ORIGINAL ARTICLE

Estrogenic activity of a hydro-alcoholic extract of *Bambusa arundinaceae* leaves on female wistar rats

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J. Adv. Pharm. Technol. Res.

ABSTRACT

To study the estrogenic activity of the hydro-alcoholic extract of *Bambusa arundinaceae* leaves (HEBA) in female Wistar rats. The dried powdered leaves were extracted with hydroalcoholic mixture (60%), and the resultant extract was subjected for phytochemical analyses to identify different phytoconstituents. HEBA were administered to ovariectomized rats for 7 days at three different doses (viz., 200, 300, 400 mg/kg body weight, p.o.) and their estrogenic activity were compared with each of daily treatment with 0.2 mg/kg body weight, i.p. conjugated equine estrogen as a positive control or olive oil as a negative control. Estrogenic activity was evaluated by doing uterotropic assay, vaginal cytology and measurement of vaginal opening in female Wistar rats. Oral administration of HEBA in ovariectomized immature and mature female Wistar rats in a dose of 400 mg/kg b.w. resulted in significant increase in the uterine wet weight (in mg) (224.82 ± 7.01) and (912.25 ± 27.22) when compared with ovariectomized control rats (111.52 ± 3.17) and (506.67 ± 21.39). HEBA (400 mg/kg b.w., p.o.) treated rats, showing only cornified epithelial cells which was an indication of the presence of the estrogen and also showed 100% vaginal opening. It was observed that HEBA possess significant estrogenic activity at 400 mg/kg b.w., p.o. which was evident by uterotropic assay, measurement of vaginal opening, and histopathological changes.

Key words: *Bambusa arundinaceae* leaves, estrogenic activity, uterotropic assay, vaginal cytology, vaginal opening

INTRODUCTION

Several hundreds of plants have been found to reveal estrogenic activity or to contain estrogenically active compounds. These plant-derived nonsteroidal compounds with estrogen-like biological activity have been defined as phytoestrogens. Lot of reports are available on the potential valuable role of nutritional phytoestrogens in human health (cancer chemoprevention, relief of postmenopausal symptoms, osteoporosis) and have a few side effects.[1,2] Phytoestrogens appear to have both estrogenic and antiestrogenic effects.[3,4] Phytoestrogens mainly belong to a large group of substituted phenolic compound known as flavonoids. The coumestans, isoflavones, lignans, and stilbens are four of the most active in estrogenic effects in this class. The best-researched phytoestrogens are isoflavones, which are commonly found in soy and red clover.[5] Phytoestrogens battle with estradiol with varying affinities to bind to estrogen receptors, induce transcription of estrogen-responsive genes[6] and depending on the outcome measured, either mimic or antagonize the action of steroidal estrogens.[7]

Bambusa arundinacea (*Poaceae*), commonly known as bans, is a thorny, tall-sized tree. A bamboo culm consists of internodes (which are hollow for most bamboo) and a node, which is solid and provides structural integrity for the plant. At the node are one or more buds (depending on the species) which produce side branches.

The different parts of this plant contain silica, betain, cynogenetic glycosides, oxalic acid, reducing sugar,

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Access this article online

Quick Response Code:

Website: www.japtr.org

DOI: 10.4103/2231-4040.150367

albuminoids, resins, waxes, benzoic acid, cysteine, arginine, histidine, isoleucine, leucine, phenylamine, threonine, lysine, methionine, valine, tyrosine, riboflavin, niacin, thiamine, protein, gluteline, betain, choline, proteolytic enzyme, nuclease and urease.[8]

Various parts of *B. arundinacea* such as leaf, root, shoot, flower, and seed showed anthelmintic,[9] antidiabetic,[10] wound healing,[11] antiinflammatory,[12] antiulcer,[12] antifertility,[13] antimicrobial,[14] antiarthritic activity,[15] antithyroid,[16] antioxidant[17] and vessel-protective[18] activities. The root (burnt root) is applied to bleeding gums, ringworm, and painful joints.[19] Seeds are acrid, laxative, and said to be useful in strangury and urinary discharge.[13] Bark is used for skin eruptions. Leaf is emmenagogue, febrifuge, antileprotic, bechic, used in hemoptysis.[19]

This study was aimed to evaluate the estrogenic effects of hydro-alcoholic extract of *Bambusa arundinacea* leaves (HEBA) on Wistar rats by performing uterotropic assay, vaginal cytology, and measurement of vaginal opening.

MATERIALS AND METHODS

Chemicals

Conjugated equine estrogen (Premarin from Wyeth Pharmaceuticals Inc., Philadelphia, PA), ketamine hydrochloride injection (Neon Laboratories Limited, Mumbai, India), chloroform (RFCL Ltd., New Delhi, India). All other chemicals used were of analytical grade.

Plant material

The matured leaves of *B. arundinaceae* used for the present studies were collected from Central Institute of Medicinal and Aromatic Plants, Lucknow, India and its identification and authentication were done from National Botanical Research Institute (Council of Scientific and Industrial Research), Lucknow - 226 001, India (Ref. No: NBRI/CIF/276/2012). Soon after authentication, all leaves were shade dried until they were free from the moisture. Finally, leaves were subjected to size reduction to get a coarse powder and then passed through Sieve No. 40 to get uniform powder. The resulting powder was then used for extraction.

Preparation of hydro-alcoholic extract

The powdered plant material was subjected to Soxhlet extraction with about 60% hydroalcoholic mixture for 6 h at 50°C. The resulting syrupy mass after evaporation of the ethanol was washed successively with petroleum ether, chloroform, and ethyl acetate. Percentage yield of thus obtained syrupy mass was calculated. The syrupy mass was subjected to phytochemical investigation and pharmacological screening for its estrogenic activity.[13]

Preliminary phytochemical screening

An attempt was made to observe the presence and absence of diverse phytochemical constituents in HEBA, viz., flavonoids (Shinoda test), steroids (Salkowski reaction), carbohydrates (Benedict's test), proteins (Biuret test), and amino acids (Ninhydrin test) according to standard methods.[20]

Animals

Mature and immature female Wistar rats weighing between (45–50 g, 250–350 g, respectively) were procured from the Central Drug Research Institute Lucknow, India. They were housed in polypropylene cages (22.5 cm × 37.5 cm) and maintained under standard laboratory environmental conditions; temperature 25°C ± 2°C, 12 h light: 12 h dark cycle and 55% ± 10% relative humidity with free access to standard pellets and water, *ad libitium*. The experimental protocols were 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 and conform to the international norms of the Indian National Science Academy. Ethical norms were strictly followed during all experimental procedures. [Hygia/M.Pharm./07/2011-12].

Preparation of test samples and dosing

The ovariectomized rats were divided into five groups each consisted of six animals.

- Group 1 (control): Olive oil
- Group 2 (standard): Conjugated equine estrogen (suspended in olive oil) 0.2 mg/kg b.w., i.p
- Group 3 (test): HEBA at a dose of 200 mg/kg b.w., p.o. for 7 days
- Group 4 (test): HEBA at a dose of 300 mg/kg b.w., p.o. for 7 days
- Group 5 (test): HEBA at a dose of 400 mg/kg, b.w., p.o. for 7 days.

All drugs are administered orally daily for 7 days.

Estrogenic parameters measurement

Uterotropic assay in immature and mature ovariectomized female rats

Immature and mature female Wistar rats weighing between (45–50 g, 250–350 g, respectively) were selected and divided into five groups, each of six rats. All rats were bilaterally ovariectomized by dorsolateral approach under light ether anesthesia and semi-sterile conditions. After 1 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 pellets and water, *ad libitium*. On the 8th day of the experiment, vaginal smears from all rats were examined. 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.[21]

Vaginal cytology

This was done by vaginal smear method in ovariectomized immature female Wistar rats. Vaginal smear was taken by introducing a few drops of saline into vagina with the help of the eye dropper. The saline was expelled into the vagina and withdrawn 2 or 3 times. The contents of the eye dropper were 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, nucleated epithelial cells, and cornified epithelial cells. The presence of only cornified epithelial cells was indicative of estrogenic activity.[22]

Measurement of vaginal opening

The vaginal opening was observed and noted daily for 7 days after oral administration of HEBA in ovariectomized immature female Wistar rats. The percentage of vaginal opening was calculated using the formula.[22]

$$\text{Percentage of vaginal opening} = \frac{\text{No. of animals with vaginal opening}}{\text{Total no. of animals in the group}} \times 100$$

Increase in vaginal opening was indicative of estrogenic activity.

Statistical analysis

The results were analyzed using One-way analysis of variance (ANOVA) with Dunnett's comparison test. $P < 0.001$ were considered statistically significant.

RESULTS

Preliminary phytochemical screening

The results revealed the presence of flavonoids, steroids, carbohydrates, amino acids, and proteins in the crude extract in Table 1.

Uterotropic assay in immature and mature ovariectomized female rats

Administration of conjugated equine estrogen (positive control) at 0.2 mg/kg, i.p. and HEBA at 200, 300 and 400 mg/kg b.w., p.o., respectively, effectively increases the uterine wet weight both in immature and mature ovariectomized rats. The increase in the uterine wet weight was dose-related [Figures 1 and 2]. HEBA at 400 mg/kg b.w., p.o. shows estrogenic activity comparable to that of standard conjugated equine estrogen.

Vaginal cytology

The vaginal smear test score report was tested:

- Di estrus smear, mainly leukocytes few epithelial cells
- Mixture of leukocytes and epithelial cells
- Pro estrus smear nucleated or nucleated + cornifield epithelial cells
- Estrus smear cornified epithelial cells only.

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 CEE treated animals as shown in Table 2 and Figures 3-7.

The vaginal smear of ovariectomized control rats did not show any vaginal cornification [Figure 3], during the treatment period. Conjugated equine estrogen (0.2 mg/kg b.w., p.o.)

Table 1: Phytochemical tests on HEBA

Compounds	Chemical tests	Results
Alkaloids	Mayer's test	Negative
Flavonoids	Shinoda test	Positive
Steroids	Salkowski reaction	Positive
Carbohydrates	Benedict's test	Positive
Proteins	Biuret test	Positive
Amino acids	Ninhydrin test	Positive
Tannins	Ferric chloride test	Negative

HEBA: Hydro-alcoholic extract of *Bambusa arundinaceae*

Table 2: Effects of HEBA on vaginal cornification in ovariectomized immature rats

Group number	Treatment	Dose	Vaginal smear test score
1	Control	10 ml/kg	Negative
2	Standard	0.2 mg/kg	Positive
3	HEBA	200 mg/kg	Negative
4	HEBA	300 mg/kg	Positive
5	HEBA	400 mg/kg	Positive

Animals showing the score 2 and 3 were considered to be positive.
HEBA: Hydro-alcoholic extract of *Bambusa arundinaceae*

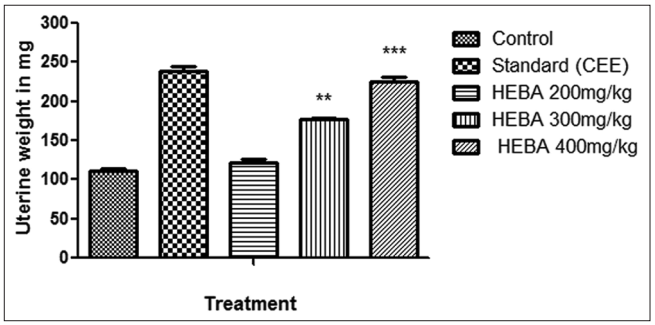

Figure 1: Effects of hydro-alcoholic extract of *Bambusa arundinaceae* leaves 200 mg/kg, 300 mg/kg and 400 mg/kg, p.o. on uterine wet weight in immature ovariectomized female rats. Conjugated equine estrogen (suspended in olive oil) 0.2 mg/kg b.w., i.p. was used as standard. Results were expressed as mean ± S.E.M ($n = 6$); $^{**}P < 0.01$ and $^{***}P < 0.001$ (significantly different from the control)

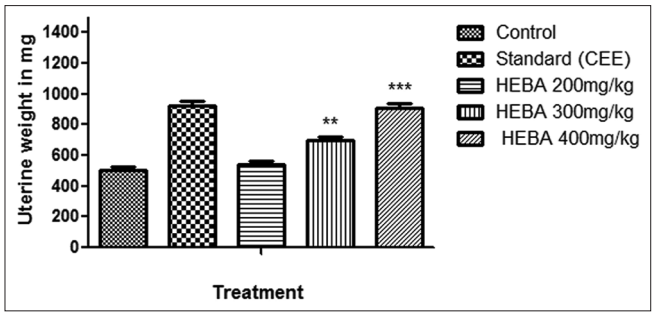

Figure 2: Effects of hydro-alcoholic extract of *Bambusa arundinaceae* leaves 200 mg/kg, 300 mg/kg and 400 mg/kg, p.o. on uterine wet weight in mature ovariectomized female rats. Conjugated equine estrogen (suspended in olive oil) 0.2 mg/kg b.w., i.p. was used as standard. Results were expressed as mean ± S.E.M ($n = 6$); $^{**}P < 0.01$ and $^{***}P < 0.001$ (significantly different from the control)

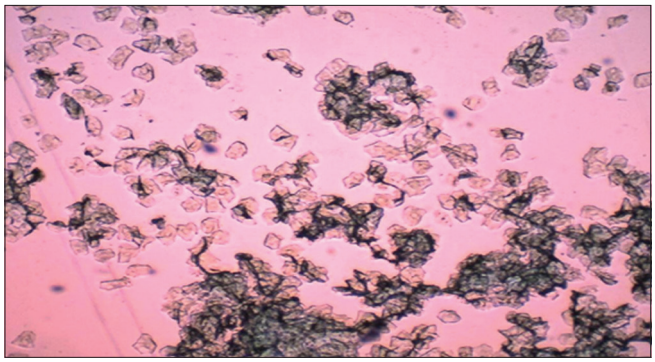

Figure 4: Photomicrograph of methylene blue stained vaginal smear (in estrous) of rat treated with conjugated equine estrogen (0.2 mg/kg b.w., i.p.) for 7 days, showing only cornified epithelial cells

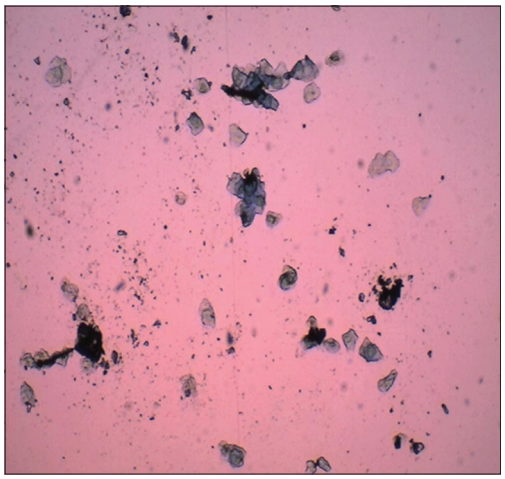

Figure 6: Photomicrograph of methylene blue stained vaginal smear (in estrous) of rat treated with hydro-alcoholic extract of *Bambusa arundinaceae* leaves (300 mg/kg b.w., p.o.), showing only few cornified epithelial cells

showing only cornified epithelial cells [Figure 4]. Whereas, HEBA showed increase in vaginal cornification, from day 4th onward for 300 mg/kg b.w. [Figure 6] and

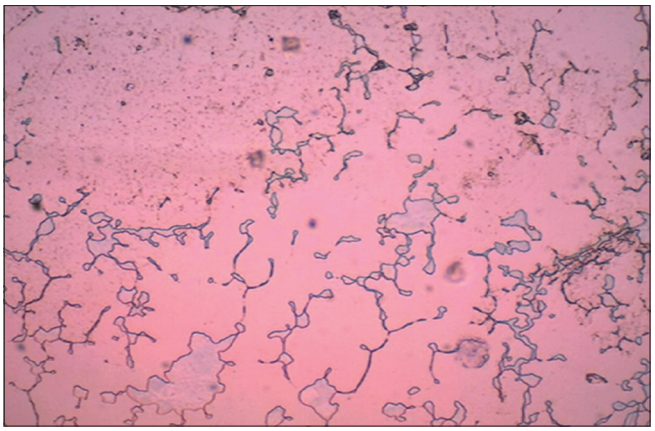

Figure 3: Photomicrograph of methylene blue stained vaginal smear (in diestrous) of rat from the control group treated with olive oil for 7 days, showing only leukocytes

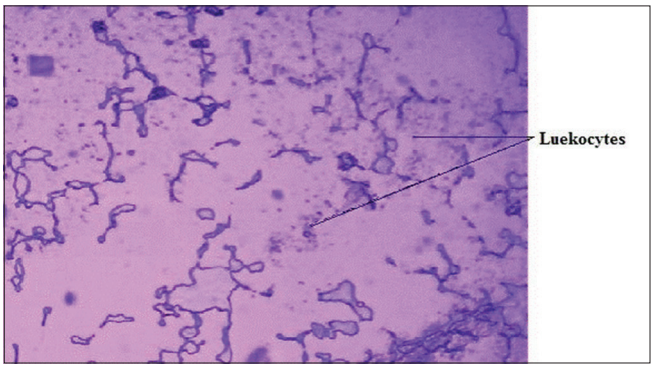

Figure 5: Photomicrograph of methylene blue stained vaginal smear (in estrous) of rat treated with hydro-alcoholic extract of *Bambusa arundinaceae* leaves (200 mg/kg b.w., p.o.), showing only leukocytes

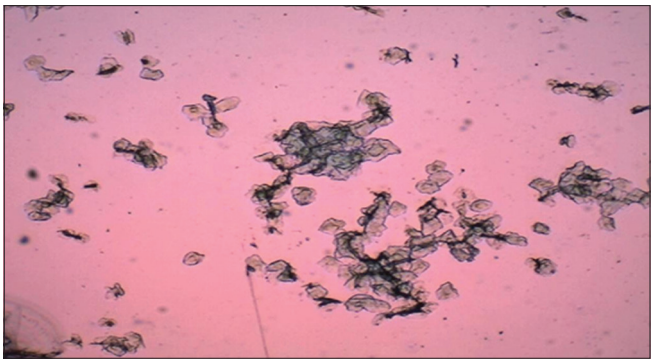

Figure 7: Photomicrograph of methylene blue stained vaginal smear (in estrous) of rat treated with hydro-alcoholic extract of *Bambusa arundinaceae* leaves (400 mg/kg b.w., p.o.), showing only cornified epithelial cells

400 mg/kg b.w. [Figure 7]. But for 200 mg/kg b.w. negligible cornification occurred [Figure 5]. (300 mg/kg b.w., p.o.) treated rats, showing both cornified and nucleated epithelial cells. HEBA (400 mg/kg b.w., p.o.) treated rats, showing only cornified epithelial cells. From this result, it clearly

Table 3: Effects of HEBA on vaginal opening in immature ovariectomized rats

Group number	Treatment	Number of animals	Dose	Vaginal opening (%)
1	Control	6	10 ml/kg	0
2	Standard	6	0.2 mg/kg	100
3	HEBA	6	200 mg/kg	50
4	HEBA	6	300 mg/kg	67
5	HEBA	6	400 mg/kg	100

HEBA: Hydro-alcoholic extract of *Bambusa arundinaceae*

indicates that HEBA at a dose of 400 mg/kg b.w., p.o. was having significant estrogenic activity.

Vaginal opening

The hydro-alcoholic extract showed a dose-dependent increase in vaginal opening from day fourth onward compared to control, which remained closed. HEBA 400 mg/kg b.w., p.o. showed significant vaginal opening. But HEBA 200 mg/kg b.w., p.o. and 300 mg/kg b.w., p.o. showed insignificant vaginal opening [Table 3]. The vaginal opening shows that HEBA at 400 mg/kg b.w., p.o. was having significant estrogenic activity.

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 hormone replacement therapy is not as safe or effective as previously considered.[23] Phytoestrogens have a 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.[24]

The aim of this study was to evaluate the estrogenic activity of the HEBA. The phytochemical analysis showed the presence of flavonoids, steroids, carbohydrates, proteins, and amino acids in the crude extract. Results of uterotropic assay revealed that HEBA showed dose-related increase in uterine wet weight after administration of 200 mg/kg, 300 mg/kg and 400 mg/kg b.w., p.o. both in immature and mature ovariectomized rats. HEBA (400 mg/kg b.w., p.o.) treated rats, showing only cornified epithelial cells. The vaginal opening also showed that HEBA (400 mg/kg b.w., p.o.) was having significant estrogenic activity.

ACKNOWLEDGMENTS

The authors are thankful to Central Drug Research Institute, Lucknow, India for providing necessary facilities to carry out this research. Authors would also like to thank National Botanical Research Institute, Lucknow, India for plant authentication.

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How to cite this article: Jawaid T, Awasthi A, Kamal M. Estrogenic activity of a hydro-alcoholic extract of *Bambusa arundinaceae* leaves on female wistar rats. J Adv Pharm Technol Res 2015;6:19-24.

Source of Support: Nil, **Conflict of Interest:** Nil.