PH.D
2015

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

G.A.Hadimani

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

Thesis submitted to the

BLDE UNIVERSITY, VIJAYAPUR KARNATAKA, INDIA

For the award of

DOCTOR OF PHILOSOPHY

In

ANATOMY

By

GAVISHIDDAPPA A HADIMANI

Reg. No-10PhD003

Department of Anatomy

Under the Guidance of

DR. S.D.DESAI & PROF. DR.K.K.DAS

Shri B M Patil Medical College,

Hospital & Research CentreVijayapur-586103

2015

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

Thesis submitted to the

BLDE UNIVERSITY, VIJAYAPUR KARNATAKA, INDIA

Under the faculty of

MEDICINE

For the award of

DOCTOR OF PHILOSOPHY

In

ANATOMY

By

Gavishiddappa A Hadimani

Reg. No-10PhD003

Department of Anatomy

Shri B M Patil Medical College, Hospital & Research Centre

Vijayapur-586103

2015

BLDE UNIVERSITY

Vijayapur, Karnataka, India

CERTIFICATE

This is to certify that this thesis entitled "*Aphrodisiac effect of mucuna pruriens & withania somnifera & their effect on male reproductive organs of albino rats*" is a bonafide work of Mr. Gavishiddappa A Hadimani and was carried out under our supervision and guidance in the Department of Anatomy, Shri B. M. Patil Medical College, Hospital & Research Centre, Vijayapur, Karnataka, India.

Dr. S D Desai
Guide
Principal
& Professor Department of Anatomy
Shridevi Institute of Medical Science
& Research Centre
Tumkur, Karnataka

Prof. Dr. Kusal K. Das
Co- Guide
Laboratory of Vascular Physiology & Medicine
Department of Physiology
Shri B. M. Patil Medical College, Hospital
& Research Centre
Vijayapur, Karnataka

Dr. B M Bannur
Professor & Head
Department of Anatomy
Shri B. M. Patil Medical College, Hospital
& Research Centre
Vijayapur, Karnataka

Dr. M. S. Biradar
Principal & Dean Faculty of Medicine
Shri B. M. Patil Medical College, Hospital
& Research Centre
Vijayapur,
Karnataka

DECLARATION

I declare that the thesis entitled "*Aphrodisiac effect of mucuna pruriens & withania somnifera & their effect on male reproductive organs of albino rats*" has been prepared by me under the guidance of Dr. S D Desai and Professor Kusal K. Das, Department of Anatomy, BLDE University's Shri B. M. Patil Medical College, Hospital and Research Centre, Vijayapur, Karnataka, India. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

Date :
Place : Vijayapur

Gavishiddappa A Hadimani
Ph.D Scholar
Department of Anatomy,
BLDE University's
Shri B. M. Patil Medical College, Hospital & Research Centre
Vijayapur, Karnataka 586103

2015

DEDICATED WITH AFFECTION & GRATITUDE

To

Lord Almighty

My Lovely Sister Late Smt. Sharanakka &
Sister in Law Late Smt. Maheswari

& My Teachers

2015

Forethought

Science is Nature's interpreter

Scientist is a 'Child' learning the language of

N A T U R E

And so, if ALL minds Reason Scientifically,

Nothing happening around us will go

UNQUESTIONED

By

James Joyce

"The Human Age"

ACKNOWLEDGEMENT

The endless thanks goes to Almighty my Lord Kalakaleswar (Shiva) for all the blessings he has showered onto me, which has enabled me to write this last note in my research work.

During the period of my research, as in the rest of my life, I have been blessed by Almighty with extraordinary people who have spun a web of support around me. Words can never be enough in expressing how grateful I am to those incredible people in my life who made this thesis possible. I would like an attempt to Thank them for making my time during my research period I will treasure.

I am deeply indebted to my research Guide Dr. S D Desai Principal, Professor Dept, of Anatomy Shridevi Institute of Medical Sciences Tumkur, for his valuable guidance, scholarly inputs and constant encouragement I received throughout the research work. This feat was possible only because of the unconditional support provided by him. He has always made himself available to clarify my doubts despite his busy schedules and I consider it as a great opportunity to do my doctoral program under his guidance and to learn from his research expertise.

It is with sincerity and deep sense of gratitude that I thankfully acknowledge my honored co-guide and beloved teacher Professor Kusal K Das Laboratory of Vascular Physiology & Medicine, Dept. of Physiology, Shri B M Patil Medical College Hospital & Research Centre Vijayapur, visiting professor School of Medicine, and University of Leeds UK for presenting me such an interesting thesis topic. Each meeting with him added invaluable aspects to the implementation and broadened my perspective. He has guided me with invaluable suggestions, lightened up the way in my darkest times and encouraged me a lot in the academic life. From him I have learned to think critically, to select problems, to solve them and to present their solutions I would like to thank him for furthering my education in research. It was a great pleasure for me to have a chance of working with him. You were the best choice I could have made for an advisor. Sometime we are just (or incredibly) lucky!!!

I owe my most sincere gratitude to Dr M. S. Biradar, Principal & Dean faculty of Medicine Shri B. M. Patil Medical College, Hospital & Research Centre

for providing an excellent research environment and facilities to work in the institute.

My sincere gratitude to the Dr Tejashwini Vallabha Vice-principal, Shri B. M. Patil Medical College, Hospital & Research Centre and Chairman of Ph.D committee, BLDE University, who was a constant source of inspiration during my entire thesis. She have rendered constant support whenever I needed, whether in academic or in administration.

I express heartfelt thanks to all the Members of PhD committee BLDE University for correcting my mistake throughout my tenure.

I would like to express the deepest appreciation to Dr. B M Bannur Professor and Head, Dept. of Anatomy, BLDEU's Shri B.M. Patil Medical College, Vijayapur for his constant support during the course of my study all along the way.

I would like express my sincere gratitude to Dr. Navanath Kalyani Principal BLDEA's College of pharmacy, Dr. Shivakumar Hugar, Mr. Prakash Biradar, Mr. Nanjappayya for providing and helping me to carry out extraction of the herbals.

I offer my gratitude to Dr. R S Wali Prof. HOD Pharmacology for providing the animals timely.

I would like to offer my special thanks to Dr. Basavaraj Devarnavadgi, Prof. HOD Biochemistry for providing the facilities to work in their laboratory. My special gratitude to Mr. Govindanagouda Naregal for his great contribution and assistance during the biochemical investigations.

I would like to offer my special thanks Dr. B R Yelikar Prof. HOD Pathology, Dr. Anita Jawalagi Asst. Prof., Mrs Jessi, Mr, Mathad of Pathology for their help in Histopathology related work. It would be injustice if I don't express my gratitude to the great contribution by Dr. Saeed Yendigeri Asst. Prof. Dept of Pathology Al- Ameen Medical College, Vijayapur for reporting the histology slides in spite of his busy schedule.

I extend my thanks to Dr. Manjunath Aithal Prof. HOD Physiology for his needed help during progress report presentations.

Advice given by Mrs Vijaya Sorganvi and Dr Shahnawaz, Statisticians of Department of Community Medicine was really a great help in data analysis and data interpretations.

Central research Lab was gift for me during my research by the BLDE University where I could to able do all my investigations. Needy help provided by Dr. Satish Patil Asst. Director Central Research Lab is greatly appreciated.

I am immensely thankful to Dr. B G Patil, Dr. R S Bulagouda for their constant support. I wish to acknowledge the help provided by my all colleagues of Dept of Anatomy. I extend my thanks to all the non-teaching staff too for assistance during my thesis.

I would like to address special thanks to members of NAAC Cri committee, Cultural Committee, Women Empowerment Committee News Bulletin committee where I have been made member, for their needed help.

I will be failing in my duty if I don't acknowledge some of my friends with whom I have shared my research experiences since it was a joy and enlightenment to me. I am fortunate to have a friends like Mr. Ishwar B Bagoji, who is there with me since childhood his help is unforgettable, Mr. Ekanath P Jadhav Mr. Venkatesh Mantur Mr. Santosh Mayannavar Dr. Bharat Ambadasu, Mr. Sateesh Patil Dy. Reg. BLDE University Mr. Venku H & Mr. R S Hiremath Mr. Santosh, Dr B S Patil for their ever needed moral support.

I would like to address special thanks to the unknown reviewers of my thesis. My utmost respect goes to my animals without which this study wouldn't have come into existence.

My parents have passed onto me a wonderful humanitarian lineage, whose value cannot be measured by any worldly yardstick. I would express my deep sense of gratitude to the affection and support shown to me by my Brothers, Sisters and in Law's they all have inspired me in all dimensions of life. The warmest of thanks to my wife Mrs. Shruti for her support in each and every way. Dozens of kids in the family including Sinchana knew only to encourage and never disturbed me to achieve this last stage of my endeavor. I owe everything to childrens crew for their everlasting love.

Thank You from my heart-of-hearts.

Gavishiddappa A Hadimani

Date:

CONTENTS

Chapter Content	Page No

List of Tables

List of Figures

List of Symbols, Abbreviations and Nomenclature

SYMBOLS	ABBREVIATIONS & NOMENCLATURE
ADP	Adenosine tri phospate
ALT	Alanine Transaminase
ANOVA	one-way analysis of variance
AST	Aspartate Transaminase
ATP	Adenosine tri phospate
B. Wt	Body weight
CC	Corpora cavernosa
CE	Cholesterol ester
CHOD	Cholesterol dehydrogenase
CLIA	Chemiluminescence immunoassay
CPCSEA	Committee For The Purpose of Control and Supervision of Experiments on Animals
CSBs	Compulsive sexual behaviors
CVD	Cardiovascular diseases
D/W	Distil Water
Dl	Deci liter
DNA	Deoxy ribo nucleic acid
ED	Erectile dysfunction
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immuno Absorbent Assay
eNOS	Endothelial Nitric Oxide Synthase
$FeCl_3$	Ferric Chloride
FFA	Free Fatty Acids
FSH	Follicle Stimulating Hormone
GAA	Glacial Acitic Acid
GK	Glycerol Kinase
GR	Glutathione Reductase
GSH	Glutathione
GSSG	Oxidized Glutathione
H_2O_2	Hydrogen peroxide
H_2So_4	Sulphuric acid
Hb	Hemoglobin
HCL	Hydrochloric acid
HDL	High Density Lipoproteins
Hr	Hour
HRP	Horse Radish Peroxidase
HSD	Hypoactive sexual desire
IAEC	Institutional Animal Ethics Committee
iNOS	Inducible Nitric Oxide
IU	International Unit
$K_2CR_2O_7$	Potassium Di Chromate
LDH	Lactate dehydrogenase
LDL	Low Density Lipoproteins
LH	Luteinizing Hormone

SYMBOLS	ABBREVIATIONS & NOMENCLATURE
LPO	Lipid Peroxidation
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
MDA	Malondialdehyde
MDH	Malate dihydrogenase
mg	Mili Gram
Min	Minute
mL	Milli Liter
MP	Mucuna pruriens
NaCl	Sodium Chloride
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide Hydrogenase
NANO2	Sodium Nitrite
NaNO3	Sodium Nitrate
NCC	Neubauer Counting Chamber
nm	nano meter
NO	Nitric oxide
NOS	Nitric oxide synthase
OSI	Organo Somatic Index
PBS	Phosphate Buffer Solution
PCV	Packed cell volume
PUPA	Poly Unsaturated Phospholipid
RBC	Red blood corpuscles
RLUs	Relative Luminescence Units
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
Sec	Second
SGOT	Serum Glutamate Oxaloacetate Transaminase
SGPT	Serum Glutamate Pyruate Transaminase
SOD	Super oxide dismutase
SOP	Standard Operating Procedure
Std.	Standard
TBARS	Thio Barbituric Acid
TCA	Tri Chloro Acitic Acid
TG	Triglycerides
U	Unit
VCL3	Venedium Chloride
Vit-C	Vitamin-C
Vit-E	Vitamin-E
VLDL	Very Low Density Lipoproteins
WBC	White blood corpuscles
WS	Withania somnifera
µL	Micro Litre
17βHSD	17β Hydroxy Steroid Dehydrogenase
3βHSD	3 β Hydroxy Steroid Dehydrogenase

ABSTRACT

Introduction: Sexual dysfunction is a common problem with increase in prevalence and etiological factors, including degenerative diseases, increase in injuries and stress associated with industrialized lifestyles. Sexual dysfunction can be treated by both medical and surgical treatment modalities; however, plant-derived and herbal remedies continue to be a popular alternative for men and women seeking to improve their sexual life. Mucuna pruriens and Withania somnifera are extensively used herbs in the Indian system of Medicine-Ayurveda. The present study was conducted to assess the male reproductive health of methanolic extract of seeds of Mucuna pruriens & methanolic extract of roots of Withania somnifera on stress induced male albino Wister rats.

Material and methods: Laboratory-bred adult male albino Wister rats weighing 150 – 220gm were used in the study. The acclimatized animals divided into six groups of six rats each. Group I rats were healthy controls. Group II rats were stress group. Group III standard group treated with stress & sildenafil citrate at a dose of 5mg/kg body weight subcutaneously. Group IV rats were treated with stress & Mucuna pruriens at a dose of 250mg/kg body weight orally Group V rats were treated with stress & Withania somnifera at a dose of 250mg/kg body weight orally, and Group VI rats were treated with stress Mucuna pruriens & withania somnifera. All the rats were treated for 45 days along with stress except controls, tested for mating behavior, hematological, biochemical, stress and reproductive profile.

Results: We found significant increase in Cortisol (30.6%), MDA (36.4%), Testicular cholesterol (78.2%), LH (70.3%) & FSH (211.3%) significant decrease in NO (21.2%), Vit-C (34.3%), Vit-E (39.2%), Testosterone (58.8%), Sperm count (30.7%) & Sperm motility (22.5%) in stress group when comparing with control group. As after treatment with Mucuna pruriens seed extract and Withania somnifera root extract we found significant decrease in Cortisol, MDA, Testicular cholesterol, LH & FSH and significant increase in NO, Vit-C, Vit-E, Testosterone (14.8%), Sperm count (22.4%) & Sperm motility (10.3%).

Conclusion: Beneficial effect of the Withania somnifera & Mucuna pruriens corrugate with a standard pharmaceutical dose of sildenafil citrate in our study. Hence these phytochemical substances may be considered as aphrodisiac as well

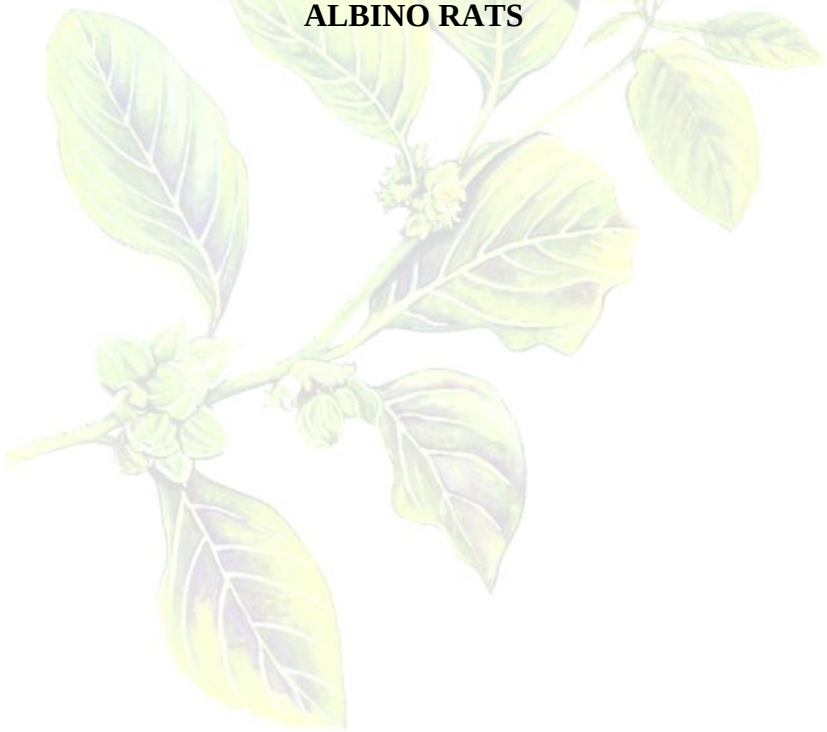
as beneficial to the overall male reproductive health like sildenafil citrate, especially during the stress induced alteration of male reproductive system in albino rats.

Key words: Aphrodisiac, Phytochemical, Extraction, Stress, Sexual dysfunction, Erectile dysfunction, Reproduction, Mating Behavior, Mount, Antioxidants.

CHAPTER 1

INTRODUCTION

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS



2015

BLDE UNIVERSITY'S

SHRI B M PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE

1.1. SEXUAL HEALTH

Sex is an architect of the ideal society on two matters: it is the means of producing new guardians, and it is emotionally charged and essential to a marriage or any other romantic relationship. One of the main aims of marriage is the procreation (reproduction) and more importantly for sexual fulfillment of both partners. Without sex no matter how much one love each other, one will feel there's something losing. Sex is an expression of your love and it releases tension and makes one feel good later on. But no matter how much you love to have sex, it wanes. For life to continue, an organism must reproduce itself before it dies. In *Homo sapiens*, reproduction is initiated by the mating of a male with a female in sexual intercourse which facilitates the coming together of sperm and egg for the purpose of fertilization[1].

1.1.1. MALE SEXUAL FUNCTION

Sexuality is a complex, multi-dimensional phenomenon that incorporates biological, psychological, interpersonal and behavioral dimensions. Sexual behavior in male rats consists of three distinct phases[2]:

- **Mount:** the animal assumes the copulatory position, but does not insert its copulatory organ (the penis) into the vagina
- **Intromission:** the copulatory organ enters the vagina during a mount
- **Ejaculation**: forceful expulsion of semen.

1.2. SEXUAL DYSFUNCTION

Sexual dysfunction is serious medical and social symptom that occurs in 10%-52% of men and 25%-63% of women. Of men aged 40-70 years, an estimated 34.8% have moderate to complete erectile dysfunction. For there to be a normal sexual intercourse and sexual fulfilment in males, the male sexual organs (the copulatory organ, the penis) and factors relating to erection must function normally. Inability to perform this function effectively is a major problem facing the reproductive process. This is known as sexual dysfunction[3].

1.3. STRESS

It has been proved through countless study that our mental attitude has powerful influence on our physical health. All kinds of mental and physical stressors simultaneously influence the body and the mind and leads to psychosomatic disease. Stress and anxiety are commonly thought to be detrimental to sexual function, and this assertion is supported by many empirical studies. Survey studies have generally found a negative relationship between psychological stress or anxiety and sexual functioning in the general population[4&5] and in clinical populations[6& 7].

☞ *'For many years people have searched for ways to achieve sexual desire, sexual health and sexual techniques. This has led to the development and use of different substances known as* ***aphrodisiacs*** *to attain the desired excitement.'*

1.4. TREATMENT OF SEXUAL DYSFUNCTION

1.4.1. APHRODISIAC

Men from different times and era would do anything to prolong their upbeat cravings for sex, hence the need of aphrodisiacs[8].

☞ *"An aphrodisiac is defined as any food or drug that arouses the sexual instinct, induces veneral desire and increases pleasure and performance. This word is derived from 'Aphrodite' the Greek goddess of love and these substances are derived from plants, animals or minerals and since time immemorial they have been the passion of man".*

Aphrodisiacs can be categorized according to their mode of action into three groups:

1. Substances that increase libido (i.e., sexual desire, arousal),
2. Substances that increase sexual potency (i.e., effectiveness of erection) and
3. Substances that increase sexual pleasure

Aphrodisiacs are substances-like food, tea or herbal products, drug or scents believed to enhance libido or sexual desires. The role of aphrodisiacs is to enhance sexual life management sex, especially those who have sexual dysfunctions.

MUCUNA PRURIENS

Scientific classification[9]

Kingdom : Plantae
Division : Magnoliophyta
Class : Magnoliopsida
Order : Fabales
Family : Fabaceae
Subfamily : Faboideae
Tribe : Phaseoleae
Genus : Mucuna
Species : M. pruriens

Figure 1: Plant & Seeds of Mucuna pruriens

Common names

Sanskrit : Kapikacchu
Marathi : Khaajkuiri
English : Velvet bean
Portuguese : Mad Bean
Botanical name : Mucuna pruriens

Mucuna pruriens is sometimes used as a coffee substitute called "Nescafe" (not to be confused with the commercial brand). Cooked fresh shoots or beans can also be eaten. This requires that they be soaked from at least 30 minutes to 48 hours in advance of cooking, or the water changed up to several times during cooking, since otherwise the plant can be toxic to humans. The above described process leaches out chemical compounds such as levodopa, making the product suitable for consumption. In history, M. pruriens has been used as an effective aphrodisiac[10&11]. It is still used to increase libido in both men and women due to its dopamine inducing properties. Dopamine has a profound influence on sexual function[12]. Mucuna pruriens seeds have also been found to have antidepressant properties when consumed. Mucuna pruriens has also recently become popular among lucid dreaming enthusiasts: when combined with other supplements that stimulate the cholinergic system, the dopamine presumably produced from the consumption of Mucuna pruriens confers upon the lucid dreamer greater motivation and confidence [13](fig-1).

For medicinal purposes, the non protein amino acid, L-DOPA is extracted from the seeds for the production of commercial drugs for treatment of Parkinson's disease. The seed powder is known to exhibit faster hypothermic[14&15]. The seed powder is known to stimulate more sexual activity in male albino rats than L-DOPA and also is reported to arouse sexual desire in patients suffering from Parkinson's disease[16].

WITHANIA SOMNIFERA

Scientific classification[17&18]

Kingdom : Plantae

Order : Solanales

Family : Solanaceae

Genus : Withania

Species : W. somnifera

Figure 2: Plant & Roots of Withania somnifera

Common names

English : Winter cherry

Hindi : Asgandh, Punir

Malayalam : Amukkiram, Pevetti

Marathi : Askandha, Kanchuki, Tilli

Sanskrit : Ashvagandha, Gandhapatri,

Botanical name: Withania somnifera (Linn.) Dunal

Withania somnifera (Ashwagandha), also known as "Indian ginseng" due to its rejuvenating effects, has been described in folk medicine as an aphrodisiac and geriatric tonic[19]. *Ashwagandha* is Sanskrit word which means "horse's smell" (*ashwa*-horse, *gandha*-smell), probably originating from the odor of its root. The species name *somnifera* means "sleep-inducing" in Latin[20].It is classified as an "adaptogen," meaning that this herb assists in combating stress and disease. To be particular the root of *Withania somnifera* is regarded as a tonic and aphrodisiac (fig-2).

Withania somnifera is rich in a wide variety of phytochemical compounds, such as alkaloids, ergostane steroids, amino acids, and neurotransmitters, which explains its numerous medicinal properties that can directly or indirectly prevent and treat a number of diseases[21].

BIBLIOGRAPHY

6

1. A. Fullick, Biology. Heinemann Advanced Science. Oxford 1994; 332

2. R. C. Kolodny, W. H. Masters & V. E. Johnson, Sexual anatomy and physiology. 2In: Textbook of Sexual Medicine. Little, Brown and Co., Boston, MA 1979; 1–228

3. A. T. Guay, R. F. Spark, S. Bansal, G. R. Cunningham, N. F. Goodman, H. R. Nankin, S. M. Petak, and J. B. Perez, American Association of Clinical Endocrinologist: Medical Guidelines for Clinical Practice for the evaluation and treatment of male sexual dysfunction. A couple's problem Endocrinol. Pract. 2003; 9(1): 78-95

4. Bodenmann G, Ledermann T, Blattner D, Galluzzo C J Nerv Ment Dis. 2006; 194(7):494-501

5. Dunn KM, Croft PR, Hackett GI Fam Pract. 1998; 15(6):519-24.

6. Figueira I, Possidente E, Marques C, Hayes K Arch Sex Behav. 2001; 30(4):369-77

7. Van Minnen A, Kampman M. The interaction between anxiety and sexual functioning: a controlled study of sexual functioning in women with anxiety disorders. Sex. Relation. Ther. 2000; 15:47–57

8. Yakubu M.T., Akanji M.A., Oladiji A.T. Pharmacog. Rev. 2007; 1: 49

9. Mucuna pruriens Retrieved 2013-05-21

http://www.tropicalforages.info/key/Forages/Media/Html/Mucuna_pruriens.html

10. Mucuna Pruriens, Dopamine, L-dopa, Growth Hormone, Mucuna http://herbalpowers.com/macunapruriens1.html Retrieved 2013-10-18

11. Mucuna Pruriens, Dopamine, L-dopa, Growth Hormone, Mucuna http://herbalpowers.com/macunapruriens1.html. herbal-powers.com Retrieved 2012-10-18

12. Giuliano F, Allard J. Dopamine and male sexual function. Eur Urol. 2001; 40:601-8

13. Giuliano F, Allard J. Dopamine and sexual function. Int. J. Impot. Res. 2001; 13(Suppl 3):18- 28.

14. C. Kalidas & v. Ramasamy. Nutritional and antinutritional composition of itching bean (mucuna pruriens (l.) Dc var. Pruriens): an underutilized tribal pulse in Western Ghats, tamil nadu. Tropical and Subtropical Agroecosystems 2011; 14: 279 - 93

15. Rajendran V., Joseph T., David J. Reappraisal of dopamineric aspects Mucuna pruriens and comparative profile with L-DOPA on cardiovascular and central nervous system in animals. Indian drugs 1996; 33: 465–72

16. Kumar K.V.A., Srinivasan K.K., Shanbhag T., Rao S.G. Aphrodisiac activity of the seeds of Mucuna pruriens. Indian Drugs 1994; 31: 321 –7

17. Gupta A., Mittal A., Jha K. K., and Kumar A. Nature's treasurer: plants acting on colon cancer. Journal of Stress Physiology & Biochemistry 2011; 7 (4): 217– 31

18. Withania somnifera (L) Dunal Germplasm Resources Information Network - (GRIN) Beltsville, Maryland: USDA, ARS, National Genetic Resources Program. National Germplasm Resources Laboratory Retrieved 2011-10-29.

19. Weiner MA, Weiner J. Herbs That Heal. Mill Valley, Calif, USA: Quantum Books Ashwagandha (Indian ginseng) 1994; 70–72.

20. Stearn *WT*. Botanical Latin: History, Grammar, Syntax, Terminology and Vocabulary. 4th edition. Portland, Ore, USA: Timber Press; 1995.

21. Mirjalili MH, Moyano E, Bonfill M, Cusido RM, Palazón J Molecules. 2009 Jul 3; 14(7):2373-93.

CHAPTER 2

REVIEW OF LITERATURE

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

2015

BLDE UNIVERSITY'S
SHRI B M PATIL MEDICAL COLLEGE, HOSPITAL & RESEARCH CENTRE

2.1. SEXUAL HEALTH

2.1.1. Anatomy of Male Reproductive Organs & Penis

Male reproductive Organs

Male genital tract has several main components. In essence, the testis produces sperm, which pass through a series of ducts and are finally expelled via the urethra together with seminal plasma produced by the accessory sexual organs[1] (fig-3).

The testes situated in the scrota and are descended from a retroperitoneal position through the inguinal canal to take its place in the scrotum during the eighth fetal month. Reasons for its unusually vulnerable position be due to the lower temperature required for spermatogenesis[2]. A countercurrent vascular heat exchange system is present to promote cooler temperatures. Seminiferous tubules comprise 95% of testicular volume, and are devoted to the production of spermatozoa. There are approximately 500 tubules per testis. The tubules are divided by fibrous septae, and surrounded by the tough tunica albuginea. Interstitial tissue located between the seminiferous tubules is comprised of connective tissue, blood vessels, lymphatics, and Leydig cells which produce testosterone[3&4].

Leydig Cells Leydig cells lie in the testicular interstitium between the seminiferous tubules, and seem primarily involved in the production of testosterone for local and distant purposes. Distant effects of testosterone include masculinization of external and internal reproductive tissues (alone or via its metabolite DHT), pubertal changes of deepening voice, facial hair pattern, etc., and CNS actions affecting libido and sexual behavior. Local effects appear directed to stimulate and support Sertoli cell function in providing the proper environment for developing germ cells. Testosterone is bound to androgen-binding protein secreted

by the Sertoli cell in the testis, and in the circulation is bound to a high affinity plasma globulin (testosterone binding globulin)[5].

The epididymis is a single convoluted duct with a length of 20 m. The epididymis has major role in the formation of semen which includes sperm conduit, Fluid resorption, Sperm reservoir & Sperm maturation.

Vas Deferens The epididymis continues as the vas deferens, which is a single straight duct transporting sperm through the upper scrotum to the seminal vesicles to form the ejaculatory ducts. The vas provides rapid transport of sperm during ejaculation[6].

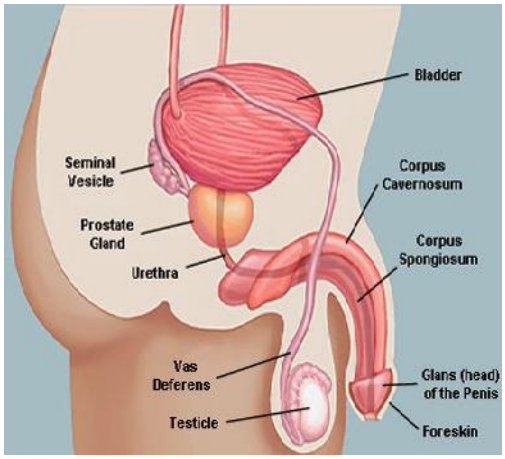


Figure 3: Male Reproductive organs

Seminal vesicles Elongated saccular organs with an irregular branching lumen. Seminal vesicle fluid notable for high fructose and prostaglandin content[7&8](fig-3).

Prostate weighs about 20 grams, with multiple excretory glands which empty into the urethra. A normal human ejaculate has a volume of 2-5 cc and contains 150-200 million sperm[9&10].

Spermatogenesis

Germ cells begin as spermatogonia, which are the stem cells lining the basement layer of the seminiferous tubule. They are small, rounded, mitotically active cells which are sensitive to chemotherapy or radiation. Type A spermatogonia develop into Type B spermatogonia, which subsequently become primary spermatocytes during the first meiotic prophase. Primary spermatocytes go through a series of stages (preleptotene, leptotene, zygotene, pachytene, diplotene) which are identified on the basis of cellular size and increasing nuclear condensation. Secondary

spermatocytes result from the first reduction division. They are diploid, in contrast to the primary spermatocytes which are tetraploid. The second meiotic prophase is very short (1 day) so secondary spermatocytes are not readily visible in tissue sections. Spermatids result from the second reduction division and are therefore haploid. They are numerous, and are found near the tubule lumen. Spermatids may be in many stages of differentiation. Spermatozoa The morphologically mature spermatozoon is released into the tubule lumen. It is a made of head, body and tail. Head consists of the condensed nucleus, the acrosome, and associated membrane structures. The tail consists of a neck, middle piece containing a sheath of mitochondria, the principal piece, and an end piece. A "9+2" axoneme extends from the neck to the end piece[1].

Hypothalamic Pituitary-Gonadal Axis

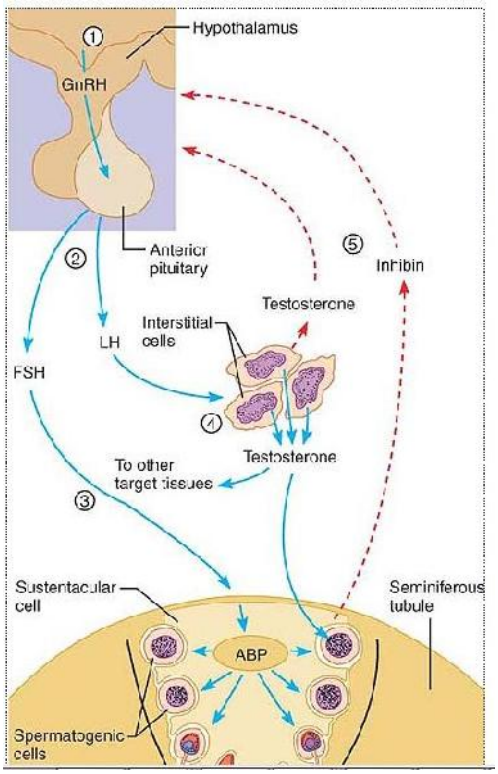


Figure 4 Spermatogenesis & HPG axis

The hypothalamic pituitary-gonadal axis controls spermatogenesis. The germinal epithelium requires high levels of testosterone, supplied by the Leydig cells, but this is not sufficient. There must also be adequate stimulation of the Sertoli cells by the pituitary peptide hormone follicle stimulating hormone (FSH). FSH binding to Sertoli cell receptors stimulates cAMP and protein kinases which lead to increased protein synthesis. Testosterone production by the Leydig cells is also under the regulation of another pituitary polypeptide hormone, luteinizing hormone (LH). LH does not seem to have any effects on seminiferous cells directly[11]. The

release of LH and FSH from the pituitary is under regulation of luteinizing hormone releasing hormone (LHRH), also known as gonadotropin releasing hormone (GnRH), a hypothalamic peptide hormone. LHRH is released in pulses averaging every70-90 minutes, and has a short half-life of 2-5 minutes. Variations in pulse frequency may be responsible for regulating the relative release of FSH or LH from the pituitary. Feedback control mechanisms play an important role in male reproductive physiology. Testosterone inhibits the release of LH from the pituitary and LHRH from the hypothalamus. Estradiol is derived from peripheral conversion of testosterone via the enzyme aromatase, and is a more potent inhibitor of both LH and FSH secretion than testosterone. FSH secretion is down-regulated by a Sertoli cell product termed inhibin. Castration results in a progressive increase of both LH and FSH serum levels since there is no negative feedback [12](fig-4).

Penile Anatomy

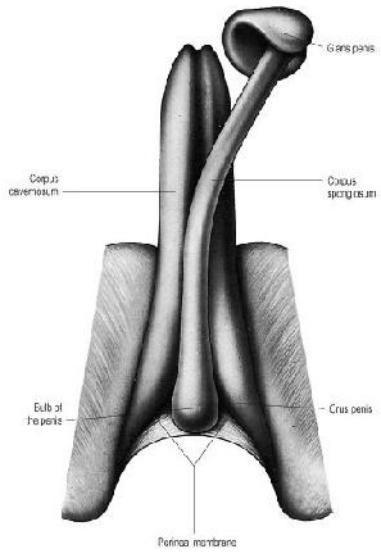


Figure 5: Penile Anatomy[13]

Penis is made up of root and body, both of them are made up of three masses of erectile tissue, root is situated in the urogenital triangle, three erectile masses of penis are namely the two crura and the bulb, firmly attached to the pubic arch and perineal membrane respectively (fig-5). The body of the penis contains three

elongated erectile masses, capable of considerable enlargement when engorged with blood during erection. The erectile masses in the body are the right and left corpora cavernosa, and the median corpus spongiosum, which are continuations of the crura and bulb of the penis respectively

The **corpora cavernosa** of the penis form most of the body. In close apposition throughout, they share a common fibrous envelope and are separated only by a median fibrous septum. On the urethral surface their combined mass has a wide median groove, adjoining the corpus spongiosum. The **corpus spongiosum** of the penis is traversed by the urethra. It adjoins the median groove on the urethral surface of the conjoined corpora cavernosa. Near the end of the penis it expands into a somewhat conical enlargement, the glans penis (fig-5).

The superficial **penile fascia** is devoid of fat, and consists of loose connective tissue, invaded by a few fibres of dartos muscle from the scrotum. Deepest layer is condensed to form a distinct tough fascial sheath known as Buck's fascia. It surrounds both corpora cavernosa and splits to enclose the corpus spongiosum, separating the superficial and deep dorsal veins. At the penile neck it blends with the fibrous covering of all three corpora. Proximally, it is continuous with the dartos muscle and with the fascia covering the urogenital region of the perineum[14](fig-6).

Penis is supplied by penile artery, posterior scrotal, inferior rectal arteries, artery of the bulb, Deep artery of penis and dorsal artery of the penis. Arteries supplying penis arises from a shared branch of the internal pudendal artery and provides an extensive anastomotic network[15]. Nowadays, there is a tendency to perform in vitro experiments using the pudendal artery instead of cavernosal tissue to investigate patophysiological aspects of ED since this artery is the major resistance to penile engogerment during sexual stimulation.

Novel findings suggest that the pudendal artery contributes 70% of the total penile vascular resistence[16]. The arterial blood supply in the CC is mainly fed from the deep penile cavernosal artery (fig-6).

The dorsal veins are superficial and deep. The superficial dorsal vein drains the prepuce and penile skin. It opens into one of the external pudendal veins. The deep dorsal vein lies deep to Buck's fascia. It receives blood from the glans penis and corpora cavernosa penis and connect below the symphysis pubis with the internal pudendal veins and ultimately enter the prostatic plexus [17](fig-6).

The nerves to the corpora cavernosa form two groups, the lesser and greater cavernous nerves, which arise from the front of the pelvic (inferior hypogastric) plexus. Lesser cavernous nerves supply the erectile tissue of the corpus spongiosum and penile urethra. Greater cavernous supply the erectile tissue: some filaments reach the erectile tissue of the corpus spongiosum. Stimulation of the sympathetic supply to the male genital organs produces vasoconstriction contraction of the seminal vesicles and prostate and seminal emission. Parasympathetic fibers are vasodilator and come from the second, third and fourth sacral spinal segments via the pudendal nerve and pelvic plexuses. On the glans and bulb of the penis some cutaneous filaments innervate lamellated corpuscles. Parasympathetic nerves are vasodilators[18&19].

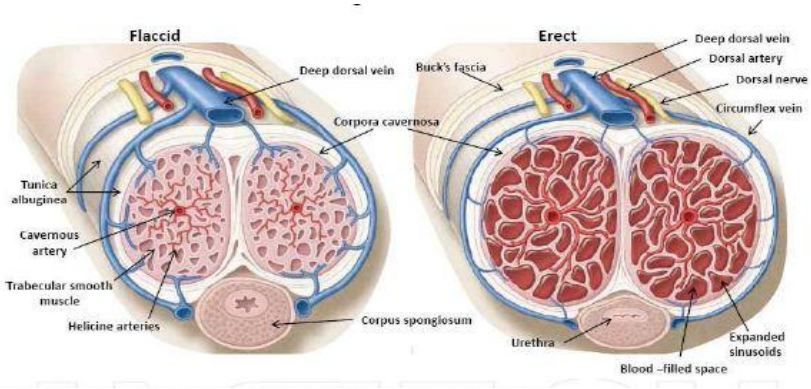


Figure 6: Cut section of Penis[20]

2.1.2. Physiology of erection & ejaculation

Leonardo da Vinci is quoted as having said, “the penis does not obey the order of its master, who tries to erect or shrink it at will, whereas, instead the penis erects freely while its master is fast asleep. The penis must be said to have its own mind[21]…”

Erection is purely vascular. It occurs in response to parasympathetic stimulation and is independent of compression by the ischiocavernosi and bulbospongiosus, although these may contribute to maximum rigidity[22]. Sexual arousal leads to rapid inflow from the helicine arteries following relaxation of the smooth muscle of the corpora cavernosa, an event which is dependent on the production of nitrous oxide and cyclic GMP. This inflow of blood fills the cavernous spaces leading to tumescence. The resulting distension converts tumescence to erection by pressure on the subtunical veins, which drain the erectile tissue, thereby obstructing them. The pressure within the corpora cavernosa is maintained at 100 mmHg to maintain penile erection[23]. Continuing cutaneous stimulation of the glans and frenulum contributes significantly to maintaining erection and initiating orgasm and ejaculation. Erection is thus dependent on a normal psychogenic response to stimulation, intact parasympathetic nerves, corporal smooth muscle capable of relaxation, patent arteries capable of delivering blood at the required rate, and a normal venous system[24](fig-4).

Ejaculation consists of two processes: **emission** and **ejaculation**. Emission is the transmission of seminal fluid from the vasa, prostate and seminal vesicles into the prostatic urethra under sympathetic control. Ejaculation is the onward transmission of seminal fluid from the prostatic urethra to the exterior. This has autonomic and somatic components. The first discernible part of the process is contraction of bulbospongiosus, which contracts about six times under somatic control. The way in

which seminal fluid crosses the external urethral sphincter into the bulbar urethra is not clear: it is known to be under autonomic control and is timed such that from the second to the final contraction of bulbospongiosus the ejaculate appears from the external meatus, in some younger men in a pulsatile fashion[25].

Failure to achieve tumescence with adequate stimulation is termed impotence or more recently and politically correctly, erectile dysfunction. The mechanism of erection is complex: failure in any of the previously mentioned components can result in impotence. The commonest causes include psychogenic disturbance with failure to relax cavernous smooth muscle; arterial insufficiency, as a result of atheromatous disease; and damage to the parasympathetic nervous system secondary to diabetes or following pelvic surgery such as radical prostatectomy, radical cystectomy or bowel resection. Pharmacotherapy is predominantly directed at achieving cavernosal smooth muscle relaxation[26].

Detumescence is affected through the sympathetic pathway. Failure of an erection to detumesce is termed priapism. This can occur spontaneously but is most commonly seen with conditions that impair blood flow by increasing its viscosity such as sickle cell anaemia or leukaemia, or as a consequence of drug treatment when given by injection. These conditions result in ischaemia of the corporal smooth muscle, which causes pain within the penis.

2.1.3. Sexual function

Sexual stimulation of the male results in a series of psychological, neuronal, vascular, and local genital changes. At least three different classifications for these changes have been described. According to Kolodny et al. psychosexual response cycle that consists of four phases; excitement, plateau, orgasm and resolution[27]. Another classification by Govier et al. psychosexual phases classified based on penodynamic changes during the sexual cycle which divides each of the psychosexual

phases into two interrelated events as excitement into latency and tumescence; plateau into erection and rigidity; orgasm into emission and ejaculation; and resolution into detumescence and refractoriness[28]. The third classification by Walsh and Wilson et al. focuses on the functional activities during the sexual cycle by adding an initial phase of desire or libido[29]. Thus, the normal male sexual response cycle can be functionally divided into five interrelated events that occur in a defined sequence: libido, erection, ejaculation, orgasm, and detumescence.

Steps involved male sexual response

1. Libido or sexual desire- Libido is defined as the biological need for sexual activity (the sex drive) and frequently is expressed as sex-seeking behavior. Its intensity is variable between individuals as well as within an individual over a given time. Higher serum testosterone appears to be associated with greater sexual activity in healthy older but not younger men[30].

2. ***Erection-*** Erection is the enlarged and rigid state of the sexually aroused penis sufficient enough for vaginal penetration. It results from multiple psychogenic and sensory stimuli arising from imaginative, visual, auditory, olfactory, gustatory, tactile, and genital reflexogenic sources.

3. Ejaculation- Ejaculation is the act of ejecting semen. It is a reflex action that occurs as a result of sexual stimulation. It is made up of two sequential processes. The first process called emission is associated with deposition of seminal fluid into the posterior urethra while the second process is the true ejaculation, which is the expulsion of the seminal fluid from the posterior urethra through the penile meatus.

4. Orgasm- This is the climax of sexual excitement. The entire period of emission and ejaculation is known as the male orgasm[31].

5. Detumescence- This is the subsidence of an erect penis after ejaculation[32].

2.2. SEXUAL DYSFUNCTION

Sex disorders of the male are classified into disorders of sexual function, sexual orientation, and sexual behaviour. In general, several factors must work in harmony to maintain normal sexual function. Such factors include neural activity, vascular events, intracavernosal nitric oxide system and androgens[33]. Thus, malfunctioning of at least one of these could lead to sexual dysfunction of any kind. Sexual dysfunction in male refers to repeated inability to achieve normal sexual intercourse. It can also be viewed as disorders that interfere with a full sexual response cycle. These disorders make it difficult for a person to enjoy or to have sexual intercourse. While sexual dysfunction rarely threatens physical health, it can take a heavy psychological toll, bringing on depression, anxiety, and debilitating feelings of inadequacy. Unfortunately, it is a problem often neglected by the health care team who strive more with the technical and more medically manageable aspects of the patient's illness[34]. Sexual dysfunction is more prevalent in males than in females and thus, it is conventional to focus more on male sexual difficulties[33]. It has been discovered that men between 17 and 96 years old could suffer sexual dysfunction as a result of psychological or physical health problems[35]. Generally, a prevalence of about 10% occurs across all ages. Because sexual dysfunction is an inevitable process of aging, the prevalence is over 50% in men between 50 and 70 years of age[36]. As men age, the absolute number of Leydig cells decreases by about 40%, and the vigour of pulsatile lutenizing hormone release is dampened. In association with these events, free testosterone level also declines by approximately 1.2% per year. These have contributed in no small measure to prevalence of sexual dysfunction in the aged[33]. Male sexual dysfunction (MSD) could be caused by various factors. These include: psychological disorders (performance anxiety, strained relationship, depression, stress, guilt and fear of sexual failure), androgen deficiencies

(testosterone deficiency, hyperprolactinemia), chronic medical conditions (diabetes, hypertension, vascular insufficiency (atherosclerosis, venous leakage), penile disease (Peyronie's, priapism, phinosis, smooth muscle dysfunction), pelvic surgery (to correct arterial or inflow disorder), neurological disorders (Parkinson's disease, stroke, cerebral trauma Alzhemier's spinal cord or nerve injury), drugs (side effects) (anti-hypertensives, central agents, psychiatric medications, antiulcer, antidepressants, and anti-androgens), life style (chronic alcohol abuse, cigarette smoking), aging (decrease in hormonal level with age) and systemic diseases (cardiac, hepatic, renal pulmonary, cancer, metabolic, post-organ transplant)[33,37 &38]. Sexual dysfunction takes different forms in men. A dysfunction can be life-long and always present, acquired, situational, or generalized, occurring despite the situation. A man may have a sexual problem if he:

- Ejaculates before he or his partner desires
- Does not ejaculate, or experiences delayed ejaculation
- Is unable to have an erection sufficient for pleasurable intercourse
- Feels pains during intercourse
- Lacks or loses sexual desire.

2.2.1. Types of sexual dysfunction

Male sexual dysfunction can be categorized as disorders of desire, disorders of orgasm, erectile dysfunction, and disorders of ejaculation and failure of detumescence.

A. Disorders of desire: Disorders of desire can involve either a deficient or compulsive desire for sexual activity. Dysfunctions that can occur during the desire phase include:

(i) Hypoactive sexual desire (HSD), defined as persistently or recurrently deficient (or absent) sexual fantasy and desire for sexual activity leading to marked

distress or interpersonal difficulty. It results in a complete or almost complete lack of desire to have any type of sexual relation[39].

(ii) Compulsive sexual behaviors (CSBs) onstitute a wide range of complex sexual behaviors that have strikingly repetitive, compelling, or driven qualities. They usually manifest as obsessive-compulsive sexuality (*e.g.* excessive masturbation and promiscuity), excessive sex-seeking in association with affective disorders (*e.g.* major depression or mood disorders), addictive sexuality (*e.g.* attachment to another person, object, or sensation for sexual gratification to the exclusion of everything else), and sexual impulsivity (failure to resist an impulse or temptation for sexual behaviour that is harmful to self or others such as exhibitionism, rape, or child molestation)[40].

B. Erectile dysfunction (ED): This is a problem with sexual arousal**.** ED can be defined as the difficulty in achieving or maintaining an erection sufficient for sexual activity or penetration, at least 50% of the time, for a period of six months[41]. It results in significant psychological, social and physical morbidity[42], and annihilates his essence of masculinity[43]. Details described under separate topic.

C. Disorders of ejaculation: There exists a spectrum of disorders of ejaculation ranging from mild premature to severely retarded or absent ejaculation. These include: (i) Premature ejaculation which is the most common male sexual dysfunction[44] and can be any of the following: a) persistent or recurrent ejaculation with minimum sexual stimulation that occurs be ore, upon, or shortly after penetration and before the person wishes it; b) marked distress or interpersonal difficulty; and c) the condition does not arise as a direct effect of substance abuse. Premature ejaculation and sexual desire disorders were the frequent reported problems in young adult males with adverse familial relationship[45]. (ii) Painful ejaculation which results from side effect of tricyclic antidepressants is a persistent and recurrent pain in the genital organs during ejaculation or immediately afterwards[46]. (iii) Inhibited or

retarded ejaculation: This is when ejaculation does not occur at all. (iv) Retrograde ejaculation: This is when ejaculation is forced back into the bladder rather than through the urethra and out of the end of the penis at orgasm.

D. Disorders of orgasm: Male orgasmic disorder is defined as a persistent or recurrent delay in or absence of orgasm after a normal sexual excitement phase during sexual activity[37].

E. Failure of detumescence: is a prolonged erection usually lasting for between 4 h or greater. It is painful and always unaccompanied by sexual desire despite the fact that it is often preceded by usual sexual stimuli. Diagnostic options for male sexual dysfunction include: patient's history which embodies medical history (evaluating historical events like chronic disease, pharmacological agents, endocrine disorders, surgeries and trauma), psychological history (assessing individual's upbringing relationships, early sexual experiences, inadequate sexual information and general psychological health), sexual history (to ascertain the time and manner of onset, its course, current status, and associated medical or psychological problems), physical examination (entails general and systemic evaluation, assessment of gonadal function, vascular competence, neurological integrity, and genital organ normalcy), diagnosis testing (include blood tests, vascular assessment, sensory testing and nocturnal penile tumescence and rigidity testing)[33&36].

2.2.2. Erectile dysfunction

Erectile dysfunction (ED) or (male) impotence is a sexual dysfunction characterized by the inability to develop or maintain an erection of the penis[47]**.** There are various underlying causes, such as cardiovascular leakage and diabetes, many of which are medically treatable. The causes of erectile dysfunction may be physiological or psychological[42]. Folk remedies have long been advocated, with some being advertised widely since the 1930s[43]**.** The introduction of the first

pharmacologically approved remedy for impotence, sildenafil (trade name Viagra), in the 1990s caused a wave of public attention, propelled in part by heavy advertising[48&49] (5)**.**

There are many herbal drugs that have been used by men with ED with varying degrees of success. Most potent herbal aphrodisiacs are available and have little or very little side effects

Erectile dysfunction (ED) is a widespread problem affecting many men across the world at all age groups. More than 30 million men suffer from ED in the U.S.[50] and it is becoming a public health issue. The prevalence of ED is very high and is expected to rise considerably over the next 25 years, impacting more than 300 million men by 2025[51]. ED is defined as the persistent inability to maintain or achieve a penile erection sufficient for satisfactory sexual performance.

2.2.3. Etiology of sexual dysfunction

Male sexual function is a product of several factors; various types of male sexual dysfunction arise from malfunctioning of any of those factors, they may be Psychological disorders, Androgen deficiencies, Pelvic surgeries to correct arterial inflow, Neurological disorders, Drugs, Life style, Aging and Chronic medical conditions like Diabetes, hypertension, vascular insufficiency (atherosclerosis, venous leakage), penile disease etc[37]. Etiological factors implicated for sexual dysfunction are summarized if fig-4[33].

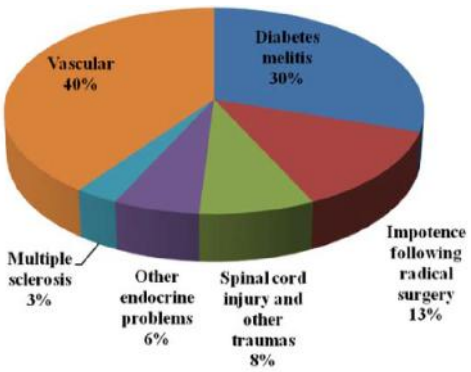


Figure 7: Factors involved in the erectile dysfunction

Various aspects affect the expression/degree of ED and risk factors include age, diabetes mellitus, neurologic diseases, smoking and cardiovascular diseases (CVD), among others[52] (fig-5). Although the disorder has been described for more than 1000 years, the molecular basis and mechanisms of ED have yet to be completely understood. In the last 4 decades, elucidation of the macroscopic structures of the erectile system 4-5 ushered in a new era of therapeutic options for erectile disorders[53&54]. Later, new insights into erectile neurotransmission[55], essentially the nitric oxide (NO) pathway[56], resulted in rational alternatives as a treatment[57]. Nowadays, advances in gene discovery and intensive research regarding different mechanisms which could lead to ED have increased the working knowledge of the pathways involved in this condition. The penis is composed of three bodies of erectile tissue running in paralel; the corpus spongiosum, encompassing the urethra and terminating in the glans penis; and the two corpora cavernosa (CC) which function as blood-filled capacitors providing structure to the erect organ[22]. The penile CC is highly specialized vascular structures that are morphologically adapted to their function of becoming engorged during sexual arousal. The trabecular smooth muscle constitutes approximately 40-50% of tissue cross-sectional area, as assessed by histomorphometric analysis[23]. There are three main arteries in the penis: cavernosal, dorsal, and bulbourethral. All three arise from a shared branch of the internal pudendal artery and provide an extensive anastomotic network[15]. Nowadays, there is a tendency to perform *in vitro* experiments using the pudendal artery instead of cavernosal.

2.2.4. Diagnosis of sexual dysfunction

Sexual dysfunction can be diagnosed by various examinations like by taking history of psychological and sexual activity, by physical examination and by diagnostic tests of blood semen etc.

2.3. Treatment of sexual dysfunction

2.3.1. Psychological/behavioral therapy with a trained counselor

2.3.2. Non-Surgical treatment

2.3.2.1. Vacuum pump Expands the penis and reduces pressure within the cavernous sinusoidal space

2.3.2.2. Constrictive rings External device used for managing erectile dysfunction in patients with mild to moderate venous leakage

2.3.3. Surgical treatment

2.3.3.1. Venous ligation Used to correct leakage of blood from the veins

2.3.3.2. Penile prosthesis Creates adequate space within the tissue of each cavernously body.

2.3.3.3. Penile implants Involves inserting a malleable or rigid substance into the penis to affect a semi-rigid state.

2.3.4. Pharmacotherapy **(Sildenafil citrate)**

There are many pharmaceutical drugs even though sildenafil citrate is considered as one of the prize winning because of its efficacy and pleiotropic effect among the phoshpodiesterases-5[58]. Sildenafil, sold as Viagra and other trade names, a medicine used for erectile dysfunction. Its effectiveness for treating sexual dysfunction in women has not been demonstrated[59]. Common side effects include headache and heart burn, as well as flushed skin. Rare but serious side effects include prolonged erection, which can lead to damage to the penis, and sudden-onset hearing loss. It was originally discovered by Andrew Bell, David Brown, and Nicholas Terrett Since becoming available in 1998[60].

2.3.5. Phytotherapy Involves the use of herbs (medicinal plants) **Aphrodisiac**

An aphrodisiac can be described as any substance that enhances sexual pleasure and/or sex drive. Aphrodisiac can be viewed as any drug, device, food, or scent that can arouse or increase sexual drive or libido[61]. Aphrodisiac substances cross the blood brain barrier and mimic or stimulate area of sexual arousal in the central nervous system. Few of nutritional foods improve the well being of the individual and consequently improving sexual performance and libido. These substances may act physiologically to increase blood flow to the penile erectile tissue, or increase the duration of sexual activity by numbing the genital area or even mimic the burning sensation of sexual inter course[34]. These substances may also limit the influence of sympathetic nervous system in order to correct erectile dysfunction.

There are two main types of aphrodisiacs, psychophysiological stimuli (visual, tactile, olfactory and aural) preparations and internal preparations (food, alcoholic drinks and love portion)**.** The increasing incidence of male sexual dysfunction is necessitating more and rapid search into plants with aphrodisiac potentials[62].

Treatment of sexual dysfunction usually involves a psycotherapetic approach; on the other hand pharmacotherapy, involves drug such as sildenafil, papvarin, alprostadil, vardnafil central stimulants like apomorphine and herbal drugs with aphrodisiac activity are also used for the treatment of sexual dysfunction.

Several food material like *turmeric, garlic, arjun, giloe Shatavari, ginger, pepper coriander, garlic, ginger* and *tulsi* etc have been reported to have beneficial effect on sexual stimulation. Badami reported that milk decoction of *coriander, garlic, ginger* and *tulsi* exhibited potent anti-oxidant activity when compared to their corresponding water decoction[63].

According to the World health organization, traditional medicines are widely used in India. Approximately 80% of the population of developing countries relies on

traditional medicines for their primary health care needs. India is one of the 12 mega-diversity countries. It is estimated that around 70,000 plant species, approximately 7500 species have been recorded to have medicinal value. The 300 species are used by 7800 medicinal drug manufacturing units in India, which consume about 2000 tons of herbs annually. In recent years, the growing demand for herbal products has led to the extinction of many important plant herbs. Medicinal plants continue to play a central role in the health care system of large proportions of the world's populations. Recognition and development of the medicinal and economic benefits of these plants are on the increase in both developing and industrialized nations. The medicinal plants contain several phytochemicals such as Vitamins (A, C, E, and K), Carotenoids, Terpenoids, Flavonoids, Polyphenols, Alkaloids, Tannins, Saponins, Enzymes, and Minerals etc. These phytochemicals possess antioxidant activities, which can be used in the treatment of multiple ailments.

Mechanism involved in Aphrodisiacs

On sexual stimulation (visual (or) otherwise the famines of the axons of parasympathetic nerves release nitric oxide (NO) gas. The gas diffuses into smooth muscle cells that line those arteries of the corpus carvenosum (spongy erectile tissue) and activates the enzyme guanylate cyclase (GC). The later converts the nucleotide guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cGMP). The cGMP in turn causes the smooth muscle cells around the penis to relax, leading to dilation and increased flux of blood into the penile tissue. This blood is essentially trapped in the penis and results in an erection. The erection ceases after a while because cGMP is hydrolyzed by phosphodiesterase type-5 enzyme (PDE-5) into inactive GMP. (The PDE-5 enzyme resides in the penile tissues). Aphrodisiac potentials inhibit the hydrolyzing action of PDE-5 with the result that active cGMP

can accumulate. ‘Undisturbed’ and prolong the erection through increased blood flow[64& 65].

2.3.5.1. Mucuna pruriens

Mucuna includes approximately 150 species of annual and perennial legumes. Among the various under-utilized wild legumes, the velvet beanMucuna pruriens is widespread in tropical and sub-tropical regions of the world. It is considered a viable source of dietary proteins[66]. Human contact results in an intensely itchy dermatitis, caused by mucunain. The nonstinging types, known as “velvet bean” have appressed, silky hairs. The velvet bean has been traditionally used as a food source by certain ethnic groups in a number of countries. M. pruriens is a popular Indian medicinal plant, which has long been used in traditional Ayurvedic Indian medicine, for diseases including Parkinsonism. M. pruriens is reported to contain L-dopa as one of its constituents[67]. The beans have also been employed as a powerful aphrodisiac in Ayurveda and have been used to treat nervous disorders and arthritis. The bean, if applied as a paste on scorpion stings, is thought to absorb the poison[68]. These factors include polyphenols, trypsin inhibitors, phytate, cyanogenic glycosides, oligosaccharides, saponins, lectins, and alkaloids. Recently, phenolics have been suggested to exhibit health related functional properties such as anti-carcinogenic, anti-viral, anti-microbial, anti-inflammatory, hypotensive, and anti-oxidant activities.

2.3.5.2. Withania somnifera

Withania somnifera is a xerophytic plant, found in the drier parts of India, Sri Lanka, Afghanistan and Sind and is distributed in the Mediterranean regions, the Canaries and Cape of Good Hope. It is found in high altitude ascending to 5,500 feet in the Himalayas. This shrub is common in Western India. It grows wildly throughout India particularly in hotter parts. It is also cultivated for medicinal purposes in fields and open grounds throughout India. Therapeutic importance of the different parts of

this plant has a long history and is mentioned in Charak Sanghita. It is an ingredient in many formulations prescribed for a variety of musculoskeletal condition (e.g., arthritis, rheumatism) and as a general tonic to increase energy, improve overall health and longevity and prevent disease in athletes, the elderly and during pregnancy. It is also used as an anti stressor and antioxidant agent[69&70]. Ashwagandha root was used historically as an aphrodisiac. This herb is mentioned in the ancient Kama Sutra as an herb to be used for heightening sexual experience, this herb has the ability to restore sexual health and improve overall vitality while promoting a calm state of mind. Laboratory studies show it can produce nitric oxide which is known to dilate blood vessels.

Sexual dysfunction can be treated by both medical and surgical treatment modalities; however, plant-derived and herbal remedies continue to be a popular alternative for men and women seeking to improve their sexual life despite the availability of effective conventional medical treatments. The study has been undertaken to prove two herbal extracts are beneficial for not only during sexual dysfunction but also during stress induced impotence.

It has been very well documented that, in addition to conventional therapies, many individuals with sexual dysfunction often seek alternative therapies. It is noteworthy that, from ancient times, Ashwagandha has been used by many practitioners as an aphrodisiac to improve on matters related to infertility and sexual activities. Numerous human and animal studies have validated the aphrodisiac and testosterone-enhancing effects of *Withania somnifera*[71].

2.4. Stress and sexual function

We all know the feeling whether it's hands trembling as after flip through a blank exam or trouble sleeping while worry about approaching deadlines. Stress is an inevitable aspect of life through college and beyond. While everyone understands the

symptoms of the stress response, Some stress, of course, can be beneficial. The pressure it exerts can be an incentive to accomplish necessary goals. Often, however, stress reaches chronic, harmful levels, and deleterious consequences follow, from compromised immune function to weight gain to developmental impairment. The intensity of the stress response is governed largely by glucocorticoids, the primary molecules involved in the stress response. Stress can be ephemeral and beneficial, or it can be long-lasting and harmful, causing suffocation, depression, and paralysis. In modern era working for long term in the office, workloads, pressure of target, all these have made the people to enjoy only on the weekends even people will have sex only during weekends. Official pressure made this condition worse; this led to development of sexual & erectile dysfunction. There may be other causes also as shown in fig- 8.

Figure 8: Probable mechanism of pathogenesis of erectile dysfunction

2.4.1. Various methods of stress induction in rats

Forced swimming test: The forced swimming test is based on the observation that animals develop an immobile posture in an in escapable cylinder filled with water. In this test, immobility is interpreted as a passive stress-coping strategy or depression-like behavior[72].

Tail suspension test: In the TST, mice are suspended by their tails using adhesive tape to a horizontal bar for a certain couple of minutes, and the time of immobility is

recorded. Typically, the suspended rodents perform immediately escape-like behaviors, followed by developing an immobile posture. A major advantage of the TST is that it is simple and inexpensive. A major disadvantage of the TST is that it is restricted to mice and limited to strains that do not tend to climb their tail[73].

Immobilization stress (Restraint stress): rats will be made immobilize in the restricted small chamber it is easy method for inducing the stress.

Elevated plus maze: For the elevated plus maze test, the rodents are placed at the intersection of the four arms of the maze (two open, two closed), facing an open arm. The number of entries and time spent in each arm is recorded and valid results are obtained in a single 5-minute testing session[74].

Chemical stress (writhing test): Stress will be inducing by giving various drugs to the animals where drug interfere with metabolic pathways and generates reactive oxygen species (ROS). Due to excessive free radical production cell membrane destruction and cell damage will occur.

2.5. Male Reproductive System and Oxidative stress

Infertility affects approximately 15% of couples trying to conceive, and a male factor contributes to roughly half of these cases. Oxidative stress (OS) has been identified as one of the many mediators of male infertility by causing sperm dysfunction. OS is a state related to increased cellular damage triggered by oxygen and oxygen-derived free radicals known as reactive oxygen species (ROS). During this process, augmented production of ROS overwhelms the body's antioxidant defenses. While small amounts of ROS are required for normal sperm functioning, disproportionate levels can negatively impact the quality of spermatozoa and impair their overall fertilizing capacity. OS has been identified as an area of great attention because ROS and their metabolites can attack DNA, lipids, and proteins; alter enzymatic systems; produce irreparable alterations; cause cell death; and ultimately, lead to a decline in the semen parameters associated with male infertility[75]. K K Das

et al reported metal (Nickel) exposer in male rats leads elevated testicular lipid peroxidation and decreased sperm count, sperm motility, alteration of steroidogenesis, and suppressed antioxidant activity[76].

2.6. Antioxidant

Popularity of antioxidant has increased in modern society and also gained publicity through its health benefits. Antioxidants are enzymes or organic substances counteracting the damaging effects of oxidation. They act as chelating agents. They are the first line of defense against free radicals and also prevent the oxidation of other molecules. Antioxidants prevent cell destruction caused by the free radicals.Dietary content of anti-oxidants like polyphenolic compounds, vitamin C, and vitamin E help in prevention of oxidative stress related diseases[77]. Anti-oxidant are of two varieties - enzymatic and non-enzymatic.

2.6.1. Non-enzymatic antioxidant

L-Ascorbic acid and alpha-tocopherol are water soluble antioxidants. L-ascrobic acid is essential for neurotrasmitter synthesis like carnitine etc. It is widely distributed in fruits and vegetables. It protects different types of oxidative stress including metal ion dependent and independent processes. Many studies have shown that physiological concetration of ascorbic acid inhibits LDL oxidation by vascular endothelial cells[78].

2.6.2. Enzymatic anti-oxidant

Super oxide dismutase (SOD), catalase, glutathione and glutathione peroxidase are enzymatic antioxidants. One of the most effective intercellular antioxidant is super oxide dismutase (SOD). It catalyzes the dismutation of super oxide into oxygen and hydrogen peroxide. Superoxide reacts with nitric oxide to form peroxynitrite which is harmful for the human body. Super oxide dismutase prevents the formation of peroxynitrite by dismutation of super oxide. Diminished levels of

total antioxidant and SOD have an important role in the development of atherosclerosis [4].

In spite numerous studies report the efficacy of Withania somnifera and Mucuna pruriens in the treatment of various diseases, specific aphrodisiac effect of Withania somnifera roots and seeds of Mucuna pruriens especially in stress induced studies assessing the effectiveness of in treating male infertility have not been found to the best of our knowledge, mostly, lacking critical data on safety and tolerability of the therapy. Hence, the present study aims to investigate the usefulness of a highly concentrated, full-spectrum root extract of Withania somnifera seed extract of Mucuna pruriens as a suitable herbal supplement in treating male infertility in stress induced rats.

BIBLIOGRAPHY

1. Standring Susan (eds), Gray's Anatomy, 40th edition, Churchill Livingstone, London 2008; 1261–77.
2. Abraham Morgentaler, Brandon C. Stahl, And Yizhong Yin Testis And Temperature: An Historical, Clinical, And Research Perspective, Sperm Physiology And Male Infertility Evaluation 1979; 12-18
3. Taylor, D. J., Green, N. P. O., Stout, G. W. and Sooper, R. Biological Science. Low Price Edition. Cambridge University Press 1998; 726
4. Johnson, L. R. Essential Medical Physiology. Raven Press 1992; 610-629.
5. Fawcett, D. W.: Perspect. Biol. Med., Winter 1979
6. Grenier, G and Byers, E. S. Rapid ejaculation: a review of conceptual, etiological and treatment issues. Arch. Sex. Behav. 1995; 22: 447-72
7. Prasad M. R. N. & Rajalakshmi M. Spermatogenesis & Accessory Gland Secretions. In: Textbook of Biochemistry and Human Biology. 2nd Ed. Prentice-Hall of India Private Limited, New Delhi 1989; 883
8. Gonzales G. F. Functional structure and ultrastructure of seminal vesicles. Arch. Androl. 1989; 22: 1-13
9. Gonzales G. F. and Villena, A. True corrected seminal fructose level: a better marker of the function of seminal vesicles in infertile men. Int. J. Androl. 2001; 24(5): 255- 66.
10. Wolff J. M., Ittel T. H., Borchers H., Brauers A. and Jakse, G. Efficacy of Skeletal Alkaline Phosphatase and Prostate-Specific Antigen in the Diagnosis of Bone Metastasis in Cancer of the Prostate. Urol. Int. 1998; 61:12-6
11. Moore K. L. & Dalley A. F. Clinical Oriented Anatomy, 4th Ed. Lippincot Williams and Williams; a Woller Klumner Corporation, Philadelphia 1999; 263-71

12. Walsh P. C. Swerdloff R. S. And Odell, W. D. Acta Endocrinol. 1973; 74:449

13. Abrahams PH, Marks S C Jr, Hutchings R. McMinn's Colour Atlas of Human Anatomy, 5th edn. London: Churchill Livingstone 2002

14. Hinman F. Jr. Structure and function of the penis and male urethra. In: Atlas of urosurgical anatomy. W. B. Saunders, Philadelphia 1993; 430-41

15. Yiee J H, Baskin L S. Penile embryology and anatomy. Scientific World Journal. 2010; 10: 1174-9

16. Manabe K, Heaton JP, Morales A. Kumon H. Adams M. A. Pre-penile arteries are dominant in the regulation of penile vascular resistance in the rat. Int J Impot Res. 2000; 12: 183-9

17. Moscovici J, Galinier P, Hammoudi S, Lefebvre D, Juricic M, Vaysse P. Contribution to the study of the venous vasculature of the penis. Surg Radiol Anat. 1999; 21: 193-9

18. Lepor H, Gregerman M, Crosby R, Mostofi FK, Walsh PC, Precise localization of the autonomic nerves of the pelvic plexus to the corpora cavernosa: a detailed anatomical study of the adult male pelvis. J. Urol. 1985; 133: 207-212

19. Dean R C, Lue T F. Physiology of penile erection and path physiology of erectile dysfunction. Urol Clin North Am. 2005;32: 379-95

20. Fazio L, Brock G. Erectile dysfunction: management update. CMAJ. 2004; 170: 1429-37

21. Goldstein I., Central Mechanisms in Erectile Dysfunction. Male sexual circuitry. Sci. Am. 2000; 283: 70-75

22. Andersson KE, Wagner G. Physiology of penile erection. Physiol Rev. 1995; 75: 191-236.

23. Nehra A, Azadzoi KM, Moreland RB, et al. Cavernosal expandability is an erectile tissue mechanical property which predicts trabecular histology in an animal modelof vasculogenic erectile dysfunction. J Urol. 1998; 159: 2229-36

24. Mundy AR, Fitzpatrick J, Neal D, George N (eds), Male sexual function. In: The Scientific Basis of Urology, Chapter 12. Isis Medical Media: 1999; 243-53.

25. Ganong, W. F. Review of Medical Physiology. 20th Edition Lange Medical Books/McGraw-Hill Medical Publishing Division, London 2001; 414-7

26. Guyton, A. C. and Hall, J. E. Textbook of Medical Physiology 10 Ed. Harcourt International Edition, W. B. Saunder Company, Philadelphia 2000; 279-81

27. R. C. Kolodny, W. H. Masters and V. E. Johnson, Sexual anatomy and physiology 16In: Textbook of Sexual Medicine. Little, Brown and Co., Boston, MA. 1979 pp. 1616–28

28. F. E. Govier, D. Asase, T. R. Hefty, R. D. McClure, T. R. Pritchett and R. M. Weissman, Timing of penile color flow duplex ultrasonography using a triple drug mixture. J. Urol. 1995; 153: 1472–75

29. P. C. Walsh and J. D. Wilson, Impotence and infertility in men In: Braunwald E, Isselbacher KJ, Petersdorf RS, Wilson JD, Martin JB, Fauci AS (eds) Harrison's Principals of Internal Medicine, ed 11. McGraw-Hill Book Co., NY, 1987; 217–220

30. B. K. Toone, M. Wheeler, M. Nanjee, P. Fenwick and R. Grant, Sex hormones, sexual activity and plasma anticonvulsant levels in male epileptics. J. Neurol. Neurosurg. Psychiatry, 1983; 46:824–826.

31. A. C. Guyton and J. E. Hall, Textbook of Medical Physiology (Tenth Edition). Harcourt International Edition, W. B. Saunder Company, Philadelphia 2000; pp. 279-281.

32. G. Wagner, Erection: physiology and endocrinology. In: Wagner, G. and Green, R. (eds) Impotence: Physiological, Psychological, Surgical Diagnosis and Treatment. Plenum Press, NY, 1981; pp. 25–36.

33. A. T. Guay, R. F. Spark, S. Bansal, G. R. Cunningham, N. F. Goodman, H. R. Nankin, S. M. Petak, and J. B. Perez, American Association of Clinical Endocrinologist: Medical Guidelines for Clinical Practice for the evaluation and treatment of male sexual dysfunction. A couple's problem. Endocrinol. Pract. 2003; 9(1): 78-95.

34. P. H. Salmon, Psychosexual dysfunction in chronic renal failure: An overview. Proc. EDTNA, 1983; 12: 209-212.

35. F. M. Joe, Erectile Dysfunction: Sexual response in men. Arch Sexual Behav, 1990; 14: 33-39.

36. M. S. Rendell, J. Rajfer and P. A. Wicker, Sildenafil for treatment of erectile dysfunction in men with diabetes. JAMA, 1999; 282: 421-426

37. H. A. Feldman, I. Goldstein, D. G. Hatzichristou, R. J. Krane, and J. B. McKinlay, Impotence and its medical and psychosocial correlates: results of the Massachusetts Male Aging Study. J Urol. 1994; 151:54–61

38. F. R. Kandeel, V. K. T. Koussa, and R. S. Swerdloff, Male sexual function and its disorders: Physiology, Pathophysiology, Clinical Investigation and, Treatment. Endocr. Rev, 2001; 22(3): 342-388.

39. American Psychiatric Association (APA), Diagnostic and Statistical Manual of Mental Disorders, ed 4. American Psychiatric Association, Washington, DC1994.

40. H. S. Kaplan, Erotic obsession: relationship to hypoactive sexual desire disorder and paraphilia. Am. J. Psychiatry, 1996; 153(Suppl 7): 30–41.

41. E. O. Laumann, A. Paik and R. C. Rosen, Sexual dysfunction in the United States: Prevalence and predictors. J. Am. Med. Assoc., 1999; 281: 537-544.

42. M. Monga, The aging penis: erectile dysfunction. Geritr. Nephol. Urol. 1999; 9(1): 27-37.

43. R. J. Bosch, F. Bernard, S. R. Aboseif, C. G. Steif, T. F. Lue and E. A. Tanagho, Penile detumescence: characterization of three phases. J. Urol. 1991; 146: 867

44. M. E. Metz, J. L. Pryor, L. J. Nesvacil, F. Abuzzahab, and J. Koznar, Premature ejaculation: a psychophysiological review. J Sex Marital Ther1997. 23:3–23.

45. J. F. Kinzl, B. Mangweth, C. Traweger, and W. Biebl, Sexual dysfunction in males: significance of adverse childhood experiences. Child Abuse Negl. 1996; 20:759–66.

46. D. Aizenberg, Z. Zemishlany, H, Hermesh, L. Karp, and A. Weizman, Painful ejaculation associated with antidepressants in four patients. J Clin. Psychiatry, 1991; 52:461–463.

47. Yakubu M.T., Akanji M.A., Oladiji A.T.: Pharmacog. Rev. 2007; 1, 49

48. Harnack L.J., Rydell S.A., Stang J.: Mayo Clin. Proc. 2001; 76, 688

49. Siegfried E.D. Jacobe G. Fatima K. Phytochemistry 2003; 62, 1019

50. Lue TF. Erectile dysfunction N Engl. J. Med. 2000; 342: 1802-13

51. Ayta I A, McKinlay J B, Krane R J. The likely worldwide increase in erectile dysfunction between 1995 and 2025 and some possible policy consequences. BJU Int. 1999; 84: 50-6

52. Hafez E S, Hafez S D. Erectile dysfunction: anatomical parameters, etiology, diagnosis, and therapy. Arch. Androl. 2005; 51: 15-31

53. Lue T F, Tanagho E A. Physiology of erection and pharmacological management of impotence J Urol. 1987; 137: 829-36

54. Mersdorf A, Goldsmith PC, Diederichs W, et al. Ultrastructural changes in impotent penile tissue: a comparison of 65 patients. J Urol. 1991; 145: 749-58

55. Andersson K E, Holmquist F. Regulation of tone in penile cavernous smooth muscle Established concepts and new findings. World J Urol. 1994; 12: 249-61

56. Ignarro L J, Bush P A, Buga G M, Wood K S, Fukuto J M, Rajfer J. Nitric oxide and cyclic GMP formation upon electrical field stimulation cause relaxation of corpus cavernosum smooth muscle. Biochem Biophys Res. Commun. 1990; 170: 843-50

57. Andersson K E. Mechanisms of Penile Erection and Basis for Pharmacological Treatment of Erectile Dysfunction. Pharmacol Rev. 2011

58. Wright PJ. Comparisionof phoshpodiesterases type 5 inhibitors. Int. J of Clin Prac 2006; 60; 967-75

59. "Sildenafil Citrate". The American Society of Health-System Pharmacists. Retrieved Dec 1 2014

60. Boolell M, Allen M J, Ballard S A, Gepi-Attee S, Muirhead G J, Naylor A M, Osterloh I H, Gingell C. Sildenafil an orally active type 5 cyclic GMP-specific phosphodiesterase inhibitor for the treatment of penile erectile dysfunction. Int. J. Impot. Res. 1996; 8 (2): 47–52

61. Rosen RC, Ashton AK. Emperical Status of New Aphrodisiacs. Arch. Sex. Behav. 1993; 22: 581-43

62. A. S. Tajuddin, A. Latif and I. A. Qasmi, Effect of 50% ethanolic extract of Syzygium aromaticum (L) Merr. & Perry. (Clove) on sexual behaviour of normal male rats, BMC Complemen. Altern. Med. 2004; 4: 17-24

63. Venkat P, Reverse Pharmacognosy: A Novel Strategy to Standardise ISM Drugs, 8th Annual Oxford-International Conference on the Science of Botanicals at Bangalore, 2009; 6-9.

64. Chew KK; Stuckey BGA; Thompson PL, Med. J. Aust. 2000, 172, 270-283.

65. Griffin P D, InDiczfalusy E, Khanna J, Research in Human Reproduction Geneva, Switzerland. Editors World Health Organization, 1988

66. Janardhanan K, Gurumoorthi P, Pugalenthi M. Nutritional potential of five accessions of a South Indian tribal pulse, Mucuna pruriens var. utilis. Part I. The effect of processing methods on the contents of L-Dopa phytic acid, and oligosaccharides. Journal of Tropical and Subtropical Agro-ecosystems 2003; 1:141–152

67. Chaudhri R.D. Herbal drug industry: a practical approach to industrial pharmacognosy. 1996

68. Jeyaweera D.M.A. Sri Lanka: National Science Council of Sri Lanka; 1981. Madicinal plants used in Ceylon Colombo

69. Bone K. Clinical applications of Ayurvedic and Chinese herbs. Monographs for the Western herbal practioner, Aust Phytotherapy Press 1996; 137-41.

70. Dadkar VN, Ranadive NU, Dhar HL. Evaluation of antistress (adaptogen) activity of Withania somnifera, Ind. J. Clin. Biochem. 1987; 2:101-8

71. Mamidi P, Thakar Efficacy of Ashwagandha (Withania somnifera Dunal. Linn.) In the management of psychogenic erectile dysfunction. A B Ayu. 2011 Jul; 32(3):322-8.

72 Benoit Petit-Demouliere, Franck Chenu, Michel Bourin. Forced swimming test in mice: a review of antidepressant activity Psychopharmacology 2005; 177: 245–255

73 Cryan JF, Mombereau C, Vassout A. The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. Neurosci Biobehav Rev 2005, 29: 571-625.

74 Crowley J J, Blendy J A, Lucki I. Strain-dependent antidepressant-like effects of citalopram in the mouse tail suspension test. Psychopharmacology 2005; 183: 257–64.

75. Ashok Agarwal, Gurpriya Virk, Chloe Ong, Stefan S du Plessis. Effect of Oxidative Stress on Male Reproduction. World J Mens Health 2014; 32(1): 1-17.

76. K.K. Das, S.N. Das, S.A. Dhundasi. Nickel its adverse health effects & oxidative stress. Indian J Med Res, 2008; pp 412-425.

77. Ames B. N., Gold L. S., Willet W. C. The causes and prevention of cancer. Proc. Natl. Acad. Sci. U.S.A. 1995; 92, 5258-65

78. Frei B: Vitamin C as an antiatherogen: mechanism of action. Vitamin C in Health and disease. Edited by Packer L, Fuchs J. Marcel and Dekker, Inc., New York; 1997; 163-182.

CHAPTER 3 & 4

HYPOTHESIS, AIMS & OBJECTIVE

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

2015

BLDE UNIVERSITY'S
SHRI B M PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

3. HYPOTHESIS

Both the substance that is seed extract of *Mucuna pruriens & root extract of Withania somnifera* can reduce stress induced alteration of endocrine profile of male albino rats.

4. AIMS & OBJECTIVE

a. To evaluate the effect of methanolic seed extract of *Mucuna pruriens* & methanolic root extract of *Withania somnifera* in stress induced effect on male reproductive organs including its aphrodisiac activity in Wistar strain of albino rats through
    i. Mating behavior test
    ii. Hematological Profile
    iii. Biochemical Profile
    iv. Stress parameters
    v. Reproductive Profile
    vi. Histopathological &
b. To find out the adverse effect (if any) of extracts on peripheral metabolically active tissue
c. Purpose of the present study was designed to address the issues to lend support to the existing information pertaining to the beneficial effect of the Mucuna pruriens & Withania somnifera plant in treating reproductive disorder.

CHAPTER 5

MATERIALS & METHOD

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

2015

BLDE UNIVERSITY

SHRI B M PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

5. MATERIALS & METHOD

5.1. Procurement and Rearing of Experimental Animal

Laboratory inbred adult male & female albino Wister rats fed with laboratory stock diet (Hindustan lever, Mumbai, India) and water *ad libitum*, male rats weighing 175 – 250g and female rats weighing around 175–200 gm were used in the study. Healthy male albino rats showing brisk sexual activity were selected for the study. Female animals showing regular oestrus cycle were used for mating behavior analysis only. The receptiveness of the female rats was confirmed before the test by exposing them to male rats. Female rats with maximum receptivity were selected for the experiment. Rats are acclimatized a week to the laboratory conditions at 22 – 24° C and a 12 h light: dark (circadian) cycle. The acclimatized animals were housed in polypropylene cages (32×40×18 cm) three animals in each.

The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC) BLDE University's Shri B M Patil Medical College Hospital & Research Centre Vijayapur & the experiments were performed as per norms of Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA).

5.2. Preparation of Extract

Seeds of Mucuna pruriens & roots of Withania somnifera were procured locally, authenticated by Dr. M B Mulimani Professor, Department of Botany SB Arts & KCP Science College, Vijayapur 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 & roots 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 & roots 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. 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 (fig 1). 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 -4^{o}. Extraction was prepared as per the standard protocol by Harborne et al.[1]

Figure 9: Extraction in soxhlet apparatus

Calculation for percentage of yield

$$\% \text{ of yield} = \frac{W2-W1 \times 100}{W0}$$

Whereas W0- initial powder weight before extraction

W1- weight of beaker

W2- weight of extraction +beaker

5.3.Preliminary Phytochemical Screening

Methanolic extract of seeds of *Mucuna pruriens* and methanolic extract of roots of *Withania somnifera* have been undergone for phytochemical constituents. Different qualitative test were performed to establish various extracts and their chemical composition. The extracts obtained were subjected to following chemical tests for identification of various phytoconstituents as per the methods given by Harborne[1].

5.3.1. Test for Steroids[2]

a. **Salkowski test:** Few drops of concentrated sulphuric acid and chloroform were added to the few drops of extract, shaken and allowed to stand, appearance of red color in lower layer indicates the presence of steroids.

b. **Liebermann-Burchard test:** Mixed extract with the chloroform and few drops of acetic anhydride was added and mixed well. 1 ml of concentrated sulphuric acid was added from the sides of the test tube, appearance of reddish brown ring indicates the presence of steroids.

5.3.2. Test for Flavonoids[3&4]

a. **Shinoda test:** To extract few fragments of magnesium ribbon and concentrated hydrochloric acid was added. Appearance of red to pink color after few minutes indicates the presence of Flavonoids.

b. **Lead acetate test:** To the extract, a few drops of aqueous basic lead acetate solution were added. Formation of yellow precipitate indicates presence of flavonoids**.**

5.3.3. Test for Alkaloids

The extract was basified with ammonia and extracted with chloroform. The chloroform solution was acidified with dilute hydrochloric acid, shaken welland filtered. The filtrate was used for testing the alkaloids.

a. **Mayer's test** (Potassium Mercuric Iodine solution): The filtrate was treated with few drops of Mayer's reagent. Formation of creamy white precipitate indicates the presence of alkaloids.

b. **Hager's test**: The filtrate was treated with few drops of Hager's reagent. Formation of yellow precipitate indicates the presence of alkaloids[5].

5.3.4. Test for Tannins

a. **Ferric chloride test:** To extracts a few drops of 1% neutral ferric chloride solution were added, formation of blackish blue color indicates the presence of tannins.

b. **Gelatin test:** To the extracts added 1% solution of gelatin containing 10% sodium chloride. Formation of white precipitate indicates the presence of tannins[6].

5.3.5. Test for Saponins[5&7]

a. **Foam test:** Small amount of extract was shaken with little quantity of water, if foam produced persists for 10 minutes; it indicates the presence of saponins.

c. **Froth test:** To 5 ml of extract of the drug added a drop of sodium bicarbonate solution. Shaken the mixture vigorously and left for 3 minutes. Honey comb like froth is formed.

5.3.6. Test for Carbohydrates

Small amount of extracts were dissolved in little quantity of distilled water and filtered separately. The filtrates were used to test presence of carbohydrates.

a. **Molisch's test:** The extract was treated with Molisch reagent and concentrated sulphuric acid was added from the sides of the test tube to form a layer. A reddish violet ring shows the presence of carbohydrates.

b. **Benedict's test:** to the filtrate added 2 ml Benedict's reagent and boiled in water bath. Green reddish brown precipitate is formed.

5.3.7. Test for Amino acid/ Protein

a. **Ninhydrin test:** Heated the 3 ml of extract and 3 drops of ninhydrin solution in boiling water bath for 10 minutes. Appearance of purple color shows the presence of amino acids.

b. **Biuret test:** To 3 ml of extract added 4% NaOH and few drops of 1% copper sulphate solution. Formation of violet color confirms the presence of protein.

5.3.8. Test for Resins

Dissolved the extract in acetone and pour the solution in to distilled water. Turbidity indicates the presence of resins [1].

5.3.9. Test for starch

Dissolved 0.015 gm of iodine and 0.075 gm of potassium iodide in 5 ml of distilled water and add 2-3 ml of extract of drug, blue color is produced.

5.4. Experimental design

The acclimatized animals divided into six groups of six animals each

Group I rats were healthy controls.

Group II rats were stress controls where restraint stress was given.

Group III rats were standard group and treated with sildenafil citrate at a dose of 5mg/kg body weight subcutaneously after giving stress.

Group IV rats were treated with *Mucuna pruriens* at a dose of 250mg/kg body weight orally after giving stress

Group V rats were treated with *Withania somnifera* at a dose of 250mg/kg body weight orally after giving stress

Group VI rats were given *Mucuna pruriens* at a dose of 250mg/kg body weight and *Withania somnifera* at a dose of 250mg/kg body weight orally after giving stress.

Animals were treated for 45 days and later they were sacrificed on day 46th. Regularly weight of the rats was measured.

5.5. Restraint stress

During 45 days of dosage period male Wister rats were assigned to a daily restraint stress (3 hours daily during day period in random times to avoid the habituation during experimental period) in a wire mesh restrainer. The wire mesh restrainer has a wooden base and stainless steel wire mesh restrainer hinged to the base. A pad lock and latch will help to secure the rat in the restrainer. The restrainer with dimensions of 17 cm (L) x 8 cm (B) x 8 cm (H) will be used to stress[15]. This type of restrainer will only restrict the animal movement without any pain, discomfort or suffocation[8].

Figure 10: Restraint stress rats

5.6. Gravimetry

Affect on body weight, reproductive and vital organ weight of all the control, standard and experimental groups of male rats were evaluated. Animals are anesthetized by euthanasia where in animals are placed in sealed chambers where high levels of anesthetic gas are introduced by soaking cotton in 40 ml of diethyl ether. Once after loss of righting reflex approximately after 60 sec rats will be removed from the chamber and continued for further process. Testis, seminal vesicles, epididymis, vas-deference, and prostate glands along with vital organ like liver,

kidney, adrenal gland, brain and heart were carefully removed and weighed using digital electronic balance. The organosomatic index was calculated.

$$\text{Organosomatic index} = \frac{\text{Weight of organ (One side/both sides) X100}}{\text{Body weight of rats}}$$

5.7. Adverse effect & Pre-coital sexual behaviors

All the group rats were observed for sign of toxicity like salivation, rhinorrhoea, lachrymation, ptosis, writhing, convulsions and tremors and also observed for characteristics of stress like erection of fur and exopthalmia and also observed for behavioral changes such as spontaneous movement in the cage, climbing and cleaning of face.

The pre-coital sexual behaviors such as chasing, nosing, anogenital sniffing and mounting were observed for up to 2 h of pairing. The values of the observed parameters for control and experimental groups were recorded.

5.8. Mating Behavior Test

Healthy male albino rats showing brisk sexual activity were selected for the study. Female animals showing regular oestrus cycle were used for mating behavior analysis. The receptiveness of the female rats was confirmed before the test by exposing them to male rats. Female rats with maximum receptivity were selected for the experiment. The tests for sexual desire will be carried out on 45th day after commencement of the treatment. The experiment will be conducted at 19:00 h in the same laboratory and under the light of same intensity. The male and receptive female rats will be introduced into the mating cages, with one female to one male ratio. The mating behaviors will be recorded and used for further analysis by giving scores for first four mating series. Test will be terminated if the male rat failed to evince sexual interest. The occurrence and disappearance of events and phases of mating will be

recorded as soon as they appear. Later, the frequencies and phases will determined by the recorded transcriptions

1. Mounting frequency (MF) : Number of mounts before ejaculation
2. Intromission frequency (IF) : Number of intromission before ejaculation
3. Mounting latency (ML) : Time from the introduction of female into the cage of the male up to then first mount
4. Intromission latency (IL) : Time from the introduction of the female up to the first intromission by the male
5. Ejaculatory latency (EL) : Time from the first intromission of a series up to the ejaculation
6. Number of mount (NM) : Number of mount in a single attempt

5.9. Method of euthanasia

Euthanasia is the act of humanely putting an animal to death or allowing it to die. Euthanasia methods are designed to cause minimal pain and distress. Euthanasia is distinct from animal slaughter and pest control, which are performed mainly for the purposes other than an act of mercy, although in some cases the procedure is the same. Gas anesthetic such as diethyl ether can be used for euthanasia of rats. The animals are placed in sealed chambers where high levels of anesthetic gas are introduced by soaking cotton in 40 ml of diethyl ether. Death may also be caused using carbon dioxide once unconsciousness has been achieved by inhaled anesthetics. Once after loss of righting reflex approximately after 60 rats will be removed from the chamber and continued for further process.

5.10. Blood & Tissue collection

5.10.1. Blood collection

Blood samples were collected by the retro-orbital route on 45th and processed for hematological, biochemical, hormonal, stress and antistress assay.

Procedure for retro orbital venous blood collection[9]

Also referred to as Peri-Orbital, Posterior-Orbital and Orbital Venous Plexus bleeding

- ✍ The animal is anaesthetized (UACC SOP # 2 Rodent anaesthesia) and at an appropriate depth of anesthesia prior to commencement or even can be euthanize, signs that indicate a satisfactory plane of anesthesia include a lack of response to a toe pinch and respirations that are relaxed and regular.
- ✍ The anaesthetized rat is laid on its back with the head toward the edge of table.
- ✍ Fix the head with thumb and forefinger, tightening the skin over the sides of the face. This retracts the eyelids and protracts the globe.
- ✍ Insert a micro hematocrit (capillary) tube into the medial canthus and twist to break through the bulbar conjunctiva. Direct the tube toward the medial aspect of the bony orbit.
- ✍ Collect blood into test tubes and seal. Continue until unable to collect. If difficulty arises change to other eye.
- ✍ Blood flow can be stopped by applying gentle finger pressure to the soft tissue. A finger should be placed over the closed eyelid for approximately 30 seconds. The rat should be checked for post-operative peri-orbital lesions approximately 30 minutes after blood sampling and on at least one more occasion within two hours of the sampling.

Advantages of retro orbital venous blood collection[9]

- ✍ Rapid – large number of animals can be bled within a short period of time.
- ✍ Obtainable volume: medium to large.
- ✍ Good sample quality. Potential contamination with topical anesthetic, if used should be taken into account.
- ✍ A minimum of 10 days should be allowed for tissue repair before repeat sampling from the same orbit. Otherwise the healing process may interfere with blood flow.
- ✍ Alternating orbits should not be attempted until the phlebotomist is proficient in obtaining samples from the orbit accessed most readily by the dominant hand ie a right handed individual should gain proficiency withdrawing samples from the right orbit before attempting to obtain samples from the left orbit.

Figure 11: Retro venous blood collection

5.10.2. Tissue collection

After euthanasia rats were kept in supine position and dissected. Male reproductive organs, liver, kidney, stomach, adrenal gland, heart and brain were collected and washed in normal saline. Histopathology of all the male reproductive organs, liver and kidney were done. Stomach was observed for ulcerogenecity. For the evaluation of histopathology testis specimens were kept in Bovine's fluid,

remaining organs were kept in 10% formalin. By following routine histological techniques, the samples were put into paraffin, and serial sections of 5 μm were taken from tissue blocks. Epididymis & ductus deferens were used for semen analysis. Liver kidney & Testis have been also used for estimation of nitric oxide concentration and lipid peroxidation; testis was used for testicular synthesis.

Figure 12: Tissue collection (Liver)

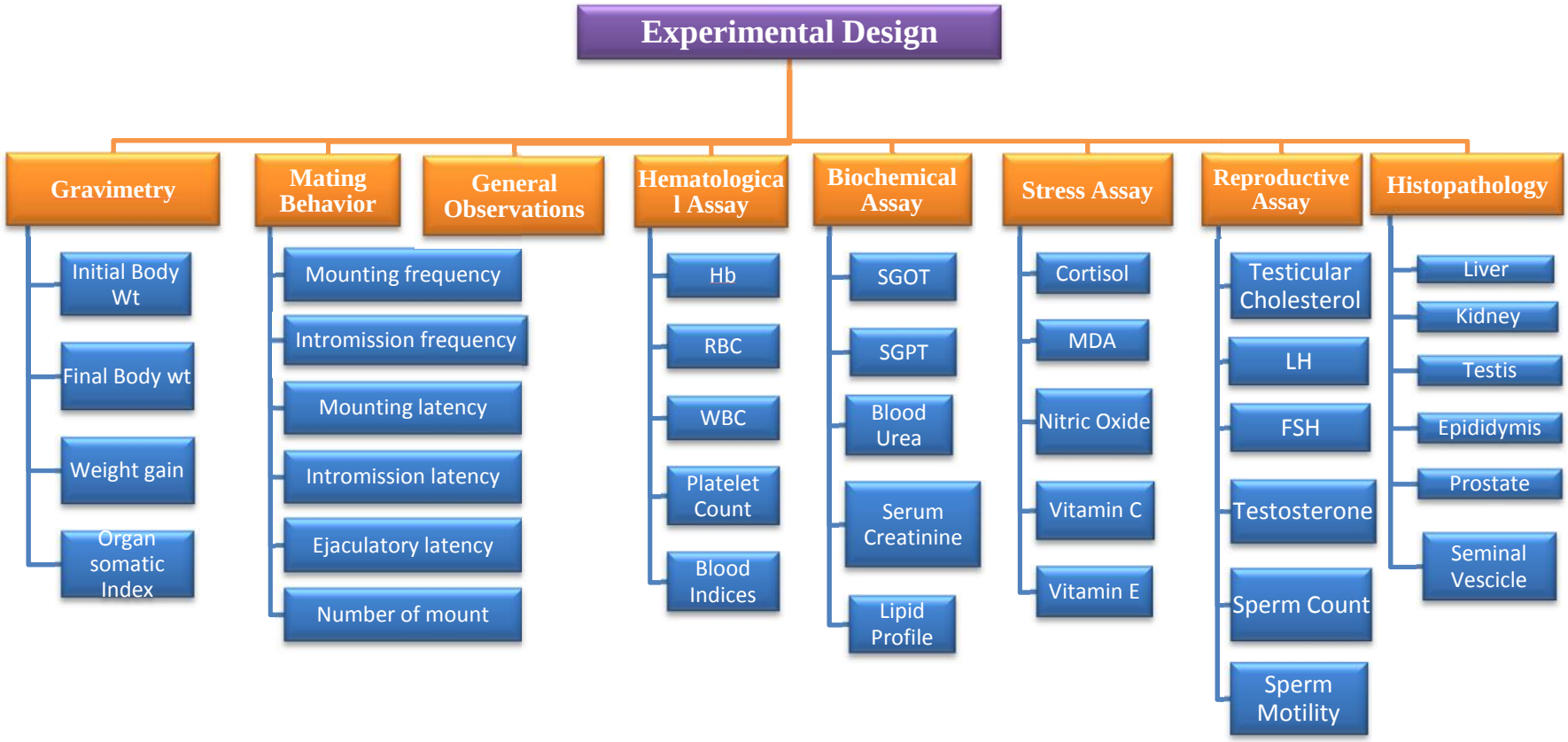


Figure 13: Flow chart showing experimental set up

5.10.3. ANALYSIS OF HEMATOLOGICAL ASSAY

All the hematological parameters like hemoglobin (Hb), red blood corpuscles (RBC) count, white blood corpuscles (WBC) count, platelet count, packed cell volume (PCV), mean corpuscular volume(MCV), mean corpuscular hemoglobin (MCH)& mean corpuscular hemoglobin concentration (MCHC) were investigated by sysmex cell counter by collecting heparinized blood.

5.10.4. ANALYSIS OF BIOCHEMICAL ASSAY

- ✓ **ORGAN FUNCTION TEST**

SERUM GLUTAMATE OXALOACETATE TRANSAMINASE (SGOT)

by IFCC method[10]

The SGOT test measures the amount of a substance called glutamic-oxaloacetic transaminase (GOT) in the blood. It is an enzyme found in the liver, muscles (including the heart), and red blood cells. It is released into the blood when cells that contain it are damaged. Both AST and ALT measurements are useful in the diagnosis of hepatocellular disease. Other names for this enzyme are aspartate aminotranskinase, aspartate transaminase, and AST.

Principle

$$\text{L-aspartate + 2 Oxoglutarate} \xrightarrow{\text{AST}} \text{Oxaloacetate + L- Glutamte}$$

$$\text{Oxaloacetate + NADH} \xrightarrow{\text{MDH}} \text{Malate + NAD}$$

$$\text{Pyruvate + NADH} \underset{}{\overset{\text{LDH}}{\rightleftharpoons}} \text{L- acetate + NAD}$$

Specimen Serum

Procedure

Dispense in tube as follows : working reagent 1000µl

Sample 50µl

Mix and incubate 60 seconds at 37^0C, then record I^{st} reading of absorbance.

Perform other 3readings at 60 seconds intervals. Calculate the delta A/minute.

Calculation

Perform claculations in units per liter. Multiflying the delta A/min by the factor.

Activity in U/L = delta A/min X 3376

SI conversion factor : 1 U/L X 0.017= 1µ Kat/ L

SERUM GLUTAMATE PYRUATE TRANSAMINASE (SGPT)

by IFCC Kinetic Method[10]

Principle

$$\text{L-alanine + 2 Oxoglutarate} \xrightarrow[\text{ALT}]{} \text{Pyruvate + L- Glutamte}$$

$$\text{Pyruvate + NADH} \xrightarrow[\text{LDH}]{} \text{L- acetate + NAD}$$

Specimen Serum or plasma.

Procedure

Dispense in tube as follows : working reagent 1000µl

Sample 50µl

Mix and incubate 60 seconds at 37^0C, then record I^{st} reading of absorbance. Perform other 3readings at 60 seconds intervals. Calculate the delta A/minute.

Calculation

Perform claculations in units per liter. Multiflying the delta A/min by the factor**.**

Activity in U/L = delta A/min x 3376

SI conversion factor : 1 U/L x 0.017= 1µ Kat/ L

✓ **LIPID PROFILE**

CHOLESTEROL

by *Modified Roeschlau's* Method[11]&[12]

Principle

The estimation of cholesterol involves the following enzyme catalyzed reactions.

1. Cholesterol ester $\xrightarrow{CE}$ cholesterol + Fatty acid

2. Cholesterol+O_2 $\xrightarrow{CHOD}$ cholest-4-en-one+H_2O_2

3. $2H_2O_2$ +4AAP+ phenol $\xrightarrow{POD}$ $4H_20$ +Quinineimine

Procedure

Table 1 Procedure for Cholesterol estimation

Reagents	Blank	Standard	Test
working reagent	1000µl	1000µl	1000µl
Distilled water	20µl	--	--
Standard	--	20µl	--
Test	--	--	20µl

TRIGLYCERIDES

This reagent is based on the method of *Wako* and the modification by *McGowan* et al *Fossati* et al [13,14&15]

Principle

Triglycerides + H_2O $\xrightarrow{LPL}$ Glycerol + Free fatty acids

Glycerol + ATP $\xrightarrow{GK}$ Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 $\xrightarrow{GPO}$ DAP +H_2O_2

H_2O_2 + 4AAP + 3, 5-DHB $\xrightarrow{Peroxidase}$ Quinoneimine dye

Procedure

Table 2 Procedure for Triglycerides

Reagent	Blank	Standard	Test
working reagent	1000 µl	1000 µl	1000 µl
Distilled water	10 µl	--	--
Standard	--	10 µl	--
Test	--	--	10 µl

Mix and incubate for 10 min. at 37°C. Read the absorbance of standard and each test at 505 nm (500-540nm) against reagent blank.

Calculation

$$\text{Triglycerides (mg/dl)} = \frac{\text{Abs. of Test}}{\text{Abs. of standard}} \text{ X Conc. Of standard}$$

HDL CHOLESTEROL

By Phospotungastic Acid Method, End point[16]

Principle

Chylomicrons, LDL and VLDL (low and very low density lipoproteins) are precipated from serum by phoshotungastate in the presence of divalent cations such as magnesium. The HDL cholesterol remains unaffected in the supernatant and is estimated using ERBA cholesterol reagent. Serum/plasma Phosphotungstate (HDL + LDL+ chylomicrons)

Procedure

Table 3 Test procedure for HDL Cholesterol

Reagents	Blank	Standard	Test
Cholesterol working reagent	1000 µl	1000 µl	1000 µl
Distilled water	50 µl	--	--
HDL Standard	--	50 µl	--
Supernatant	--	--	50 µl

Mix well; incubate for 10min.at 37°c, or 12min. at 30°C.Read the absorbance of the standard and each test at 505nm.

Calculation

HDL cholesterol= Abs. of Test X Conc. Of standard X dilution factor Abs. of Test
Abs. of Std.

HDL cholesterol = Abs. of Test X 25 X 3
Abs. of Std.

HDL cholesterol = Abs. of Test X 75
Abs.of Std.

5.10.5. STRESS ASSAY

CORTISOL ASSAY

By Chemiluminescence immunoassay (CLIA)[17&18]

Principle

Competition occurs between an unlabeled antigen and an enzyme-labeled antigen for a limited number of antibody binding sites on the microwell plate. The washing and decanting procedures remove unbound materials. After the washing step, the luminescence substrate solution is added. The relative luminescence units (RLUs) are measured on a microtiter plate luminometer. The RLU values are inversely proportional to the concentration of cortisol in the sample

Reagents & Preparation

Anti-Cortisol Antibody Coated Microwell Plate, Cortisol-Horse Radish Peroxidase (HRP), Conjugate Concentrate, Cortisol Calibrators, Control, Wash buffer, Assay Buffer, Substrate Reagent A, Substrate Reagent B & Substrate Reagent C

Working Conjugate: Dilute conjugate concentrate 1:100 in assay buffer before use.

Wash buffer working solution: Dilute wash buffer concentrate 1:10 in distilled or deionized water before use.

Working Substrate: Mix 1 part of substrate reagent A with 1 part of substrate reagent B and dilute this mixture 1:5 with substrate reagent C.

Procedure

Prepare working solutions of the conjugate, wash buffer and LIA substrate (refer to reagents provided and preparation section). Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator. Pipette 25 µL of each calibrator, control and specimen sample into correspondingly labelled wells in duplicate. Pipette 100 µL of the conjugate working solution into each well. Incubate on a plate shaker (approximately 200 rpm) for 1 hour at room temperature. Wash the wells 5 times with 300 µL of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry. Pipette 100 µL of working substrate solution into each well. Shake for 5 seconds. Incubate for 15 minutes at room temperature without shaking. Measure the RLUs in each well on a microplate luminometer within 10-30 minutes after addition of the substrate.

MELONDIALDEHYDE

By *Kei Satoh Method*[19]

Principle

MDA formed by the breakdown of poly unsaturated fatty acids (PUFA) serves as a convenient index to determine the extent of lipid peroxidation. It reacts with TBA to give a pink colour which is read at 535nm.

Chemicals Required

Trichloro acetic acid, 2-Thiobarbituric acid, Hydrochloric acid & Malonaldehydebis (dimethyl acetal)

Reagent Preparation

- TCA-TBA-HCl REAGENT
  - 0.25N HCl: 2.21mL of conc. HCl is made upto 100mL with distilled water.

- 15% TCA and 0.375% TBA - 15g TCA and 0.375g of TBA is dissolved in 100mL of 0.25N HCl the reaction mixture is warmed to dissolve the contents and stored at 4^{o}C.

➢ MDA STANDARD (STOCK-164µg/mL)

- 16.4µL of the standard malonaldehyde solution is taken and made up to 100mL with distilled water.

➢ MDA STANDARD (WORKING-1.64µg/mL)

- 100µL of the stock is made up to 10mL with distilled water.

➢ 0.1M PHOSPHATE BUFFER pH 7.4

- Solution a: 2.723gm of potassium di-hydrogen phosphate (KH_2PO_4) was dissolved in 200ml of distilled water.
- Solution b: 2.849gm of di- sodium hydrogen phosphate (Na_2HPO_4) was dissolved in 200ml distilled water.
- Add 19 ml solution 'a' and 81 ml of solution 'b' adjust the pH to 7.4 by adding acids or bases.

Procedure for standard curve

➢ STANDARDISATION (RANGE: 2-10µM/L)

The standardisation is carried out referring to Table 4 and all the reaction mixture are added according to the measures given below.

Table 4 Procedure for MDA standardization & absorbance

Sl. No.	Vol. of MDA (mL)	Vol. of Dis. water (mL)	Conc. Of MDA (µM/L)	TBA-TCA-HCl (mL)		Absorbance
B	0.0	1.0	0.0	1ml to all tubes	Keep in a boiling water bath for 15 minutes. Read OD at 535nm	0.030
1	0.2	0.8	2			0.151
2	0.4	0.6	4			0.318
3	0.6	0.4	6			0.488
4	0.8	0.2	8			0.650
5	1.0	-	10			0.793

The optical densities obtained are plotted against the concentrations on a graph.

Figure 14: Standard Operating Procedure (SOP) of Melondealdehyde, showing gradual colour change indicate the presence of concentration of Melondialdehyde in S1- S5 test tubes by using Shimadzu UV -VIS Spectrophotometer 1700

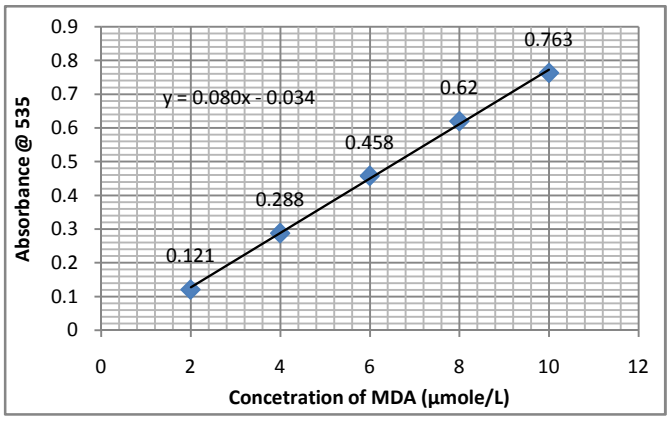


Figure 15: Standard Curve for Melondialdehyde(MDA). The X axis shows the concentration in micromole/L and Y axis showing absorbance at 535 nm by using Shimadzu UV -VIS Spectrophotometer 1700

Procedure for Sample

SAMPLE PREPARATION

Serum-100µL serum is diluted to 500µL with distilled water.

Tissue homogenate- 500µL of the 10% tissue homogenate is taken. MDA was estimated in Liver Kidney and testis.

TISSUE HOMOGENISATION

10 % tissue homogenate is prepared by adding 500mg of tissue to 5ml of 0.1M phosphate buffer solution, homogenise for few minutes, centrifuge and supernatant was used for estimation.

TREATMENT

To the diluted sample 1mL of TCA-TBA-HCl reagent is added. The samples are kept in boiling water bath for 15 minutes. The reaction mixture is cooled and centrifuged. The supernatant is taken and the optical density of the pink colour formed is read at 535nm. The concentration of Melondialdehyde in the sample is got by plotting the obtained absorbance against the standard graph. The optical density of the pink colour formed is directly proportional to the concentration of Melondialdehyde in the given sample.

Calculation

The optical densities of the test samples is directly proportional to the concentration of MDA in the sample and calculated by the plotting against the standard graph and multiplied by the respective dilution factors the final concentration is expressed as µM/L.

Calculation for Serum

$$\text{Conc}^{n} \text{ of MDA } \mu\text{Moles/L} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{Slope}} \text{ X Dilution factor}$$

Where

$$\text{Slope} = \frac{Y_2 - Y_1}{X_2 - X_1}$$

X & Y are concentration and absorbance of standards respectively

$$\text{Slope} = \frac{0.650 - 0.151}{8 - 2}$$

$$= 0.0832$$

Ex; Suppose a test reading is 0.156 calculation is

$$\text{Conc}^{n} \text{ of MDA } \mu\text{Moles/L} = \frac{0.156 - 0.030}{0.0832} \text{ X } 5$$

$$\text{Conc}^{n} \text{ of MDA} = 7.57 \ \mu\text{Moles/L}$$

Calculation for Tissue

Homogenization of tissue is prepared by adding 500mg of tissue in 5 ml of 0.1M PBS

So, 5ml Phosphate buffer saline contains 500 mg of tissue

1 ml homogenate contains 100mg of tissue,

So, after homogenization from the supernatant 0.5ml is used for test

1ml=100mg
0.5ml= ? $\frac{0.5X100}{1}$ =50mg of tissue used for testing

Conc^n of MDA = $\frac{\text{OD of Test – OD of Blank}}{\text{Slope}}$

Where Slope = $\frac{Y_2-Y_1}{X_2-X_1}$

X & Y are concentration and absorbance of standards respectively

Slope = $\frac{0.650-0.151}{8-2}$

= .0832

Ex; Suppose a test reading is 0.369 calculation is

Conc^n of MDA= $\frac{0.369 -0.030}{0.0832}$

Conc^n of MDA= 4.07 µMoles of MDA for 50 mg of tissue

So, 50mg=4.07 µMoles

1mg = ? $\frac{1X4.07}{50}$ =0.0814 µMoles / 1mg of tissue

0.0814X1000=81.49µMoles/gm of tissue

NITRIC OXIDE

by Griess (Mosssage) Method[20]

Principle

This assay determines nitric oxide based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by a colorimetric detection of nitrite as an azo dye product of the Griess reaction. The Griess reaction is

based on the two-step diazotization reaction in which acidified $NO2^{-}$ produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl) ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540 nm.

Nitric Oxide Assay includes Standardization of chemicals & Analysis Nitric Oxide in sample

Reagents & Preparation

- ⇨ Griess reagent:
  - o Sulphanil amide: 2.5gmSulphanil amide+250 ml 3mHCL
  - o 50 mg Naphtyl ethylene di-amine di-hydrochloride (NED) +250ml DW
- ⇨ Vanadium chloride (VCl_3): 8 mg of vanadium chloride in 1ml DW
- ⇨ Standard Solution
  - o Sodium nitrate ($NaNO_3$):21.2475mg/250ml DW
  - o Sodium nitrite ($NaNO_2$):17.25mg/250ml DW
- ⇨ Ethanol

Precautions: Griess reagent contain sulfa, a common allergen, use caution while handling, Vanadium chloride is highly toxic and inflammable

Procedure for standardization

Table 5: Procedure for Nitric oxide standardization & their absorbance

Sl. No	Working Standard		Vanadium chloride(ml)	Griess reagent		Standard readings	
	Standard(µl)	DW(µl)		Sulphanil amide(ml)	NED(ml)	OD	Con^{n}
1	10	490	0.5	0.25	0.25	0.078	0
2	20	480	0.5	0.25	0.25	0.142	10
3	30	470	0.5	0.25	0.25	0.241	20
4	40	460	0.5	0.25	0.25	0.344	30
5	50	450	0.5	0.25	0.25	0.450	40
6	60	440	0.5	0.25	0.25	0.55	50

Incubate for 30minute at 37°C Read it at 540nm

Figure 16: Standard Operating Procedure of Nitric Oxide showing gradual increase in Violet color indicate the presence of concentration of Nitric oxide within the test tubes by using Shimadzu UV -VIS Spectrophotometer 1700

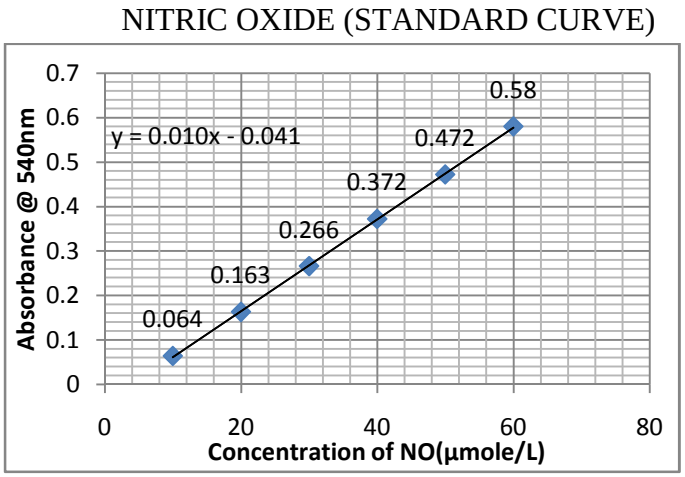


Figure 17: Standard Curve for Nitric Oxide. The X axis, indicate concentrations and the Y axis indicates the absorbance (540 nm) by using Shimadzu UV -VIS Spectrophotometer 1700

Analysis of Nitric Oxide in sample

Sample required: Serum (non haemolysed 1000 µl) & tissue homogenate, nitric oxide estimated in Liver Kidney and testis.

Sample preparation

Serum: 0.5 ml serum + 1 ml ethanol (1:2) Mix well & centrifuge for 10min use 0.5 ml Supernatant.

Tissue: 500mg tissue + 5ml normal saline, mix well centrifuge, use 1 ml of tissue homogenate + 2 ml ethanol Mix well & centrifuge & take 0.5ml of supernatant.

Procedure

1st step: Deprotinization of the sample as explained above for sample preparation

2nd Step: Reagents and Incubation

Table 6: Steps to show the procedure for Nitric oxide estimation

SN	Reagents	T(ml)	S(ml)	B(ml)	
1	Supernatant	500µl	-	-	Incubation For 30min. At 37°C
2	Working std.	-	500µl	-	
3	DW	-	-	500µl	
4	Vanadium chloride	500µl	500µl	500µl	
5	Griess Reagent (Sulphanil amide + NED)	500µl (250µl+250µl)	500µl (250µl+250µl)	500µl (250µl+250µl)	

3rd Step: Spectrophotometer

Absorbance of the product read spectrophotometrically by using 540nm filter.

Calculation for Serum:

Method 1

$$\text{Conc}^{n} \text{ of NO } \mu\text{Moles/L} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{Slope}} \times \text{Dilution factor}$$

Where

$$\text{Slope} = \frac{Y_2 - Y_1}{X_2 - X_1}$$

X & Y are concentration and absorbance of standards respectively

$$\text{Slope} = \frac{0.450 - 0.344}{40 - 30} = \frac{0.106}{10} = 0.0106$$

Ex; Suppose a test reading is 0.142 calculation is

$$\text{Conc}^{n} \text{ of NO } \mu\text{Moles/L} = \frac{0.142 - 0.078}{0.0106} \times 4$$

$$NO_x = 24.15 \ \mu\text{Moles/L}$$

Method 2

$$\text{Conc}^{n} \text{ of } NO_x \ \mu\text{Moles/L} = \text{Mean Factor} \times \text{Absorbance} \times \text{Dilution factor}$$

Where

$$\text{Mean factor} = \frac{\text{Con}^{n} \text{ of Std}}{\text{Absorbance of Std}}$$

Method 3

By solving equation y = 0.010x + 0.036 on graph

Calculation for Tissue:

Homogenization of tissue is prepared by adding 500mg of tissue in 5 ml of normal saline (0.9% NaCl)

So, 5ml normal saline contains 500 mg of tissue

1ml of homogenate is mixed with 2ml of ethanol,

1 ml homogenate contains 100mg of tissue,

So, after mixing with ethanol (1+2=3) 3ml contains 100mg of tissue, from the supernatant 0.5ml is used for test

3ml=100mg

0.5ml= ? $\frac{0.5X100}{3}$ =16.67mg of tissue used for testing

Conc^n of NO_x = $\frac{\text{OD of Test – OD of Blank}}{\text{Slope}}$

Where

Slope = $\frac{Y_2-Y_1}{X_2-X_1}$

X & Y are concentration and absorbance of standards respectively

Slope = $\frac{0.450-0.344}{40-30}$

= $\frac{0.106}{10}$

= 0.0106

Ex; Suppose a test reading is 0.142 calculation is

Conc^n of NO_x = $\frac{0.142-0.078}{0.0106}$

Conc^n of NO_x = 6.04 µMoles of nitric oxide for 16.67 mg of tissue

So, 16.67mg=6.04 µMoles

1mg = ? $\frac{1X6.04}{16.67}$ =0.362 µMoles / 1mg of tissue

0.362X1000=362.32 µMoles /gm of tissue

ESTIMATION OF VITAMIN-C

by *Roe & Kuether Method*[21]

Vitamin C Assay includes Standardization of chemicals & Analysis Vitamin C in sample

Standardization of vitamin-C (Ascorbic acid):

Principle

The ascorbic acid is oxidized to diketogulonic acid in presence of strong acid solution which reacts with 2, 4, dinitrophenyl hydrazine to form diphenylhydrazone which dissolves in strong H_2So_4 solution to produce red color which can be measure at 505nm (range of vitamin C 500-520nm) spectrophotometrically.

Reagents

- TCA: 10gm TCA dissolved in DW and make volume up to 100ml
- 2, 4-DNPH: 2gm 2, 4-DNPH dissolved in $9NH_2SO_4$ and volume up to 100ml.
- Thiourea:10gm Thiourea dissolved in 100ml of 50% ethanol (store in 4°C)
- $CuSO_4$:1.5gm $CuSO_4$ dissolved in DW up to 100ml
- Combined color reagent: 5ml 2, 4-DNPH +0.1ml Cu $(SO_4)_3$ + 1ml Thiourea
- 85% H_2SO_4:85ml H_2SO_4 added in DW up to 100ml
- Stock solution of vitamin C: 1gm Vit-C makes it 100ml with DW

Procedure for standardization

Table 7: Procedure for standardization of vitamin C

Sr no.	Vol. of standard in ml	D/W (ml)	TCA(ml)	Color Reagent(ml)
1	0	0.5	0.5	0.4
2	0.1	0.4	0.5	0.4
3	0.2	0.3	0.5	0.4
4	0.3	0.2	0.5	0.4
5	0.4	0.1	0.5	0.4
6	0.5	0	0.5	0.4

Mix all the test tube kept in warm water bath at 56° degree C for 1 hour then cooled in ice bath for 5 min. Add 2 ml of 85% H_2SO_4 to all the test tubes, mix well & after 20 minute, take the reading, wave length 505nm

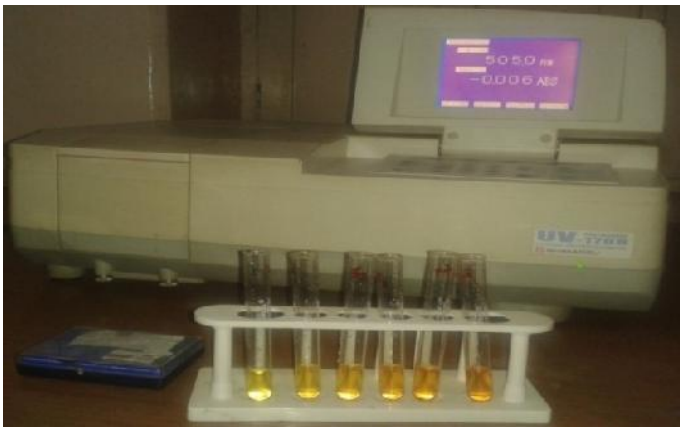


Figure 18: Standard Operating Procedure of Vitamin C showing gradual increase in color by using Shimadzu UV -VIS Spectrophotometer 1700

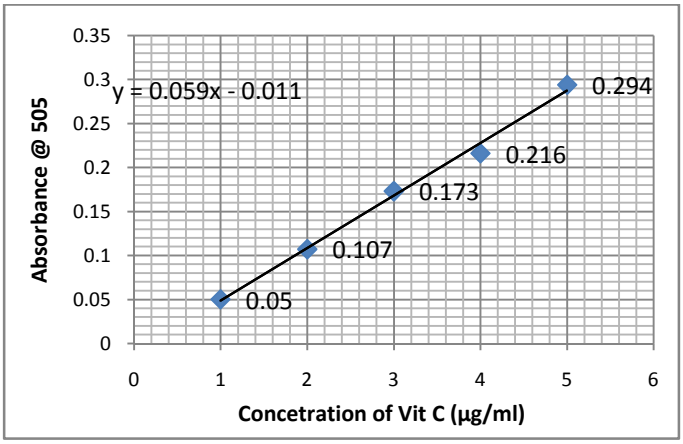


Figure 19: Showing Standard Curve for Vit C, X axis showing the concentration (µg/ml) of Reagent and Y axis showing the absorbance at 505 nm by using Shimadzu UV -VIS Spectrophotometer 1700

Procedure for Estimation of serum vitamin C

1st step: Deprotinization of the sample

For plasma/serum sample: 1 ml sample + 1ml 10% TCA (1:1v/v) accordingly 1ml plasma/serum in dry centrifuge + 1ml 10% TCA, Mix well (10-15 sec.) Centrifuge for 10min at 3500rpm take supernatant

Note: 1.plasm should be prepared using heparin as anticoagulant

2. for tissue: 1ml tissue homogenate +2ml 10% TCA (1:2 v/v)

(For tissue homogenation take 0.5gm tissue + 5ml 0.9%NaCl)

2nd Step: Reagents and Incubation

Table 8: Procedure for estimation of vitamin C

Sr. No	Reagents	T(ml)	S(ml)	B(ml)
1	Supernatant	1	-	-
2	Working std.(gm./ml)	-	0.5(stock :DW)	-
3	10%TCA	-	0.5	0.5
4	DW	-	-	0.5
5	Color reagent(fresh)	0.4	0.4	0.4

Mix & all test tubes kept in warm water bath at 56 degree C for 1 hr. then cooled in ice bath for 5 min. Add 2 ml of 85% H_2SO_4 to all the test tubes mix well & after 20 minute, take the reading.

3rd Step: **Spectrophotometer**

Absorbance of the product read spectrophotometrically by using 505nm filter.

Calculation

$$\text{Conc}^n \text{ of Vit C } \mu\text{gm/ml} = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{Slope}} \times \text{Dilution factor}$$

Where,

$$\text{Slope} = \frac{Y_2 - Y_1}{X_2 - X_1}$$

X & Y are concentration and absorbance of standards respectively

$$\text{Slope} = \frac{0.382 - 0.261}{5 - 3}$$

$$= 0.0605$$

Ex; Suppose a test reading is 0.204 calculation is

$$\text{Conc}^n \text{ of Vit C } \mu\text{gm/ml} = \frac{0.204 - 0.088}{0.0605} \times 2$$

$$\text{Conc}^n \text{ of Vit C} = 3.835 \ \mu\text{gm/ml}$$

VITAMIN E ESTIMATION

by Jameel Jargar & Das K K Method[22]

Reagents

Absolute ethanol (aldehyde free), N-propanol, Xylene (extra pure), Ferric chloride (0.12%), Distilled water, DL α-tocopherol acetate, & 2, 2'-Bipyridyl

Preparation of reagents

Stock standard of α-tocopherol (0.27% w/v): 270 mg of α-tocopherol acetate dilute in 100mL absolute ethanol and mix thoroughly.

2, 2'-Bipyridyl (0.12% w/v): 120 mg 2, 2'-bipyridyl dissolved and made volume up to 100ml of n-propanol & Kept in brown bottle.

Ferric chloride (0.12% w/v): 120 mg $FeCl_3.6H_2O$ dissolved in 100mL ethanol & Kept in brown bottle. All the solutions are stable at room temperature.

Preparation of standard curve

Working standard of α-tocopherol (27µg/mL): Dilute 1mL of stock standard to 100mL absolute ethanol to obtain concentration 27µg/mL (2.7mg/100mL). This solution is stable at room temperature. In six centrifuged tubes labeled as (blank) B, S_1, S_2, S_3, S_4 and S_5 place 00, 150, 300, 450, 600 and 750 µL of working standard α-tocopherol (27µg/mL) in respectively and add absolute ethanol to make the volume of each tube equal as 750µL. These solutions (S_1- S_5) are equivalent to 4µg/mL, 8µg/mL, 12µg/mL, 14µg/mL, 16µg/mL and 20µg/mL of α-tocopherol respectively. Use these solutions in the routine procedure shown in following table-9. Read the absorbance by using 200µl of solutions prepared above including blank putting on plain ELISA micro plate (non antibody coated) and read in ELISA reader at 492nm. Plot a standard curve absorbance *vs* α-tocopherol (µg/mL).

Table 9: Procedure for standardization of vitamin E

S. no	Working Standard		DW (µL)	Xylene (µL)		2,2'-Bipyridyl (µL)	$FeCl_3$ (µL)	
	α-tocopherol	Ethanol						
B	0	750	750	750	Centrifuge for 10min at 3000 rpm. 500µL Xylene layer taken out	500	100	After 2 min read OD at 492 nm
S_1	150	600	750	750		500	100	
S_2	300	450	750	750		500	100	
S_3	450	300	750	750		500	100	
S_4	600	150	750	750		500	100	
S_5	750	0	750	750		500	100	

The curve is drawn to determine the extent of adherence to the Beer-Lambert law with various photoelectric instruments.

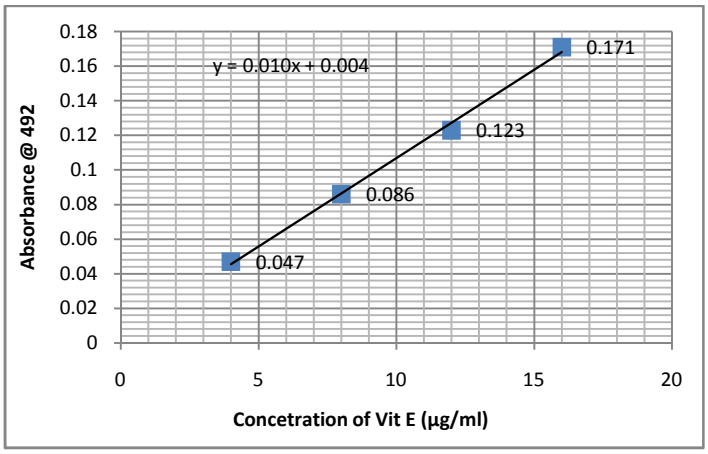


Figure 201: Showing Standard Curve for Vit E, X axis showing the concentration (µg/ml) of Reagent and Y axis showing the absorbance at 492 nm by using Shimadzu UV -VIS Spectrophotometer 1700

Analysis of serum α-tocopherol

Sample preparation: Allow the 3mL blood to clot in centrifuge tube for 2hrs at room temperature and centrifuge at 3000rpm for 15min to get serum. Serum for the analysis of α-tocopherol should be protected from sunlight and undue agitation. α-tocopherol darkens on exposure to light and slowly oxidized by atmospheric oxygen. α-tocopherol was found stable in separated serum at 25°C for 1day, at 4°C for 2 weeks and at -20°C for 2 months.

Procedure Step-1: two centrifuge tubes label as T and B (*i.e.* sample and blank). To the sample tube add 750µL absolute ethanol and 750µL serum. Add the serum slowly

with shaking to obtain a finely divided protein precipitate. To the blank tube add 750µL distilled water and 750µL absolute ethanol. Stopper the tubes tightly by wrap paper and shake vigorously for at least 30 sec. To all these tubes add 750µL xylene. Again stopper the tubes tightly by wrap paper and shake vigorously for at least 30 sec and centrifuge all tubes for 10 min at 3000 rpm (Table-10).

Step-2: Transfer 500µL of the xylene layer (supernatant) into properly labeled clean small size test tubes. To each tube add 500 µL of α,α –dipyridyl solution and 100 µL ferric chloride solution and wait for 2 min (Table-11).

Step-3: Transfer 200µL solution from these tubes to unlabeled microwells respectively. Readings are made in ELISA Reader (Erba, Transansia), with the rapid measure mode. Set the primary wavelength as 492 nm and measure the absorbance within 4 min (Table-12).

Table 10: Analysis of α-tocopherol procedure

	T (µL)	B (µL)	
Serum	750	-	Mix for 30 sec & centrifuge for 10 min at 3000 rpm
DW	-	750	
Ethanol	750	750	
Mix for 30 sec			
Xylene	750	750	

Table 11: Analysis of α-tocopherol procedure

	T (µL)	B (µL)	
Supernatant	500	500	Wait for 2 min
α-α dipyridyl	500	500	
$FeCl_3$	100	100	

Table 12: Analysis of α-tocopherol procedure

	T (µL)	B (µL)
ELISA	200	200
Read the absorbance at 492nm within 2 min		

Note: we can take the serum 350µL (in case of less availability) by reducing all other reagents by half.

Calculation

$$\text{Conc}^{n} \text{ of Vit E } \mu g/ml = \frac{\text{OD of Test} - \text{OD of Blank}}{\text{Slope}} \times \text{Dilution factor}$$

Where, $\text{Slope} = \frac{Y_2 - Y_1}{X_2 - X_1}$

X & Y are concentration and absorbance of standards respectively

5.10.6. REPRODUCTIVE ASSAY

TESTICULAR CHOLESTEROL

By Zak's ferric chloride Method

Testicular cholesterol Assay includes Standardization of chemicals & Analysis of Testicular cholesterol in sample

Standardization of chemicals

Principle

Cholesterol in acetic acid reacts with ferric chloride and sulfuric acid to produce a red color. Absorbance of color read at 560 nm.

Sample: Testis homogenate (250µL).

Chemicals Required

Ethanol, Acetone, Glacial acetic acid (aldehyde free), 10% ferric chloride, Concentrated H_2SO_4, Cholesterol Standard, 0.9% NaCl

Preparation of reagents

- Ethanol acetone mixture: equal volume of Ethanol and acetone mixed (1:1)
- Preparation of colour reagent: 0.5ml of 10% ferric chloride + 50 ml H_2SO_4

- Stock Cholesterol Standard: (100mg/100ml in acetic acid) dissolve exactly 100mg cholesterol in 100ml glacial acetic acid. Keep in cool dry place; reagent is stable for one month.
- Working standard cholesterol: dilute 1ml of stock standard cholesterol solution to 24ml of 0.05% $Fecl_3$ with ferric chloride reagent.
- 0.9% NaCl: 0.9g NaCl in 100 ml water.

Standardisation procedure

Table 13: Standardization procedure for Testicular cholesterol

Reagent	Blank	S1	S2	S3	S4	S5
Std(ml)	-	1	2	3	4	5
Conc of Std(mg)		40	80	120	160	200
0.05% $FeCl_3$(ml)	5	4	3	2	1	-
0.9% NaCl (ml)	0.05	0.05	0.05	0.05	0.05	0.05
Con H_2So_4(ml)	3	3	3	3	3	3

Mix and keep at room temperature for 30 min read the optical density between 560-570 nm

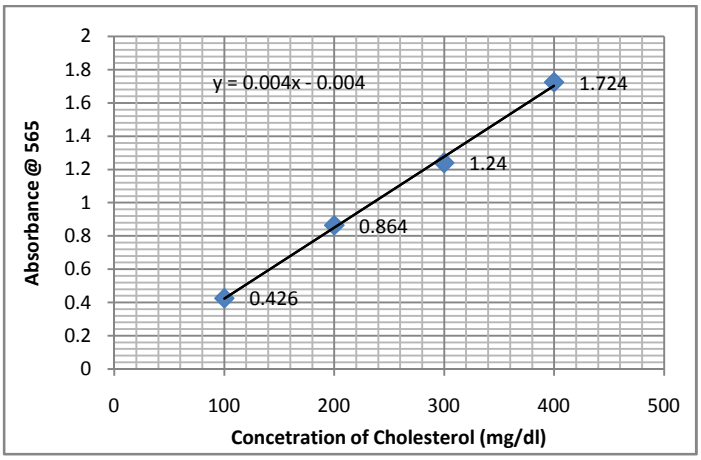


Figure 21: Standard Operating Procedure of Testicular cholesterol, showing gradual colour change indicate the presence of concentration of cholesterol in S1- S5 test tubes, where B Tube indicates Blank by using Shimadzu UV -VIS Spectrophotometer 1700

TISSUE HOMOGENISATION

Tissue homogenate is prepared by adding 500mg of testis to 5ml of Acetone ethanol mixture (1:1), homogenised for few minutes then whole mixture was centrifuged and 0.25ml of supernatant was used for estimation.

Procedure

Table 14: Procedure for testicular cholesterol estimation

	Reagent	**T**	**B**
1	Homogenate	0.25	0
2	Acetone: Ethanol(1:1) (mL)	5	5
	Water bath for 10 minutes at 60°-70°C then volume make upto 5mL by acetone:ethanol (1:1)		
	Cooling Centrifuge for 15 min at 3500-4000 rpm. Take 0.1mL DW for blank		
3	Test tube for supernatant (mL)	0.1	0.1
	Dry test tubes in boiling water bath then keep tubes at room temperature to cool		
4	Glacial acetic acid (aldehyde free) (mL)	3	3
	Wait for 5 minutes at room temperature		
5	Colour Reagent (mL) 0.5mL 10% $FeCl_3$ + 50mL conc. H_2SO_4	2	2
	Measure the optical density against blank at 560-570nm on Shimadzu UV -VIS Spectrophotometer 1700.		

Calculation

Homogenization of tissue is prepared by adding 500mg of tissue (testis) in 5 ml of acetone ethanol (1:1) mixture

So, 5ml acetone ethanol mixture contains 500 mg of tissue

1 ml homogenate contains 100mg of tissue,

0.125 ml homogenate contains 12.5mg of tissue,

So, after homogenization from the supernatant 0.125ml was diluted with 2.5ml of acetone ethanol (1:1) mixture. (0.125+2.5=2.625 ml)

2.625 ml of mixture contains 12.5mg of testis,

0.1ml of above mixture used for test

So, 2.625=12.5mg

0.1ml= ? $\frac{0.1X12.5}{2.625}$ =0.476mg of testis is present in 0.1ml of mixture

According to equation of graph **y = 0.004x - 0.004**

Where, y= absorbance (ex, 0.125)

$y = 0.004x - 0.004$

$0.125=0.004x - 0.004$

$0.125+0.004=0.004x$

$x=\frac{0.129}{0.004}$

$x=32.25$mg of cholesterol

So, 0.1ml of mixture contains 0.476mg of testis, which contains 32.25mg of cholesterol

0.476mg=32.25.5gm

1mg =? $\frac{32.25X1}{0.476}$ =67.75mg of cholesterol is present in1mg of tissue

67.75mg of cholesterol is present in1mg of tissue

LUTEINIZING HORMONE

by Chemiluminescence immunoassay (CLIA)[23, 24&25]

Principle

The LH CLIA Kit is based on a solid phase enzyme-linked immunosorbent assay. The assay system utilizes one anti-LH antibody for solid phase immobilization and another mouse monoclonal anti-LH antibody in the antibody-enzyme conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in the LH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 60 min incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of chemiluminescent substrate is then added and read relative light units in a Luminometer. The intensity of

the emitting light is proportional to the amount of enzyme present and is directly related to the amount of LH in the sample.

Reagents

Anti-LH Antibody Coated Microtiter Wells, Enzyme Conjugate Reagent, Reference Standard, Wash Buffer, Chemiluminescence Reagent A, Chemiluminescence Reagent B

Reagent Preparation

Substrate solution- 1:1 mixing of Reagent A with Reagent B. Mix gently

Reconstitute each lyophilized standard and controls with distilled water. Allow to stand for 20 minutes.

Dilute 1 volume of Wash Buffer with 49 volumes of distilled water. Mix well before use.

Procedure

Secure the desired number of coated well in the holder. Make data sheet with sample identification. Dispense 50 µl of LH standard, specimens, and controls into appropriate wells. Dispense 100 µl of Enzyme Conjugate Reagent into each well. Thoroughly mix for 30 seconds. Incubate at room temperature (18-25°C) for 60 minutes. Remove the incubation mixture by flicking plate contents into a waste container. Rinse and flick the microtiter wells 5 times with washing buffer. Strike the wells sharply onto absorbent paper to remove residual water droplets. Dispense 100 µl chemiluminescence substrate solutions into each well. Gently mix for 5 seconds. Read wells with a chemiluminescence microwell reader 5 minutes later. (Between 5 and 20 min. after dispensed the substrates).

FOLLICLE STIMULATING HORMONE

By Chemiluminescence immunoassay (CLIA)[19,26&27]

Principle

The assay system utilizes a polyclonal anti-FSH antibody for solid phase immobilization and a mouse monoclonal anti-FSH antibody in the antibody- enzyme conjugate solution. The test sample is allowed to react simultaneously with the antibodies, resulting in FSH molecules being sandwiched between the solid phase and enzyme-linked antibodies. After 60 minute incubation at room temperature, the wells are washed with water to remove unbound labeled antibodies. A solution of chemiluminescent substrate is then added and read relative light units (RLU) in a Luminometer. The intensity of the emitting light is proportional to the amount of enzyme present and is directly related to the amount of FSH in the sample.

Reagents

Anti-FSH Antibody Coated Microtiter Wells, Enzyme conjugate reagent, Reference Standard, Wash Buffer, Chemiluminescence Reagent A & Chemiluminescence Reagent B

Reagent Preparation

Substrate solution- 1:1 mixing of Reagent A with Reagent B. Mix gently

Dilute 1 volume of Wash Buffer with 49 volumes of distilled water. Mix well before use.

Procedure

Secure the desired number of coated well in the holder. Make data sheet with sample identification. Dispense 50 µl of FSH standard, samples, and controls into appropriate wells. Dispense 100 µl of Enzyme Conjugate Reagent into each well. Thoroughly mix for 30 seconds. Incubate at room temperature (18-25°C) for 60 minutes. Remove the incubation mixture by flicking plate contents into a waste

container. Rinse and flick the microtiter wells 5 times with washing buffer. Strike the wells sharply onto absorbent paper to remove residual water droplets. Dispense 100 µl Chemiluminescence substrate solutions into each well. Gently mix for 5 seconds. Read wells with a chemiluminescence microwell reader 5 minutes later (Between 5 and 20 min. after dispensing the substrates).

TESTOSTERONE

By Chemiluminescence immunoassay (CLIA)[28]

Principle

The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the Testosterone molecule. Endogenous Testosterone of a donor sample competes with a Testosterone horseradish peroxidase conjugate for binding to the coated antibody. After incubation the unbound conjugate is washed off. The amount of bound peroxidase conjugate is reverse proportional to the concentration of Testosterone in the sample. After addition of the substrate solution, the intensity of emitted light is inversely proportional to the concentration of Testosterone in the donor sample

Reagents & preparation

Microtiterwells coated with a monoclonal anti-Testosterone antibody, Standard, Enzyme Conjugate, and Substrate Solution Reagent A, Reagent B, Reagent C, Wash Solution

Working Substrate Solution: Mix 1 part of the chemiluminescence Reagent A with 1 parts of Reagent B and dilute this mixture 1:1.5 with Reagent C.

Wash Solution: Dilute 30 mL of concentrated Wash Solution with 1170 mL deionized water to a final volume of 1200 mL.

Procedure

Secure the desired number of Microtiter wells in the holder. Dispense 20 µL of each Standard, Control and samples into appropriate wells. Dispense 200 µL Enzyme Conjugate into each well. Thoroughly mix for 10 seconds. Incubate for 60 minutes at room temperature. Rinse the wells 5 times with diluted Wash Solution. Strike the wells sharply on absorbent paper to remove residual droplets. Add 100 µL of the freshly prepared Substrate Solution to each well. Incubate for 10 minutes at room temperature. Read the RLU with a microtiter plate luminometer within 20 minutes after incubation time of substrate.

SEMEN ANALYSIS

Sperm Concentration ("Sperm Count") is the number, in millions, of spermatozoa per milliliter of semen. Sperm are immobilized, diluted and counted in a quality-verified chamber such as a hemocytometer, Makler chamber, or fixed-cover slip slide[29].

Collection of specimen

Sperm count was performed on semen samples collected from the cauda epididymis. The cauda portion was separated and placed in 0.25 M sucrose solution. The epididymal tubes were punctured with a fine hypodermic needle and spermatozoa were extruded by squeezing. Semen also can be collected from the vas deferens by squeezing. 10µl of semen in 990µl of sucrose solution and mix well. Take 100µl from mixture and charge neubauer counting chamber.

Preparation of .25M sucrose solution

Add 0.342(molecular weight of sucrose) gms of sucrose in one ml of distilled water. This will form 1M sucrose solution. Dilute the 1M sucrose with 4 ml of distilled water it will become 0.25M sucrose solution.

Sperm Count

Sperm Concentration ("Sperm Count") is the number, in millions, of spermatozoa per milliliter of semen. Sperm are immobilized, diluted and counted in a quality-verified chamber such as a hemocytometer, Makler chamber, or fixed-cover slip slide. Sperm heads within a 4 corner square area were counted in the same manner as blood cells counted in a hemocytometer; their number represents their concentration in millions/mL.

Procedure (Neubauer Counting Chamber Method)

Clean and dry chamber completely. Mix properly & place a drop (8 to 10µ) on to the center of the lower disk. Place the cover glass on four posts semen should spread over the entire area of the lower glass disk avoid adding excess semen. View under X20 objective and 10X eyepiece. Count the number of sperms within a strip of 10 squares. Repeat this count from 2 to 3 other strips and average the counts. If sperms are highly concentrated it may be difficult to count the motile sperms. Sperms can be immobilized by heating an aliquote at 50-60 ^{0}Cfor 5 min or placing the chamber at -20 ^{0}C for10min and then proceeding with the count.

Calculations:

Length of each square = 1mm

Width of each square = 1mm

Height of the Neubauer chamber = 1/10mm

Dilution Factor = $\frac{\text{Final Volume}}{\text{Initial Volume}} = \frac{1000\ \mu l}{10\ \mu l} = 100\mu l$

No of Sperm/ml of semen = $\frac{\text{N X Dilution factor X 1000}}{\text{Areas counted X depth}}$

Where

N= Number of sperms counted in 4 squeres

Dilution factor= 100µl (10µl semen in 990µl sucrose solution)

Areas counted= 4 corners of WBC area in Neubauer Counting chamber

Depth = Depth of chamber 0.1mm

1000=is conversion from µl to ml

I.e. No of Sperm/ml of semen = $\frac{N \times 100 \times 1000}{4 \times 0.1}$

Now $= \frac{N \times 25 \times 1000}{1/10}$

$= N \times 25 \times 1000 \times 10$

$= N \times 2{,}50{,}000$

= cells/ml

Sperm motility:

Percentage of motile sperm with time and speed of motility of individual sperm will be assessed semi quantitatively on a scale of 0-5 and the spermatozoa will be evaluated for the forward movement & graded accordingly.

Grading of sperm motility

0= No movement

1= sluggish or tail movement alone

2= intermittent sluggish movement

3-4= fair-good movement

5= maximum direction in forward direction

5.10.7. HISTOPATHOLOGY

MANUAL TISSUE PROCESSING

Procedure is fallowed as per the Bancroft procedure[30]

Fixation: Fixation is the first step towards the preparation of a histological section from a dead biological specimen. The substances used for fixation are called as fixatives. Tissues will be kept in fixatives for 24 hrs.

Common Fixatives, Formalin, Zenker's fluids, Bouin's fluid (Preparation)

Bouin's fluid Preparation

a) Saturated picric acid 1500.0 ml
b) Formaldehyde 500.0 ml
c) Glacial acetic acid 100.0 ml

Mix well, stable for 1 year. Bouin's fluid is used to preserve testicular tissue.

Washing: After fixation tissue is washed under running tap water one to two hours, it removes the fixative from tissue.

Dehydration, Clearing and Impregnation: Our aim is to imbibe the tissue with paraffin; this cannot be done directly because paraffin is not miscible with water of the tissue. Dehydration is done in stages, first the tissue is immersed in alcohol which replaces water of tissue (dehydration). Paraffin is not miscible with alcohol also; Xylene benzene, chloroform and toluene are substances which get mixed both the alcohol and paraffin therefore after alcohol the tissue is treated with one of these liquids (called clearing agents) and then tissue is impregnated in melted paraffin. Following is the list of time for manual tissue processing.

Table 15: Procedure for Manual Tissue Processing

Step	Treatment	Hours
Fixation	Formalin	24
Dehydration	30% Alcohol	1
	50% Alcohol	1
	80% Alcohol	1
	80% Alcohol	1
	90% Alcohol	1
	100% Alcohol	1
	100% Alcohol	1
	100% Alcohol	1
Clearing	Xylene	1
	Xylene	1
Infiltration	Paraffin at 50-56^{o} C	1
	Paraffin at 50-56^{o} C	1
	Paraffin at 50-56^{o} C	1

Embedding: The process of embedding enables specimens too small and/or too delicate to be surrounded with some suitable material example paraffin that will support them on all sides with firmness but without producing any injurious effect on the specimen the embedded tissue may then be section into sufficiently thin slices without distortion.

Preparation of Blocks: It is done with the help of L-blocks; L-blocks are L-shaped metallic pieces. Two L-blocks are placed on glass plate, so that they enclose a rectangular space. The tissue is put in it over which the melted paraffin is poured which solidifies slowly. The L-blocks are removed.

Section cutting: For histological preparation tissues cut (sectioned) into thin slices. Trim the upper or cutting surface of the block parallel to the knife edge until the surface of the tissue gets exposed. Attach the block holder to the microtome. Clamp the knife firmly in position in microtome. Adjust the desired thickness scale (8-12m)

in the microtome. Move the microtome fairly constant and fast. Pick up the end of short ribbon with a soft brush, and gently pull it away from the knife as you continue sectioning.

Haematoxylin & Eosin Staining

1. Xylene 2 Minutes
2. Xylene 2 Minutes
3. Absolute alcohol 1 Minute
4. Absolute alcohol 1 Minute
5. 90% Alcohol 2 Minutes
6. 70% alcohol 2 Minutes
7. 50% alcohol 2 Minutes
8. Distilled Water 5 Minutes
9. Haematoxyllin 2 to 5 minutes depending on which of the above types of Haematoxyllin solution is used (Stain for 20 minutes with Ehrlich's and for 2-5 minutes with Harris' Haematoxyllin).
10. Wash well in running tap water for 2-3 minutes sections may be examined with a microscope to confirm a sufficient degree of staining
11. Remove excess stain by differentiation in acid alcohol (1% HCL in 70% alcohol) for a few seconds. Blue staining of haematoxylin stained section is changed to red by the action of the acid
12. Immediately wash in alkaline tap water for at least 5 minutes to regain the blue colour ('bluing sections') (a few drops of saturated lithium carbonate may be added to tap water in a large beaker and the bluing of section done in this. After blueing, the slides should be thoroughly washed in running rap water to remove excess of lithium carbonate).if necessary, examine microscopically.
13. 1% aqueous Eosin 1 to 3 minutes

14. Wash of surplus eosin in water.

15. Examine the sections microscopically. Cytoplasm and muscle cell should be deep pink, collagen fibres a lighter pink, Erythrocytes and eosinophil granules should be bright orange-red

16. 90% alcohol for 10 to 15 seconds

17. Absolute alcohol I agitate for 10 to15 seconds

18. Absolute alcohol II for 30 seconds

19. Xylene I—1 to 2 Minutes

20 Xylene II --- 1 to 2 Minutes or until completely clear.

21. Mount in D.P.X. or any other synthetic resin medium.

Results

Muscle cell - deep pink, Collagen fibers - lighter pink, Erythrocytes and eosinophil granules - bright orange red, Cytoplasm – Pink, Nuclei - Blue of blue-back

5.11. Ulcerogenecity

Ulcerogenecity of stomach will be done on 46th day by dissecting the stomach. After removal of stomach, interior of which will be washed with normal saline completely and fixed to slide, interior of stomach will be observed by dissection microscope. Grading of ulcers will be done in the following manner.

Grading of Ulcerogenecity

0= absence of lesion, vasodilatation or up to 3 pin point ulcers

1= more than 3 pin point ulcers

2= 1-5 small ulcers (<2mm)

3= more than 5 small ulcers (<2mm)

4= 1 or more giant ulcers

5.12. Statistics Analysis

Data obtained from all control and experimental samples has been subjected to statistical analysis for evaluating the range of significance. After collecting all the data was filled in Microsoft excel, data was presented with tables and diagrams. Mean $\pm$ SD (standard Deviation) values were calculated for each group. To determine the significance of inter-group differences, each parameter will be analyzed separately. All the parameters were compared by using proper statistical tests using software SPSS V 20 32bit, values were analyzed by one-way analysis of variance (ANOVA) using post hoc Tukey HSD test to study the differences between the groups. The level of statistical significance was set at $p<0.05$

BIBLIOGRAPHY

1. Herborne J B. Phytochemical Methods: A guide to modern techniques of plant analysis, 3rd Edn. Chapman and Hall Ltd. London, 1973: 135-203

2. R D Gibbs Chemotaxonomy of Flowering Plants, Vol. 1, McGill Queen's University Press, Montreal and London; 1974

3. K Peach, M V Tracey. Modern methods of plant analysis, Vol 3, Springer Verlag, Berlin; 1956

4. A M Rizk. Fitoterapia, 1982, 52(2), 35-42

5. GE Trease, WC Evans. Pharmacognosy, 15th edition, WB Saunders Publishers, London, 2002

6. CK Kokate. Practical Pharmacognosy. 4th edition, Vallabh Prakashan, New Delhi, India, 1994

7. A Sofowara. Medicinal plants and traditional medicine in Africa. Chichester John Wiley & Sons, New York, 1993

8. Zaidi S M K R & Banu N Clin. Chim. Acta. 2004; 340: 229-33

9. Van Herck H. Baumans V. Brandt C J. Hesp A P. Sturkenboom J H. van Lith H A. et al. Orbital Sinus Blood Sampling in Rats as Performed by Different Technicians: the Influence of Technique & Expertise. Lab Anim. 1998; 32, 377-86

10. Burtis C A. ashwood, E. R. editors. Tietz textbook of clinical chemistry. 2nd ed. Philadelphia. W. B. Saunders company 1994;790-5

11. Allain C. C. Poon L. S. Chan C. S. G. Richmond W. and Fu p. clin. Chem. 1974; 20 (470)

12. Roeschlau P. Bernt E. & gruber W A. clinchem. clinBiochem 1974;12(226)

13. Product Data sheet, Triglycerides-G code no. 997-69801 WAKO pure chemical industries Ltd. Dallas
14. McGown M W et al. clinchem 1983; 29; 538
15. Trider p. Fossati p. Ann clin Biochem 1969; 6, 24-7
16. Rurstein M. Scholnic H. R. Morfin R. L Lipid Res. 1970
17. Check J. H. et al. Falsely elevated steroidal assay levels related to heterophile antibodies against various animal species. Gynecol. Obstet. Invest. 1995; 40: 139-40.
18. Poland R. E. et al. Life Sci. 1982;30:177
19. Buege, J. A. Aust, S. D. Microsomal lipid peroxidation. Methods Enzymol 1978, 52: 302-10.
20. Mosage H, kok B, Huzenya JR, Jansen PL. Nitrite & nitrate determination in plasma critical evaluation clin. Chem 1995; 4:892-96
21. Roe J. H. Koether C. A. The determination of ascorbic acid in whole blood & urine through the 2, 4-dinitrophenylhydrazine derivative of dehydroascorbic acid J Biol Chem. 1943; 147:399 – 407
22. Jameel G J, Shaheenkousar H. H. Swastika D. Salim A. D & Kusal K. Das. A modified simple method for determination of serum α -tocopherol (vitamin E) Basic clin physiology pharmacology Boston 2012; 23(1):45-48
23. Knobil E. The neuroendocrine control of the menstrual cycle Rec. Prog. Horm. Res. 1980; 36: 52-88
24. Harris, G. W. Naftolinf. The hypothalamus and control of ovulation, Brit. Med. Bullet. 1970; 26: 1-9
25. Uotila, M. Ruoslahti E and Engvall, E. J. Immunol. Methods 1981;42: 11-5
26. Marshall J. C. Clinic in Endocrinol. Metab. 1975; 4:545
27. Rebar R. W. Erickson G. F. and Yen S. S. C. Fertil. Steril. 1982; 37: 35

28. Tietz, N. W. Textbook of Clinical Chemistry. Saunders, 1986

29. Praful B G, Darshan P G; textbook of medical laboratory technology, 2nd edition Mumbai India: 957-970

30 . John D Bancroft, Marilyb Gamble-Theory and Practice of Histological Techanique.6th edition Churchill Livingstone Elsevier

CHAPTER 6

RESULTS

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

2015

BLDE UNIVERSITY
SHRI B M PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

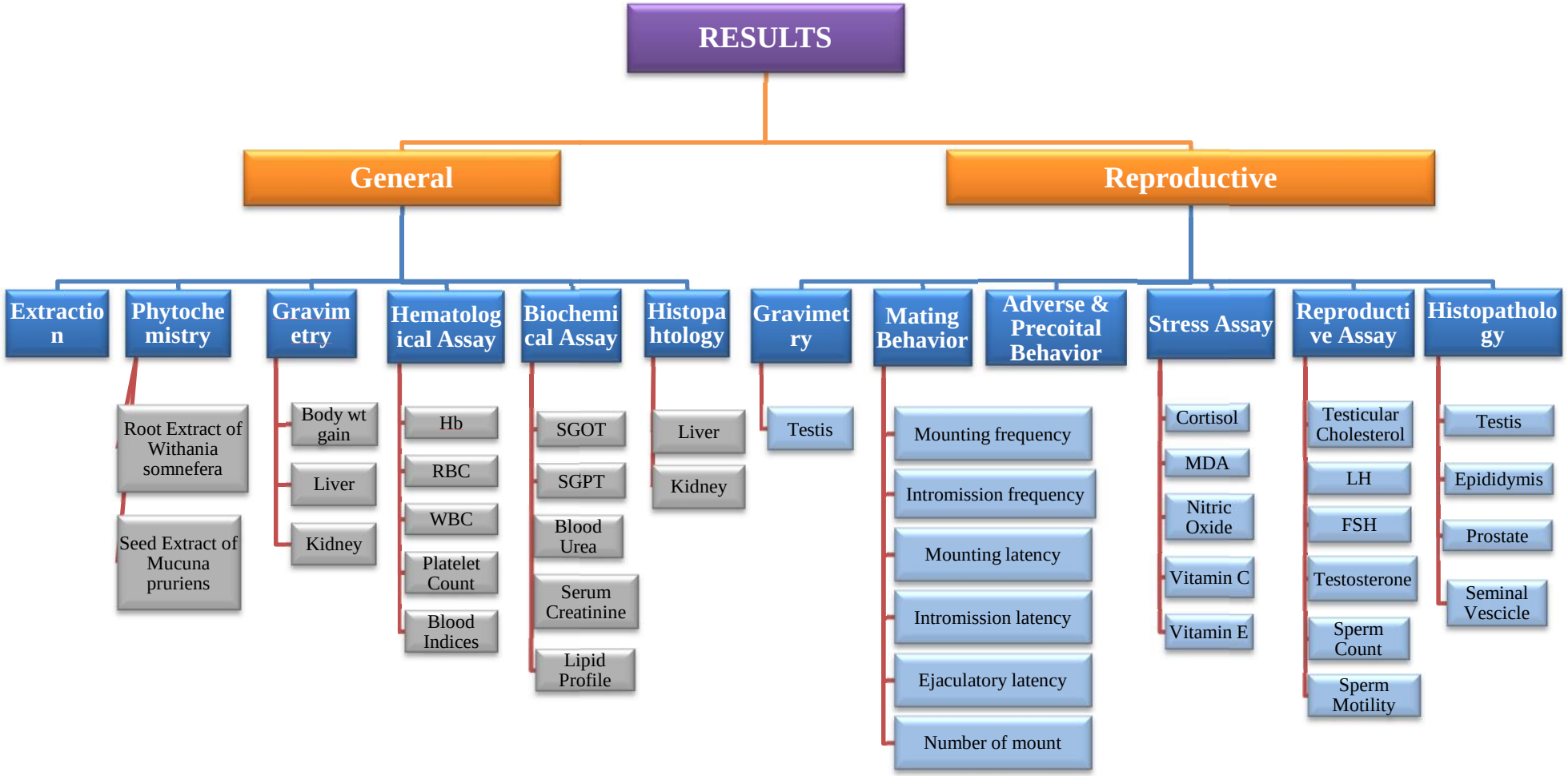


Figure 22: Flow chart showing results of all parameters

CHAPTER 6.1

GENERAL

6.1. GENERAL

1. Extraction

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 percentage of yield was calculated.

Yield for methanolic seed extract of Mucuna pruriens was: 9.534%

Yield for methanolic root extract of Withania somnifera was: 10.682%

2. Preliminary Phytochemical Screening

Both the drugs have been undergone for phytochemical constituents. Different qualitative test were performed to establish various extracts and their chemical composition. The extracts obtained were subjected to following chemical tests for identification of various phytoconstituents.

Preliminary phytochemical screening of methanolic seed extracts of *Mucuna pruriens* shows presence of Flavonoids, Steroids, Alkaloids, Tannins, Carbohydrates, Starch, Phenols, Amino acids & Resins and absence of Glycosides & Saponins

Preliminary phytochemical screening of methanolic root extracts of *Withania somnifera* shows the presence of Steroids, Alkaloids, Tannins, Flavonoids, Carbohydrates, Starch, Glycosides & Phenols and absence of Saponins & Amino acids. Complete results have been shown in table no 16.

Table 16: Results of phytochemical screening for both the extract

Preliminary phytochemical screening				
S.N	**Phytochemical**	**Test**	**In Withania somnifera**	**In Mucuna pruriens**
1	Test for Steroids	Salkowski test	Present	Present
		Liebermann-Burchard test	Present	Present
2	Test for Flavonoids	Shinoda test	Present	Present
		Lead acetate test	Present	Present
3	Test for Alkaloids	Hager's test	Present	Present
		Mayer's test	Present	Present
4	Test for Tannins	Gelatin test	Present	Present
		Ferric chloride test	Present	Present
5	Saponins	Foam test	Absent	Absent
		Froth test	Absent	Absent
6	Carbohydrates	Molisch's test	Present	Present
		Benedicts test	Present	Present
7	Starch		Present	Present
8	Glycosides	Foam test	Present	Absent
9	Phenols	Lead Acetate test	Present	Present
10	Amino acid/ Protein	Ninhydrin test	Absent	Present
		Biuret test	Absent	Present
11	Resins		Absent	Present

3. Gravimetry

3.1. Weight gain

Regular weight of the rat was measured during dosage period, initial & final weight was considered to calculate the weight gain. All the rats in groups I, III, IV, V and VI 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. However, in case of group IV (stress + *Mucuna pruriens*) V (stress + *Withania somnifera*) and VI (stress + *Mucuna pruriens* + *Withania somnifera*) an improvement in body weight was observed, as compared to the group II (stress)

Rats, while in group III (stress + Sildenafil citrate) very slight improvement in the body weight was observed(table 17 & Fig 23-25).

Table 17: Comparison of initial, final body weight & weight gain in each group

	Control Group Mean±SD	Stress Control Mean±SD	STD Group Mean±SD	Mucuna pruriens Mean±SD	Withania somnifera Mean±SD	MP+WS Mean±SD	Anova	p-value
Initial body Weight	210.5±11.31	181±10.31	182.83±16.59	215.83± 14.29	212.33±13.21	232±22.79	10.198	.000
Final body Weight	355.17±21.09	286±23.02	293.67±25.37	331±43.53	334.17±40.14	340±45.25	3.751	.009
Weight gain	144.67±17.34[b]	98.33±3.14[a]	110.83±10.61[a]	129.67±24.63	126.83±26.65	115.17±26.70	3.874	.008

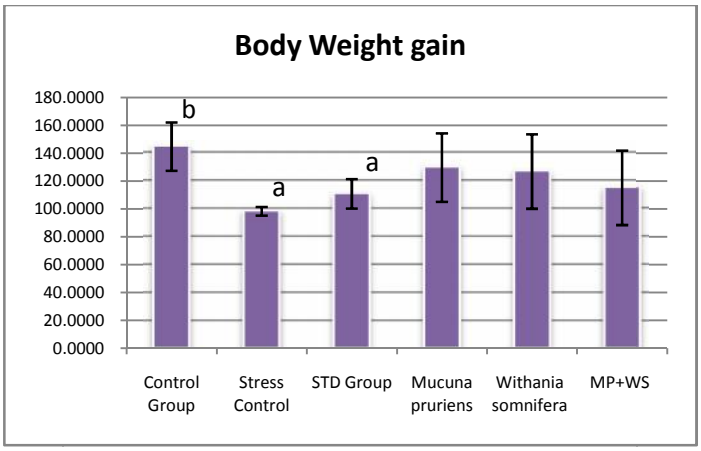


Figure 23: Body weight gain

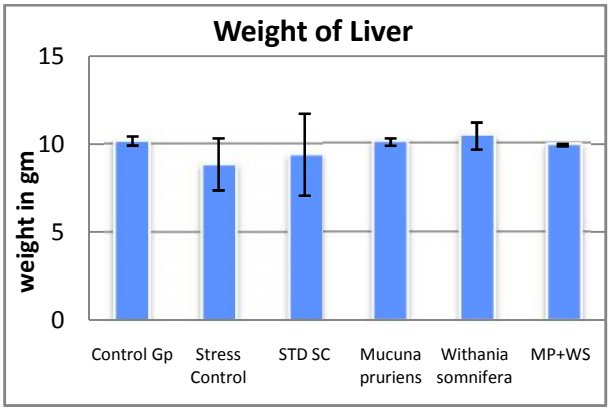


Figure 24 Weight of Liver

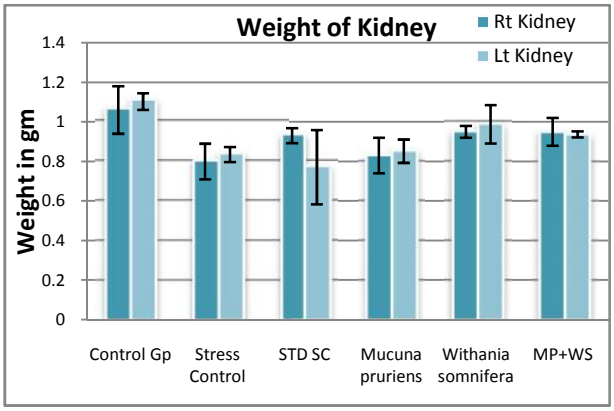


Figure25 Weight of Kidney

Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared to control, b-compared to stress group & c-compared to standard group.

4. Hematology

Hematological profile reveals that there was significant increase in the level of hemoglobin in group IV, V & VI when comparing with group I. There was significant increase in the level of WBC count of group IV & VI as compared to group I, even though there was increase in the level of WBC count in group V the increase was not significant statistically. There was significant increase in the level of RBC count in group IV, V & VI as compared to group I. Blood indices shows not much difference in any of the groups (Table 18).

Table 18: Shows results of hematological parameters

	Control Gp Mean±SD	Stress Control Mean±SD	STD Group Mean±SD	Mucuna pruriens Mean±SD	Withania somnifera Mean±SD	MP+WS Mean±SD	Anova	p-value
Hb (gm/dl)	13.33±1.17	14.75±0.52	15.43±1.46	15.83±0.14^{ab}	16.72±0.69^{ab}	16.95±0.83^{ab}	13.085	.000
WBC (cells/cumm)	8033.3±1440	9966.7±3859	13916.7±4206	15466.7±1842^{ab}	11433.3±2989^{a}	15383.3±3194^{ab}	5.856	.001
RBC (mill/cumm)	7.61±.098	7.35±.071	8.28±0.75	8.780.04^{ab}	9.57±0.37^{abc}	9.183±0.51^{ab}	11.252	.000
Platelet(lakhs /cumm)	8.22±1.83	8.91±0.57	7.57±2.85	8.9±0.25	10.25±2.36	8.030.67	1.783	.147
PCV (%)	48.68±5.67	49.67±2.33	52.32±6.10	51.78±2.94	53.98±5.63	55.38±2.15	1.921	.120
MCV (fl)	63.23±4.73	59.20.18	61.88±2.05	60.25±1.68	62.82±3.7	59.85±1.10	2.261	.074
MCH (pg)	18±1.58	17.73±0.51	18.32±0.44	18.1±0.61	18.32±1.12	17.72±0.49	.532	.750
MCHC (gm/dl)	28.53±2.11	29.70.99	29.57±095	30.08±0.71	29.18±1.97	30.03±0.78	1.081	.391

Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared to control, b-compared to stress group & c-compared to standard group.

5. Biochemistry

Biochemical profile was estimated to observe the effect of extracts on the organs where liver function was monitored by SGOT & SGPT and kidney function was monitored by urea & creatinine estimations. Levels of urea & creatinine were significantly increased in group II & III as compared with group I, the same levels were significantly decreased in group IV as compared with group II & III. Even though there was an increase in the level of urea in the group II creatinine level were similar to the control group. Levels of SGOT & SGPT were not much different when compared within the group. Levels of triglycerides, cholesterol, LDL & VLDL were significantly increased in group IV & VI (Table 19).

Table 19 Shows results of Biochemical parameters

	Control Gp Mean±SD	Stress Control Mean±SD	STD Group Mean±SD	Mucuna pruriens Mean±SD	Withania somnifera Mean±SD	MP+WS Mean±SD	Anova	p-value
Liver function tests								
SGOT (U/L)	28.67±6.77	47.67±5.39[a]	38.17±12.61	47±5.58[a]	34±16.02	45.33± 4.37[a]	4.045	.006
SGPT (U/L)	40.67±5.42	44.5±7.18	38.83±11.29	51.53.73	40.33±16.7	43.17± 3.19	1.469	.229
Kidney function tests								
Blood Urea mg%	34.5±0.90	55±1.41[a]	52.83±5.5	61±4.24[a]	39.5±13.84	50.17± 4.36[a]	12.102	.000
Serum Creatinine (mg%)	1.40±0.32	1.6±0.14	1.02±0.44	1.55±0.19	1.2±0.33	1.4±0.14	3.571	.012
Lipid profile								
TGL (mg/dl)	140.17±5.27	162±7.02[ac]	114.5± 37.79[b]	188±2[abc]	134± 26.72	180.83± 7.47[abc]	12.916	.000
Total Cholesterol (mg/dl)	183.67± 34.24	167.67± 12.66	166.83± 14.63	210.17± 3.97	167.83± 28.91	194±11.71	4.474	.004
HDL (mg/dl)	35.5±2.59	33.67±2.07	29.83±3.6	37±1.79	36.33±4.97[c]	33.5±3.39	3.851	.008
LDL (mg/dl)	119.63± 30.55	116.6± 26.03	102.43± 13.35	137.5± 3.89	114.57± 35.95	132.8± 3.400	1.816	.140
VLDL (mg/dl)	28.7±1.76	31.47±4.49	22.9±7.56	37.4±0.79	28.6±9.42	36.27±2.07	6.018	.001

6. Stress in Liver & Kidney

There was significant increase in the concentration MDA and NO levels in group II rat compared with control group I, and group IV (Mucuna pruriens) rats showed significant improvement towards control group when compared with the group II rats (Table 20).

Table 20 Results of Tissue MDA & NO

	Control Gp Mean±SD	Stress Control Mean±SD	STD Group Mean±SD	Mucuna pruriens Mean±SD	Withania somnifera Mean±SD	MP+WS Mean±SD	Anova	p-value
Liver MDA (µMoles/g)	90.05± 26.73[b]	166.96± 3.58[ac]	128.43± 18.53[b]	69.128± 10.34[bc]	76.81± 7.85[bc]	71.95± 3.14[bc]	44.200	.000
Kidney MDA (µMoles/g)	81.51± 3.91	108.42± 17.46	93.61± 4.12	94.85± 7.56	96.33± 19.43	89.49± 19.19	2.467	.055
Liver NO (µMoles/g)	211.92± 24.69[b]	459.83± 186.49[a]	283.89± 175.82	217.19± 85.26	165.80± 88.71	183.29± 42.82	5.116	.002
Kidney NO (µMoles/g)	378.81± 28.23[bc]	179.79± 27.52[a]	276.54± 70.6[a]	252.64± 31.19[ab]	374.71± 35[bc]	303.97± 27.80[b]	21.841	.000

Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared to control, b-compared to stress group & c-compared to standard group.

7. Histopathology

7.1. Liver

Group 1 Control rats

Section studied under H&E stain shows normal hepatic parenchymal tissue which is composed of numerous hexagonal to pyramidal 'Lobules'. Each lobule consists of a central vein from which the hepatic plates radiate outwards towards the portal areas; 3 to 5 portal triads are located at the periphery of the lobule; containing branches of bile duct, portal vein and hepatic artery; and occasional mononuclear cells. Cords of hepatocytes and blood containing sinusoids radiate from central vein to the peripheral portal triads.

The sinusoids are lined by both endothelial c ells and Kupffer's cells both of which have inconspicuous flattened nuclei and ill-defined cytoplasmic margins. The central veins are lined by endothelial cells surrounded by a ring of collagen fibres.

The hepatocytes are polygonal in shape with well-defined borders. The nucleus is single, round and has a fine chromatin pattern with 1 to 2 clearly defined amphophilic prominent nucleoli. The cytoplasm is eosinophilic and finely granular. Zone 1 (Periportal), Zone 2 (mid-zone) & Zone 3 (Centrilobular) appears normal

Group 2 Stress group

Section studied under H&E stain shows hepatic parenchymal tissue consisting of a central vein, hepatic plates, and portal areas containing branches of bile duct, portal vein and hepatic artery. The hepatocytes are **appear to be little swollen** having ill-defined cell borders with variation in cellular size and shape. The nuclei are large, more vesicular with variable size and shape and contain multiple 3 – 4 prominent nucleoli. The cytoplasm is **vacuolated and microvesicular**. There are foci of **fatty change** and **ballooning degeneration** and **necrosis** of hepatocytes in zone 3 (centrilobular) areas. The portal area appears **mildly enlarged** with **mild proliferation fibrous tissue** with **infiltration of mixed acute and chronic inflammatory cells.**

Group 3 Standard group

Section studied under H&E stain shows hepatic parenchymal tissue **distorted *'Lobular'* architecture** which is consisting of a central vein, hepatic plates, and portal areas containing branches of bile duct, portal vein and hepatic artery. The hepatocytes are **appear to be little swollen** having ill-defined cell borders with variation in cellular size and shape. The nuclei are large, more vesicular with variable size and shape and contain multiple 3 – 4 prominent nucleoli. The sinusoidal spaces are variably **widened with increase in number of Kupffer's cells.** Central vein shows features of **dilatation and congestion.**

Group 4 Study group treated with Mucuna pruriens

Section studied under H&E stain shows hepatic parenchymal tissue. Foci of **fatty change** of hepatocytes in zone 3 (centrilobular) areas. Central vein shows features of **dilatation and congestion.**

Group 5 Study group treated with Withania somnifera

Section studied under H&E stain shows hepatic parenchymal tissue. Hepatocytes **appear to be little swollen** having ill-defined cell borders. The portal area appears **mildly enlarged** with **mild proliferation fibrous tissue with infiltration of mixed acute and chronic inflammatory cells.** The sinusoidal spaces are variably **widened** with increase in number of **Kupffer's cells.**

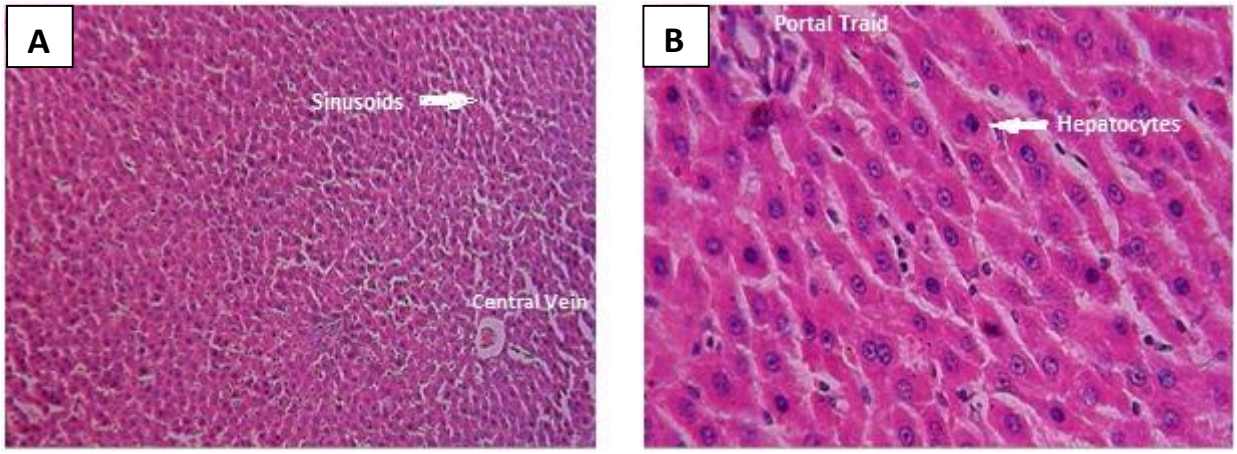
Group 6 Study group treated with Mucuna pruriens& Withania somnifera

Section studied under H&E stain shows hepatic parenchymal tissue which is consisting of a central vein, hepatic plates, and portal areas containing branches of bile duct, portal vein and hepatic artery. The hepatocytes are **appear to be little swollen** having ill-defined cell borders with variation in cellular size and shape. The portal area appears **mildly enlarged** with **mild proliferation fibrous tissue** with infiltration of mixed **acute and chronic inflammatory cells.** The sinusoidal spaces are variably **widened** with **increase in number of Kupffer's cells.** Central vein shows features of **dilatation and congestion.**

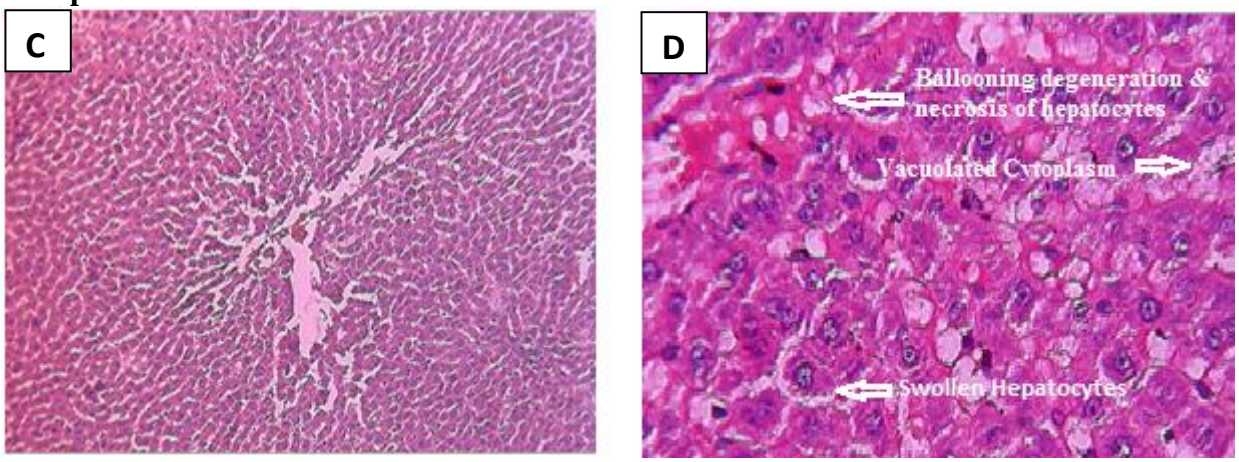
Histopathology of Liver

Low power **High power**

Group 1



Group 2



Group 3

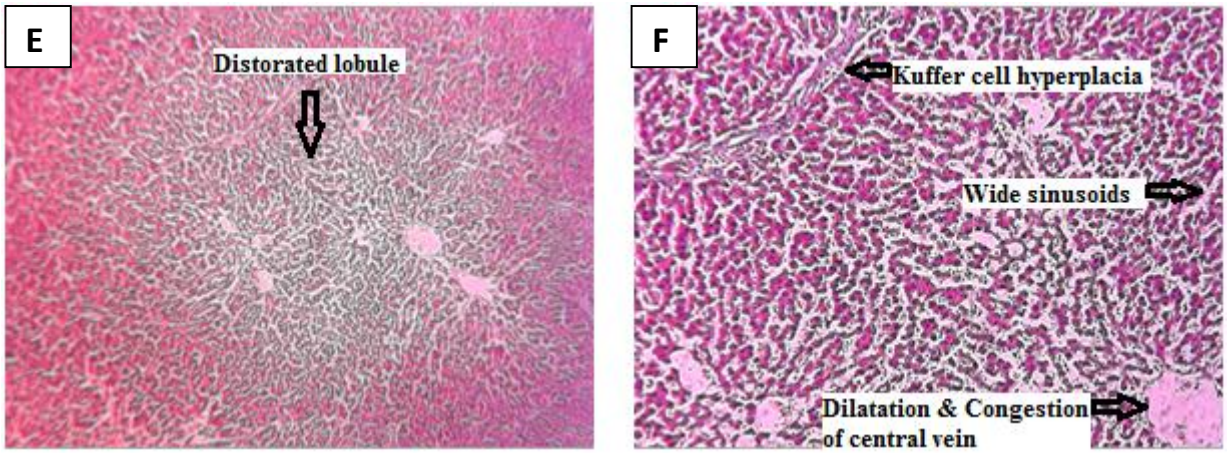
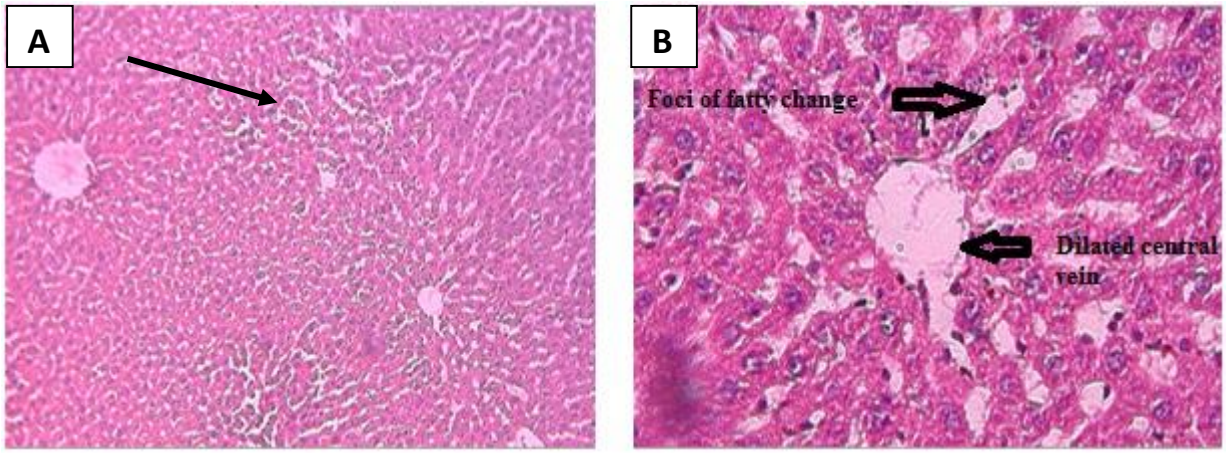


Figure 26 Plate showing histopathology of Liver in low (labeled A,C&E) & high power(labeled B,D&F) for 1, 2 & 3 group

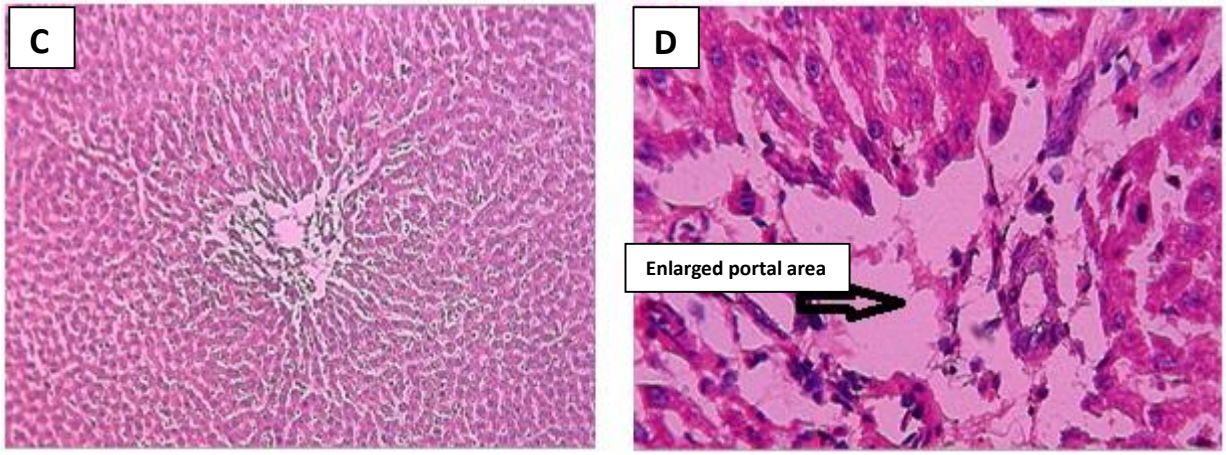
Histopathology of Liver

Low power **High power**

Group 4



Group 5



Group 6

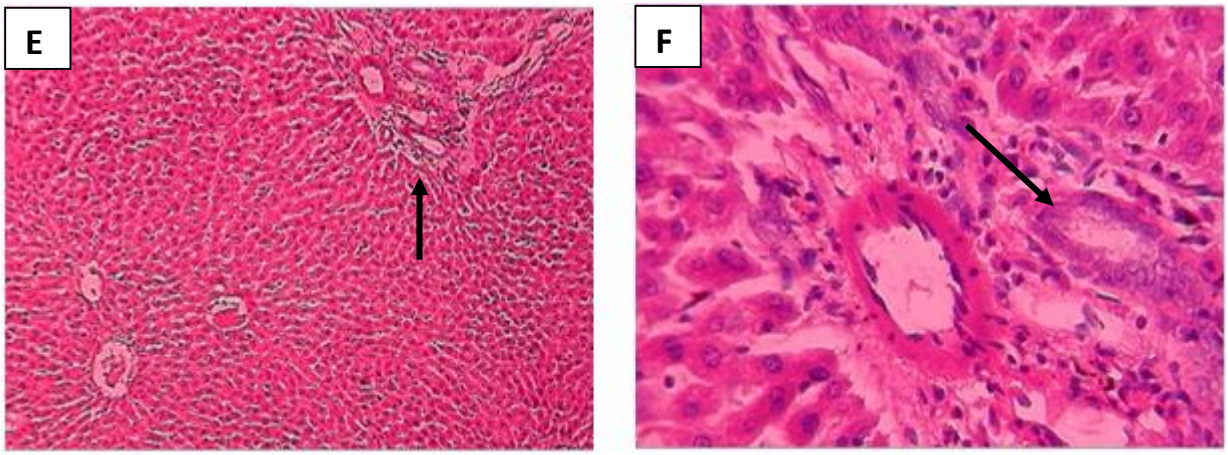


Figure 27 Plate showing histopathology of Liver in low (labeled A,C&E) & high power(labeled B,D&F) for 4, 5 & 6 group

7.2. Kidney

Group 1 control rats

Section studied under H&E stain shows normal renal parenchymal tissue which is composed of glomeruli and tubules separated by small amount of interstitial connective tissue containing peritubular capillaries.

Each glomerulus is spherical collection of interconnected capillaries within a Bowman's space lined by flattened parietal cells. The outer aspects of the glomerular capillaries are covered by a layer of visceral epithelial cells (podocytes).The capillary tufts are supported by the mesangium. Tubules appear normal. Glomeruli appear normal in morphology and in Cellularity, Interstitium appears normal, Vessels appear normal

Group 2 Stress group

Section studied under H&E stain shows renal parenchymal tissue which is composed of glomeruli and tubules. Glomeruli are **hypercellular** with **thickening of glomerular basement membrane** and **mesangial proliferation**. Tubules show focal **tubular basement membrane mild thickening** with **cloudy swelling (coagulation necrosis)**. Interstitium is edematous with infiltration of **inflammatory cells**. Vessels are **congested** and **sclerotic**.

Group 3 Standard group

Section studied under H&E stain shows renal parenchymal tissue which is composed of glomeruli and tubules. Glomeruli are **hypercellular** with **thickening of glomerular basement membrane** and **mesangial proliferation**. Tubules show focal **tubular basement membrane mild thickening**. The lumen shows eosinophilic protienacious (pink body), suggestive of **Acute Tubular Necrosis (ATN)**

Group 4 Study group treated with Mucuna pruriens

Section studied under H&E stain shows renal parenchymal tissue which is composed of glomeruli and tubules. Glomeruli are **hypercellular** and show **mesangial proliferation**. Tubules show focal **degenerative changes**. Interstitium shows infiltration of **inflammatory cells**. Vessels are **congested** and **sclerotic**

Group 5 Study group treated with Withania somnifera

Section studied under H&E stain shows renal parenchymal tissue which is composed of glomeruli and tubules. Glomeruli are **hypercellular** and show **mesangial proliferation**. Tubules show focal **degenerative changes**. Interstitium shows infiltration of **inflammatory cells**. Vessels are **congested** and **sclerotic.**

Group 6 Study group treated with Mucuna pruriens& Withania somnifera

Section studied under H&E stain shows renal parenchymal tissue which is composed of glomeruli and tubules. Glomeruli are **hypercellular** and show **mesangial proliferation**. Tubules show focal **degenerative changes**. Interstitium shows infiltration of **inflammatory cells**.

6.1.8 Ulcerogenecity

In case both the extract after treatment for 45 days stomach shown no ulcer

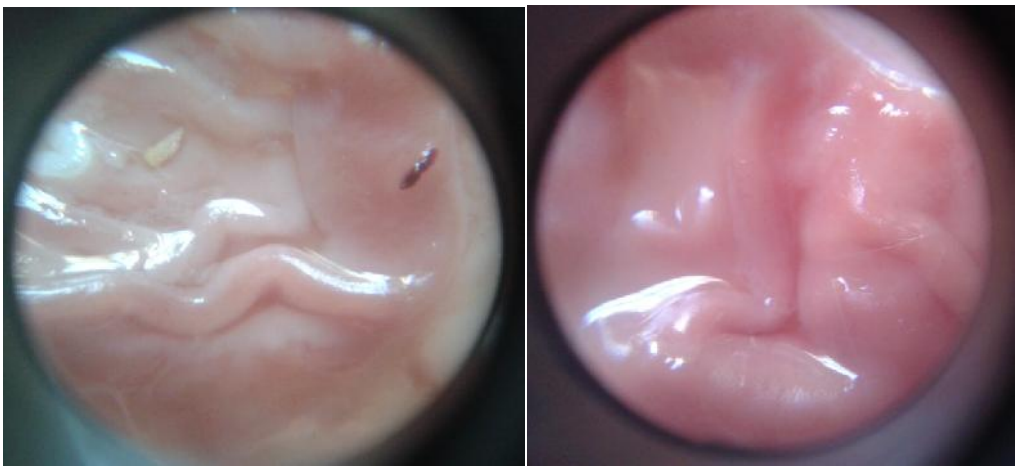
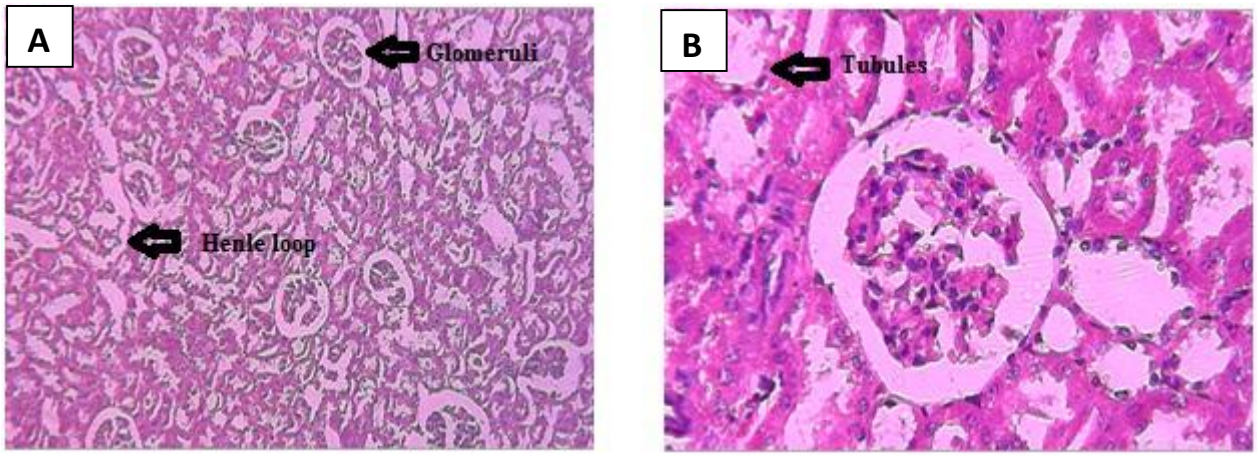


Figure 28.a Interior of Stomach

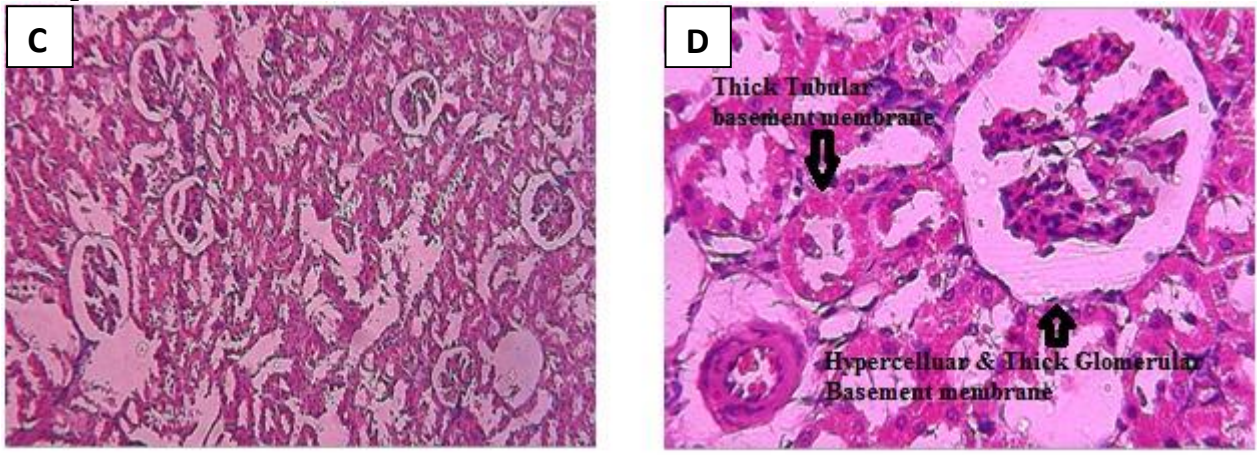
Histopathology of Kidney

Low power **High power**

Group 1



Group 2



Group 3

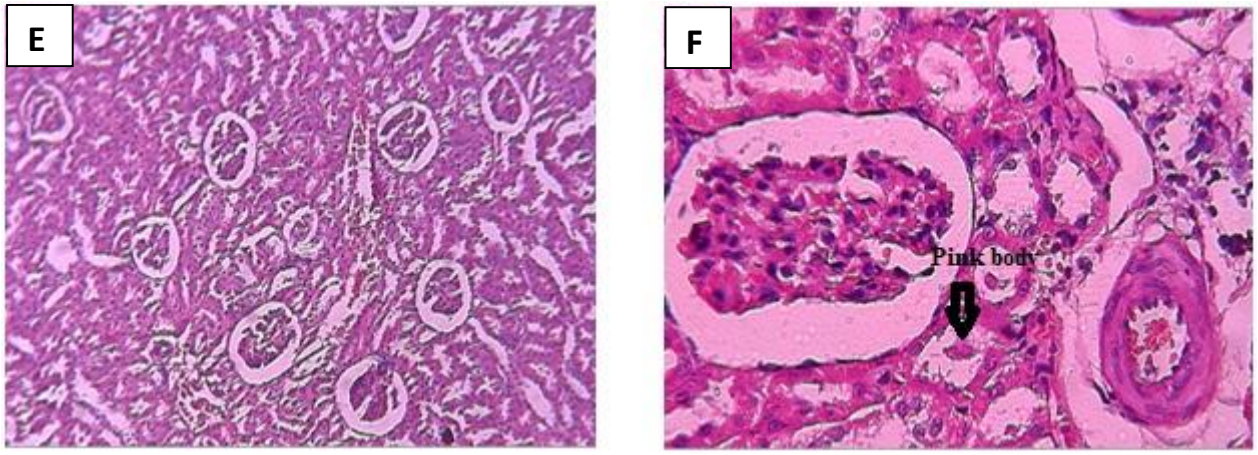
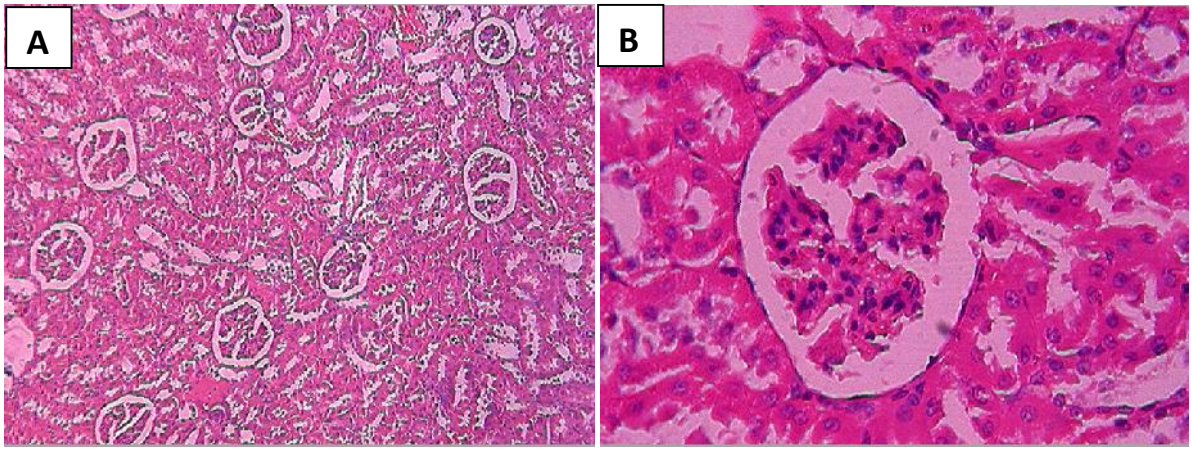


Figure 28 Plate showing histopathology of Kidney in low (labeled A,C&E) & high power(labeled B,D&F) for 1, 2 & 3 group

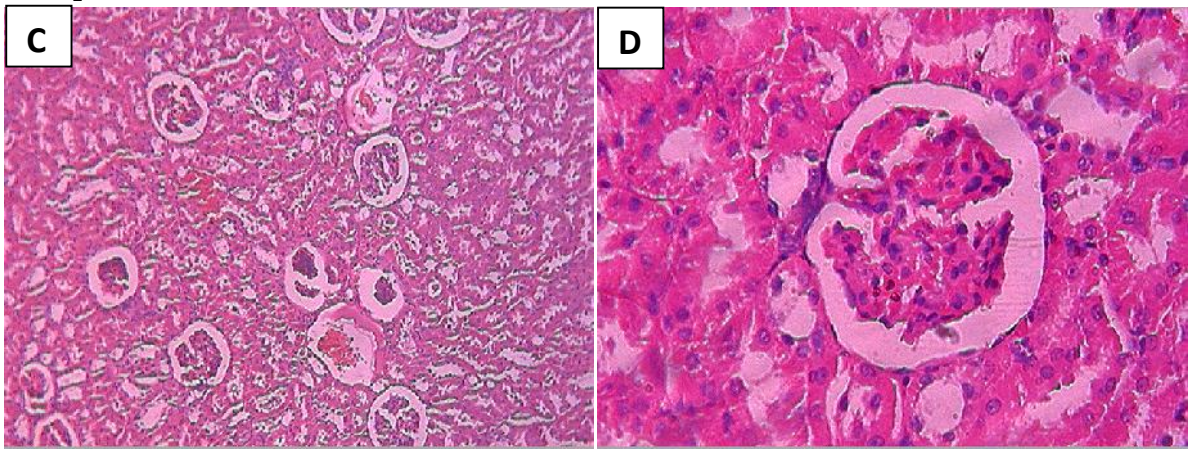
Histopathology of Kidney

Low power **High power**

Group 4



Group 5



Group 6

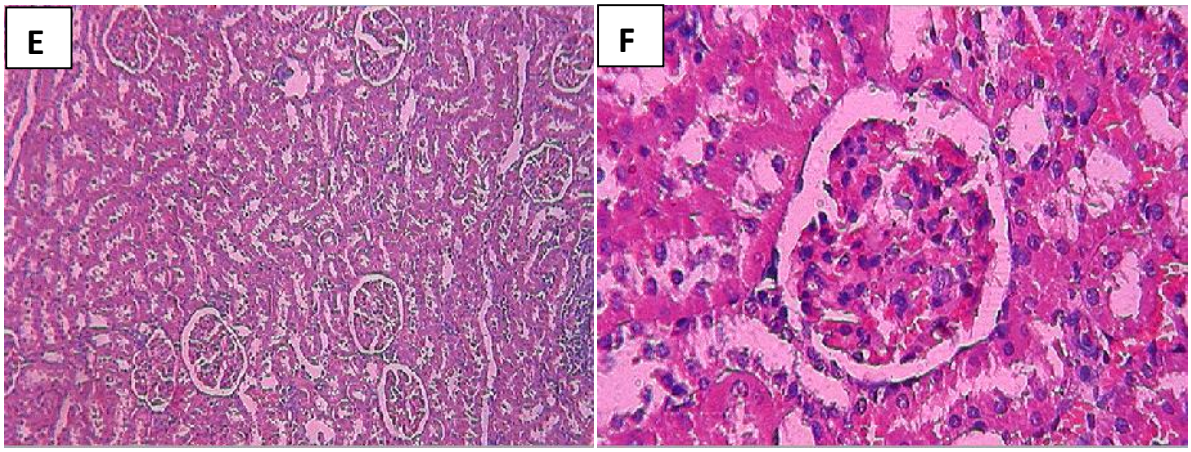


Figure 29 Plate showing histopathology of Kidney in low (labeled A, C&E) & high power(labeled B,D&F) for 4, 5 & 6 group

CHAPTER 6.1

REPRODUCTION

6.2. REPRODUCTION

1. Gravimetry & Organosomatic 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 21 Results of testiculosomatic Index

	Control Group	Stress Control	STD Group	Mucuna pruriens	Withania somnifera	MP+WS	Anova	p-value
Testiculosomatic Index	0.71±.06	0.86±.16	0.82±.10	0.81±.10	0.80±.11	0.80±.12	1.078	.392

Each value is Mean±SD of six observations in each group for testiculosomatic Index. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared with control, b-comparing with stress group & c-comparing with standard group.

2. Mating Behavior Test

All the rats in groups I, III, IV, V and VI showed healthy and active with increased aphrodisiac behavior. However, rats of group II acted as stress 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 & VI 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 22).

Table 22: Results of Mating behavior analysis

	Control Gp Mean±SD	Stress Control Mean±SD	STD Group Mean±SD	Mucuna pruriens Mean±SD	Withania somnifera Mean±SD	MP+WS Mean±SD	Anova	p-value
Mounting Frequency	53.67±9.29	48.17±2.04[c]	61.17±5.42[b]	58.5±8.69[b]	58.17±8.54[b]	58.5±3.83[b]	2.797	.034
Intromission frequency	5.83±1.47	5.17±1.17	7.83±2.14	9.5±2.74[b]	9.83±3.25[b]	8.17±2.79[b]	3.804	.009
Mounting latency (Sec)	523.5 ±54.13[b]	834± 101.79[a]	663.67± 123.38	728.83 ±132.09[a]	606.33± 85.06[b]	812.5± 120.48[a]	3.851	.008
Intromission latency (Sec)	592.5 ±30.11[b]	879.33 ±109.10[a]	708.83± 126.71	777.83± 130.59	712± 73.39	873.5± 99.5[a]	7.103	.000
Ejaculatory latency (Sec)	409.83 ±38.8[b]	230.83 ±52.75[ac]	445.17± 88.95[b]	464.33± 75.23[b]	612.17 ±95.07[abc]	397.33± 72.95[b]	16.910	.000
Number of mount	4±0.89	2.5±1.05	4.33±1.63	3.67±1.37	3.67±1.21	3±0.89	1.858	.132

Each value is Mean±SD of six observations in each group for mating behavior. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared with control, b-comparing with stress group & c-comparing with standard group.

3. Adverse effect & Pre-coital sexual behaviors

None of the rats showed the sign of toxicity (salivation, rhinorrhoea, lachrymation, ptosis, writhing, convulsions and tremors) and stress (erection of fur and exopthalmia) in the stress group rats some behavioral changes were noted (such as spontaneous movement in the cage, climbing and cleaning of face).

The test for pre-coital sexual behaviors such as chasing, nosing and anogenital sniffing, were well performed in the Group I, III, IV & V whereas in Groups II and VI the behaviors were not to the extent seen in Group I, III, IV & V. However, effect of Group I & IV showed less effect than Group III & V.

4. Stress Assay

Serum Cortisol, MDA, NO, Vitamin C & E

Levels of Cortisol were significantly increased in stress induced rats (group II & III) as compared to control rats and levels of Cortisol were significantly decreased in group IV, V & VI as compared to group I, II & III (Table 23, Fig 30A &B)

Levels of MDA were significantly increased in stress induced rats (group II) as compared to control and standard group rats. There was an significant decrease in MDA level was noticed in the remaining all group rats. Administration of both the extracts in treated rats showed a significant decrease in the levels of Cortisol & MDA contents, especially in group V Cortisol levels were very much close to the levels of control (group I) rats. Group III, IV, V& VI show better results in MDA levels which came back to the level of control groups (Table 23, Fig 30 C &D)

Concentration of Nitric oxide levels were significantly decreased in group II & increased in group III, IV, V &VI as compared to control rats (Table 23, Fig 30 E&F).

Concentration of vitamin C were significantly decreased in group II, IV & VI as compared to group I & significantly increased in group III & V as compared to group I & II. Concentration of vitamin E were significantly decreased in group II & increased in remaining all group (Table 23, Fig 31A, B, C &D).

.

Concentration in each group **Percentage of change**

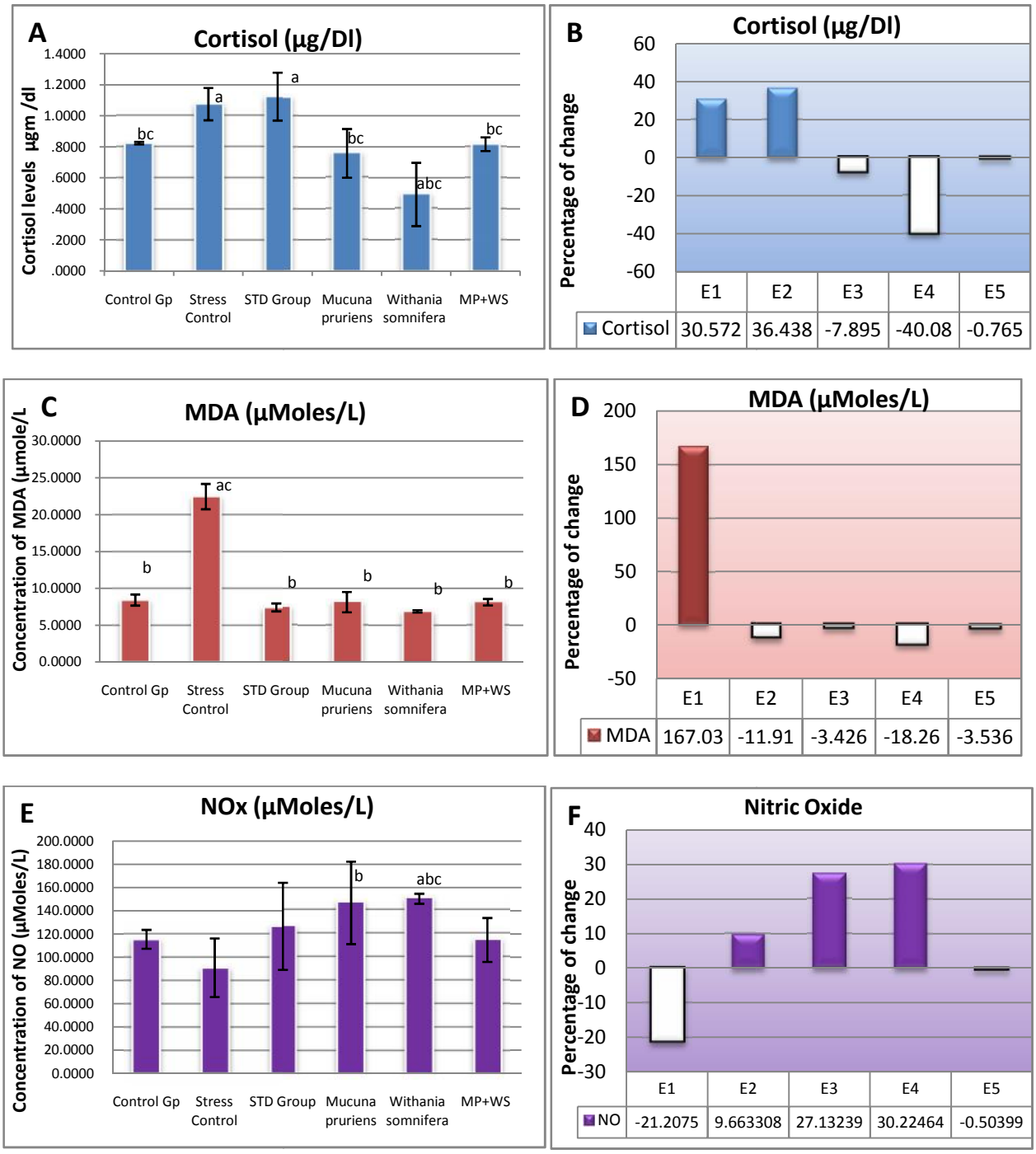


Figure 30 Plate showing result graph (Labeled A, C & E) and percent change (Labeled B, D & F) of Cortisol, MDA & Nitric oxide
Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared with control, b-comparing with stress group & c-comparing with standard group. E is effect, E1- % change in group 2, E2- % change in group 3, E3- % change in group 4, E4- % change in group 5 & E5- % change in group 6.

Concentration in each group **Percentage of change**

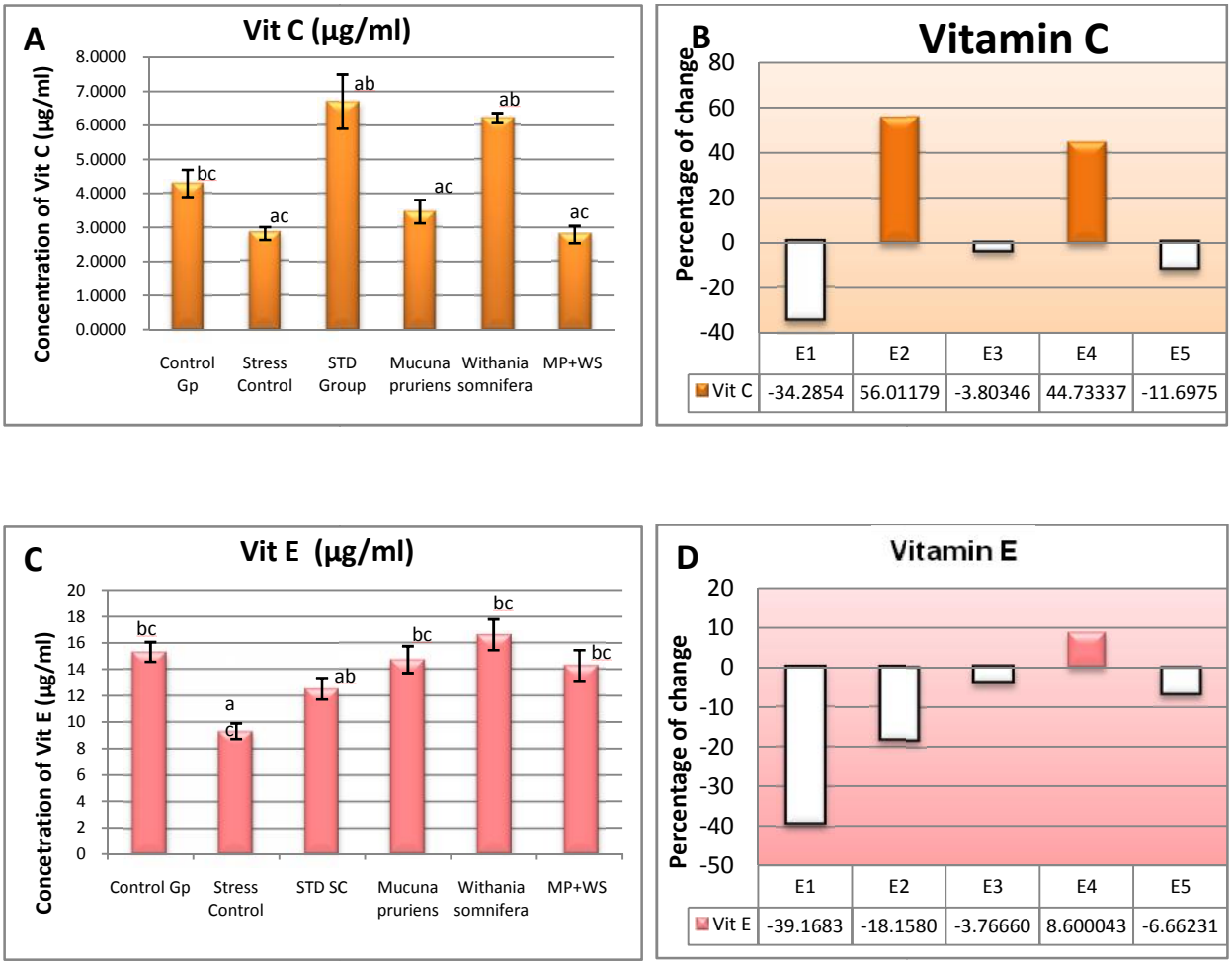


Figure 31 Plate showing result graph (Labeled A & C) and percent change (Labeled B & D) of Vit C & E

Table 23 Results of Stress parameters

	Control Gp Mean±SD	Stress Control Mean±SD	STD Group Mean±SD	Mucuna pruriens Mean±SD	Withania somnifera Mean±SD	MP+WS Mean±SD	Anova	p-value
Cortisol (µg/Dl)	0.823± 0.008^{bc}	1.075± 0.104^{a}	1.123± 0.154^{a}	0.758± 0.157^{bc}	0.493± 0.204^{abc}	0.817± 0.044^{bc}	18.990	.000
MDA (µMoles/L)	8.41± 0.75^{b}	22.45± 1.72^{ac}	7.41± 0.54^{b}	8.12± 1.38^{b}	6.87± 0.16^{b}	8.11± 0.44^{b}	219.363	.000
NO (µMoles/L)	115.56± 8.14	91.05± 25.18	126.72± 37.47	146.91± 35.55^{b}	150.48± 4.34^{abc}	114.97± 18.9	30.954	.000
Vit C (µg/ml)	4.29± 0.4^{bc}	2.82± 0.19^{ac}	6.7± 0.8^{ab}	4.131± 0.97^{ac}	6.215± 0.15^{ab}	3.792± 0.64^{ac}	101.352	.000
Vit E (µg/ml)	15.31± 0.75^{bc}	9.31± 0.6^{ac}	12.53± 0.82^{ab}	14.73± 1.02^{bc}	16.63± 1.17^{bc}	14.29± 1.16^{bc}	44.583	.000

Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared with control, b-comparing with stress group & c-comparing with standard group. E is Effect, E1- % change in group 2, E2- % change in group 3, E3- % change in group 4, E4- % change in group 5 & E5- % change in group 6.

5. 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 (Table 24 & Fig 32 A & B).

Results of LH in group II, III, IV & VI rats significantly increased when compared with group I and V rats. (Table 24 & Fig 32 C & D)

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 (Table 24 & Fig 32 E & F).

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 & VI was higher and in group IV was lower when compared to the standard reference drug group (Group III) (Table 24 & Fig 33 A & B).

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 & VI, which was statistically significant while comparing with group I & II (Table 24 & Fig 33 C, D, E & F).

Concentration in each group **Percentage of change**

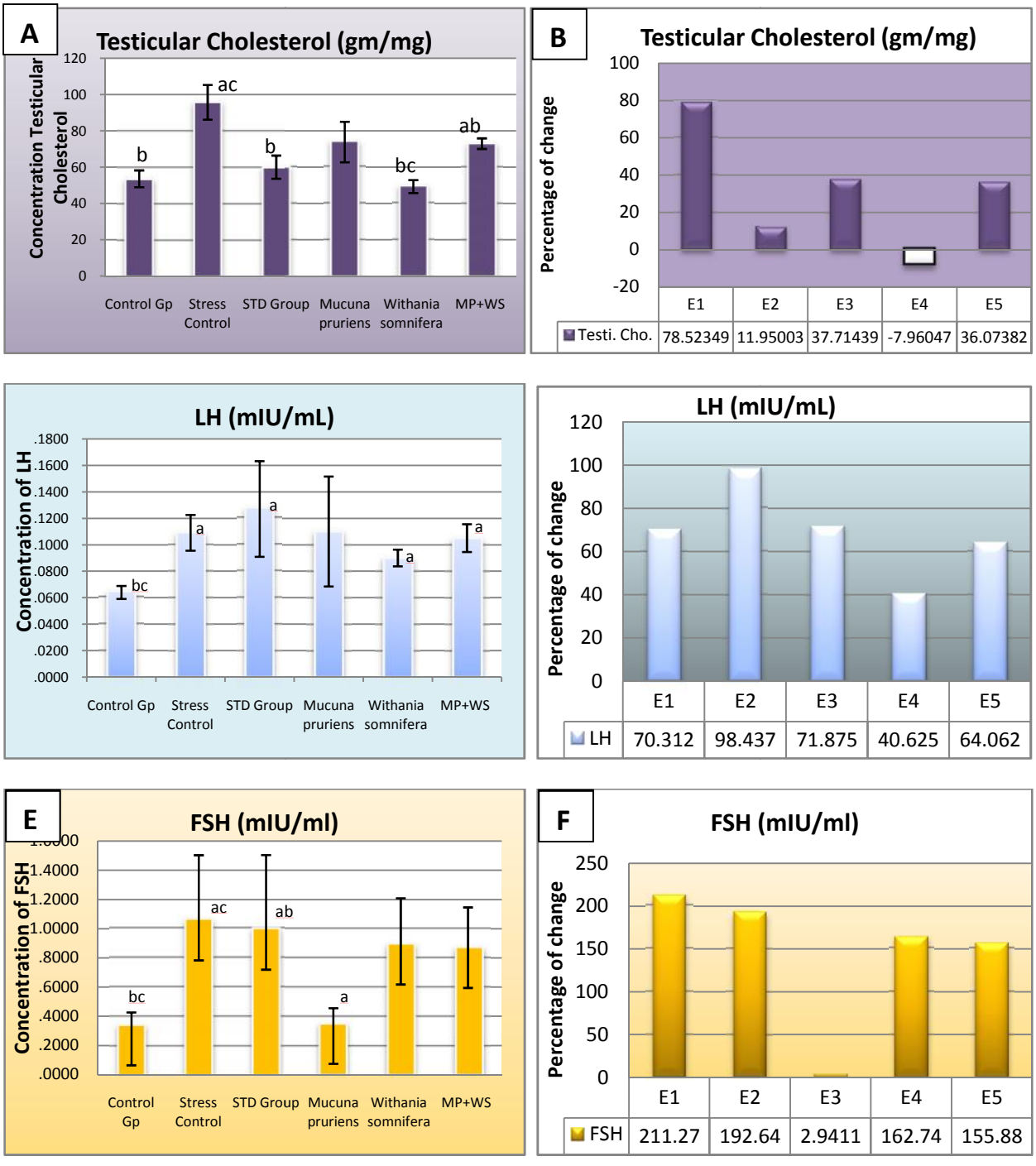


Figure 32 Plate showing result graph (Labeled A, C & E) and percent change (Labeled B, D & F) of Testicular cholesterol, LH & FSH

Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared with control, b-comparing with stress group & c-comparing with standard group. E1- % change in group 2, E2- % change in group 3, E3- % change in group 4, E4- % change in group 5 & E5- % change in group 6.

Concentration in each group **Percentage of change**

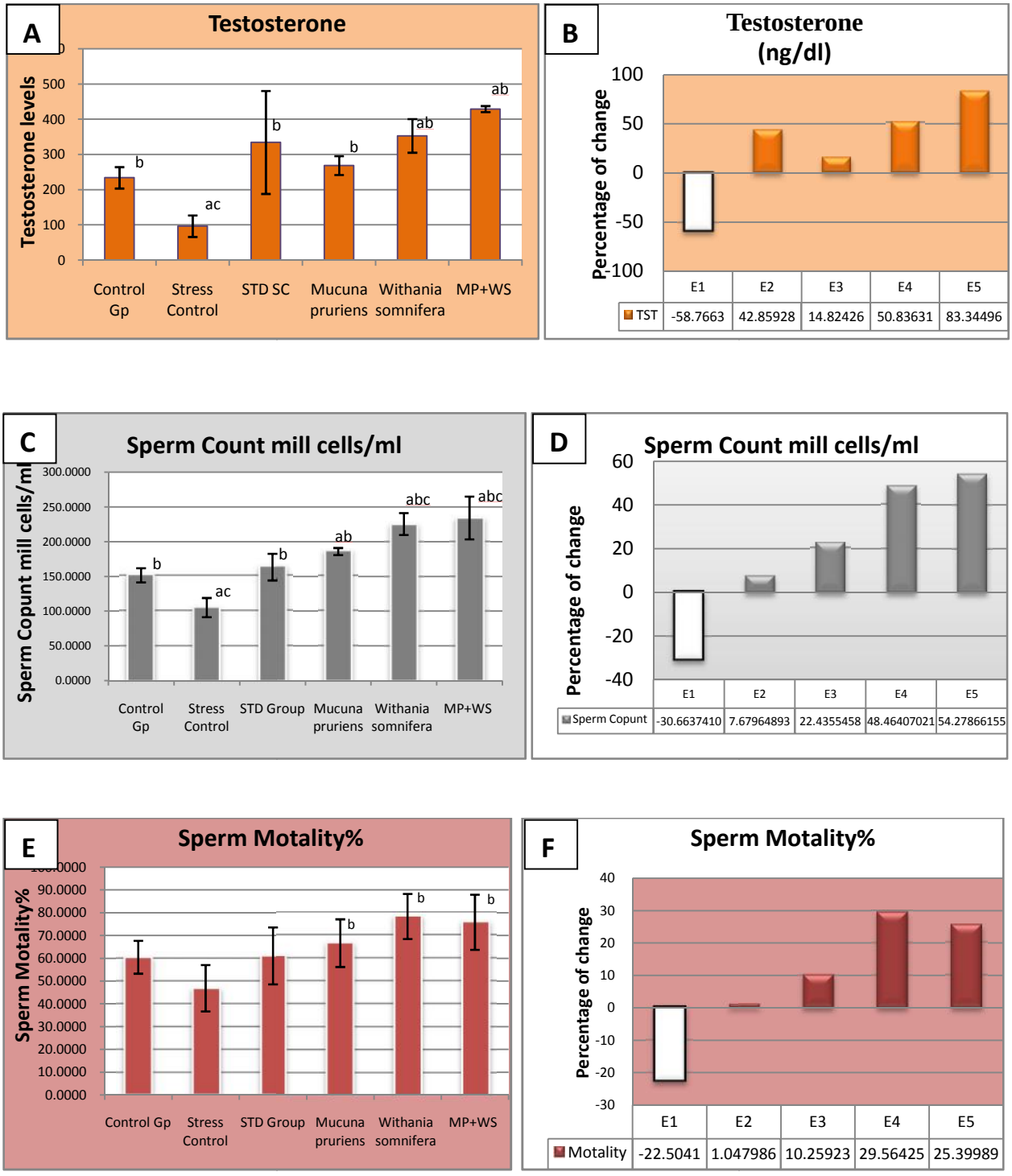


Figure 33 Plate showing result graph (Labeled A, C & E) and percent change (Labeled B, D & F) of Testosterone, Sperm count & Motility

Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared with control, b-comparing with stress group & c-comparing with standard group. E1- % change in group 2, E2- % change in group 3, E3- % change in group 4, E4- % change in group 5 & E5- % change in group 6.

Table 24 Results of Reproductive profile

	Control Gp Mean±SD	Stress Control Mean±SD	STD Group Mean±SD	Mucuna pruriens Mean±SD	Withania somnifera Mean±SD	MP+WS Mean±SD	Anova	p-value
Testicular Cholesterol (gm/mg)	53.64± 4.61[b]	95.76± 9.59[ac]	60.05± 6.4[b]	73.87± 11.15	49.37± 3.55[bc]	72.99± 2.92[ab]	34.745	.000
LH (mIU/mL)	0.064± 0.005[b]	0.109± 0.01[c]	0.127± 0.036[a]	0.11± 0.04	0.09± 0.006[a]	0.105± 0.0[a]	4.892	.002
FSH (mIU/ml)	0.34± 0.086[bc]	1.058± 0.445[ac]	0.995± 0.509[ab]	0.35± 0.105[a]	0.893± 0.316	0.87± 0.28	5.726	.001
Testosterone (ng/dL)	233.96± 30.4[b]	96.47± 30.4[ac]	334.24± 146.1[b]	268.65± 26.7[b]	352.90± 47.8[ab]	428.96± 8.7[ab]	18.135	.000
Sperm Copunt mill cells/ml	151.92± 10.2[b]	105.33± 13.8[ac]	163.58± 19.1[b]	186± 5.2[ab]	225.54± 15.7[abc]	234.38± 30.8[abc]	44.627	.000
Sperm Motality%	60.43± 7.19	46.83± 10.15	61.07± 12.5	66.63± 10.5[b]	78.3± 9.9[b]	75.78± 12.[b]	3.862	.007

Each value is Mean±SD of six observations in each group. In each row values with different superscripts (abc) were significantly different from each other (P<0.05), a-compared with control, b-comparing with stress group & c-comparing with standard group.

6. Histopathology of male reproductive organs

6.1. Histopathology of Testis

Group 1 Control Group: Section studied under H&E stain shows testicular parenchymal tissue whose architecture appears normal and is divided into lobules containing many seminiferous tubules. the tubules consist of several layers of spermatogenic cells (spermatogonia, spermatocytes, spermatids and mature spermatozoa) and sertoli (supportive) cells which are large, round, contain pale nuclei and prominent nucleoli, located next to the basement membrane. The fibrovascular stroma present between the seminiferous tubules contains the varying number of interstitial cells of Leydig. Spermatogenesis appears normal (Fig-34 A&B).

Group 2 Stress Group: Section studied under H&E stain shows testicular parenchymal tissue and is divided into lobules which contain many seminiferous tubules comprising of several layers of cells spermatogenic cells (spermatogonia, spermatocytes, spermatids and mature spermatozoa) and sertoli cells. Majority of the tubules show **focal atrophic and degenerative changes** and marked thickening of Tubular lining basement membrane. There are many foci of loss of spermatogenesis > 75% and loss of germ cell layer. The fibrovascular stroma present between the seminiferous tubules; shows marked decrease in number of interstitial cells of Leydig

and been replaced by many foci of congestion, edema, necrosis and interstitial fibrosis (Fig-34 C&D).

Group 3 Standard Group: Section studied under H&E stain shows testicular parenchymal tissue and is divided into lobules which contain many seminiferous tubules comprising of several layers of cells spermatogenic cells (spermatogonia, spermatocytes, spermatids and mature spermatozoa) and sertoli cells. Majority of the tubules show **focal atrophic and degenerative changes.** There are many foci of loss of spermatogenesis. The fibrovascular stroma present between the seminiferous tubules; shows marked decrease in number of interstitial cells of Leydig and been replaced by many foci of congestion, edema, necrosis and interstitial fibrosis (Fig-34 E&F).

Group 4 Study group treated with Mucuna pruriens: Section studied under H&E stain shows testicular parenchymal tissue whose architecture appears normal and is divided into lobules which contains many seminiferous tubules comprising of several layers of cells spermatogenic cells and sertoli cells. Foci of spermatogenesis >75% with adequate germ cell layer, The fibrovascular stroma present between the seminiferous tubules; shows mild decrease in number of interstitial cells of Leydig and been replaced by interstitial fibrosis (Fig-35 A&B).

Group 5 Study group treated with Withania somnifera: Section studied under H&E stain shows testicular parenchymal tissue whose architecture appears normal and is divided into lobules which contains many seminiferous tubules comprising of several layers of cells spermatogenic cells and sertoli cells. Foci of spermatogenesis > 75% with adequate germ cell layer, the fibrovascular stroma contains adequate number of interstitial cells of Leydig (Fig-35 C&D).

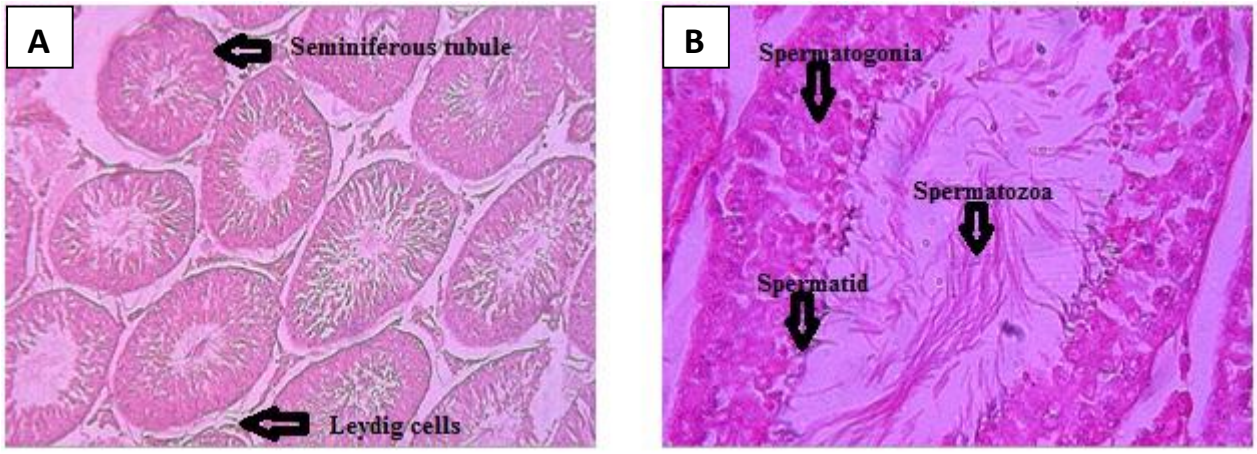
Group 6 Study group treated with Mucuna pruriens& Withania somnifera: Section studied under H&E stain shows testicular parenchymal tissue whose architecture appears normal and is divided into lobules which contains many seminiferous tubules comprising of several layers of cells spermatogenic cells and sertoli cells. Foci of spermatogenesis > 50% with adequate germ cell layer, The fibrovascular stroma present between the seminiferous tubules; shows mild

decrease in number of interstitial cells of Leydig and been replaced by interstitial fibrosis (Fig-35 E&F).

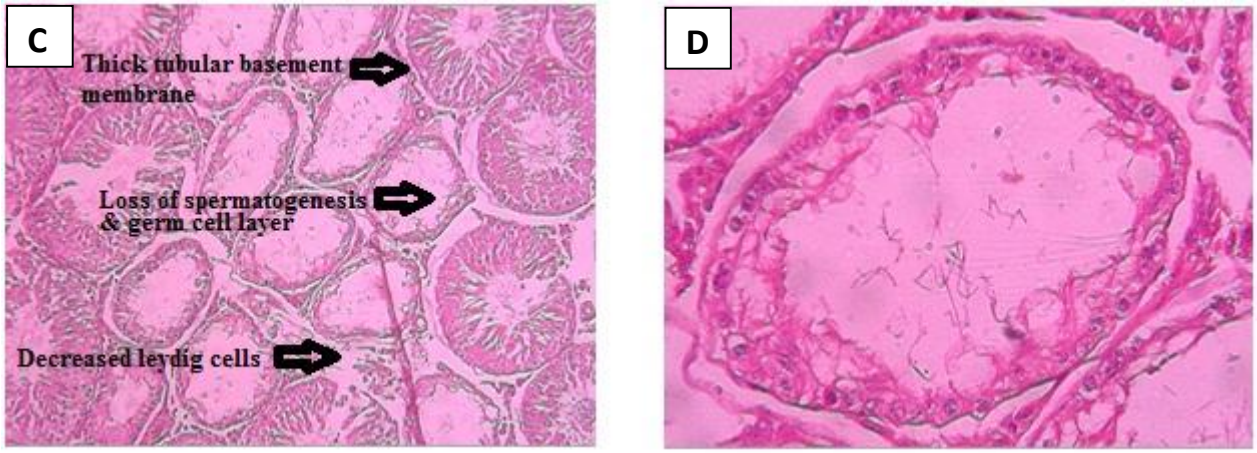
Histopathology of Testis

Low power **High power**

Group 1



Group 2



Group 3

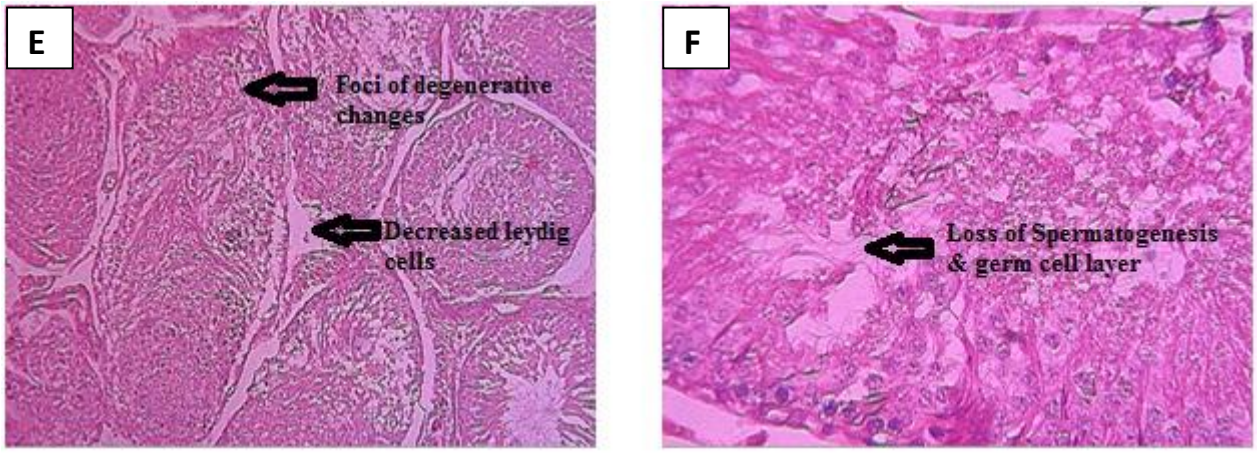
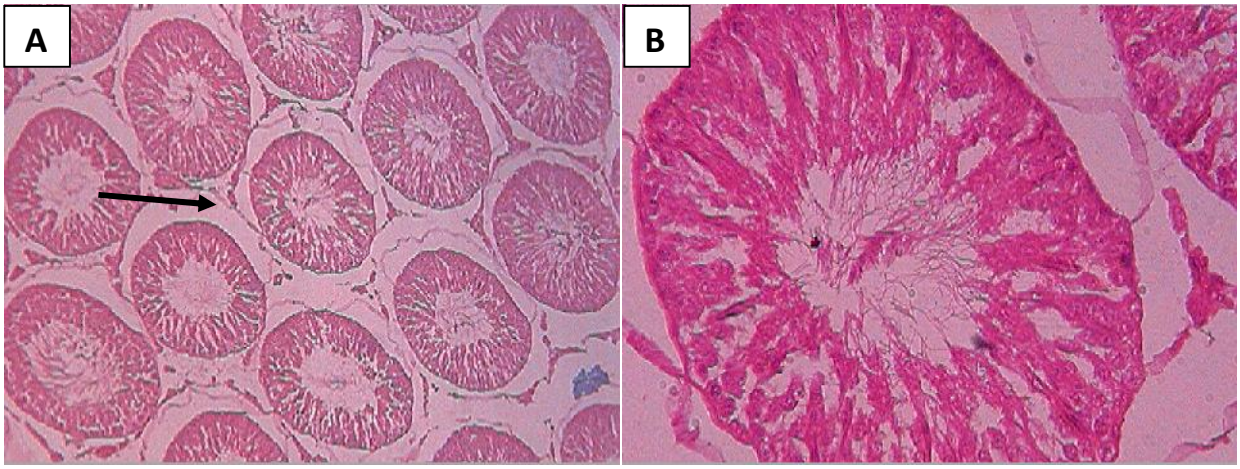


Figure 34 Plate showing histopathology of Testis in low (labeled A, C &E) & high power (labeled B,D&F) for 1, 2 & 3 group

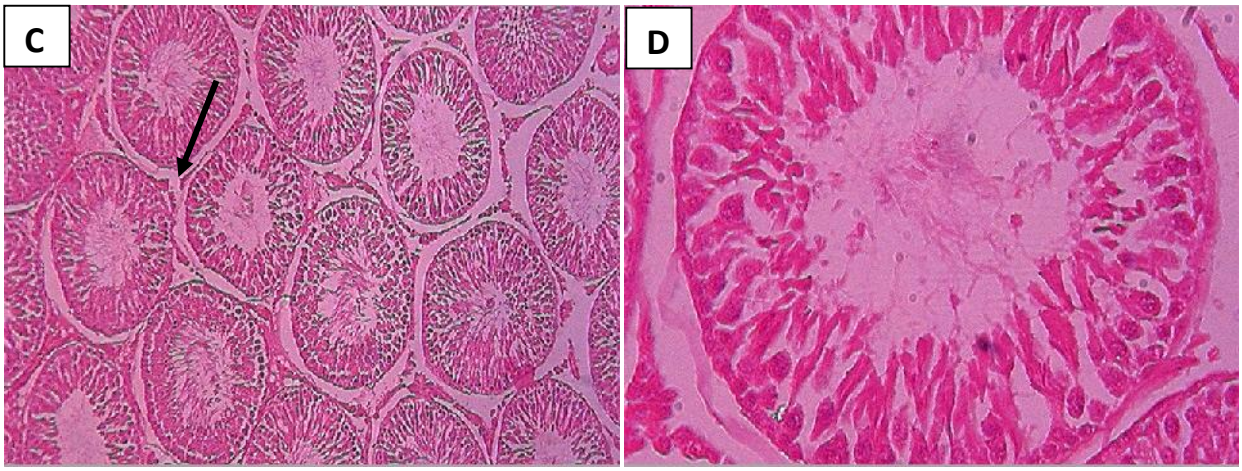
Histopathology of Testis

Low power **High power**

Group 4



Group 5



Group 6

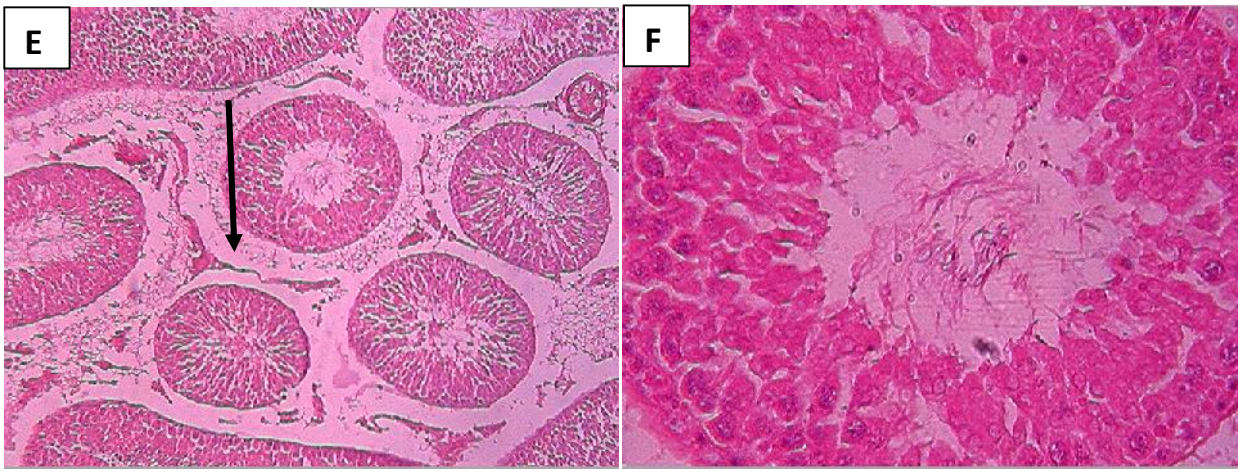


Figure 35 Plate showing histopathology of Testis in low (labeled A,C&E) & high power(labeled B,D&F) for 4, 5 & 6 group

6.2. Histopathology of Epididymis

Group 1 Control Group: Section studied under H&E stain shows normal epididymal tissue which is formed by continuations of efferent ductules and duct of epididymis which is highly convoluted and coiled on itself. Clump of spermatozoa are seen in the lumen (Fig-36 A&B).

Group 2 Stress Group: Section studied under H&E stain shows normal epididymal tissue which is formed by continuations of efferent ductules and duct of epididymis. There are features of focal tubular **dilatation, stratification, tortousity and atrophy** with decreased spermatogenesis. The fibrovascular stroma present between the efferent ductules shows foci of edema, fibrosis and acute inflammatory infiltration (Fig-36 C&D).

Group 3 Standard Group: Section studied under H&E stain shows normal epididymal tissue which is formed by continuations of efferent ductules and duct of epididymis. There are features of focal tubular **dilatation and stratification**. The fibrovascular stroma present between the efferent ductules shows foci of acute inflammatory infiltration (Fig-36 E&F).

Group 4 Study group treated with Mucuna pruriens: Section studied under H&E stain shows normal epididymal tissue which is formed by continuations of efferent ductules and duct of epididymis. There are features of focal tubular **dilatation** (Fig-37 A&B).

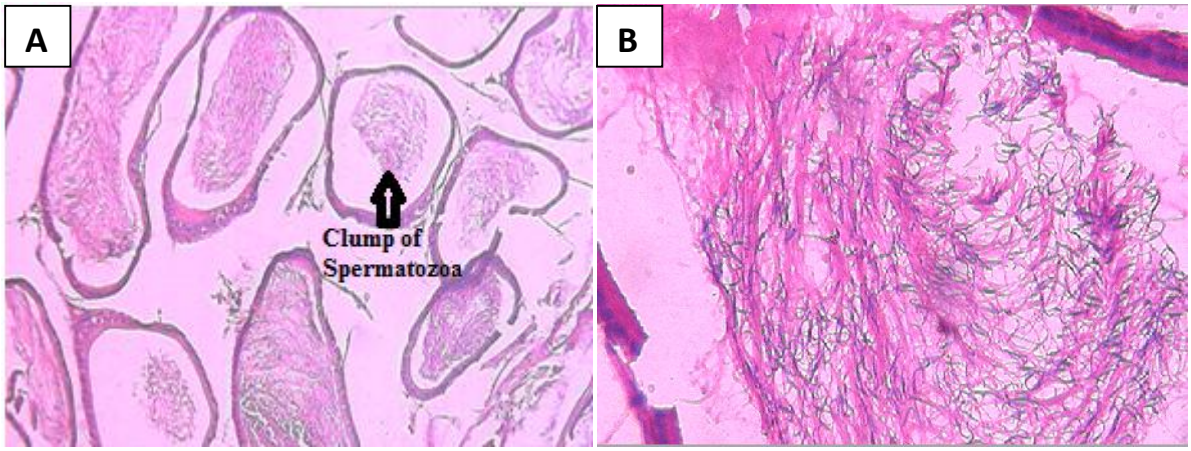
Group 5 Study group treated with Withania somnifera: Section studied under H&E stain shows normal epididymal tissue which is formed by continuations of efferent ductules and duct of epididymis. There are features of focal tubular **dilatation** (Fig-37 C&D).

Group 6 Study group treated with Mucuna pruriens& Withania somnifera: Section studied under H&E stain shows normal epididymal tissue which is formed by continuations of efferent ductules and duct of epididymis. There are features of focal tubular **dilatation** (Fig-37 E&F).

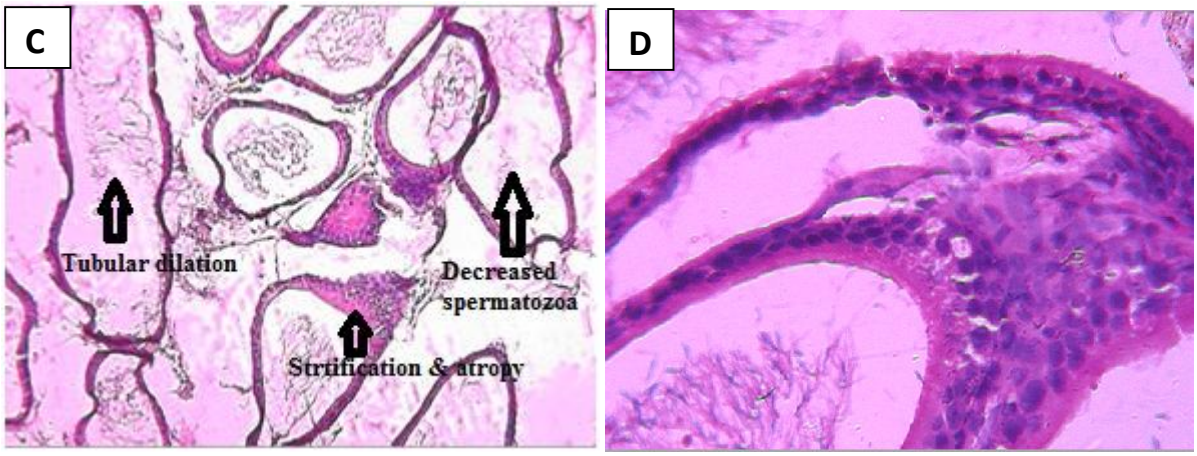
Histopathology of Epididymis

Low power **High power**

Group 1



Group 2



Group 3

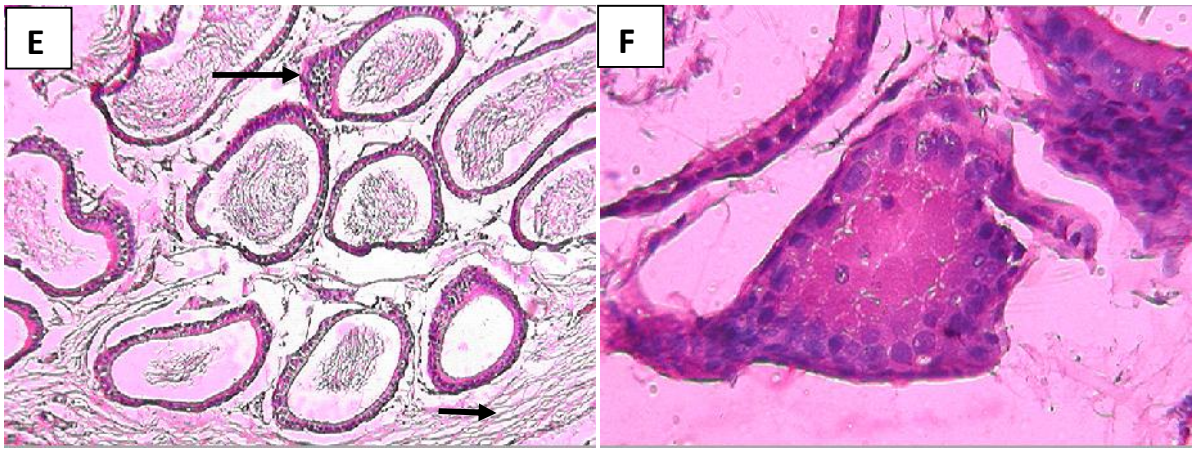
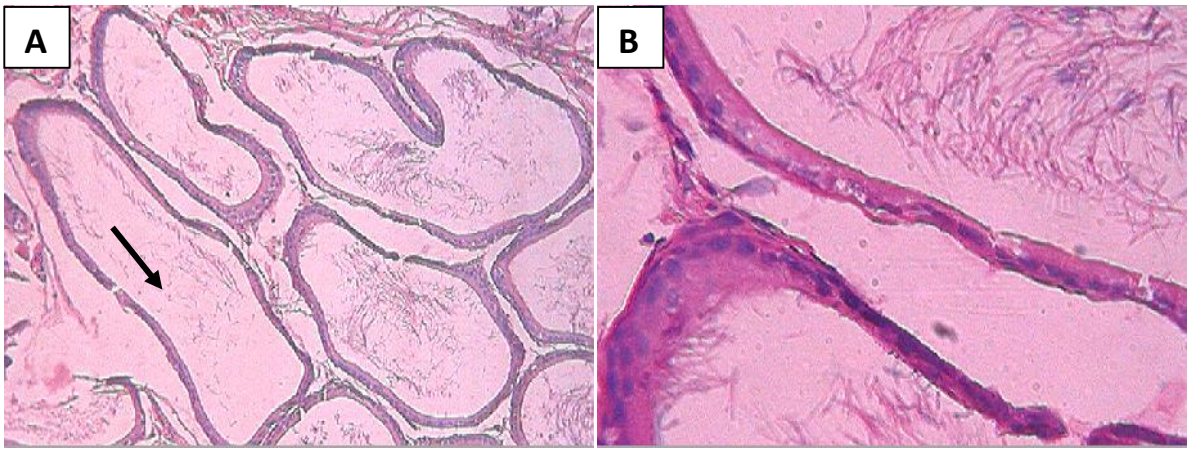


Figure 36 Plate showing histopathology of Epididymis in low (labeled A,C&E) & high power(labeled B,D&F) for 1, 2 & 3 group

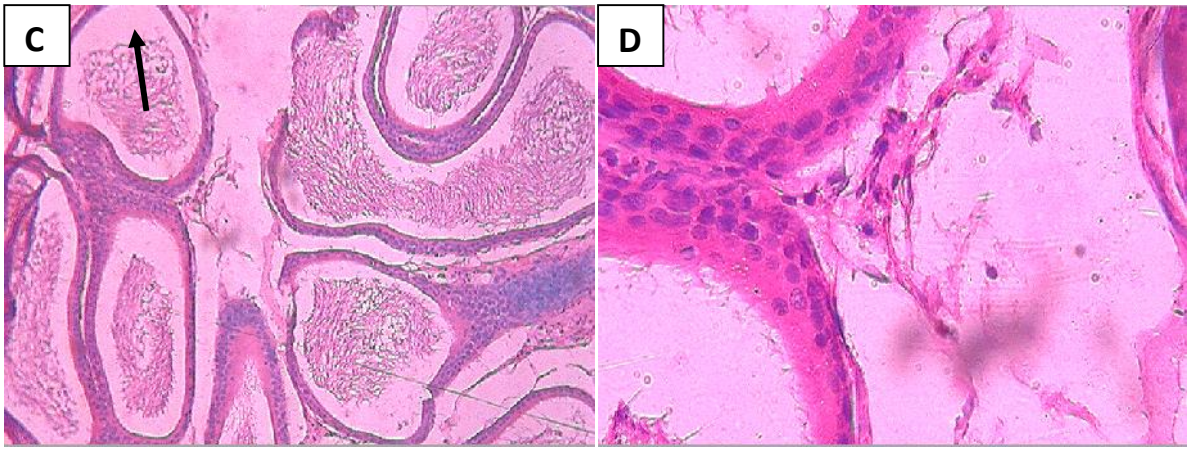
Histopathology of Epididymis

Low power **High power**

Group 4



Group 5



Group 6

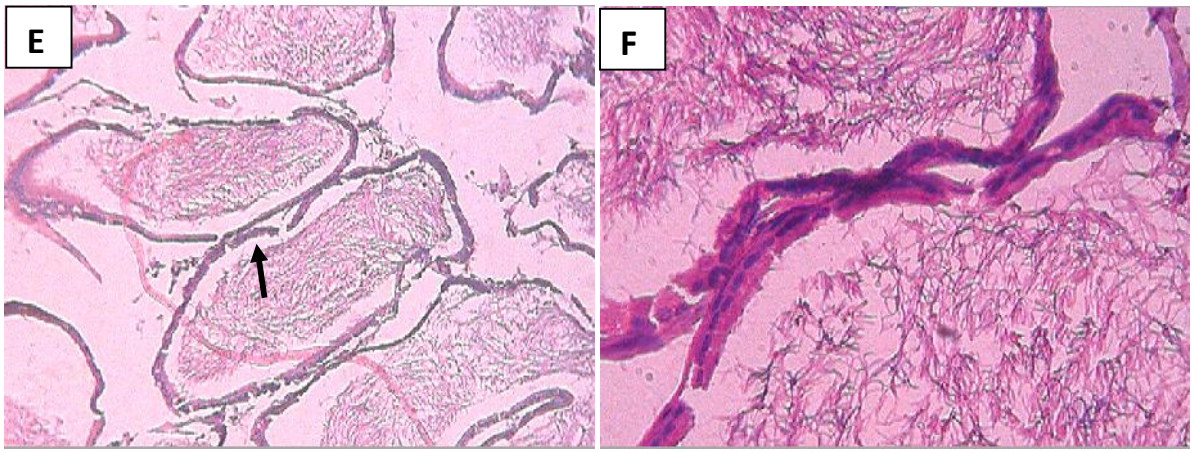


Figure 37 Plate showing histopathology of Epididymis in low (labeled A,C&E) & high power(labeled B,D&F) for 4, 5 & 6 group

6.3. Histopathology of Prostate

Group 1 Control Group: Section studied under H&E stain shows prostate which is composed of tubular alveoli (acini) embedded in fibromuscular tissue mass. The glandular epithelium forms infoldings and consists of two layers – a basal layer of low cuboidal cells and an inner layer of mucous secreting tall columnar cells. The alveoli are separated by thick fibromuscular septa containing abundant smooth muscle fibres. Lumen of the follicle contains amyloid bodies (Fig-38 A&B).

Group 2 Stress Group: Section studied under H&E stain shows prostate which is composed of tubular alveoli embedded in fibromuscular tissue mass. The glandular epithelium forms infoldings**.** Tubular focal hyperplasia. Few acini are dilated and filled with exudates material. The fibromuscular septa are edematous and show diffuse acute inflammatory infiltrate (Fig-38 C&D).

Group 3 Standard Group: Section studied under H&E stain shows prostate which is composed of tubular alveoli embedded in fibromuscular tissue mass. The glandular epithelium forms infoldings. Tubular focal hyperplasia. Few acini are dilated and filled with exudates material. The fibromuscular septa are edematous and show diffuse acute inflammatory infiltrate (Fig-38 E&F).

Group 4 Study group treated with Mucuna pruriens: Section studied under H&E stain shows prostate which is composed of tubular alveoli embedded in fibromuscular tissue mass. The glandular epithelium forms infoldings. The alveoli are separated by thick fibromuscular septa containing abundant smooth muscle fibres (Fig-39 A&B).

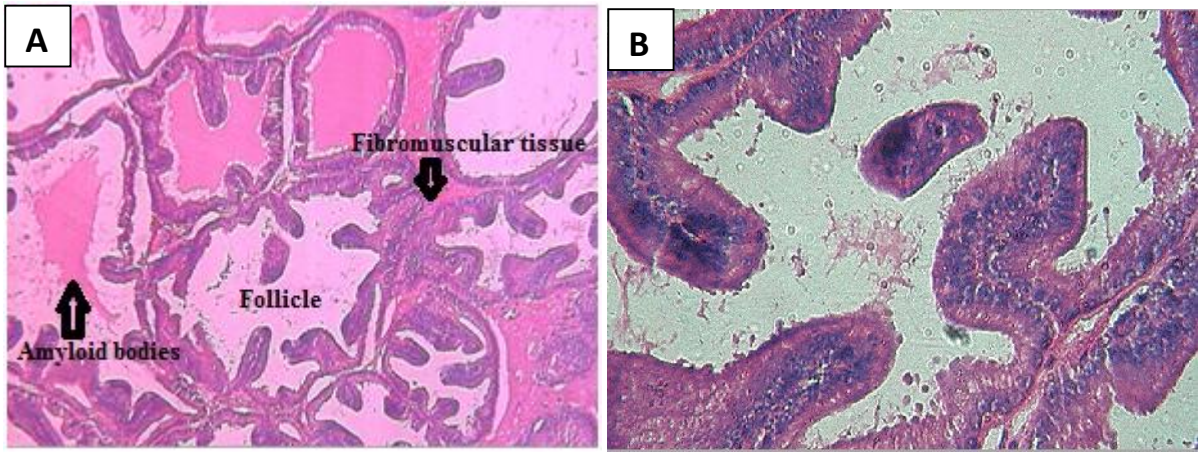
Group 5 Study group treated with Withania somnifera: Section studied under H&E stain shows prostate which is composed of tubular alveoli embedded in fibromuscular tissue mass. The glandular epithelium forms infoldings. The fibromuscular septum is edematous and shows diffuse acute inflammatory infiltrate (Fig-39 C&D).

Group 6 Study group treated with Mucuna pruriens& Withania somnifera: Section studied under H&E stain shows prostate which is composed of tubular alveoli embedded in fibromuscular tissue mass. The glandular epithelium forms infoldings. The fibromuscular septum is edematous and shows diffuse acute inflammatory infiltrate (Fig-39 E&F).

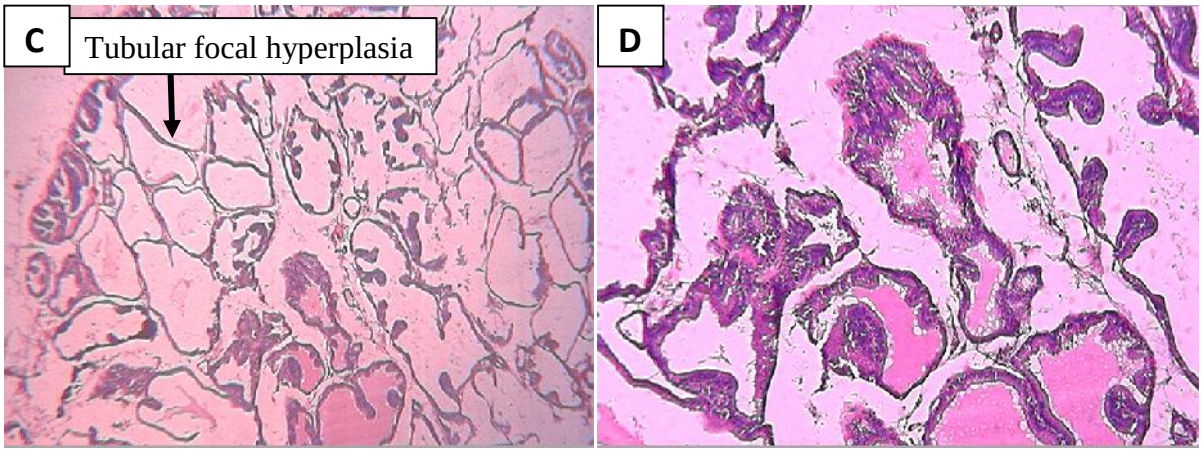
Histopathology of Prostate

Low power **High power**

Group 1



Group 2



Group 3

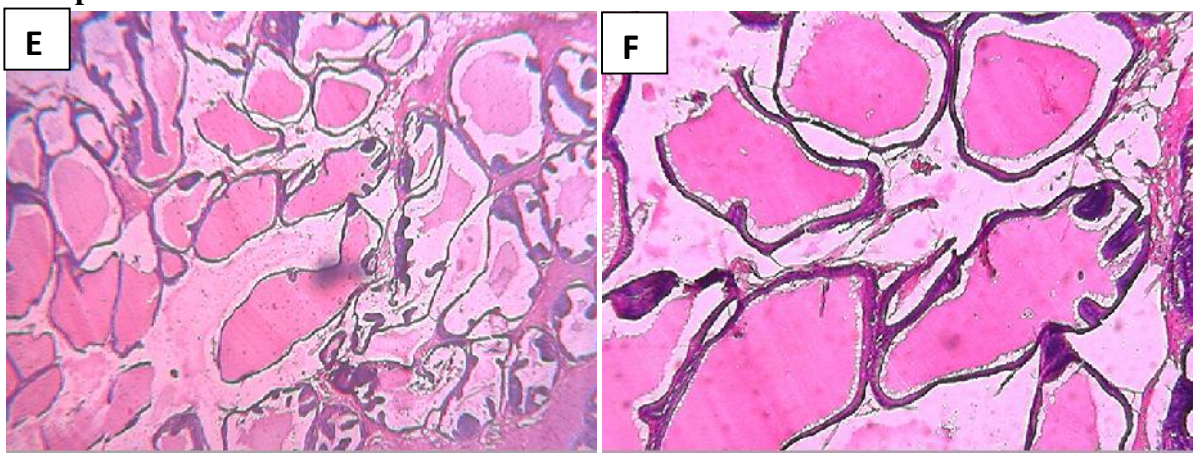
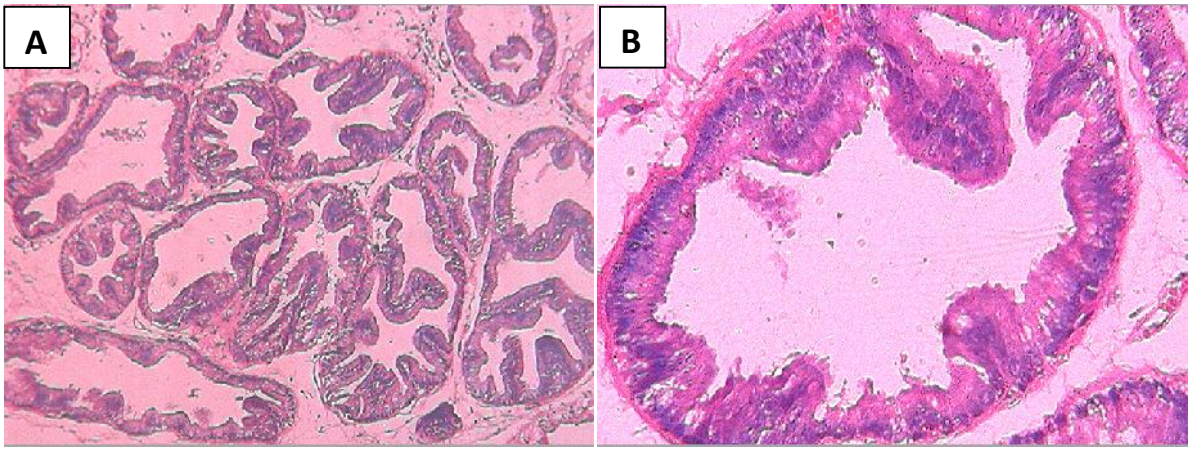


Figure 38 Plate showing histopathology of Prostate in low (labeled A,C&E) & high power(labeled B,D&F) for 1, 2 & 3 group

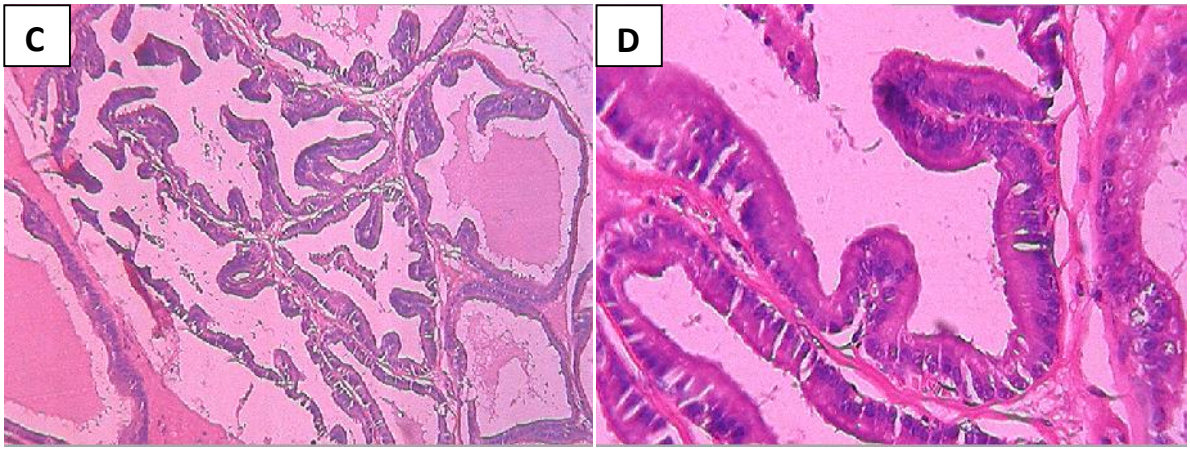
Histopathology of Prostate

Low power **High power**

Group 4



Group 5



Group 6

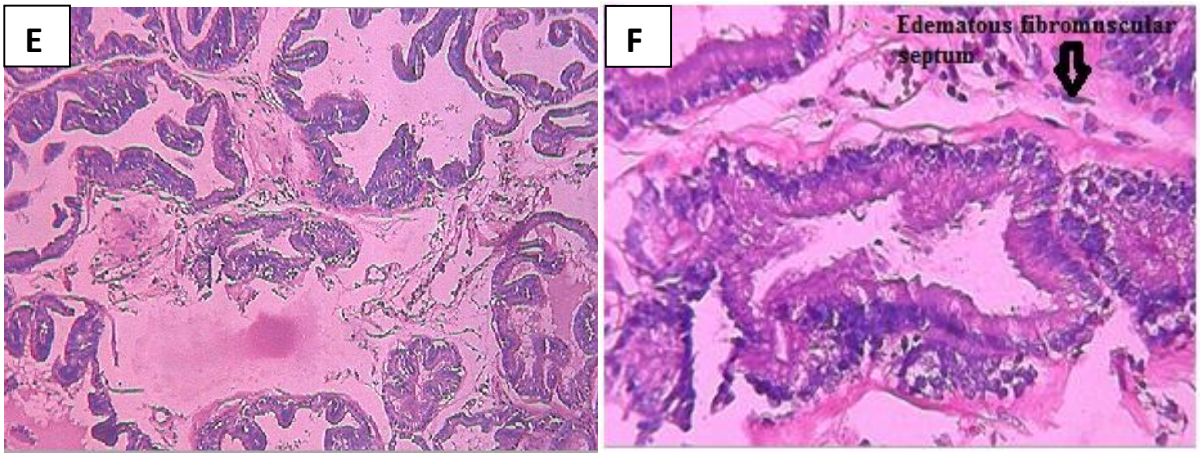


Figure 39 Plate showing histopathology of Prostate in low (labeled A,C&E) & high power(labeled B,D&F) for 4, 5 & 6 group

6.4. Histopathology of Seminal vesicle

Group 1 Control Group: Section studied under H&E stain shows seminal vesicles with convoluted tubule consists of mucosa, laminar propria; and a thick muscular wall. The lumen of the glands is irregular and contains folding. The mucousa is thin, has branching anastomosing folds and is lined by pseudo-stratified columnar epithelium. The muscular layer is consisting of an inner circular and outer longitudinal layer of smooth muscle (Fig-40 A&B).

Group 2 Stress Group: Section studied under H&E stain shows seminal vesicles consisting of mucosa, laminar propria; and a thick muscular wall. The lumen of the glands is irregular and contains folding. The lamina propria is loose, edematous and shows infiltration of mixed inflammatory cells. Foci of mucousal degeneration and ulceration (Fig-40 C&D).

Group 3 Standard Group: Section studied under H&E stain shows seminal vesicles with convoluted tubule consists of mucosa, laminar propria; and a thick muscular wall. The lamina propria is edematous and shows infiltration of mixed inflammatory cells. Foci of mucosal degeneration (Fig-40 E&F).

Group 4 Study group treated with Mucuna pruriens: Section studied under H&E stain shows seminal vesicles with convoluted tubule consists of mucosa, laminar propria; and a thick muscular wall (Fig-41 A&B).

Group 5 Study group treated with Withania somnifera: Section studied under H&E stain shows seminal vesicles with convoluted tubule consists of mucosa, laminar propria; and a thick muscular wall (Fig-41 C&D).

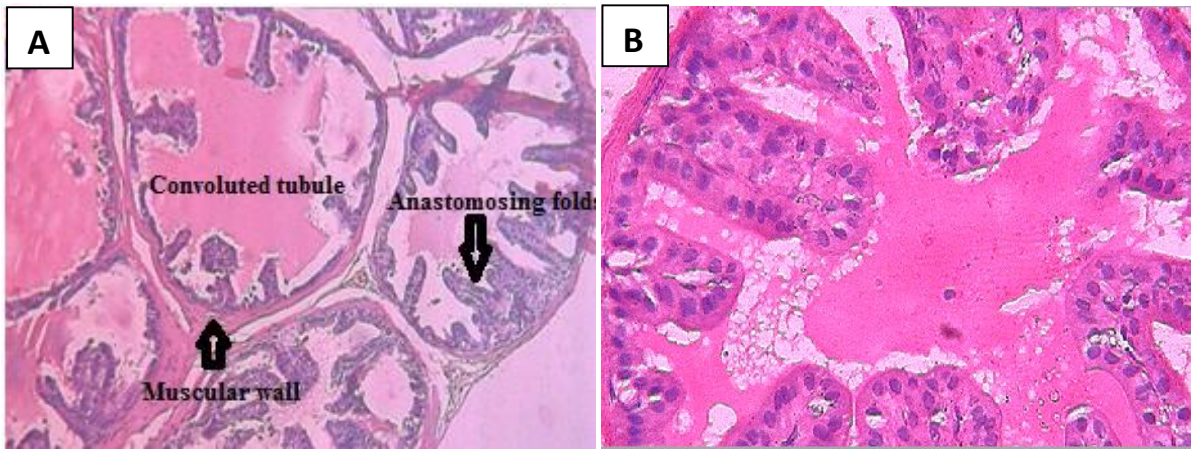
Group 6 Study group treated with Mucuna pruriens& Withania somnifera: Section studied under H&E stain shows seminal vesicles with convoluted tubule consists of mucosa, laminar propria; and a thick muscular wall. The lamina propria is loose and shows infiltration of mixed inflammatory cells (Fig-41 E&F).

.

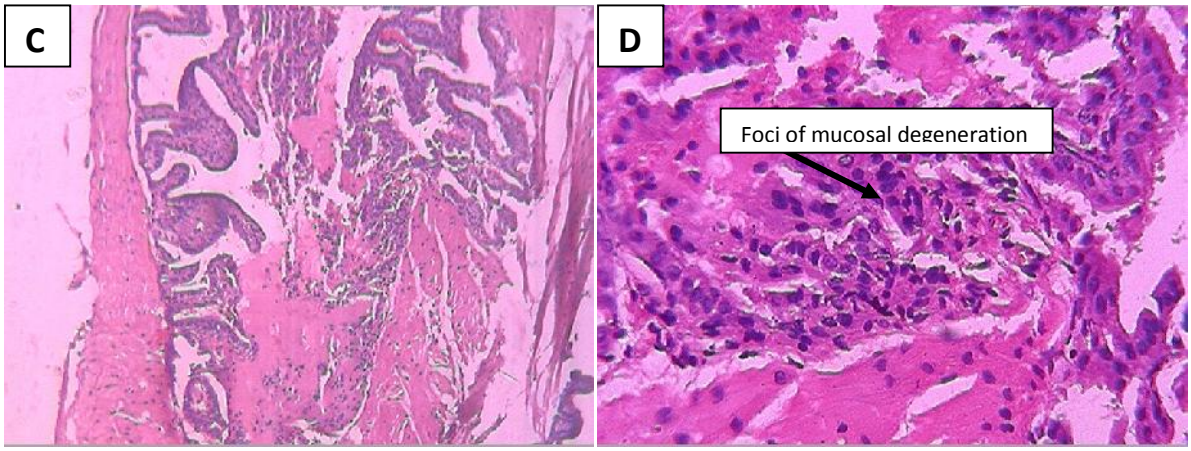
Histopathology of Seminal vesicle

Low power **High power**

Group 1



Group 2



Group 3

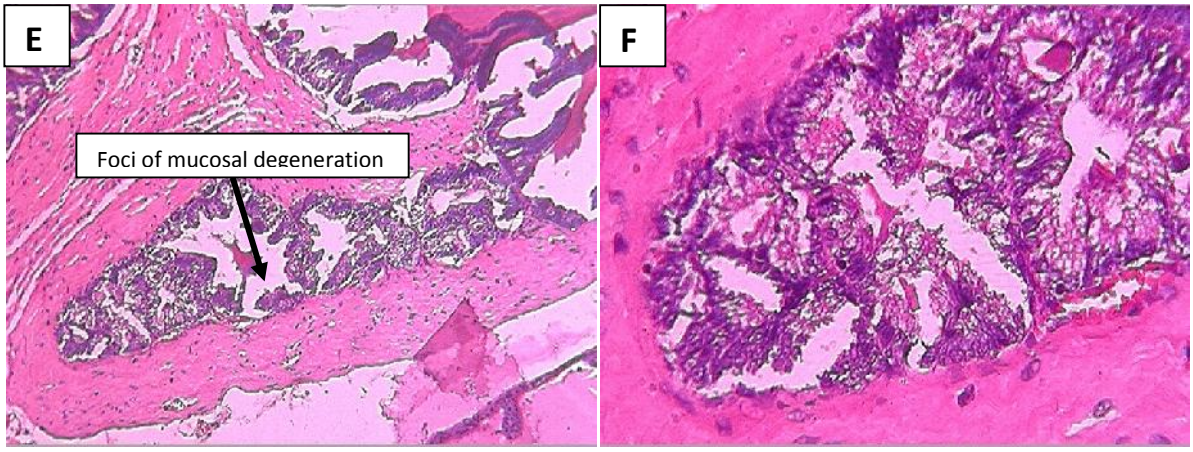
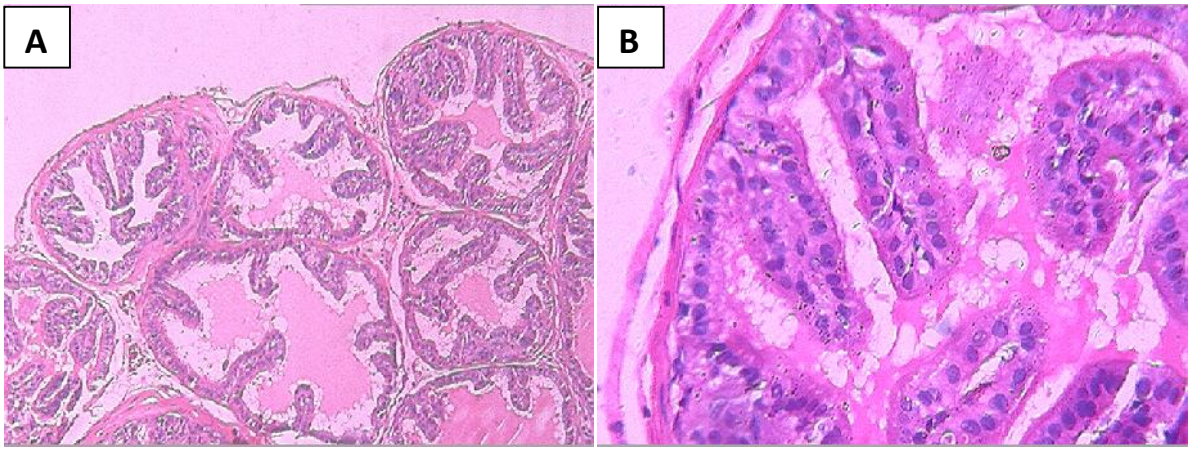


Figure 40 Plate showing histopathology of Seminal vesicle in low (labeled A,C&E) & high power(labeled B,D&F) for 1, 2 & 3 group

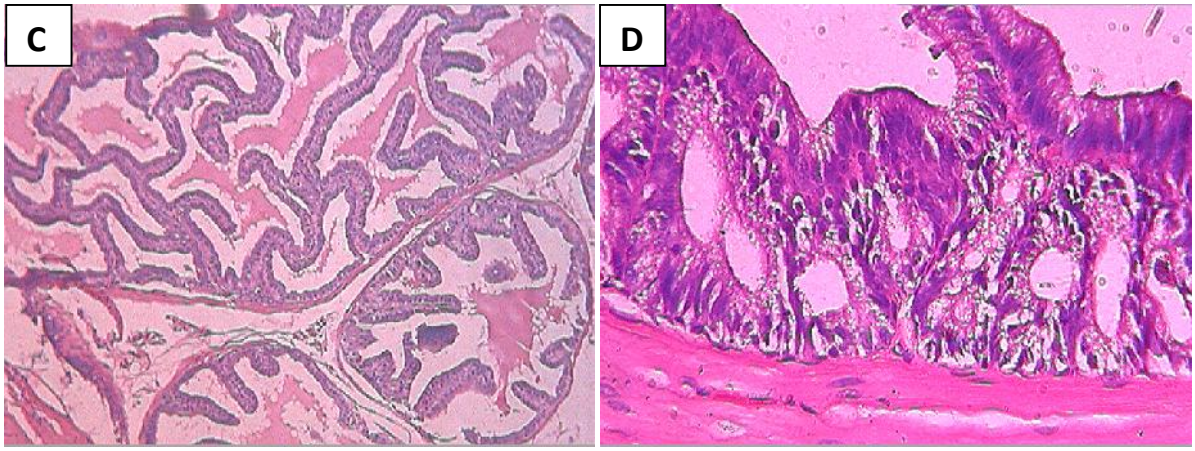
Histopathology of Seminal vesicle

Low power **High power**

Group 4



Group 5



Group 6

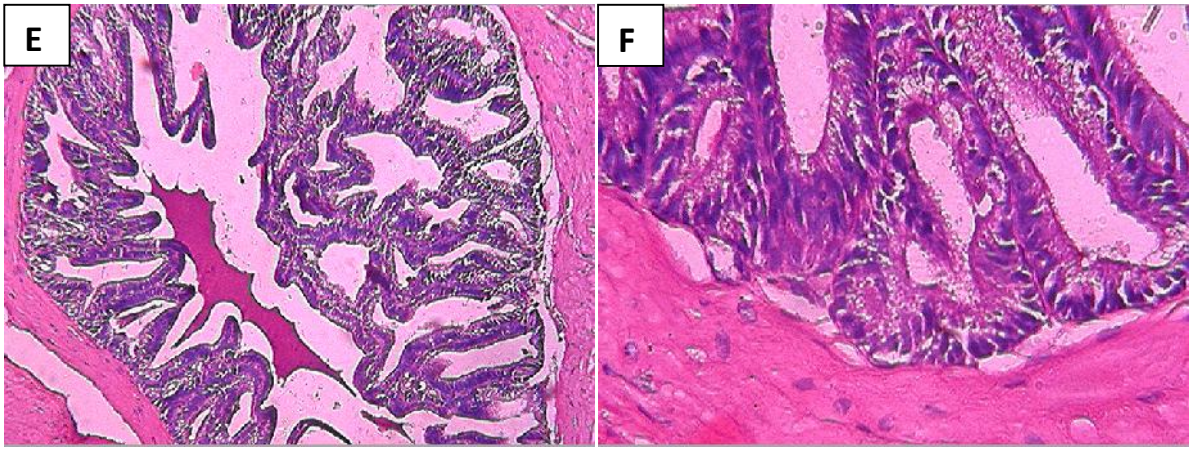


Figure 41 Plate showing histopathology of Seminal vesicle in low (labeled A, C&E) & high power (labeled B,D&F) for 4, 5 & 6 group

CHAPTER 7

DISCUSSION

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

2015

BLDE UNIVERSITY
SHRI B M PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

7.1.1. GENERAL

7.1.2. Phytochemistry

Phytochemical estimation indicate that M. pruriens seed extract and W. somnifera root extract contains significant amount of phenolic, flavonoids, carbohydrate, tannin and alkaloid content (Table 16) which confirms its antioxidant property. Phenolics content are very important plant constituents because they can act as reducing agents, hydrogen donors and metal chelator[1]. They also act as radical scavenger due to their hydroxyl groups. Flavonoids show their antioxidant action through scavenging or chelating process[2]. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity[3]. Also, the carbohydrates in food are of major interest in relation to chronic diseases. Different types of carbohydrates give rise to different glycaemic responses, and also able to stimulate lipogenesis[4&5]. Moreover, in the medicinal effects described in the ayurvedic, siddha, folk and chinese traditional recipe tannins, phenolic acids, flavonoids and alkaloids are the important ingredients to prevent against oxidative stress and decrease the activity of cholinesterase and xanthine oxidase and also alleviating the mucus secretion in the airway glands[6]. The results acquired in this study thus suggest the identified phytochemical compounds may be the bioactive constituents and this plant is proving to be a valuable reservoir of bioactive compounds.

7.1.3. Gravimetry

Result shows least percentage of body weight gain in case of stress (group II) rats. It clearly indicates stress might be interfering metabolic pattern of experimental animals. A significant improvement of percentage of body weight gain in case of

Mucuna pruriens, *Withania somnifera* and combined doses after stress treated rat indicates a beneficial role of both the extracts to overcome from the adverse effect of stress. Probably stress induced adrenergic hyperactivity of liver function may alter the glucose homeostasis which will decrease the activity of hunger centre and decrease in the food intake which intern body weight will be decreased[7].

7.1.4. Hematological assay

Changes in hematology picture reveal that no such specific alteration either in stress or *Mucuna pruriens & Withania somnifera* group, change in the levels of hemoglobin, RBC & WBC in group IV, V & VI may have indication that both the extracts helpful on the general health[8]. An effect on blood hemostatic mechanisms is assessed by determination of platelet count. Decreased production or increased consumption of platelets may lead to fall of platelet count.

7.1.5. Biochemical assay

The primary function of the liver is to maintain body homeostasis, besides it plays a key role in metabolism, detoxification, and inflammatory response[9]. The liver is a versatile organ in the body concerned with regulation of internal chemical environment. Therefore, damage to the liver inflicted by a hepatotoxic agent is of grave consequence[10]. It is known that many toxic compounds accumulate in the liver where they are detoxified[11]. Liver transaminases such as AST (aspartate transaminase) or SGOT (serum glutamic oxaloacetate transaminase), and ALT (alanine transaminase) or SGPT (serum glutamic pyruvate transaminase) have still remained the gold standards for the assessment of liver injury, and have been used as biomarkers of choice for decades (Table 19).

Results from the liver function test & kidney function test indicates both the phytochemicals are safe in consumption and do not have any adverse impact on liver

and kidney by keeping SGOT & SGPT remain unchange. Results clearly indicated that there was no much effect on the liver with any of the groups. Even though there was slight increase in the urea levels in group IV simultaneous increase in creatinine was not shown, which again indicate that there was no much adverse effect on the kidney also.

Our observation on lipid profile reveal dislipidemia in stress group (II) rats, possibly stress induced alteration of glucose metabolism like gluconeogenesis or impaired insulin sensitivity may be the cause of such changes[12&13]. Treatment with both the phytochemicals induces beneficial effect on altered lipid profile may be due to their antioxidant properties (Table 19).

Treatment with Withania somnifera was found to be effective to reduce stress induced dislipidemia apparently but no such beneficial effect was noticed in case of Mucuna pruriens. Our results corroborated with findings of Visavadya & Narasinhcharya[14]**.** Perhaps Withania somnifera inhibit HMG-CoA reductase activity or increases cholesterol absorption or increase lecithin cholesterol acyl trasferase activity in hepatocellular microenvironment. But Mucuna pruriens might have not able to perform such activity in stress induced alteration in dislipidemia. In case of lipid profile both the extracts are no additional beneficial effect on regulating lipid profile alteration induced by stress[15].

7.1.6. Stress in Liver & Kidney

Nitric oxide (NO) is a gaseous signaling molecule and effecter in various biological processes produced by NO synthases (NOS). Three NOS isoforms have been identified as: neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS)[16]. It has recently been appreciated that altered function of endothelial nitric oxide synthase (eNOS) and/or decreased availability of nitric oxide (NO) can

account for a broad array of clinical manifestations in patients with endothelial dysfunction (Table 20).

Both Withania somnifera & Mucuna pruriens are found to be effective to combat stress induce increase in Liver MDA & NO which indicates both the drugs are potential antioxidants which effectively reduces hepatic oxidative and nitrosative stress[17&18]. Higher liver nitrite/nitrate in this study indicate overproduction of MDA & NO in response to stress, probably iNOS might have played important role. Nitric oxide its metabolite peroxinitrate ($ONOO^-$) cross hepatocellular membrane through anion channel that produces nitration of thyrosin in activation of biological important proteins & enzymes. Mucuna pruriens & Withania somnifera induce decrease level of nitrate & nitrite in stress induce rat might be due to inhibition of hepatic cell lysolic iNOS activity[19&20] .

7.1.7. Histopathology

The histopathlogical structure of untreated rat liver (group I) showed normal architecture. Stress has major impact on liver where it shows majority of the distorted 'lobular' architecture of liver parenchyma, little swollen hepatocytes, vacuolated microvesicular and eosinophilic cytoplasm. Foci of fatty change and ballooning degeneration with necrosis of hepatocytes were found in zone 3 (centrilobular) areas. Moderate proliferation of portal area along with fibrous tissue and infiltration of mixed acute and chronic inflammatory cells were also noticed. Stress also shows variable widening of sinusoidal spaces and Kupffer cells with abnormal dilatation and congestion of the central vein. In group III showed little swollen hepatocytes, moderate proliferation of the portal area with fibrous tissue with infiltration of mixed acute and chronic inflammatory cells, variable widening of sinusoidal spaces, kupffer cell hyperplasia, dilatation and congestion of the central vein (Fig 26&27).

Group IV, V & VI showed near normal architecture of liver parenchymal tissues. Hepatocytes also showed mild variation in cellular size and shape, foci of fatty changes of hepatocutes in zone 3 along with mild dilatation and congestion of central vein.

Supplementation of Mucuna pruriens and Withania somnifera markedly ameliorated the histopathological changes by stress. Our study demonstrated that Mucuna pruriens administration in combination with Withania somnifera minimized its hazards. Both the extracts lowered the effect of stress toxicity by lowering the free radicals and increasing the level of antioxidants, this helped the hepatic architecture to come to near normal.

The histological structure of normal untreated rat kidney (group I) showed a normal architecture. In group II rat's kidney showed hyper cellular glomeruli with thickening of glomerular basement membrane and mesangial proliferation. Mild thickening with cloudy swelling of tubular basement membrane. Interstitium shows infiltration of focal inflammatory cells, blood vessels are congested and sclerotic, features of Acute Tubular Necrosis (ATN). Group III rats show hypercellular glomeruli, mesangial proliferation, focal tubular degenerative changes, interstitium shows infiltration of focal inflammatory cells, vessels are congested and sclerotic**.** Group IV, V & VI rats showed near normal features where interstitium shows infiltration of focal inflammatory cells. Supplementation of Mucuna pruriens and Withania somnifera markedly ameliorated the histopathological changes by stress not only in the liver but also in the kidney (Fig 28&29).

7.2. REPRODUCTION

7.2.1. Testiculosomatic Index

Increase in the testiculosomatic index in group II may be due to decreased body weight. Similarly, significant decreased liver, kidney and testicular weight in group II

rat indicate an alteration of metabolism in liver kidney and testis. The improvement of organ weight in liver, kidney and testis after treating with seed extracts of *Mucuna pruriens* & root extract of *Withania somnifera* on group IV, V & VI rats indicates a beneficial effect in this regard possibly by alteration of testicular androgen.

7.2.2. Mating behavior

Mating behavior test revealed that both the extract of *Mucuna pruriens* & *Withania somnifera* at a dose of 250mg/kg body weight either individually or combination significantly increased the MF, IF, EL and NM in treated group during all the experimental days when compared to control & stress group. Both the test extracts not only significantly increased the MF, IF, EL and NM but also significantly reduced the ML and IL compared to stress control (II) group, which indicates the aphrodisiac nature of MP & WS. ML and IL were considered inversely proportional to arousal or motivational effects[18]. Similarly, all treated rats including standard (sildenafil citrate) group copulated more vigorously than stress control group (II), especially group I, III & V was very good in overall performance. These results thus provide experimental support to the folk reputation of MP& WS as sexual stimulating drugs without any toxic effect. For penile erection, a well-coordinated system of vascular, endocrine and neural networks are required. Hence, a drug that brings about changes in erection and sexual behavior would induce changes in neurotransmitter levels or at cellular levels.

7.2.3. Adverse effect & pre-coital sexual behavior

In the present study, sexual behavior tests showed that the methanolic seed extract of *Mucuna pruriens* and methanolic root extract of *Withania somnifera* possesses significant sexual function enhancing activity. None of the rats showed the sign of toxicity (salivation, rhinorrhoea, lachrymation, ptosis, writhing, convulsions

and tremors) and stress (erection of fur and exopthalmia), in the stress group rats some behavioral changes were noted (such as spontaneous movement in the cage, climbing and cleaning of face).

Pre-coital sexual behaviors such as chasing, nosing and anogenital sniffing, were well performed in group I, III, IV & V whereas in groups II and VI the behaviors were not to the extent seen in group I, III, IV & V, which indicate stress has reduced the pre-coital sexual activity, as after treatment with both the extracts rats have shown increases in the activity.

7.2.4. Stress assay

Cortisol

Significant increase of Cortisol in stress group (II) reflects the experimental protocol for stress induction was appropriate. It is also interesting that sildenafil citrate does not have any impact on stress; whereas induction of two extracts individually and in combination clearly indicates as potential antistressor as part blood Cortisol concentration concern[21&22] (Table 23, Fig 30A &B).

Lipid peroxidation

Stress group show significant increase in lipid peroxidation by increased levels of MDA which indicates generation of reactive oxygen species, that means restrained to animal generates oxidative stress but interestingly both the extract supplementation reduces oxidative stress & proved to be act as potential antioxidants[23,24&25] (Table 23, Fig 30 C &D).

Nitric oxide

Stress induced decrease in the nitric oxide concentration reflects a possible suppression of eNOS activity and impairment of vascular integrity[26&27] , interestingly

both extracts found to have ameliorative effect against stress induced alteration of nitric oxide concentration generation[28&29](Table 23, Fig 30 E&F).

Antioxidants

Similarly reduction of Vitamin C & E during stress clearly indicates either over utilization of vitamin C & E or lack of biosynthesis of vitamin E/over degradation of vitamin C during metabolism[30] (Table 23, Fig 31A, B, C &D). Rise of both vitamin C and E level indicate after supplementation of both the extract are active antistressor perhaps the supplement itself is having good concentration of vitamin C which facilitate and interactive metabolic pathway for vitamin C synthesis[31& 32](Table 23, Fig 31A, B, C &D).

Incase of Withania somnifera vitamin C and E concentration markedly improved in stress group as compared to Mucuna pruriens group. Hence Withania somnifera found to be protective against stress induced alteration exogenous antioxidant concentration.

Vitamin E is a powerful lipophilic antioxidant that is absolutely vital for the maintenance of mammalian spermatogenesis[33]. It is present in particularly high amounts in Sertoli cells and pachytene spermatocytes and to a lesser extent round spermatids. Vitamin C also contributes to the support of spermatogenesis at least in part through its capacity in reduced α-tocopherol and maintain this antioxidant in an active state[34]. Deficiencies of vitamins C or E leads to a state of oxidative stress in the testes that disrupts both spermatogenesis and the production of testosterone.

7.2.5. Reproductive assay

Testicular secretary constituents like cholesterol and glycogen can be used to determine the functional capacity of the testes[35,36&37]. Cholesterol is the precursor in

the synthesis of steroid hormone and its requirement for normal testicular activity has been well established[12&38](Table 24 & Fig 32 A & B).

An increased level of testicular cholesterol is due to the unutilized cholesterol present in the interstitial cells which is an important precursor for the testosterone synthesis.

Decrease in testosterone concentration with a concomitant increase of LH & FSH in the stress group clearly indicates impaired function of hypothalamic-pituitary-testicular axis in group II rats. Treatment with these two phytochemical *Mucuna pruriens & Withania somnifera* particularly recovered the altered hypothalamic-pituitary-testicular axis, perhaps counter acting with stress induced ROS generation. The result indicates both phytochemicals are potent anti-stressor & have a beneficial effect on steroidogenesis particularly testosterone pathways, these observations further supported by changes of MDA and NOx level.

Results from our observation also reveal decrease vit C & E in the stress group (II) which are back to near normal with the supplementation of *Mucuna pruriens* & *Withania somnifera*.

Sexual behavior, performance extremely dependent on the endocrine profile besides overall metabolic activity altered MDA and NOx level due to stress not only indicate generation of free radicals and cellular damage but also indicates reduction sperm production sperm motility and impaired sexual performance. Supplementation of both the phytochemicals partially recovers stress induced reproductive function (behavioral & physiological) this beneficial action may be due to capability of antioxidant or direct beneficial action on metabolic regulation. Results from our study have been supported by histological reports.

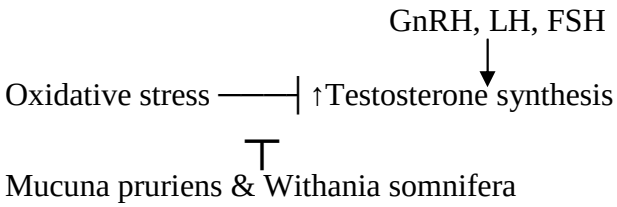


Figure 42 Hypothetical scheme illustrating oxidative inhibition of testosterone synthesis and its prevention by Mucuna pruriens and Withania somnifera

Healthy cellular metabolism requires the generation of metabolic energy within mitochondria without the production of collateral oxidative damage caused by oxidizing product of metabolism including ROS such as superoxide, hydrogen peroxide, hydroxyl radicals, nitric oxide, peroxynitrate etc. ROS and other oxidizing molecule generated by stress add to testicular oxidative stress[39&40]. Dependence on the mitochondrion electron transport chain system for the energy to drive testosterone synthesis exposes Leydig cell mitochondria to oxidative stress and the generation of ROS within the Leydig cell increases during testosterone synthesis, which leads to reduce the activity of antioxidant enzyme (catalase, SOD, etc) and increases the synthesis of testicular MDA product of lipid peroxidation[41]. Such type of oxidative damage in Leydig cell or over all testicular histology or less sensitive to LH with fewer LH receptors expressed in each cell and alter activities of several testicular biosynthetic enzymes like 3βHSD or 17βHSD ultimately the stress causes decreases the testosterone synthesis[42]. Thus stress associated decline in testosterone production and circulation concentration may considered as consequences of cumulative oxidative stress during stress within Leydig cell.

Treatment with Mucuna pruriens and Withania somnifera reverse the testicular adversity due to stress and brought back normal gonadal homeostasis due to presence of large number of nutritional antioxidants, including ascorbic acid, α-tocopherol and large polyphenol compounds which are stable at stomach pH and passes to small

intestine unchanged. The polyphenolic compounds in Mucuna pruriens and Withania somnifera reduces stress induced oxidative stress and stimulate testosterone within the testis. Our observation may be further supported by our findings on vitamin E concentration which shows remarkable decrease in vitamin E during stress and revert it back after Mucuna pruriens and Withania somnifera supplementation. Vitamin E sensitizes Leydig cell to LH and increases testosterone synthesis. Hence we can conclude that Mucuna pruriens and Withania somnifera might augmented the antioxidant defense system in the testis and mitochondrion protective nutrients and keep healthy redox balance in leydig cell and prevent leydig cell from oxidative inhibition of testosterone biosynthesis and increases rate of testosterone secretion[43].

Our results clearly indicate stress induces decrease sperm count & motility which may be due to overproduction of ROS and compromising the concentration of vit E level in the testis. Perhaps stress induces overproduction of Cortisol concentration in our study may be another reason for alteration of seminiferous tubular microenvironment which directly affect spermatogenesis (Fig 34& 35).

It is worthy to note that high level of reactive oxygen species (ROS) in the semen induce oxidative damage to the sperm and are associated with abnormal semen parameters leading to infertility. Withania somnifera has been found to counteract the formation of ROS in infertile men[44].

Figure 43 depicts the overall impact of stress on male reproductive system and amelioratic effect of both Mucuna pruriens and Withania somnifera on stress induced alteration of spermatogenesis

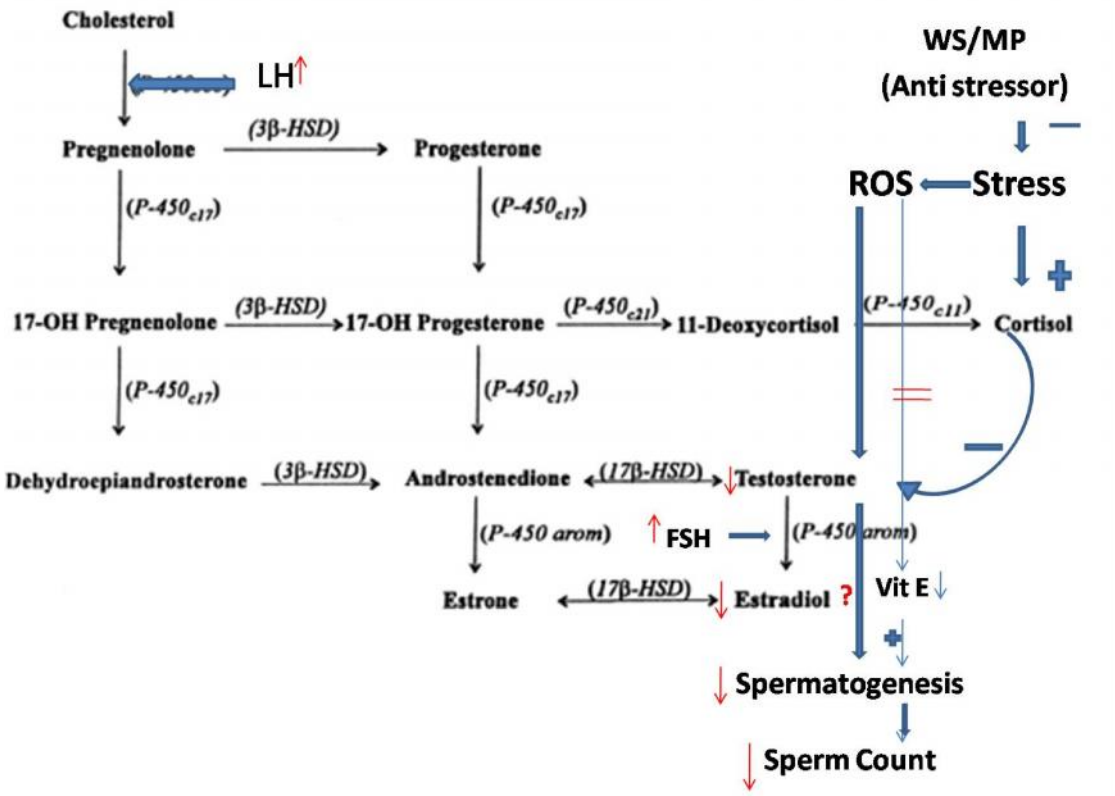


Figure 43 Impact of Stress Mucuna pruriens & Withania somnifera on spermatogenesis

7.2.6. Histopathological changes in male reproductive organs

The histological structure of normal untreated rat testis (group I) showed a normal architecture. Group II rats showed majority of atrophic seminiferous tubules, marked thickening of tubular basement membrane with the many foci of loss of spermatogenesis up to 75%, loss of germ cell layer decrease in the number of interstitial cells of Leydig and many foci of congestion, edema, necrosis and interstitial fibrosis. Group III rats showed seminiferous tubules with Focal atrophic changes of tubules, foci of spermatogenesis > 50%, adequate germ cell layer. Group IV, V & VI rats showed a normal testicular parenchymal architecture with seminiferous tubule, spermatogenesis up to 75% and an increase in germ cell layer.

The results clearly indicate that stress has an adverse effect on testicular cells, like less no Leydig cells and fibrosis like appearance. The degeneration of sertoli cells, and dilated interstitial space lumen of seminiferous tubules in group II rats

clearly indicate toxic insult of stress on seminiferous tubules and probably disrupt blood testes barrier (Foley G et al 2001). After treatment with both the extracts testis showed a remarkable improvement of pathophysiology of testis.

Histopathological architecture of epididymis, prostate, seminal vesicle further supports the above results. Hence it may be concluded.

BIBLIOGRAPHY

1. Rice-Evans C A. Miller N J, Bolwell P G. Bramley P M. Pridham J B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free Rad Res. 1995; 22 (4): 375-83.

2. Kessler M, Ubeaud G, Jung L. Anti- and pro-oxidant activity of rutin and quercetin derivatives. J Pharm Pharmacol. 2003; 55 (1): 131-142.

3. Nobori T. Miurak K. Wu D. J. Takabayashik L. A Carson D. A. Deletion of cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. Nature 1994; 46: 753-6

4. Weisburger J H. Eat to live, not live to eat. Nutrition 2001; 16: 767-73.

5. Parks E J. Dietary carbohydrate's effects on lipogenesis and the relationship of lipogenesis to blood insulin and glucose concentrations. Br J Nutr. 2002; 87 (Suppl. 2): S247-S253.

6. Franova S. Nosalova G. Mokry J. Mahmud T H K. Ather A. Phytotherapy of cough. In Iwu M (ed.) Advances in phytomedicine, U. S. A. Elsevier; 2006. p. 111-31.

7. Bar-Tal Y. Cohen-Mansfield J. and Golander H. which stress matters? The examination of temporal aspects of stress, Journal of Psychology 1998; 132(5); 569-76

8. G. K. A. Adepoju, O. O. Odubena. Effect of Mucuna pruriens on some haematological and biochemical parameters J. of Medicinal Plants Res. 2009; 3(2), 73-6

9. Tacke F., Luedde T., Trautwein C. Inflammatory pathways in liver homeostasis and liver injury. Clin. Rev. Allergy Immunol. 2009; 36:4–12

10. Senthilkumar R. Rahul C. Thangaraj P. Hepatoprotective effect of Rhodiola imbricata rhizome against paracetamol-induced liver toxicity in rats. Saudi Journal of Biological Sciences 2014; 21, 409–16
11. Clarke E. G., Clarke M. L. Cassel and Collier Macmillan Publishers; London: 1977. Veterinary Toxicology pp. 268– 77
12. Day C. Metabolic syndrome or what you will: definitions and epidemiology. Diab. Vasc. Dis. Res. 2007; 4:32-8
13. Seematter G, Binnert C, Martin JL, Tappy L, Relationship between stress, inflammation and metabolism. Curr Opin Clin Nutr Metab Care 2004; 7:169-73
14. Visavadiya NP, Narasimhacharya AV. Hypocholesteremic and antioxidant effects of Withania somnifera (Dunal) in hypercholesteremic rats. Phytomedicine. 2007; 14 (2-3):136-42
15. P. Usharani, P.V. Kishan, Nishat Fatima and C. Uday Kumar. A Comparative Study To Evaluate The Effect Of Highly Standardised Aqueous Extracts Of Phyllanthus Emblica, Withania Somnifera And Their Combination On Endothelial Dysfunction And Biomarkers In Patients With Type II Diabetes Mellitus Int. J. of Pharmaceutical Sci. & Res. 2014; 5(7): 2687-97.
16. Luo S, Lei H, Qin H, Xia Y. Molecular mechanisms of endothelial NO synthase uncoupling. Curr Pharm Des. 2014; 20(22):3548-53.
17. Vidyasagar J, Karunaka N, Reddy MS, Rajnarayan K, Surender T, Krishna DR. Oxidative stress and antioxidant status in acute organophosphorous insecticide poisoning. Indian J of Pharmacol. 2004; 36(2): 76-79.
18. Katrina MM, Michael GE, David AW. A rapid simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Biology and Chemistry. 2001; 5(1): 62–71.

19. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol. Rev. 2007; 87(1):315-424.

20. Yokozawa T, Kim HY, Kim HJ, Okubo T, Chu DC, Juneja LR. Amla (Emblica officinalis Gaertn.) prevents dyslipidaemia and oxidative stress in the ageing process. Br J Nutr. 2007; 97(6):1187-95.

21. Obminski Z, Wojtkowiak M, Golec L, Hockney AC. Effect of acceleration stress on salivary cortisol and plasma Cortisol and testosterone levels in cadet pilots. Journal of Physiology & pharmacology 1997; 48(2): 193-200.

22. Wheeler G, Gumming D, Burnham R. Testosterone, Cortisol and catecholamine response to exercise stress and autonomic dysreflexia in elitequadriplegic athletis. Paraplegia 1994; 32(5): 292-9.

23. Sampath Madhyastha, Rachana Chauhan, Gayathri M. Rao, Hema Umesh. Neuroprotective Effects of Mucuna pruriens Against Stress-Induced Oxidative Damage. J. Physiol. Biomed. Sci. 2011; 24(2): 28-33

24. Zaidi SM, Banu N. Antioxidant potential of vitamins A, E and C in modulating oxidative stress in rat brain. Clin Chim Acta. 2004; 340: 229-33

25. Zaidi SM, Al-Qirim TM, Banu N. Effects of antioxidant vitamins on glutathione depletion and lipid peroxidation induced by restraint stress in the rat liver. Drugs R D. 2005; 6(3): 157-65.

26. Argacha, J.F., Egrise, D., Pochet, S., Fontaine, D., Lefort, A., Libert, F., Goldman, S., vande Borne, P., Berkenboom, G., Moreno-Reyes, R. Vitamin D deficiency-induced hypertension is associated with vascular oxidative stress and alteredheart gene expression. J. Cardiovasc. Pharmacol. 2011; 58, 65–71.

27. Schiffrin EL. Vascular endothelin in hypertension. Vascul Pharmacol. 2005; 43: 19-29

28. Rebeca Santiso, María Tamay, Jaime Gosalvezc, Steve Johnstond, Alfonso Marinoe, Carlos Fernández, Carlos Losadaf, José Luis Fernández. Mutation Research DNA fragmentation dynamics allows the assessment of cryptic sperm damage in human: Evaluation of exposure to ionizing radiation, hyperthermia, acidic pH and nitric oxide. Mutation Research. 2012 ;734: 41– 49,
29. Prabhu LV, Rai R, Ranade AV, Kini H, Krishnamurthy A, Bernhardt KL. Effect of sildenafil-induced nitric oxide on the histomorphology of cardiomyocytes in male rats. Int J App Basic Med Res 2013; 3: 84-7.
30. Ching Kuang Chow Vitamin E and oxidative stress Free Radical Biology and Medicine 1991; 11(2), 215-232
31. Jean-Marc Zingg Vitamin E: An overview of major research directions, Molecular Aspects of Medicine. 2007; 28(5-6);400-22
32. A. Ipek, O. Canbolat and A. Karabulut, The Effect of Vitamin E and Vitamin C on the Performance of Japanese Quails (Coturnix Coturnix Japonica) Reared under Heat Stress during Growth and Egg Production Period. Asian-Aust. J. Anim. Sci. 2007; 20(2):252-256
33. Johnson FC et al. The antioxidant vitamins CRC. Crit Rev Food Sci Nutr. 1979; 11:217–309.
34. Yoganathan T, Eskild W, Hansson V. Investigation of detoxification capacity of rat testicular germ cells and Sertoli cells. Free Radic Biol Med. 1989; 7:355–359
35. Gupta, R.S., Sharma, R., Sharma, A., Bhatnager, A.K., Dobhal, M.P., Joshi,Y.C., Sharma, M.C., Effect of Alstonia scholaris bark extract on testicular function of wistar rats. Asian Journal of Andrology. 2002; 4, 175–8

36. Kamatchouing, P., Fandio, Y. M., Dimo, T., Jatsa, H.B., Evaluation of androgenic activity of Zingiber officinale and Pentadiplandra brazzeana in male rats. Asian Journal of Andrology. 2002; 4, 299–301

37. Watcho, P. , Kamtchouing, P., Sokeng, S.D., Moundipa, P.F., Tantchou, J., Essame, J.L., Koueta, N. Androgenic effect of Mondia whitei roots in male rats. Asian Journal of Andrology. 2004; 6, 269–72

38. Bedwall, R.S., Edwards, M. S., Katoch, M., Bahuguna, A., Dewan, R., Histological and biochemical changes in testes of zinc deficient BALB/C strain of mice. Indian Journal of Experimental Biology. 1994; 32, 243–7

39. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev. 2008; 1: 15-24.

40. Glade MJ and Smith K. Oxidative Stress, Nutritional Antioxidants, and Testosterone Secretion in Men. Ann Nutr Disord & Ther. 2015; 2(1): 1019.

41. Ceribasi AO, Sakin F, Türk G, Sönmez M, Atessahin A. Impact of ellagic acid on adriamycin-induced testicular histopathological lesions, apoptosis, lipid peroxidation and sperm damages. Exp Toxicol Pathol. 2012; 64: 717-724

42. Akazawa N, Mikami S, Kimura S. Effects of vitamin E deficiency on the hormone secretion of the pituitary-gonadal axis of the rat. Tohoku J Exp Med. 1987; 152: 221-229

43. Omu AE, Al-Azemi MK, Al-Maghrebi M, Mathew CT, Omu FE, Kehinde EO, et al. Molecular basis for the effects of zinc deficiency on spermatogenesis: An experimental study in the Sprague-Dawley rat model. Indian J Urol. 2015; 31: 57-64.

44. Ahmad MK, Mahdi AA, Shukla KK, Islam N, Rajender S, Madhukar D, Shankhwar SN, Ahmad S Fertil Steril. 2010; 94(3):989-96.

CHAPTER 8

SUMMARY & CONCLUSION

APHRODISIAC EFFECT OF MUCUNA PRURIENS & WITHANIA SOMNIFERA & THEIR EFFECT ON MALE REPRODUCTIVE ORGANS OF ALBINO RATS

2015
BLDE UNIVERSITY'S

SHRI B M PATIL MEDICAL COLLEGE HOSPITAL & RESEARCH CENTRE

The figure depicted below summarizes stress induced alteration of reproductive and sexual dysfunction in male albino rats. Figure also reveals the possible antistress mechanism of Mucuna pruriens and Withania somnifera.

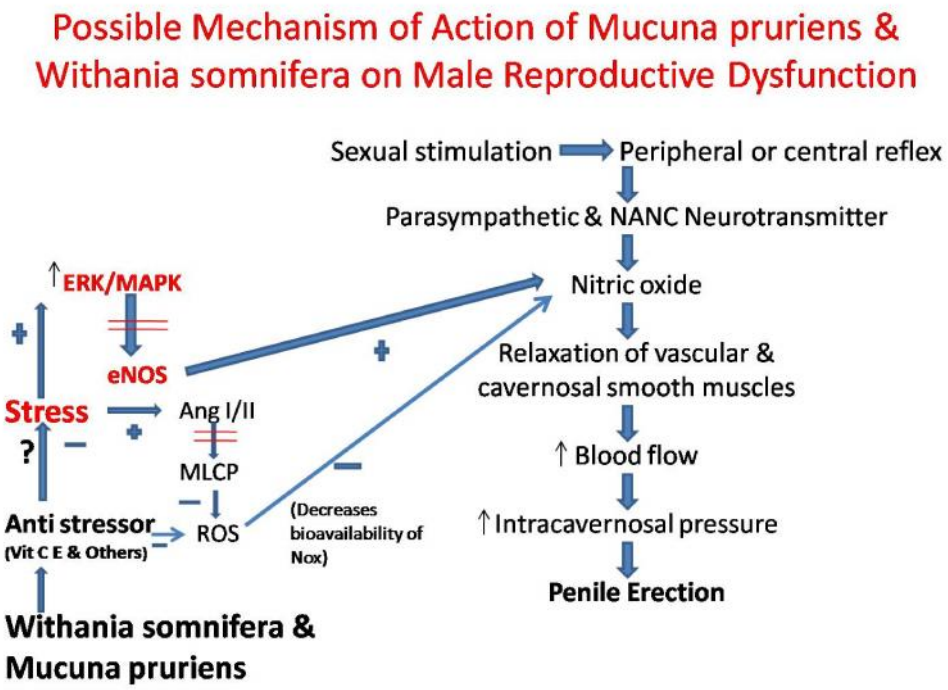


Figure 44 Possible mechanism of action

It may be concluded from above figure that stress alters eNOS mechanism, angiotension system and ROS generation which in turn develop oxidative and nitrosative stress, autonomic function and vascular integrity. Possibly erectile dysfunction due to stress follows these pathways.

The testicular dysfunction and altered spermatogenesis, endocrine profile and testicular histology clearly indicate stress induced adverse effect on hypothalamo-pituitary-gonadal axis. Low sperm count and motility, low testosterone concentration with high LH, FSH concentration and altered histological architecture of testis due to restraint stress on male albino rats clearly reflects stress in general affects male reproductive system. Low testosterone with high LH and FSH also indicate a possible negative feedback on LH secretion and Leydig cell mal function. Supplementation of

Mucuna pruriens and Withania somnifera contributes its antioxidant potentiating effect on Leydig cell testosterone production. Possibly antioxidants characteristic of Mucuna pruriens and Withania somnifera protects mitochondrial function of testicular cells and reduces cell-white oxidative damage, support redox balance within Leydig cell and increase testosterone production.

In brief fallowing are the summery and conclusions:-

- ☞ Results clearly indicate both pharmaceutical components are beneficial on male reproductive health in albino rats exposed to physical stress.
- ☞ Withania somnifera better beneficial effect as compared to Mucuna pruriens in controlling stress induced impairment.
- ☞ It has also shown that Mucuna pruriens increased blood urea level but its adverse effect on kidney has not been proved with other findings.
- ☞ Results also indicate a combination of both drugs did not give any additional benefit as compared to single compound on overall reproductive health of male albino rats exposed to physical stress.
- ☞ All results of the study clearly indicate there is an improvement of the neuroendocrine profile for functional improvement of the male reproductive system. Our histopathological findings further support this observation.
- ☞ As the beneficial effect of the Withania somnifera & Mucuna pruriens corrugate with a standard pharmaceutical dose of sildenafil citrate in our study. Hence these phytochemical substances may be considered as aphrodisiac as well as beneficial to the male reproductive system like sildenafil citrate.

LIMITATIONS OF THE STUDY

- ☞ We could not evaluate exact active compounds Mucuna pruriens & Withania somnifera
- ☞ We also didn't assay 3βHSD and 17βHSD on steroidogenic pathways

SCOPE FOR FUTURE

- ☞ More refine screening of Mucuna pruriens and Withania somnifera to be done for further conclusion perhaps screening through HPLC will be the better option.

PUBLICATIONS

- ☞ Gavishiddappa A. Hadimani, S D Desai, P Biradar, Nanjappaiah H M, S Hugar, I B Bagoji, Evaluation of Acute oral toxicity and Phytoconstituents of Methanolic extract of Mucuna pruriens, Journal of Pharmaceutical Science & Research 2015, 7(1): 33-36
- ☞ S D Desai, Gavishiddappa A Hadimani, I B Bagoji, P Biradar, Nanjappaiah H M, S Hugar, Evaluation Of Phytoconstituents Of Methanolic Root Extract Of Withania Somnifera, Journal of Advanced Scientific Research 2015;6(1):27-30

PRESENTATIONS

- ☞ "Aphrodisiac Effect of Mucuna pruriens on Male reproductive organs of albino rats" 2nd National Seminar- Applications of Nanotechnology in Health Care, 30/06/2012, BLDEA's College of Pharmacy
- ☞ "Aphrodisiac effect of methanolic extract of roots of Withania somnifera on male albino rats and their effect on male reproductive organs" 14th Conference of Karnataka Chapters of Anatomist, 8 &9 Sep 2012 Dept. of Anatomy Shri B M Patil Medical College Vijayapur
- ☞ "Effect of methanolic extract of Withania somnifera on hematological parameters of male albino rats", 16th Conference of Karnataka Chapters of Anatomist, 23-24/9/2014, SDM College of Medical Science Dharwad

ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Evaluation of Acute Oral Toxicity and Phytoconstituents of Methanolic Extract of *Mucuna pruriens*

Gavishiddappa A. Hadimani[1]*, S D Desai[2], Prakash Biradar[3],Nanjappaiah H M[3], Shivakumar Hugar[4],Ishwar B. Bagoji[1]

[1]*PhD Scholar, Dept. of Anatomy, Sri B M Patil Medical College, Bijapur.*
[2]*Principal, Shridevi Institute of Medical Science & Research Hospital, Tumkur.*
[3]*Lecturer, Dept. of Pharmacology, BLDEA's College of Pharmacy, Bijapur.*
[4]*Prof. & Head, P. G. Dept. of Pharmacology, BLDEA's College of Pharmacy, Bijapur.*

Abstract

The present research work was designed to identify the phytochemical constituents and acute oral toxicity of methanolic extract of seeds of *Mucuna pruriens*. Fresh mature seeds were shade dried at room temperature, coarse powdered and extracted with metahanol by Soxhlet's extraction method. Thereafter, the extract was concentrated using rotary flash evaporator to obtain semisolid crude extract with the yield of 09.534 %. *Mucuna pruriens* seed extract was investigated for the presence of phytochemical constituents and oral acute toxicity study. Preliminary phytochemical tests was conducted on test extract to detect the presence of phytochemicals by the standard methods described in the Pharmacognosy text book of Trease and Evans. The acute toxicity of test extract was determined in mice weighing between 20 – 25 g following fixed dose method of CPCSEA, OECD (Organization for Economic Cooperation and Development), guideline No. 420. The preliminary phytochemical evaluation of the *Mucuna pruriens* seed extract revealed the presence of steroids, alkaloids, tannins, carbohydrates, amino acid, resins and starch. In an acute toxicity studies, test extract of title plant did not cause any mortality of the animals at dose of 2000 mg/kg.

Key words: *Mucuna pruriens*, acute oral toxicity, phytochemical constituents.

INTRODUCTION

Nature always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. The biotic and abiotic elements of nature are all interdependent. The quest for long, healthy and happy life is as old as man himself. Nature has provided a complete storehouse of remedies to relieve the ailments of mankind. The consistent effects have resulted in many effective means of ensuring health care. The seers of Ayurveda were able to understand and record the various aspects regarding the drugs that even today are difficult to understand with modern available parameters [1, 2].

The medicinal plant products, which are derived from plant parts such as stem bark, leaves, fruits and seeds have been part of phytomedicine that produce a definite physiological action on the human body. The most important of these natural bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds [3].

Itching bean Mucuna pruriens an underutilized legume species grown predominantly in Asia, Africa and in parts of America [4]. Mature seeds, seeds from unripe pods and young pods of itching bean, Mucuna pruriens are soaked and boiled/roasted and eaten as such or mixed with salt by the North-East Indian tribes; North-Western parts of Madhya Pradesh tribes; South Indian tribes [5-7]. To make this less- known legume palatable, tribal people follow a special processing method of continuous boiling and draining for about eight times until the boiled water changes from black to milky white. Consumption of improperly boiled seeds of itching bean is known to cause increase in body temperature and skin eruptions [8]. It is attributed to the presence of high levels of 3, 4- dihydroxy-L-phenylalanine, L-Dopa, the aromatic non-protein amino acid [9].

Hence, in the present study, the seeds of Mucuna prurienswere investigated and their chemical composition was investigated with a view to assess their phytochemical potential.

MATERIALS AND METHODS

Plant materials:

The seeds of Mucuna pruriens were procured locally after the seeds was authenticated by Dr. M B Mulimani Professor, Department of Botany. SB Arts & KCP Science College, Bijapur by the studies include organoleptic tests and macroscopic and microscopic observations. A voucher specimen has been deposited in our department.

Preparation of extract

Seeds were washed twice using tap water and then washed again in distilled water to remove the dust. The seeds were shade dried for 7–12 days at room temperature, until they were free from the moisture and then pulverized into coarse powder. The powdered material was extracted with methanol by Soxhlet's extraction method. Thereafter, the extract was concentrated using rotary flash evaporator to obtain semisolid crude extract. The percentage yield of the extract was found to be 09.534 %. The extract was stored in airtight container in refrigerator below 10^0C. Desired concentration of stock solution was prepared using distilled water for the following studies.

01. Preliminary phytochemical investigation.
02. Acute toxicity study in mice.

Preliminary phytochemical screening

Preliminary phytochemical tests were conducted on test extract to detect the presence of phytochemicals by following below mentioned the standard methods described in the Pharmacognosy text book of Trease and Evans.

1. **Test for Steroids** [11]

   Salkowski test: 2-3 drops of concentrated sulphuric acid was added to chloroform solution, shaken and allowed to stand, appearance of red color in lower layer indicates the presence of sterols.

   Liebermann-Burchard test: Extract was mixed with the chloroform and few drops of acetic anhydride and mixed well. Concentrated sulphuric acid was added from the sides of the test tube slowly until the ring appears; appearance of reddish brown ring indicates the presence of steroids.

2. **Test for Flavonoids** [12, 13]

   Shinoda test**:** To the extract a few fragments of magnesium ribbon and concentrated hydrochloric acid was added. Appearance of red to pink color after few minutes indicates the presence of Flavonoids.

   Lead acetate test: To the extract added few drops of aqueous basic lead acetate solution. Formation of yellow precipitate indicates presence of flavonoids**.**

   Alkaline reagent test/ NaOH test: few drops of sodium hydroxide solution was added to extract. Intense yellow color disappeared after adding dilute HCl which indicates the presence of flavonoids.

3. **Test for Alkaloids**: The extract was basified with ammonia and extracted with chloroform. The chloroform solution was acidified with dilute hydrochloric acid, shaken well and filtered. The filtrate was used for testing the alkaloids.

   Hager's test*:* The filtrate was treated with few drops of Hager's reagent. Formation of yellow precipitate indicates the presence of alkaloids [14].

   Wagner's test (Iodine in Potassium iodide): The acid layer was treated with few drops of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

   Mayer's test (Potassium Mercuric Iodine solution): The acid layer was treated with few drops of Mayer's reagent. Formation of creamy white precipitate indicates the presence of alkaloids.

   Dragendorff's reagent (Potassium Bismuth Iodide): The acid layer was treated with few drops of Dragendorff's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

4. **Test for Tannins**[15]

   Gelatin test: To the extracts of the drug added 1% solution of gelatin containing 10% sodium chloride. Formation of white precipitate indicates the presence of tannins.

   Ferric chloride test: To extracts few drops of 1% neutral ferric chloride solution were added, formation of blackish blue color indicates the presence of tannins.

5. **Test for Saponins** [14, 16]

   Foam test**:** Small amount of extract of the drug was shaken with little quantity of water, if foam produced persists for 10 minutes; it indicates the presence of saponins.

   Froth test**:** To 5 ml of extract of the drug added single drop of sodium bicarbonate solution. Shaken the mixture vigorously and left for 3 minutes. Formation of honey comb like froth indicates presence of saponins.

6. **Test for Carbohydrates:** Small amount of extracts of the drug were dissolved in little quantity of distilled water and filtered separately. The filtrates were used to test presence of carbohydrates.

   Molisch's test: The filtrate of the drug was treated with Molisch reagent and concentrated sulphuric acid was added from the sides of the test tube to form a layer. A reddish violet ring shows the presence of carbohydrates.

   Benedicts test: to the filtrate added 2 ml Benedict's reagent and boiled in water bath. Formation of Green or reddish brown precipitate indicates presence of carbohydrates.

   Fehlings test: Filtrates were hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with equal amount of Fehling's A and B solutions. Formation of green to yellow to red precipitate indicated the presence of reducing sugars.

7. **Test for Amino acid/ Protein**

   Ninhydrin test: Heated the 3 ml of extract of the drug and 3 drops of ninhydrin solution in boiling water bath for 10 minutes. Appearance of purple color shows the presence of amino acids.

   Biuret test: To 3 ml of extract of the drug added 4% NaOH and few drops of 1% copper sulphate solution. Formation of violet color confirms the presence of protein.

   Millon's reagent test: Mixed the extract with millon's reagent. Formation of brick red precipitate indicates the presence of protein.

8. **Test for Resins:** Dissolved the extract in acetone and pour the solution in to distilled water. Turbidity indicates the presence of resins [10].

9. **Test for starch:** dissolved 0.015 gm of iodine and 0.075 gm of potassium iodide in 5 ml of distilled water and add 2-3 ml of an aqueous extract of drug, blue color is produced.

Experimental animals

Female albino Swiss mice (20 - 25 g) were used in the experiments. Animals were procured from central animal house, BLDEU Sri B M Patil Medical College, Bijapur, India. After randomization into various groups and before initiation of experiment, the animals were acclimatized for a period of 10 days. Animals were housed in polypropylene cages and maintained under standard environmental conditions such as temperature (26 ± 2^0C), relative humidity (45 - 55%) and 12 hr. dark/light cycle. The animals were fed with rodent pellet diet and water *ad libitum*. The study protocol was approved from the Institutional Animal Ethics Committee (IAEC) before commencement of experiment.

Determination of acute toxicity (LD_{50})

The acute toxicity of test extract was determined in mice weighing between 20 - 25g following fixed dose method of CPCSEA, OECD (Organization for Economic Cooperation and Development), and guideline No. 420; (Annexure-2d) [17].

RESULTS

Preliminary phytochemical screening

Preliminary Phytochemical screening of methanolic extract of seeds of Mucuna pruriens revealed the presence of different kind of phytochemical components that are summarized in table 1

Table 1: Preliminary phytochemical screening of Methanolic Mucuna pruriens seed extract

S. No	Phytochemical	Test	Result
1	Test for Steroids	Salkowski test:	Present
		Liebermann-Burchard test	Present
2	Test for Flavonoids	Shinoda test	Absent
		Lead acetate test	Absent
		Alkaline reagent test/ NaOH test	Absent
3	Test for Alkaloids	Hager's test	Present
		Wagner's test	Present
		Mayer's test	Present
		Dragendorff's reagent	Present
4	Test for Tannins	Gelatin test	Present
		Ferric chloride test	Present
5	Test for Saponins	Foam test	Absent
		Froth test	Absent
6	Test for Carbohydrates	Molisch's test	Present
		Benedicts test	Present
		Fehlings test	Present
7	Test for Amino acid/ Protein	Ninhydrin test	Present
		Biuret test	Present
		Millon's reagent test	Present
8	Test for Resins		Present
9	Test for starch		Present

Determination of acute toxicity (LD_{50})

In an acute toxicity studies, test extract of title plant did not cause any mortality of the animals at dose of 2000 mg/kg.

DISCUSSION:

These results obtained in the present study are in good consonance with the earlier reports of *Mucuna pruriens*. Steroids, alkaloids, tannins, carbohydrates, amino acid and resins were present in methanolic extract of seeds of *Mucuna pruriens*. The medicinal values of the seeds may be related to their constituent phytochemicals. According to Varadarajan et al., the secondary metabolites (phytochemicals) and other chemical constituents of medicinal plants account for their medicinal value [18]. For example, saponins are glycosides of both triterpene and steroids having hypotensive and cardiodepressant properties, while anthraquinones posses' astringent, purgative, anti-inflammatory, moderate antitumor, and bactericidal effects [19&20].

In the present study methanolic extract of seeds of *Mucuna pruriens* have significant amount of alkaloids and those are responsible for most varied type of pharmacological actions and have the effect on central nervous system probably may help in relieving the causes of infertility and maintenance of pregnancy with overall influence on the body and fetus. Presence of steroids indicates that these drugs may have influence on endocrine system. As steroids are precursors for synthesizing sex hormones especially progesterone and estrogen which are basic factors for infertility. Tannins were positive in Mucuna pruriens and contributory to overcome the possible haemorrhagic or other discharges of yoni andhelps conception. Mucuna pruriens indicates presence of both carbohydrates and starch this indicate they are polysaccharides. They are nutritive, help for conception and maintain pregnancy and promote normal delivery. Because of presence of resins in some Mucuna pruriens it has been used as antibacterial and anti-fungal. There are conditions in infertility which are due in inflammatory lesions which may be overcome by presence of resins. Presence of proteins in Mucuna pruriens has been described by Fathima et al. Mucuna pruriens contain higher crude protein when compared with commonly consumed pulse crops such as black gram, green gram, pigeon pea, chick pea and cow pea [21-22].

CONCLUSION

In conclusion, the findings of the present study suggest that seeds extract of the Mucuna pruriens possesses steroids, alkaloids, tannins, carbohydrates, amino acid, resins and starch. In an acute toxicity studies, test extract of title plant did not cause any mortality of the animals at dose of 2000 mg/kg.

ACKNOWLEDGEMENT

The authors are thankful to Hon'ble Vice Chancellor and Registrar BLDE University Principal and Vice Principal of Shri B M Patil Medical College Hospital and Research Centre, Bijapur, Karnataka, India for providing necessary facilities to carry out research work, and also thankful to Principal of BLDEA's College of Pharmacy, Bijapur for providing facility to carry out extraction and phytochemistry.

REFERENCES:

1. Anonymous. Charaka Samhita, Chakrapani Datta Teeka Chowkambha Prakashan, Varanasi, 1992.
2. S R Govind Das. Bhaishajya Ratnavali. Chowkambha Sanskrit Series office, Varanasi, 1961.
3. Hill AF. Economic Botany. A textbook of useful plants and plant products. 2nd edn. New York: McGraw Hill Book Company Inc; 1952. P. 205
4. Vadivel, V., and Janardhanan, K. Nutritional and anti-nutritional composition of velvet bean: an underutilized food legume in South India. Int. J. Food Sci.Nutr., 2000.51: 279-287
5. Arora, K. R. Native food plants of the northeastern India. In: Contributions to Ethnobotany of India (Ed. SK Jain), Scientific Publishers, Jodhpur, and India. 1991. P.137-152.
6. Sahu, T. R. Life support promising food plants among aboriginal of Baster (M.P.), India. In: Ethnobiology in Human Welfare (Ed. SK. Jain), Deep Publications, New Delhi, India, 1996. P. 26-30.
7. Jain, S. K. Glimpses of Indian ethnobotany. New Delhi, India: Oxford and IBH Publishing Co. 1981. P. 1-230
8. Shankaranarayanan, A.S. Studies in the chemistry and pharmacology of Indian medicinal plants. Ph.D. Thesis, Madras University, Madras, India. 1978
9. Jabadhas, A. W. Ethnobotanical studies on some hill tribes of South India. Ph.D. Thesis, Madras University, Madras, India. 1980
10. JB Harborne, Phytochemical methods: A guide to modern techniques of plant analysis, 3rd edition, Chapman and Hall, London, 2007: 135-203.

11. RD Gibbs. Chemotaxonomy of Flowering Plants, Vol.1, McGill Queen's University Press, Montreal and London. 1974.
12. K Peach, MV Tracey. Modern methods of plant analysis, Vol 3, Springer Verlag, Berlin, 1956.
13. AM Rizk. Fitoterapia, 1982, 52;2: 35-42.
14. GE Trease, WC Evans. Pharmacognosy, 15th edition, WB Saunders Publishers, London, 2002
15. CK Kokate. Practical Pharmacognosy. 4th edition, Vallabh Prakashan, New Delhi, India, 1994.
16. A Sofowara. Medicinal plants and traditional medicine in Africa. Chichester John Wiley & Sons, New York, 1993.
17. Prema Veeraraghavan, Expert consultant, CPCSEA, OECD Guideline no. 420; 2000.
18. P. Varadarajan, G. Rathinaswamy, and D. Asirvatahm, "Antimicrobial properties and phytochemical constituents of Rheo discolor," Ethnobotanical Leaflet, 2008,12: 841–845.
19. M. T. Olaleye, "Cytotoxicity and antibacterial activity of methanolic extract of Hibiscus sabdariffa," Journal of MedicinalPlants Research, 2007, 1:1: 9–13.
20. R. A. Muzychkina, Natural Anthraquinoues: Biological and Physiological Properties, G. A. Tolstikov, Ed., PHASIS, Moscow, Russia, 1998.
21. Nagamani, J Suresh, J Ahuja, V Reddy; Comparative phytochemical screening of Vatashunga, Shatavari and Shatapushpa claimed for Prajasthapana activity; Annals of Biological Research, 2012, 3:3:1294-1304
22. K. R. Fathima, P. Tresina Soris, and V. R. Mohan; Nutritional and Antinutritional Assessment of Mucuna pruriens Adv. Biores. December 2010 vol 1:2:79-83.

ScienSage

Journal of Advanced Scientific Research

Available online through http://www.sciensage.info/jasr

ISSN
0976-9595
Short Communication

EVALUATION OF PHYTOCONSTITUENTS OF METHANOLIC ROOT EXTRACT *OF WITHANIA SOMNIFERA*

Shenkreppa Doddappa Desai[1], Gavishiddappa Andanappa Hadimani[2]*, Ishwar Basvantappa Bagoji[2], Prakash Biradar[3], Nanjappaiah H M[3], Shivakumar Hugar[3]

[1]*Shridevi Institute of Medical Science & Research Hospital, Tumkur*

[2]*Dept. Of Anatomy, Shri B M Patil Medical College, Hospital and Research Centre BLDE University, Bijapur*

[3] *Dept. of Pharmacology BLDEA's College of Pharmacy, Bijapur*

Corresponding author: gavish.hadimani@yahoo.com, gavish.hadimani@bldeuniversity.ac.in

ABSTRACT

The present research work was designed to identify the phytochemical constituents of methanolic extract of roots of *Withania somnifera*. *Withania somnifera* is widely used in Ayurvedic medicine, the traditional medical system of India. It is a member of the family Solanaceae. Therapeutic importance of the different parts of this plant has a long history. Fresh mature roots were shade dried at room temperature, coarse powdered and extracted with methanol by Soxhlet's extraction method. Thereafter, the extract was concentrated using rotary flash evaporator to obtain semisolid crude extract with the yield of 10.682%. *Withania somnifera* root extract was investigated for the presence of phytochemical constituents. The preliminary phytochemical evaluation of the *Withania somnifera* root extract revealed the presence of alkaloids, flavonoids, phenolics, carbohydrates, tannins, steroids and starch.

Keywords: *Withania somnifera*, Methanolic extraction, Phytochemicals

1. INTRODUCTION

Nature always stands as a golden mark to exemplify the outstanding phenomenon of symbiosis. The biotic and abiotic elements of nature are all interdependent. The quest for long, healthy and happy life is as old as man himself. Nature has provided a complete storehouse of remedies to relieve the ailments of mankind. The consistent effects have resulted in many effective means of ensuring health care. The seers of Ayurveda were able to understand and record the various aspects regarding the drugs that even today are difficult to understand with modern available parameters [1, 2].

Withania somnifera (*W. somnifera*, Ashwagandha) is widely used in Ayurvedic medicine, the traditional medical system of India. Its height is 3-4 feet and grows into a bush and is a member of the family Solanaceae. In India, its growth is maximum and at present this plant is cultivated for medicinal purpose. Therapeutic importance of the different parts of this plant has a long history and is mentioned in Charak Sanghita. It is an ingredient in many formulations prescribed for a variety of musculoskeletal condition (e.g., arthritis, rheumatism) and as a general tonic to increase energy, improve overall health and longevity and prevent disease in athletes, the elderly and during pregnancy. It is also used as an anti stressor and antioxidant agent [3, 4]. Number of reactive molecules generated through various biological redox reaction such as superoxide radicals (O_2-), hydroxyl radicals (OH), hydrogen peroxide (H_2O_2) and nitric oxide $(NO)^2$ which can directly react with biological macromolecule such as proteins lipid and DNA of health human cells and cause cell membrane disintegration, DNA mutation and protein damage deregulation of these reactive oxygen species (ROS) can further create cancer, atherosclerosis, cardiovascular disease, liver injury, ageing and inflammatory disease [5]. Antioxidants act as oxygen scavengers by interrupting the oxidation process by reacting with free radical, chelating catalytic metals [6]. Some synthetic antioxidants were developed in the past few decades but they are suspected of having some adverse effects. Therefore in search of suitable alternative natural antioxidants has received much attention to identify and develop more potent antioxidants of natural origin to replace synthetic ones. Different kinds of plant material have already been reported as natural antioxidants [7].

Ashwagandha root was used historically as an aphrodisiac. This herb is mentioned in the ancient Kama Sutra as an herb to be used for heightening sexual experience, this herb has the ability to restore sexual health and improve overall vitality while promoting a calm state of mind. Laboratory studies show it can produce nitric oxide which is known to dilate blood vessels. Hence, in the present study, the roots of *Withania somnifera* were investigated and their chemical composition was investigated with a view to assess their phytochemical potential.

2. MATERIAL AND METHOD

2.1. Drug preparations

2.1.1. Preparation of plant material

The roots of *Withania somnifera* were procured locally; plant was authenticated by Dr. M B Mulimani Professor, Department of Botany. SB Arts & KCP Science College, Bijapur by the studies include organoleptic tests and macroscopic and microscopic observations. After authentication the voucher specimen was deposited in the Department.

2.1.2. Methanolic extraction of Withania somnifera

Roots of *Withania somnifera* were washed twice using tap water and then washed again in distilled water to remove the dust. The roots were shade dried for 7–12 days at room temperature, until they were free from the moisture and then pulverized into coarse powder. The powdered root extracted with methanol by Soxhlet's extraction method. Thereafter, the extract was concentrated using rotary flash evaporator to obtain semisolid crude extract. The percentage yield of the extract was found to be 10.682%. The extract was stored in airtight container in refrigerator below 10^0C. Desired concentration of stock solution was prepared using distilled water for preliminary phytochemical investigation.

2.2. Preliminary phytochemical screening

A systematic and complete study of crude drugs includes a complete investigation of both primary and secondary metabolites derived from plant metabolism. Different qualitative test were performed for establishing profiles of various extracts for their nature of chemical composition. The extracts obtained were subjected to following chemical tests for identification of various phytoconstituents as per the methods given by Harborne [9]. There were no previously isolated compounds.

2.2.1. Test for starch

Dissolved 0.015 gm of iodine and 0.075 gm of potassium iodide in 5 ml of distilled water and add 2-3 ml of an aqueous extract of drug, blue color is produced.

2.2.2. Test for Steroids [10]

Salkowski test: 2-3 drops of concentrated sulphuric acid was added to chloroform solution, shaken and allowed to stand, appearance of red color in lower layer indicates the presence of sterols.

Liebermann-Burchard test: Extract was mixed with the chloroform and few drops of acetic anhydride and mixed well. Concentrated sulphuric acid was added from the sides of the test tube slowly until the ring appears; appearance of reddish brown ring indicates the presence of steroids.

2.2.3. Test for Flavonoids [11, 12]

Shinoda test: To the extract a few fragments of magnesium ribbon and concentrated hydrochloric acid was added. Appearance of red to pink color after few minutes indicates the presence of Flavonoids.

Lead acetate test: To the extract added few drops of aqueous basic lead acetate solution. Formation of yellow precipitate indicates presence of flavonoids**.**

Alkaline reagent test/ NaOH test: few drops of sodium hydroxide solution were added to extract. Intense yellow color disappeared after adding dilute HCl which indicates the presence of flavonoids.

2.2.4. Test for Alkaloids

The extract was basified with ammonia and extracted with chloroform. The chloroform solution was acidified with dilute hydrochloric acid, shaken well and filtered. The filtrate was used for testing the alkaloids.

Hager's test: The filtrate was treated with few drops of Hager's reagent. Formation of yellow precipitate indicates the presence of alkaloids [13].

Wagner's test: (Iodine in Potassium iodide): The acid layer was treated with few drops of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

*Mayer's test***:** (Potassium Mercuric Iodine solution): The acid layer was treated with few drops of Mayer's reagent. Formation of creamy white precipitate indicates the presence of alkaloids.

*Dragendorff's reagent***:** (Potassium Bismuth Iodide): The acid layer was treated with few drops of Dragendorff's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

2.2.5. Test for Tannins [14]

Gelatin test: To the extracts of the drug added 1% solution of gelatin containing 10% sodium chloride. Formation of white precipitate indicates the presence of tannins.

Ferric chloride test: To extracts few drops of 1% neutral ferric chloride solution were added, formation of blackish blue color indicates the presence of tannins.

2.2.6. Test for Saponins [13, 15]

Foam test: Small amount of extract of the drug was shaken with little quantity of water, if foam produced persists for 10 minutes; it indicates the presence of saponins.

Froth test: To 5 ml of extract of the drug added single drop of sodium bicarbonate solution. Shaken the mixture vigorously and left for 3 minutes. Formation of honey comb like froth indicates presence of saponins.

2.2.7. Test for Carbohydrates:

Small amount of extracts of the drug were dissolved in little quantity of distilled water and filtered separately. The filtrates were used to test presence of carbohydrates.

Molisch's test: The filtrate of the drug was treated with Molisch reagent and concentrated sulphuric acid was added from the sides of the test tube to form a layer. A reddish violet ring shows the presence of carbohydrates.

Benedicts test: to the filtrate added 2 ml Benedict's reagent and boiled in water bath. Formation of Green or reddish brown precipitate indicates presence of carbohydrates.

Fehlings test: Filtrates were hydrolyzed with dilute hydrochloric acid, neutralized with alkali and heated with equal amount of

Fehling's A and B solutions. Formation of green to yellow to red precipitate indicated the presence of reducing sugars.

2.2.8. Test for Phenols

Phenolic compounds: Extract was dissolved in alcohol and 1 drop of neutral ferric chloride was added to this. The intense color indicates the presence of phenolic compound.

2.2.9. Glycosides

0.5ml of extract was taken in a test tube and added with 1 ml glacial acetic acid containing traces of ferric chloride. To this solution 1 ml of concentrated sulphuric acid was added and observed for the formation of reddish brown color at the junction of two layers and the upper layer turned bluish green in the presence of glycosides.

3. RESULTS

3.1. Preliminary phytochemical screening

Preliminary Phytochemical screening of methanolic extract of roots of *Withania somnifera* revealed the presence of different kind of phytochemical components that are summarized in table 1.

Table 1: Preliminary phytochemical screening of Methanolic root extract of *Withania somnifera*

S. N.	Phytochemical	Test	Result
1	Test for starch	Iodine test	Present
2	Test for Steroids	Salkowski test	Present
		Liebermann-Burchard test	Present
3	Test for Flavonoids	Shinoda test	Present
		Lead acetate test	Present
		Alkaline reagent test/ NaOH test	Present
4	Test for Alkaloids	Hager's test	Present
		Wagner's test	Present
		Mayer's test	Present
		Dragendorff's reagent	Present
5	Test for Tannins	Gelatin test	Present
		Ferric chloride test	Present
6	Test for Saponins	Foam test	Absent
		Froth test	Absent
7	Test for Carbohydrates	Molisch's test	Present
		Benedicts test	Present
		Fehlings test	Present
8	Test for Phenolics	Lead acetate test	Present
9	Test for Glycosides	Foam test	Absent

4. DISCUSSION

The phytochemical screenings of chemical constituents related to biological activity of the plant root extract are alkaloids, flavonoids, phenolics, carbohydrates and tannins (Table 1). The quantitative phytochemical estimation indicate that *W. somnifera* root extract contains significant amount of phenolic, flavonoids, carbohydrate, tannin and alkaloid content which confirms its antioxidant property [16]. Phenolics content are very important plant constituents because they can act as reducing agents, hydrogen donors and metal chelator [17]. They also act as radical scavenger due to their hydroxyl groups. Flavonoids show their antioxidant action through scavenging or chelating process [18]. Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity [19]. Also, the carbohydrates in food are of major interest in relation to chronic diseases. Different types of carbohydrates give rise to different glycaemic responses, and also able to stimulate lipogenesis [20, 21]. Moreover, in the medicinal effects described in the ayurvedic, siddha, folk and chinese traditional recipe tannins, phenolic acids, flavonoids and alkaloids are the important ingredients to prevent against oxidative stress and decrease the activity of cholinesterase and xanthine oxidase and also alleviating the mucus secretion in the airway glands [22]. The results acquired in this study thus suggest the identified phytochemical compounds may be the bioactive constituents and this plant is proving to be a valuable reservoir of bioactive compounds of substantial medicinal merit.

5. CONCLUSION

In conclusion, the findings of the present study suggest that roots extract of the *Withania somnifera* possesses alkaloids, flavonoids, phenolics, carbohydrates, tannins, steroids and starch.

6. ACKNOWLEDGEMENTS

The authors are thankful to Principal of BLDEA's College of Pharmacy, Bijapur for providing facility to carry out extraction and phytochemistry also thankful to Dr. M B Mulimani Professor, Department of Botany, S B Arts & KCP Science College, Bijapur for authenticating the drug. Our special thanks to Prof. Dr. Kusal K Das for his constant support and guidance.

7. REFERENCES

1. Charaka S, Chakrapani DTCP, Varanasi; 1992.
2. Govind DSR. Bhaishajya R, Chowkambha Sanskrit Series office, Varanasi; 1961.
3. Bone K. Clinical applications of Ayurvedic and Chinese herbs. Monographs for the Western herbal practioner. Aust Phytotherapy Press; 1996; p. 137-41.
4. Dadkar VN, Ranadive NU, Dhar HL. *Ind J Clin Biochem,* 1987; **2:**101-108.
5. Aruoma OI, Halliwell B, Hoey BM, Butter J. Free *Radic Biol Med*, 1989; **6(6):**593-597.
6. Braca A, Sortino C, Politi M, Morelli I, Mendez J. *J Ethnopharmacol*, 2002; **79(3):**379-381.
7. Gulcin I, Buyukokuroglu ME, Oktay M, Kufrevioglu OI. *J Pineal Res*, 2002; **33(3):**167-171.

8. Packer L, Ong ASH. Biological oxidants and antioxidants: Molecular mechanisms and health effects. Champaign, USA: AOCS Press; 1997.
9. Harborne JB, Phytochemical methods: A guide to modern techniques of plant analysis, 3rd edition, Chapman and Hall, London; 2007: 135-203.
10. Gibbs RD. Chemotaxonomy of Flowering Plants, Vol.1, McGill Queen's University Press, Montreal and London; 1974.
11. Peach K, Tracey MV. Modern methods of plant analysis, Vol 3, Springer Verlag, Berlin; 1956.
12. Rizk AM. Fitoterapia; 1982:52(2): 35-42.
13. Trease GE, Evans WC. Pharmacognosy, 15th edition, WB Saunders Publishers, London; 2002.
14. Kokate CK. Practical Pharmacognosy. 4th edition, Vallabh Prakashan, New Delhi, India; 1994.
15. Sofowara A. Medicinal plants and traditional medicine in Africa. Chichester John Wiley & Sons, New York; 1993.
16. Dipankar C, Nikhil BG, Rhitajit S, Nripendranath M. *Asian J. Pharm Clin Res*, 2012; **5(4):**193-199.
17. Rice-Evans CA, Miller NJ, Bolwell PG, Bramley PM, Pridham JB. *Free Rad Res*, 1995; **22(4):**375-383.
18. Kessler M, Ubeaud G, Jung L. *J Pharm Pharmacol*, 2003; **55(1):**131-142.
19. Nobori, T., Miurak, K., Wu, D.J., Takabayashik, LA, Carson DA. *Nature*, 1994; **46:**753-756.
20. Weisburger JH. *Nutrition*, 2001; **16:**767-773.
21. Parks EJ. *Br J Nutr,* 2002; **87 (Suppl.2):** S247-S253.
22. Franova S, Nosalova G, Mokry J, Mahmud THK, Ather A. Phytotherapy of cough. In Iwu M (ed.) Advances in phytomedicine, U.S.A. Elsevier; 2006. p. 111-131.

MASTER CHART

	Control Gp		Stress Control		STD Group		Mucuna pruriens		Withania somnifera		MP+WS		ANOVA		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F	p-value	
Initial BW (Gm)	210.5000	11.30929	181.0000	10.31504	182.8333	16.59418	215.8333	14.28869	212.3333	13.21615	232.0000	22.79474	10.198	.000	Weight
Final BM (gm)	355.1667	21.09423	286.0000	23.20345	293.6667	25.36664	331.0000	43.53389	334.1667	40.14681	340.0000	45.25041	3.751	.009	
OS Index	.7120	.05617	.8558	.15970	.8176	.10260	.8107	.10390	.8001	.10600	.8046	.11917	1.078	.392	
Weight Gain	144.6667	17.33974	98.3333	3.14113	110.8333	10.60974	129.6667	24.63060	126.8333	26.65646	115.1667	26.70892	3.874	.008	
Hb (gm/dl)	13.3333	1.16562	14.7500	.52440	15.4333	1.45556	15.8333	.13663	16.7167	.68823	16.9500	.82644	13.085	.000	Hematology
WBC (cells/ cumm)	8033.3333	1440.37032	9966.6667	3859.87910	13916.6667	4206.85948	15466.6667	1842.46212	11433.3333	2989.09128	15383.3333	3194.00480	5.856	.001	
RBC (mill/ cumm)	7.6083	.98170	7.3467	.70580	8.2750	.75453	8.7800	.04099	9.5467	.37259	9.1833	.51317	11.252	.000	
Platelet(lakhs /cumm)	8.2233	1.83268	8.9133	.56797	7.5700	2.85353	8.9017	.24943	10.2517	2.35999	8.0300	.65830	1.783	.147	
PCV (%)	48.6833	5.67113	49.6667	2.32780	52.3167	6.10096	51.7833	2.94171	53.9833	5.63149	55.3833	2.15445	1.921	.120	
MCV (fl)	63.2333	4.73357	59.2000	.17889	61.8833	2.05272	60.2500	1.68375	62.8167	3.69941	59.8500	1.10408	2.261	.074	
MCH (pg)	18.0000	1.58493	17.7333	.50859	18.3167	.43551	18.1000	.61319	18.3167	1.11609	17.7167	.48751	.532	.750	
MCHC (gm/dl)	28.5333	2.10966	29.7000	.99599	29.5667	.94798	30.0833	.70828	29.1833	1.97324	30.0333	.77632	1.081	.391	
BLOOD UREA MG%	34.5000	5.89067	55.0000	1.41421	52.8333	5.49242	61.0000	4.24264	39.5000	13.83835	50.1667	4.35507	12.102	.000	OFT
SERUM CREATININE	1.4000	.32249	1.6000	.14142	1.0167	.44460	1.5500	.18708	1.2000	.32863	1.4000	.14142	3.571	.012	
SGOT (U/L)	28.6667	6.77249	47.6667	5.39135	38.1667	12.60820	47.0000	5.58570	34.0000	16.02498	45.3333	4.36654	4.045	.006	
SGPT (U/L)	40.6667	5.42832	44.5000	7.17635	38.8333	11.28568	51.5000	3.72827	40.3333	16.69331	43.1667	3.18852	1.469	.229	
TGL (mg/dl)	140.1667	5.26941	162.0000	7.04273	114.5000	37.78756	188.0000	2.00000	134.0000	26.72078	180.8333	7.46771	12.916	.000	Lipid Profile
Total cholesterol (mg/dl)	183.6667	34.23838	167.6667	12.65965	166.8333	14.63443	210.1667	3.97073	167.8333	28.91655	194.0000	11.71324	4.474	.004	
HDL (mg/dl)	35.5000	2.58844	33.6667	2.06559	29.8333	3.60093	37.0000	1.78885	36.3333	4.96655	33.5000	3.39116	3.851	.008	
LDL (mg/dl)	119.6333	30.55472	116.6000	26.03198	102.4333	13.35450	137.5000	3.89204	114.5667	35.94850	132.0000	8.33954	1.816	.140	
VLDL (mg/dl)	28.7000	1.75613	31.4667	4.49296	22.9000	7.55751	37.4000	.79246	28.6000	9.42168	36.2667	2.07429	6.018	.001	
Testicular Cholesterol (gm/mg)	53.64	4.61	95.76	9.59	60.05	6.41	73.87	11.15	49.37	3.55	72.99	2.92	34.745	.000	Reproductive Assay
LH (mIU/mL)	.0640	.00492	.1090	.01357	.1270	.03615	.1100	.04147	.0900	.00632	.1050	.01049	4.892	.002	
FSH (mIU/ml)	.3400	.08626	1.0583	.44544	.9950	.50895	.3500	.10488	.8933	.31570	.8700	.27568	5.726	.001	
Testosterone (ng/dL)	233.9633	30.37381	96.4717	30.43703	334.2383	146.12329	268.6467	26.69707	352.9017	47.75993	428.9600	8.69783	18.135	.000	
Sperm Copunt mill cells/ml	151.9167	10.22212	105.3333	13.82269	163.5833	19.10606	186.0000	5.15752	225.5417	15.69985	234.3750	30.80939	44.627	.000	
Sperm Motality%	60.4333	7.19463	46.8333	10.14646	61.0667	12.49555	66.6333	10.46779	78.3000	9.89929	75.7833	12.13267	3.862	.007	

MASTER CHART

	Control Gp		Stress Control		STD Group		Mucuna pruriens		Withania somnifera		MP+WS		ANOVA		
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	F	p-value	
Cortisol (µg/Dl)	.8233	.00816	1.0750	.10407	1.1233	.15423	.7583	.15664	.4933	.20403	.8170	.04401	18.990	.000	Stress Assay
MDA (µMoles/L)	8.4063	.75274	22.4480	1.72053	7.4050	.53668	8.1183	1.37873	6.8707	.16385	8.1090	.43888	219.363	.000	
NO (µMoles/L)	115.5567	8.13570	91.0500	25.17784	126.7233	37.46613	146.9100	35.54525	150.4833	4.33893	114.9743	18.90524	30.954	.000	
Vit C (µg/ml)	4.2943	.39945	2.8220	.19198	6.6997	.79675	4.131	0.97	6.2153	.14758	3.792	0.64	101.352	.000	
Vit E (µg/ml)	15.3100	.75387	9.3133	.59547	12.5300	.81922	14.7333	1.01958	16.6267	1.16757	14.2900	1.16306	44.583	.000	
Liver NO (µMoles/g)	211.9167	24.68620	459.8333	186.48946	283.8933	175.82423	217.1867	85.25606	165.8033	88.71760	183.2867	42.81555	5.116	.002	
Kidney NO (µMoles/g)	378.8067	28.23358	179.7900	27.51855	276.5367	70.63271	252.6433	31.19651	374.7067	35.00441	303.9733	27.80398	21.841	.000	
Testis NO (µMoles/g)	120.8533	19.71648	475.4400	26.81692	296.7900	45.74661	248.4933	31.91682	224.0267	29.29341	206.9833	33.77058	82.609	.036	
Liver MDA (µMoles/g)	90.0500	26.72913	166.9600	3.58168	128.4300	18.52679	69.1283	10.34392	76.8083	7.84720	71.9450	3.13577	44.200	.000	
Kidney MDA (µMoles/g)	81.5100	3.91126	108.4233	17.46610	93.6050	4.11757	94.8500	7.56372	96.3333	19.43148	89.4783	19.19151	2.467	.055	
Testis MDA (µMoles/g)	82.6200	10.38643	142.6800	12.57884	132.1300	18.20304	81.3700	16.78005	122.6133	21.30026	124.0250	28.80976	11.236	.000	
Mounting Frequency (MF)	53.6667	9.28799	48.1667	2.04124	61.1667	5.41910	58.5000	8.68907	58.1667	8.54205	58.5000	3.83406	2.797	.034	Mating behavior
Intromission frequency (IF)	5.8333	1.47196	5.1667	1.16905	7.8333	2.13698	9.5000	2.73861	9.8333	3.25064	8.1667	2.78687	3.804	.009	
Mounting latency (ML)Sec	523.5000	54.13225	834.0000	101.78605	663.6667	123.38179	728.8333	132.08697	606.3333	85.06390	812.5000	120.47863	3.851	.008	
Intromission latency (IL) Sec	592.5000	30.11810	879.3333	109.10301	708.8333	126.70662	777.8333	130.58854	712.0000	73.39210	873.5000	99.49824	7.103	.000	
Ejaculatory latency (EL) Sec	409.8333	38.80421	230.8333	52.74625	445.1667	88.95036	464.3333	75.22677	612.1667	95.06507	397.3333	72.94838	16.910	.000	
Number of mount (NM)	4.0000	.89443	2.5000	1.04881	4.3333	1.63299	3.6667	1.36626	3.6667	1.21106	3.0000	.89443	1.858	.132	

Aphrodisiac Effect Of Mucuna Pruriens Seed On Sexual Behavior Of Male Albino Rats

G A Hadimani, S D Desai, I B Bagoji, B S Patil
Department of Anatomy Shri B M Patil Medical College Hospital & Research Centre, BLDE University Bijapur 9901352604 gavish.hadimani@gmail.com

Introduction

Sexual dysfunction is a common problem with increase in prevalence and etiological factors, including degenerative diseases, increase in injuries and stress associated with industrialized lifestyles. Sexual dysfunction can be treated by both medical and surgical treatment modalities; however, plant-derived and herbal remedies continue to be a popular alternative for men and women seeking to improve their sexual life despite the availability of effective conventional medical treatments(1). In many countries, different varieties of plants have been used as sexual stimulants in traditional medicine. Indian Systems of Medicine use Mucuna pruriens(MP), a leguminous plant, for improving fertility. The plant is being cultivated in India, Sri Lanka, South East Asia and Malaysia (2). MP has been recognized as an aphrodisiac agent. Saksena and Dixit have reported that the number of spermatozoa increases when the rats were treated with bark extract of MP (3). Further, it has been reported that the sexual and androgenic activities in adult male rats were sustained while improving the mass of the muscles (4&5)

Methods

Animals: Twelve-week-old female (body weights around 175–200 gm) and male (body weights around 225–250 gm) albino rats of Wistar strain were used for the present study. The rats were housed singly in separate standard cages and maintained under standard laboratory conditions (temperature 24–28 ◦C, relative humidity 60–70%, 12 h light–dark cycle) with free access to solid pellet diet and water ad libitum throughout the study. Animals were randomly divided into five groups with six animals per group. Group I represented the control animal, animals in Groups II, III and IV were given oral suspension of MP extract for 45 days at 18:00 h, at doses of 150, 200 and 250 mg/kg, respectively, and Group V rats received Sildenafil citrate (SC) (5 mg/kg body weight) reference drug, which served as a positive control.

Mating behavior test: The test was carried out in accordance with the method of Agmo (6). Healthy male albino rats showing brisk sexual activity were selected for the study. Female animals showing regular oestrus cycle were used for mating behavior analysis. The mating behaviors were recorded and used for further analysis by giving scores for first four mating series. Test was terminated if the male rat failed to evince sexual interest. The occurrence and disappearance of events and phases of mating were recorded as soon as they appeared. Later, the frequencies and phases were determined by the recorded transcriptions: number of mounts before ejaculation or mounting frequency (MF), number of intromission before ejaculation or intromission frequency (IF), time from the introduction of female into the cage of the male up to the first mount or mounting latency (ML), time from the introduction of the female up to the first intromission by the male or intromission latency (IL), time from the first intromission of a series up to the ejaculation or ejaculatory latency (EL), number of intromission in a single attempt or number of intromission (NI). The pre-coital sexual behaviours such as chasing, nosing, anogenital sniffing and mounting were observed for up to 2 h of pairing. The values of the observed parameters for control and experimental groups were recorded.

Test for libido: Libido was assessed according to the method described by Davidson (7), later modified by Amin et al (5). This test was done using the MF of the mating behaviour test during 15th, 30th and 45th day. The number of mountings along with intromission and ejaculation were analysed.

Hormonal analyses: The blood was collected from retro orbital venous plexus of all animals at the 15th, 30th and 45th day of the experiment. The serum was separated, and testosterone and estradiol were measured by using RIA.

Results

Mating behaviour, test for libido and test for potency The test for libido showed that the pre-coital sexual behaviors, such as chasing, nosing and anogenital sniffing, were well performed in the Group III (200mg/kg) whereas in Groups I, II and IV the behaviours were not to the extent seen in Group III (200mg/kg). However, effect of Group III showed less effect than Group V. The test for potency has shown that the 200mg/kg dose of the test drug significantly increased the frequency of erections, quick flip, long flip and total reflex. Nevertheless, the effect was less when compared with Group V.

Adverse effects and acute toxicity

An increase in the body weights of the animals in all the groups was observed, whereas in Group III (200mg/kg) there was a gradual increase in body weight during the complete course of the study. When compared to control, marked histological changes were not observed in kidney and liver of the drug-treated groups (Groups II–V). Acute toxicity studies showed no mortality, and normal behaviour was observed in all the treated and control groups.

Hormonal analysis

Hormonal analysis revealed that the levels of testosterone and estradiol increased gradually in all the experimental groups.

Discussion and conclusion

In the present study, sexual behaviour tests showed that the ethanolic seed extract of MP possesses significant sexual function enhancing activity. Mating behaviour test revealed that the test drug at a dose of 200mg/kg significantly increased the MF, IF and EL in all the experimental days when compared to control. The test drug (200mg/kg) not only significantly increased the EL but also significantly reducted the ML and IL compared to control, which indicates the aphrodisiac nature of MP. A significant decrease in PEI (the potency and libido) was observed with the administration of MP extract at a dose of 200mg/kg in all three testing days. The test drug decreased PEI by enhancing either the potency or the libido. Similarly, all treated rats copulated more vigorously than controls, especially Group III was very good in overall performance. These results thus provide experimental support to the folk reputation of MP as sexual stimulating drugs without any toxic effect. A marked increase in potency in all experimental groups with a profound increase seen in Group III (200mg/kg). Further, the morphological study revealed an increase in testicular and epididymal weights, along with an increase in sperm count and motility. It was clear that the administration of MP not only increases aphrodisiac activity but also enhances the spermatogenic potential, as the action may be in the hormonal level. With Group III (200mg/kg) showing best results, it was concluded that changes were dose dependent. The result was close to the effects produced by SC (Group V), which was used as the standard reference drug in the experiment. From the present investigation, we conclude that the ethanolic extract of MP 200mg/kg body weight possesses potent aphrodisiac activity in normal male albino rats without any gastric ulceration and adverse effects. This result is the scientific evidence in favor of the claims made in Indian Systems of Medicine that the MP is clinically useful as sexual invigorator in males.

References

Rowland, D.L., Tai, W., 2003. A review of plant-derived and herbal approaches to the treatment of sexual dysfunctions. Journal Sex and Marital Therapy 29, 185–205.

Kharelep, C.P., 2004. Encyclopedia of Indian Medicinal Plants (Regional Western Therapy Ayurvedic and Other Traditional Usage, Botany). Springer-Verlag, Berlin Heidelberg, New York, pp. 318–319.

Saksena, S., Dixit, V.K., 1987. Role of total alkaloids of Mucucna pruriens bark in spermatogenesis in albino rats. Indian Journal of Natural Products 3, 3–37.

Rao, M.R., Parakh, S.R., 1978. Effect of some indigenous drugs on the sexual behavior of male rats. Indian Journal of Pharmacological Sciences 40, 236.

Amin, K.M.Y., Khan, M.N., Rahman, S.Z., Khan, N.A., 1996. Sexual function improving effect of Mucuna pruriens in sexually normal male rats. Fitoterapia 67, 53–58.

Agmo, A., 1997. Male rat sexual behaviour. Brain Research Protocols 1, 203–209.

Davidson, J.M., 1982. In: Zewi, H. (Ed.), and Sexology, sexual biology, behaviour and therapy: selected papers of Fifth World Congress of sexology erusalem 1981. Amsterdam, Holland. Excerpta Medica, Princeton, Oxford, pp. 42–47.

EFFECT OF METHANOLIC EXTRACT OF WITHANIA SOMNIFERA ON HEMATOLOGICAL PARAMETERS OF MALE ALBINO RATS

GAVISHIDDAPPA A. HADIMANI1, Dr. S D DESAI2, PRAKASH BIRADAR3, ISHWAR B. BAGOJI1,
1-Lecturer Dept. of Anatomy, 2- Principal SIMS Tumkur, 3- Lecturer Dept of Phamacology,
BLDE UNIVERSITY'S SHRI B M PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE
BIJAPUR, KARNATAKA, INDIA

INTRODUCTION:

Withania somnifera is an extensively used herb in the Indian system of Medicine-Ayurveda. This herb is being used traditionally since more than 4000 years in India. Ashwagandha(Withania somnifera) is used as a tonic in the treatment of spermatopathia, impotence and seminal depletion (Nadkarni) and the men who used the herb enjoyed higher vigour performance (Boone, 1998).

Objective: The present study was conducted to assess the effect of methanolic extract of Withania somnifera on albino wister rats, to see its effect on various hematological parameters.

MATERIAL AND METHODS:

Laboratory-bred adult male albino Wister rats fed with laboratory stock diet and water ad libitum, and weighing 150 200 g were used. Rats are acclimatized a week to the laboratory conditions at 22 24° C and a 12 h light: dark (circadian) cycle. The acclimatized animals divided into four groups of six animals each. Group I rats were healthy controls (treated with normal saline). Group II rats were standard and treated with sildenafil citrate at a dose of 5mg/kg body weight subcutaneously. Group III rats were treated with low dose of withania somnifera at a dose of 250mg/kg body weight orally, and Group IV rats were given high dose of withania somnifera at a dose of 500mg/kg body weight orally. Animals were treated for 45 days and later they were sacrificed on day 46th. 4ml of Blood samples were collected in EDTA tube by the retro-orbital route on 15th and 45th and processed for hematological, assay.

Statistical Analysis: Collected data was analyzed for mean ± SD (SE). All the parameters were compared by using proper statistical tests. Parametric unpaired't' test and non-parametric Mann Whitney test to study the differences between groups, and parametric paired 't' test/ non-parametric wilcoxon matched paired signed rank test for comparing within group. The level of statistical significance was set at $p<0.05$

RESULTS

Group I: Control, Group II: Standard group treated with sildenafil citrate, Group III: treated with Withania somnifera 250mg/Kg body weight, Group IV: treated with Withania somnifera 500mg/Kg body weight. Each value is mean + SD (SEM) of six observations in each group. Results are tabulated according to group and displayed in the form of graph. Inference column indicates statistical significance, S- Significant, NS- Not Significant, HS- Highly Significant.

Gravimetry

Fig 1 Shows comparison of weight of rats before and after giving drug, all the groups shows there is significant increase in the body weight, group 1(control rats) and group III treated with Withania somnifera 250mg/kg body weight shows highly significant increase in the final body weight in comparison with the initial body weight.

Hematology parameters

Hemoglobin estimation

While comparing hemoglobin parameters of control rats with other groups and standard group with other groups on 45th day it was noticed that there was increase in the level of hemoglobin in group II, group III and group IV when comparing with control rats, increase was highly significant statistically in group II and III. There was increase in the level of hemoglobin in group III and decrease in hemoglobin in group IV when comparing with group II rats, increase & decrease was not significant statistically. (fig 2).

Red Blood Corpuscles Count

Comparison of RBC count of control rats with other groups and standard group with other groups on 45th day. There was significant increase in the level of RBC count in group II, group III and group IV when comparing with control rats, and increase was highly significant statistically in group II & group III. There was increase in the level of RBC count in group III and decrease in RBC count when comparing with standard group rats, increase and decrease was not significant statistically(fig 3).

White Blood Corpuscles Count

Comparison of WBC count of control rats with other groups and standard group with other groups on 45th day. There was significant increase in the level of WBC count in group II (treated with sildenafil citrate), group III treated with Withania somnifera 250mg/kg body weight and group IV treated with Withania somnifera 500mg/kg body weight when comparing with control rats, and increase was highly significant statistically in the entire group. There was increase in the level of WBC count in group III and group IV when comparing with standard group rats, increase was not significant statistically (fig 4).

Platelet Count

Comparison of Platelet count of control rats with other groups and standard group with other groups on 15th day. There was increase in the level of platelet count in group II, group III and IV when comparing with control, but the increase was highly significant statistically. There was increase in the level of platelet count in group III and decrease in platelet count when comparing with standard group rats, increase was significant statistically but the decrease was not significant statistically (fig.5)

CONCLUSION

All the parameters clearly indicate there is increase in the levels of hematological parameters in group II, group III, and group IV. Group II treated with Sildenafil citrate which is used as aphrodisiac drug so as been taken as standard drug. Supplementations low dose of Withania somnifera i.e. 250mg/kg body weight improve the hematological parameters in Group III. This indicates improvement in the general health. Supplementations high dose of Withania somnifera i.e. 500mg/kg body weight also increases hematological parameter but not much affective like low dose. This clearly indicate dose of 250mg/kg body weight of Withania somnifera is effective than 500mg/kg bw

REFERENCE

1. Nadkarni, K.M. Indian Materia Medica; Popular Prakshan Limited: Bombay, India, 1976; p. 1291.
2. Boone K (1998) Withania-The Indian ginseng and anti-aging adaptogen. Nutr. Healing. 5, 5-7.
3. Rohatash k. Bhardwaj, Aditi bhardwaj & Gangwar, S. K. Efficacy of Ashwagandha supplementation on hematological and immunological parameters of Japanese quails, Int. J. Of sci & nat, 2012: 3(2): 476-478
4. Bhardwaj, R.K. and Gangwar, S.K. (2011) Effect of dietary supplementation of Withania somnifera on egg production and egg quality parameters in Japanese Quails. International Journal of Advanced Biological Research. I.J.A.B.R., 2011, 1(1):32-34.
5. Arun Kumar, Mohammad Ali, Ranjit Kumar, Swapnil Suman, Hemant Kumar, A. Nath, J. K. Singh Dhyanendra Kumar Withania Somnifera Protects The Haematological Alterations Caused By Sodium Arsenite In Charles Foster Rats, Int. J. Res. Ayurveda Pharm. 2013 ; 4(4), 491-4.

Gravimetry

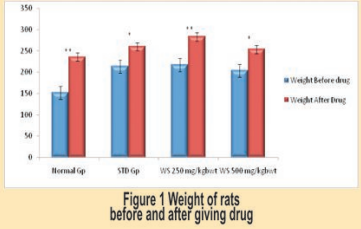


Figure 1 Weight of rats before and after giving drug

Hemoglobin estimation

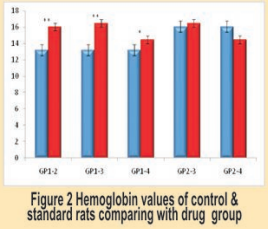


Figure 2 Hemoglobin values of control & standard rats comparing with drug group

Red Blood Corpuscles Count

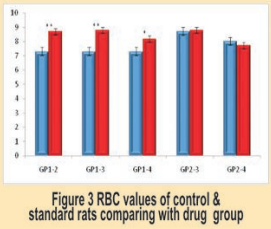


Figure 3 RBC values of control & standard rats comparing with drug group

White Blood Corpuscles Count

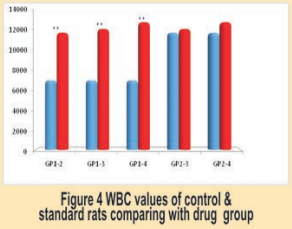


Figure 4 WBC values of control & standard rats comparing with drug group

Platelet Count

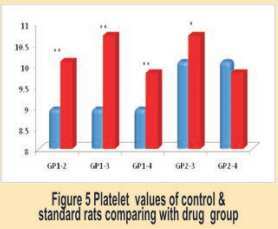


Figure 5 Platelet values of control & standard rats comparing with drug group

From

Dr.R.S. Wali
Chairman,
Institutional Animal Ethics Committee (IAEC),
Prof. & HOD, Dept. of Pharmacology,
BLDEU's Shri. B.M.Patil Medical College,
BIJAPUR.

To,

Mr. Gavishiddappa A Hadimani,
Tutor, Dept. of Anatomy,
BLDEU's Shri. B.M.Patil Medical College,
BIJAPUR.

ETHICAL CLEARANCE CERTIFICATE

The Institutional Animal Ethics Committee (IAEC) of this College met on 31.05.2011 at 10.30am to scrutinize the Research Project submitted by faculty member of this College.

After scrutiny the following research project has been accorded ethical clearance.

Title: "Aprodiasic effect of mucuna pruriens & withania somnifera & their effect on male reproductive organs of albino rats".

Principal Investigator: Mr. Gavishiddappa A Hadimani, Tutor, Dept. of Anatomy.

23/8/2012

Dr. R. S. Wali
Chairman, (IAEC)
Prof. & HOD,
Dept. of Pharmacology
BLDEU's Shri. B. M. Patil
Medical College,
BIJAPUR.

Professor & HOD
Dept. of Pharmacology
BLDEU's Shri B.M. Patil
Medical College, BIJAPUR.

23.08.2012

B.L.D.E.U'S SHRI.B.M.PATIL MEDICAL COLLEGE, BIJAPUR-586103

INSTITUTIONAL ETHICAL COMMITTEE

Ethical Committee
BIJAPUR-586103
OUT WARD
No 13/2011
Date 05/04/11
B.L.D.E.U's Shri B. M. PATIL Med.col College

DR.M.S.BIRADAR
CHAIRMAN .I.E.C.
BLDEU'S SHRI: B.M.PATIL MEDICAL COLLEGE
BIJAPUR-586103

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this college met on 31-03-2011 at 10-30am to scrutinize the Synopsis/Research projects of post graduate student/undergraduate student/Faculty members of this college from ethical clearance point of view. After scrutiny the following original/corrected & revised version Synopsis of the Thesis/Research project has been accorded Ethical Clearance.

Title "Aphrodisiac effect of Mucuna pruriens and Withania somnifera and their effect on Male Reproductive organs of albino rats"

Name of P.G. /U.G.Student /Faculty member Mr. G.A Hadimani
Lecturer in Anatomy

Name of Guide Dr. S.D. Desai, Prof & HOD. Anatomy

DR.M.S.BIRADAR
CHAIRMAN
INSTITUTIONAL ETHICAL COMMITTEE

Following documents were placed before E.C.for securitization:
1) Copy of Synopsis/Research project
2) Copy of informed consent form
3) Any other relevant document's