ijlpr 2020; doi 10.22376/ijpbs/lpr.2021.11.1.P94-99

International Journal of Life science and Pharma Research

Research Article

Pharmacology for Better Medicial Drug

Investigation of Toxic Effect of Ethanolic Extract of *Carthamus tinctorius* Seeds on Spermatogenesis in Male Rats

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Abstract: *Carthamus tinctorius* (Family: Asteraceae) is a medicinal plant and many reported for the treatment of different diseases namely hypertension, arthritis, neurological, kidney disorders, etc. The higher dose (500 mg/kg) or long-term use of herbal medicines may cause serious toxic effects. The toxicity of the medicinal plant is an important concern to the researchers, and they investigate the harmful effects of the natural products for the safety purpose. Hence, the present study was aimed to assess the toxic effects of ethanolic extract (Containing semipolar and polar phytoconstituents) of *Carthamus tinctorius* seeds on spermatogenesis of rats. The seeds of *Carthamus tinctorius* collected from Bareilly, India. The ethanolic extract was prepared from *Carthamus tinctorius* seeds by macerating with 70% ethanol, and extract obtained was used for further studies. The ethanolic extract of *Carthamus tinctorius* was administered to rats at the dose of 100 mg/kg and 200 mg/kg for 45 days, and its toxic effect was determined by assessing the autopsy, body weight, testis, epididymis, seminal vesicle, number of pups, litter weight, serum testosterone, sperm motility and sperm count. The testis were isolated from the exploratory animals for the histopathological studies. The rats treated with the extract showed significant alteration in the autopsy, body weight, testis, epididymis, seminal vesicle, number of pups, litter weight, serum testosterone, sperm motility and sperm count compared to the control group of experimental animals. The histopathological observation of extract treated rats showed variations in testis morphology, supporting the failure in spermatogenesis. The findings concluded that the *Carthamus tinctorius* extract has a toxic effect on the rat testicular tissues without any signs of clinical toxicity.

Keywords: *Carthamus tinctorius*, spermatogenesis, testis, histopathology, sperm motility

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Recieved On 25 June 2020
Revised On 09 September 2020
Accepted On 23 September 2020
Published On 03 October 2020

Funding This research did not receive any specific grant from any funding agencies in the public, commercial or not for profit sectors.

Citation Himanshu Gupta, Dinesh Kumar Sharma, Kamal Kishore Maheshwari , Investigation of Toxic Effect of Ethanolic Extract of Carthamus tinctorius Seeds on Spermatogenesis in Male Rats.(2021).Int. J. Life Sci. Pharma Res.11(1), P94-99 http://dx.doi.org/10.22376/ijpbs/lpr.2021.11.1.P94-99

1. INTRODUCTION

The seminiferous tubules present in the testis produces sperms through spermatogenesis. The spermatogenesis process takes place when male germ cell's evolve through the three particular stages. In the initial stage, spermatogonia multiply to offer ascent to spermatocytes and at the same time keep up their number by replenishment. During the subsequent stage, spermatocytes experience two meiotic divisions promoting the development of haploid spermatids. At the last stage, spermatids experience broad morphological rebuilding to deliver the exceptionally separated germ cells, the spermatozoa. Moreover, the seminiferous tubules experiences a progression of changes in the improvement of spermatozoa inside a given zone of seminiferous epithelium.[1] Sertoli cells create a particular microenvironment for the turn of events and reasonability of occupant germ cells by discharging hormonal and sustenance factors into the adluminal compartment. Also, these give a proficient paracrine flagging component and physical help for them. Some poisonous plant material upset spermatogenesis through a multifaceted guideline and cell collaborations that initiate testicular tissue destruction.[2,3] Presently, the prescriptions of medicinal plants are increased for the healing of various diseases due to fewer side effects. Conversely, it has been reported in many studies that the use of natural products may lead to adverse effects and drug interaction. Hence, medicinal plant toxicity is the chief concern to the researchers to evaluate the adverse effects of plants.[3,5] *Carthamus tinctorius* are deliberated for medicinal plants, and used for the ailment of various diseases. The seeds of *Carthamus tinctorius* (Family: Asteraceae) are opulent in edible oil. This oil has a high nutritive value incorporating palmitic acid, stearic acid, oleic acid (omega 9) and linoleic acid (omega 6). *Carthamus tinctorius* reported innumerable biological and pharmacological activity such as analgesic, purgative, antipyretic, chronic bronchitis, whooping cough, rheumatism, antihypertensive, neuroprotective, anticancer, anticoagulant and immunosuppressive, etc.[6,7] The medicinal properties of herbs depend upon the presence of secondary metabolite. Many studies documented *Carthamus tinctorius* have flavonoids, phenylethanoid glycosides, safflomin C, pre carthamin, carthamin, carbamidine, coumarin, caryophyllene, isocarthamidin, fatty acids, hydroxysafflor yellow A, p-allyl toluene, safflor yellow A, safflamin C, luteolin, 1-acetyl tetralin, heneicosane and steroids as a phytochemical.[8-10] However, some studies show that *Carthamus tinctorius* associated with adverse effects, and sometimes it leads to damage the kidney and brain. On looking above data of toxic effects of *Carthamus tinctorius* seeds, it was planned to evaluate the toxic effect of the Ethanolic Extract of *Carthamus tinctorius* Seeds on the spermatogenesis of rats.

2. MATERIALS AND METHODS

2.1 Animals

The twenty-four Swiss albino rats were selected for studies, which were weighing between 175 to 250 gm with ascertained fertility. The study was approved by the Institutional Animal Ethics Committee (IAEC). The study was conducted under the guideline of CPCSEA with approval No.711/02/a/CPCSEA dated 2002). Before initiation of experiment, the animals were kept at room temperature (22±2˚C) with humidity (50±10%) and a 12 h light and dark cycle.

2.2 Collection and authentication of the crude drug

Seeds of *Carthamus tinctorius* were collected from the local market of Bareilly, India in the month of September 2018 and were identified taxonomically in the Department of Botany, M.J.P. Rohilkhand University Bareilly (U.P) India (Voucher specimen number MJP/Bot/Auth/2018/34).

2.3 Preparation of ethanolic extract

The extract of the seeds of *Carthamus tinctorius* was prepared by maceration of seeds in 70% ethanol solution at 50 °C for 2 hours. The extractions were performed thrice and were filtered and combined. The collected menstrum were evaporated to dryness in a rotary evaporator under reduced pressure. The dried extract was stored under the refrigerator at 4-8 °C until its use.

2.4 Experimental design

The experimental animals were divided into 4 groups, and containing six animals in each group: Group I received saline and served as control. Groups II received an ethanolic extract of *Carthamus tinctorius* (100 mg/kg) for 45 days Group III received an ethanolic extract of *Carthamus tinctorius* (200 mg/kg) for 45 days Group IV also received the ethanolic extract of *Carthamus tinctorius* at 200 mg/kg for 30 days and served as the recovery group The rats of recovery group received the ethanolic extract for the full treatment period of 45 days but for assessing the reversible effect of extract a washout period of 30 days was given.[1]

2.5 Autopsy and organ weights

After completion of the experiment, each rat was sacrificed by dislocating the cervical disengagement. The testicles, seminal vesicles and epididymis were analyzed, liberated from disciple tissue and weighed precisely up to milligram level.[12-15]

2.6 Fertility test

Effective mating (male female proportion 1:2) was completed with all the experimental animals, five days preceding penance period. The effective mating was affirmed in the expected mornings by vaginal plug and spermatozoa in the vaginal smear. The inseminated females were isolated and after the incubation period the number of females delivered, number of litter conceived and fruitfulness rate was noted.[12-15]

2.7 Sperm motility and count

The sperm fluid was withdrawn from rats by making 1 mm incision in the caudal epididymis, and the characteristics of fluid were studied under the microscope. The percentage of Epididymal sperm motility was evaluated by calculating motile spermatozoa per unit area. The counting of Epididymal sperm was done by homogenizing the epididymis in HBSS and counting the sperm by using haemocytometer.[12-15]

2.8 Sperm characteristic analysis

The sperm fluid was withdrawn from rats by making 1 mm incision in the caudal epididymis. The morphology of sperm heads was studied by making sperm suspension in HBSS and stained with a 2% eosin solution, kept for 1 hr. After that the

prepared smear of the above solution by fixing with absolute methanol for 5 min. The two hundred sperms of each rat were studied to determine the morphological abnormalities.[12-15]

2.9 *Testosterone assay*

Serum testosterone assay was carried out using the enzyme immunoassay (EIA) method. The within assay variation was 8.1 % and the sensitivity was 0.3 ng/mL. For the estimation of testosterone blood samples were collected by retro orbital plexus and serum samples were separated by standard procedures and stored at -20°C for subsequent analysis.[16,17]

2.10 *Tissue biochemistry*

The protein and glycogen of the testis and epididymis were estimated, according to methods discussed by Lowry[18] and Montgomery[19].

2.11 Histopathological studies

The testis dissected from rats were fixed in 10% formalin, dehydrated in increasing concentrations of ethanol and then embedded in paraffin. The blocks were cut into different sections by microtone and stained to colour the cells present in the testis. The section of testis were examined under the microscope.[20-22]

3. STATISTICAL ANALYSIS

The findings were presented in Mean ± standard error of mean (S.E.M.) for each group. The statistical analysis of the values were done by SigmaStat (Version 2.03) software. The multiple comparisons, One-way analysis of variance (ANOVA) was used. The significance difference P≤0.05, P≤0.01 and P≤0.001 compared with control group were considered.

4. RESULTS

4.1 Autopsy and organ weights

Ethanolic extracts of *Carthamus tinctorius* at 100 mg/kg and 200 mg/kg showed a significant change (p≤0.01) in the weight of seminal vesicle and epididymis after the treatment period of 45 days compared with the control group (Table 1). However, no significant changes were observed after 30 days of withdrawing the treatment.

4.2 Fertility test

Table 2 exhibited the decline in a ratio between delivered and inseminated females in extract-treated groups, while no major changes observed in the recovery group compared to control groups. However, it was found that normal and healthy delivered pups in rats.

4.3 Effect on spermatozoa indices

4.3.1 Motility

Table 3 illustrated the significant decrease in motility in the extract-treated at a dose of 200 mg/kg compared to the control group. While the rats were treated with 100 mg/kg extract and recovery groups, no significant changes in sperm count were observed.

4.3.2 *Count of Epididymal sperm and sperm head morphology*

The significant decrease (p <0.01) in sperm count was observed in the extract treated rats compared to the control group (Table 3). However, no significant change in sperm count was seen in the recovery group. Further findings showed abnormalities in the sperm head of treated groups compared to control group (Fig 1).

4.3.3 *Serum testosterone levels*

The rats treated with the extracts showed significantly decreased levels of serum testosterone compared to the control group. However, the recovery group exhibits no significant changes in the serum testosterone level.

4.4 Glycogen and protein levels

The rats treated with the extract at the dose of 100 mg/kg showed significant deceased (p<0.01) in glycogen level in testis and epididymis compared to control group rats. The rats administration of the extract at the dose of 200 mg/kg showed significant deceased (p<0.01) in glycogen level in the epididymis compared to control group rats, while slight changes in glycogen of the testis. The rats treated with the extract at the dose of 100 mg/kg and 200 mg/kg showed significant deceased (p<0.001) in protein level in testis and epididymis compared to control group rats. There were no significant changes in the recovery group (Table 5).

4.5 Histopathology of Testis

The histology of *Carthamus tinctorius* treated groups at doses of 100 mg/kg and 200 mg/kg showed maturation distress of spermatozoa at different stages as compared to control groups in which varied stages of spermatogenesis can be seen. But the effects produced in both the groups were patchy. The seminiferous tubules showed the lack of spermatozoa along with the necrosis of the germinal epithelium. Furthermore, there was a reorganization of plasmalemma in the basal portion of a few seminiferous tubules. However, the histological slides of the recovery group showed all the stages of spermatogenesis (Fig 2).

Table 1: Data of *Carthamus tinctorius* extract on body, testicular, epididymal and seminal vesicle weights in wistar rats

Treatment Group	Body weight (gm)		Testis (gm)	Epididymis (gm)	Seminal vesicle (gm)
	Initial	Final			
Group I (Control)	249±17.19	252±11.61	1.98±0.11	0.43±0.03	0.38±0.19
Group II *Carthamus tinctorius* (100 mg/kg)	243±13.02	245±16.21	1.94±0.07	0.21±0.05**	0.17±0.13**
Group III *Carthamus tinctorius* (200 mg/kg)	243±13.34	251±12.81	1.89±0.13	0.19±0.03**	0.21±0.09*

Group IV (Recovery)	245±12.12	249±13.13	1.91±0.08	0.43±0.09	0.36±0.17

*Results are stated as Mean ± SEM (n=6), * p≤0.01 when compared with control group*

Table 2: Efficacy of *Carthamus tinctorius* extract on no. of females delivered/no. of inseminated females, total no. of pups, litter weight and mean percentage fertility (Male:female ratio, 1:2)

Treatment groups	No. of females delivered/no. of inseminated females	Total no. of pups	Litter weight (gm)	Mean percentage fertility (%)
Group I (Control)	12/12	75	9.3±0.83	100.0
Group II *Carthamus tinctorius* (100 mg/kg)	06/12	35	9.1±0.63	50.0
Group III *Carthamus tinctorius* (200 mg/kg)	04/12	43	8.8±0.61	33.33
Group IV (Recovery)	11/12	65	9.1±0.41	90.3

*Results are stated as Mean ± SEM (n=6), * p≤0.01 when compared with control group*

Table 3: Efficacy of *Carthamus tinctorius* extract on sperm motility and sperm count in wistar rats

Treatment Group	Sperm motility (%)	Sperm counts (×10^6/mL)
Group I (Control)	83.58±2.54	61.25±7.45
Group II *Carthamus tinctorius* (100 mg/kg)	82.31±1.41	42.17±5.62**
Group III *Carthamus tinctorius* (200 mg/kg)	72.88±4.31*	50.88±3.62**
Group IV (Recovery)	80.19±2.22	64.67±6.43

*Results are stated as Mean ± SEM (n=6), * p≤0.05, ** p≤0.01, ***p≤0.001 when compared with control group*

Table 4: Effects of *Carthamus tinctorius* extract on serum testosterone level in male rats

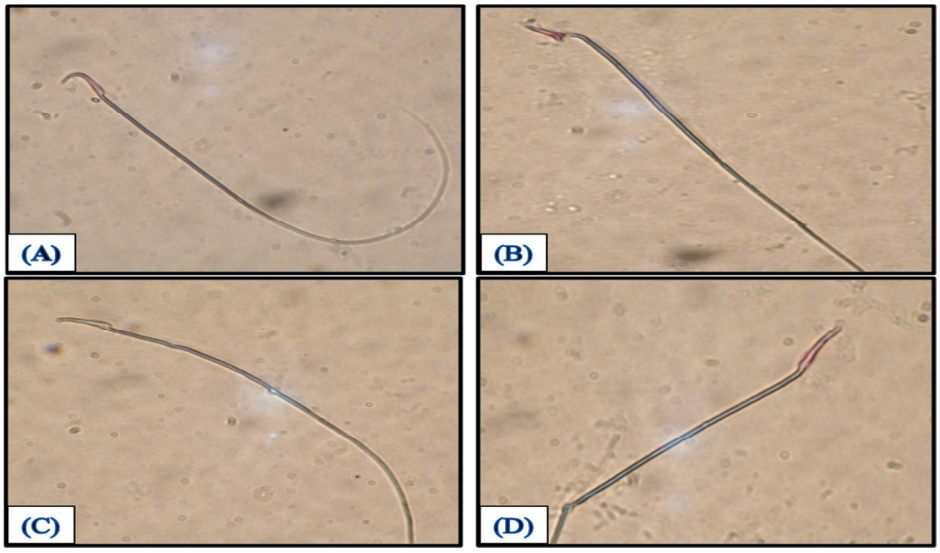
Treatment Group	Serum testosterone level (ng/dL)
Group I (Control)	257±1.8
Group II *Carthamus tinctorius* (100 mg/kg)	89.871±1.3***
Group III *Carthamus tinctorius* (200 mg/kg)	68.13±2.2***
Group IV (Recovery)	255±2.2

*Results are stated as Mean ± SEM (n=6), ***p≤0.001 when compared with control group.*

Table 5: Efficacy of *Carthamus tinctorius* extract on glycogen and protein level in reproductive organs of wistar rats

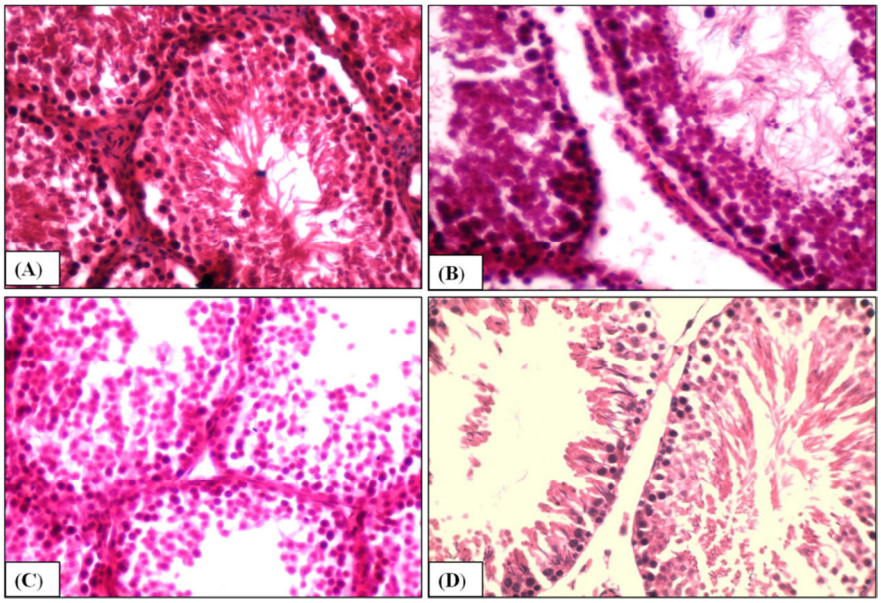
Treatment Group	Glycogen level (µg/mg tissue)		Protein level (µg/mg tissue)	
	In testis	In epididymis	In testis	In epididymis
Group I (Control)	5.71±0.14	2.75±0.11	8.91±0.98	4.61±0.2
Group II *Carthamus tinctorius* (100 mg/kg)	4.02±0.75**	1.92±0.21**	6.64±0.64***	2.42±0.1***
Group III *Carthamus tinctorius* (200 mg/kg)	5.33±0.53	1.84±0.57**	6.42±0.44***	2.85±0.55***
Group IV (Recovery)	5.82±0.81	2.70±0.69	8.86±0.78	4.66±0.2

*Results are stated as Mean ± SEM (n=6), **p≤0.01, ***p≤0.001 when compared with control group.*



Normal sperm (A), sperm with triangular head (B), sperm without hook (C), banana head sperm (D)

Fig 1: Representative photomicrographs of sperms.



a) Group I (Control), b) Group II (Carthamus tinctorius 100mg/kg) shows absence of seminiferous tubules wit necrosis (Arrow), c) Group III (Carthamus tinctorius 200mg/kg) shows absence of seminiferous tubules wit necrosis (Arrow), d) Recovery group display repossession of seminiferous tubules (star)

Fig 2: Photomicrographs of stained histological slides of the testis after 45 days of treatment

5. DISCUSSIONS

In developing countries, more than 80% populations are using herbal drugs due to low toxicity and cost effectiveness. However, distinctive studies reported that the medicinal plants may cause adverse effects. The unusual types of chemical constituents were present in medicinal plants and impart certain medicinal properties to specific organs. Even so, sometimes the phytoconstituents at the higher dose or longer time uses may lead adverse effects. The present study was planned to evaluate the toxic effect of ethanolic extract of *Carthamus tinctorius* on rats spermatogenesis. The rats treated with the ethanolic extract of *Carthamus tinctorius* demonstrated decrease in the weight of seminal vesicles, and epididymis indicates the improper production of semen and sperm cells in the testis. In addition, decreased in the testis weight, litter weight and fertility support the above statement. Morphometric examinations indicated that seminiferous distances across were altogether declining in the extract-treated group. The various studies showed that expanded seminiferous tubule measurement is demonstrative of fluid maintenance coming about because of impeded purging through the efferent channels, though diminished seminiferous distance across may demonstrates germ cell degenerations. The extract initiated the development of multinucleated giant cells in the germinal epithelium. The nearness of multinucleated mammoth cells is demonstrative of backward changes in the germinal epithelium and is brought about by annihilation and loss of the intercellular extensions that are basic for the procedure of spermatogenesis, spermiogenesis and synchronization of germ cell development.[23-25] The toxic substance of extract follows up on three principles testicular objective cells to disturb the spermatogenesis incorporated in the somatic cells, the Leydig, Sertoli cells, and the germ cells. All these cells can be specifically focused by some particular toxic substance that prompts germ cell demise and spermatogenic failure in rodent[26]. But the actual mechanism of testicular toxicity by the ethanolic extracts of *Carthamus tinctorius* was unknown. The current consequence of juvenile germ cells sloughing from the seminiferous tubules demonstrates that this plant may influence sertoli cell capacities. Consequently, the findings of toxicity of spermatogenic, indicates not only from the undeviating effect of ethanolic extract of *Carthamus tinctorius* on germ cell death, but also from alterations to the Sertoli and Leydig cells' function.

6. CONCLUSION

The present study was designed to investigate the toxic effect of ethanolic extract of *Carthamus tinctorius* seeds on spermatogenesis of rats. The experimental animals treated with the ethanolic extract of *Carthamus tinctorius* alters the autopsy, body weight, testis, epididymis, seminal vesicle, number of pups, litter weight, serum testosterone, sperm motility and sperm count along with testis histological structure leads to failure in spermatogenesis. The above findings suggest the toxic effect of *Carthamus tinctorius* on sperm functions and fertility of rats without signs of clinical toxicity. Further in a future, the researchers can perform a study to determine the exact mechanism of testicular toxicity of *Carthamus tinctorius* seeds.

7. AUTHORS CONTRIBUTION STATEMENT

Himanshu Gupta and Dinesh Kumar Sharma conducted the experimental work, while Kamal Kishore Maheshwari has done statistical analysis of experimental results. All authors equally contributed for the preparation of manuscript and approved for the publication.

8. CONFLICT OF INTEREST

Conflict of interest declared none.

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