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Estrogenic effect of *Erythrina variegata* L. in prepubertal female rats

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Alcoholic extract of *Erythrina variegata* L. leaves was evaluated by uterotrophic assay in 17 day old prepubertal female rats and was chromatographically processed for compound isolation. The animals divided into 3 (n=6) groups were treated for 7 days as follows: Control group (Grp I) –vehicle, 0.5% Na CMC, 5 mL/kg bw p.o, *E. variegata* alcohol extract group (Grp II) – 250 mg/kg bw/day in vehicle p.o., Ethinyl estradiol group (Grp III) - 30µg/kg bw/day in vehicle, p.o. 24 h post last exposure to extract. Animals were sacrificed and the uterus and ovaries examined for classical morphological and histomorphometric changes induced by estrogen stimulation. Extract treatment increased the absolute and normalized uterine weight, uterine diameter, endometrial thickness, luminal epithelial cell height, diameter of ovary and the number of primary and secondary ovarian follicles relative to vehicle control. Presence of ciliated epithelial cells in the oviduct and signs of vascularization in the cortex of ovarian sections in this group similar to EEG is indicative of estrogenic activity of the tested extract. This is consequent to the antioxidant activity of β-sitosterol, daucosterol and oleanolic isolated from the extract. This study supports the earlier reported hypolipidaemic and anti-atherosclerotic activities, lending scientific validity to anti-obesity claims in traditional medicine.

Keywords: β-sitosterol, Daucosterol, *Erythrina variegata*, Estrogenic activity, Fabaceae, Oleanolic acid.

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Introduction

Erythrina variegata L. (Family-Fabaceae) or Indian coral tree, is a medium sized quick growing tree found in deciduous forests throughout India[1]. Locally called 'kalyana murungai', its leaves are eaten as a pot herb in Tamil Nadu and it is prescribed for its hypolipidaemic, anti- obesity effect in Siddha system of medicine[2]. Several parts of the plant have folkloric reputation as an anti-inflammatory in India, China and South East Asia. Anti-inflammatory, haemagglutinating, insecticidal, skeletal muscle relaxant effects are the activities reported for the plant [3]. Stem bark extract is reportedly estrogenic[4]. Tetracyclic alkaloids of erythrina type have been isolated from the bark, wood, root and flowers. They are also a prolific source of iso- flavones, pterocarpans and biphenyls[5]. Isoflavones are a class of phytoestrogens reported with profound physiological effects and their dietary intake is associated with a reduction of cardiovascular diseases[6]. Apart from reports of nematicidal activity, the leaves of *E. vareigata* have not been studied for biological activity nor have they been examined phytochemically. We have reported the nutritive value, hypolipidaemic and anti-atherosclerotic activity of the leaves[7,8]. Our preliminary phytochemical screening of the leaves has not indicated the presence of isoflavones. However, in present report the estrogenic activity of the alcohol extract of the leaves and the compounds isolated from the same are reported.

Materials and Methods

Plant Material

Fresh green leaves of *E. variegata* were obtained locally and authenticated by Dr Sasikala Ethirajulu, Research officer (Botany), CSMDRIAS, Chennai and a voucher specimen (COP, M/23/07) has been deposited in the Herbarium of Department of Pharmacognosy, SRU.

Extraction

Collected leaves were shade dried for four days and milled into a coarse powder. Cold maceration of the

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size reduced, dried leaves in alcohol to complete exhaustion, followed by filtration and evaporation of the filtrate under vacuum yielded a dark greenish pasty residue (Ev) of 1.3% w/w yield. It was dried in a desicator and stored until needed for analysis. The extracts and ethinyl estradiol dissolved in 0.5% sodium carboxymethyl cellulose (Na CMC) were used for animal experimentation.

Animals

Female wistar foster dams with 9-11 day old, fostered pups (minimum of 10 pups/female) from Centre for Toxicology and Developmental Research, Sri Ramachandra University were housed in plastic cages containing paddy husk bedding in separate environmentally controlled rooms for the duration of the study. On day 17, i.e., the first day of dosing, all female pups were weighed and those weighing 25-40 g were included in the study and identified uniquely. The acclimatization period was 5 days and they were caged with dams until weaning. The experimental procedure was carried out in accordance with the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India and the study was approved by our Institutional Animal Ethics Committee (Xth IAEC / SRMC & RI /57 /1.9.2006).

Chemicals

Ethinyl estradiol (EE>99 % purity) and reference compounds were purchased from Sigma (St Louis, USA), precoated silica gel plates $60F_{254}$ of 0.2 mm thickness from E Merck (Mumbai, India) and silica gel G 60-120 mesh for column chromatography from SISCO Research (Mumbai, India). Samples were scanned between 600 & 4000/cm. All solvents and chemicals were of analytical grade.

Estrogenic activity

A version of the uterotrophic assay in immature rats, one of the tier I screening assays recommended for detecting the estrogenic properties of endocrine disrupting chemicals[9] was adopted for evaluation of estrogenic activity. This assay administered on prepubertal female rats prior to endogenous estrogen priming, ascertains the ability of the extract to elicit biological activities consistent with agonists or antagonists of neutral estrogen. *E. variegata* was found to be non toxic up to 2g/kg bw in acute toxicity studies on wistar rats [8].

The animals divided into 3 groups of 6 animals each were treated as follows: Control group (CG) – vehicle, 0.5% Na CMC, 5 mL/kg bw p.o, alcohol extract of *E. variegata* leaves group (EvG) – 250 mg/kg bw/day in vehicle p.o, Ethinyl estradiol treated group (EEG) - 30µg/kg bw/day in vehicle, p.o. All these treatments were given each day at the same time for 7 days, during which mean daily body weights were measured to the nearest 0.1 g.

On day 8 the rats were sacrificed, uteri and ovaries removed as one piece caudal to the cervix, adhering fat and mesenchymal tissue dissected out, ovaries dissected free and the uteri weighed after blotting out surface fluid. Care was taken to ensure that any fluid in the uterus was not disturbed during trimming and weighing procedures. Left ovary and the left uterine horn with cervix from all the pups in each group was fixed in Bouin's fixative (Picric acid-0.75g, formaldehyde-2.5g, glacial acetic acid-0.5 g, water- 97.25 mL)[10] for 20 h and then in 10% formalin buffer for 24 h. They were then dehydrated in ethanol and processed to paraffin blocks.

Histomorphometric analysis

The tissues were sectioned (5 µm), stained with H&E and examined microscopically for histomorphological changes on a Nikon eclipse TE 2000S microscope and morphometric measurements were made with the help of Image ProPlus® Software (Version 6.0). Uterine diameter, thickness, epithelial cell height and heights of endometrium and myometrium were measured. Diameter of the ovary, relative follicle count (numbers representing the average counts of each follicle type per section) and primordial oocyte diameter were the measurements made with respect to ovary.

Isolation & physicochemical characterization

Ev (30g) was subjected to column chromatography on silica gel (60-120 mesh) using a step gradient of hexane, hexane: chloroform, chloroform, chloroform: methanol (19:1, 9;1) to yield 45 fractions. These were collected and similar fractions pooled, monitored by TLC. They were further processed and recrystallized to yield compounds that gave single spot on TLC. Further their melting points were determined. Identity of the compounds was confirmed based on confirmatory chemical tests, Co-TLC and superimposability of IR with reference compounds. IR analysis was undertaken on ALPHA FT-IR (Bruker Optik, GmbH- Ettlingen, Germany) spectrometer equipped with a versatile high throughput ZnSe ATR crystal, using OPUS software version 6.5.

Statistical analysis

Statistical calculations were carried out with Medcalc software. Results are expressed as mean ± S.E.M. Treatment effects were assessed using one way ANOVA followed by Dunnett's 't' test for comparison of treatment groups with control. $P < 0.05$ was considered statistically significant.

Results

From Table 1 it can be seen that body weight gain was maximal in Group III, followed by those of Group II and they differed significantly from vehicle control. Uterus from Group II animals showed visible signs of fluid accumulation, imbibition and vascularization. There has been a statistically significant increase in normalized tissue weight (uterine weight/body weight) in this group (Table 1). The increase has been maximal in Group III and it differed significantly from normal control in these groups.

Photomicrographs of uterine cross sections exhibit the differential ability of the treatment effects on uterine histomorphology (Plate 1). Overall uterine diameter increased greatly in Groups II and III. Their uteri exhibited endometrial epithelial cell pseudo stratification, hypertrophy and development of endometrial glands.

It can be seen from Table 2, that there has been a statistically significant increase in uterine diameter, wall thickness, luminal epithelial cell height and endometrial thickness in Group II. Epithelial cell height has been maximal in Group III. Ev has been most effective in stimulating all the 3 layers of the uterus.

In Plate 2 numerous ovarian follicles in various stages of development located in the stroma of the cortex are seen in Group I. Primordial follicles are more numerous and located in the periphery of the cortex. These follicles are smallest and most simple in structure. Few mature follicles and many atretic follicles are also seen. Ovarian sections from Group II and Group III show greater numbers of larger follicles with antral cavities of various sizes. These secondary or vesicular follicles are situated deeper in the cortex. Several atretic follicles are also present and there are the signs of vascularization in the cortex of sections from Group II. A large tertiary follicle with typical cumulus oophorus is seen in this group in Plate 2.

Diameter of the ovary is largest in Group III ($P < 0.01$) (Table 2). There has been a pervasive activation of follicular growth and progression of increased numbers of follicles to more advanced stages of follicular development in this Group compared to Group I. In Group II there has been a 1.5 to 1.7 fold increase in the number of primary and secondary follicles, respectively. Uterine tube histology also corroborated with the uterine changes (Plate 2). Presence of ciliated lining epithelium is marked in Group II (Plate 2). They appear hypertrophied and the proportion of ciliated to non ciliated cells is higher.

Table 1—Body and uterine weight of experimental animals

Group/treatment	Body weight (g)			Uterine wet weight (mg)	Uterine weight/body weight (mg/g)
	Initial	Final	% Increase		
I (vehicle control)	26 ± 2.4	34 ± 3	31	83 ±1.69	2.44 ±0.12
II (Ev-250mg/kg)	29 ± 3.2	39 ± 2.8	35[a]	100 ±1.82[a]	2.56 ±0.16[a]
III (EE- 30µg/kg)	35 ± 2.1	52 ± 1.9	49[a]	157 ±1.82[a]	3.02 ±0.16[a]

Immature female wistar foster dams were weighed on the Ist day of dosing and prior to necropsy; values represent mean ± SEM of 6 animals; [a] Statistically different from controls ($P < 0.05$)

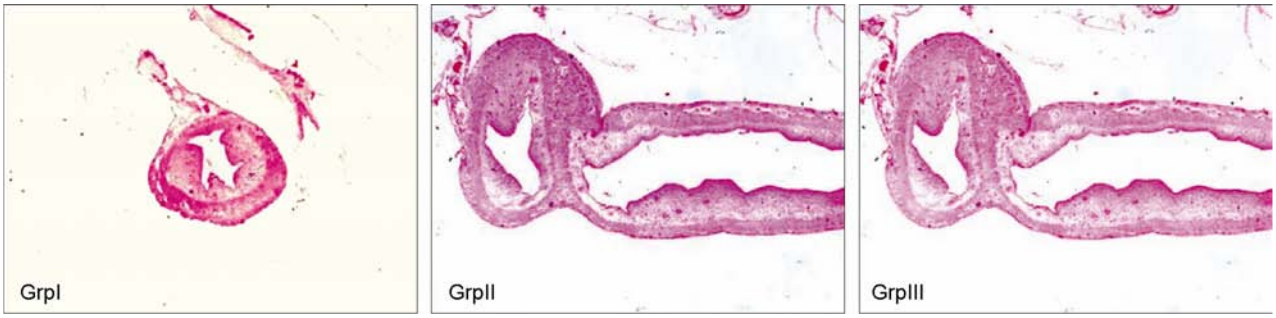

Plate 1—Photomicrographs of uterine sections from experimental animals (H & E X 4)

Table 2—Histomorphometric analysis of uterus and ovary of experimental animals[a]

Grp	Uterus #					Ovary				
	Diameter (µm)II	Wall thickness (µm)	Endometrialheight(µm)	Myometrial height (µm)	Luminal epithelial cell height (µm)	Diameter[b] of Ovary (µm)	Primary follicle[b] oocyte dia (µm)	Number[c] of		
								PF	SF	TF
I	857 ± 3.87	333.6 ± 1.23	165.4 ± 2.45	100.9 ± 1.74	15.2 ± 0.03	1275.3 ± 3.74*	27.8 ± 0.16*	19	9	-
II	990.5 ± .12	344.4 ± 2.4	268.1 ± 2.64	90.7 ± 1.35*	15.8 ± 0.15	1364.9 ± 4.86	59.1 ± 0.06	29	16	2
III	812.5 ± 4.72	324.3 ± 1.62	218.1 ± 0.91	135.1 ± 0.89	16.2 ± 0.11	2177.2 ± 4.45	23.7 ± 0.02*	17	15	4

Grp, I -Normal control, II - Ev-250mg/kg, III - EE- 30 µg/kg; I-PF-primary follicles, SF- secondary follicles, TF- tertiary follicles; [a] values represent mean ± SEM of 6 animals; [#] numbers represent average of 30 measurements from 3 fields per animal, $P < 0.05$ statistically significant;* Non significant; [b] numbers represent average of 15 measurements from every 5th section from serially sectioned ovary from each animal, numbers represent average counts of each follicle type per section.

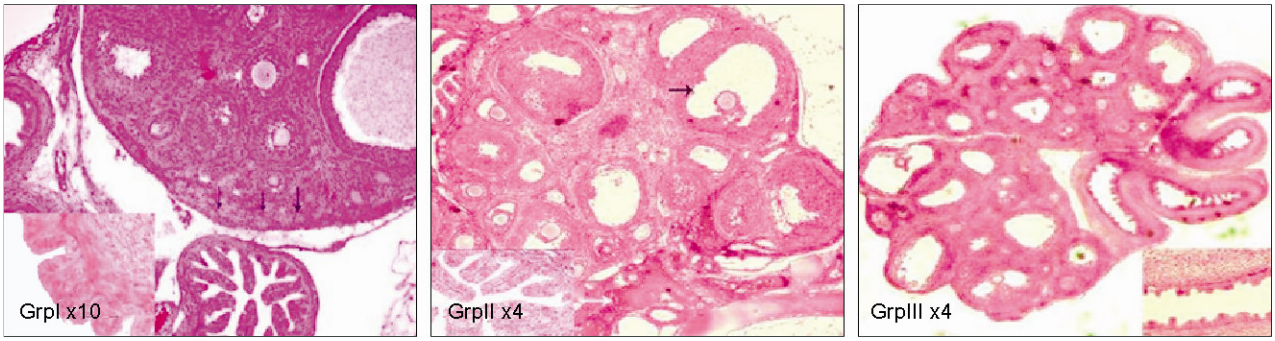

Plate 2—Micrographs of left ovary from experimental animals, H & E, Inserts: sections of oviduct X 20, Grp I- black arrows- primordial follicles, Grp II- black arrow- tertiary follicle with cumulus oophorus, white arrow- signs of vascularizations.

Identical chloroform fractions from chromatographic processing of Ev yielded a solid material which upon crystallization gave a white waxy solid of melting point 136° C. It answered Leibermann Burchard reaction and gave on TLC in hexane: ethyl acetate (9:1), a single spot of R_f 0.46. Melting point, mixed melting point determination (mmp), Co-TLC and superimposable IR with authentic sample confirm the identity of the compound as β-sitosterol.

Early fractions from chloroform: methanol (19:1) upon concentration and recrystallisation gave a white amorphous solid which on TLC in the same solvent system gave a single spot of R_f 0.48 and had a sharp melting point of 306° C. It gave a pink colour with tin and thionyl chloride, showing it to be a triterpenoid. It was identified as oleanolic acid by comparison with an authentic sample by its mmp, Co-TLC and superimposable IR.

Fractions from chloroform: methanol (9:1) gave a white powdery substance of 284°C, which gave a single spot on TLC in the same system. It tested positive for Leibermann burchard reagent and anthrone/sulphuric acid test. The compound was identified as β–sitosterol- β-D-glucoside by mmp, Co-TLC and superimposable IR with authentic sample.

Discussion

It can be seen from the results that growth of the animals was affected by drug treatment. It has been reported that some estrogenic compounds are potent inducers of imbibition than cell proliferation and vice versa[11]. The studied extract has caused fluid imbibition of the uterus. Uterine changes mediated by the extract are evident from Table 1 and Plate 2. Changes in uterine epithelial cell height and wall thickness are induced by estrogens and are considered highly specific for estrogenic activity[11]. When both the endpoints are affected by treatment, there is a high degree of confidence that estrogenic processes have mediated the event. Thus from the results it is evident that Ev and EE have brought about uterotrophic estrogenic changes. Also ovarian changes seen in Plate 2 are representative of early proliferative phase, possibly signs of estrogen priming. These changes are indicative of follicular initiation possibly due to estrogen mimicking constituents present in the extracts. All changes are characteristic of early proliferative phase indicating estrogen priming to some extent in this group.

Thus the histological and histomorphometric observations for potential treatment related effects have demonstrated estrogenic activity of Ev akin to EE in immature female Wistar rats. Ovarian follicular changes have corroborated the conclusion arrived at. Thus alcohol extract of *E. vareigata* has exhibited estrogenic activity in the tested prepubertal female rats. Compounds oleanolic acid, β-sitosterol (BSS) and β-sitosterol-3-O-β-D-glucopyranoside (BSSG) in significant yield have been isolated from Ev. Literature abounds in the antioxidant related activities[12-15] of these compounds. This is supportive of the observed estrogenic activity.

Unlike in adult rats where ovarian follicular atresia is initiated at low FSH concentration, oxidative damage by macrophage invasion initiates the atretic process in larger follicles in immature rats[16]. On the contrary, macrophages are absent in early adult atretic follicles in adult rats invading them only in advanced stages of atresia. In this study, at the dose tested, antioxidant principles in the extract have possibly neutralized the pro oxidant damage caused by macrophages in the immature female rats. Follicular atresia being prevented, they have progressed to advanced stages of growth relative to CG. Ovarian follicular growth has triggered the observed estrogenic changes in the treated animals.

Conclusion

Demonstration of estrogenic activity of *E. variegata* leaves is significant in view of its earlier reported hypolipidaemic and anti-atherosclerotic activity. Powerful antioxidant constituents in the extract explain the demonstrated estrogenic activity and the reported vascular protective effects. Our study has also elucidated the possible action mechanism of the observed activities lending scientific validity to the anti-obesity, hypolipidaemic claims for the leaves in traditional medicine. The tender leaves are consumed as a green leafy vegetable and their nutritive value has been reported. In view of the reported estrogenic activity, leaves of this pot herb may also be explored for their utility as a potential health food in menopausal symptoms.

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