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Estrogenic activity of *Citrus medica* L. leaves growing in Egypt

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ABSTRACT

The estrogenic activity of petroleum ether extract of *Citrus medica* L. leaves as well as the chemical constituents responsible for the biological activity was studied. The petroleum ether extract proved to retain high estrogenic activity in immature female rats. The extract was saponified and its components (saponifiable part 23% and the unsaponifiable matter 77%) were identified using GC/MS technique. The extract proved to be safe ($LD_{50}< 2g/kg.bw$). Oral administration of petroleum ether extract of *C. medica* in ovariectomized immature female Wistar rats for 7 days in a dose of 400 mg/kg resulted in significant increase in the uterine weight (g) (1.7±0.11) when compared with ovariectomized control rats (1.3±0.07). GC/MS analysis of both saponifiable and unsaponifiable matters revealed the presence of thirty three components (28 hydrocarbons and 5 sterols) in the unsaponifiable fraction, the major hydrocarbon was n-Heneicosane (16.7%) while the major sterol was β-sitosterol (4.03%) and 15 components in the saponifiable matter it's major component was hexadecanoic acid (19.93%). As a conclusion petroleum ether extract of *Citrus medica* L. leaves possess a significant estrogenic activity.

Keywords: *Citrus medica*, GC/MS, toxicity, estrogenic effect.

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INTRODUCTION

Citrus medica L. (Citron), (Fig.1), is native to Persia or the land of the Medes (wikipedia.org/wiki/Citron). Africa is the chief continent for its production, especially Nigeria (http://www.hort.purdue.edu/new crop/tropical/ lecture_32/lec 32.html). It is reported that Petroleum ether extract of *Citrus medica* displayed anthelmintic activity against earthworm (Bairagi et al., 2011). The fruit juice exerts antimutagenicity and anticancer effect (Entezari et al., 2009). The aqueous and alcoholic extracts of *Citrus medica* were found to be active as anthelmintic with reference to both the paralysis and death times as compared to the piperazine citrate. The aqueous extract shows more activity than alcoholic extract. Phytochemical screening states that it contains fixed oils, volatile oils, citric oxide and flavanone glycosides are abundant constituents of citrus leaves and fruits (Manoj and Kumar; 2011).

Fig. 1: Fruits and leaves of *Citrus medica* L., Rutaceae.

Hexadecanoic acid (palmitic acid), major fatty acid

Heneicosane, major hydrocarbon
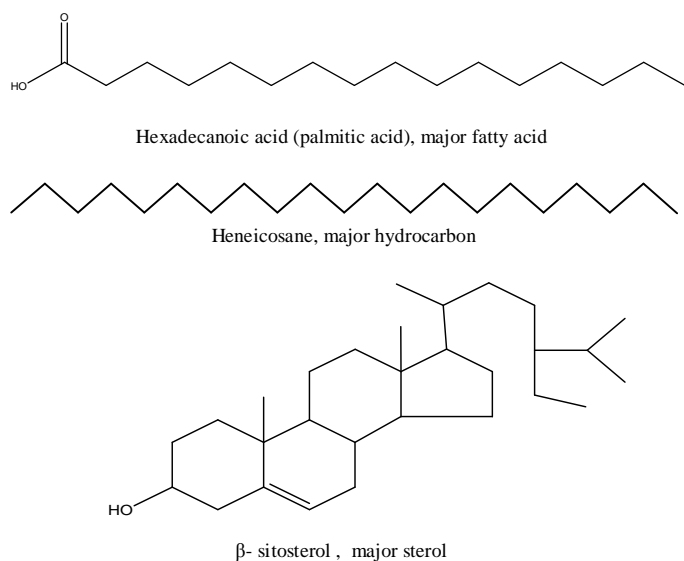

β- sitosterol , major sterol

Fig. 2: Structures of major components detected by GC/MS in unsaponifiable and saponifiable fractions .

MATERIAL AND METHODS

Phytochemical study

Plant material

The leaves of *C. medica* L. were collected from El Qualyobia Governorate, 2008; and the study was carried out immediately after the plant material was dried. It was authenticated by Thérèse Labib, specialist in El Orman Garden, Guiza, Egypt. A voucher specimen is deposited in Pharmacognosy Department, Faculty of Pharmacy, Beni Suef University.

Preparation of the extract

Two kilograms of *Citrus medica* L. fresh leaves were air – dried in shade, pulverized and weighed to give 500g powder. It was macerated with petroleum ether, concentrated at low temperature and reduced pressure to give 9.0 g residue (18%w/v).

Saponification of Petroleum ether

About 1g of Petroleum ether extract of *Citrus medica* L. was saponified by reflux overnight with 50ml of 20% alcoholic potassium hydroxide at room temperature. The fatty solution was acidified with hydrochloric acid (5N), followed by extraction with ether. The ether extract was collected, washed three times with water, dried over anhydrous sodium sulfate and evaporated to dryness (yield: 0.23g; 23%).

Preparation of the fatty acid methyl esters by diazomethane

0.5g Fatty acids produced from lipid fraction were dissolved in a little anhydrous methanol and ethereal solution of diazomethane (Vogel, 1975) was added in a small portion until gas evolution ceased. The reaction mixture was left for 10 min and ether was evaporated under nitrogen stream at room temperature. Two drops of redistilled chloroform solution was added to dissolve the fatty acids methyl esters and 10 µl of this solution were injected into GC/MS instrument.

Condition of GC/MS

GC-MS analyses were carried out on a Hewlett-Packard 5973 Mass Selective Detector (MSD), directly coupled to HP 6890 Series GC – System. Capillary column (30.0m x 250µm x 0.25 µm film thickness) model: Thermo 260 M142P TR-FAME was used, using Helium carrier gas. The oven temperature program was 80°C (2 min), 3°C/min to 230°C (5min) and flow rate is 2.0 ml/min. For USM capillary column of HP-5 5% phenyl methyl siloxane (HP 6890 GC), 30 m length, 320.00 µm in diameter and 0.25 µm thickness was used; carrier gas, N2 30.0 ml/ 3min, He 30.0ml/min, air 300 ml/ min oven initial temperature 120°C for 2 min, at a rate of 4°C/min., 250°C for 10 min; injector temperature 250°C detector temperature 280°C.

Biological Study

Acute toxicity studies

Acute oral toxicity study of petroleum ether extract of leaves of *C. medica* L. was determined in mature albino mice of 20–25 g b.wt. each, of both sexes according to Lorke; 1983. According to the results of the preliminary test; mice were divided into 3 groups, each of 10 mice. Mice were given oral doses of 1, 1.5 and 2 g/kg b.wt. of the tested extracts, respectively. Control mice received the vehicle and kept under the same conditions without any treatment. Mice were observed for 24 hours for signs of toxicity or deaths. Two weeks later, blood samples from the retro-orbital plexus of all mice were obtained, for the estimation of blood hemoglobin (Hb), red blood cell count (RBCs) and total leukocytic count (TLC). Animals were sacrificed; the livers and kidneys were collected and kept in 10% formalin solution for histopathological investigations.

Estrogenic activity

For studying the estrogenic activity, immature female Wistar rats (25 days old, weighing between 50-60 g), were utilized. Animals were allowed to adapt to the laboratory environment for one week before experimentation. A minimum dose of 200 and 400 mg/kg b.wt. of the petroleum ether extract were used (1/10 and 1/5 of the minimum lethal dose). The tested extract was freshly prepared by dissolving 1.5g of the petroleum ether extract in 25 ml of 1% solution of tween 80 in distilled water volume per volume (v/v). The final solution concentration was 6%.

The rats were divided into five groups, each of seven rats. All rats; except the normal (-ve control), group 1, were bilaterally ovariectomized by dorso-lateral approach under light, ether anesthesia and semi-sterile conditions. Afterward, all rats were housed under standard conditions of natural 12 h light and dark cycle with free access to food and water.

Group 2 was kept as ovariectomized (+ve control group). Groups 3 & 4 were orally administered with petroleum ether extract at doses of 200 & 400 mg/kg for 7 successive days. Group 5 was subcutaneously injected with Estriol® (1 µg/rat/day), as a standard drug, for 7 successive days. On the 8th day of the experiment, vaginal smears from all rats were examined and cornification of vaginal epithelial cells as the points of evaluation as previously described by Sharaf, 1951. Animals were then sacrificed by cervical decapitation and the uteri were dissected out and separated from the adherent tissues and weighed up to the nearest on an electronic balance. The relative uterus weight, of each rat was calculated according to Chavalittumrong et al., 2004, using the following formula:

Relative organ weight (kg) = [organ weight (g)/body weight (g)] × 1000

Experimental animals were obtained from the Animal House Colony at the National Research Centre (NRC), Egypt.

Ethics
All animal procedures were performed after approval from the Ethics Committee of The National Research Centre- Egypt and in accordance with the recommendations of the proper care and use of laboratory animals.

Statistical analysis
The results were analyzed using one way analysis of variance (ANOVA) with Dunett's comparison test p-values <0.05 were considered statistically significant. Statistical analysis of results, was done using analytical software named SPSS statistics 17.0, release (Aug. 23, 2008), Chicago, USA.

RESULTS

GC/MS analysis
The residues obtained after the evaporation of each of the saponifiable and unsaponifiable fractions weighed 0.23g and 0.77 g respecting 23% and 77% respectively of the total lipoidal matter of *Citrus medica* L. Results of GC/MS analysis of the unsaponifiable matter is presented in Table 1. It revealed the presence of 33 components (28 hydrocarbons and 5 sterols). Total identified hydrocarbons represent (63%), the major of which being Heneicosane (16.7%). Total sterols represent (7.77%), the major of which is β-sitosterol (4.03%). The saponifiable constituents identified by GC/MS technique (Table 2), showed the presence of 15 components representing 92.56%. The major component is hexadecanoic acid (19.93%).

Table. 1: Unsaponifiable components of *C. medica* leaves detected by GC/MS.

Peak No	Identified hydrocarbons/sterols	Rt (min.)	Molecular formula	Molecular Weight	Base peak	%
1.	2,4-bis(1,1-dimethylethyl) phenol	25.04	$C_{14}H_{22}O$	206	191	0.81
2.	4,5,7,7-tetrahydro-4,4,7-trimethyl-2(6H) benzofuranone	25.36	$C_{11}H_{16}O_2$	180	111	1.70
3.	1-Hexadecene	26.34	$C_{16}H_{32}$	224	41	1.0
4.	Heptadecane	28.96	$C_{17}H_{34}$	238	57	0.38
5.	1-Octadecene	31.05	$C_{18}H_{36}$	252	41	5.42
6.	Anthracene	31.26	$C_{14}H_{10}$	178	178	0.5
7.	2(4H)-benzofuranone, 5,6,7,7-tetrahydro-6-hydroxy-4,4,7-trimethyl	32.06	$C_{11}H_{16}O_3$	196	111	1.9
8.	2-pentadecanone, 6, 10, 14-trimethyl	32.62	$C_{18}H_{36}O$	268	43	4.86
9.	Nonadecane	33.47	$C_{19}H_{40}$	268	57	0.3
10.	Normuscone	34.23	$C_{15}H_{28}O$	224	43	0.32
11.	Cyclopentanemethanol, 2-methyl-5-(1-methylethenyl)	34.40	$C_{10}H_{18}O$	154	43	0.96
12.	9-Heneicosene	37.45	$C_{21}H_{42}$	294	41	0.66
13.	Tetrahydropyran-4-yl-[exo-(2-norbornyl)]-ether	37.72	$C_{12}H_{20}O_2$	196	85	0.4
14.	1-decanol,10-[(tetrahydro-2H-pyran-2-yl)oxy]	37.99	$C_{15}H_{30}O_3$	258	85	0.7
15.	Phytol	38.97	$C_{20}H_{40}O$	296	71	2.37
16.	17-pentatriacontene [1-docosene]	39.31	$C_{35}H_{70}$	490	97	3.01
17.	Trans decahydro-1-naphthalene[L-trans-1-decalone]	40.29	$C_{10}H_{16}O$	152	71	0.67
18.	n-Eicosane	41.24	$C_{20}H_{42}$	282	57	1.96
19.	n-Heneicosane	53.08	$C_{21}H_{44}$	296	57	16.70
20.	Docosane	51.94	$C_{22}H_{46}$	310	57	2.04
21.	Tricosane	42.96	$C_{23}H_{48}$	324	43	2.18
22.	Pentacosane	44.64	$C_{25}H_{52}$	352	57	0.95
23.	Heptacosane	47.79	$C_{27}H_{56}$	380	57	1.72
24.	Nonacosane	50.56	$C_{29}H_{60}$	408	57	5.17
25.	Triacontane	52.73	$C_{30}H_{62}$	422	57	0.69
26.	14-β-4-pregnene	52.87	$C_{21}H_{34}$	286	124	0.61
27.	4-methylcholesta-8,14,24-trien-3-O	53.83	$C_{28}H_{43}O$	395	135	0.24
28.	Triacontanol	55.68	$C_{30}H_{62}O$	438	57	5.02
29.	Ergost-22-en-3-ol	56.83	$C_{28}H_{48}O$	400	289	0.96
30.	Non identified	56.96	--------	--------	------	0.55
31.	Stigmasterol	57.35	$C_{29}H_{48}O$	412	57	0.38
32.	β-sitosterol	58.37	$C_{29}H_{50}O$	414	57	4.03
33.	γ-sitosterol	58.11	$C_{29}H_{50}O$	414	57	2.16

Table. 2: Components of fatty acid methyl ester (FAME) of leaves of *Citrus medica* L. detected by GC/MS.

Peak No	Identified FAME	Retention time	Molecular formula	Molecular Weight	Area%
1.	Dodecanoic acid methyl ester	12.82	$C_{13}H_{26}O_2$	214	3.19
2.	Tridecanoic acid methyl-ester	18.30	$C_{14}H_{28}O_2$	228	6.43
3.	Pentadecanoic acid, methyl ester	20.73	$C_{16}H_{32}O_2$	256	0.92
4.	Pentadecanoic acid 14-methylester	23.59	$C_{17}H_{34}O_2$	270	16.41
5.	Hexadecanoic acid, methyl ester	23.97	$C_{17}H_{34}O_2$	270	19.93
6.	9-Ethyl hexadecenoic acid methyl ester	24.38	$C_{18}H_{34}O_2$	282	1.70
7.	14-Methylhexadecanoic acid methyl ester	25.07	$C_{18}H_{36}O_2$	284	1.24
8.	Heptadecanoic acid methyl ester	25.76	$C_{18}H_{36}O_2$	284	1.09
9.	Octadecanoic acid methyl ester	28.44	$C_{19}H_{38}O_2$	298	9.26
10.	9-Octadecenoic acid methyl ester,	28.86	$C_{19}H_{36}O_2$	296	5.88
11.	9,12-Octadecadienoic acid methyl ester	30.20	$C_{19}H_{34}O_2$	294	10.81
12.	9,12,15-Octadecatrienoic acid methyl ester	31.80	$C_{19}H_{32}O_2$	292	12.78
13.	Eicosanoic acid methyl ester	32.41	$C_{21}H_{42}O_2$	326	1.25
14.	Docosanoic acid methyl ester	36.36	$C_{23}H_{46}O_2$	354	1.07
15.	Tetracosanoic acid methyl ester	40.07	$C_{25}H_{50}O_2$	382	0.60

Table. 3: Effect of petroleum ether extract on hemoglobin (Hb), red blood cell count (RBCs) and total leukocytic count (TLC) in mice.

Groups	Dose (g/kg) p.o.	Hb (g/dl)	RBCs (10^6/mm^3)	TLC (10^3/mm^3)
Control	Saline	11.2± 0.14	3.8±0.03	5.1±0.29
C. medica (Petroleum ether extract)	2	10.9± 0.18	3.7±0.05	4.9±0.25

Values represent the mean ± S.E. of five mice for each group.
No significant difference from control (Dunnett's test, P< 0.05).
No significant difference from control (Independent sample t-test, P< 0.05).

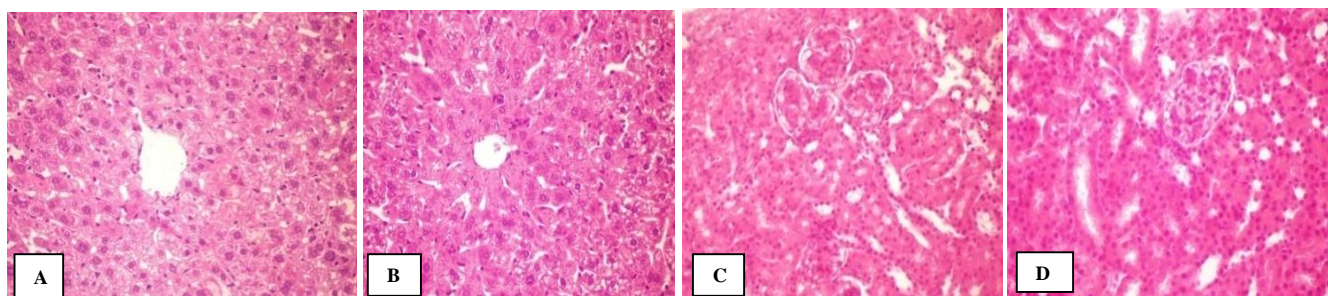**Table. 4:** Effect of petroleum ether extract on relative uterine weight in rats.

Groups	Dose / Route of administration	Relative Uterine Weight (g)
Control	1% Tween 80	1.6 ± 0.06
Ovariectomized control	Tween 80	1.3 ± 0.07
Petroleum ether extract	200 mg/kg/day, p.o	1.4 ± 0.13
Petroleum ether extract	400 mg/kg/day, p.o.	1.7 ± 0.11*
Estriol	1 µg/rat/day, s.c.	1.5 ± 0.07

Values represent the mean ± S.E. of seven rats for each group.
* P< 0.05: Statistically significant from ovariectomized control (Dunnett's test).

Table. 5: Effect of petroleum ether extract on oestrus cycle phases in rats.

| Groups	Dose / Route of administration	Oestrus cycle stage
Control (-ve control)	Saline	Estrus (5/7), Metoestrus (2/7)
Ovariectomized (+ve) control	saline	Anoestrus (7/7)
Petroleum ether extract	200 mg/kg/day, p.o	Proestrus (2/7), Metoestrus (2/7), Estrus (3/7)
Petroleum ether extract	400 mg/kg/day, p.o.	Metoestrus (6/7), Dioestrus (1/7)
Estriol	1 µg/rat/day, s.c.	Proestrus (2/7), Metoestrus (3/7), Estrus (2/7)

Fig. 3: Histopathological investigations of the effect of petroleum ether extract on liver in acute toxicity study in mice.
Fig. (3a): Liver of mice from control group showing normal histological structure of hepatic lobule (H&E×400).
Fig . (3b): Liver of mice from petroleum ether extract group showing no histological changes (H&E×400).
Fig . (3c): Kidney of mice from control group showing normal histological structure of hepatic lobule (H&E×400).
Fig. (3d) Kidney of mice from petroleum ether extract group showing no histological changes (H&E×400).
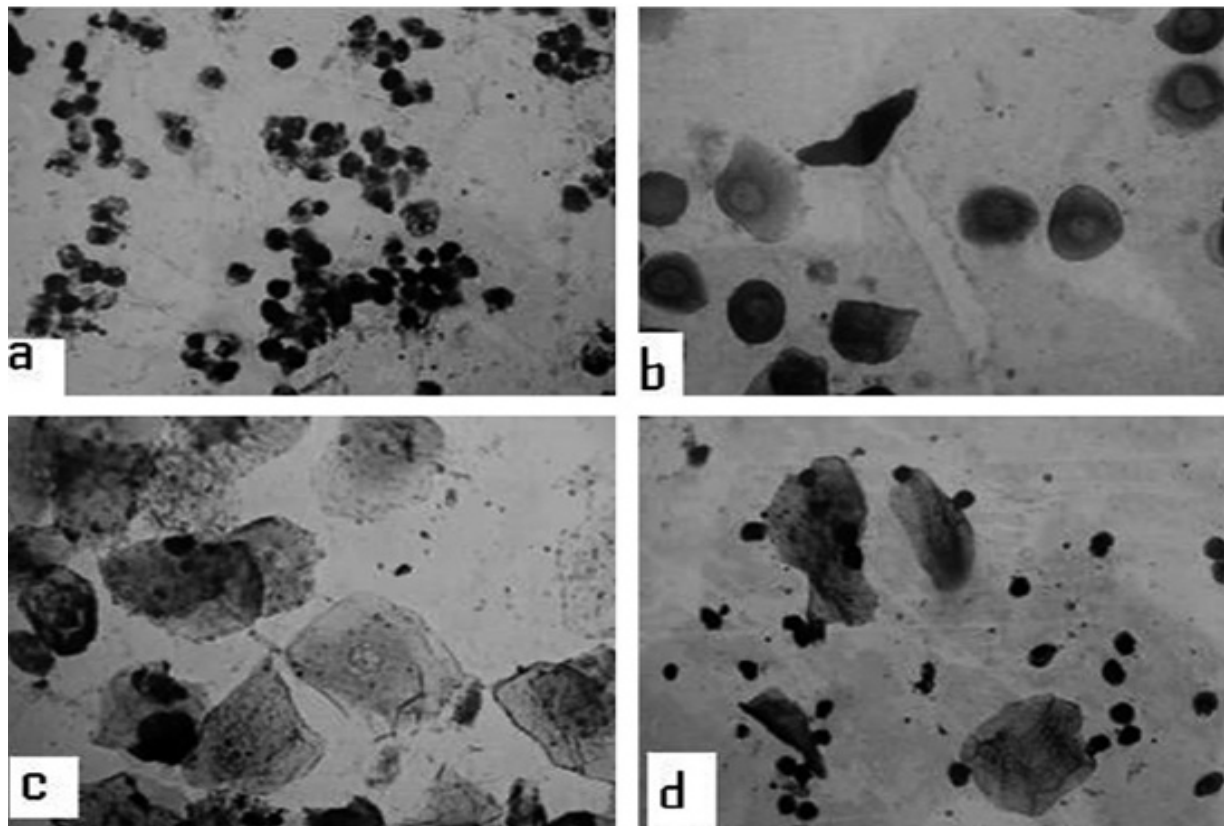
Fig. 4: four phases of estrus cycle of rats; a: the majority of cells are leucocytes then it was labeled as in diestrus phase; b: presence of large no. of nucleated cells estrus indicating proestrus phase; c: estrus phase was confirmed when the smear showed more than 50% of cornified epithelial cells; d: metestrus phase indicated by presence of nutreophils and scattered squamous epithelial cells in the smear.

Biological study

Acute oral toxicity

No signs of toxicity or mortality were recorded in mice following single administration petroleum ether extract of *C. medica* L. leaves at oral doses up to 2 g/kg. Histopathological examination of liver and kidney (Fig. 3) of mice showed no histopathological alterations. Additionally, estimation of hemoglobin, red blood cell count and total leukocytic count of mice showed no significant difference from control (Table 3). Results indicated that the petroleum ether extract was non toxic at doses up to 2 g/kg and accordingly, the safe therapeutic doses used for the evaluation of its possible estrogenic activity are 400 and 200 mg/kg.

Histopathological investigations (Effect of petroleum ether extract on liverand kidney in acute toxicity study in mice)

Estrogenic activity

Oral administration of *C. medica* extract to ovariectomized immature female Wistar rats for 7 days in a dose of 400 mg/kg resulted in significant increase in the uterine weight (g) (1.7 ± 0.11) when compared with ovariectomized control rats (1.3 ± 0.07) (Table 4, Fig 4).

Examination of vaginal smears obtained from rats administered either *C. medica* extract or estriol; showed different phases of oestrus cycle following (Table 5). Untreated ovariectomized control rats (+ve control) revealed anoestrus (no cornified cells). Petroleum ether extract when administered orally at 400mg/kg/day, exhibited a significant change in estrus cycle, 6 rats from the 7 were in Metestrus stage and one in Diestrus, while the group receiving the standard Estriol, showed 2 rats in Proestrus, 3 rats in Metestrus and 2 in Estrus cycle stage (Table 5). These results denote that *C. medica* extract has estrogenic activity.

DISCUSSION

Petroleum ether extract of *Citrus medica* leaves exhibited a significant estrogenic activity $(P<0.05)$ at a dose of 400mg/kg b.wt. The duration of estrus cycle in rats is normally 4-5 days. During a normal rat estrus cycle, there are three cell types. Presence or absence of these types of cells and their relative proportions determine the stage of estrus cycles phase. Estrus cycle is controlled by the synthesis of estrogen in the ovary. Oral administration of petroleum ether extract at 400mg/kg b.wt increased the weight of the uterus and showed estrogen like activity in the ovariectomized rats in 2 cell types phases (Metestrus, During this phase, the signs of estrogen stimulation subside and the corpus luteum starts to form and Diestrus, which is characterised by the activity of the corpus luteum). Earlier studies have shown that unsaturated fatty acids, but not saturated fatty

acids, modulate estrogen and/or ER(s) by alterations in estradiol binding to receptors and/or by cleaving native ER(s) (Borras and Leclercq; 1992). Phytoestrogens are of biological interest because they exhibit oestrogenic activity, both in vitro and in vivo, by weakly binding to oestrogen receptors (Adlercreutz, 1990).

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 (Sharangouda, 2007), this agree with our finding which showed an increase in uterine weight by both doses and more with the higher (400mg/kg b.wt) and the results indicated the potent estrogenic nature of petroleum ether extract of *Citrus medica* seeds as antifertility agent. Petroleum ether extract has been shown to have the most potent antifertility effect out of the three extracts tested in our laboratory (Sharangouda; 2007). Nonesterified fatty acids may influence cell growth and proliferation by modifying membrane fluidity (Burns *et al;* 1979). 10-hydroxy-trans-2-decenoic acid, 10-hydroxydecanoic acid, trans-2-decenoic acid and 24-methylenecholesterol were reported to induce mild hypertrophy of the luminal epithelium of the uterus, but were not associated with an increase in uterine weight. These findings provide evidence that these compounds contribute to estrogenic activity (Kazu-Michi *et al;* 2008). It has been reported that estrogen administration leads to increase in synthesis of nuclear mRNA, and increased concentration of specific mRNA (O'Malley and Means, 1974).

Evidence from experiments on a number of organs responsive to steroid hormones indicates that steroids bring about their effects on protein synthesis by specifically increasing the concentration of mRNA for these proteins mainly by transcriptional regulation (Walker and Kaye, 1981).

Petroleum ether (hydrocarbons) may exert their estrogenic effects by inhibiting E2 metabolism. As sulfation of E2 by estrogen sulfotransferase (SULT1E1) is an important pathway for E2 inactivation, inhibition of SULT1E1 may lead to an increased bioavailability of estrogens in tissues expressing this enzyme. They may increase the bioavailability of E2 through inhibition of E2 inactivation in target tissues (Kester *et al;* 2002).

Other properties include inhibition of tyrosine kinase, epidermal growth factor, malignant cell proliferation, differentiation and angiogenesis. These properties make them strong candidates for a role as natural protective compounds against cancer (Setchell *et al;* 1981).

Phytoestrogens represent just one of many important bioactive non-nutrients found in many plants commonly consumed in the human diet. The myriad of biological properties that have been associated with phytoestrogens has resulted in the current euphoria over their potential for the prevention and/or treatment of many hormone-dependent diseases. Animal and in vitro studies convincingly argue a case for positive effects from phytoestrogens in many disease states. However, the clinical data supporting many of the currently claimed health benefits of phytoestrogens remain to be established definitively. Nevertheless, the limited studies thus far performed in humans clearly confirm that diet can have

significant hormonal effects and that these may be of benefit in the prevention of many of the common diseases.

CONCLUSION

Petroleum ether extract of *Citrus medica* L. leaves could be useful as a safe natural source for estrogenic activity for Post-menopausal women.

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