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Antiovulatory Activity of Petroleum Ether Extract of Chromatographic Fractions of *Citrus medica* Seeds in Albino Rats

Sharangouda J. Patil and Saraswati B. Patil

In India the control of fertility is based on the folk use of numerous traditional antifertility plants that has been practiced for many years. The petroleum ether extract of *Citrus medica* seeds which showed promising antiovulatory activity in female albino rats was examined for the isolation of its active fractions. Two fractions were obtained using Thin Layer Chromatography (TLC) of the extract. Both fractions were subjected for testing their anti-ovulatory activity and estrous cycle in rats. After preliminary trials, the fraction II showed maximum antiovulatory activity when administered orally to the rats for 30 days. At autopsy on day 31st, chromatographic fractions treated rats showed increased ovarian weight and histological changes of the ovary indicate increases in the number of atretic follicles but decreases in the number of healthy developing follicles, *Graafian* follicles and corpora lutea. The total cholesterol, activity of acid and alkaline phosphatase content of the ovary were increased, whereas, protein, glycogen and alkaline protease content were decreased. The estrous cycle of these rats was irregular with prolonged proestrus and estrous, reduced metestrus and diestrus phase during the experimental period. These results suggest that a chromatographic fraction II of petroleum ether extract of *Citrus medica* might be used as a contraceptive in the females.

Key words: *Citrus medica*, antiovulatory activity, estrous cycle, antifertility

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For further information about this article or if you need reprints, please contact:

Sharangouda J. Patil
NAIP Livelihood Project,
National Institute of Animal Nutrition and Physiology (NIANP and ICAR), Adugodi,
Bangalore-560 030, India

Tel: +91-9742069766

ANSInet
Asian Network for Scientific Information

Laboratory of Reproductive Biology, Department of Zoology, Gulbarga University Gulbarga-585106, Karnataka, India

INTRODUCTION

The national family planning program which was established in 1952, has played an important role in India's fertility decline. When the program began, there was little awareness or use of modern birth control methods. Four decades later, in 1992-93 National Family Health Survey (NFHS) (IIPS, 1995) found nearly universal knowledge of family planning, with 96% of married women aged between 13-49 years having heard of at least one modern method and almost 41%, or almost 70 million women, using contraception.

Hence, the fertility control is the most important and urgent mainstay of all biomedical and biosocial problems. The need for evolving more acceptable and effective means of contraception with nil or minimal side effects is more actually felt now, than ever before, in view of the frightening rate at which population is growing. Different contraceptive methods in male and female are already in practice, but the important factor is that the available methods and services of family planning should be in expensive and sophisticated to meet the demand of developing countries as evidenced by the number of unwanted births and high rates of population growth (Fathalla, 1983). In this direction, modernization and updating the research in human reproduction and fertility regulation in order to improve the safety and efficacy of Family Planning Methods both in male and female is very essential.

Due to severe and long lasting side effects of modern medicines including contraceptive, the people are now looking for using herbal medicines for curing various diseases and also for fertility control. Fortunately India has rich heritage of use of medicinal plants for fertility control. 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 (Chopra *et al*., 1958; Nadkarni and Nadkarni, 1976; Satyavati *et al*., 1987). 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 approach is being pursued to identify antifertility agent from the seeds of *Citrus medica*. In our previous findings on the petroleum ether extract of *C. medica* seeds demonstrated their anti-implantation, estrogenic, antiovulatory, abortifacient activities (Sharangouda and Patil, 2007, 2009, 2008, 2010) and toxicity studies (Patil and Patil, 2010, 2011) in rats and mice. In the present study, we have undertaken the investigation of the antiovulatory activity of chromatographic fractions of crude petroleum ether extract of *C. medica* seeds to elucidate its active ingredient.

MATERIALS AND METHODS

Collection of seeds: 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).

Preparation of test materials and soxhlet extraction of seed constituents: The shade dried, powdered 100 g seed material was soxhleted with petroleum ether (b.p. 60-80°C) in a soxhlet extractor for 48 h. The extracts 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 obtained by using 1:1 chloroform : benzene. The same was further processed on preparative TLC slides to obtained good concentration of the pure separated compounds (Sadasivam and Manickam, 1991). Over 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 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.

Animals: Colony bred adult, virgin female albino rats of Wistar strain weighing 150-180 g body weight with normal estrous cycle were used for the experimentation. The rats were housed in polypropylene cages measuring 12"×10"×8", under well ventilated animal house conditions (Ambient temperature: 28-31°C, photoperiod: 12 h natural light and 12 h 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 (NIH, 1985).

Experimental protocol: The fractions were prepared in Tween-80 (1%) in distilled water for complete dissolution and were administered orally for 30 days to the experimental rats by using intragastric catheter at different doses. The control animals received an equivalent amount of vehicle only. The animals were divided into 5 groups consisting of 6 animals in each and treated as follows:

Group I: Control, received 0.2 mL Tween-80 (1%) orally
Group II: Received 50 mg fraction I/kg b.wt. in 0.2 mL Tween-80 (1%) orally
Group III: Received 100 mg fraction I/kg b.wt. in 0.2 mL Tween-80 (1%) orally
Group IV: Received 50 mg fraction II/kg b.wt. in 0.2 mL Tween-80 (1%) orally
Group V: Received 100 mg fraction II/kg b.wt. in 0.2 mL Tween-80 (1%) orally

All the treatments were given for 30 days to cover six regular cycles. Vaginal smear was observed throughout the experimental period. All the animals were sacrificed 24 h after the last treatment. The ovaries were dissected out immediately and separated from the adherent tissue and weighed to the nearest mg on an electronic balance. Organs from one side of each animal were fixed in Bouin's fluid, embedded in paraffin wax, sectioned at 5 μ and stained with Ehrlich's haematoxylin and eosin for histological studies. Number of developing follicles, *Graafian* follicles, corpora lutea and atretic follicles were counted from stained serial section of the ovary from each rat. Organs from the other side were used for biochemical estimations like protein (Lowary *et al*., 1951), glycogen (Carroll *et al*., 1956), cholesterol (Peters and Vanslyke, 1946), acid phosphatase and alkaline phosphatase (Bessey *et al*., 1946) and alkaline protease (Davis and Smith, 1955).

Statistical analysis: The data were statistically analyzed and expressed as Mean±SE. Statistical analysis of the variance between control and experimental values was done using Students't-test using SPSS package (SPSS, 2001).

RESULTS

Antiovulatory activity of fraction I and II of petroleum ether extract of *C. medica* seeds

Changes in body weight: Oral administration of fraction I and II of petroleum ether extract to rats has shown non significant change in the body weight. The treated rats were healthy and maintained normal growth rate throughout the experiment (Table 1). Hence, the body weight of the treated rats showed slight fluctuation and produce no significant change when compared to control rats. The percent growth in experimental animals ranges from 12.30-18.61 whereas, it is 22.70 in control rats.

Changes in the estrous cycle Administration of fraction I and II has increased the duration of proestrus and estrus phases and decreased the metestrus and diestrus phases. The increase in the duration of proestrus and diestrus is significant ($p<0.01$) with high dose of fraction I and highly significant ($p<0.001$) with both the doses of fraction II. But, the decrease in the duration of metestrus and diestrus is highly significant ($p<0.001$) with both the doses of fraction I and II (Table 2).

Table 1: Changes in the body weight due to administration of TLC fraction I and II of petroleum ether extract of *C. medica* seeds for different periods in rats

Treatment	Dose (mg kg^{-1} b.wt.)	Initial weight (g)	Weight after 1 week (g)	Weight after 2 weeks (g)	Weight after 3 weeks (g)	Weight after 4 weeks (g)	Final body weight (g)	Percent change (%)
Control	Tween-80 (1%)	141.66±2.10	145.16±2.44	147.83±2.08	151.50±2.04	155.00±1.82	178.83±1.89	22.70
Fraction-I	50	140.83±1.53	148.33±105	154.16±1.53	161.66±1.05	167.50±1.70	170.00±1.82	18.61
Fraction-I	100	151.6±1.05	154.16±0.83	160.00±0.00	163.33±1.05	168.33±1.05	172.50±1.11	12.95
Fraction-II	50	153.33±1.05	162.50±1.11	166.66±1.66	170.83±1.53	174.16±0.83	175.00±0.00	12.97
Fraction-II	100	141.66±2.47	144.16±1.53	148.33±1.66	150.83±1.53	155.83±1.53	160.00±1.82	12.30

M±SE: Mean±Standard error, Duration: 30 days, Six animals were maintained in each group

Table 2: Effect of TLC fraction I and II of petroleum ether extract *C. medica* seeds on various stages of estrous cycle in rats

		Duration of stages of the estrous cycle in days			
Treatment	Dose (mg kg^{-1} b.wt.)	Proestrus	Estrus	Metestrus	Diestrus
Control	Tween-80 (1%)	4.50±0.22	4.33±0.21	4.00±0.00	17.16±0.40
Fraction-I	50	4.83±0.47	5.83±0.47	8.83±0.60***	10.50±0.99***
Fraction-I	100	5.33±0.61**	6.16±0.74**	6.66±0.71***	10.83±1.93***
Fraction-II	50	13.33±0.80***	7.50±1.02***	3.83±0.47**	8.33±1.45***
Fraction-II	100	13.66±1.70***	8.16±0.60**	3.50±0.42**	7.66±0.49***

M±SE: Mean±Standard error, Duration: 30 days, Six animals were maintained in each group, *p<0.05, **p<0.01, ***p<0.001 when compared with control

Table 3: Gravimetric and biochemical changes of the ovary due to the administration of TLC fraction I and II of petroleum ether extract of *C. medica* seeds

Treatment	Dose (mg kg^{-1} b.wt.)	Weight (mg/100 g b.wt.)	Protein (µg/100 mg)	Glycogen (µg mg^{-1})	Cholesterol (µg mg^{-1})	Acid phosphatase (µ moles of P-nitro phenol released/100 mg/30 min)	Alkaline phosphatase (µ moles of p-nitro phenol released/100 mg/30min.)	Alkaline protease (µ moles of tyrosine liberated/g/mg protein)
Control	Tween-80 (1%)	55.28±3.17	15.93±0.10	2.12±0.04	3.26±0.01	17.66±1.04	11.00±0.23	0.16±0.02
Fraction-I	50	58.32±1.86	16.17±0.41	1.99±1.14	3.84±0.03	17.0±0.73	11.5±0.40	0.16±0.01
Fraction-I	100	59.61±1.98	17.85±0.39	2.02±2.24	3.62±0.03	19.0±0.26	12.2±0.32	0.16±0.02
Fraction-II	50	52.29±01.13	13.27±0.17**	1.82±0.03***	6.62±0.03***	22.6±0.81***	18.1±0.93***	0.12±0.01***
Fraction-II	100	50.95±00.48**	9.61±0.76***	1.80±0.06***	7.30±0.05***	25.42±1.06***	19.2±0.11***	0.12±0.00***

M±SE: Mean±Standard error, Duration: 30 days, Six animals were maintained in each group, *p<0.05, **p<0.01, ***p<0.001 when compared with control

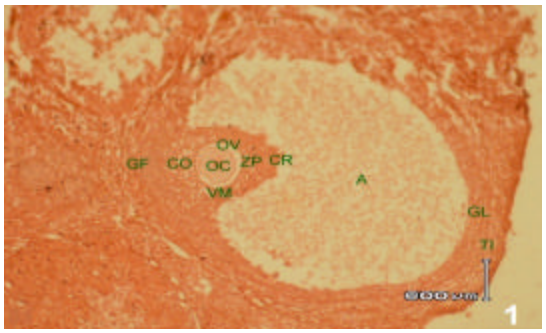

Fig. 1: Cross section of the ovary treated with vehicle showing normal fully developed *Graafian* follicle with healthy oocyte (x400)

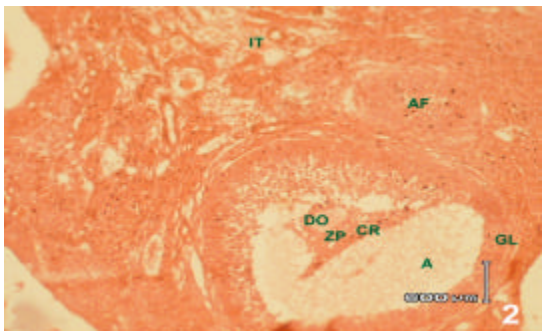

Fig. 2: Cross section of the ovary treated with fraction I petroleum ether extract of *C. medica* seeds showing degeneration in ovarian components and atresia in antral follicle (x400)

Changes in the ovary

Gravimetric changes: The administration of fraction I at both the dose level has increased the ovarian weight non-significantly when compared with that of control. But the administration of fraction II at both the dose level has decreased the ovarian weight significantly ($p<0.01$) only with high dose of fraction II (Table 3).

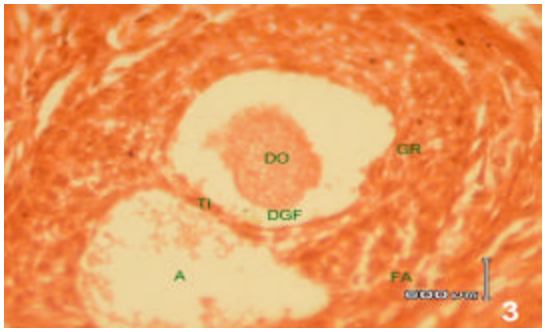

Fig. 3: Cross section of the ovary treated with fraction II petroleum ether extract of *Citrus medica* seeds showing more advanced degenerative changes in ovarian components (x400). A: Antrum; O: Oocyte; GF: Graafian Follicle, CR: Corona Radiata, GL: Granulosa Layer, GC: Granulosa Cells, ZP: Zona Pellucida, VM: Vitelline Membrane, CO: Cumulus Oophorus, IT: Interstitial Tissue, TI: Theca Interna, AF: Atretic Follicle, DGF: Degenerative Graafian Follicle, DO: Degenerative Oocyte, FA: Follicular Atresia

Histological changes: The administration of both the doses of fraction I of petroleum ether extract has increased the *Graafian* follicles and developing follicles. But, the number of developing follicles, *Graafian* follicles, corpora lutea are decreased and that of atretic follicles are increased with both the dose of fraction II administration (Fig. 1-3).

Biochemical changes

Protein: Non-significant increase in the protein content is observed with both the doses of fraction I. But, significant reduction is seen with low ($p<0.01$) and high dose ($p<0.001$) of fraction II (Table 3).

Glycogen: Administration of both the doses of fraction II has caused significant ($p<0.001$) reduction

in the glycogen, whereas, fraction I treatment at both the dose level has shown non-significant reduction.

Cholesterol: The cholesterol content is increased significantly ($p<0.001$) with both the doses of fraction II, but it is non-significant with the treatment of both the doses of fraction I.

Acid phosphatase: The administration of both the doses of fraction I has caused non-significant increase and that of fraction II has caused significant ($p<0.001$) increase in the acid phosphatase activity.

Alkaline phosphatase: The alkaline phosphatase activity is increased non-significantly with both the dose of fraction I and significantly ($p<0.001$) with both doses of fraction II when compared to control.

Alkaline protease: The alkaline protease activity is more or less same with both the doses of fraction I, but fraction II has reduced the activity significantly ($p<0.001$) with both the doses when compared to control.

DISCUSSION

Antiovulatory activity of fraction I and II of petroleum ether extract of *C. medica* seeds: The estrous cycle of mammals reflects the changes in the ovary and their components. The cyclic changes that occur in these organs are under the synergistic influence of many hormones and factors. Any abnormality or dysfunction of the organs or their synchronization directly affects the reproductive phenomenon in the particular individual.

Ovarian changes: The time sequence of various events occurring during estrous cycle under controlled conditions has been studied with events occurring during entire reproductive cycle, particularly the "critical period" for the release of pituitary Luteinizing Hormone (LH) on the afternoon of proestrus (Kobayashi *et al*., 1969). This LH is responsible for the initiation and differentiation of follicular elements towards ovulation. During the process of differentiation, unfavorable conditions bring dedifferentiation of follicles, blockade of LH release and inhibition of ovulation in the ovaries. Estrogen secretion declines a few hours prior to the ovulatory surge of gonadotrophins (Hori *et al*., 1970). In the present investigation, as our plant extract is confirmed to be an estrogenic, the administration of the extract or its fractions having high estrogenic effect in experimental rats which might be one of the reasons for the blockade of ovulation.

Gravimetric changes: The ovary may be considered to be an aggregate of mainly three endocrine tissues: the stroma, follicle and the corpora lutea. The healthy functioning of these tissues constitutes the net weight of the ovary. Weight of the ovarian tissue increased under the influence of gonadotrophic and steroidal hormones. (Peters and McNatty, 1980). The decrease in the weight of ovaries in chromatographic fraction II of petroleum ether extract treated rats indicates the decrease in the activity of stroma, follicles formation and corpora lutea which indicates the non-availability of gonadotrophins.

Histological changes: Follicles which contain ova, are the functional units of ovaries. The decrease in the number of *Graafian* follicles in the groups which received fraction II, indicates that there is a disruption in their growth and differentiation (Peters and McNatty, 1980; Knobil and Neil, 1994; Chabbart-Buffet and Burchard, 2002). The induction in the growth and differentiation of preovulatory follicle to *Graafian* follicles requires pituitary gonadotrophins and responsible for the follicular growth, differentiation and synthesis of estrogen by granulosa cells (Byskow, 1979; Peters, 1979).

The decrease in the number of *Graafian* follicles, increase in the number of atretic follicles and decrease or absence in the number of corpora lutea in the ovaries of extract treated animals compared to those of control group clearly indicates that the growth, differentiation and ovulation are inhibited due to the effect of the treatment of *C. medica* seeds chromatographic fractions of petroleum ether extract which might have resulted in loss of hormonal receptors (Guyton and Hall, 1996).

Biochemical changes: Protein is considered to be the building material and is responsible for growth of organ. In the present study the low protein content of the ovary indicates the retarded ovarian growth. It is well understood that FSH is essential for protein synthesis in gonads (Means, 1975). The inhibited pituitary FSH release in extracts treated rats might have resulted in low protein content.

Glycogen is involved in providing energy to various processes like ovulation, transportation, survival of eggs and implantation (Walaach, 1952). For the decreased ovulatory index and inhibiting of implantation lowered availability of glycogen may also be one of the factors.

Cholesterol derived from the different sources is the precursor for the steroidogenesis of ovarian endocrine tissues (Veldhuies *et al*., 1982; Rajendran *et al*., 1983). The increased ovarian cholesterol could be attributable to a probable alteration in its synthesis of steroids or transport to gonads. It is evident that biosynthetic capacity of the

ovary is influenced by FSH, LH and prolactin (Purandare *et al*., 1974; McNatty *et al*., 1975). As FSH and LH levels are reduced in the rats those received the fractions of *C. medica* seeds, the cholesterol, a precursor level is increased. Similar results have been obtained by the administration extracts of *Nelumbo nucifera* (Mazumder *et al*., 1992), *Hibiscus rosa sinensis* (Murthy *et al*., 1997) and *Crotalaria juncea* (Vijaykumar *et al*., 2004; Malshetty and Patil, 2007) to rats and mice.

Increase in the acid and alkaline phosphatases in granulosa and thecal cells precede histological changes leading degeneration of follicles (Lobel *et al*., 1961). Alkaline protease is a proteolytic enzyme, produced in response to LH and responsible for breakdown of collagen fibres (Peters and McNatty, 1980). Therefore, it can be interpreted that increased ovarian phosphatases and decreased alkaline protease have brought disintegration in follicles and reduction in proteolytic activity. As a result increase in follicular atresia and decrease in ovulation and corpora lutea formation is observed in the ovaries treated with fraction II of *C. medica* seeds.

Changes in the duration of estrous cycle: On the basis of cytological observations of the vaginal smears the different phases of the estrous cycle can be decided unambiguously in lower mammals like rodents which in turn helpful to predict the effect of other factors that influence the structure and function of the ovaries.

A cyclic change in the vaginal smear observed gives a reasonable index of the ovarian activity and its steroidal hormone synthesis. The levels of these hormones (estrogen and progesterone) are controlled by pituitary gonadotrophins and in turn by hypothalamic releasing hormone (Lerner, 1969). The cornification in the vaginal epithelial cells is mainly due to high levels of estrogen secreted by the matured follicles. It is also known that exogenous administration of estrogen consistently stimulates the proliferation of the vaginal epithelium in adult spayed animals (Mandle, 1951).

Normally in the rats, estrogen level increases during estrus phase and decreases gradually during diestrus phase (Michel *et al*., 1969; Smith *et al*., 1975). The progesterone hormone is low during estrus phase and high during diestrus phase and highest during the proestrus phase (Smith *et al*., 1975). The increase in the duration of estrus and proestrus phases in the treated rats indicates the induced estrogenecity upon administration of the fraction II. The significant proliferation of vaginal epithelial cells during proestrus phase in treated rats might be due to surplus availability of estrogen and progesterone in required concentration to pass to the next phase of the cycle. Moreover, the chromatographic fraction II of petroleum ether extract of *C. medica* seeds possesses estrogenic effect.

CONCLUSION

The fraction II reduced the number of healthy follicles and corpora lutea but increased in the number of regressing follicles, whereas, prolonged duration of proestrus and estrus phase is possibly due to direct estrogen effect. This indicates nonavailability of gonadotrophins for follicular development and ovulation. Hence, compare to other fraction, fraction II 100 mg kg^{-1} body weight of petroleum ether extract of *C. medica* seeds has strong antiovulatory activity.

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