How to Cite:
Yadav, P., & Bhawar, S. B. (2022). Activity guided isolation and characterization of bioactive constituents from Mucuna pruriens. *International Journal of Health Sciences*, *6*(S4), 11065–11081. https://doi.org/10.53730/ijhs.v6nS4.10930

Activity guided isolation and characterization of bioactive constituents from Mucuna pruriens

Piyush Yadav
Research Scholar, Bhagwant University, Rajasthan, India
Corresponding author email: piyush61184@gmail.com

Dr. Sanjay B. Bhawar
Professor, Bhagwant University, Rajasthan, India
Email: sbbhawar@gmail.com

***Abstract*---**There are roughly 150 species of annual and perennial legumes in the genus Mucuna, which belongs to the Fabaceae family, subfamily Papilionaceae. The velvet bean *Mucuna pruriens* is widespread in tropical and sub-tropical parts of the world, and is one of the many undervalued wild legumes. Velvet bean, Florida velvet bean, Bengal velvet bean, Mauritius velvet bean, Yokohama velvet bean, cowhage, cowitch, lacuna bean, and Lyon bean are some of the other frequent names for this unusual plant. Atmagupta, which means "hidden self," and Kapikacchu, which means "one begins scratching like a monkey," are two Sanskrit names for it. Levodopa, often known as L-dopa, is found in high amounts in the seeds of the *Mucuna pruriens* plant, ranging from four to seven percent. It also includes tryptamines, phenols, and tannins, which can cause hallucinations. Its high L-dopa concentration is the primary rationale for its usage in Parkinson's disease therapy. Seeds of *Mucuna pruriens* was procured locally, authenticated by Professor, Department of Botany, by the studies include organoleptic tests, macroscopic and microscopic observations were done. The animal used for the work were Healthy wister rats(male) weighing about 150-200g used for the study were procured from local animal house. The animals were caged individually and kept in air conditions room at temperature of 22±20C with 50%±10% relative humidity with 12hrs light and dark cycle. The methanol extract was found to have good properties, so for the further studied methanol extract will be taken. The Weight Gain. All The Rats In Groups I, III, IV, V Remained Healthy And Active With Normal Feeding Behaviour. All the Body weight after 14 days and 30 days are noted in table no (Weight gain in each study). All the rats in groups I, III, IV, V showed healthy and active with increased aphrodisiac behaviour. Reproductive profile analysis observed in

Manuscript submitted: 9 April 2022, Manuscript revised: 27 June 2022, Accepted for publication: 18 July 2022

group II rats showed significant decrease in the testosterone levels in the group II rats when compared with group I and Group III rats.

***Keywords*---**Mucuna pruriens (MP), ayurvedic medicinal, aphrodisiac, velvet beans.

Introduction

Many medicinal plants require a special climate for growth. Secondary compounds aresynthesized in certain types of cells during a particular development stage of the plant and are usually stored in the vacuoles of the plant cell or in other differentiated tissues, e.g. glandular hairs. Thus, the optimal storage of the compounds is dependent on the age of the plant and it sometimes takes several years before the plants can be harvested. There is also a risk of over-collecting endangered species (e.g. Taxus brevifolia). Alternative methods for producing these plant-derived drugs are therefore desirable.[8] All Secondary metabolic pathways originate from primary precursors. Most enzymes in a given pathway of secondary metabolism are coordinately regulated and speculated that there are no clear rate-limiting enzymes as is the case for primary metabolism.

The genus *Mucuna*, belonging to the Fabaceae family, sub family Papilionaceae, includes approximately 150 species of annual and perennial legumes. Among the various under-utilized wild legumes, the velvet bean *Mucuna pruriens* is widespread in tropical and sub-tropical regions of the world. It is considered a viable source of dietary proteins due to its high protein concentration (23–35%) in addition its digestibility, which is comparable to that of other pulses such as soybean, rice bean, and lima bean. It is therefore regarded a good source of food.[1,2]

The dozen or so cultivated *Mucuna* spp. found in the tropics probably result from fragmentation deriving from the Asian cultigen, and there are numerous crosses and hybrids.[3] The main differences among cultivated species are in the characteristics of the pubescence on the pod, the seed color, and the number of days to harvest of the pod. “Cowitch” and “cowhage” are the common English names of *Mucuna* types with abundant, long stinging hairs on the pod. Human contact results in an intensely itchy dermatitis, caused by mucunain.[4] The nonstinging types, known as “velvet bean” have appressed, silky hairs. The plant *M. pruriens*, widely known as “velvet bean,” is a vigorous annual climbing legume originally from southern China and eastern India, where it was at one time widely cultivated as a green vegetable crop.[5] It is one of the most popular green crops currently known in the tropics; velvet beans have great potential as both food and feed as suggested by experiences worldwide. The velvet bean has been traditionally used as a food source by certain ethnic groups in a number of countries. It is cultivated in Asia, America,

Africa, and the Pacific Islands, where its pods are used as a vegetable for human consumption, and its young leaves are used as animal fodder. The plant has long, slender branches; alternate, lanceolate leaves; and white flowers with a bluish-purple, butterfly-shaped corolla. The pods or legumes are hairy, thick, and

leathery; averaging 4 inches long; are shaped like violin sound holes; and contain four to six seeds. They are of a rich dark brown color, and thickly covered with stiff hairs. In India, the mature seeds of *Mucuna* bean are traditionally consumed by a South Indian hill tribe, the *Kanikkars*, after repeated boiling to remove anti-nutritional factors. Most *Mucuna* spp. exhibit reasonable tolerance to a number of abiotic stresses, including drought, low soil fertility, and high soil acidity, although they are sensitive to frost and grow poorly in cold, wet soils.[5] The genus thrives best under warm, moist conditions, below 1500 m above sea level, and in areas with plentiful rainfall. Like most legumes, the velvet bean has the potential to fix atmospheric nitrogen via a symbiotic relationship with soil microorganisms.

Material& Method

Collection of plant material

All the medicinal plant materials were collected around, Uttar Pradesh. Fresh, healthy seeds of *Mucuna pruriens* (L) which were free of diseases were collected from local market, garden and seeds of Mucuna were purchased from local market.

Preparation of Extract

Seeds of *Mucuna pruriens* was procured locally, authenticated by Professor, Department of Botany, by the studies include organoleptic tests, macroscopic and microscopic observations. They were washed twice using tap water and then washed again in distilled water to remove the dust. The seeds were dried in the shade for 7–12 days at room temperature, until they were free from the moisture and then pulverized into coarse powder. The powdered seeds was added separately in soxhlet tube, methanol was also added to both soxhlet tube and round bottom flask, it was subjected to continuous hot extraction (soxhlet) with methanol at 50-60^{o} Celsius for 16 to 20hrs. Ethanol for ethanolic extract, water for aqueous extract and water and alcohol in a ratio of 50:50 for hydroalcoholic extract respectively. The complete extraction was confirmed by taking about 5 ml solvent from the thimble and evaporated to check for the absence of residue and solvent in siphon was colorless. The extracts were concentrated using Rotary evaporator under reduced pressure below 40°C to get reddish-brown semi solid extract. The obtained mass for each crude drug was weighed, subjected for lyophilized to get free from methanolic solvent and kept in vacuum desiccators. Procedure was repeated to get sufficient amount of extract. Weight of round bottom flask with extract was measured every time and noted for calculation of percentage of yield. Later it was transferred into a container and the filtrate was vacuum concentrated to remove the moisture content. Finally the extract was stored at -4oc.[6]

Animal Used

Laboratory rats are one of the most commonly used species for biomedical research, second only to mice. Developing an adequate institutional animal care and use program requires that the institution's occupational health and safety

program perform risk assessment at the interface between humans and rats. The human-rat interface must be characterized for the presence of risk agents that may adversely affect human health.

Healthy wister rats(male) weighing about 150-200g used for the study were procured from local animal house. The animals were caged individually and kept in air conditions room at temperature of 22±20C with 50%±10% relative humidity with 12hrs light and dark cycle. Throughout the study, Animals were maintained at normal laboratory conditions. Animals were maintained at standard rat pellet diet and pure drinking water ad libitum.[7,8]

Evaluation of Acute toxicity

The animals were kept under fasting for overnight but allowed water ad libitum before drug administration. The weight of the fasting animal was taken and drug dosages were calculated. The extracts were orally administered in a single dose by oral gavage for the experimental animals. Single animal was dosed in sequence, usually at 48 hrs intervals. The dosage was initiated at 150mg / kg, then 250 mg / kg, and preceded with 500mg/kg body weight as recommended in OECD Guidelines. Signs of toxicity and mortality were observed for the first 30 minutes followed by 1, 6 and 24 hours and thereafter twice daily until 2nd -14th day. [9]The wellness parameters and mortality were recorded for each animal. The wellness parameters such as skin looked for pigmentation, discoloration, furloss, nasal and oral mucous membrane for any ulceration, respiratory rate, heart rate, salivation, lacrimation, lethargy, urinary incontinence, defecation, sleep, gait, tremors, convulsion and mortality were all observed, recorded for each animal and compared between control and experimental groups for assessment of individual organ system. The hematological parameters and biochemical were analysed at the end of 2 weeks. Animals were kept under close observation for 4 hours after administering the extract, and then they were observed daily for seven days for any change in general behavior and/or other physical activities.[10] Objective of this Acute oral toxicity study is to assess toxic effects of sample materials that is seed powder extracts of *Mucuna pruriens*, in rats. In this study assessment was to see and record the toxic characteristics shown by them when sample material was administered as a single oral dose as per the OECD 423 guidelines

Principle of the test

It is the principle of the test that is based on a stepwise procedure with the use of a minimum number of animals per step. Sufficient information is obtained on the acute toxicity of the sample material to enable its classification. The substance is administered orally to a group of experimental animals at one of the defined doses. The substance is tested using a stepwise procedure, each step using three animals of a single sex (normally females). Absence or presence of sample material related mortality of the animals dosed at one step will determine the next step, that is

- no further testing is needed,
- dosing of three additional animals, with the same dose

- dosing of three additional animals at the next higher or the next lower dose level.

Single animals were dosed in sequence usually at 48 hrs intervals. However, the time intervals between dosing were determined by the onset, duration, and severity of toxic signs. Dosing of an animal at the next dose was delayed until one was confident of survival of the previously dosed animal. The time interval adjusted as appropriate, example in case of inconclusive response. The test is simpler to implement when a single time interval is used for making sequential dosing decisions.

Observations

Mortality

On the day of dosing, all animals were observed for mortality at 30 min, 1, 2, 3, 4 and 6 hours after dosing and thereafter they were observed once in a day for 14 days.

Clinical signs

The treated animals were observed for signs of intoxication, at 30 min, 1, 6 and 24 hours after dosing and thereafter once a day for 14 days. The appearance, progress and disappearance of these signs were recorded.(Respiratory rate, Heart Rate)

Body weights

The body weights of rats were individually recorded before dosing and at weekly intervals thereafter.

Skin & Fur

The Skin & Fur of rats were individually recorded before dosing and at weekly intervals thereafter. For checking skin irritation Problems.

Sexual behavior of male rats

The sexually active male rats were selected for testing and trained for sexual behavior 2 times a day for 10 days initially before administering the drugs.

Preparation of male rats

The sexually active male rats were selected for testing and trained for sexual behavior 2 times a day for 10 days initially before administering the drugs.

Preparation of female rats

Adult healthy young female rats of 8 weeks old weighing about 130 – 140 gm were selected and administered benzoate oestradiol 10 µg /100 gm body weight to

bring the female rats for oestrous phase 48 hours before copulatory study and progesterone 500 µg /100gm body weight was administered through subcutaneous route, 4 hours before the copulatory studies.

Copulatory study of the rats

The experiment was carried out in a specially designed box measuring 50×30×30 cm with a dim yellow light. One male rat selected randomly from each group, marked by picric acid was kept initially in the box and two female rats which were prepared as explained above were introduced into the box. After a 15 minutes acclimatization period, the following sexual behaviour parameters were recorded for one hour with video feeds.

- Mount frequency (MF): The number of mounts without intromission from the time of introduction of the female until ejaculation,
- Intromission frequency (IF): The number of intromissions from the time of introduction of the female until ejaculation,
- Mount latency (ML): The time interval between the introduction of the female and the first mount by the male,
- Intromission latency (IL): The interval from the time of introduction of the female to the first intromission by the male,
- Ejaculation latency (EL): The time interval between the first intromission and ejaculation,
- Post-ejaculatory interval (PEI): The time interval between ejaculation and the first intromission of the following series. A total of 5 series observed.
- Total sexual behavior (TSB) includes genital grooming, ano-genital sniffing, chasing of female rats were observed for one hour, till ejaculation of semen into any one of the female.

Semen analysis

Sperm count

The semen samples were then collected from the cauda epididymis by milking out on a glass slide and mixed with one drop of 2.9% sodium citrate selfkept at 37^{o}C[11]. The semen was collected from any one epididymis, either right or left randomly. The other side epididymis was utilized for semen smear preparation. Improved double Neubauer ruling Chamber (Depth 1/10 mm) was used for counting the spermatozoa. The spermatozoa samples was diluted 1:10 with normal saline and drawn to 0.5 mark in the WBC diluting pipette, then the diluting fluid was drawn to the mark 11. The contents of the bulb were mixed for 5 minutes, then the first few drops were discarded before charging the counting chamber with a cover slip over it and the sperms were counted under high power in the 4 WBC squares (Fig. 17) as done for Total leucocyte count[12].
Calculation for sperm count[13]:
Number of sperms in 64 square = N (Volume = 4/10 mm3)
Number of sperms in 1 mm3 of undiluted sperm = N x 10/4 x 20
Sperm count in 1 ml = N x 50 x 1000

The sperm counts were tabulated

Morphological analysis of sperm:
Sperm motility and viability:
The cauda epididymis was minced in 5ml of 0.9% sodium chloride (normal saline) and incubated for 15min at 32°C. A drop of the sample placed in Neubauer ruling Chamber and the motile and immotile sperm were counted per unit area. The percentage was calculated out of a total of 100 spermatozoa and noted. [14]

Preparation of smear

Smears were prepared from the spermatozoal samples. Three smears were prepared from each rat's spermatozoal samples. After milking out the sperm on a glass slide, one drop of 2.9% sodium citrate was mixed well with seminal fluid, then the spreader (slide) placed at an angle of 45o to the slide, drawn back to touch the drop which then spreads along the edge of the spreader (Fig. 18). The spreader was pushed towards the left from the right side with a quick uniform motion with a light but even pressure. The spermatozoal suspension follows the spreader to form a film. It was dried quickly by waving in the air. The slides were stained by Papanicolaou stain. Procedure for staining the smear: The smears were fixed in alcohol and taken through descending grades of alcohol (80%, 70% and 50%) and then to water followed by staining in Harris Hematoxylin for 3 minutes and then rinsed in tap water for 1-2 minutes. The smears were differentiated in acid alcohol and in running tap water for few seconds and blued the nuclei in slightly ammoniated water and again rinsed in water and transferred to 70% alcohol for few seconds, then to 95% alcohol.[15] The smear was then transferred for staining in Orange G6 for approximately 3 minutes, again rinsed in two changes of alcohol followed by staining in Eosin Ayure 50 for about 2- 4 minutes until the desired intensity of the colour obtained, then rinsed in 2 changes of 95% alcohol for a few seconds on each. The smear at last dehydrated in alcohol, cleared in xylene, and mounted in neutral synthetic resin medium (DPX).

Morphology of Sperm Cells

The morphological characteristic of the sperm cells in all the smears were observed under oil immersion (100 X). The following abnormalities were noticed both in the control and experimental groups such as headless tail, rudimentary tail, curved mid piece, curved tail, looped tail, bent mid piece, tailless head, bent tail.[16] The total numbers of normal and abnormal sperms were tabulated for both control and experimental groups, by counting 50 sperm cells per smear and three smears per rat, a total of 900 sperms were counted per group. The data's were analyzed by Chi-square test.

Estimation of serum testosterone level

Serum was separated by centrifugation for about 3000 rpm after the coagulation of blood for 40 minutes. The serum was collected using micropipette from the blood samples. Procedure for testosterone hormone estimation: The serum samples were added to each wells (25 µl) followed by adding of conjugate (100 µl) to each wells and then mixed. The wells were incubated at 37 °c for one hour and

the contents were removed from the wells, then the wells were washed with (300 µl) distilled water (2 times) and TMB-Substrate (100 µl) was added to all the wells, followed by incubation of wells for 15 minutes in dark and stop solution (100 µl) was added to all the wells. After completing all the above procedures readings were taken using ELISA analyzer.[17,18]. The readings were noted down and tabulated.

Result & Discussion

Wellness parameter observation for acute toxicity study

The wellness parameters such as skin looked for pigmentation, discoloration and fur loss, nasal and oral mucous membrane for any ulceration, respiratory rate, heart rate, salivation, lacrimation, lethargy, pilo erection, urinary incontinence, defecation, sleep, gait, tremors, convulsion and mortality were all observed, recorded for each animals and compared between control and experimental groups and found to be normal in all the animals. The Hematological parameters such as haemoglobin concentration, red blood cell count, white blood cell count, platelet count of all rats, were found out. Data's were tabulated as Mean ± SEM (Table below) and compared between control and experimental rats by one way ANOVA, p value for all hematological parameters were insignificant and show only less difference among the groups and the values are approximately within same range to the study done for some Siddha formulations. The biochemical parameters such as total protein, creatinine, alkaline Phosphatase, were within same ranges with a slight difference between control and experimental rats. P value for all markers was not significant among the groups.

Table 1: Wellness parameters observed for acute toxicity study

S.no	Parameters	0 Min	6 Hrs	24Hrs
1.	Skin & Fur	Normal	Normal	Normal
2.	Respiratory rate	Normal	Normal	Normal
3.	Clinical signs	Normal	Normal	Normal
4.	Mortality	No	No	No
5.	Body weights	Normal	Normal	Normal
6.	Lethargy	Normal	Normal	Normal

The methanol extract was found to have good properties, so for the further studied methanol extract will be taken:

Pharmacological study

Weight gain

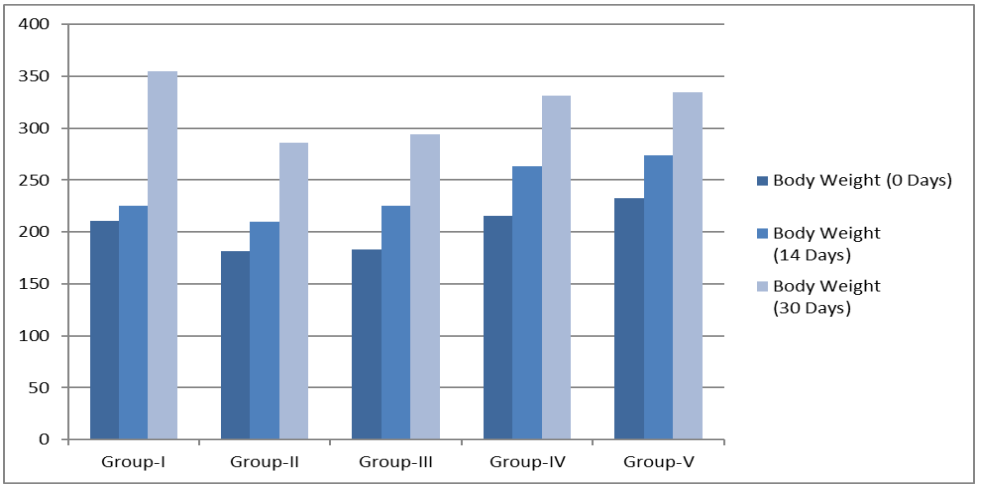
Regular Weight Of The Rat Was Measured During Dosage Period, (0 Days), (14 Days),(30 Days) Weight Was Considered To Calculate The Weight Gain. All The Rats In Groups I, III, IV, V Remained Healthy And Active With Normal Feeding Behavior. However, Rats Of Group II Acted As Stress Control Where The Only Stress Was Given Without Any Treatment Were Found To Be Lethargic And Their

Body Weight Gain Was Found To Be The Lowest Among All Groups, Which Was Statistically Significant When Compared With The Control Group.

Table 2: Weight gain in each group

STUDY GROUP	Body Weight (0 Days)	Body Weight (14 Days)	Body Weight (30 Days)
Group-I	210.5	255.21	355.17
Group-II	181	210.23	256.12
Group-III	182.83	225.12	293.67
Group-IV	215.82	263.12	331.2
Group-V	232.83	274.12	334.17

Figure 1: Body weight after 14 days and 30 days



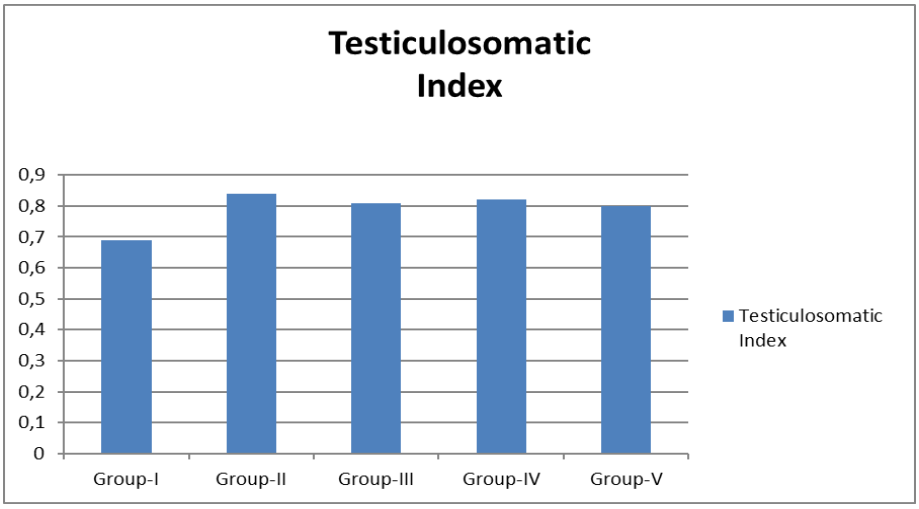
Testiculosomatic index

Testiculosomatic index was calculated by weight of both the testis multiplied by 100 and divided by total body weight. Testiculosomatic index was increased in the group ii rats when compared with the control groups.

Table 3: Testiculosomatic index of different groups

STUDY GROUP	Testiculosomatic Index
Group-I	0.69
Group-II	0.84
Group-III	0.81
Group-IV	0.82
Group-V	0.80

Figure 2: Graph of testiculosomatic index of different groups



Mating Behaviour of Animals

All the rats in groups I, III, IV, V showed healthy and active with increased aphrodisiac behavior. However, rats of group II acted as control where the only stress was given without any treatment were found to be less aphrodisiac it was found to be the lowest among all groups. There was a significant decrease in the levels of Mounting Frequency (MF), Intromission frequency (IF), Ejaculatory latency (EL) & Number of mounts (NM) was noted in the group II rats as compared to control rats, above mentioned parameters were significantly increased in group III, IV, V rats as compared to group I & II rats. There was an increase in the time of mounting latency (ML) & Intromission latency (IL) in the group II rats when compared with control group rats and. There was a decrease in the time of mounting latency (ML) & Intromission latency (IL) in the group III, IV, V & VI rats when compared with group II rats (table below).

Table 4 : Mating behavior of different groups

STUDY GROUP	Mounting Frequency	Intromission Frequency	Number of Mount
Group-I	54.25	5.71	4
Group-II	49.12	5.17	3
Group-III	62.14	8.59	4
Group-IV	55.98	7.86	3
Group-V	58.65	8.21	3

Figure 3 : Graph showing mating behaviour of different groups

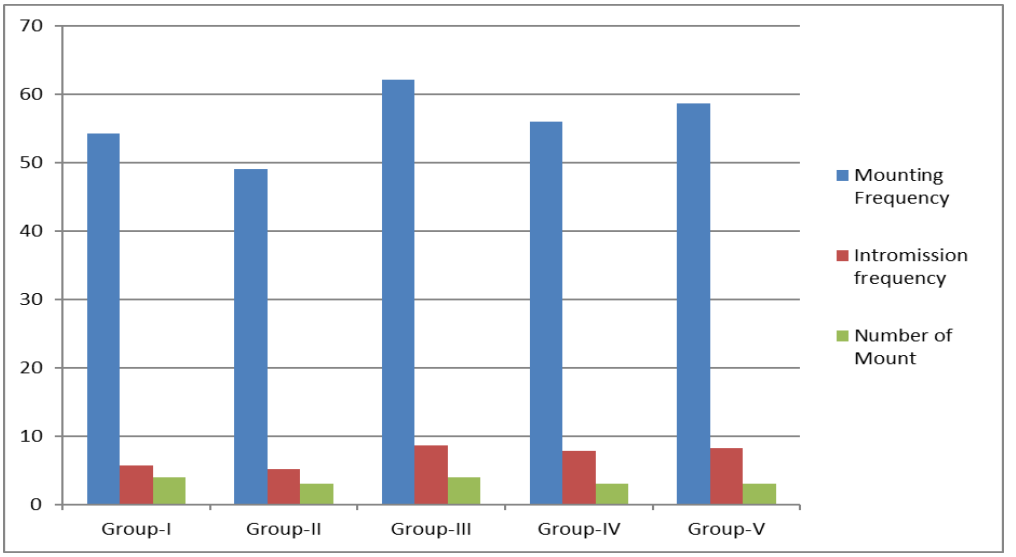
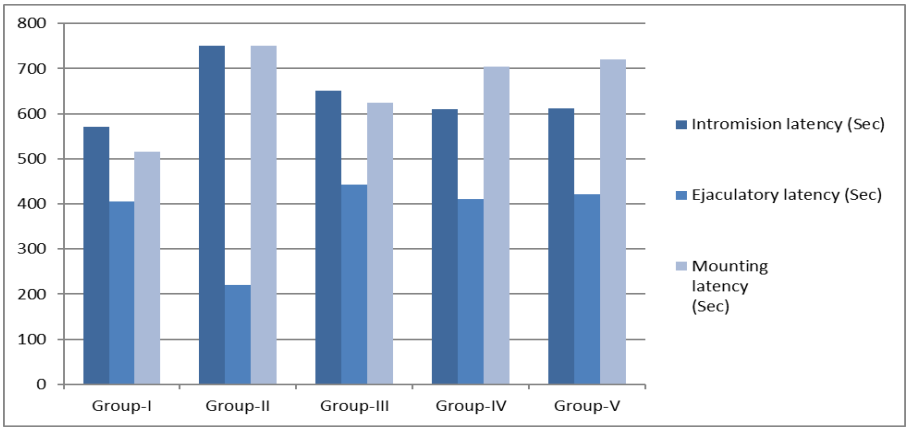


Table 5 : Intromission latency, ejaculatory latency and mounting latency of different groups

STUDY GROUP	Intromision latency (Sec)	Ejaculatory latency (Sec)	Mounting latency (Sec)
Group-I	570.25	405.40	515
Group-II	750.50	220.73	750
Group-III	650	442.45	624
Group-IV	610	410.30	704
Group-V	612	421.50	720

Figure 4 : Graph showing intromission latency, ejaculatory latency and mounting latency of different groups



Reproductive Assay

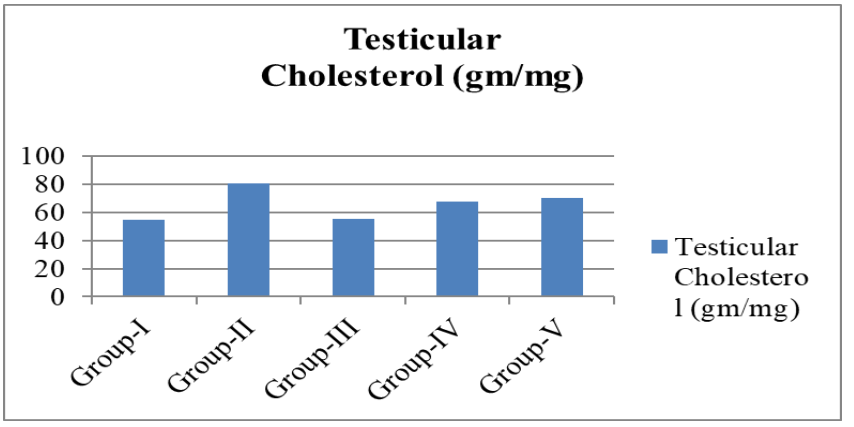
Analysis of reproductive profile revealed that the levels of testicular cholesterol were significantly increased in group II & VI and decreased in group V when compared with control group (I). Even though there was slight increase in testicular cholesterol level in group III & IV which was not significant statistically. Results of LH in group II, III, IV,V rats significantly increased when compared with group I rats. Results observed in group II & IV showed significant increase in the FSH levels when compared with group I & IV rats. Group III & V rats showed improvement in the concentration of FSH levels compared with group I & II rats.

Reproductive profile analysis observed in group II rats showed significant decrease in the testosterone levels in the group II rats when compared with group I and Group III rats. Group IV, V & VI rat showed improvement in the concentration of Testosterone levels. However, the increase in testosterone in groups V was higher and in group IV was lower when compared to the standard reference drug group (Group III). There was significant decrease in the number of sperms & sperm motility in the group II as compared to group I. An increase in the number of sperm count & motility was observed in the group III, IV, V which was statistically significant while comparing with group I & II.

Table 6: Testicular Cholesterol of different groups

Study Group	Testicular Cholesterol (gm/mg)
Group-I	54.64
Group-II	80.65
Group-III	55.35
Group-IV	67.45
Group-V	69.98

Figure 5 : Graph showing testicular Cholesterol of different groups



LH : Luteinizing hormone (LH, also known as luteinising hormone, lutropin and sometimes lutrophin) is a hormone produced by gonadotropic cells in the anterior pituitary gland.

Table 7 : LH of different groups

STUDY GROUP	LH (mIU/mL)
Group-I	0.056
Group-II	0.178
Group-III	0.110
Group-IV	0.091
Group-V	0.101

Figure 6 : Graph showing LH of different groups

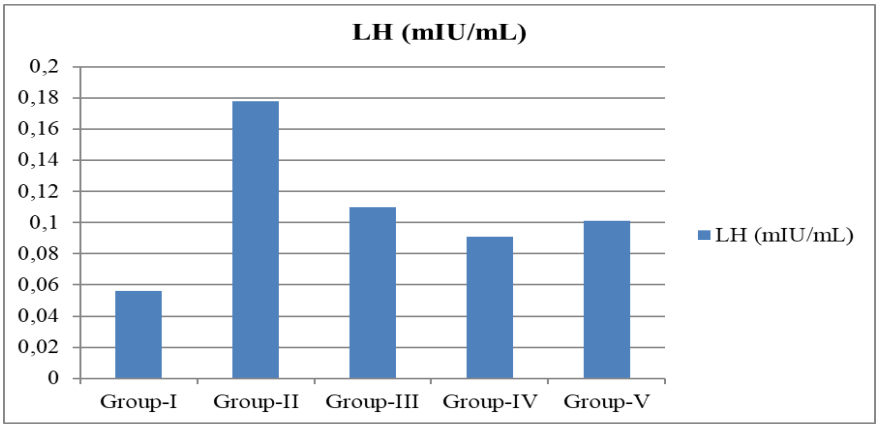


Table 8 : Testosterone (ng/dL) level of different groups

STUDY GROUP	Testosterone (ng/dL)
Group-I	220.68
Group-II	94.35
Group-III	274.62
Group-IV	245.45
Group-V	255.87

Figure 7 : Graph showing testosterone level of different groups

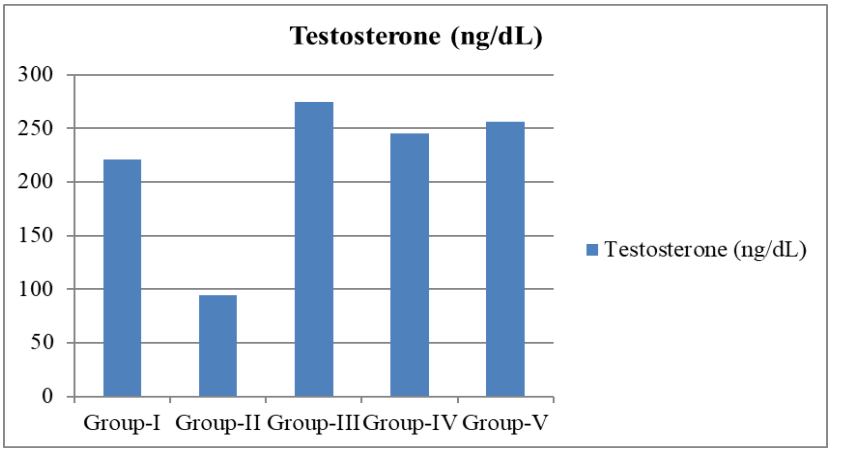


Table 9 : Sperm Count mill cells/ml of different groups

Study Group	Sperm Count mill cells/ml
Group-I	149.02
Group-II	102.49
Group-III	163.43
Group-IV	155.23
Group-V	165.27

Figure 8 : Graph showing sperm Count mill cells/ml of different groups

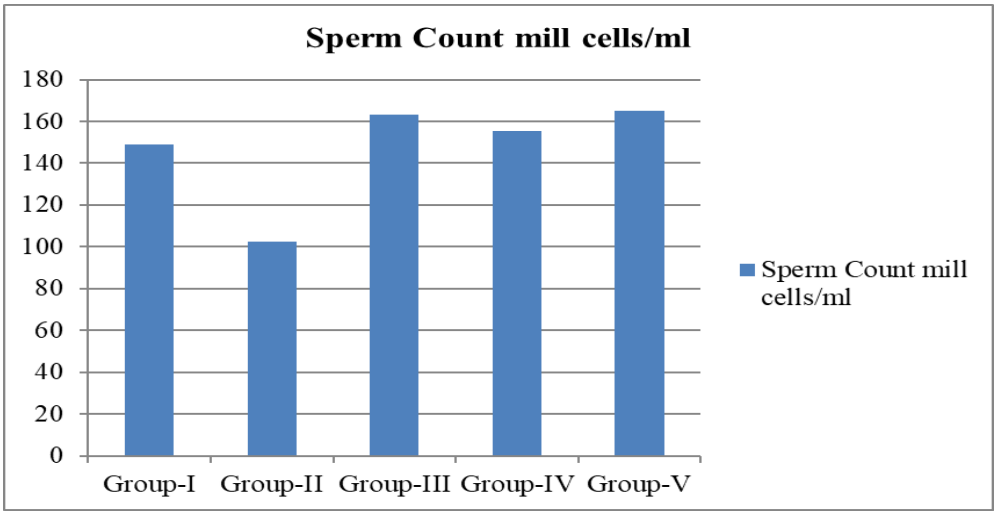
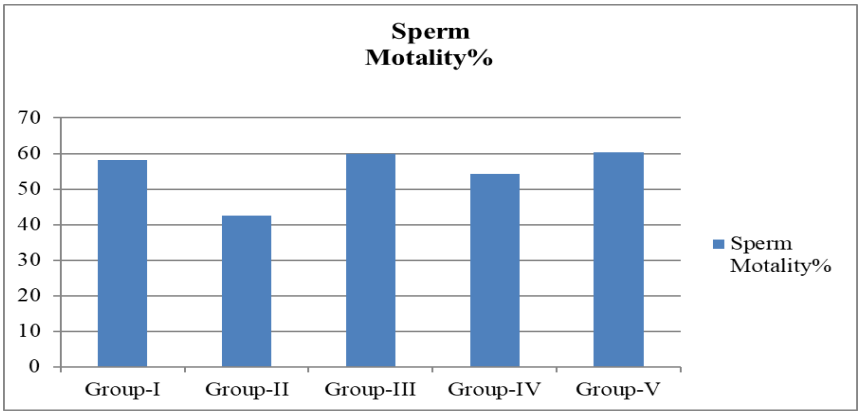


Table 10 : Sperm Motality of different groups

Study group	Sperm Motality%
Group-I	58.23
Group-II	42.43
Group-III	59.98
Group-IV	54.43
Group-V	60.25

Figure 9 : Graph showing sperm motality of different groups



Conclusion

A Boon for Healthy Human Life provides a comprehensive overview of the role of herbal medicines for treating a broad variety of human diseases, from neurological disorders to cancer and major disorders such as infectious diseases, metabolic disorders, and more. The wellness parameters such as skin looked for pigmentation, discoloration and fur loss, nasal and oral mucous membrane for any ulceration, respiratory rate, heart rate, salivation, lacrimation, lethargy, pilo erection, urinary incontinence, defecation, sleep, gait, tremors, convulsion and mortality were all observed, recorded for each animals and compared between control and experimental groups and found to be normal in all the animals. All the wellness parameters were found normal during the study. No mortality was noticed during the acute toxicity study.

The methanol extract was found to have good properties, so for the further studied methanol extract will be taken. The Weight Gain. All The Rats In Groups I, III, IV, V Remained Healthy And Active With Normal Feeding Behaviour. All the Body weight after 14 days and 30 days are noted in table no (Weight gain in each study). All the rats in groups I, III, IV, V showed healthy and active with increased aphrodisiac behaviour. Reproductive profile analysis observed in group II rats showed significant decrease in the testosterone levels in the group II rats when compared with group I and Group III rats. Group IV, V & VI rat showed improvement in the concentration of Testosterone levels. However, the increase in testosterone in groups V was higher and in group IV was lower when compared to

the standard reference drug group (Group III). There was significant decrease in the number of sperms & sperm motility in the group II as compared to group I. An increase in the number of sperm count & motility was observed in the group III, IV, V which was statistically significant while comparing with group I & II.More research is needed to determine the lead chemicals derived from the active principle of this plant extract. For the lead compounds, the development of drug designing may be clarified.

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