RESEARCH ARTICLE

Estrogenic activity of petroleum ether extract of seeds of *Citrus medica* on immature albino rats

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Estrogenic/anti-oestrogenic activities of petroleum ether extract of *Citrus medica* seeds was studied in albino rats. The extract at the dose level of 200 and 400 mg/kg body weight was administered for seven days to immature ovariectomised rats, along with or without 1 µg ethinyl estradiol. Suitable controls were maintained. The extract-treated rats exhibited estrogenic effects, which include increase in uterine weight and vaginal epithelial cell cornification. The micrometric measurements of the uterus and its components were increased and glands showed high secretory activity. When the above extract was tested in 30-day-old immature rats, they exhibited opening of vagina on the fifth day and cornification of vaginal epithelial cells, which is about 10 days earlier compared to controls, further supporting the estrogenic activity of the extract. Hence, these results strongly indicate the potent estrogenic nature of petroleum ether extract of *Citrus medica* seeds, which may be used as an antifertility agent. Phytochemical studies of the petroleum ether extract of *Citrus medica* seeds were also carried out to find the chemical nature of the extract.

Key words: *Citrus medica*, estrogenic, ethinyl estradiol, rats

INTRODUCTION

Phytoestrogens are plant substances that have structural and functional similarity to estradiol-17B.[1] Plant-derived isoflavonoids, coumestans and ligands compete with estradiol with varying affinities to bind to oestrogen receptors, induce transcription of oestrogen responsive genes[2] and depending on the outcome measured, either mimic or antagonize the action of steroidal oestrogens.[3] The impact of dietary phytoestrogens on normal biological processes was first recognized in sheep.[4] Therefore, the fact that these compounds function as phytoestrogens might be an important dietary factor affecting human health.[5-9] Among their widespread clinical effects dietary phytoestrogen is purported to reduce the risk of cancer, have antioxidant and free radical scavenger activity, reduce serum cholesterol, induce cellular differentiation and inhibit angiogenesis.[8,9] Phytoestrogens can therefore potentially benefit humans, and yet there are probably many more unidentified phytoestrogens in nature.

Herbal therapies are unconventional treatments in wide use for many diseases. It can have different estrogenic effect on both animal and human.[10-14]

The reduction of ovulation rate in mice[15] and disruption of reproductive hormones and ovarian function in cyclic women.[16-18] In addition, epidemiological studies have shown that women who consumed high amount of isoflavones from soy had a decrease in serum levels of estrone and estradiol.[19,20]

Therefore, in the present investigation, efforts have been made to test the effect of petroleum ether extract of seeds of *Citrus medica* in immature (ovariectomised) albino rats. Petroleum ether extract has been shown to have the most potent antifertility effect out of the three extracts tested in our laboratory.[21] Emphasis has been placed on studying the estrogenic/antiestrogenic activity of the extract, as its antiimplantation property is an important step in the contraceptive nature of the compound. The biological nature of the extract has also been studied.

MATERIALS AND METHODS

Plant Material

The seeds of the plant *Citrus medica* (Lemon) were collected from the fruits grown in the fields in and around Gulbarga (Karnataka) during summer (January-May) and authenticated at the herbarium, Department of Botany, Gulbarga University, Gulbarga, India, where voucher specimens were deposited.

Extraction of Plant Material

The seeds were shade-dried, chopped into small pieces, powdered and subjected to soxhlet extraction successively with petroleum ether (60-80°C), benzene and ethanol (95%) for 18-20 h. The extracts were concentrated to dryness in a flash evaporator under reduced pressure and controlled temperature (50-60°C). All the extracts were preserved in the refrigerator at 6°C used for experimental studies.

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Phytochemical Screening

In the present study phytochemical tests were carried out and the presence of various chemical constituents in plant extracts were determined by preliminary phytochemical screening.[22,23]

Estrogenic/antiestrogenic Activity

Colony-bred immature female albino rats (Wistar strain), 25 days old, weighing between 30-35 g were bilaterally ovariectomised by dorsolateral approach under light ether anaesthesia and semi-sterile conditions. Rat cages were arranged randomly to limit variation based on temperature and light. They were maintained in barrier rooms under 12: 12 - light: dark cycle, with a temperature of 22 ± 1°C and relative humidity of 50%.

The animals were divided into six groups consisting of six rats in each group.

Group-I: Control, received 0.2 ml Tween-80 (1%) orally.
Group-II: Treated, received 1 μg ethinyl estradiol/rat/day in olive oil subcutaneously.
Group-III: Treated, received 200 mg petroleum ether extract/kg body weight in 0.2 ml Tween-80 (1%) orally.
Group-IV: Treated, received 400 mg petroleum ether extract/kg body weight in 0.2 ml Tween-80 (1%) orally.
Group-V: Treated, received 200 mg petroleum ether extract/kg body weight in 0.2 ml Tween-80 (1%) orally + 1 μg ethinyl estradiol/rat/day in olive oil subcutaneously.
Group-VI: Treated, received 400 mg petroleum ether extract/kg body weight in 0.2 ml Tween-80 (1%) orally + 1 μg ethinyl estradiol/rat/day in olive oil subcutaneously.

All the above treatments were given for seven days. On the eighth day of the experiment, the animals were sacrificed by cervical decapitation and the uteri and adrenals were dissected out and separated from the adherent tissues and weighed up to the nearest mg on an electronic balance. Estrogenic activity was assessed according to the method of Edgren and Calhoun,[24] taking uterine wet weight, opening of the vagina, and cornification of vaginal epithelial cells as the points of evaluation. Additionally, the uterine tissue of rats from each group was fixed in Bouin's fluid and processed for histological preparation. Haematoxylin-eosin[25] stained slides were examined for changes in tissues and cellular organization.

Statistical Analysis

The gravimetric and histometric measurements were analyzed by using standard method described by Snedcor and Cochran.[26] For estrogenic/antiestrogenic analysis all the doses of extracts were compared with the vehicle and ethinyl estradiol-treated groups. All the values were statistically analyzed by using student's ‚t' test. The values were judged as almost significant if $P < 0.05$, significant if $P < 0.01$ and highly significant if $P < 0.001$.

RESULTS

Results of the phytochemical screening of the petroleum ether extract from the seeds of *Citrus medica* are presented in Table 1.

Estrogenic/antiestrogenic activity of the seeds' extract of *Citrus medica* [Tables 2 and 3]

The petroleum ether extract when administered orally at 200 and 400 mg/kg body weight caused a significant increase in the uterine weight in immature ovariectomised rats versus control ($P < 0.001$) (Table 2). The uterotrophic changes such as the diameter of the uterus ($P < 0.001$), thickness of the endometrium ($P < 0.001$) were significantly increased

Table 1: Phytochemical investigation of the petroleum ether extract of *Citrus medica* seeds

Extract	Phytochemical tests								
	Alkaloids	Steroids	Glycosides	Amino acids and proteins	Saponins	Flavones	Carbohydrates	Fats and oils	Phenols
Petroleum ether	+ve	–ve	+ve	+ve	–ve	+ve	–ve	+ve	–ve

+ = Positive; – = Negative

Table: 2: Effect of petroleum ether extract of *C. medica* seeds on uterine weight, vaginal opening and cornification of vaginal epithelial cells in bilaterally ovariectomised immature rats

Group	Treatment	Dose (kg)	Uterine wet weight (mg/100g)	Vaginal opening/cornification
I	Control	Tween-80 (1%)	260.55 ± 8.22	-/-
II	Ethinyl estradiol	1 μg	329.44 ± 3.79***	6/6
III	Petroleum ether	200mg	331.71 ± 7.86**	6/6
IV	Petroleum ether	400mg	347.77 ± 5.81***	6/6
V	Ethinyl estradiol + Petroleum ether	1 μg + 200mg	320.55 ± 11.9***	6/6
VI	Ethinyl estradiol + Petroleum ether	1 μg + 400mg	335.55 ± 5.55***	6/6

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

Table: 3: Histometric changes in the uterus due to the administration of petroleum ether extract of *C. medica* seeds in bilaterally ovariectomised immature rats

Group	Treatment	Dose (kg)	Diameter of uterus (µm)	Thickness of myometrium (µm)	Thickness of endometrium (µm)	Epithelial cell height (µm)
I	Control	Tween-80 (1%)	363.86 ± 5.53	25.60 ± 2.83	58.76 ± 4.22	24.86 ± 2.25
II	Ethinyl estradiol	1 µg	885.92 ± 9.17***	130.78 ± 4.21***	250.86 ± 9.71***	51.98 ± 2.76***
III	Petroleum ether	200 mg	510.76 ± 2.25**	40.87 ± 5.76*	85.88 ± 2.76**	33.9 ± 0.0*
IV	Petroleum ether	400 mg	780.68 ± 2.78***	76.34 ± 3.76***	198.87 ± 3.54***	40.21 ± 7.34**
V	Ethinyl estradiol + Petroleum ether	1 µg + 200 mg	928.86 ± 2.25***	96.6 ± 3.73***	260.1 ± 4.83***	33.0 ± 1.59**
VI	Ethinyl estradiol + Petroleum ether	1 µg + 400 mg	970.8 ± 5.75***	151.5 ± 3.10***	293.8 ± 6.18***	54.24 ± 2.25***

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

when compared with control rats. The uteri of these rats were inflated and full of fluid resembling the proestrous or oestrus uterus. The epithelial layer of the endometrium consisted of spindle-shaped cells with basal nuclei. The stroma was represented by fibroblast type of cells and was loose and oedematous.

The experimental animals showed an open vagina and an oestrous smear. The number of cornified cells in the vaginal smears was considerably higher than that of controls, but notably less than of the ethinyl estradiol-treated animals (Table 2).

Simultaneous administration of 400 mg of petroleum ether extract/kg body weight and ethinyl estradiol caused a highly significant increase in the uterine weight versus control ($P < 0.001$). The degree of uterotrophic response was greater than that produced by ethinyl estradiol alone ($P < 0.001$). It also caused a highly significant increase in uterine diameter, thickness of the endometrium and height of the endometrial epithelium versus control ($P < 0.001$).

These results indicate that though both the doses are estrogenic in nature 400 mg/kg body weight dose level is more potent. The petroleum ether extract and ethinyl estradiol are synergetic in their action, as their combination increased all the parameters of the uterus more than their individual administration.

DISCUSSION

The indication of estrogenic activity is the opening of the vagina and of vaginal cells. In this study, the petroleum ether extract of *C. medica* seeds were tested for their estrogenic activity. 200 and 400 mg/kg of Petroleum ether extract of *C. medica* was tested for the cornification of vaginal cells (estrogenic effect). The results are consistent with the trend for uterine weight gain in these rats. Cornification was not obvious until one week after beginning treatment, which indicates that the estrogenic effects of both the extracts may result from short-term administration. Other studies have shown that the post coital effectiveness of plant agents is roughly parallel to their estrogenecity.[27]

Administration of both the doses to ovariectomised rats tended to increase uterine weight, indicating that both the doses contain oestrogen-like compounds. The uterine weight increase was significantly higher than that of the ethinyl estradiol-treated group, indicating that petroleum ether extract at 400 mg/kg body weight dose level is only high estrogenic. Due to the increase in uterine weight, other parameters like diameter & thickness of the endometrium, myometrium and epithelial cell height also increased in both the extracts as compared with the control. Oestrogen levels are lowest during the oestrous phase and increased gradually during the dioestrous phase and reached to a peak at the proestrous phase.[28,29]

In this study, petroleum ether extract affected the uterus and its parameters. Estrogenic stimuli are highly complex and a variety of promoter regions upstream from oestrogen responsive genes confer specificity of tissue activation.[30] *In vitro* studies have confirmed that isoflavones tend to have a higher affinity for oestrogen receptor-β not for oestrogen receptor-α, thus a simple interpretation of oestrogen receptor specificity as the key determinate of tissue-specific estrogenic proportion of *C. medica* seeds extract is sufficient studying mixture of phytoestrogens like isoflavones, coumestans, lignans, resveratrol and 8-prehylnarinagenin such as found in *C. medica* extract might help uncover potential mechanisms whole by exogenous estrogens confirm tissue selectivity. Flavonoids have been reported to possess antifertility activity,[27,31-34] the estrogenic activity of the petroleum ether of *Citrus medica* seeds might be due to the presence of flavonoids or other constituents. In addition, using a standardized extract to study will help eliminate questions concerning the dose of the active compounds and their mixed estrogenic behaviour.

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