Journal of Infertility and Reproductive Biology

Journal of Infertility and Reproductive Biology, 2015, Volume 3, Issue 1, Pages: 136-144

Effect of isolated chromatographic fractions of *Citrus medica* seeds: *In vivo* study on anti-implantation and estrogenic activity in albino rats

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Abstract

The therapeutic effect of medicinal plants for the treatment of various diseases is based on the presence of chemical constituents in the plants. The present investigation is aimed to justify the active principle of *Citrus medica* seeds by isolating chromatographic fractions of petroleum ether extract and studied for anti-implantation, pregnancy interruption and estrogenic activities in albino rats. Two fractions were obtained from thin layer chromatography (TLC) and subjected for testing to know their anti-implantation, pregnancy interruption and estrogenic activities in *in vitro*. Each isolated fraction (I & II) at the dose level of 50mg and 100mg/kg body weight were administered orally for 7 days in 5 groups (from day 1 to 7 of pregnancy) to pregnant rats for anti-implantation, pregnancy interruption on day 10. Also non-pregnant rats in 6 groups for estrogenic activity. Among the two isolated TLC fractions, fraction II at 50 and 100mg/kg body weight is highly significantly effective in reducing mean number of implants and exhibited 71.65 & 80.17 percent inhibition of implantation respectively. Estrogenic activity of fraction II at the dose level of 100mg/kg body weight exhibited highly significant increase in the wet weight of uterus and adrenals diameter of uterus, thickness of myometrium & endometrium and epithelial cell height. Vaginal cornification and premature opening of vagina in 6 out of 6 rats exhibited positive result and almost similar to Ethinyl estradiol administration. Furthermore, histological changes of uterus and adrenals were support the anti-implantation and estrogenic study. Hence, the results concluded that estrogenic nature of the fraction II, at 100mg/kg body weight level, possessing active constituents present in petroleum ether extract and have been proved to have significant antifertility activity in *Citrus medica* seeds.

Keywords: *Citrus medica*, Anti-implantation, Pregnancy, Estrogenic, Antifertility, Rats

1. Introduction

The therapeutic effects of medicinal plants, which are used as a food-relish in folk medicine, are well documented. Ayurvedic medicine is still in the mainstay of about 75–80% of the world's population, mainly in developing countries, for primary health care because of better cultural acceptability, better compatibility with the human body and lesser side effects. It is estimated that approximately one quarter of prescribed drugs contain plant extracts or active ingredients obtained from or modelled on plant substances. Aspirin, atropine, artimesinin, colchicine, digoxin, ephedrine, morphine, physostigmine, pilocarpine, quinine, quinidine, reserpine, taxol, tubocurarine, vincristine and vinblastine are a few important examples of what medicinal plants have given us in the past. Most of these plant-derived drugs were originally discovered through the study of traditional cures and folk knowledge of indigenous people and some of these could not be substituted despite the enormous advancement in synthetic chemistry.

The Citron plant, sister concern of the Lemon plant, scientifically known as *Citrus medica* belongs to Rutaceae family. Runnebaum et al., reviewed the biological evaluation of some medicinal plant extracts

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for contraceptive efficacy in female (1), Piyachaturawat et al., also shown antifertility effect of *Citrus hystrix* on peel (2), Mallikarjuna et al., reviewed the evaluation of some indigenous plant extracts for anti-implantation activity (3) and Kulkarni et al., also shown in their study antifertility effect of lemon seeds (*Citrus limonum*) (4). As earlier studies in our laboratory have reported (5-7) partial inhibition of implantation, pregnancy interruption and estrogenic activity in rats with petroleum ether extract of *C. medica* seeds, a lot of interest has been generated to determine the active principle in this extract of *C. medica* seeds that has antifertility effects.

In this article we are presenting the data of the antifertility characteristics and associated effects of chromatographic fractions of crude petroleum ether extract to elucidate the active principle. For further investigation on this plant extract, isolation of novel constituents were undertaken and carried out through thin layer chromatography and two fractions were found from potent petroleum ether extract of *C. medica* seeds.

For preliminary trials, both the fractions of petroleum ether extract of *C. medica* were studied for antiovulatory activity and resulted in innovation of the constituents present in it (8). Similarly, now our aim is to explore these fractions for *in vivo* study on the activity of anti-implantation, pregnancy interruption in pregnant rats and estrogenic in non-pregnant immature bilaterally ovariectomy (OVX) rats.

2. Materials and methods

2.1. Collection of seed material

The fresh seeds of *Citrus medica* were collected from Hyderabad Karnataka areas of northern region of Karnataka, during fruiting season i.e., in the month of July to October and authenticated at the herbarium, Department of Botany, Gulbarga University, Gulbarga, Karnataka, India. Collected seeds material was immediately sprayed with ethanol to cause the enzymatic degradation of secondary metabolites. The seeds were shade dried, chopped into small fragments and powdered inside the laboratory within 10-15 days at room temperature (28-30°C).

2.2. Preparation of seed material and Soxhlet extraction of constituents

The shade dried, powdered 100gm seed material was soxhleted with petroleum ether (b.p. 60-80°C) in a soxhlet extractor for 48 hours. The extract was concentrated to dryness in a flash evaporator (Buchi) under reduced pressure and controlled temperature (50-60°C) to obtain the crude extract. The petroleum ether extract was chromatographed by initially analytic Thin Layer Chromatography method for solvent standardization using silica gel as stationary phase. Better resolution of the compounds has obtain by using 1:1 chloroform: benzene. The same was further processed on preparative TLC slides to obtained good concentration of the pure separated compounds (9).

TLC is performed on a sheet of glass, plastic or aluminium foil, which is coated with a thin layer of adsorbent material, usually silica gel G. 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 retention power (Rf) was designated as fraction I and the compounds having low Rf value was designated as fraction II. The fraction I of the petroleum ether extract yielded brownish semi liquid material and the fraction II yielded dark brown semi liquid material when the silica gel was washed and filtered with methanol.

2.3. Animals

Colony bred virgin female albino rats of Wistar strain (150 -180g) with normal estrous cycle were selected for the experimentation. The rats were housed in polypropylene cages measuring 12”x10”x8”, under well ventilated animal house conditions (Ambient temperature: 28-31°C, photoperiod: 12h natural light and 12h darkness, relative humidity: 50-55%). The rats were given pelleted feed (Hindustan Unilever Limited, India) and water *ad libitum*. 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 (10).

2.4. Experimental protocol of anti-implantation activity of fraction I and II of petroleum ether extract of *C. medica* seeds

The female rats were caged with male rats of known fertility in the ratio of 2:1 on the evening of proestrus and the vaginal smear was examined following morning for the evidence of copulation. Rats showing thick clumps of spermatozoa in their vaginal smear were separated and that day was designated as day 1 of pregnancy and pregnant rats were divided into 5 groups consisting of 6 rats in each group:

Group I: Control, received 0.2 ml Tween-80 (1%) orally, Group II: Received 50mg fraction I/kg body weight in 0.2 ml Tween-80 (1%) orally, Group III:

Received 100mg fraction I/kg body weight in 0.2ml Tween-80 (1%) orally, Group IV: Received 50mg fraction II/kg body weight in 0.2 ml Tween-80 (1%) orally and Group V: Received 100mg fraction II/kg body weight in 0.2 ml Tween-80 (1%) orally.

All the above treatments were given from day 1 to 7 of pregnancy and on day 10, laparotomy was performed under light ether anaesthesia and semi sterile conditions. The uteri were examined to determine the number of implantation sites. The abdominal wound was sutured and animals were allowed to recover and deliver after full term.

2.5. Experimental protocol of estrogenic activity of fraction II of petroleum ether extract of *C. medica* seeds

The colony bred immature albino female rats of Wistar strain (30-35g) aged 25 days old, were bilaterally ovariectomy (OVX) by dorsolateral approach under light ether anaesthesia in sterile conditions. They were divided into six groups consisting of 6 rats in each as shown below:

Group I: Control, received 0.2ml tween-80 (1%) orally, Group II: Received ethinyl estradiol in olive oil, 1µg/rat/day subcutaneously, Group III: Received 50mg fraction II/kg body weight in 0.2 ml Tween-80 (1%) orally, Group IV: Received 100mg fraction II/kg body weight in 0.2 ml Tween-80 (1%) orally, Group V: Received 50mg fraction II/kg body weight in 0.2ml Tween-80 (1%) orally+1µg ethinyl estradiol /rat/day subcutaneously and Group VI: Received 100mg fraction II/kg body weight in 0.2ml Tween-80 (1%) orally+1µg ethinyl estradiol/rat/day subcutaneously.

All the treatments were given for 7 days, on 8th day the rats were sacrificed by decapitation. The uteri and adrenals were dissected out and separated from the adherent tissues and weighed to the nearest mg in an electronic balance. Estrogenic activity was assessed according to the method of Edgren and Calhourn (11) taking uterine wet weight, opening of the vagina and cornification of vaginal epithelial cells as the points of evaluation. The uteri and adrenals were fixed in Bouin's fluid dehydrated in alcohol and embedded in paraffin wax. The paraffin blocks were sectioned at 5µ stained with Ehrlich's haematoxylin and eosin for histological observations (12).

Using ocular and stage micrometer the micrometric measurements of the uteri and adrenals were made. Vaginal smear was recorded daily from these animals during experimental period.

2.6. Statistical analysis

The data were statistically analysed and expressed as mean ± S.E. Statistical analysis of the variance between control and experimental values were done by Student's-*t* test using SPSS package (13).

3. Results

3.1. Anti-implantation activity of TLC fraction I and II petroleum ether extract of *C. medica* seeds

As shown in table 1, administration of the fraction I at the dose of 50 & 100 mg/kg body weight and fraction II at both the dose level resulted in significantly reduction in the number of implantation sites compared to control group. The percent inhibition of implantation activity in fraction I and II respectively with low and high dose of the extract, among the two fractions at different doses, fraction II at 100mg/kg body weight is highly effective in inhibiting implantation activity.

Fraction I did not show pregnancy interruption activity, mean number of litters ($P<0.01$) and mean weight of litters were not different and normal litters were born when these rats were allowed for parturition. Though small number of implantation were seen in rats treated with low and high dose of fraction II, no one rats were delivered at full term of parturition, indicating the pregnancy interruption activity by partial resorption of embryos.

3.2. Estrogenic activity of TLC fraction II of petroleum ether extract of *C. medica* seeds

The fraction II of petroleum ether extract was found to be more active than fraction I according to early opening in vagina, significant changes in gravimetry and hisometry studies. Therefore, fraction II was subjected to in vivo study for testing the estrogenic activity in OVX immature rats.

3.2.1. Vaginal opening and cellular cornification.

Control immature rats did not show vaginal opening after 7 days of vehicle treatment. But the rats treated with ethinyl estradiol showed vaginal opening and cornified vaginal epithelial cells. Four out of six rats treated with low dose level of fraction II showed vaginal opening on 3rd day of administration and showed cornified epithelial cells. All the rats treated with high dose of fraction II only or along with ethinyl estradiol showed vaginal opening and cornification of vaginal epithelial cells Table 2.

3.2.2. Changes in the uterus

3.2.2.1. Gravimetric changes

Administration of both the dose level of fraction II alone or along with ethinyl estradiol caused highly significant ($P<0.001$) increase in the uterine wet weight of immature OVX rats when compared to

control group. These results indicate that the fraction II exhibit estrogenic property when treated alone or along with ethinyl estradiol. It does not possess antagonistic property with other estrogen (Table 2).

Table 1. Anti-implantation activity of TLC fractions of petroleum ether extract of *Citrus medica* seeds in pregnant rats

Group	Treatment	Dose (mg/kg body wt.)	No. of rats without implantation sites on day 10	Mean no. of implants ± S.E.	% inhibition of implantation	% rats delivered on full term	Mean no. of litters born ± S.E.	Mean weight of litters	Mean no. of litters died within one week of parturition
I	Control	Tween-80 (1%)	0	11.75±0.25	0	100	11.75±0.25	5.97±0.06	0
II	Fraction I	50	0	8.66±0.61**	26.29	100	8.66±0.61**	5.63±0.21	0
III	Fraction I	100	1	8.83±1.16**	24.85	100	8.83±1.16**	5.70±0.07	0
IV	Fraction II	50	0	3.33±0.55***	71.65	000	----------	----------	----------
V	Fraction II	100	1	2.33±0.71***	80.17	000	---------	---------	---------

M±S.E. = Mean ± Standard error
Duration: 07 days, received the treatment from day1 to 7 and laparotomized on day 10 of pregnancy
Six animals were maintained in each group
* P <0.05; **P<0.01; ***P<0.001 when compared with control

Table 2. Effect of TLC fraction II of petroleum ether extract of *C. medica* seeds on uterine, adrenal wet weight, vaginal opening and cornification of vaginal epithelial cells in OVX immature rats
Data showed as Mean±SD
Duration: 07 days, six animals were maintained in each group
*P <0.05; ** P <0.01; *** P <0.001 when compared with control

Group	Treatment	Dose (mg/kg body wt.)	Uterine wet weight	Adrenal wet weight	Vaginal opening and cornification
I	Control	Tween-80 (1%)	260.55±8.22	26.66±5.96	-/-
II	Ethinyl estradiol	1µg/rat/day	329.44±3.79***	41.66±2.39***	6/6
III	Fraction II	50mg	317.77±4.60***	49.44±1.02***	4/6
IV	Fraction II	100mg	331.66±4.21***	63.32±4.47***	6/6
V	Ethinyl estradiol + Fraction II	1µg/rat/day + 50mg	321.66±4.19***	50.55±1.59***	6/6
VI	Ethinyl estradiol + Fraction II	1µg/rat/day + 100mg	324.99±2.68***	74.44±4.09***	6/6

3.2.2.2. Histometric changes

The histometric changes like diameter of the uterus (P<0.001), thickness of the myometrium and endometrium (P<0.001) and epithelial cell height (P<0.001) increased significantly in both the doses of fraction II treated groups alone when compared to control rats. Ethinyl estradiol alone treated groups caused highly significant increase in (P<0.001) all the histometric changes. Administration of ethinyl estradiol and fraction II at both the dose levels also caused a highly significant (P<0.001) increase in uterine diameter 1113.82 ± 10.56 & 1342.25 ± 13.17, thickness of myometrium and endometrium and epithelial cell height showing the synergistic action of both compounds. These changes exhibit the estrogenic potency of the fraction II of petroleum ether extract of *C. medica* seeds (Table 3; Figure 1-4).

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Table 3. Histometric changes in the uterus due to the administration of TLC fraction II of petroleum ether extract of *C. medica* seeds in OVX immature rats

Group	Treatment	Dose (mg/kg body wt.)	Diameter of uterus (µm)	Thickness of myometrium (µm)	Thickness of endometrium (µm)	Epithelial cell height (µm)
I	Control	Tween-80 (1%)	423.64 ± 7.42	56.48 ± 1.59	242.70 ± 2.90	22.56 ± 1.01
II	Ethinyl estradiol	1µg/rat/day	942.04 ± 10.09***	114.45 ± 2.18***	641.92 ± 4.63***	38.35 ± 0.94***
III	Fraction II	50mg	645.01± 6.41***	93.67 ± 1.00***	420.09 ± 6.93***	30.55 ± 1.45***
IV	Fraction II	100mg	897.58± 5.33***	107.17 ± 2.63***	459.27 ± 7.03***	32.31 ± 0.81***
V	Ethinyl estradiol + Fraction II	1µg/rat/day + 50mg	1113.82± 10.56***	129.08 ± 1.65***	653.06 ± 2.16***	46.53 ± 1.36***
VI	Ethinyl estradiol + Fraction II	1µg/rat/day + 100mg	1342.25± 13.17***	197.55± 2.00***	815.63 ± 4.89***	59.54 ± 1.65***

M±S.E. = Mean ± Standard error
Duration: 07 days, six animals were maintained in each group
* P <0.05; ** P <0.01; *** P <0.001 when compared with control

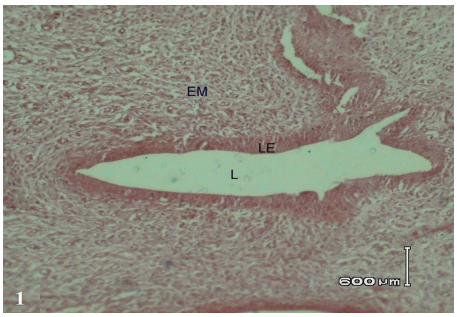

Figure 1. Photomicrograph of the cross section of the uterus of OVX immature rats treated with vehicle showing normal endometrium and luminal epithelium (X100). L: Lumen; LE: Luminal Epithelium; EM: Endometrium.

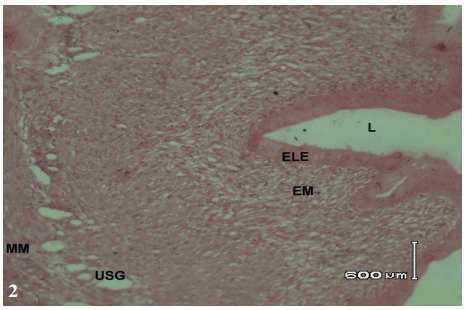

Figure 2. Photomicrograph of the cross section of the uterus of OVX immature rats treated with ethinyl estradiol showing hypertrophied endometrium with conspicuous uterine gland and secretory luminal epithelium cells (X100). L: Lumen; EM: Endometrium; USG: Uterine Secretory Gland; MM: Myometrium; ELE: Enlarged Luminal Epithelium.

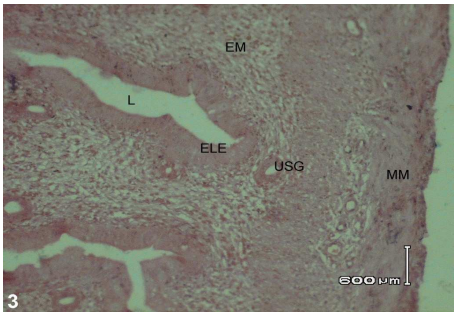

Figure 3. Photomicrograph of the cross section of the uterus of OVX immature rats treated with fraction II showing hypertrophy in endometrium, endometrial gland and luminal epithelium (X100). L: Lumen; EM: Endometrium; USG: Uterine Secretory Gland; MM: Myometrium; ELE: Enlarged Luminal Epithelium.

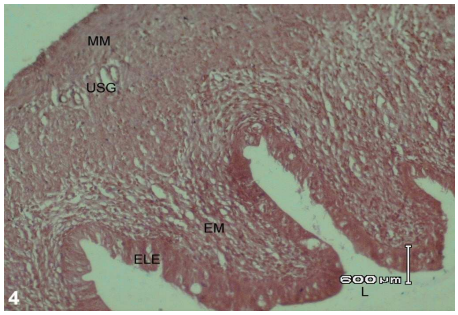

Figure 4. Photomicrograph of the cross section of the uterus of OVX immature rats treated with ethinyl estradiol + fraction II of petroleum ether extract showing enlargement of endometrium, endometrial gland and luminal epithelium. Note the enlargement of endometrial villi (X400). L: Lumen; EM: Endometrium; USG: Uterine Secretory Gland; MM: Myometrium; ELE: Enlarged Luminal Epithelium.

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3.2.3. Changes in the adrenal gland

3.2.3.1. Gravimetric changes

Administration of both the doses of fraction II alone caused a highly significant (P<0.001) increase in the adrenal wet weight in immature OVX rats when compared to control. Ethinyl estradiol treatment or combined administration of ethinyl estradiol and fraction II at both the dose levels caused a highly significant (P<0.001) increase in the adrenal wet weight. The degree of ACTH (Adrenocorticotropic hormone) response was greater with the administration of combined ethinyl estradiol and fraction II than that produced by ethinyl estradiol or fraction II of petroleum ether extract alone (Table 4).

3.2.3.2. Histometric changes

The histometric changes like diameter of cortex and medulla was significantly increased (P<0.01 and P<0.001) with both the dose of fraction II treatment compare to control. Administration of ethinyl estradiol alone or in combination with fraction II also caused highly significant increase in the diameter of cortex and medulla (P<0.001). These results indicate that fraction II at both the dose level may stimulate the hypothalamic releasing factors responsible for ACTH (Table 4; Figures 5-8).

Table 4. Histometric changes in the adrenal due to the administration of TLC fraction II of petroleum ether extract of *C. medica* seeds in OVX immature rats

Group	Treatment	Dose (mg/kg body wt.)	Diameter of cortex (µm)	Diameter of medulla (µm)
I	Control	Tween-80 (1%)	877. 3±22.2	557.6±16.2
II	Ethinyl estradiol	1µg/rat/day	995.4±20.2***	612.6±16.4***
III	Fraction II	50mg	953.3±19.2**	587.9±24.2***
IV	Fraction II	100mg	967.4±17.4***	597.1±17.4***
V	Ethinyl estradiol + Fraction II	1µg /rat/day + 50mg	982.2±16.2***	632.1±36.0***
VI	Ethinyl estradiol + Fraction II	1µg /rat/day + 100mg	999.0±12.2***	647.7±16.6***

M±S.E. = Mean ± Standard error
Duration: 07 days, six animals were maintained in each group
*P<0.05; **P<0.01: ***P<0.001 when compared with control

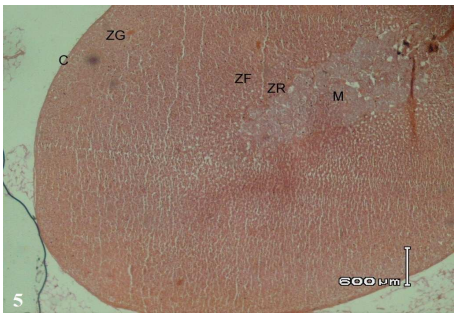

Figure 5. Photomicrograph of the cross section of the uterus of OVX immature rats treated with vehicle showing normal cortex and medulla (X100). C: Cortex/Capsule; M: Medulla; ZR: Zona Reticularis; ZF: Zona Fasciculata; ZG: Zona Glomerulosa.

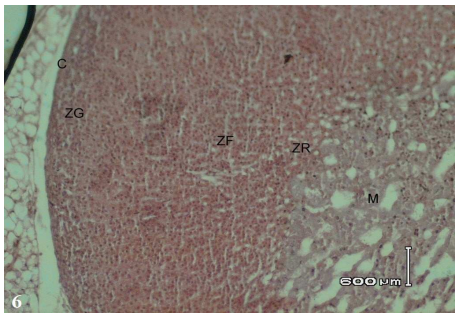

Figure 6. Photomicrograph of the cross section of the adrenal gland of OVX immature rats Treated with ethinyl estradiol showing hypertrophied medulla and cortex (X100). C: Cortex/Capsule; M: Medulla; ZR: Zona Reticularis; ZF: Zona Fasciculata; ZG: Zona Glomerulosa.

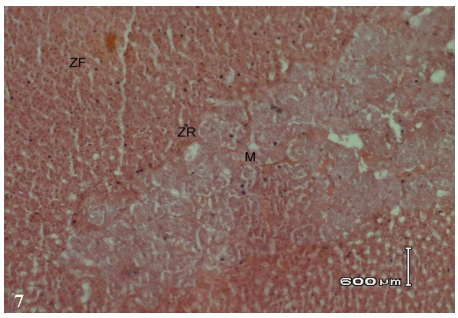

Figure 7. Photomicrograph of the cross section of the adrenal gland of OVX immature rats treated with fraction II showing more advanced growth in medulla and cortex (X100). M: Medulla; ZR: Zona Reticularis; ZF: Zona Fasciculata.

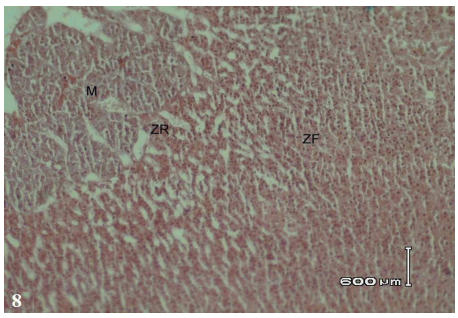

Figure 8. Photomicrograph of the cross section of the adrenal gland of OVX immature rats treated with ethinyl estradiol + fraction II of petroleum ether extract showing hypertrophied and highly secretory medulla and cortex (X100). M: Medulla; ZR: Zona Reticularis; ZF: Zona Fasciculata.

4. Discussion

4.1. Anti-implantation activity of TLC fraction I and II petroleum ether extract of *Citrus medica* seeds

In the present investigation the TLC fraction of petroleum ether extract of the *C. medica* seeds were tested for their anti-implantation and pregnancy interruption activities. Between the two fractions were tested, the fraction II at the dose level of 100mg/kg body weight was found to be the most effective in reducing the implantation sites and interrupting the pregnancy. The loss of implantation caused by the administration of fractions of petroleum ether extract may be due to antizygotic, blastocytotoxic or anti-implantation activity as described by Hafez (14).

In the present investigation, inhibition of implantation sites on 10^{th} day of pregnancy in the rats received TLC fractions of petroleum ether extract act as anti-implantation agent due to presence of active constituents and may altered in hormone secretion during pregnancy maintenance. Further action of fractions of petroleum ether extract might have imbalanced the biosynthesis of hormones such as progesterone, estrogen and glucocorticoides by blocking the enzymes necessary for implantation.

Our preliminary phytochemical studies showed the presence of alkaloids, glycosides, flavonoids and fixed oils in petroleum ether extract of *C.medica seeds*. Several reports revealed that alkaloids, flavonoids and coumarins have antifertility potency (15) and also flavonoids isolated from *Striga lutea* and *Striga orobanchioides* possessed strong estrogenic and antifertility properties (16).

In several species (fishes, amphibians, reptilians and mammalian) including non-human, progesterone is essential for blastocyst implantation (17, 18) and for the maintenance of pregnancy in all phases (19-21). Inhibition of progesterone synthesis or a blockade or receptor building will result in the failure of blastocyst implantation and interruption of early pregnancy (20, 18), which might have resulted due to administration of TLC fractions of petroleum ether extract of *C. medica* seeds.

It is also well established that estrogen secretion during implantation is much lower compared to progesterone, as the former is in the range of nanogram and latter is in microgram (22, 23). In the present study, as the fractions of petroleum ether extract of *C. medica* seeds has proved to possess estrogenic activity, the imbalance caused in progesterone and estrogen levels might be the reason for anti-implantation activity. Withdrawal of these treatments from adult rats has resulted in normal reproductive activities. Hence, fraction II obtained from active petroleum ether extract, after subjecting to TLC has shown remarkable potency of anti-implantation activity.

4.2. Estrogenic activity of TLC fraction II of petroleum ether extract of *C. medica* seeds

The premature vaginal opening, cornification of vagina and vaginal epithelial cells in the rats treated with TLC fraction II of petroleum ether extract of *C. medica* seeds indicates its estrogenic activity. Reproductive and general metabolic effects in mature and immature rats are manipulated with the ingestion of phytoestrogenic compounds that produce effects similar to that of gonadal steroid 17β-estradiol (24). Phytoestrogen stimulates the growth and development of the uterus and other sexual organs.

The observed changes like increase in the weight

of the uteri and adrenals, histological changes and micrometric measurements of the petroleum ether extract fraction II treated rats clearly indicates estrogenic nature of the extract (25). In several species, including man, the adrenals of the female are heavier than those of the male and this sexual dimorphism reflects the endocrine balance and adrenocortical activity of the animals (26). Oral administered rats with the fraction II of *C. medica* seeds for 7 days have shown significant increase in adrenal weight and its enlargement. The pituitary releases increasing amounts of ACTH when the blood levels of glucocorticoides are low and diminishes the output as the plasma glucocorticoides are elevated (27). Under stress, the hypothalamus secrets corticotropic-releasing hormone which stimulates the anterior pituitary to release corticotropin in to the blood (28). Hypertrophy of adrenal cortex and medulla observed in the present experimental indirectly indicates the increased corticotrophin level. Many steroidal and non-steroidal plant constituents have estrogenic activity.

The most potent naturally occurring estrogen is 17β-estradiol followed by estrone. One of the first non-steroidal estrogens is diethylstilbestrol, which is structurally similar to estradiol. The search of potent and orally active natural products with estrogenic activity is a milestone in the development of effective endocrine therapy. In addition, it has also been proved that several commonly occurring flavonoids mimic the biological effects of 17β-estradiol by virtue of their ability to bind and activate the nuclear estrogen receptors (29). Exploration of natural products reveals that there are number of plants which possess the potentiality of preventing implantation or may cause fetal resorption. Although their exact mechanism for antifertility action is not fully understood, these agents elicit effect through variety of actions. Extracts of number of plants are known to possess estrogenecity (30), which increase in contractility of the smooth muscles of uterus along with increase in vascular permeability. These changes may force to expel the fertilized eggs without making any contact with the uterus (31).

In other category there are plants extracts antagonize the estrogenic action of the estradiol dipropionate and thus deplete the estrogenic response (32). By this finding the fertilized eggs do not get stable equilibrium of estrogen and fail to invade luminal epithelium. The non-steroidal compounds with estrogenic activity such as flavones, isoflavones, alkaloids, phenolics and fatty acids occur naturally in a wide variety of explored plants. There are few reported plants extracts elicit their action through the antiprogestional activity (33, 34) but they also possess other additional activity like estrogenic and hence their antifertility mode of action remains uncertain.
Due to difficulty in isolation of compound from oil or semi liquid material, the further process of column chromatography is avoided. The TLC fraction II was processed for further analysis of spectral study.

5. Conclusion

The antifertility property of the *Citrus medica* may be due to an estrogenic property and which may be due to the presence of alkaloids, glycosides, flavonoids and fixed oils in the plants. With these findings, we can conclude that the TLC fraction II of petroleum ether extracts showed significant *in vivo* antifertility activities by means of potent anti-implantation, pregnancy interruption and estrogenic activity in a dose-dependent manner.

References

1. Runnebaum B, Rabe T, Kiesel L, et al. Biological evaluation of some medicinal plant extracts for contraceptive efficacy in female, Future aspects in contraception, Boston USA, MTP Press Ltd, 1984; 115-128.
2. Piyachaturawat P, Glinsukon T, Chanjarnee A. Antifertility effect of *Citrus hystrix* D.C. J Ethnopharm. 1985; 13(1): 105-110.
3. Mallikarjun AP, Sharanabasappa A, Vijaykumar BM, et al. Evaluation of some indigenous plant extracts for antiimplantation activity in albino rats. Ori Pharm Exp Med. 2005; 5(4): 347-351.
4. Kulkarni TR, Kothekar MA, Mateenuddin M. Study of antifertility effect of lemon seeds (*Citrus limonum*) in female albino mice. J Phys Pharm. 2005; 49(3): 305-312.
5. Sharangouda, Patil SB. Phytochemical screening and antifertility activity of various extracts of *Citrus medica* (Lemon) seeds in albino rats. Adv Pharm Tox. 2007; 2: 71-74.
6. Sharangouda, Patil SB. Post-coital antifertility activity of Citrus medica seeds. The Bioscan. 2007; 2(4): 305-310.
7. Sharangouda, Patil SB. Estrogenic activity of petroleum ether extract of seed of Citrus medica on immature albino rats. Int J Green Pharm. 2008; 2(2): 91-94.
8. Patil SJ, Patil SB. Antiovulatory activity of petroleum ether extract of chromatographic fractions of Citrus medica seeds in albino rats. J Med Sci. 2013; 13(6): 410-417.
9. Sadasivam S, Manickam, Biochemical methods, 2nd ed, New Delhi, New Age International Publishers, 1991; 222-228.

10. National Institutes of Health, Guide for the Care and Use of Laboratory Animals. Bethesda, USA, NIH Publication no. 85-23, 1985.
11. Edgren RA, Calhoun DW. The biology of steroidal contraception, In: Edgren RA, ed. Contraception, New York, The Chemical Control of Fertility, 1957.
12. Gurr E. "Staining animal tissues, Practical and theoretical, Leonard Hill Limited, London, 1962; 233.
13. SPSS. "SPSS for Windows," Rel. 11.0.1. 2001, Chicago SPSS Inc. New York.
14. Hafez ESE. In "Reproduction and Breeding Techniques for Laboratory Animals. Lea and Febiger, Philadelphia, 1970.
15. Sudhir S, Singh MM, Kulsheshtha DK. A new alkaloid and other antiimplantation principles from *Tabernacemontana heyneana*. Planta Medica. 2001; 67(6): 577-579.
16. Hiremath SP, Hanumantha Rao S. Antifertility efficacy of the plant *Striga lutea* (Scrophulariaceae). Contraception. 1990; 42(4): 467-477.
17. Murray RK, Granner DK, Mayes PA, et al. Harpers Biochemistry, In: Hormones of the Gonads, 21st ed, Englewood Cliffs NJ, Practice Hall International Inc, 1988.
18. Ghosh D, Nayak NR, Lalitkumar PGL, et al. Hormonal requirement for blastocyst implantation and a new approach for antiimplantation strategy. Ind J Phys Pharm. 1997; 41(2): 101-108.
19. Caspo AI. The sea saw theory of the regulatory mechanism of pregnancy. Am J Obst Gyn. 1975; 123(4): 578-582.
20. Caspo AI, Erdos T. The critical control of progesterone levels of pregnancy by antiprogesterone. Am J Obst Gyn. 1976; 126(5): 598-608.
21. Caspo AI, Pulkkinen M. Indispensability of the human corpus luteum in the maintenance of early pregnancy luteoectomy evidence. Obst Gyn Survey. 1978; 33(2): 69-72.
22. Eto TH, Masuda Y, Suzuki et al. Progesterone and pregn-4-one-20 –ol-3-one in rat ovarian blood at different stages in reproductive cycle. The Jap J Ani Rep. 1962; 8: 34-40.
23. Hashimoto, Henricks DM, Anderson LL, et al. Progesterone and pregn-4-en-20-10 in ovarian venous blood during various reproductive states in rat. Endocrinol. 1968; 82(2): 333-341.
24. Fowier MD. Plant poisoning in free-living wild animals: A review. J Wild Life Dis. 1983; 19(1): 34-39.
25. Hardman JG, Limbard JE, Gilman AG. The pharmacological basis of therapeutics, 10th Ed, McGraw-Hill, Book Company, 2002.
26. Greep RO, Astwood EB. Handbook of Physiology, Section 7, Endocrinology, vol. II, part 2, Washintgon, American Physiological Society, 1973.
27. Turner CD, Bagnara JT. General Endocrinology, 6th Ed, Philadelphia, W. B. Saunders Company, 1976.
28. Lehninger AL, Nelson DL, Cox MM. Principles of Biochemistry, 2nd Ed, New Delhi, CBS Publishers and Distributers, 1993.
29. Jain S, Chodhury GP, Jain D. Pharmacological evaluation and antifertility activity of *Jatropha gossypifolia* in rats. BioMed Res Int. 2013; 5.
30. Pathak S, Prakash AO. Post-coital contraceptive effect of *Ferula jaeschkeana* Vatke. and its hormonal properties. Phyt Res. 1989; 3(2): 61-66.
31. Bennet JP, Kendle KE, Vallance DK, et al. A comparison of the antifertility and hormonal activities of some new synthetic oestrogens. Acta Endocrinol (Copenh). 1966; 53(3): 453-455.
32. Kholkute SD, Munshi SR. Antifertility activity of indigenous plants *Sida carpinifolia* and *Podocarpus brevifolius* Stapf in female rats. Ind J Exp Biol. 1978; 16(6): 696-698.
33. Shukla S, Mathur R, Prakash AO. Antifertility profile of the aqueous extract of *Moringa oleifera* roots. J Ethnopharmacol. 1988; 22(1): 51-62.
34. Prakash AO, Saxena V, Shukla S, et al. Contraceptive potency of *Pueraria tuberosa* D.C. and its hormonal status. Acta Eur Fertil. 1985; 16(1): 59-65.