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Research Article

ANTIFERTILITY EFFICACY OF CANNABIS SATIVA LEAVES ON FEMALE ALBINO RATS

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ABSTRACT

According to folklore medicine, the plant *Cannabis sativa* (Cannabinaceae) possesses antifertility activity. The present study was carried out in female albino rat to explore the abortifacient activity of the *Cannabis sativa* leaves. Pregnant rats weighing 140 -210 gm were randomized into four groups of six animal each. Rats were laprotomised on 10th day of pregnancy and the two horns of uteri were examined to determine the implantation sites. Aqueous, alcoholic and chloroform extract of *Cannabis sativa* exhibited significant abortificient activity (9% to 42%). The alcoholic extract at a dose of 400 mg/kg body weight was found to be most effective in causing strong abortifacient activity. The extract also showed estrogenic activity and prolonged the estrous cycle in experimental animal. The extract of *Cannabis sativa* caused a significant decrease in the ovarian and uterine weight, while a non-significant increase in the body weight. There was a slight decrease in the serum estrogen level and an increase in serum progesterone level, while the level of LH and FSH were found to be significantly reduced. The phytochemical screening of the leaves of *Cannabis sativa* revealed the presence of flavonoids, simple phenolics, alkaloids,steroids and saponins.

Keywords: Antifertility Activity, Estrous Cycle, *Cannabis sativa*, Female Albino Rats, Estrogenic Activity.

INTRODUCTION

Since ancient times, plants have been a source of drugs, but scientific medicines tend to ignore the importance of herbal medicine[1]. The World Health Organization suggested that effective, locally available plants can be used as substitutes for drugs. Since the population explosion is a leading cause of poverty and pollution in developing countries, they created a population control programme, which includes studies of traditional medical practices[2].

Cannabis sativa is a member of the family Cannabinaceae. *Cannabis sativa* preparation is known by various names worldwide. It is called Marijuana in America; Bhang, Ganja and Charas in India; Kif in North Africa; Dogga in South Africa; Krori in Tunisia, Habak in Turkey; Hashish in Middle East; Djomba or Liamba in Central Africa and Brazil; Sodom, Tampl, Gum, Gauge and stuff in Kinshasa, Swala and Whiskt in Ghana; Grifa in Mexico and Ma- cohna in some parts of South America[3].

Cannabis sativa leaves are bitter, astringent, tonic, aphrodisiac, alterative, intoxicating, stomachic, analgesic and abortifacient. They are used in convulsions, otalgia, abdominal disorders, malarial fever, dysentery, diarrhoea, skin diseases, hysteria, insomnia, gonorrhoea, colic, tetanus and hydrophobia. Its excessive use causes dyspepsia, cough, impotence, melancholy, dropsy, restlessness and insanity. The bark is tonic, and is useful in inflammations, haemorrhoids and hydrocele. The inflorescence of female plant is intoxicating, stomachic, soporific, abortifacient and useful in convulsions. Seeds are carminative, astringent, aphrodisiac, antiemetic and antiinflammatory. The resin is smoked to allay hiccough and bronchitis. It is useful in insomnia, sick headaches, neuralgia, rnigrain, mania, whooping cough, asthma, dysuria and in relieving pain in dysmenorrhoea and menorrhagia[4, 5, 6].

MATERIALS AND METHODS

Identification and Collection of Plant Material:

Collection of plant was done from the Melghat forest region Amravati. The plant *Cannabis sativa* was identified and authenticated by experts from Botanical Survey of India, Pune where a voucher specimen with herbarium accession number (MAWCASA5) was deposited.

Preparation of Extract:

The leaves of *Cannabis sativa* was collected, shade dried, powdered and subjected to soxhlet extraction with aqueous, alcohol, and chloroform as solvent. The extract was evaporated to near dryness on a water bath, weighed and kept at 4^0C in refrigerator until the experimental testing.

Experimental Animals:

Wistar albino male and female rats approximately 8 weeks of age and weighing 120 to 170 gm were

purchased from theSudhakarraoNaik Institute of Pharmacy,Pusad (M.S). They were housed in a room, maintained at approximately 25 ±2^{0}C. The photoperiod was 12 hrs light and 12 hrs dark cycles. The animals were provided with standard pelleted diet (Trimurti Lab Feeds, Nagpur) and water ad libitum. They were allowed a one week acclimatization period before the experimental session.

All the experimental protocols were met with the approval of Institutional Animal Ethics Committee with registration no. 1060/ac/09/CPCSEA (IAEC/01/2009).

Phytochemical Screening:

The presence of various plant constituents in the extracts was determined by preliminary phytochemical screening as described by Thimmah[7].

Acute Toxicity Study:

The healthy female albino rats, starved for 3- 4 hr were subjected to acute toxicity studies as per OECD 423 guideline[8]. The rats were observed continuously for 2 hrs for behavioral, neurological and autonomic profiles and intermittently after or 24 and 72 hrs for any lethality or Death.

Abortifacient Activity:

The plant extracts were tested in female albino rats for abortifacient activity by the method described by Khanna and Chaudhury[9]. The vaginal smears of caged female rats of known fertility were monitored daily. Unstained material was observed under a light microscope. The proportion among the cells observed was used for the determination of the estrous cycle phases[10]. Female rats were caged with males of proven fertility in the ratio of 2:1, in the evening of proestrous and examined the following day for the evidence of copulation. Rats exhibiting thick clumps of spermatozoa in their vaginal smears were separated and that day was designated as day one of pregnancy. These rats were randomly distributed into 4 groups, a control group and 3 experimental groups of 6 animals each. Group I received vehicle only and served as control. Groups II, III, and IV received aqueous, alcohol and chloroform extracts. On the 10th day of pregnancy the animals were laparotomized under light ether anesthesia using sterile conditions. The two horns of uteri were examined to determine the implantation sites. Thereafter the abdominal wound was sutured in layers. Post operational care was taken to avoid any infection.

The extract to be tested were then fed to operated pregnant rats, at the dose of 100mg / kg, 200 mg/kg and 400 mg/kg body weight specified by an intragastric soft rubber catheter from day 11 upto the 15th day of pregnancy. The animals were allowed to go to full term. After delivery the pups were counted and the antifertility activity of extract was evaluated. Litters were examined for any malformation.

Effect on Estrogenic and Antiestrogenic Activity:

The alcoholic extract at 400 mg/kg was found to be the most active amongst the three treatments in antifertility testing. Hence it was subjected to a detailed investigation for study of estrogenic and 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 under semi-sterile conditions. They were divided into four groups consisting of six rats each. The group I served as control and received vehicle only (olive oil). The group II received ethinyl estradiol in olive oil (1ug/rat/day) orally. The group III received most effective extract at the dose of 400 mg/kg body weight. The group IV received, in addition to ethinyl estradiol a test dose of effective extract. All the above treatments were given for 7 days. On day 8, the rats were sacrificed, the uteri were dissected out, and surrounding tissue was removed. The uteri were blotted on filter paper and weighed quickly on an electronic balance. Estrogenic and antiestrogenic activity was assessed according to the method of Edgren and Calhoun[11], by considering 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 experimental group was fixed in 10% formaline for 24h.The tissue were dehydrated in alcohol, and then embedded in paraffin. The paraffin blocks were sectioned at 6 um and stained with haematoxylin-eosin for histological observation. The diameter of the uterus & thickness of endometrium were measured using an ocular micrometer.

Effect on Estrous Cycle:

The alcoholic extract at 400 mg/kg was found to be the most active amongst the three treatments in antifertility testing. Hence it was also subjected to a detailed investigation for study of estrous cycle, adult female rats (140-180 gm) for 30 days. To study the estrous cycle pattern, animal showing regularity in the normal cycle were separated and were divided in two groups of 6 animals each; Group I (control), received distilled water (Vehicle) and Group II (treated), received alcoholic extract at dose of 200 mg/kg body weight. Vaginal smear using saline solution were taken twice daily during the entire treatment period. Observation of the vaginal opening and the cell type obtained in the vaginal smear was also done. The duration of estrous cycle together with that of various phases was determined[10, 12].

Statistical Analysis:

The data is expressed as mean±SE. Statistical analysis was done by using paired and unpaired Student´s t-test and by using ANOVA[13].

RESULTS

Preliminary phytochemical screening of the pod extract of *Cannabis sativa* revealed the presence of alkaloids, flavonoids, steroids and saponines whereas anthraquinone and tannins were not detected.

Clinical toxicity symptoms such as respiratory distress, salivation, weight loss and change in appearance of hair as well as maternal mortality were not observed at any period of the experiment. Similarly no mortality and changes in the behavioural, neurological and autonomic profile were observed in treated groups of the rats up to highest dose of 4000 mg/kg body weight. Based on acute toxicity result the doses of 100 mg/kg, 200 mg/kg and 400 mg/kg for body weight were selected antifertility evaluation.

Administration of aqueous extract of *Cannabis sativa* leaves at a dose of 100 mg/kg, 200mg/kg and 400 mg/kg body weight showed 9.09%, 15.25% and 23.72% abortifacient activity respectively, However administration of alcoholic extract showed maximum abortifacient activity as compared to aqueous and chloroform extract. The alcoholic extract at a dose level of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight produced 10.90%, 20.00%, and 42.00% abortion. Similarly the chloroform extract also caused pregnancy interceptive activity at a dose level of 100 mg/kg, 200 mg/kg and 400 mg/kg body weight producing 13.20%, 23.52% and 34.92% abortifacient activity respectively (Table 1). Significant abortifacient activity was observed at higher dose of all the extracts as compared to lower dose. The numbers of litters delivered within the experimental group were compared with the control group. No teratogenic effect of drug in the litters or single post natal mortality was observed after the delivery.

Table 1:

Treatment groups (dose, mg/kg body wt)		No. of foetus individual rats on day 10	No. of rats delivered (litter size)	No. of resorption in individual rats	No. of resorption (mean±SE)	Abortifacient activity (%)
Control	Vehicle	7,7,9,8,7,4	6(7,7,9,8,7,4)	0,0,0,0,0,0,0	0	Nil
Aqueous extract	100	11,12,6,9,8,9	6(10,11,5,9,7,8)	1,1,1,0,1,1	0.83±0.16***	9.09%
	200	12,9,8,9,10,11	6(10,8,6,8,9,9)	2,1,2,1,1,2	1.5±0.22***	15.25%
	400	12,11,10,9,8,9	6(10,8,8,6,6,7)	2,3,2,3,2,2	2.3±0.21***	23.72%
Alcoholic extract	100	10,9,11,9,8,8	6(9,8,10,9,7,6)	1,1,1,0,1,2	1..00±0.25***	10.90%
	200	11,9,12,10,9,9	6(9,8,9,8,7,7)	2,1,3,2,2,2	2.00±0.25***	20.00%
	400	9,8,9,9,8,7	6(5,4,6,5,5,4)	4,4,3,4,3,3	3.5±0.22***	42.00%
Chloroform extract	100	9,9,8,8,10,9	6(8,8,6,7,9,8)	1,1,2,1,1,1	1.16±0.16***	13.20%
	200	10,9,9,7,7,9	6(8,7,6,5,6,7)	2,2,3,2,1,2	2.00±0.25***	23.52%
	400	12,11,10,9,9,12	6(7,8,7,5,6,8)	5,3,3,4,3,4	3.66±0.33***	34.92%

Table 1: Effect of aqueous, ethanol, ethyl acetate and chloroform extract of *Cannabis sativa* leaves on fertility of female rats fed orally from day 11 to 15 of pregnancy

Values are from 6 animals in each group, P values*:* *<0.05, **<0.01, ***<0.001, when compared between group.

The effect of *Cannabis sativa* extract on the immature rat uterus of control animal presented a typical infantile condition. The administration of ethinyl estradiol provoked significant increase in the uterine wet weight (P<0.001),while the administration of alcoholic extract of *Cannabis sativa* at 400 mg/kg body weight for 7 days did not increase uterine wet weight significantly(P<0.01) as compared to control. Simultaneous administration of ethinyl estradiol and the alcoholic extract of *Cannabis sativa* leaves caused a highly significant increase in uterine weight but the extent of the uterotrophic response was less than that produced by ethinyl estradiol alone(P<0.001). Vaginal opening of alcoholic extract treated animals was slightly open, while all the control rats had closed vaginas. The vaginal smear predominantly showed cornified and nucleated cells in ethinyl estradiol treated rats. The uterotropic changes such as, the diameter of the uterus and thickness of the endometrium was significantly increased in treated rats as compared to control

(P<0.001) (Table 2, 3).

Table 2:

Treatment groups dose, mg/kg body weight		Uterine weight (mg) Mean±S.E	Vaginal cornification (vaginal opening)
Group- I	Control	56.24±1.36	Nil(closed)
Group- II	Ethinyl estradiol(1ug/rat)	166.61 ± 4.80***	+++(open)
Group-III	Alcoholic extract(400mg/kg)	64.32 ± 1.59**	+ to ++(open)
Group- IV	Ethinyl estradiol (1ug/kg)+ Alcoholic extract(400mg/kg)	100.58 ± 1.49***	+++(open)

Table 2: Effect of alcoholic extract of *Cannabis sativa* leaves on uterine weight of female albino rats

Values are from 6 animals in each group, P values*:* *<0.05, **<0.01, ***<0.001, when compared between group.

Table 3:

Treatment groups dose, mg/kg body weight		Diameter of uterus (um) Mean± S.E	Thickness of endometrlum (um) Mean±S.E
Group- I	Control	353.33±1.36	58.83±2.23
Group- II	Ethinyl estradiol (1ug/rat)	636.00±5.66***	244.00±5.75***
Group-III	Alcoholic extract (400 mg/kg)	389.50±2.95***	68.00±2.22**
Group- IV	Ethinyl estradiol (1ug/kg)+ Alcoholic extract (400mg/kg)	527.83±3.51***	209.67±3.24***

Table 3: Effect of alcoholic extract of *Cannabis sativa* leaves on diameter and thickness of endometrium of female albino rats

Values are from 6 animals in each group, P values*:* *<0.05, **<0.01, ***<0.001, when compared between group.

Administration of 400 mg/kg alcoholic extract of *Cannabis sativa* leaves caused an irregular estrous cycle (Table 4), with an increase in the diestrus phase (P<0.001) while the proestrous (P<0.05) and metaestrous phase (P<0.01) was found to be significantly reduced as compared to the normal. Withdrawal of the treatment did not indicate any significant change either in the four phases of the estrous cycle or in the duration of the cycle.

Table 4:

Phases	Proestrus	Estrous	Metestrus	Diestrus	Estrous cycle
Vaginal opening/ cell type obtained in a vaginal smear	25% to 40% Epithelial cells	Above 40% Cornified cells	50%to70% Cornified plus leukocyte	50% to70% Leukocytes plus epithelial cells	----
Group- I Control	0.64± 0. 04	0.80±0.02	0.98±0.02	1.83±0.01	4.21±0.03
Group- II Alcoholic extract 400 mg/kg	0.53+0.02*	0.71+0.01**	0.78±0.02***	3.45±0.10***	5.47±0.05***

Table 4: Effect on estrous cycle of female albino rats after the administration of 400 mg/kg alcoholic extract of *Cannabis sativa* leaves.

Values are from 6 animals in each group, P values*:* *<0.05, **<0.01, ***<0.001, when compared between group.

DISCUSSION

Cytotoxic agents can disrupt pregnancy possibly by interfering with the mitotic division of the fetus, chemical insults both before and after the implantation process can result in pre and post implantation embryonic loss[14, 15]. Therefore, the increase in the number of dead fetus as well as reduced survival ratio is an indication of the abortifacient activity of the extract. Such a high number of dead fetuses suggest a more potent abortifacient activity of the extract.

The implantation index, resorption index and pre- implantation loss are useful indices for evaluating the number of blastocyst implanted on the uterus and the underdeveloped[16]. Therefore, the increase in the resorption index by the extract is an indication of failure in the development of the embryo, a rate which was dose dependent in this study. Such occurrence of fetal resorption suggests that interruption of pregnancy occurred after implantation[14]. All these are indications of the pregnancy terminating potential of the extract

of the Cannabis sativa leaves.

In ovariectomized immature rats, oral administration of alcoholic extract of Cannabis sativa leaves did not altered the weight of the uterus but the provoked vaginal opening which could possibly indicate the estrogenic activity of the extract. This may, however contribute at least in part to the mechanism of abortifacient activity of the plant. The extract also caused a significant increase in the diameter and thickness of endometrium when compared with the control. It was also observed that the alcoholic extract suppressed the action of ethinyl estradiol when administered together. The extract showed a estrogen like activity when given alone but, with ethinyl estradiol, it exhibited a slight antiestrogenicnature. This indicates that the extract acted as a competitive antagonist to the more potent ethinyl estradiol which is in accordance with findings of Badamiet al[17].

In the present study prolongation of diestrus phase suggests the negative influences of the extract on the estrous cycle as this reduces the number of days/ova ovulated during the proestrus and estrous phase[18]. The disruption of the cycle due to the effect of the Riveahypocrateriformis extract on the ovarian and extra-ovarian hormones was reported by Shivalingappaet al[19].Similar observation of prolonged diestrus phase and reduced proestrus phase was also reported by Bakare et al[20]on administration of Citrus aurantifolia in rats, which is consistent with our present findings.

Preliminary phytochemical studies indicated the presence of saponin, flavonoid, alkaloids, steriods and phenolics in Cannabis sativa leaves. Several of these compounds are known to exhibit antifertility activity in rats.[21- 23].

CONCLUSION

In conclusion, all the three extract i.e. aqueous, alcoholic and chloroform of *Cannabis sativa* leavesadministered orally possess antifertility activity. Further studies to identify the bioactive principle of abortifacient activity of the extract are in progress.

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