*-estradiol in 0.6% (w/v) sodium CMC (2 mg/kg, *po*), Group 3 (test) aqueous suspension of HEAL in 0.6% (w/v) sodium CMC (200 mg/kg, *po*), Group 4 (test) aqueous suspension of HEAL in 0.6% (w/v) sodium CMC (400 mg/kg, *po*) and Group 5 (test) aqueous suspension of HEAL in 0.6% (w/v) sodium CMC (600 mg/kg, *po*).

MEASUREMENT OF ESTROGENIC PARAMETERS

UTEROTROPIC ASSAY IN IMMATURE AND MATURE OVARIECTOMIZED FEMALE RATS

Immature and mature female SD rats were divided into five groups, each of five rats. All rats were bilaterally ovariectomized by dorso-lateral approach under anesthesia and semi-sterile conditions. After one day of ovariectomization, the dose treatment was started. All the above mentioned dosages were administered orally daily for 7 days. During this period of treatment, the rats were maintained under standard pellet diet and water. Vaginal cornification was examined daily. After 24 h of last treatment, hysterectomy was performed in all rats under ketamine anesthesia. Harvested uteri were cleaned carefully from adhering connective tissue and weighed.[22]

VAGINAL CYTOLOGY

This was performed by vaginal smear method in ovariectomized immature female SD rats. Vaginal smear was taken by introducing a few drops of saline into vagina with the help of eye dropper. The saline was expelled into the vagina and withdrawn two or three times. The contents of the eye dropper was placed and spread on a glass slide, the smear was immediately fixed with 1% w/v aqueous methylene blue for 5-6 min. The smear was examined under microscope to check the

presence or absence of leukocytes and cornified epithelial cells. The presence of only cornified epithelial cells is indicative of estrogenic activity.[23]

The vaginal smear test score report was tested.

0 Di estrus smear, mainly leucocytes few epithelial cells.

1 Mixture of leucocytes and epithelial cells.

2 Pro estrus smear nucleated or nucleated+cornifield cells.

3 Estrus smear, cornified cells only.

MEASUREMENT OF VAGINAL OPENING

The vaginal opening was observed and noted daily for 7 days after oral administration of HEAL in ovariectomized immature female SD rats. Increase in vaginal opening is indicative of estrogenic activity.[24]

STATISTICAL ANALYSIS

The results were analyzed using one way analysis of variance (ANOVA) with Dunnett's comparison test. *P* values<0.01 were considered statistically significant.

RESULTS

PRELIMINARY PHYTOCHEMICAL SCREENING

HEAL showed the presence of carbohydrates, saponins, triterpenes, tannins, flavonoids, alkaloids and steroids.

UTEROTROPIC ASSAY IN IMMATURE AND MATURE OVARIECTOMIZED FEMALE RATS

Oral administration of *β*-estradiol (positive control) at 2 mg/kg, and HEAL at 200, 400 and 600 mg/kg, *po*, respectively, effectively increased the uterine wet weight both in immature and mature ovariectomized rats. Increase in uterine wet weight was dose-related (**Figure 1** and **Figure 2**). HEAL at the dose of 600 mg/kg, *po* showed estrogenic activity comparable to that of standard *β*-estradiol.

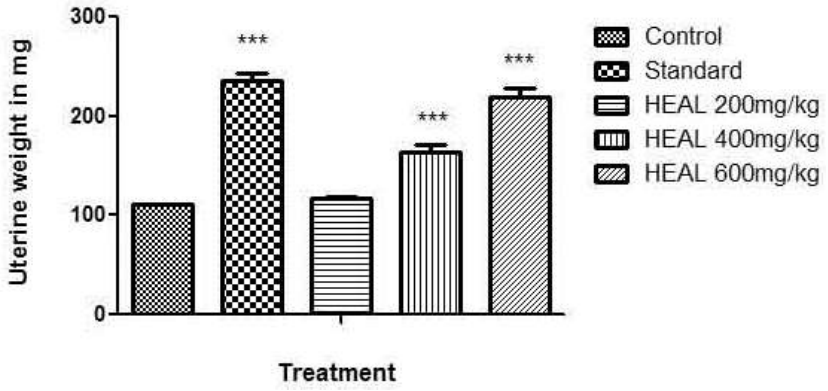


Figure 1 Effects of HEAL 200 mg/kg, 400 mg/kg and 600 mg/kg, *po* on uterine wet weight in immature SD rat. β-estradiol was used as standard at 2 mg/kg b.w. Data were expressed as mean±S.E.M. ***P<0.01 (significantly different from the control)

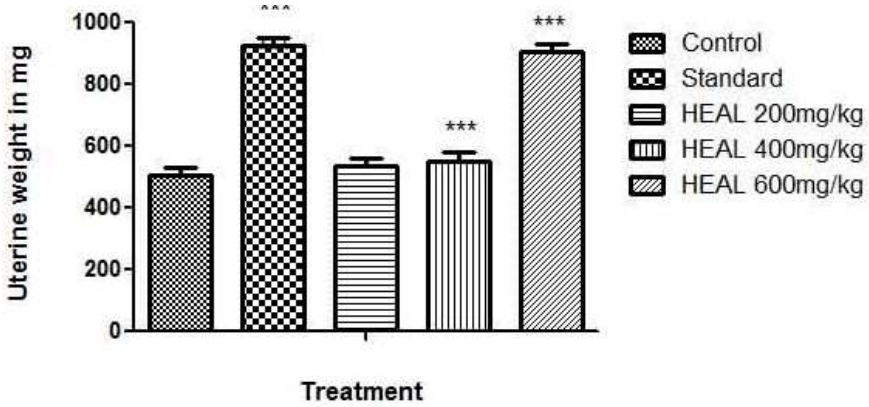


Figure 2 Effects of HEAL 200 mg/kg, 400 mg/kg and 600 mg/kg, *po* on uterine wet weight in mature SD rat. β-estradiol was used as standard at 2 mg/kg b.w. Data were expressed as mean±S.E.M. ***P<0.01 (significantly different from the control)

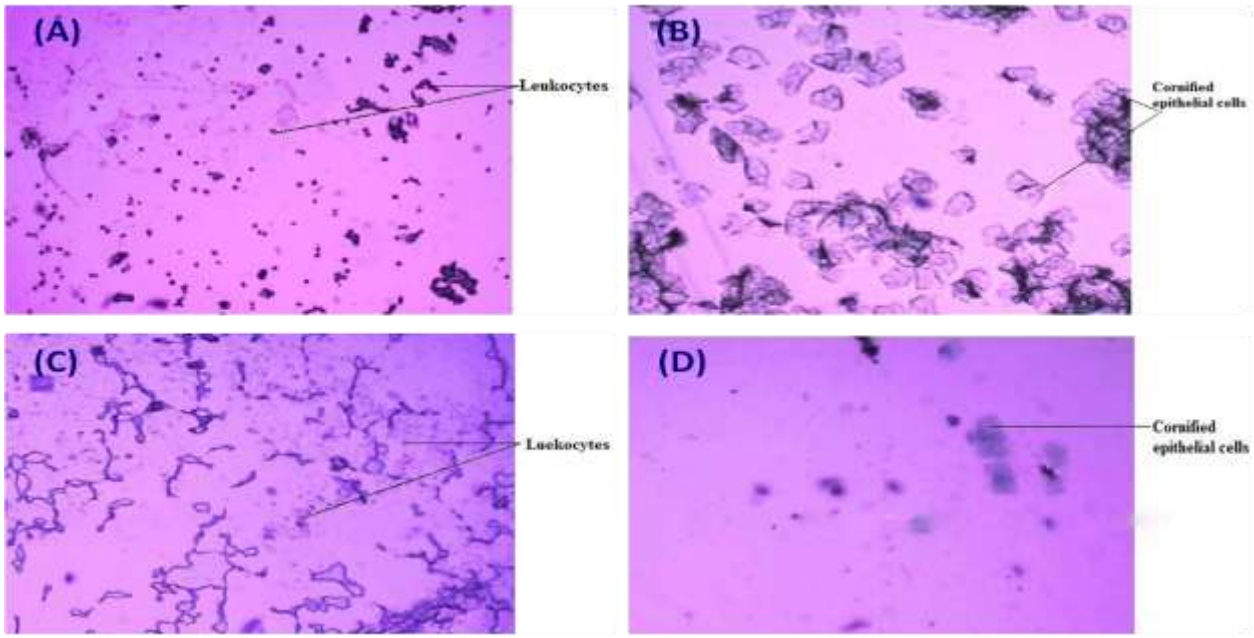
VAGINAL CYTOLOGY

Animals showing the score 2 and 3 were considered to be positive. The vaginal smear test score of cornification of epithelial cells was positive because the number of cornified cells in the vaginal smear was considerably higher than the control and less than *β*-estradiol treated animals as shown in **Table 1** and **Figure 3**. HEAL treated rats (at the dose of 600 mg/kg, *po*) showed only cornified epithelial cells. This result clearly indicated that HEAL at a dose of 600 mg/kg, *po* is having significant estrogenic activity.

Table 1 Effects of HEAL on vaginal cornification in ovariectomized immature rats

Group	Drug treatment	Dose	Vaginal smear test score
1.	Control	10 ml/kg	Negative
2.	Standard	2 mg/kg	Positive
3.	HEAL	200 mg/kg	Negative
4.	HEAL	400 mg/kg	Negative
5.	HEAL	600 mg/kg	Positive

Animals showing the score 2 and 3 are considered to be positive.



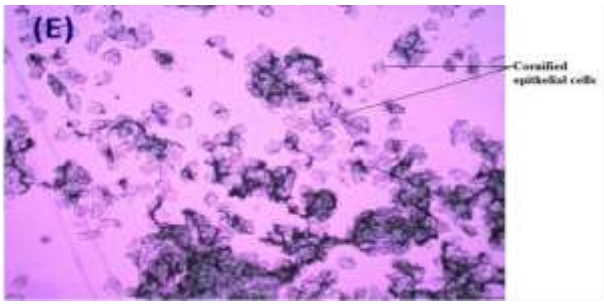


Figure 3 Photomicrograph of methylene blue stained vaginal smear **(A)** in diestrous of control rat, showing only leukocytes. **(B)** in estrous of *β*-estradiol (2 mg/kg, *po*) treated rat, showing only cornified epithelial cells. **(C)** in estrous of HEAL (200 mg/kg, *po*) treated rat, showing only leukocytes. **(D)** in estrous of HEAL (400 mg/kg, *po*) treated rat, showing only few cornified epithelial cells. **(E)** in estrous of HEAL (600 mg/kg, *po*) treated rat, showing only cornified epithelial cells

VAGINAL OPENING

HEAL showed a dose dependent increase in vaginal opening from day fourth onwards compared to control which remained closed. HEAL at dose of 600 mg/kg, *po* showed significant vaginal opening. But, HEAL at dose of 200 mg/kg, *po* and 400 mg/kg, *po* showed insignificant vaginal opening (**Table 2** and **Figure 4**). It showed that HEAL at dose of 600 mg/kg, *po* is having significant estrogenic activity.

Table 2 Effects of HEAL on vaginal opening in immature ovariectomized rats

Group	Drug treatment	Dose	Vaginal opening (%)						
			Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
1.	Control	10 ml/kg	0	0	0	0	0	0	0
2.	Standard	2 mg/kg	0	0	0	70	100	90	80
3.	HEAL	200 mg/kg	0	0	0	5	5	6	8
4.	HEAL	400 mg/kg	0	0	0	45	55	50	60
5.	HEAL	600 mg/kg	0	0	0	60	85	75	70

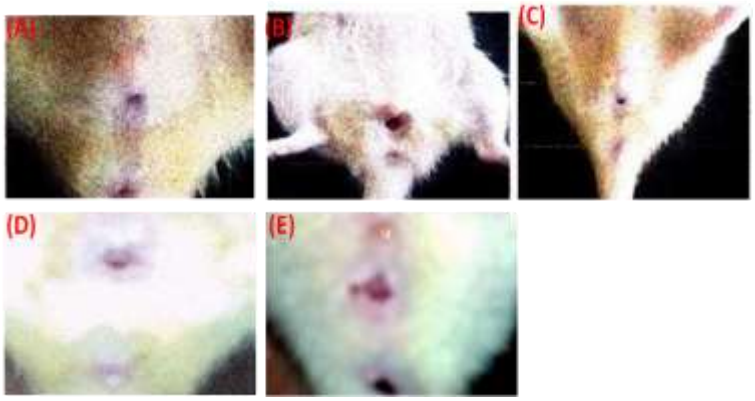


Figure 4 Effects of HEAL on vaginal opening in ovariectomized immature SD rats. **(A)** Control **(B)** Standard **(C)** HEAL 200mg/kg, *po* **(D)** HEAL 400mg/kg, *po* **(E)** HEAL 600mg/kg, *po*

DISCUSSION

The excess of estrogen can cause breast, endometrial, ovarian, and prostate cancer and its deficiency can result in menopausal symptoms, cardiovascular disease and osteoporosis. The major causes of estrogen deficiency in females are menopause and ovariectomy. Interest in plant derived estrogens or phytoestrogens has recently been amplified by the realization that HRT is not as

safe or effective as previously considered.[25,26] Phytoestrogens have negative impact on the development of human reproductive organs and male fertility, unlike xenobiotic estrogens (environmental pollutants with estrogenic activity), they are believed to have primarily beneficial effects on the health.[27] Results of uterotropic assay revealed that HEAL showed increase in uterine wet weight after administration of 200 mg/kg, 400 mg/kg and 600 mg/kg, *po* dose both in immature and mature ovariectomized rats. It clearly indicated that all the doses contain estrogen like compound. HEAL (600 mg/kg, *po*) treated rats showed only cornified epithelial cells. The vaginal opening showed that HEAL (600 mg/kg, *po*) is having significant estrogenic activity.

CONCLUSION

HEAL possess significant estrogenic activity at dose of 600 mg/kg, *po* which was evident by uterotropic assay, measurement of vaginal opening and vaginal cytology in female rats. HEAL could be useful as a safe natural source for estrogenic activity for postmenopausal women.

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