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OVARIAN ANTISTEROIDOGENIC EFFECT OF THREE ETHNOMEDICINAL PLANTS IN PREPUBERTAL FEMALE MICE

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ABSTRACT

This study is a part of an integrated systematic approach to develop effective oral contraceptive agents from ethnomedicinal plants which are traditionally used to induce sterility in women. In the present investigation, the effect of ethanol extracts of *Mitragyna parvifolia* bark (EEMP), *Plumeria rubra* flowers (EEPR), and *Zizyphus xylopyrus* fruits (EEZX) at two different dose levels were studied on the onset of reproductive maturity and the ovarian steroidogenesis. All three plants caused remarkably ($P<0.01$) a dose-dependent delay in sexual maturation in as evidenced by the age at vaginal opening and appearance of first estrus. Further, statistically ($P<0.05$) a dose-dependent elevation of the ovarian cholesterol, ascorbic acid and protein contents were noted elevated. At the same time, three plants treatment also resulted in significantly ($P<0.05$) a dose-dependent diminution of Δ^5-3β-hydroxysteroid dehydrogenase (Δ^5-3β-HSD) and glucose-6-phosphate dehydrogenase (G-6-PDH) activities along with a reduction in the weight of ovary and uterus. Of the three plants, the EEZX showed produced more effects followed by EEMP. On the basis of above said results, it is suggested that the probable cause of delayed maturation in selected ethnomedicinal plants-treated immature mice is due to the suppressed ovarian steroidogenesis which, further, supports the traditional use of these plants as contraceptives.

Key words: Ethnomedicinal plants, ovarian steroidogenesis, G-6-PDH, Δ^5-3β-HSD, prepubertal mouse.

INTRODUCTION

Natural product research can often be guided by ethnopharmacological knowledge, and it can give substantial contribution to drug innovation by providing novel chemical structures and/or mechanisms of actions (Harvey, 1999). It is well established that the development of sexual organs in early life and the onset of puberty is closely related with ovarian steroidogenesis (Armstrong *et al.*, 1964) under the influence of gonadotrophins (Horikoshi and Wiest, 1971).

Mitragyna parvifolia (Roxb.) Korth (Rubiaceae) is commonly known as Kaim. The plant grows throughout India, in deciduous and evergreen forests (Prajapati *et al.*, 2003). The bark and root are used to treat fever, colic, muscular pain, burning sensation, poisoning, gynecological disorders, cough and edema. The bark and roots of this plant are used to treat fever, colic, muscular pain, burning sensation, poisoning, gynecological disorders, cough,

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edema and as an aphrodisiac (Panwar and Tarafdar, 2006). In local folklore medicines, the bark used as contraceptive in female, in fever, leucorrhoea, muscular pain, stomachache and syphilis (Jain, 1991).

Plumeria rubra Linn. (Apocynaceae) is called as a Temple tree in India although the flowers are not used as much as temple offerings. This plant is native to Mexico and grown throughout India (Warrier *et al.,* 1995). Traditionally, this plant is used to control fertility in women (Tiwari *et al.*, 1982). In local folklore medicines, the decoction of flowers is taken daily for seven days to induce sterility in women (Personal communications).

Zizyphus xylopyrus (Retz.) Willd. (Rhamnaceae) has a restricted global distribution occuring only in India and Sri Lanka. Within India, it found in North-western India, Uttar Pradesh, Bihar, Central and South India. It is quite common in dry, open deciduous or scrub forests and grasslands (Anonymous, 1976). In local folklore medicines, the crushed fruit powder is dipped in water and kept overnight and this extract is taken by the women early in the morning for 7 days to check oogenesis. Fruit decoction is used to develop sterility in women (Jain *et al.,* 2004). Traditionally, the fresh fruits crushed with water and taken twice a day to get relief from urinary problems (Jagtap *et al.,* 2006).

In our earlier studies, we reported the anti-steroidogenic activity of *Mitragyna parvifolia* bark, *Plumeria rubra* flowers, and *Zizyphus xylopyrus* fruits in mature female mice (under review)**.** However, the precise mode of action and their effects on reproductive maturation processes in mice is not known. Therefore, on continuation of our earlier work, an experimental design was made to elucidate the possible mode of action and the influence of these three plants on the onset of sexual maturity and the ovarian steroidogenesis in immature female mice and the reports are presented herein.

MATERIALS AND METHODS

Plant materials

The specified parts of the selected three plants were collected different places of Tamilnadu, and Andhra Pradesh, India, in the year of 2006 (March-August). The plant specimen was authenticated by Dr.P.Jayaraman, M.Sc., Ph.D, Plant Anatomy Research Centre (PARC), Chennai Tamil Nadu, India. The parts of the plants were separately dried in shade, pulverized by a mechanical grinder and passed through 40-mesh sieve and stored in airtight container for further use.

Preparation of plant extract

The powdered dry parts of the selected plants about 500 g were individually soxhlated successively with 70%v/v ethanol at 68°C. The extracts were collected in 5 liter individual conical flasks, filtered, and the solvent was evaporated to dryness under reduced pressure in an Eyela Rotary Evaporator (Japan) at 40-45°C and were stored in vacuum desiccators for further use.

Animals

Sixty-four closed colony, randomly bred albino female mice were weaned at 31 days of age and selected for the present investigation. Standard laboratory diet (Hindustan Lever Ltd.) and drinking water were continuously made available to mice and they were acclimatized to normal laboratory conditions. All the animal experiments were performed according to the guidelines of university animal ethics committee.

Experimental design

This experiment was designed and carried out according to Gupta et al [5].

The mice were divided into eight groups and each group containing eight and given the following treatment:

Group-1 (Normal control): Distilled water at the dose level of 10 ml/kg. b.w

Group-2 (Vehicle control): Acacia mucilage (0.5% w/v) at the dose level of 10 ml/kg b.w

Groups-3 and 4 Ethanolic extract of bark of *M.parvifolia* (EEMP) at the dose levels of 300 and 600 mg/kg b.w, respectively, suspended in 0.5% acacia mucilage

Groups-5 and 6: Ethanolic extract of flowers of *P.rubra* (EEPR) at the dose levels of 200 and 400 mg/kg b.w, respectively, suspended in 0.5% acacia mucilage

Groups-7 and 8: Ethanolic extract of fruits of *Z.xylopyrus* (EEPR) at the dose levels of 250 and 500 mg/kg b.w, respectively, suspended in 0.5% acacia mucilage

The vehicles and plant drugs were given orally from 40 days of age of mice on every day for 18 days. Initial body weight before treatment (i.e. at the age of 40 days) and final body weight during sacrifice (i.e. at the age of 58 days) were recorded. Four animals of each group were carefully noted to observe the onset of sexual maturity and left intact. Two measures of reproductive maturity were made: age at vaginal opening, age at first estrus. The mice were inspected between 7-9 a.m. and 6-7 p.m. for vaginal opening and after vaginal introitus, a daily vaginal lavage was taken from each mouse to determine the age at first estrus (cornified smear). The remaining four mice from each group were sacrificed by cervical dislocation 24 h after the last dose (i.e. at the age of 58 days) and 18 h fasting condition. Ovaries and uterus were dissected out, freed from fatty materials, weighed and kept on ice for further processing.

Biochemical estimation

Ovaries were homogenized in a Potter Elvehjem homogenizer using chloroform ethanol mixture (2:1) and nonpolar part was extracted out and total cholesterol quantified according to the methods of Kingsley and Roscoe (Kingsley and Roscoe, 1949).

About 5mg of tissue was homogenized in a Potter Elvehjem homogenizer using 45µl ice cold 5% metaphosphoric acid and centrifuged for 20 min at 3500×*g*. Then, 30µl supernatant, 15µl acetate buffer and 15µl, 2,6-dichlorophenolindophenol sodium (0.1 mg/ml) were mixed and optical density was measured against water at 520nm. Standard curve was drawn against known concentrations and ascorbic acid content was calculated (Omaye *et al.*, 1979).

Ovaries were homogenized in a Potter Elvehjem homogenizer using 0.1 M phosphate buffer (pH 7.4) and centrifuged. The activity of Δ5-3β-HSD was estimated as described by Rabin et al (Rabin *et al.*, 1961). Ovaries were homogenized in a Potter Elvehjem homogenizer using 0.5 M Tris–HCl (pH 8.3) and centrifuged to estimate G-6-PDH (Lohr and Waller, 1974).

Protein was estimated with folin phenol reagent and activity of enzymes was expressed in unit per mg of protein (Lowry *et al.*, 1951).

Statistical analysis

Statistical comparison was performed by using GraphPad Prism 3.0 (GraphPad Software Inc, San Diego, CA). Results were compared using one-factor analysis of variance (ANOVA) with Dunnett's post-hoc test. All the results are expressed as mean ± standard error of the mean (S.E.M). Values were considered significant at $P<0.05$ or less.

RESULTS

The oral administration of EEMP, EEPR, and EEZX at two different dose levels significantly ($P<0.05$) retarded the onset reproductive maturity as indicated by the age at vaginal opening and appearance of first estrus in a dose-dependent manner when compared with vehicle control group (Figure-1). In the same condition, a dose-dependent reduction in the rate of body growth (weight gain) accompanied the delay in sexual maturation (Table-1). Moreover, among three plants tested, the EEZX at both the dose levels produced more reductions in the rate of body growth, followed by EEMP.

Marked dose-dependent reductions were observed in the weights of ovary and uterus pituitary of all three plants-treated immature mice when compared with vehicle control (Figure-2). Moreover, among three plants tested, the EEMP at both the dose levels produced more elevations followed by EEZX.

When compared to vehicle control group, significantly ($p<0.05$) a dose-dependent elevation of ovarian cholesterol and ascorbic acid contents were noted in the immature mice after treated with all the three plants at both the dose levels (Figure-3). Moreover, among three plants tested, the EEMP at both the dose levels produced more elevations followed by EEZX. At the same time, The EEMP, EEPR and EEGV significantly brought down the ovarian protein content in a dose-dependent manner as compared to vehicle control group (Figure-4).

The oral administration of EEMP, EEPR and EEGV significantly ($P<0.05$) inhibited the ovarian G-6-PDH and Δ^5-3β-HSD activities in a dose-dependent manner when compared with vehicle control (Figure-5). Moreover, among three plants tested, the EEZX at both dose levels remarkably produced more diminutions of these enzymes activities, followed by EEMP.

Table-1: Rate of body growth after treated with the selected ethnomedicinal plants

Treatment Design	Dose (mg/kg b.w)	Body weight		
		Initial body weight (g)	**Final body weight** (g)	**% increase in b.w**
Group-1: DW	10 ml	10.1 ± 0.8	23.2 ± 0.8	129.20
Group-2: AM	10 ml	10.2 ± 0.3	24.4 ± 0.3	139.21
Group-3: EEMP	300 mg	10.1 ± 0.7	21.6 ± 0.6**	113.86
Group-4: EEMP	600 mg	10.3 ± 0.6	17.2 ± 0.2**	66.99
Group-5: EEPR	200 mg	10.2 ± 0.4	22.4 ± 0.3**	119.60
Group-6: EEPR	400 mg	10.6 ±0.7	18.1 ± 0.5**	70.75
Group-7: EEZX	250 mg	10.3 ± 0.8	19.3 ± 0.2**	75.72
Group-8: EEZX	500 mg	10.3 ± 0.6	16.7 ± 0.3**	62.13

[*Results presented as mean ± SEM; n = 4 mice in each group; (**) = P < 0.01 when compared with vehicle control*]

Figure-1: Mean age in days of immature female mice at two measures of sexual maturity after treated with the selected ethnomedicinal plants [Results presented as mean ± SEM*; n* = 4 mice in each group; P < 0.01 when compared with vehicle control]

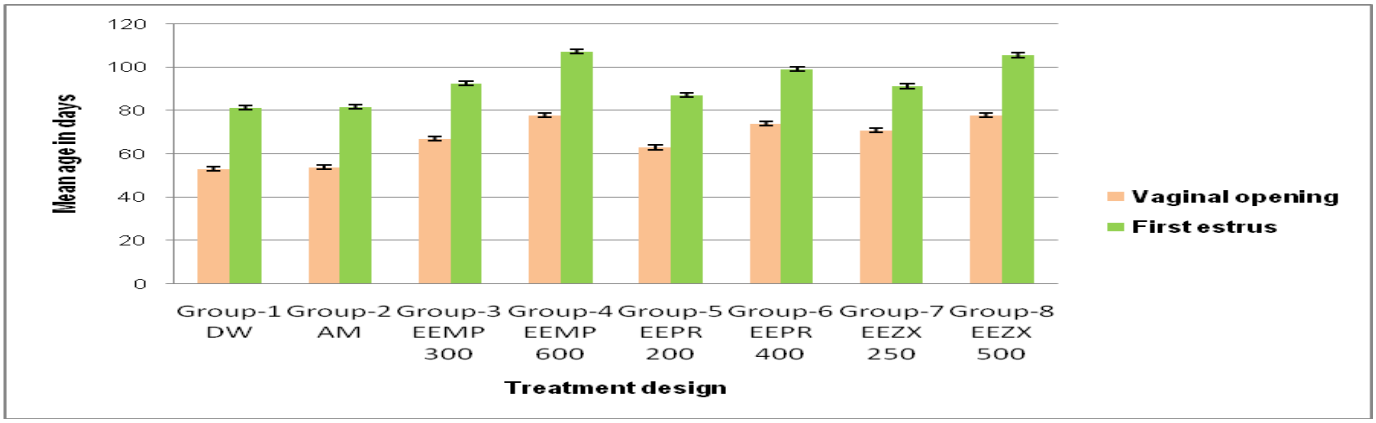


Figure-2: Weights of ovary and uterus of immature female mice after treated with the selected ethnomedicinal plants [Results presented as mean ± SEM*; n* = 4 mice in each group; P < 0.01 when compared with vehicle control]

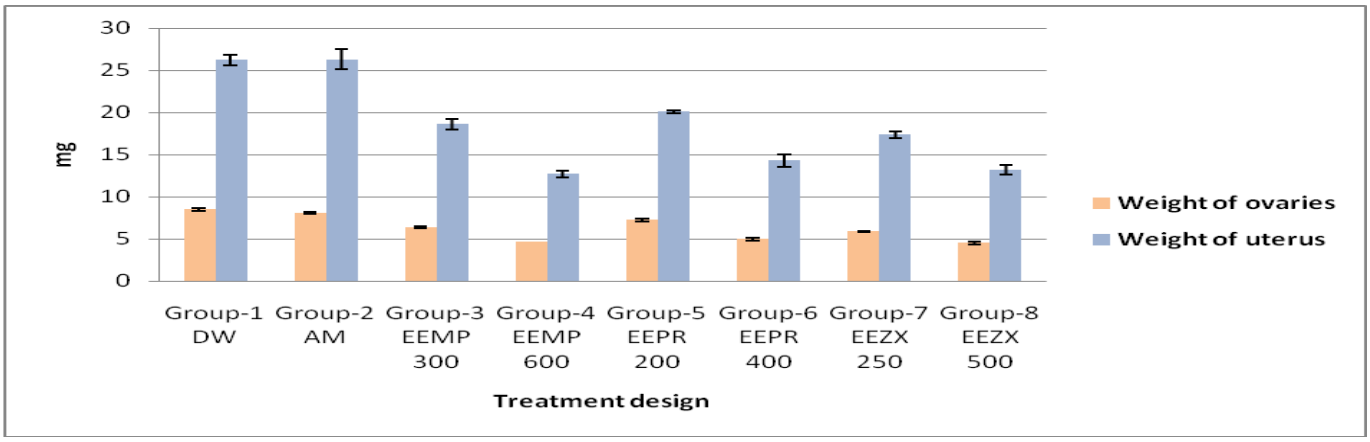


Figure-3: Effect of the selected ethnomedicinal plants on contents of ascorbic acid, cholesterol and protein in immature mice ovaries [Results presented as mean ± SEM*; n* = 4 mice in each group; *P* < 0.05 when compared with vehicle control]

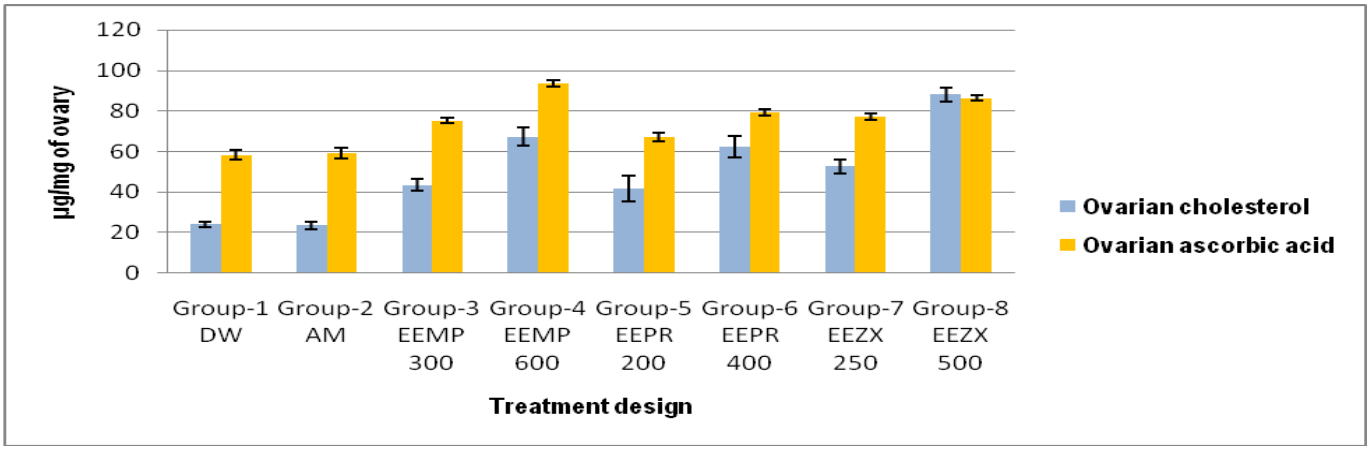


Figure-4: Effect of the selected ethnomedicinal plants on protein content in immature mice ovaries [Results presented as mean ± SEM*; n* = 4 mice in each group; *P* < 0.05 when compared with vehicle control]

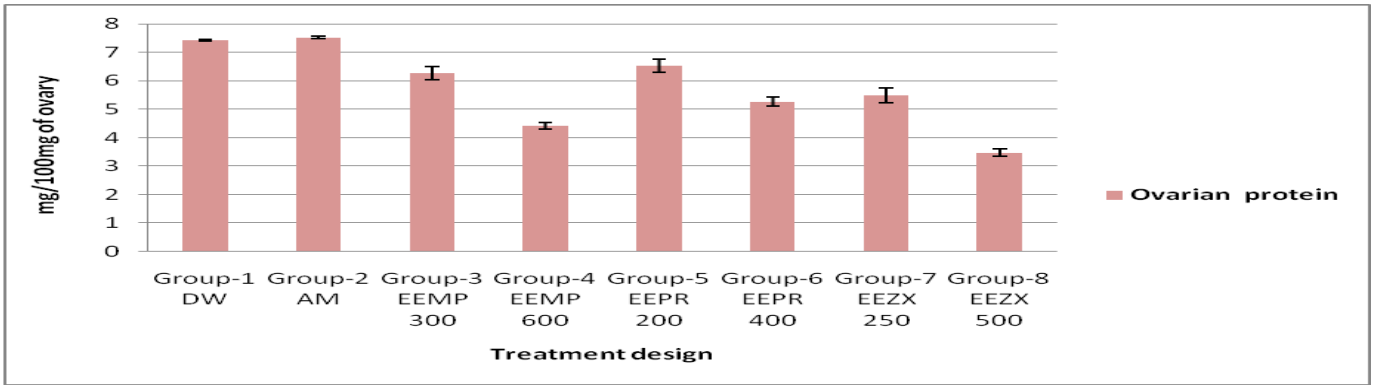
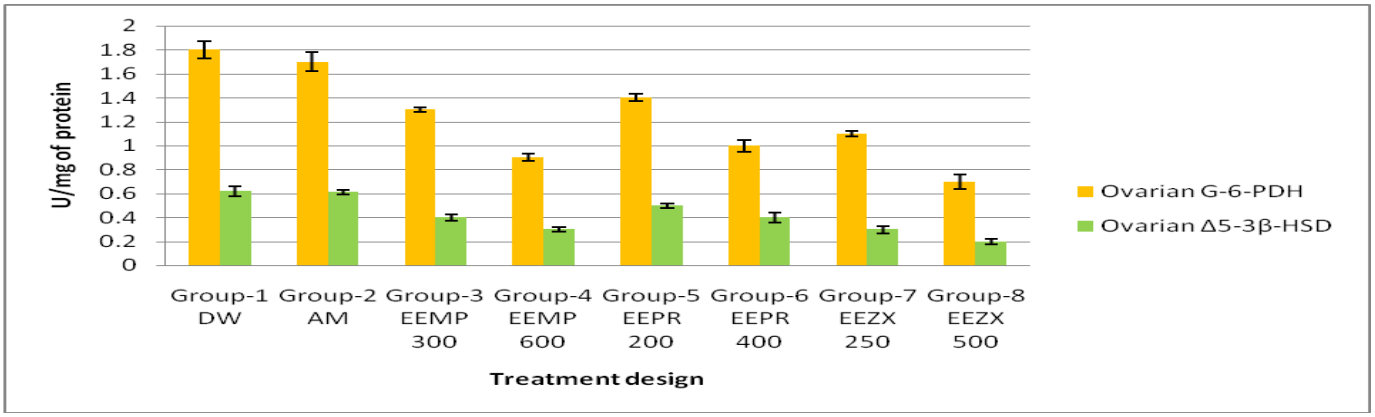


Figure-5: Effect of the selected ethnomedicinal plants on G-6-PDH and Δ^5-3β-HSD activities in immature mice ovaries [Results presented as mean ± SEM*; n* = 4 mice in each group; *P* < 0.05 when compared with vehicle control]



DISCUSSION

Figure-1 confirmed that the oral administration of EEMP, EEPR and EEZX remarkably delayed the onset of sexual maturity as evidenced by the age at vaginal opening (Elbetieha *et al.*, 1998) and appearance of first estrus. These disturbances in the reproductive cycle and the decrease in the weight of the ovary in the present investigation may be related with the diminution of ovarian steroidogenesis (Rindi *et al.*, 1963). This was associated with an elevation in the level of cholesterol which serves as a precursor for the synthesis of steroid hormone in ovaries (Marcus *et al.*,1996; Rang *et al.*, 1999; Wilks *et al.*,1970) and also precursor for the steroidogenesis of ovarian endocrine tissue (Strauss *et al.*, 1981) suggesting thereby that cholesterol was not utilized. The depressed ovarian steroidogenic activity and hypofunctioning of the gland was evident by increase in ascorbic acid level after treatment with extracts of selected plants (Deane, 1952). Of the three plants tested, however, the EEMP and EEZX at the both dose levels produced the more accumulation of cholesterol.

To substantiate these facts, the estimation of G-6-PDH and Δ^5-3β-HSD, the two key enzymes involved in steroidogenesis was performed (Armstrong, 1982; Suzuki *et al.*, 1984). The importance of G-6-PDH of pentose phosphate pathway in the synthesis of estrogen in the sexually immature animals has been reported earlier (Dey *et al.*, 1972). It is also well established that Δ^5-3β-HSD is associated with steroid biogenesis (Knorr *et al.*, 1970). In the present study, tested extracts inhibited the activity of two key steroidogenic enzymes significantly when compared with control groups. Therefore, in the present investigation a fall of G-6-PDH and Δ^5-3β-HSD after treatment with EEMP, EEPR, and EEZX suggests a diminution of ovarian steroidogenesis (Dhanapal *et al.*, 2005; Gupta *et al.*, 2003; Mazumder et al., 1997; Gupta et al., 1980) and which may be the possible mechanism of action of this these plants in reducing fertility.

Overall, the tested plants delayed the onset of puberty and suppressed the ovarian steroidogenesis in the following order of *Z. xylopyrus* > *M. parvifolia* > *P. rubra*, which confirms the effect observed with individual plant drugs in our earlier study.

CONCLUSION

It is suggested that the delay of the onset of puberty following treatment with *Mitragyna parvifolia* bark, *Plumeria rubra* flowers, and *Zizyphus xylopyrus* fruits is possibly due to the depression and reduction in ovarian steroidogenesis. However, possible site of action of the selected plants either directly on the ovary or via gonadotrophins secretion is subject to further clarification.

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