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Post-coital antiimplantation and pregnancy interruption potency of the seeds of *Crotalaria juncea* Linn

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SUMMARY

Petroleum ether, benzene and alcohol extracts of the seeds of *C. juncea* were tested for antiimplantation and pregnancy interruption activities in female albino rats. Of these three extracts, the alcohol extract was found to be the most effective in causing antiimplantation and pregnancy interruption activities. These adverse effects on fertility are reversible upon withdrawal of the extract treatments. The alcohol extract was found to possess estrogenic activity. After subjecting to preliminary phytochemical screening, the alcohol extract showed positive tests for alkaloids, steroids, glycosides, saponins, flavonoides, fixed oils, phenols and tannins.

Key words: *Crotalaria juncea*; Rat; Antiimplantation; Pregnancy; Estrogenic activity

INTRODUCTION

The planetary crisis that is upon us has the population explosion as a major component, and the W. H. O. has put great attention on the search for a safe, cheap and socially acceptable form of contraception. Part of this vital work has focused upon the folk use of antifertility herbs. Many plant preparations are reported to have antifertility regulating properties in ancient Indian literature (Kirtikar and Basu, 1935; Nadkarni and Nadkarni, 1954; Chopra *et el.*, 1956; Anonymous, 1996). A large number of plants have been tested for their antifertility activity in laboratory animals (Dhar *et al.*, 1968; Bhakuni *et al.*, 1969; Garg *et al.*, 1978; Dhawan *et al.*, 1980; Murthy *et al.*, 1997; Sharanabasappa *et al.*, 2002), but so far no single plant is available which can be developed further as a potent antifertility agent. Hence the search continues.

Crotalaria juncea Linn. (Papilionaceae) is a perennial herb, cultivated throughout India. Indian-Ayurvedic medicines state that various parts of *C. juncea* have properties such as analgesic, astringent, emmenagogue, abortifacient and are also used for treatment of skin diseases. It is also mentioned that the seeds are known to have various medicinal properties (Kirtikar and Basu, 1935; Anonymous, 1996; chopra *et al.*, 1956). Ayurvedic physicians have used the seeds of *C. juncea* to prevent fertility in women. But so far, no systematic biological or chemical investigation has been carried out. Hence in the present investigation the various solvent extracts of the seeds of *C. juncea* were subjected to systematic antifertility activities in female albino rats.

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MATERIALS AND METHODS

Plant material

The fresh seeds of *C. juncea* were obtained from a local source during October and November 2000, and authenticated at the Herbarium, Department of Botany, Gulbarga University, Gulbarga (HGUG No. 141), India, where voucher specimens were deposited.

Extraction of plant material

The seeds were shade dried, powdered and subjected to Soxhlet extraction successively and separately from nonpolar to polar solvents i.e., petroleum ether (b.p. 60-80°C), benzene and alcohol (95%). The extracts were concentrated to dryness in

a flash-evaporator (Buchi type) under reduced pressure and controlled temperature (50-60°C). The extracts were stored in refrigerator at 6°C until used for treatment.

Phytochemical tests

The presence of various chemical constituents in plant extracts were determined by preliminary phytochemical screening as described by Kokate (1985).

Animals

Healthy, colony bred, virgin adult female albino rats, Wistar strain (150-200 g) were maintained under controlled standard animal house conditions of temperature, relative humidity, light/dark cycle and fed with commercial diet (Hindustan Lever Ltd., Bombay) and water *ad libitum*. All the extracts were prepared in Tween-80 (1%) in distilled water for complete dissolution and were administered orally to the experimental rats by using intragastric catheter at desired doses. The control animals received an equivalent amount of vehicle only.

Antiimplantation and pregnancy interruption activity

Proven fertile female Wistar rats, with normal estrous cycle (Hariharan, 1980) were selected for this study. Antifertility activity was determined as described by Khanna and Choudhury (1968). The female rats were caged with male rats of known fertility in the ratio of 2:1 on the evening of proestrus and the vaginal smear was examined following morning for the evidence of copulation (Wiest *et al.*, 1964; Hariharan, 1980). The animals exhibiting thick clumps of spermatozoa in their vaginal smear were separated and that day was designated as day 1 of pregnancy. These rats were divided into 8 groups consisting of 5 rats in each group. Group I received vehicle only (Tween-80, 1%) and served as control. Groups II and III received petroleum ether extract at doses of 200 and 400 mg/kg body weight respectively. Groups IV and V received benzene extract at doses of 200 and 400 mg/kg body weight respectively. Groups VI and VII received alcohol extract at doses of 200 and 400 mg/kg body weight respectively. All the above treatments were given from day 1 to 7 of pregnancy and on day 10, laparotomy was performed under light ether anesthesia and semisterile conditions. The uteri were examined to determine the number of implantation sites.

Group VIII received the alcohol extract at a dose of 400 mg/kg body weight orally from day 8 to 14 of pregnancy and was laparotomised on day 16 of pregnancy. The abdominal wound was sutured in layers and the animals were allowed to recover and deliver after full term (Hariharan, 1980). Each pup was weighed and examined for gross defects. The litters were allowed to grow to check post-natal growth and monitered for congenital abnormalities.

Estrogenic/antiestrogenic activity

The alcohol extract was found to be the most active extracts of *C. juncea;* hence it was subjected for detailed investigation for potential estrogenic/ antiesrtogenic activity. Bilaterally ovariectomized immature female rats (Wistar strain) of 25-30 days old, weighing between 30-40 g were divided into 4 groups, each consisting of 6 animals. The first group served as control and received the vehicle only (Tween-80, 1%). The second group received ethinyl estradiol 1 μg/ rat/day in olive oil, subcutaneously. The third group received the alcohol extract at the dose level of 400 mg/kg body weight and the fourth group received, in addition to ethinyl estradiol, above test dose of the alcohol extract. All the above treatments were given for 7 days. On the 8^{th} day of experiment, the animals were sacrificed by decapitation and the uteri were dissected out from surrounding tissues, blotted on filter paper and weighed quickly on a sensitive balance. The tissues were fixed in Bouins fluid, dehydrated and embedded in paraffin. The paraffin sections were cut at 6 μm and stained with Haematoxylin-eosin (Gurr, 1962) for histological observations. The diameter of uterus, thickness of endometrium and height of endometrial epithelium were measured in 20 randomly selected sections using an ocular and stage micrometers by the method described by Deb *et al.* (1946).

Statistical analysis

The statistical analysis was done to determine significant difference of results between treated and control groups using Students *t*-test.

RESULTS

The results of antiimplantation and pregnancy interruption study were given on Table 1.

Effect on implantation and early pregnancy

Among the three extracts of *C. juncea* evaluated for antiimplantation and pregnancy interruption activity, the alcohol extract was found to be more effective. The alcohol extract at a dose of 200 mg/kg body weight inhibited pregnancy in 5/5 rats with mean number of implants 4.6±0.24 ($P<0.001$) and interrupted pregnancy in 5/5 rats. The same extract at a dose of 400 mg/kg body weight significantly inhibited pregnancy in 5/5 rats with mean number of implants 0.6±0.40 ($P<0.001$) and interrupted the pregnancy in 5/5 rats. Benzene extract at the dose level 200 and 400 mg/kg body weight inhibited pregnancy in 5/5 with mean number of implants 7.4±0.24 and 6.0±0.31 respectively. However both the doses of petroleum ether extract was found to be ineffective as the number of implantation sites in these cases were comparable with the control rats.

Effect on late pregnancy

The alcohol extract at a dose of 400 mg/kg body weight was also found to be effective in interruption of pregnancy in rats, which received the treatment from day 8 to 14 of pregnancy.

Terratogenic effects

The decreased weight of the litters and their maximum death within 1 week of parturition was observed in both the doses of benzene extract treated animals. No toxic effects were observed in the animals and their pups either by gross visual examination or in the weight due to petroleum ether and alcohol extract treatments soon after the parturition. Further these experimental animals were exhibited the normal estrous cycle and underwent normal pregnancy when left for breeding and delivered normal litters, indicating that the toxic effects of the extracts on reproductive activities of these animals is reversible.

Estrogenic and antiestrogenic activity of the alcohol extract (Table 2, Figs. 1-4)

The oral administration of the alcoholic extract at 400 mg/kg body weight caused a significant increase in the uterine weight in immature rats (versus control, $P<0.001$). The uterotrophic potency, as judged by the weight of the uterus, was 72% that of ethinyl estradiol. The uterotrophic changes, such as the diameter of the uterus ($P<0.001$), thickness of the endometrium ($P<0.001$) and height of the endometrial epithelium ($P<0.001$), were significantly increased when compared with control rats. The uteri of these rats were inflated and full of fluid resembling the proestrus/estrus uterus. The epithelial layer of the endometrium consisted spindle-shaped cells with basal nuclei. The stroma was represented by oedematous fibroblast type of cells. The treated rats showed open vaginas, while all the control had closed vaginas. Examination of the vaginal smears of the treated rats revealed cornified cells. However, their number was less than that in ethinyl estradiol treated rats.

Simultaneous administration of ethinyl estradiol and alcohol extract 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, $P<0.001$). It appears that the alcohol extract of *C. juncea* seeds has estrogenic activity.

DISCUSSION

In the present study, the petroleum ether, benzene and alcohol extracts of the seeds of *C. juncea* were tested for their antiimplantation, pregnancy interruption and estrogenic properties. Among the three extracts tested, the alcohol extract at dose level of 400 mg/kg body weight was found to be the most potent in reducing the implantation sites and interrupting the pregnancy. The loss of implantation caused by the administration of alcohol extract may be due to antizygotic, blastocytotoxic or antiimplantation activity as described by Hafez (1970).

It is well known that for implantation exact equilibrium of estrogen and progesterone is essential, and any disturbance in the level of these hormones may cause infertility (Psychoyos, 1966). The compound of hormonal values usually disturbs the hormonal milieu in the uterus and provokes an infertility

Table 1. Antiimplantation and pregnancy interruption effect of extracts of *C. juncea* during pregnanc

Group	Treatment (Dose)	No. of rats without implantation sites on laparotomy day	Mean no. of implants ±S.E.	% inhibition of implantation	% rats delivered on full term	Mean no. of litters born on parturition±S.E.	Mean wt. of Litters±S.E.	Mean no. of litters died within 1 week of parturition±S.E.
I	Control	Nil	10.0±0.31	Nil	100	10.0±0.31	5.92±0.08	Nil
II	Petroleum ether Extract (200 mg/kg)	Nil	9.8±0.20	2.0	100	9.8±0.20	5.84±0.19	Nil
III	Petroleum ether Extract (400 mg/kg)	Nil	9.6±0.24	4.0	100	9.2±0.20	5.76±0.20	Nil
IV	Benzene extract (200 mg/kg)	Nil	7.4±0.24**	26.0	100	6.6±0.24**	4.86±0.33	2.4±0.24
V	Benzene extract (400 mg/kg)	Nil	6.0±0.31***	40.0	100	4.6±0.24***	4.76±0.34	3.0±0.31
VI	Alcohol extract (200 mg/kg)	Nil	4.6±0.24***	54.0	100	1.2±0.37***	5.70±0.07	Nil
VII	Alcohol extract (400 mg/kg)	Nil	1.8±0.37***	82.0	000	---	---	---
VIII	Alcohol extract•(400 mg/kg)	Nil	5.2±0.37***	---	000	---	---	---

**$P<0.01$; ***$P<0.001$ when compared to control.
Five animals were maintained in each group.
•Received the treatment from day 8 to day 14 and laparotomised on day 16 of pregnancy.

Table 2. Histometric changes of the uterus and endometrium after treatment with alcohol extract of *C. juncea* (400 mg/kg body weigh)

Group	Treatment	Diameter of uterus (μm)	Thickness of endometrium(μm)	Height of endometrial epithelium (μm)	Uterine weight (mg/100 g)	Vaginal cornification
I	Control (vehicle only) p.o.	396.4±5.33	64.2±2.69	30.5±2.32	50.2±2.06	Vagina not open
II	Ethinyl estrdiol (1 μg/rat s.c.)	1040.6±14.19***	238.8±3.84***	61.2±3.43***	213.4±4.17	Cornified cells
III	Alcohol extract (400 mg/kg p.o.)	608.2±6.47***	173.6±4.30***	45.8±2.36**	154.4±3.99***	Cornified cells
IV	Ethinyl estradiol (1 μg/rat s.c.)+ alcohol extract (400 mg/kg p.o.)	1108.6±6.61***,†††	293.8±6.18***,†††	77.4±5.22***,†	297.4±4.92***,†††	Cornified cells

**$P<0.01$; ***$P<0.001$, when compared to control.
†$P<0.05$; ††$P<0.01$; †††$P<0.001$, when compared with ethinyl estradiol.

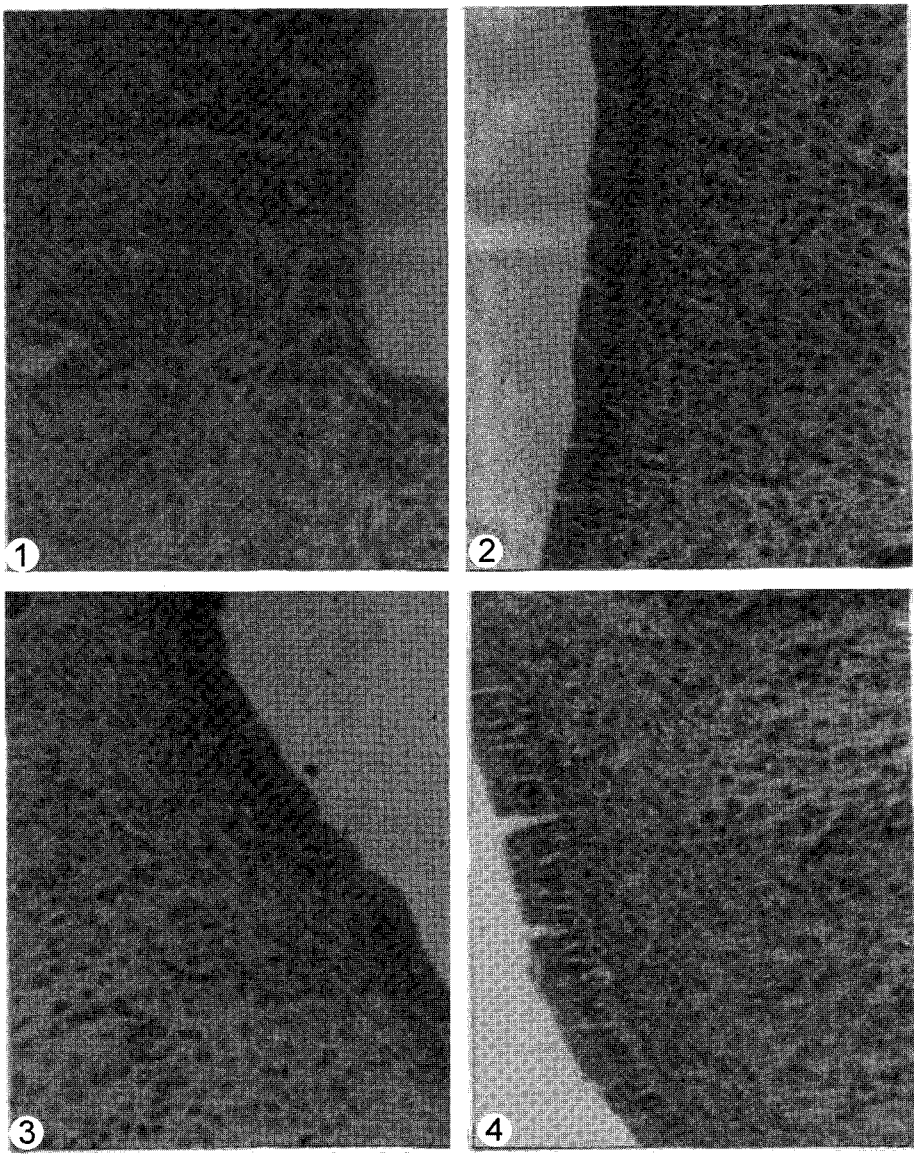

Fig. 1. Control rats showing indistinct and unconspicuous luminal epithelium X 200.
Fig. 2. Rats treated with ethinyl estradiol. Note increase in the height of endometrial epithelium with basal nuclei X 200.
Fig. 3. Rats treated with alcohol extract of *C. juncea* at 400 mg/kg showing well-organized endometrium with loose stroma X 200.
Fig. 4. Rats treated conjointly with ethinyl estradiol and alcoholic extract of *C. Juncea* at 400 mg/kg showing hyper activity of luminal epithelium and stroma X200.

effect. In this study, the histological evidence of the uterus treated with alcohol extract clearly supports an unfavourable uterine milieu. Therefore, antiimplantation activity may be due to estrogenic activity, causing expulsion of ova from the tube, disrupting the luteotrophic activity of the blastocyst (Pincus, 1965; Anderson, 1972).

Testing the alcohol extract further evidences the estrogenic activity. Administration of the alcohol extract to immature ovarictomised rats has caused significant increase in uterine weight, diameter of uterus, thickness of endometrium, height of endometrial epithelium and vaginal epithelial cornification.

In several species including many non-human, progesterone is essential for blastocyst implantation (Murray *et al.*, 1988; Ghosh *et al.*, 1997) and for the maintenance of pregnancy in all phases (Caspo, 1975; Caspo and Erdos, 1976; Caspo and Pulkkinen,

Table 3. phytochemical screening of various extracts of *C. juncea* seeds

Plant constituents	Petroleum ether	Benzene	Alcohol
Alkaloids	--	--	+
Steroids	--	--	+
Glycosides	+	+	+
Saponins	--	--	--
Flavonoides	--	--	+
Fixed oils	+	+	+
Phenols and tannins	--	--	+

+, positive
--, negative

1978; Neef *et al.*, 1984). Inhibition of progesterone synthesis or a blockade of receptor binding will result in the failure of blastocyst implantation and interruption of early pregnancy (Neef *et al.*, 1984; Ghosh *et al.*, 1997), which might have resulted due to administration of various extracts of *C. juncea* seeds.

It is also well established that estrogen secretion during pregnancy is much lowered compared to progesterone, as the former is in the range of nanogram and latter is in microgram (Eto *et al.*, 1962; Hashimoto *et al.*, 1968). In the present study, as the alcohol extract of *C. juncea* has proved to possesses estrogenic activity, the imbalance caused in progesterone and estrogen levels might be the reason for interruption of pregnancy. Withdrawal of these treatments to adult rats has resulted in normal reproductive activities. The decreased weight of newborn litters and their spontaneous death in the benzene extract treated rats may be due to the presence of toxic constituents in that particular extract.

Since various flavonoids have been reported to possess antifertility activity (Khanna and Choudhary, 1968; Bhargava, 1984; Pincus, 1965; Anderson. 1972; Psychoyos, 1966; Hafez, 1970), the antiimplantation activity of the alcohol extract of *C. juncea* seeds might be due to the presence of flavonoids, which possess estrogenic activity.

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