EFFICACY OF *CITRUS MEDICA* SEEDS EXTRACTS ON REPRODUCTIVE ACTIVITIES IN FEMALE ALBINO RATS

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Summary

Petroleum ether, benzene and ethanol extracts of the seeds of *C. medica* administered orally at the dose level of 200 mg and 400 mg/kg body weight to adult female albino rats for 30 days. The estrous cycle of these rats was irregular with prolonged proestrus and estrous, reduced metestrus and diestrus phase during the experimental period. At autopsy on day 31st , petroleum ether extract treated rats showed reduced ovarian weight, benzene extract treated rats showed increased ovarian weight and ethanol extract treated rats showed non-significant change in the weight of ovary. 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 and ascorbic acid content of the ovary are increased, whereas, protein and glycogen content were decreased. The uterine weight and its micrometric measurements in all experimental rats were increased significantly. However, petroleum ether extract of *C. medica* seeds was more effective in causing these changes comparing to other extracts. Based on these results the reproductive activities of *C. medica* seed extracts are discussed.

Key Words: Reproductive, Rat, Ovary,Uterus,*Citrus medica.*

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Introduction

Plants have served as a natural source of antifertility substances. The women of rural natives have used medicinal plants especially by the tribes before coitus for interruption of pregnancy. Kirtikar and Basu, [1], Nadakarni and Nadakarni, [2] and Chopra et al., [3] have all reported many such plants. Research on Indian plants with antifertility activity has been exhaustively reviewed by Satyavati, [4], Kamboj, [5] and Bhargava, [6]. In recent years work is going on in our laboratory has investigated the antifertility activity of some indigenous plants [7-10]. In the same direction present approach is being pursued to identify antifertility agent from the seeds of *Citrus medica*. In and around Gulbarga (South India) Ayurvedic physicians are using *Citrus medica* (Rutaceae) to prevent fertility. Though, the *Citrus hystrix* DC and *Citrus limonum* have been reported for antifertility activity [11-12]; so far, no systematic biological and pharmacological investigation has been carried out. Therefore, in the present study efforts have been made to test the effect of the various extracts of *C. medica* seeds on estrous cycle and reproductive activities in rats.

Material and methods

Plant material

Fresh seeds of *Citrus medica* were collected from the fruits grown in the fields in and around Gulbarga, Karnataka, India during January-May 2004 and authenticated at the herbarium, Department of Botany, Gulbarga University, Gulbarga, where voucher specimens are deposited.

Extraction of plant material

The seeds were shade dried, chopped into small pieces, powdered and subjected to soxhlet extraction successively and separately from non-polar to polar solvents i.e., petroleum ether (b.p.60-80˚C), benzene and ethanol (95%) for 18-20 hours. The decoction so obtained was evaporated under reduced pressure and controlled temperature (50-60˚C). The dried mass considered as the extract, preserved at 6˚C in refrigerator until used and diluted as required for experimental studies.

Animals

Mature, healthy, virgin adult female albino rats of Wistar strain (140-160 g) with normal estrous cycle were kept under controlled conditions of light (12hr) and temperature (24±3ºC) with free access to rats chow pellets (CFTRI, Mysore, India) and water *ad libitum* All the extracts were prepared in Tween-80 (1%), suspended in distilled water and administered orally to the animals with the help of intragastric catheter at desired doses. The control animals received an equivalent amount of vehicle only.

Experimental protocol

The animals were divided into seven groups consisting of six rats in each group.
Group– I: Control, received 0.2ml Tween-80 (1%) orally.

Group– II: Received 200mg petroleum ether extract /kg body weight in 0.2ml Tween-80 (1%) orally.

Group– III: Received 400mg petroleum ether extract /kg body weight in 0.2ml Tween-80 (1%) orally.
Group– IV: Received 200mg benzene extract /kg body weight in 0.2ml Tween-80 (1%) orally.
Group– V: Received 400mg benzene extract /kg body weight in 0.2ml Tween-80 (1%) orally.
Group– VI: Received 200mg ethanol extract /kg body weight in 0.2ml Tween-80 (1%) orally.
Group– VII: Received 400mg ethanol extract /kg body weight in 0.2ml Tween-80 (1%) orally.

All treatments were given for 30 days to cover six regular estrous cycles. Vaginal smear of all the animals were taken daily during morning and the stage of estrous cycle was identified microscopically [14]. The normal treated animals were sacrificed on day 31st by cervical dislocation, 24 hour after the last treatment. Ovaries and uteri were dissected out, freed from surrounding tissues, blotted on filter paper and weighed quickly to the nearest milligram on an electronic balance. Tissues from one side of each animal were fixed in Bouin's fluid and processed for histological preparation. Haematoxylin-Eosin stained slides were examined microscopically. Number of developing follicles, Graafian follicles, corpora lutea and atretic follicles were observed from stained serial sections of the ovary from each rat [15]. The micrometric measurements like diameter of uterus, thickness of endometrium and myometrium and height of epithelial cells were calculated by the method described by Deb et al., [16]. Organs from the other side were used for biochemical estimations like protein [17], glycogen [18], cholesterol [19], ascorbic acid [20], acid and alkaline phosphatase [21].

Statistical analysis:

The mean and standard error of mean (SEM) were calculated and the significance of difference analysed by applying Student's *t*-test as described by Snedchor [22].

Results

Changes in duration of estrous cycle

The results are detailed in table-1. Administration of petroleum ether extract of *C. medica* seeds at both the dose level showed significantly increased the duration of the proestrus and estrous ($P<0.01$) to ($P<0.001$) phases and decreased metestrus and diestrus ($P<0.001$) phases when compared to that of controls. Changes observed due to benzene extract are non-significant at all the phases of estrous cycle at both the dose levels. The ethanol extract has significantly increased the duration of proestrus, estrous and metestrus ($P<0.01$) but decreased the diestrus ($P<0.01$) to ($P<0.001$), when compared with controls.

Changes in the ovary

Gravimetric changes

The results are detailed in table 2. Administration of both the doses of petroleum ether extract of *C. medica* seeds to the adult female rats decreased the weight of ovary significantly (*P<0.001*). The benzene extract at both the dose levels increased the weight of ovary, but it is significant (*P<0.05*) only with low dose. The ethanol extract at both the doses have shown non-significant changes in the weight of ovary.

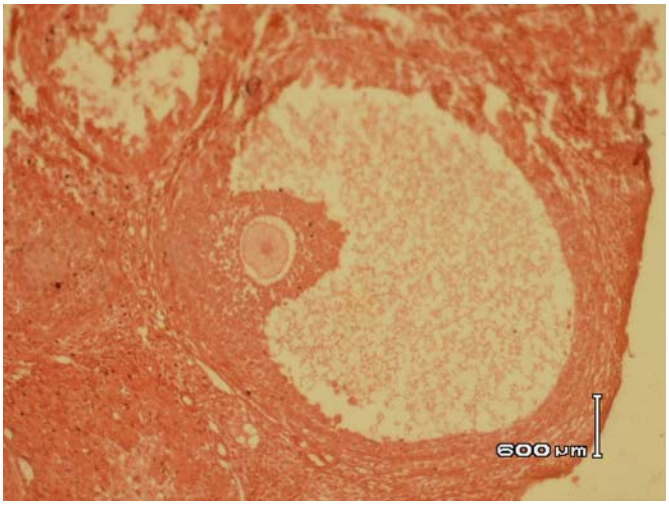
Histological changes

The number of healthy follicles and corpora lutea are reduced in both the doses of petroleum ether extract treated rats. These changes are also seen in benzene & ethanol extract administered rats at both the dose levels. The numbers of regressing follicles and atretic follicles are observed due to treatment of both the doses of petroleum ether extract. The ovaries of both the doses of benzene and ethanol extract administered rats showed slight non-significant change in ovarian components.

Biochemical changes

In the present study biochemical assay of protein, glycogen, cholesterol and ascorbic acid contents of the ovary are estimated. The acid phosphatase and alkaline phosphatase activities are evaluated and results are detailed in table 2.

Administration of petroleum ether extract at both the dose levels has decreased the protein and glycogen content highly significantly (*P<0.001*). Similarly highly significant (*P<0.001*) increase in the cholesterol, acid & alkaline phosphatase activity ascorbic acid contents are observed. Benzene extract at high dose level has increased the protein content (*P<0.05*) at low dose level decreased ascorbic acid content (*P<0.05*) significantly and other biochemical parameters have shown non-significant changes. Ethanol extract at both the dose level has decreased protein (*P<0.01*) and glycogen content (*P<0.05*) and increased the cholesterol (*P<0.05*), acid and alkaline phosphatase activity (*P<0.01*) and ascorbic acid content (*P<0.01*) significantly.



1. Photomicrograph of ovary treated with vehicle showing normal fully developed Graafian follicle with healthy oocyte (x 400).

Table 1. Effect of various extracts of *C. medica* seeds on the duration of various stages of estrous cycle in rats.

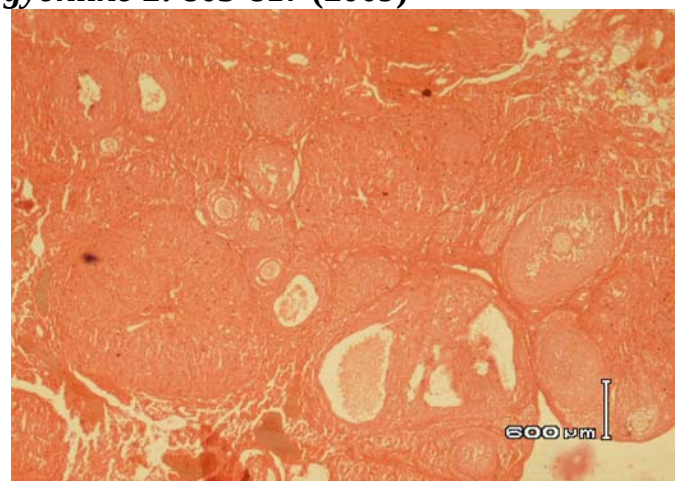
Treatment	Dose (mg/kg body wt.)	Duration of stages of the estrous cycle in days			
		Proestrus	Estrus	Metestrus	Diestrus
Control	Tween-80 (1%)	4.50±0.22	4.33±0.21	4.00±0.00	17.16±0.40
Petroleum ether	200	6.83±0.98*	9.83±0.94**	5.66±0.91*	7.33±0.84**
	400	11.66± 1.49***	8.83±0.60**	4.50±0.84	5.33±1.22***
Benzene	200	4.00±0.25	4.16±0.30	4.33±0.55	17.50±0.67
	400	4.33±0.42	4.33±0.21	4.83±0.47	16.50±0.56
Ethanol	200	7.16±0.83*	8.50±0.84**	4.50±0.56	10.00±1.23**
	400	8.33±0.66**	8.33±0.55**	7.16±0.60**	6.16±0.30***

M±S.E. = 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

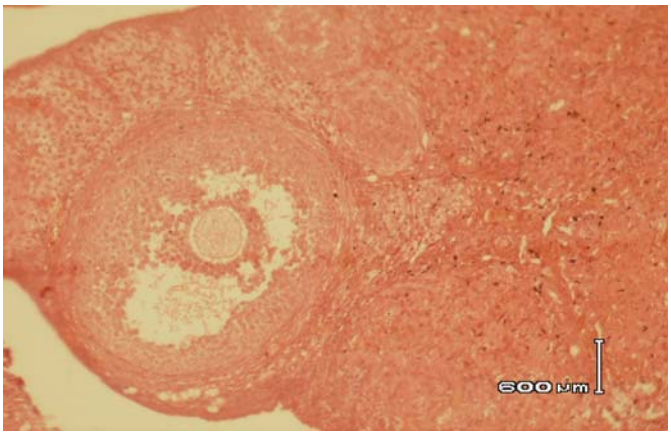
Table 2. Gravimetric and biochemical changes of the ovary due to the administration of various extracts of *C. medica* seeds.

Treatment	Dose (mg/kg body wt..)	Weight (mg/100g body wt.)	Protein (µg/100mg)	Glycogen (µg/mg)	Cholesterol (µg/mg)	Acid phosphatase (µ moles of P-nitro phenol released/100mg/30 min.)	Alkaline phosphatase (µ moles of P-nitro phenol released/100mg/30 min.)	Ascorbic acid (µg/mg)
Control	Tween-80 (1%)	55.28±3.17	15.93±0.10	4.11± 0.25	14.30 ± 0.27	17.66±1.04	11.00±0.23	1.24±0.10
Petroleum ether	200	53.21±1.27**	6.50±1.11***	2.50± 0.28**	20.37±0.47***	21.58±0.46***	18.12±0.18***	1.66± 0.08**
	400	48.99±1.86**	9.33±0.84***	2.20± 0.32***	22.03± 0.88***	25.78±0.39***	22.45±0.78***	1.72± 0.09***
Benzene	200	58.99±0.76*	14.22±1.22	3.88± 0.15	14.67± 0.24	16.68±0.21	11.05±0.33	0.94± 0.07*
	400	57.49±1.83	18.16±0.40*	4.01±0.17	14.96± 0.66	16.28±0.15	11.07±0.43	1.26± 0.02
Ethanol	200	54.46±1.40	8.06±0.57**	3.77± 0.19*	15.68± 0.55*	18.70±0.71*	14.41±0.42**	1.41± 0.07*
	400	56.36±0.90	7.42±0.21**	3.70± 0.20*	15.14± 0.53*	19.27±0.45*	15.79±0.85**	1.48± 0.04*

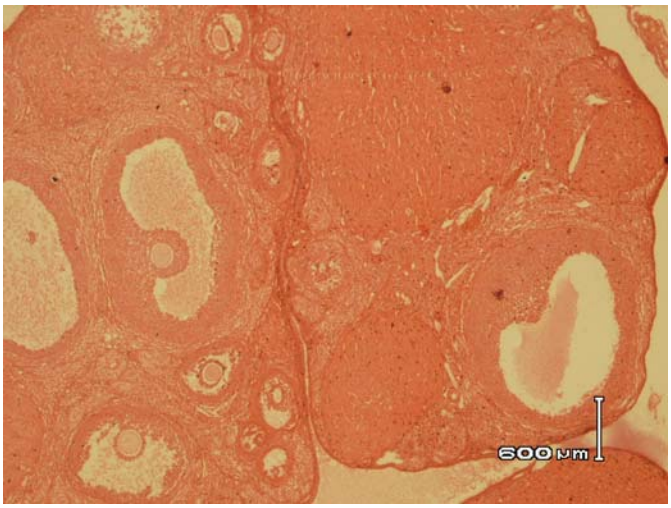
M±S.E. = Mean ± Standard error
Duration: 30 days
Six animals were maintained in each group
**p<0.05*; ***p<0.01*: ****p<0.001* when compared with control



2. Photomicrograph of ovary treated with petroleum ether extract of *Citrus medica* seeds showing degenerative follicles (x 100).



3. Photomicrograph of ovary treated with benzene extract of *Citrus medica* seeds showing atretic Graafian follicle. Note the infiltration of granulosa, cumulus oophorus and corona radiata cells (x 400).



4. Photomicrograph of ovary treated with ethanol extract of *Citrus medica* seeds showing under developed and degenerating follicles (x 100).

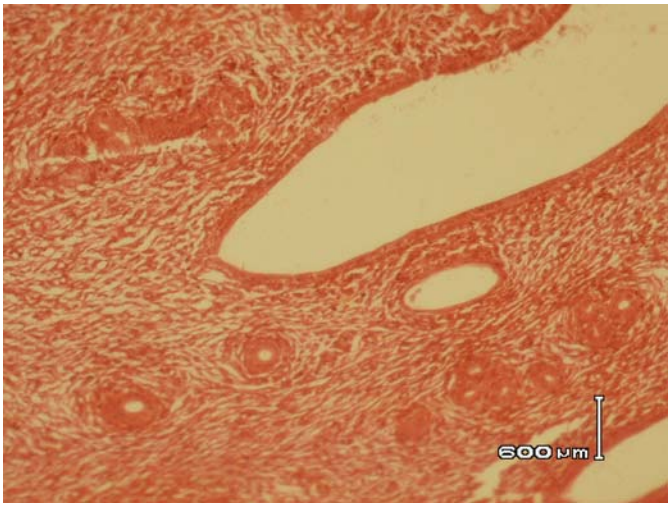
Changes in the uterus

Gravimetric changes

Administration of all the three extracts at high dose level has increased uterine weight highly significantly (*P<0.001*) and at low dose level all the three extracts have shown almost significant (*P<0.05*) increase in uterine weight.

Histometric changes

The diameter of uterus, thickness of endometrium and myometrium and surface epithelial cell height are increased highly significantly (*P<0.001*) due to the administration of both the dose level of petroleum ether extract. Benzene extract at both the dose level has increased the diameter of uterus and epithelial cell height (*P<0.05*) to (*P<0.01*) but, other parameters are non-significant increase. Ethanol extract at both the dose level are effective in increasing diameter of uterus (*P<0.01*) and thickness of endometrium (*P<0.05),* myometrium (*P<0.01*) and surface epithelial cell height (*P<0.05*) significantly. These changes observed in uterus indirectly exhibited the estrogenecity of extracts of *C. medica* seeds.

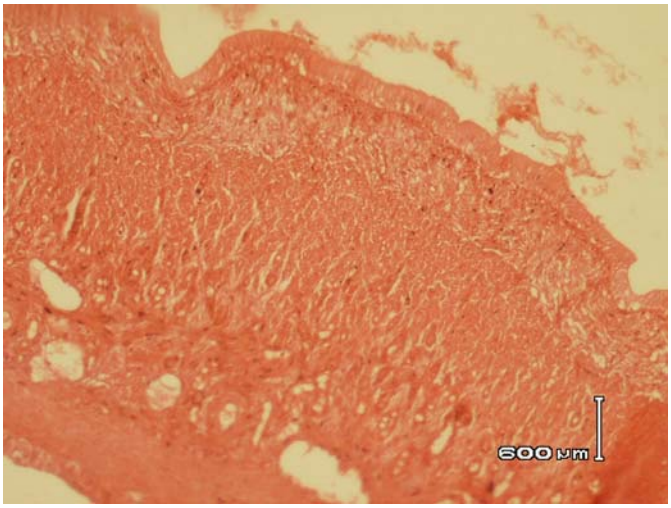


5. Photomicrograph of uterus treated with vehicle showing normal endometrium with endometrial glands and luminal epithelial cells (x 100).

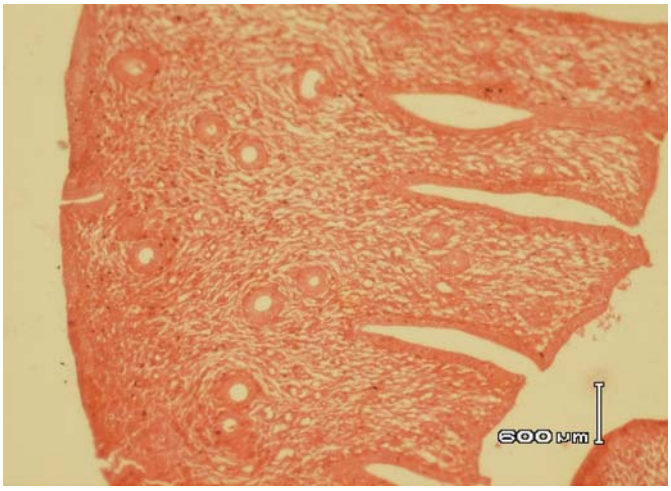
Table 3. Gravimetric and biochemical changes of the uterus due to the administration of various extracts of *C. medica* seeds.

Treatment	Dose (mg/kg body wt.)	Weight (mg/100g body wt.)	Protein (µg/100mg)	Glycogen (µg/mg)	Cholesterol (µg/mg)	Acid phosphatase (µ moles of P-nitro phenol released/100mg/30 min.)	Alkaline phosphatase (µ moles of P-nitro phenol released/100mg/30 min.)	Ascorbic acid (µg/mg)
Control	Tween-80 (1%)	112.25±3.09	11.53±3.10	1.5±0.04	4.38±0.12	3.83±0.72	15.66±1.06	1.23±0.10
Petroleum ether	200	138.02±6.27*	16.80±0.71*	3.9±0.06***	7.20±0.06**	12.23±0.86***	26.16±0.82***	1.10±0.02***
	400	239.87±23.32***	27.0±0.36***	4.1±0.09***	9.65±0.12***	18.73±1.26***	29.06±0.24***	1.02±0.05***
Benzene	200	133.77±4.11*	11.47±0.30	1.5±0.07	4.28±0.02	3.93±0.82	15.06±1.06	1.22±0.06
	400	193.22±5.67***	11.70±0.33	1.6±0.03	4.35±0.06	4.41±0.34*	15.60±0.86	1.23±0.04
Ethanol	200	137.66±7.27*	12.83±0.40	2.7±0.16**	5.48±0.12*	5.46±0.30*	16.93±1.54	1.19±0.07*
	400	194.66±2.48***	16.34±0.81*	2.9±0.26***	7.80±0.06**	6.63±0.77**	18.66±0.22**	1.15±0.03**

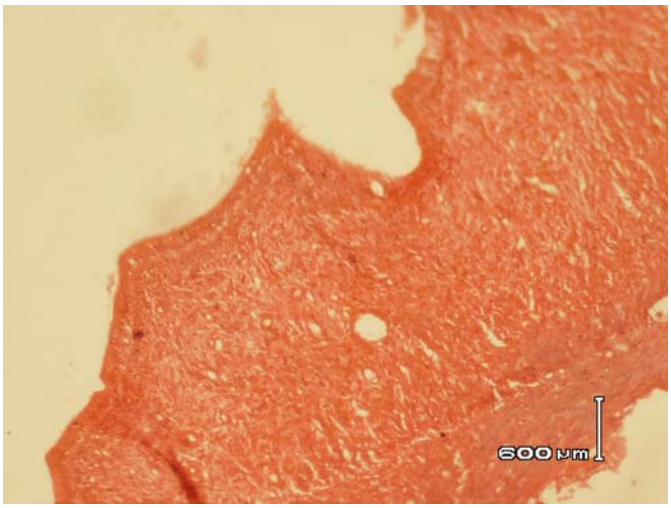
M±S.E. = Mean ± Standard error
Duration: 30 days
Six animals were maintained in each group
*$p<0.05$; **$p<0.01$: ***$p<0.001$ when compared with control



6. Photomicrograph of uterus treated with petroleum ether extract of *Citrus medica* seeds showing hypertrophied endometrium, endometrial glands and luminal epithelium (x 100).



7. Photomicrograph of uterus treated with benzene extract of *Citrus medica* seeds showing normal uterine components (x 100).



8. Photomicrograph of uterus treated with ethanol extract of *Citrus medica* seeds showing slightly hypertrophied endometrium, endometrial glands and luminal epithelium (x 100).

Biochemical changes

Administration of low dose of petroleum ether extract has increased protein content almost significantly (*P<0.05*) and cholesterol content significantly (*P<0.01*). The glycogen, acid and alkaline phosphatase activity increased highly (*P<0.001*) significantly, contrarily highly significant (*P<0.001*) decrease in ascorbic acid content is observed. Highly significant increase (*P<0.001*) in the protein, glycogen, cholesterol content and acid and alkaline phosphatase activity and decreased ascorbic acid content (*P<0.001*) is observed due to administration of high dose level of petroleum ether extract. Benzene extract administration at both the dose level is non-effective in causing any significant changes. The low dose level of ethanol extract has shown increase in glycogen (*P<0.01*), cholesterol content and acid phosphatase (*P<0.05*) activity, but, decrease in ascorbic acid content (*P<0.05*) significantly. At high dose level it has shown significant increase in protein (*P<0.05*), glycogen (*P<0.001*), cholesterol (*P<0.01*) content, acid and alkaline phosphatase activity (*P<0.001*) significantly. But, ascorbic acid content is decreased (*P<0.01*) significantly.

Discussion

The ancient Indian literature mentions the use of a number of plant/preparations for fertility regulation particularly as antiovulatory, antiimplantation, abortifacient and emmenogogues. According to ancient Ayurvedic concepts the systematic contraceptives had to be administered during the period of menstrual blood flow as this was supposed to be accompanied by ovulation [23]. Extracts of *Hibiscus rosa sinensis* flowers [7], seeds of *Momordica charantia* [8], aerial parts of *Rivea hypocrateriformis* [9], *Crotalaria juncea* [10] and many other plants have been reported for this antiovulatory activity in our laboratory. Similarly the stem bark of *Alangium salvifolium* [24], the roots of *Calotropis procera* [25], *Azadirachta indica and Melia azedarach* seeds [26] have also been investigated.

In this investigation petroleum ether, benzene and ethanol extracts of seeds of *C. medica* administered for 30 days increased the duration of estrous and proestrus and decreased the duration of metestrus and diestrus significantly during experimental period indicates the induced estrogenecity of the extracts as estrogen are necessary for cornification of vaginal epithelial cells [7]. It is well documented that FSH is essential for follicular growth and LH is necessary for ovulation and corpora lutea formation [27] which are responsible for the growth and weight of ovary. Therefore, observed reduction in the ovarian weight after the treatment of *C. medica* seed extracts may be due to reduction in the follicular growth and ovulation which are dependent on availability of gonadotrophins.

The lowered protein content of the gonads indicates the retarded ovarian growth which is dependent on the availability of pituitary FSH, FSH is essential for protein synthesis in gonads [28]. The ovarian glycogen which is an energy source for various processes like ovulation, transformation, survival of egg and implantation [29].

Table 4. Histometric changes of the uterus due to the administration of various extracts of *C. medica* seeds.

Treatment	Dose (mg/kg body wt.)	Diameter (µm)	Thickness of endometrium (µm)	Thickness of myometrium (µm)	Epithelial cell height (µm)
Control	**Tween-80 (1%)**	2096.10 ± 3.78	604.91 ± 9.45	110.90 ± 3.10	20.08 ± 0.52
Petroleum ether	200	2401.38 ±3.77***	660.34 ± 7.25***	128.19 ± 1.70***	27.57 ± 0.64***
	400	2546.03 ± 10.23***	677.02 ± 5.75***	141.28 ± 0.73***	33.61 ± 1.29***
Benzene	200	2134.89 ±16.45*	604.09 ± 12.71	111.36 ± 2.83	21.57 ± 0.79*
	400	2154.60 ± 13.53**	615.70 ± 10.29	118.05 ± 2.34	21.92 ± 0.70*
Ethanol	200	2150.00 ± 6.63**	626.65 ± 3.55*	122.59 ± 1.43**	23.98 ± 1.52*
	400	2175.86 ± 8.83**	631.65 ± 1.58*	125.12 ± 1.57**	21.92 ± 0.70*

M±S.E. = 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

Therefore, decreased ovarian glycogen content observed may be due to reduced availability of ovarian estrogens. The increased ovarian cholesterol could be attributable to a probable alteration in its synthesis of steroids or transport of gonads. It is evident that biosynthetic capacity of the ovary is influenced by FSH, LH and prolactin [30, 31]. Increase in the acid and alkaline phosphatases in granulose and thecal cells precedes histological changes leading degeneration of flollicles [32]. The ability of LH to block the uptake of ascorbic acid by gonadotrophin primed rat ovaries provided the basis for a bioassay [33]. The high concentration of ascorbic acid accumulated in the ovary of experimental rats may be due to reduced availability of LH. Though, the follicular atresia is common in rat ovary. The increased number of atretic follicles of experimental animals indicates the non-availability of required amount of gonadotrophins for follicular growth and ovulation [34].

Uterine growth depends on the availability of ovarian steroid hormones particularly estrogens [35]. The possibility of these changes may be due to the progestogenic and estrogenic effect of the extract of *C. medica* seeds as uterine growth and secretion depends on the availability of ovarian steroid hormones [36, 37] The increase in the protein concentration, glycogen content, cholesterol level, acid and alkaline phosphatase of the uterus and decrease in ascorbic acid are responsible for changing the uterine milieu [39] which are unfavorable for implantation and pregnancy maintenance.

Therefore, increased uterine weight and prolonged duration of proestrus and estrous phase is possibly due to direct estrogen effect of plant extracts. However, it may be concluded that petroleum ether extract of *Citrus medica* seeds have potent antiovulatory activity more effective than other extracts.

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