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STUDY OF ANTI-FERTILITY EFFECT OF LEMON SEEDS *(CITRUS LIMONUM)* IN FEMALE ALBINO MICE

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Abstract : Among the citrus species, *Citrus-limonum* is native of North West region of India. The petroleum ether, alcoholic and aqueous extracts of *Citrus-limonum* (lemon) seeds were investigated for anti-fertility effect in female albino mice. The extracts were administered orally for 7 days after insemination (i.e. post-ovulatory test). The control group received 4% gum acacia. The animals were examined for implantation sites on 10th day of pregnancy and the number of pups delivered at term for each group was recorded. The alcoholic extract showed significant anti-fertility effect as compared to petroleum ether and aqueous extracts. The alcoholic extract was subjected for fractionation and the fractions were again tested for their anti-fertility effect. The fraction of ethyl-acetate (12–25 fractions) showed most encouraging anti-fertility activity. In second part of the study, the alcoholic extract and its ethyl-acetate fraction (12–25 fractions) were subjected to evaluation of their mechanism of action and it was found that their principal mode of action is as an anti-zygotic agent. Withdrawal of the test drug, resulted in complete restoration of fertility. Thus the ethyl-acetate fraction (12–25 fractions) of alcoholic extract of lemon seeds exerted reversible anti-fertility effect in female mice by virtue of its anti-zygotic action.

Key words : *citrus-limonum* post ovulatory test anti-zygotic activity

INTRODUCTION

Exploration of drugs having anti-fertility activity is the need of current time, and many time plant extracts have been investigated for their anti-fertility effect in female and male albino mice.

Citrus-limonum commonly known as lemon, belongs to the citrus-Linn (Ruteaceae) family. Lemon peel is used as stomachae and carminative. Anti-pneumonia factor is said to be present in lemon juice and it is also used in the treatment of acute tropical dysentry and diarrhoea. Citrus-fruits are rich in vitamin C and in mineral salts (1).

The species *Citrus-hystrix DC, and Citrus-medica* have been studied for their

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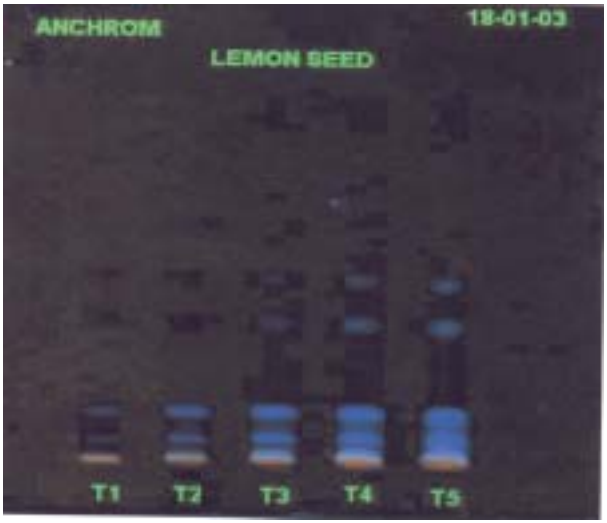
anti-fertility activity (2, 3). In the present study various extracts i.e. petroleum ether, alcoholic and aqueous extracts of seeds of the native species *Citrus-limonum* are investigated for their anti-fertility activity in female mice. The most promising extract was subjected for fractionation, and the fractions obtained were further tested for anti-fertility activity. The extract and the fractions showing maximum anti-fertility activity were further assessed for anti-zygotic, blastocytotoxic, and anti-implantation activity, so as to know the mechanism of their anti-fertility effect.

MATERIAL AND METHODS

Lemon seeds were obtained, after authentication by a pharmacognosist. The air dried powder of seeds of citrus lemon *(Citrus-limonum)* was successively extracted from petroleum ether (b.p 60 to 80 c), 95% of alcohol and distilled water in a soxhlet apparatus. The plant material was extracted for minimum period of 36 to 48 hours with each solvent. The solvent from each extract was recovered by distillation apparatus. They were dried on water bath. The dried extract was cooled at room temperature and weighed (4). Their suspensions was made in 4% gum acasia in concentration of 20 mg/ml and were screened in female albino mice for their anti-fertility activity. The extract showing maximum anti-fertility activity was further subjected to HPTLC, at M/s Anchrom HPTLC specialists Mumbai on Win-cat-planner Chromatography Camag, Switzerland. The different concentrations showed different intensities confirming the presence of various components in the tracks (Fig. 1), so to separate these components on the basis of HPTLC analysis liquid – liquid fractionation was done by using separating funnel. The fractionation was carried out according to polarity by using various solvents i.e. benzene, ethyl-acetate, and water. The different fractions so obtained were made into suspensions in 4% gum acacia and were again tested for anti-fertility activity. The fractions which showed same components in TLC plates were combined together.

ALCOHOLIC EXTRACT OF LEMON SEED [366 NM]



T1 = Alcoholic Extract of Lemon Seed = 128.80 µg.
T2 = Alcoholic Extract of Lemon Seed = 322.00 µg.
T3 = Alcoholic Extract of Lemon Seed = 644.00 µg.
T4 = Alcoholic Extract of Lemon Seed = 966.00 µg.
T5 = Alcoholic Extract of Lemon Seed = 1288.00 µg.

Fig. 1 : Showing various components in alcoholic extract of lemon seed when HPTLC analysis was done.

Adult albino mice of Wistar strain weighing between 10–20 gms housed in clean environment under 12 hour light and

12 hour dark cycle, having free access to food pellet which contains 15% protein, 5.5% fat, and 70% carbohydrate with adequate quantities of Vitamins and Minerals, and water *ad-libitum*, were used after approval of the protocol by the Institutional Animal Ethical Committee.

Estrous cycle of each animal was followed for two cycles before including animal in to the study. This ensured that animals were of proven fertility.

For establishing male breeder colony, males were mated with fertile females. Autopsy of mated females were done on 10th day of gestation. Only males that sired normal litters were designated as proven breeder males. These male and female mice used in the study.

Anti-fertility testing in female mice was done by the post-ovulatory test (5, 6). Vaginal smears were examined daily and the animals in pro-estrous phase of the estrous cycle were kept overnight or cohabited in a ratio of 1:2 with known fertile males. The females were examined for evidence of copulation every-day morning. Those mice which showed a vaginal plug or thick clumps of spermatozoa in their vaginal smears, were separated and the day was labeled DAY-ONE of pregnancy.

Seventy such selected animals were divided into seven groups of 10 animals each. The groups received following treatment orally from day 1 to day 7 of pregnancy.

No.	Group	Treatment
1.	Group I	The control group received gum-acacia 4% from day 1 to day 7 of pregnancy.
2.	Group II (a)	Petroleum ether extract 200 mg/kg/day.
3.	Group II (b)	Petroleum ether extract 500 mg/kg/day.
4.	Group III (a)	Alcoholic extract 200 mg/kg/day.
5.	Group III (b)	Alcoholic extract 500 mg/kg/day.
6.	Group IV (a)	Aqueous extract 200 mg/kg/day.
7.	Group IV (b)	Aqueous extract 500 mg/kg/day.

Oral feeding was done by a feeding needle. The animals were laparotomised under light ether anaesthesia on 10th day of pregnancy and two horns of the uterus were examined for implantation sites. The abdominal wound was sutured in layers and the animals were allowed to go to full term. After delivery the number of pups born was noted and examined for evidence of teratogenicity. The same procedure was repeated for various fractions of the most effective extract.

In second phase of the study, forty adult female albino mice of proven fertility weighing between 10 to 20 gms were included in the study, these were divided into four groups of 10 animals each. The groups were treated as under.

No.	Group	Treatment
1.	Group I	Received gum acacia 4% from day 1 to day 7 of pregnancy.
2.	Group II	Received alcoholic extract 200 mg/kg/day from day 1 to day 3 of pregnancy (to detect anti-zygotic or early abortificient effect).
3.	Group III	Received alcoholic extract 200 mg/kg/day on day 4 and day 5 of pregnancy (to detect blastocytoxic effect).
4.	Group IV	Received alcoholic extract 200 mg/kg/day on day 6 and day 7 of pregnancy (To detect anti implantation effect).

Implantation sites and number of pups born were recorded as described above.

The same procedure was repeated for the (most effective fraction i.e.) the ethyl-acetate fraction (12–25 fractions) of alcoholic extract of lemon seeds.

After a washout period of one month, female mice were mated to examine reversibility of anti-fertility action of the test drugs.

The ethyl acetate fraction (12–25 fractions) of alcoholic extract was again subjected to HPTLC to identify the number of components.

RESULTS

In the control group receiving gum acacia 4% suspension from day 1 to day 7 of pregnancy, all mice showed implantation sites and all were delivered.

The petroleum ether extract at the two dose levels of 200 mg/kg and 500 mg/kg body weight showed 20% reduction in pregnancy. The aqueous extract showed 40%

TABLE I: Effect of various extracts of lemon seeds on fertility in female albino mice when fed orally from day 1 to day 7 of pregnancy.

Group	*Treatment*	*Dose (mg/kg)*	*No. of mice pregnant/treated*	*No. of implants in individual mouse*	*(No. of mice delivered) No. of pups*	*Percentage of reduction in pregnancy*
Group I	Control	–	10/10	5,6,8,4,7 6,6,7,7,5	(10),5,6,8,4,7 6,6,7,7,5	0%
Group II (a)	Petroleum ether extract	200	8/10	5,0,8,4,7 9,6,0,8,10	(8),5,0,8,4,7 9,6,0,8,10	20%
Group II (b)	Petroleum ether extract	500	8/10	0,6,7,7,6 8,0,10,4,11	(8),0,6,7,7,6 8,0,10,4,11	20%
Group III (a)	Alcoholic extract	200	2/10	0,0,0,4,0 6,0,0,0,0	(2),0,0,0,4,0 6,0,0,0,0	80%
Group III (b)	Alcoholic extract	500	2/10	0,0,0,0,7 10,0,0,0,0	(2),0,0,0,0,7 10,0,0,0,0	80%
Group IV (a)	Aqueous extract	200	6/10	5,0,6,8,0 12,7,0,5,7	(6),5,0,6,8,0 12,7,0,5,7	40%
Group IV (b)	Aqueous extract	500	6/10	0,6,7,0,7 10,0,0,4,8	(6),0,6,7,0,7 10,0,0,4,8	40%

reduction at both the dose levels. While the alcoholic extract showed 80% reduction in pregnancy at both the doses (Table I).

Results obtained with various fractions of the alcoholic extract revealed that the fraction of ethyl acetate (12–25 fractions) was effective in reducing 80% pregnancy when administered from day 1 to day 7 of pregnancy. Where as the ethyl acetate fraction (1–7 fractions) showed 20% and (8–11 fractions) showed 40% reduction in pregnancy respectively, the benzene fraction (21–40 fractions) showed 20% reduction in the pregnancy and the benzene fraction (1–20 fractions) showed no reduction in the pregnancy (Table II).

Results of second phase of the study revealed that the alcoholic extract in the dose of 200 mg/kg reduced pregnancy by 80% when administered from day 1 to day 3 of pregnancy, while in the other two groups i.e. Group III and Group IV, there was only 40% and 60% reduction in pregnancy respectively (Table III).

Similarly ethyl acetate fraction (12–25 fractions) when administered from day 1 to day 3 of pregnancy resulted in 80% reduction in pregnancy, where as administration on 4th to 5th and 6th to 7th day of pregnancy led to only 40% and 20% reduction in pregnancy respectively (Table IV).

TABLE II: Effect of various fractions of alcoholic extract of lemon seeds on fertility in female albino mice when fed orally at a dose of 200 mg/kg from day 1 to day 7 of pregnancy.

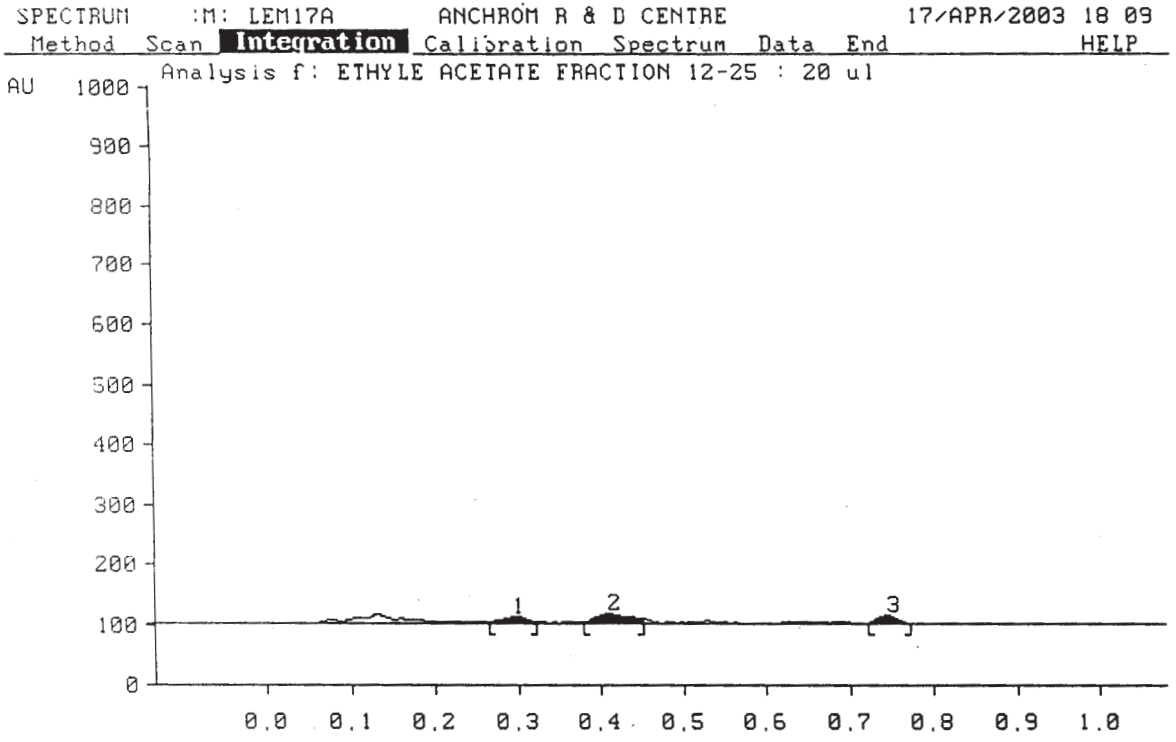
Treatment	*No. of mice pregnant/treated*	*No. of implants in individual mouse*	*(No. of mice delivered) No. of pups*	*Percentage of reduction in pregnancy*
Control	5/5	6,5,8,5,6	(5),6,5,8,5,6	0%
Benzene fraction (1-20 fractions)	5/5	7,5,4,5,8	(5),7,5,4,5,8	0%
Benzene fraction (21-40 fractions)	4/5	4,9,0,5,7	(4),4,9,0,5,7	20%
Ethyl acetate fraction (1-7 fractions)	4/5	6,0,5,5,7	(4),6,0,5,5,7	20%
Ethyl acetate fraction (8-11 fractions)	3/5	8,0,0,6,5	(3),8,0,0,6,5	40%
Ethyl acetate fraction (12-25 fractions)	1/5	4,0,0,0,0	(1),4,0,0,0,0	80%

TABLE III: Effect of alcoholic extract of lemon seeds on fertility in female albino mice when fed orally in a dose of 200 mg/kg for various days of pregnancy.

Group	*Treatment*	*Period (days) of treatment during pregnancy*	*No. of mice pregnant/ treated*	*No. of implants in individual mouse*	*(No. of mice delivered) No. of pups*	*Percentage of reduction in pregnancy*
Group I	Control	1–7	10/10	6,5,8,5,6 7,8,8,7,6	(10),6,5,8,5,6 7,8,8,7,6	0%
Group II	Alcoholic extract	1–3	2/10	4,0,0,0,0 0,6,0,0,0	(2),4,0,0,0,0 0,6,0,0,0	80%
Group III	Alcoholic extract	4–5	6/10	0,5,7,0,8 8,10,0,0,9	(6),0,5,7,0,8 8,10,0,0,9	40%
Group IV	Alcoholic extract	6–7	4/10	6,0,8,0,0 0,0,5,10,0	(4),6,0,8,0,0 0,0,5,10,0	60%

TABLE IV: Effect of ethyl acetate fractions (12-25 fractions) of alcoholic extract of lemon seeds on fertility in female albino mice when fed orally in a dose of 200 mg/kg for various days of pregnancy.

Group	*Treatment*	*Period (days) of treatment during pregnancy*	*No. of mice pregnant/ treated*	*No. of implants in individual mouse*	*(No. of mice delivered) No. of pups*	*Percentage of reduction in pregnancy*
Group I	Control	1–7	10/10	5,6,8,4,7 6,6,7,7,5	(10),5,6,8,4,7 6,6,7,7,5	0%
Group II	Ethyl acetate fraction (12-25)	1–3	2/10	5,0,0,0,0 0,8,0,0,0	(2),5,0,0,0,0 0,8,0,0,0	80%
Group III	Ethyl acetate fraction (12-25)	4–5	6/10	0,9,0,5,7 0,0,6,8,11	(6),0,9,0,5,7 0,0,6,8,11	40%
Group IV	Ethyl acetate fraction (12-25)	6–7	8/10	6,0,6,7,7 0,8,11,6,12	(8),6,0,6,7,7 0,8,11,6,12	20%



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Track 6, Analysis f: ETHYLE ACETATE FRACTION 12–25 : 20 µl

Peak	start		max			end		area	
#	Rf	H	Rf	H	[%]	Rf	H	F	[%]
1	0.27	3.2	0.29	12.9	28.07	0.32	3.2	319.5	25.30
2	0.38	2.9	0.41	18.6	40.46	0.45	8.5	645.6	51.13
3	0.72	1.1	0.75	14.5	31.48	0.78	0.3	297.5	23.56

Total height = 46.0 total area = 1262.6

In all the groups the number of pups delivered was equal to the number of implantation sites found in each mice. There was no evidence of teratogenicity.

All the female mice used in the experiment showed 100% fertility when they were mated with males one month after the experiment.

HPTLC of the ethyl acetate fraction (12–25 fractions) showed presence of three components (Fig. 2).

DISCUSSION

In the present investigation petroleum ether, alcoholic and aqueous extracts of *Citrus limonum* seeds were studied for their anti-fertility activity in female albino mice. The alcoholic extract was found to be most effective amongst the three extracts. Results of present study are in conformation with results obtained with *Citrus-hystrix DC,* which is one of the species of citrus (Rutaceae) family. Ethanolic extract of *Citrus-hystrix DC* in a dose of 1 gm/kg resulted in abortificient effect in female albino rats (2). *Citrus medica* a species of the same family, however failed to show anti-fertility effect in female albino rats in a dose of 200 mg/kg (3).

The ethyl acetate fraction (12–25 fractions) was found to be the most active fraction of the alcoholic extract.

The sequence of events in mice after mating consists of following stages.

Stage	2 Cell Stage	4 Cell Stage	8 Cell Stage	Blastocyst	Implantation
Day	2 & 3	Late 3	Late 4	5	Late 5

Thus zygote is formed during first 3 days of pregnancy, blastocyst formation takes place within 5 days and by the end of 5th day of pregnancy implantation is completed (7). Hence to detect the exact mode of anti-fertility action i.e. anti-zygotic, blastocytotoxic, or anti-implantation, the mice were given the test drugs for different periods of pregnancy. Results revealed that the mechanism of anti-fertility acitivity of the alcoholic extract and its ethyl-acetate fraction (12–25 fractions) is their anti-zygotic or early abortificient action, as maximum reduction in pregnancy was seen when the extract or the fraction was administered for first three days of pregnancy, while administration for subsequent days of pregnancy failed to produce significant reduction in the pregnancy. Moreover, there was no post implantation loss of conceptus as number of pups delivered was found to be equal to number of implantation sites in individual mice. There was no evidence of teratogenicity.

Reversibility after stopping the treatment is one of the major criteria of a good contraceptive agent. In the present study complete recovery of fertility was observed following withdrawal of the drug.

The different extracts and the different fractions of the alcoholic extract of *Citrus limonum* seeds showed variable efficacy in anti-fertility action. This conforms with the fact that extraction and fractionation leads to change in pharmacological activity of a plant (8). Similar variations in anti-fertility activity of various extracts of the same plant were observed in case of *Daucus corota, Sapindus trifoliatus,* and *Polygonum hydropiper* (9). Ethanolic extract of *Daucus corota* when administered in a dose of 500

mg/kg prevented implantation in 66% of animals where as 50 mg/kg of the chloroform methanol fraction of the same alcoholic extract prevented implantation in 80% of the animals (9). The alcoholic extract of *Polygonum hydropiper* prevented implantation in 60% of animals at a dose of 200 mg/kg, while the petroleum ether fraction of the same alcoholic extract prevented implantation in 80% of the animals at half of the dose 100 mg/kg (9). Similarly alcoholic extract of the seeds of *Butea monosperma*, when administered in a dose of 100 mg/kg inhibited implantation in all animals, while the petroleum ether, benzene, chloroform, acetone and methanol fractions of the extract did not demonstrate activity comparable to that shown by the total alcoholic extract (10).

The three components observed on HPTLC of ethyl acetate fraction (12–25 fractions) may have a major role in anti-fertility effect of the alcoholic extract. Isolation of these three components however requires a huge quantity of the extract.

Further phytochemical analysis of alcoholic extract of *Citrus limonum* seeds after trying in more species using more number of animals will possibly make this a prospective anti-fertility agent for clinical trial.

Conclusion

Alcoholic extract of *Citrus-limonum* seeds exerted reversible anti-fertility action on female albino mice. The ethyl acetate fractions (12–25 fractions) turned out to be the most active fraction of the extract. Anti fertility activity of the extract and its fractions was found to be due to their anti-zygotic action.

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