International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2015): 78.96 | Impact Factor (2015): 6.391

Anti-implantation and Antiestrogenic Activity of Ethyl Acetate Extract of Chromatographic Fractions of *Eugenia Jambolana* Seeds in Female Albino Rats

Sarita M[1], Manjunath M J[2]

[1]Regional Institute of Education, Mysore

[2]Maharani's Science College for Women, Mysore

Abstract: ***Plants are a valuable source of new natural products. Ancient literature mentions the use of a number of plants preparations for fertility regulation. In the present study, the ethyl acetate extract of Eugenia jambolana seeds which showed promising antiimplantation and antiestrogenic activity in female albino rats was examined for the isolation of its active fractions. Two fractions (I & II) were obtained using Thin Layer Chromatography (TLC) of the extract. Both fractions were subjected for testing their antiimplantation and antiestrogenic activity in rats. The data revealed that the fraction I of ethyl acetate extract treatment at both dose levels decreased the number of implants compared to control group. The fraction I exhibited a strong antiestrogenic activity, when administered alone. It also inhibits the estrogen induced gain in the uterine weight when administered along with ethinyl estradiol. Histological studies of the uterus were carried out to confirm this activity. These findings suggest that a chromatographic fraction I of ethyl acetate extract of E. Jambolana seed might be used as a contraceptive in the females.***

Keywords: Implantation, *Eugenia jambolana*, Antiestrogenic, Pregnancy, Female rats

1. Introduction

Population explosion has created a grave set back in the economic growth and all round human development in developing countries. Current pandemic population explosion demands an immediate betterment of new potential contraceptives [1]. Thus, control of human fertility in the sense of its limitation is the most important and urgent of all biosocial and medical problems confronting mankind today [2]. In recent years there has been a considerable interest in plant with possible antifertility effect [3]. In the modern system of medicine, about 25% of prescriptions contain active principle derived from plants. Plant kingdom therefore holds a great promise for the discovery of new and effective antifertility agents [5]. In this context, it is appropriate to locate the large number of indigenous plants that are used as oral contraceptives by tribal and other section of people. Many such plants are recommended in Ayurvedic, Yunani and Folk medicines [5] - [7]. A good number of scientific papers have been already published related to the use of medicinal plants for antifertility. However, still many more medicinal plants are either less investigated or left investigated. In the same direction present study is being carried out to identify antifertility agent from the seeds of *E. Jambolana*. Earlier studies of our laboratory have shown that the ethyl acetate extract of *E. Jambolana* seed demonstrated their antiovulatory, anti-implantation, antiestrogenic and abortifacient activities [8] in rats and mice. In the present study, we have undertaken the investigation of the anti-implantation and antiestrogenic activity of chromatographic fractions of crude ethyl acetate extract of *E. Jambolana* seeds to elucidate its active ingredients.

2. Materials and Methods

(1) Collection of Seeds

The fully matured fresh seeds of *Eugenia jambolana* were collected from fields in and around Madikeri district of Karnataka, India, during fruiting season ie., in the month of June to August. The seeds were identified and authenticated by Dr. Sudarshan, Professor. Department of Botany, University of Mysore, Manasagangotri and the plant bearing herbarium number of 1634, where voucher specimens were deposited.

(2) Extraction of Seed and Preparation of Test Material

The seeds were shade dried; powdered and 100 gm seed powdered was subjected to extraction with ethyl acetate (76-80^{o}C) in a soxhlet extractor for 72 hr. The extract so obtained was concentrated to dryness in a flash evaporator under reduced pressure and controlled temperature (50-60^{o}C) to obtain crude extract. The ethyl acetate extract was subjected to Thin Layer Chromatography using silica gel 'G' as absorbent. The extract was loaded on the preparative plates developed with solvent system. Two major bands were observed by exposing the plates to Iodine vapors. The compounds having high Rf was designated as fraction I and the compounds having low Rf value was designated as fraction II. The fractions were prepared in DMSO (1%) in distilled water for complete dissolution.

(3) Animals

Female wistar rats weighing 150-200g were obtained from the animal house of the University of Mysore. Mysore showing regular estrous cycle were used for the experimentation. The rats were housed in polypropylene cages under well ventilated animal house. The rats were given pelleted feed and water ad libitum. Immature ovariectomized female weighing 30-35 g was also employed for the study of the estrogenic/antiestrogenic effect. The experimental protocol was approved by the Animal Ethical

Committee in accordance with the guidelines for care and use of laboratory animals prepared by the Institutional Animal Ethics Committee (NIH, 1985).

3. Experimental Protocol

3.1 Anti-implantation Activity

Anti-implantation activity was determined following the method [9]. The female rats showing proestrous stage in the vaginal smear were caged with male rats of known fertility in the ratio of 2.1 and examined for the following morning for the evidence of copulation. The animals exhibiting thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day 1 of pregnancy and those rats were divided into 5 groups containing 6 rats in each group.

Group I: control received the vehicle (0.2ml 1% DMSO)
Group II: Received 100mg fraction-I extract / kg b w/0.2 ml/day/rat.
Group III: Received 300mg fraction-I extract / kg b w/0.2ml/day/rat.
Group IV: Received 100mg fraction-II extract / kg b w/0.2ml/day/rat.
Group V: Received 300mg fraction-II extract / kg b w/0.2ml/day/rat.

The fractions were administered orally from 1-7 days using intragastric catheter and laparotomy was performed on day 10 of pregnancy under ether anesthesia and determine the number of implantation sites on both uterine horns. Samples of fresh ovary were removed, fixed in Bouin's fluid for 24 h, dehydrated in alcohol, cleared in xylene and embedded in paraffin wax. Routine 5µm sections were then cut and stained using Haematoxylin-Eosin method and count the number of corpora lutea.

3.2 Estrogenic/Antiestrogenic Activity

The fraction I of ethyl acetate extract found to be most active fraction; hence it was subjected for potential estrogenic/antiestrogenic activity. Colony bred immature albino rats, 21-23 days old, weighing between 30-35g were bilaterally ovariectomized by dorsolateral approach under light ether anesthesia in sterile conditions. They were divided into six groups consisting of 6 rats in each group.

Group I: control and received the vehicle (0.2 ml 1%DMSO)
Group II: Received 1 µg ethinyl estradiol/rat/day in olive oil subcutaneously.
Group III: Received 100mg fraction I extract/kg b w/0.2ml/day/rat.
Group IV: Received 300mg fraction I extract/kg b w/0.2ml/day/rat
Group V: Received 100mg fraction I extract / kg b w/0.2 ml/day/rat orally + 1 µg ethinyl estradiol/ rat/ days subcutaneously.
Group VI: Received 300mg fraction I extract / kg b w/0.2 ml/day/rat orally + 1 µg ethinyl estradiol/ rat/ days subcutaneously.

All the above treatments were given for 7 days. On the 8th day, the rats were sacrificed by ether anesthesia, the uteri dissected out and separated from the adherent tissues and weighed up to the nearest milligrams on sensitive balance. Estrogenic activity was assessed according to the method of taking uterine wet weight, opening of the vagina and cornification of vaginal epithelial cells as the points of evaluation [10]. Additionally, the uterine tissue of rats from each group was fixed in Bouin's fluid and processed for histological preparation using haematoxylin-eosin stain. The diameter of the uterus, thickness of the endometrium and myometrium and the epithelial cell height were measured on 20 randomly sectioned sections using stage and ocular micrometer.

Statistical Analysis

One way analysis of variance [ANOVA] followed by Duncan's multiple test were used to find out significant difference among mean values of each parameter of different experimental groups by fixing minimum significance level at $P<0.05$. Values with same superscript letters are not significantly [$P<0.05$] different whereas those with different superscript letters are significantly [$P<0.05$] different when compared to control.

Table 1: Effect of fraction I and II of ethyl acetate extract of E. jambolana seeds on implantation in female albino rats

Treatment	Dose (mg/kg bw)	No of rats with implantation sites	No of rats without implantation sites	No of corpora lutea	Fertility Index (%)	No of implantations/rat		Mean number of implants±SE
Control	1% DMSO	6	00	15.56±0.50	100	6+6=12 6+5=11 6+6=12	7+7=14 5+7=12 7+5=12	12.16 ± 0.40^{a}
Fraction I	100	2	4	4.32±0.71	33.34	2+4=6 0+0=0 0+0=0	0+0=0 3+3=6 0+0=0	2.00 ± 1.26^{c}
	300	1	5	2.24±0.25	16.66	2+3=5 0+0=0 0+0=0	0+0=0 0+0=0 0+0=0	0.83 ± 0.83^{d}
Fraction II	100	6	00	10.79±0.60	100	6+4=10 5+5=10 4+5=9	6+6=12 6+4=10 6+3=9	9.93 ± 0.47^{a}
	300	5	1	7.10±0.41	83.33	4+4=8 0+0=0 4+3=7	5+4=8 5+6=11 4+5=9	8.98 ±1.42^{b}

All values are expressed as Mean ± Standard error. The data was analyzed by one way ANOVA, Analysis of Variance. Values with same superscript letters are not significantly [$P<0.05$] different whereas those with different superscript letters are significantly [$P<0.05$] different as judged by Duncan's Multiple Test.

4. Results

Effect of fractions (I & II) of ethyl acetate extract of *E. jambolana* seeds on Implantation

The laparotomy study showed that on day 10 of pregnancy, the control groups exhibited 12.16±0.60 mean number of implants. The administration of fraction I of ethyl acetate extract at 100 and 300mg/kg body weight significantly inhibited pregnancy in four out of six rats with a mean number of implants of 2.00 ± 1.26 (P<0.01) and five out of six rats with a mean number of implants 0.83 ± 0.83 (P<0.001) respectively. Fraction I of ethyl acetate extract was also showed reduction in the number of corpora lutea of pregnancy as well as the pre-coitum fertility index.

However, both the doses of fraction II of ethyl acetate extract were found to be ineffective and the number of implantation sites and the number of corpora lutea in these cases were comparable with the control rats **(Table -1)**.

Table 2: Effect of fraction I of ethyl acetate extract of E. jambolana seed on antiestrogenic activity in bilaterally ovariectomized immature rats

Treatment	Dose (mg/kg b w)	Uterine Weight (mg/100 b w)	Vaginal Status
Control	1% DMSO	72.01 ±1.72	Not opened
Ethinyl estradiol	1µg/rat/day	165.42 ± 1.87	Opened
Fraction I	100	59.16 ± 2.54	Not opened
	300	50.46 ± 1.92	Not opened
Ethinyl estradiol + Fraction I	1µg/rat/day + 100mg	42.34 ± 2.38	Not opened
	1µg/rat/day + 100mg	34.66 ± 1.17	Not opened

All values are expressed as Mean ± Standard error. The data was analyzed by one way ANOVA, Analysis of Variance. Values with same superscript letters are not significantly [P<0.05] different whereas those with different superscript letters are significantly [P<0.05] different as judged by Duncan's Multiple Test.

No toxic effects were observed either by gross visual examination or in the weight of animals. After discontinuation of treatment, all the animals were mated. This resulted in pregnancy and delivery of normal litters, indicating that the action of the fraction I extract was reversible.

Effect of fraction I of ethyl acetate extract of *E. jambolana* seeds on Estrogenic/Antiestrogenic activity

Table 3: Effect of fraction – I of ethyl acetate extract of E. jambolana seeds on uterine histometric parameters in bilaterally

Treatment	Dose (mg/kg b w)	Diameter of Uterus (µm)	Thickness of Myometrium (µm)	Thickness of endometrium (µm)	Epithelial cell height (µm)
Control	1% DMSO	594.32 ± 4.21 [b]	78.47 ± 2.95 [b]	298.07 ± 1.92 [b]	32.61 ± 1.30 [b]
Ethinyl estradiol	1µg/rat/day	1201.3 ± 4.09 [a]	187.53 ± 2.31 [a]	499.11 ± 4.31 [a]	61.52 ± 0.75 [a]
Fraction I	100	527.16 ± 6.41 [c]	59.21 ± 1.07 [c]	230.14 ± 3.16 [c]	24.61 ± 1.07 [c]
	300	483.03 ± 3.76 [d]	50.64 ± 2.67 [d]	182.98 ± 2.86 [d]	20.41 ± 0.25 [d]
Ethinyl estradiol + Fraction I	1µg/rat/day + 100mg	402.32 ± 6.33 [e]	40.49 ± 1.89 [e]	173.66 ± 3.30 [e]	18.94 ± 0.44 [e]
	1µg/rat/day + 300mg	316.61 ± 6.42 [f]	36.34 ± 1.76 [f]	151.61 ± 2.91 [f]	16.83± 2.41 [f]

All values are expressed as Mean ± Standard error. The data was analyzed by one way ANOVA, Analysis of Variance. Values with same superscript letters are not significantly [P<0.05] different whereas those with different superscript letters are significantly [P<0.05] different as judged by Duncan's Multiple Test.

As fraction I of ethyl acetate extract of *E. jambolana* seeds proved to be more potent in having contraceptive properties, the antiestrogenic activity of fraction I has also been tested. The uterine weight of rats when received fraction I of ethyl acetate seed extract at 100mg/kg body weight induced a significant decrease (P<0.05) and at 300mg/kg body weight it caused a highly significant decrease (P<0.001) in the weight of the uterus when compared to control. The uterine weight is still more decreased when administered fraction I of ethyl acetate seed extract along with ethinyl estradiol, indicating the high antiestrogenic activity of the fraction I of ethyl acetate seed extract. The fraction I of ethyl acetate extract at both the dose level either along with ethinyl estradiol or alone has caused highly significant (P<0.001) decrease in the diameter of uterus, thickness of myometrium and endometrium and surface epithelial cell height in overiectomized immature rats. Also all the experimental rats treated with fraction I of ethyl acetate extract alone or in combination with ethinyl estradiol were showed closed vagina and the absence of cornified epithelial cells in the vagina. **Table 2 & 3.**

5. Discussion

The use of plant preparations for antifertility activities especially for prevention / interruption of pregnancy have been practiced since ancient time in India [11]. Implantation in rat depends on the completion of basic sequence of events occurring both at the fertilized egg and endometrium. The endometrium needs 48hrs period of progesterone preparation and presence of estrogen at the end, leading to the formation of high sensitive decasualized endometrium [12]. The implantation index, reduced number of corpora lutea and pre-implantation loss are useful indices for evaluating the number of blastocyst implanted in the uterus [13].

A number of plants possessing antiestrogenic activities have also been reported to interrupt pregnancy [14] - [18]. An active antiestrogen has been reported to decrease the wet weight of uterus. The antiestrogenic effect also supported by decrease in diameter of uterus, thickness of myometrium, endometrium and the epithelial cell height, closed vagina and the absence of cornified epithelial cells in vagina.

It is well known that for implantation exact equilibrium of estrogen and progesterone is essential and any disturbance in the balance of these hormones may cause infertility [19]. In the present study the fraction I of ethyl acetate extract at 100 and 300mg/kg body weight shows highly potent anti-implantation activity. The high rate of implantation losses may be due to direct/indirect effect on corpus luteum resulting in inhibited synthesis or secretion of progesterone [20] [21] that creates an imbalance in progesterone and estrogen ratio [22] necessary for implantation. The anti-implantation activity of fraction I of ethyl acetate extract could involve antiestrogenic action, since estrogen induced uterine hypertrophy is inhibited by fraction I. The decrease in the number of corpora lutea and graafian follicles and increase in the number of atretic follicles in the treated rats indicate that the development of preovulatory follicles and their conversion of corpora lutea are completely inhibited. The cornification in the vagina is mainly due to the level of stimulation of estrogen which acts directly on the vaginal epithelium [23] [24]. But in the present study, the absence of cornified epithelial cells in the vagina is due to the decreased level of estrogen which acts directly on the vaginal epithelium. The decrease in the wet weight of uterus in the immature rat shows antiestrogenic nature of fraction I of ethyl acetate extract of *E. jambolana* seed.

Similar observations have been reported with sesquiterpene isolated from roots of *Aristolochia indica* showed 91.7% anti-implantation and antiestrogenic activity in female mice [25]. The methanolic root extract of *Boerhaavia diffusa* at 400mg/kg were found to possess anti-implatation and antiestrogenic activity [26]. Embelin isolated from dried berries of *Embelia ribes* inhibited pregnancy and also possess antiestrogenic and 85.71% anti-implantation activity in rats when administered at 50mg/kg body weight for 7 days [27]. The benzene, hexane and alcohol extracts of *Echinops echinatus* root showed to possess rich antiestrogenic active principle and also inhibited pregnancy [28]. The methanolic extract of *Melia azedarach* Linn. showed to possess high anti-implantation and antiestrogenic activity in female rats [29].

6. Conclusion

The present investigation suggest that fraction I of ethyl acetate seed extract of *Eugenia jambolana* can be used as a fertility regulating agent as it possess anti-implantation activity that might be due to its antiestrogenic property. Preliminary phytochemical analysis and TLC revealed that fraction I contains more of flavanoids and a mixture of compounds. The activity of the fraction I is owing due to synergistic action of multiple metabolites. Therefore, it is hoped that this fraction can be further purified to get a potent compound and may be brought out as an effect contraceptive in near future.

References

[1] Ghosh K and Bhattacharya TK. Preliminary study on the anti-implantation activity of compounds from the extracts of seeds of *Thespesia populinea*. Indian Journal of Pharmacology. 2004; Vol. 36, No 5, PP. 288 – 291.
[2] Darney PD. Oral contraceptive practice guidelines; minimizing side effects. Inter J of Fertility, 1997; 42 (supp 1); 158 -169.
[3] Vishnukantha, Rana AC. Antifertility activity of the leaves of *Plumbago zeylanica* L. in female albino rats. Indian J. Pharm. Educ Res., 2010; 44(1); 49 -55.
[4] Rahaman NSA, Azahari B. Effect of calcium hydrofiller loading on the properties on Banana stem handset. Bioresources 2012; 7(3): 4321-4340.
[5] Chopra, RN, Chopra IC, Handa KL and Kapur LD. Chopra's Indigenouse Drugs of India. UN Dhursons Pvt. Ltd., Calcutta, 1958; PP, 49.
[6] Nadkarni, AK and Nadkarni KM. Indian Medicinal Plants. 1976: Vol 2, Popular Book Depot, Bombay.
[7] Satyavathi GV, Gupta AK and Tandon N. Medicinal plants of India. Vol I (Publications and information Directorate CSIR, New Delhi). 1987; 254.
[8] Sarita M. and Bhagya M. Anti-implantation and Antiestrogenic activity of *Eugenia jambolana* seed. Journal of Pharmacy Research. 2012; 5(5), 2607 -2609.
[9] Khanna U, Chaudhury RR. Antifertility screening of plants, Part –I: Investigations of *Butea monosperma* Lam (Kuntze). Indian J. Med Res 1968; 56; 1575-80.
[10] Edgren RA and Calhoun DW. The biology of steroidal contraception. In: Edgren, RA., ed, Contraception, The chemical control of fertility. New York.
[11] Kamboj VP and Dhawan BN. Research on Plants for fertility regulation in India. Journal of Ethnopharmacology. 1982; 6. 191-226.
[12] Psychoyos A. Recent research on egg implantation. CIBA Found study groups. 1966: 23.
[13] Hafez EE. Reproduction and Breeding, Techniques for Laboratory Animals. Philadelphia, PA; Lea and Febiger. 1970.
[14] Almeida FCG, Lemonica IP. The toxic effects of Coleus barbatus B on the different periods of pregnancy in rats. J. Ethnopharmacology. 2000; 73. 53 – 60.
[15] Dhaliwal PK, Roop JK, Guraya SS. Antifertility activity of neem-seed oil in cyclic female albino rats. In: Dhaliwal GS, Randhawa NS, Arora R, Dhawan AK (eds) Ecological agriculture and sustainable development. 1997; Vol. 2: 340-346. Indian ecological society, Punjab Agricultural University, Ludhiana, and Centre for Research in Rural and Industrial Development, Chandigarh, India.
[16] Badami S, Aneesh R, Sankar S, Satishkumar MN, Suresh B, Rajan S. Antifertility activity of Derris brevipes variety cariacea. J. Ethnopharmacol. 2003; 84:99-104.
[17] Sharma JD, Binda A. Antifertility activity of steroidal extract of *Trigonella foenum graceum* (seeds) in female rats. Asian J. Exp. Sci. 2005; 19:115-120.
[18] Gbotolorun SC, Osinubi AA, Noronha CC, Okanlawon AO. Antifertility potential of neem flower extract on adult female Sprague- Dawley rats. African Health Sciences. 2008; 8:168-173.
[19] Mustapha AR, Bawa EK, Ogwu D, Abdullahi US, Kaikabo AA, Diarra SS. Effects of ethanolic extract of *Rhynchosia sublobata* (Schumach) Meikle on estrous cycle in wistar rats. Int. J. Med. Arom. Plants. 2011; 1: 122-127
[20] Psychoyos A. Recent research on egg implantation. CIBA Found study groups. 1966: 23.

[21] Finn CA, Martin L. The control of Implantation. J. Reprod. Fertil. 1974; 39. 195-206.
[22] Hiremath SP, Badami S, Swamy HKS, Patil SB, Londonkar RL. Antifertility activity of *Striga orobanchioides*. Biol. Pharmaceutical Bull. 1994; 17. 1029-103.
[23] Hiremath SP, Rao SH, Jain PK, Jaya Y, Sembulingham. Antifertility activity of *Striga lutea* – Part I. Indian. J. Physiol. Pharmacol. 1990; 34. 23- 25.
[24] Turner RA and Hebborn P. Screening methods in Pharmacology. Academic Press, New York and London. 1971; 2. 94 – 95.
[25] Pakrashi A and Shaha C. Effect of sesquiterpene from *Aristolochia indica* Linn.on fertility of female mice. Experentia. 1977; 33, 1498.
[26] Neetesh KJ. Suman J. Mehta SC and Tonpay SD. Anti-implantation and Antiestrogenic activity of *Boerhaavia diffusa* root extract in female albino rats. American Journal of Pharmacological Sciences. 2016; Vol 4, No.2, 15-29.
[27] Prakash AO. Antifertility investigation in Embelin. Planta Medica. 1981; 41, 259.
[28] Sharma K, Mishra S and Mehata, BK. Antifertility activity of *Echinops echinatus* in albino rats. Indian J. Med. Sci. 1988; 42, 23.
[29] Roop Jk. Antifertility and Anti-implantation activity of Methanol Fraction of *Melia azedarach* Linn seed extract in Female albino rats. International Journal of Science and Research. 2015;4(5), 548-552.