Form B (per rule 8(a)*

APPLICATION FOR PERMISSION FOR ANIMAL EXPERIMENTS

Application to be submitted to the CPCSEA, New Delhi after approval of Institutional Animal Ethics Committee (IAEC)

Part A

1. **Name and address of establishment:**
   VIGNAN PHARMACY COLLEGE,
   Vadlamudi post, Chebrolu Mandal, Guntur District. AP. 522213. India.
2. **Registration number and date of registration.**
   1499/PO/Re/S/2011/CPCSEA is valid upto 26th July, 2019.
3. **Name, address and registration number of breeder from which animals acquired (or to be acquired) for experiments mentioned in parts B & C.**
   Mahaveer enterprises, Hyderabad. (146/99/CPCSEA)
4. **Place where the animals are presently kept (or proposed to be kept).**
   In-house Animal house of VIGNAN PHARMACY COLLEGE, Guntur.
5. **Place where the experiment is to be performed (Please provide CPCSEA Reg. Number)**
   Pharmacology Laboratory of VIGNAN PHARMACY COLLEGE, Guntur. 1499/PO/Re/S/2011/CPCSEA is valid upto 26th July, 2019
6. **Date on which the experiment is to commence and duration of experiment.**
   Approximate date: dd/mm/yyyy; 000 Days/months/years
7. **Type of research involved (Basic Research / Educational/ Regulatory/ Contract Research)**

Signature

Name and Designation of Investigator
Vara Prasad Saka
Assistant Professor,
Vignan Pharmacy College, Vadlamudi.

Date:
Place:

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*The filled in Form B having above information / details / supporting documents (1 original + 14 copies and 1 soft copy in CD) should be sent to: -

The Member Secretary,
CPCSEA, Ministry of Environment, Forests & Climate Change
5th Floor, Vayu Wing, Indira Paryavaran Bhawan,
Ali Ganj, Jor Bagh Road, New Delhi-110 003.

PART B

Protocol from for research proposals to be submitted to the committee / Institutional Animal Ethics Committee, for new experiments or extensions of ongoing experiments using animals other than non-human primates.

1. **Project / Dissertation / Thesis Title:**
   Effect of *Avena sativa* (oats) on fluoride induced infertility in male albino rats.

.Principal Investigator / Research Scholar / Research Guide / Advisor:

a. **Name** : Vara Prasad Saka
b. **Designation** : Assistant Professor
c. **Dept / Div/ Lab** : Department of Pharmacology
d. **Telephone No.** : +91 8019152501
e. **Experience** : 3 years

2. **List of names of all individuals authorized to conduct procedures under this proposal.**
   Co-guides

a. **Name** Dr. P. Srinivasa Babu

b. **Address** Vignan Pharmacy College, Vadlamudi.

c. **Experience** 20 years

3. **Funding source with complete address (Please attach the proof)**
   Self-finance
4. **Duration of the project**

a. **Number of months** : < 1 months
b. **Date of initiation (Proposed)** : 01/01/2017
c. **Date of completion (Proposed)** : 07/02/2017

5. **Detailed study plan may be given (Not more than one page)**
   Attached as annexure 1
6. **Animals required**

a. Species / Common name : Albino rats
b. Age/ weight/ size : 250 gm
c. Gender : male
d. Number to be used : 36
   (Year-wise breakups and total figures needed to be given)
e. Number of days each animal will be housed : 1 month
f. Proposed source of animals. : Mahaveer Enterprises.

7. **Rationale for animal usage**

a. Why is animal usage necessary for these studies?
In-vitro models are complicated and costly, the reproducibility is also poor. We are taking into consideration an artificial condition. In vivo is better as it will give brief idea about the pharmacodynamics and pharmacokinetics behavior of molecule in a living organism.

b. Why are the particular species selected required?
Appreciable historical data is available on these species

c. Why is the estimated number of animals essential?
This is the minimum number to arrive at a meaningful statistical conclusion of acceptable standards and to avoid errors.

d. Are similar experiments conducted in the past? If so, the number of animals used and results obtained in brief.
No similar experiments has been conducted for this test compound.

e. **If yes, why new experiment is required?** N/A

f. **Have similar experiments been made by any other organization agency? If so, their results in your knowledge.**
Many extracts were already tested by other organizations in same models. These models are already well established and mortality rate is also less.

8. **Description the procedures to be used.**
List and describe all invasive and potentially stress full non-invasive procedures that animals will be subjected to in the course of the experiments.
Furnish details of injections schedule
Substances : Fluoride, plant extracts, testosterone
Doses : 40 mg/kg, 200 and 400 mg/kg, and 20 mg/kg respectively
Sites : P.O and I.P
Volumes : according to their body weights (< 1 mL)
Blood withdrawal
Volumes : N/A
Sites : N/A
Radiation (dosage and schedules): N/A

9. **Please provide brief descriptions of similar studies from invitro / invivo (from other animal models) on same / similar test component or line of research. If, enough information is available, justify the proposed reasons.**
N/A

10. **Does the protocol prohibit use of anesthetic or analgesic for the conduct of painful procedures (any which cause more pain than that associated with routine injection or blood withdrawal)? If Yes, explanation and justification.**
NO

11. **Will survival surgery be done?** NO
If Yes, the following to be described.
a. List and description of all such surgical procedures (including methods of asepsis)
b. Names, qualifications and experience levels of operators
c. Description of post-operative care
d. Justification in major survival surgery is to be performed more than once on a single individual animals.

12. **Methods of disposal post-experimentation**
a. Euthanasia (Specific method) : Cervical dislocation
b. Method of carcass disposal : Burial
c. Rehabilitation : N/A

13. **Animal transportation methods if extra-institutional transport is envisaged.**
Air Conditioned vehicle with provisions for proper caging, bedding, feed and water shall be used for the transportation of the laboratory animals, if required.

14. **Use of hazardous agents** (use of recombinant DNA-based agents or potential human pathogens requires documented approval of the Institutional Biosafety Committee (IBC). For each category, the agents and the biosafety level required, appropriate therapeutic measures and the mode of disposal of contaminated food, animal wastes and carcasses must be identified)
(a) Radionuclides: N/A
(b) Microorganisms / Biological infectious Agents: N/A
(c) Hazardous chemicals or drugs: N/A
(d) Recombinant DNA: N/A
(e) Any other (give name): N/A

If, your project involved use of any of the above, attach copy of the minutes of IBC granting approval.

Investigator's declaration.

1. I certify that I have determined that the research proposal herein is not unnecessarily duplicative of previously reported research.

2. I certify that, I am qualified and have experience in the experimentation on animals.

3. For procedures listed under item 11, I certify that I have reviewed the pertinent scientific literature and have found no valid alternative to any procedure described herein which may cause less pain or distress.

4. I will obtain approval from the IAEC/ CPCSEA before initiating any significant changes in this study.

5. Certified that performance of experiment will be initiated only upon review and approval of scientific intent by appropriate expert body (Institutional Scientific Advisory Committee / funding agency / other body (to be named).

6. Institutional Biosafety Committee's (IBC) certification of review and concurrence will be taken (Required for studies utilizing DNA agents of human pathogens).

7. I shall maintain all the records as per format (Form D)

8. I certify that, I will not initiate the study unless approval from CPCSEA received in wiring. Further, I certify that I will follow the recommendations of CPCSEA.

9. I certify that I will ensure the rehabilitation policies are adopted.

Signature

Name of Investigator

Date:

Certificate

This is certify that the project title *EFFECT OF AVENA SATIVA (OATS) ON FLUORIDE INDUCED INFERTILITY IN MALE ALBINO RATS* has been approved by the IAEC meeting held on 21.11.2015 with approval number 004/IAEC/VPC/2015

Name of Chairman/ Member Secretary IAEC:
Dr. P.Srinivasa Babu
Principal
Vignan Pharmacy College, Vadlamudi, Guntur.

Name of CPCSEA nominee:

Signature with date

Chairman/ Member Secretary of IAEC:

CPCSEA nominee:

ANNEXURE I

PROJECT PROTOCOL

EFFECT OF AVENA SATIVA (OATS) ON FLUORIDE INDUCED INFERTILITY IN MALE ALBINO RATS

INTRODUCTION:

Infertility is one of the major health problems in modern life (Vikas Sharma, etal., 2013). Infertility can be defined as a lack of pregnancy after one year of regular unprotected intercourse. (Ashok Agarwal, Ph.D., et al., 2004). Male factor infertility accounts for up to half of all cases of infertility and affects one man in 20 in the general population(Tremellen, 2008)*. The total sperm count, motile sperm count, and normal morphologic features have been reported as the indices of fertility in men(Vikas Sharma, et al., 2013). Fluoride interferes with the structural and functional integrity of testis, internal milieu of epididymis, vas deferens and also affected the metabolism and morphology of spermatozoa of mice, rats and rabbits and reduces fertility.(Sharma, 1998). The present work focused on the treatment of fluoride induced infertility in male albino rats by *Avena sativa*.

PLANT REVIEW:

Botanical Name : *Avena sativa*
Common Name : oats
Family : Poaceae
Use : Nutritional supplement
Useful parts : Whole grains and barn

CHEMICAL CONSTITUENTS:

It has chemical constituents like carbohydrates, proteins, Avenanthramides, lipids (9 glycolipids and 11 phospholipids), an indole alkaloid- gramine, number of flavonoids, 3 flavonolignans, saponins, and sterols have been reported from A. sativa. Avenacin, a triterpenoid saponins, has been isolated from the roots while Avenacoside A and B are present in leaves. The oat is rich in protein, has lots of beneficial minerals such as iron, calcium, potassium, magnesium, copper, zinc, silicon, selenium and also number of vitamins like Vitamin B1, B2, B6, B12, Niacin, Vitamin C, Vitamin A, Vitamin E.

TRADITIONAL USES:

In folk medicine, oats are used to treat nervous exhaustion, insomnia, and weakness of the nerves. They are considered as antispasmodic, antitumor, cyanogenetic, demulcent, diuretic, Neurotonic etc.

PHARMACOLOGICAL ACTIVITIES:

Oats and it constituents are reported to possess varied pharmacological activities like lowering of blood cholesterol and blood sugar, immunomodulatory, anticancer, antioxidant, antiatherogenic, topical anti-inflammatory, useful in controlling childhood asthma, body weight, etc.

EXPERIMENTAL PROTOCOL:

Main objective of the project:

The main objective of the proposed project is to evaluate the spermatogenic activity of avena sativa (oat) on fluoride induced infertility in male albino rats.

MATERIALS AND METHODS:

PLANT MATERIAL: *Avena sativa* (oats)

EXTRACTION PROCESS:

Kernal (oat grout) was dried, weighed,(in g) ,and powdered in a grinder. Then, the powder wa s subjected to successive extractions starting with 70% ethanol at 80°C for 20 min, and water using soxhlet apparatus ,(Lim et al., 1992; Manthey et al.,1999; Marketta et al., 2004). Following centrifugation(5 min),the supernatants were combined and solvent was moved under vacuum at 40 °C. Filtered through 0•2-m filter paper, and stored at −20 °C until analysed & then Preliminary screening is carried out .

CHEMICALS:

DRUGS ADMINISTERED	*DOSAGE (mg/kg body weight)*	*ROUTE OF ADMINISTRATION*
Sodium fluoride	10	oral
Testosterone propionate	0.5	Subcutaneous
Oat extract	Based on acute toxicity studies	oral

ANIMALS:

The 30 adult Wister albino male rats were 8 weeks old and weighed 250 ± 10 g. The male rats were housed in temperature controlled rooms (25^{o}C) with constant humidity (40–70%) and 12 h/12 h light/dark cycle prior to experimental protocols.

EXPERIMENTAL PROCEDURE:

ACUTE TOXICITY STUDIES:

As per the OECD guidelines 423 , the acute toxicity of the Hydroalcoholic / ethanolic extract of Avena sativa was tested on different groups of 10 mice each receiving different doses of 50, 100, 250 and 500…1500 mg/kg body weight. The number of deaths in each group was recorded with in 48 h. The lethal dosage (LD50) was estimated , based on which dose selection is carried out ,different groups will be given orally, the A. sativa extract (suspension in water) consisting of diff concentrations. Blank group served as control and received equivalent volumes of water, this is for preliminary estimation of ED50 , there by further doses are selected.

METHODS:

All rats were fed a standard diet and water. The daily intake of animal water was monitored at least one week prior to the start of treatment, in order to determine the amount of water needed per experimental animal. Then the animals are divided into 5 groups of 6 animals each (Table no: 1) Group II animals were given subcutaneously 0.5 mg/Kg dose of Testosterone propionate suspension twice weekly. Group III were given Sodium fluoride (NaF) was administered orally at a dose of 10 mg/kg body weight for 30 days,

Group	*Treatment*	*Duration of treatment (Days)*	*Day of autopsy*	*Number of animals per group*
I (C)	Normal	28	29	6
II (SF)	Control (NaF)	28	29	6
III(OE)	Negative control(Oat extract)	28	29	6
IV (SOE-I)	Treatment group I (Oat Extract low dose+NaF)	28	29	6
V (SOE-II)	Treatment group II (Oat Extract high dose+NaF)	28	29	6
VI (TP)	Standard (testosterone propionate)	28	29	6

Surgical procedure: The rats were at the time of sacrifice first weighed and then cervical dislocation was carried out. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. The testis were detached and cleared free of the surrounding tissue(Godson Gabriel ., et al., 2012).

Organ weight:

The testis was weighed with an electronic analytical and precision balance, while the testis volumes were measured using immersion method.(Godson Gabriel AKUNNA, Oluwaseyi Samson OGUNMODEDE , Chia Linus SAALU , Babatunde OGUNLADE, Ayomide Joel BELLO 2012)

SEMEN ANALYSIS:

Sperm count(Rahul B. Patil, 2012): The sperm count was carried out by using Haemocytometer. The epididymis was removed and placed in a pre-chilled Petri-plate. 2 ml. of 0.9% saline was added to it and the epididymis was gently minced with the help of sharp razor. The sample was pipette out with the help of pipette provided in the Haemocytometer. A clean and dry cover slip was kept on the Neaubaur's ruling. The ruling was loaded with the sample by touching the tip of the pipette to the slide. The slide was kept on a bench for 2 min. to allow the sperms to settle down. The sperms were counted in four squares at the corner of the ruling covering an area of 4 sq. mm. under high power objective. The spermatozoa with head and tail were counted.

Calculation:

Total sperm count/epididymis = (sperm count/0.1)*1000

Sperm number=averaged no: of spermatozoa counted x multiplication factor (10'000) dilution factor (20) = N x 10'000 x 20 = N x 0:2 x 10^6spermatozoa

Sperm motility(Vikas Sharma, Jente Boonen, 2013): A drop of sperm suspension was placed on a clear glass slide and then covered with a cover slip. The slide was then examined under the microscope at 400X, and the motility was scored in different fields of view. Spermatozoa showing any degree of movement were considered to be motile. All spermatozoa (motile as well as immotile) were counted with the help of a blood cell counter. Sperm motility was calculated using the following formula:

Motility % = number of motile spermatozoa/total number of spermatozoa (motile + immotile) X 100

Sperm viability(Vikas Sharma, Jente Boonen, 2013): Sperm viability was assessed using a supravital staining technique based on the principle that cells with damaged plasma membrane take up the stain, whereas viable ones do not. All glass wares as well as the eosin–nigrosin stain were maintained at 37 °C. A drop of sperm suspension and a drop of eosin–nigrosin stain (1% eosin + 5% nigrosin, 1:1) were placed on a clear glass slide and mixed thoroughly with the help of a fine glass rod. A portion of the mixture was transferred to a second slide, and a thin film was prepared. The slide was then examined under the microscope (400X), and about 100 spermatozoa (viable and dead) were counted from different fields of the slide. Spermatozoa appearing pinkish (stained) were considered to be dead, whereas those appearing colorless (unstained) were counted as viable.

Sperm viability was calculated in percent by using the following formula:

Viability %= number of viable spermatozoa/ total number of spermatozoa (viable + dead) X 100

Sperm morphology test(Rekha D Kini, et al., 2012)**:**

For the evaluation of the sperm morphology the filtrate obtained was stained with 1% eosin Y or periodic acid-Schiff's reaction and morphological defects were analyzed. Briefly, the sperms in the smears were visualized under oil immersion objectives and any abnormalities of either heads or tails were noted. The microcephaly, which was also a type of head abnormality, and cephalo-caudal junction defects (CC), which were a type of tail defects have been classified separately. Two hundred sperms were screened for each animal and total abnormality was expressed as incidence/200 sperms/animal.

Histological studies(Vikas Sharma, Jente Boonen, 2013):

After 28 days of treatment to animals of all respective groups, the testis of animals from each group was dissected. The testis was fixed in Bouin's fluid for histological studies. Twenty-four hours after fixation, the tissues were dehydrated in an ascending series of alcohol, treated with xylene, and embedded in paraffin wax. Sections of 6-mm thickness were cut by a rotary microtome and stained with Hematoxylin and Eosin. Histological sections of the testis were viewed in a microscope, and images were captured with a digital camera. For the determination of the percentage of affected seminiferous tubules, all the tubules in a randomly selected section of the testis from three rats of each group were counted.

The seminiferous tubules were considered affected if they showed any of the following details: Regenerated appearance of germ cells; condition of germinal epithelium; presence of spermatids of different stages of spermatogenic cycle in the same tubule; diameter of the seminiferous tubule; presence

of Spermatogonia; presence of primary and secondary spermatocytes; differentiation of spermatids; lumen of the seminiferous tubule; Sertoli cell cytoplasm; presence of stages of spermatogenesis; formation of clones of germ cells structure; and distribution of Leydig cells.

BIO CHEMICAL ESTIMATIONS:

HORMONAL ASSAYS:

Serum testosterone assay(N. Ralebona, 2012):

Serum collected at termination was used for assaying for total testosterone. Testosterone was measured using a commercial ELISA kit (IBL) which is based on competitive binding of testosterone on immobilized antibody. Horse radish peroxidase was used for color development and absorbance at 420 nm measured on a plate reader (Multiskan EX). Values are reported as ng/ml of serum. (Sanghishetti Vijay Prasad et al., 2012)

FSH and LH assay: Assay to be done by using RIA and ELISA kits

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1) Ashok Agarwal, Ph.D., HCLD. Shyam S.R. Allamaneni, M.D. 2004. "Oxidants and Antioxidants in Human Fertility." *Middle East Fertility Society Journal* 9 (3): 187–197.
2) Godson Gabriel AKUNNA, Oluwaseyi Samson OGUNMODEDE , *, Chia Linus SAALU , Babatunde OGUNLADE, Ayomide Joel BELLO, Emmanuel Oluwatobi SALAWU. 2012. "Ameliorative Effect of Moringa Oleifera (Drumstick) Leaf Extracts on Chromium- Induced Testicular Toxicity in Rat Testes." *World Journal of Life Sciences and Medical Research* 2 (20): 20–26.
3) N. Ralebona, C. R. Sewani-Rusike and B. N. Nkeh-Chungag. 2012. "Effects of Ethanolic Extract of Garcinia Kola on Sexual Behaviour and Sperm Parameters in Male Wistar Rats." *African Journal of Pharmacy and Pharmacology* 6 (14) (April 15): 1077–1082. doi:10.5897/AJPP11.652. http://www.academicjournals.org/ajpp/abstracts/abstracts/abstract 2012/15 Apr/Ralebona et al.htm.
4) Rahul B. Patil, Shreya R. Vora and Meena M. Pillai. 2012. "Protective Effect of Spermatogenic Activity of Withania Somnifera (Ashwagandha) in Galactose Stressed Mice." *Annals of Biological Research* 3 (8): 4159–4165.
5) Rekha D Kini, Yogesh Tripathi , C V Raghuveer , Sheila R Pai, Nayanatara AK. 2012. "EFFECT OF α-TOCOPHEROL PRETREATMENT ON EPIDIDYMAL WEIGHT, SPERM COUNT & SPERM MORPHOLOGY ON CADMIUM INDUCED TESTICULAR DAMAGE IN MALE ALBINO RATS." *International Journal of Plant, Animal and Environmental Sciences* 2 (3): 110–114.
6) Sanghishetti Vijay Prasad1, Ghongane BB2, Nayak BB1, Raul AR1, Vijay Kumar AN1, Mutalik MM1, Kapure NL1. 2012. "An Experimental Study on Effect of Antioxidant Vitamin E in Stress and Alcohol Induced Changes in Male Fertility in Albino Rats." *International Journal of Medical Research & Health Sciences* 1 (1): 1–6.
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8) Tremellen, Kelton. 2008. "Oxidative Stress and Male Infertility — a Clinical Perspective." *Human Reproduction Update* 14 (3): 243–258. doi:10.1093/humupd/dmn004.
9) Vikas Sharma, Jente Boonen, Bart De Spiegeleer and V. K. Dixit. 2013. "Androgenic and Spermatogenic Activity of Alkylamide-Rich Ethanol Solution Extract of Anacyclus Pyrethrum DC." *PHYTOTHERAPY RESEARCH* 106 (April 2012): 99–106. doi:10.1002/ptr.4697