Lepedium meyeni Based Green Synthesized Silver Nanoparticles: A Potential Therapeutic Approach for Improving the Reproductive Functions in Adult Male Sprague Dawley Rats

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A dissertation submitted in the partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY IN

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2023

Dedicated to My Father

Who gave me the opportunity to

Study from the best institutions and

Supported me at every endeavor

DECLARATION

I hereby affirm that the work presented in the following thesis is my individual effort, and all the content within this thesis is of my own creation. I have not previously submitted any portion of this work for consideration towards any other academic degree or elsewhere.

Ameer Hamza

CERTIFICATE

This dissertation "*Lepedium meyeni* Based Green Synthesized Silver Nanoparticles: A Potential Therapeutic Approach for Improving the Reproductive Functions in Adult Male Sprague Dawley Rats", submitted by **Mr. Ameer Hamza** is accepted in its present form by Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of Master of Philosophy in Zoology (Reproductive Physiology)

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List of Abbreviations

Abbreviation	Full Form
LM@AgNPs	*Lepedium meyeni* based green synthesized silver nanoparticles
LM	*Lepedium meyeni*
$AgNO_3$	Silver Nitrate
UV	Ultraviolet
FTIR	Fourier Transform Infra-Red Spectroscopy
PXRD	Powder X-ray Diffraction
SEM	Scanning Electron Microscopy
EDX	Energy Dispersive X-Ray Spectroscopy
Kg	Kilogram
µl	Microliter
µg	Microgram
g	Gram
ml	Milliliter
%	Percentage

1: Abstract

Male infertility, a prevalent and unrecognizable health issue, is affecting human beings at an alarming rate. Chemically synthesized drugs to treat the infertility are associated with severe complications. Potent versatile therapeutic agents with minimum side effects are required to overcome reproductive health challenges. The current research work aims at synthesis of a novel silver nanoparticles (LM@AgNPs), its characterization with evaluation of its effects on reproductive organs, hormone synthesis, and semen parameters in adult male Sprague Dawley rats. LM@AgNPs were synthesized from *Lepedium meyeni* (LM) using coprecipitation method and characterized with UV Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR), Powder X-ray Diffraction (PXRD), Energy Dispersive X-ray Spectroscopy (EDS), and Scanning Electron Microscopy (SEM). The rats (n=15) were divided into three groups and administered with distilled water, LM, and LM@AgNPs for 28 days through oral gavage for evaluation of their effects on reproductive system. The UV-Visible Spectrum gave a characteristic surface plasmon resonance band peak at 41 5nm. FTIR highlights major functional groups fabricated on metallic silver. The newly synthesized crystals exhibit Face Centered Cubic symmetry with crystallite size 15.57nm based on PXRD analysis while SEM measurements indicate the particle size of 21.126 with highest proportion of reduced silver confirmed through EDS. LM@AgNPs induced morphometric and morphological changes in seminiferous tubular structure with fertility enhancing properties. There was a significant increase in testosterone concentration ($P=0.0003$) along with significant improvement in semen profile ($P \leq 0.0001$) following treatment with LM@AgNPs. In conclusion, LM@AgNPs demonstrated fertility enhancing properties with minimal side effects highlighting their potential as a potent therapeutic agent in male reproductive system of adult male Sprague Dawley rats.

2: Introduction

Male infertility is an issue of grave concern in the current global arena (Zhu *et al.*, 2022). A male is marked infertile if he is unable to achieve pregnancy in a reproductively active fertile female for one year (Olooto, 2012). Infertility impacts more than 180 million people globally and 15% couples in the United States (Leslie *et al.*, 2023).In Pakistan male partner related infertility contributes up to 21% in overall infertility cases (Javaid *et al.*, 2022). According to researchers on male fertility, male factors contribute to 40-50 % of human fertility problems (Kurkowska *et al.*, 2020). Many couples have been facing compromised fertility as shown by epidemiological survey (Abdalla *et al.*, 2023). According to estimates: 60% of married females who engage in regular unprotected sexual activity become pregnant after six months of living together, 90% by twelve months, and 95% between 18 to 24 months (Sulivan *et al.,* 2023). One couple out of ten experiences infertility and looks for medical assistance (Wischmann *et al.*, 2022). It is estimated that Infertility afflicted 8–12% of couples worldwide and male component was the primary contributing factor in 50% of those cases(Agarwal *et al.*, 2021). Poor Semen quality and deficiencies in semen are indicators of male infertility (Zańko *et al.*, 2022). Low motility, insufficient spermatozoa (azoospermia/oligospermia), and abnormal morphology have all been associated to male infertility.(Okonofua *et al.*, 2022). Infertility impacts reproductive health as individuals grow older(Chaudhuri *et al.*, 2022).

Although male infertility is common, relatively very little is known about its etiology There are several risk factors associated with day by day increase in male infertility including genetic (Abdul-Razzaq, 2023), endocrinological (Ghosh *et al.*, 2022),and physiological(Swathi Krishna *et al.*, 2022). Researchers have shown that 10 % of the human genome is linked with reproductive processes (Choy *et al.*, 2018). There are hundreds of examples of genes associated with infertility. Out of genetic mutations pool, Cystic fibrosis transmembrane conductance regulator gene mutation is an example of the phenomenon in this regard due to which bilateral absence of vas deferens occurs leading to male infertility (Kamiński *et al.*, 2020). Mutations in other genes (for example: MLH1, ERCC1, MSH2) lead to reproductive disorders(Burke *et al.*, 2022; Mukherjee *et al.*, 2023). Epimutations and mutations in genes associated with male germ lines can affect the functionality and production of spermatozoa (Aitken *et al.*, 2020). Advancement in next generation sequencing has enabled reproductive physiologists to

highlight abnormalities in the genetic makeup of individuals causing infertility (Dai *et al.*, 2021). Whole Exome Sequencing (WES), a high throughput sequencing technique, has been used to highlight the genes responsible for human infertility (Jiao *et al.*, 2021). Similarly, the functionality of different hormones influences male fertility (Bhattacharya *et al.*, 2019; Dutta *et al.*, 2019; Mann *et al.*, 2020; Salas-Huetos *et al.*, 2021). Hypogonadism, Hyperprolactinemia, thyroid disorders, pituitary disorders, adrenal gland disorders, varicocele, endocrinological gland tumors, and obesity results in hormonal imbalances. Among Physiological factors exposure to diseases such as diabetes, cardiovascular diseases, and long-term fever may affect the fertility of male. In addition to these, exposure to different endocrine disrupters (Martínez *et al.*, 2022), environmental pollutants (Selvaraju *et al.*, 2021), pesticides (Reshi *et al.*, 2022), and insecticides (Kara *et al.*, 2020) affected the fertility rate across the globe. Modern lifestyle accompanied with unhealthy diets, stress, and sedentary habits impacted the fertility in men badly (Gallo, 2022; Gantenbein *et al.*, 2021; Osadchuk *et al.*, 2023). Similarly mental health plays a significant role in fertility. Mental disorders such as depression, anxiety, and social isolation significantly are contributing to male infertility(Hanna *et al.*, 2020; Öztekin *et al.*, 2020; Simionescu *et al.*, 2021).

Only a small portion of male factor infertility can be treated successfully using conventional procedures (Dabaja *et al.*, 2014). Contrarily, secondary methods like Invitro Fertilization (IVF) (Iketubosin *et al.*, 2018), Intra Cytoplasmic Sperm Transfer (Sutcliffe, 2000), Artificial Insemination (Ombelet *et al.*, 2015), Intra Uterine Insemination(Abdelkader *et al.*, 2009), and foster adoption(Chandy, 2016; Shiraishi *et al.*, 2021) are typically considered as alternatives to male infertility. Medications (Fang *et al.*, 2022), adoption of healthy lifestyles (Tomada *et al.*, 2023), Sperm retrieval techniques(Chu *et al.*, 2022), and control of serious medical conditions e.g., cardiovascular disorders and diabetes (Burke *et al.*, 2022)etc. have been employed in treating male infertility. Since last four decades, there has been decline in the sperm count and sperm quality alarmingly leading to a condition where 1/20 person is facing infertility (Mustafa *et al.*, 2022). So, there is a dire need to prepare cost effective and inexpensive agents to treat male infertility.

Nanotechnology is a diverse field and has emerged as a revolutionary scientific discipline in the modern era (Omran, 2020; Tawade *et al.*, 2023). Nanotechnology provides

potential solutions to various problems in different branches of science(Aithal *et al.*, 2015; Shah *et al.*, 2021) Nanomaterials such as nanotubes, quantum dots, fullerene derivatives, and nanowires have received greater attention recently due to their application in life sciences(Braydich-Stolle *et al.*, 2005; Kashyap *et al.*, 2023; Kucherenko *et al.*, 2019). Nanotechnology involves understanding of properties, production, manipulation and applications of structures and devices; their control on nanoscale presenting new opportunities to develop novel medicines (Haleem *et al.*, 2023). Subjects on nanoscales have completely different properties than that of larger scales(Liu *et al.*, 2022). Since last few decades, scientists are paying special attention to this field dealing with reactions at atomic and molecular level (Lu *et al.*, 2011).

Nanoparticles are particles having dimensions between 1-100 nm ranges of diameter (Anzini *et al.*, 2022; Hamawand *et al.*, 2020; Lasenko *et al.*, 2022). Metal nanoparticles exhibit unique magnetic, thermal, mechanical, electrical, and optical properties(Aziz *et al.*, 2019; Khan *et al.*, 2022). Nanoparticles find its applications in different fields due to their sizes and shapes (Yousaf *et al.*, 2020). Nanoparticles have various morphological forms such as nanobelts, nanorods, nanosheets(Periyasamy *et al.*, 2020; Ponzoni, 2020) which find their applications in solar cells (Singh *et al.*, 2022), gas sensors (Raturi *et al.*, 2023), light emitting diodes(V. Chauhan *et al.*, 2023) and biological probes (Loos, 2023). Production of silver nanoparticles can be carried out employing various chemical, biological, and physical methods (Ijaz *et al.*, 2022). Both chemical and physical methods involve utilization of toxic chemicals which act as toxicant to the environment (Islam *et al.*; Ren *et al.*, 2016; Viswanath *et al.*, 2017). Moreover, these techniques are expensive, energy consuming, and unsustainable (De *et al.*, 2021). Silver is an attractive metal for nanoscale due to its biosensing (Loiseau *et al.*, 2019), antimicrobial (Chahande *et al.*, 2020; Gharpure *et al.*, 2020), and imaging applications (Anderson *et al.*, 2019). Silver nanostructures demonstrate plasmonic waveguiding potentials (Shenashen *et al.*, 2014), chemical and biological sensing (Jaswal *et al.*, 2023), catalysis (Araujo *et al.*, 2019) and surface enhanced Raman spectroscopy(Chen *et al.*, 2020). Excessive exposure to nanoparticles may prove hazardous to human health (Mohajerani *et al.*, 2019). Nanoparticles can enter the bodies of human beings through gastrointestinal, dermal, pulmonary (Dreno *et al.*, 2019). Owing to their tiny nature, they can easily pass through blood testes barriers (Iftikhar *et al.*, 2021) and blood brain barriers (Terstappen *et al.*, 2021). Nanoparticles also exhibit

toxicological effects on various organs such as testes(Torabi *et al.*, 2017), liver (Boey *et al.*, 2020), and kidney(Wu *et al.*, 2018).

Among metal nanoparticles silver particles (AgNPs) have gained attention of scientists due to their properties (Chugh *et al.*, 2021). AgNPs find its applications in clothing (Ahmed *et al.*, 2022), household water filters (Ndebele *et al.*, 2021), cosmetics (Xi *et al.*, 2022), detergents (Adelere *et al.*, 2020), cooking utensils (Ali *et al.*, 2023), cell phones (Merghni *et al.*, 2022; Peng *et al.*, 2021), and medical devices (Jaswal *et al.*, 2023). Silver nanoparticles have demonstrated antibacterial (Bruna *et al.*, 2021), antiviral (Jeevanandam *et al.*, 2022) and anticancerous activities (Oves *et al.*, 2022). Chemically synthesized nanoparticles due to their widespread applications have created certain environmental issues of grave concerns which not only impacted human beings but also affected marine and biological systems (Wani *et al.*, 2011). AgNPs toxicity impacts include lethality (Ellegaard-Jensen *et al.*, 2012), cytotoxicity (You *et al.*, 2012), oxidative stress, cell membrane damage, DNA damage (Ahn *et al.*, 2014; Olugbodi *et al.*, 2023; Ramzan *et al.*, 2022; Zareii *et al.*, 2023), mitochondrial malfunctioning (Suthar *et al.*, 2023), cellular proliferation(Pang *et al.*, 2020), and inflammation(Ferdous *et al.*, 2020).

Synthesis of nanoparticles using biological materials has emerged as an important branch of nanotechnology named, "**Biogenic synthesis or green synthesis**" (Rana *et al.*, 2020). Synthesis of silver nanoparticles can be carried out using plant's, animal and microorganism material (Pirtarighat *et al.*, 2019). Biogenic synthesis has received great attention due to their advantages including scalability, low cost, and safety(Hano *et al.*, 2021). Molecules of plant such as sugars, terpenoids, proteins, alkaloids, polyphenols and phenolic acids play a pivotal role in the bio reduction of silver (P. Chauhan *et al.*, 2023). Variations in the sizes and morphologies of nanoparticles can be achieved using various kind of bio extracts (Kumar *et al.*, 2020).

***Lepedium meyeni* (LM)** also named Peruvian Maca, an ancient root, is native to Andean region which has been cultivated for two thousand years (Todorova *et al.*, 2021). The root is a source of multiple essential nutrients such as essential amino acids, fibers, fatty acids, Calcium, Iron, Copper, Zinc, and vitamin C(Carvalho *et al.*, 2020). Maca is a rich reservoir of bioactive substances which have medicinal properties with health effects (Peres *et al.*, 2020). LM has

anticancerous, anti-inflammatory, antioxidant, neuroprotective, memory enhancement, antidepressant, and skin protectant impacts (Yang *et al.*, 2023). LM is characterized by different colors of hypocotyls viz red, black, and yellow demonstrating different biological properties (Tafuri *et al.*, 2021). The edible part of the plant is its hypocotyl which can be used in dried and wet forms. The root of LM has long been used as an active ingredient in chocolate, coffee, and oils due to its fatigue relieving, body strengthening, and fertility improving impacts (Ibrahim *et al.*, 2022). Moreover, LM roots can reach up to 20 cm in circumference while plant can attain a height of 10-20 cm (Peñaloza *et al.*, 2023). LM biocomponents are unable to cross the blood brain barrier(Yu *et al.*, 2021). LM has positive effects on the reproductive system of males as it improves human semen parameters (Tafuri *et al.*, 2021).

Owing to the threatening nature of infertility, there is a dire need of finding inexpensive and cost-effective drugs to treat male infertility with minimum side effects. The current research work aims at synthesis of novel LM@AgNPs using LM extract with evaluation of its effects on reproductive system of adult male Sprague Dawley rats. The main objectives of our present investigation are characterization of LM@AgNPs and estimation of its impact on the reproductive organs focusing on spermatogenesis, hormonal profiling of hypothalamus pituitary gonadal axis and semen analysis.

3: Methodology

3.1: Study Site

The current experiment was performed in Reproductive Physiology Lab, Department of Zoology, Quaid-i-Azam University, Islamabad and Inorganic Chemistry Lab, Institute of Chemistry, Islamia University Bahwalpur.

3.2: Plant Collection

The plant was collected from a local pinsar store,grinded into fine powder, and aqueous extract was prepared by adding 10 g of LM powder into 100 ml of distilled water.

3.3: Chemicals

All the chemicals used in experimentation were of analytical grade (99% Pure) and they were bought from Sigma Aldrich chemical company.

3.4: Biogenic Synthesis of LM@AgNPs

Initially, 100 ml of silver nitrate solution (concentration 1mM) with 50 ml aqueous plant extract was taken in a beaker and mixed thoroughly with continues stirring (200 rpm) at 72 °C. The color change from yellow to black solution showed the formation of LM @AgNPs. To separate newly formed LM@AgNPs from the solution, centrifugation at 6000 rpm for 17 minutes was carried out. The obtained precipitates were separated from the liquid, washed with ethanol, dried in a furnace at 100 °C and stored for further use.

3.4: Characterization of LM@AgNPs

3.4.1: UV-Visible Spectroscopy

UV-Visible spectroscopy revealed the synthesis of LM@AgNPs by reducing silver nitrate to silver (Thermoscientific spectrophotometer UV-3100PC). The data was collected in wavelengths ranging from 300 to 700 nm.

3.4.2: Fourier Transform Infra-Red Spectroscopy

The reduction of $AgNO_3$ into LM@AgNPs by reducing agents found in LM aqueous extract was determined by using a Fourier Transform Infrared Spectrometer (Thermo Scientific USA FTIR, Model: Nicolet Summit Lite). The measurements were taken between 400 and 4000 cm^{-1}

3.4.3: Powder X-Ray Diffraction

Powder x-ray Diffractometer (Bruker D8 Advance PXRD) with Cu radiation source and high-resolution Lynx Eye detector was used to evaluate the crystallite size of freshly prepared LM@ AgNPs.

3.4.4: Scanning Electron Microscopy

The surface morphology of prepared LM@AgNPs was examined using scanning electron microscope (SEM) (Model: MIRA3 TESCAN). SEM pictures were captured at various magnifications. The elemental makeup of LM@AgNPs was also analyzed using energy dispersive spectroscopy (EDS). The Fiji Image J software program was used to determine the grain size.

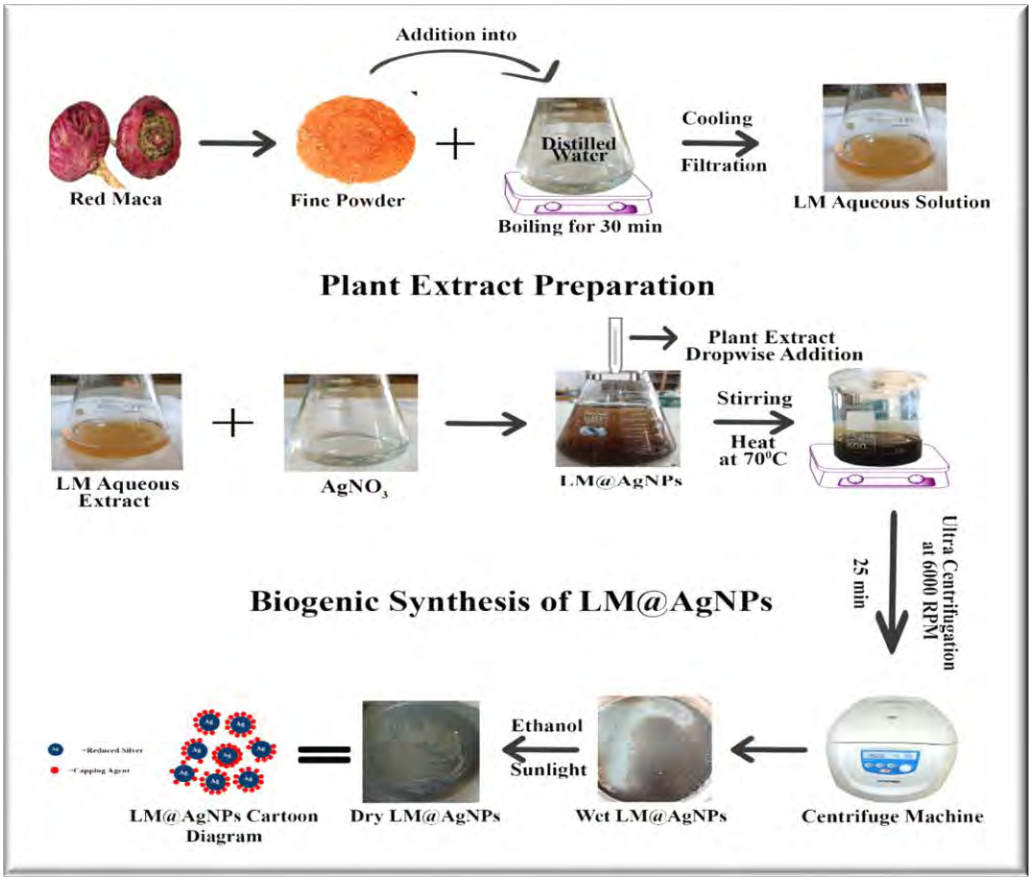

Figure 1:Biogenic Synthesis of *Lepedium meyeni* Based Green Synthesized Silver Nanoparticles

3.5:Animals

Fifteen adult male Sprague Dawley rats (75-95 days old) were taken from the primate house of Zoology Department,Quaid-i-Azam University, Islamabad. Animals were aclimitized in the laboratory for seven days before the start of experiment. The animals were kept in stainless steel cages and provided with standard laboratory feed. Water was available in excess in glass bottles.All the protocols were approved by Bioethical Committee of Quaid-i-Azam University, Islamabad.

3.6: Experimental Design

Animals (n=15) were divided into three groups. The control group (n=5) received one ml of distilled water/rat /day. LM treatment group (n=5) received a dosage of 200 mg/kg/rat/day. LM@AgNPs treatment group (n=5) received a dosage of 200 mg/kg/rat/day. All animals received the same treatment for 28 days. After dissections, blood and organs were collected on the 29^{th} day for further analysis.

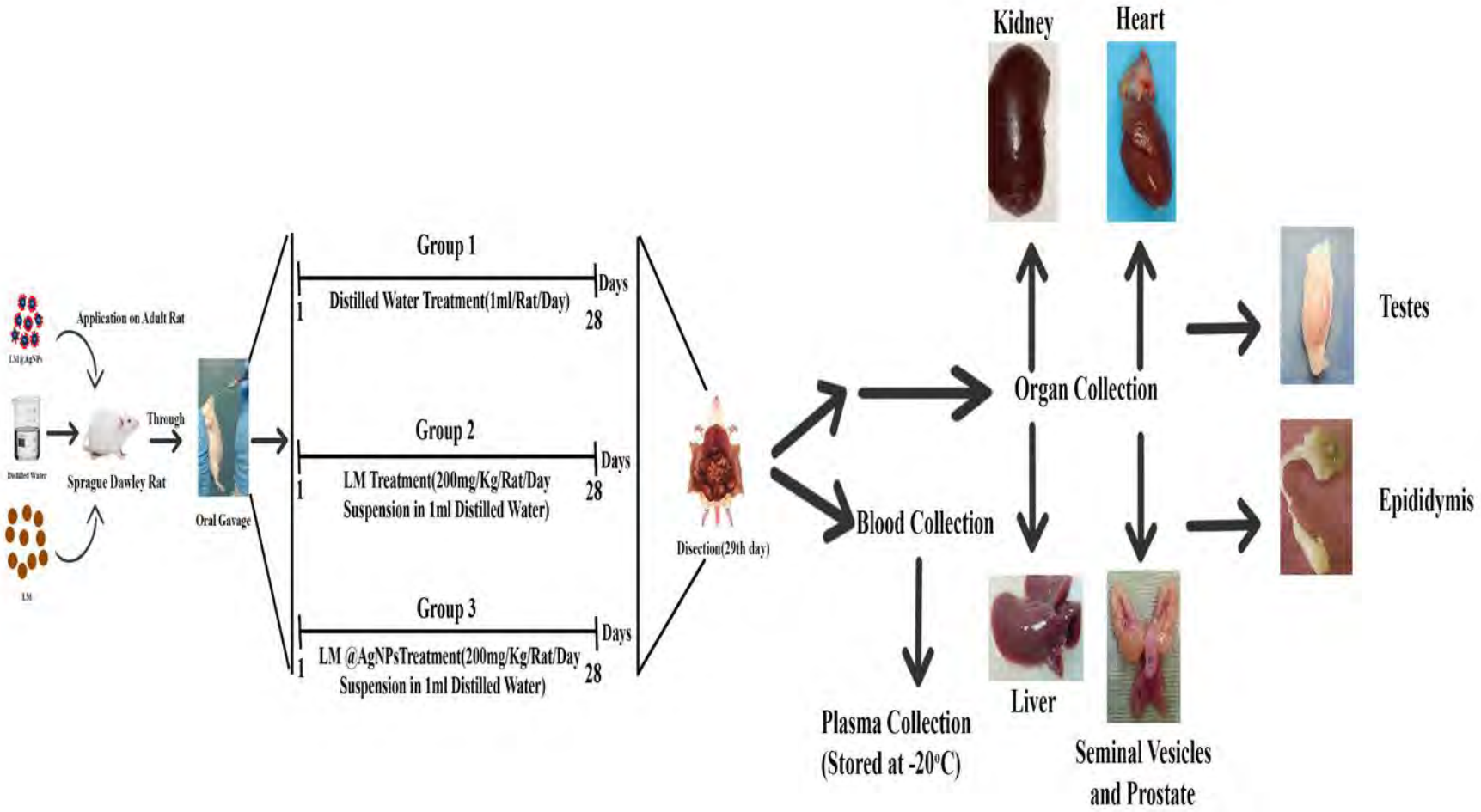

Figure 2:Schematic Representation of Experimental Design

3.7: Histology

Morphological changes in testicular tissues after application of LM and LM@AgNPs were examined. After collection of tissues the tissues were undergone these treatments:

3.7.1: Fixation

The tissues were placed in sera for 5 hours.

The tissues were immersed in 10% formalin for 24 hours.

3.7.2: Dehydration

Subsequently, the tissues underwent dehydration employing progressive increase in alcohol concentrations.

Table 1: Dehydration process with progressive increase in alcohol concentration

Time(minutes)	Alcohol Treatment
60	70% ethanol
60	80% ethanol
60	90% ethanol
60	95 % ethanol
90	100% ethanol I
90	100% ethanol II
90	100 % ethanol III

3.7.3: Embedding

Subsequent steps were carried out for embedding purposes. Tissues were placed in xylene and fixed in paraffin in the following order:

Table 2: Sequential embedding treatments	
Time (minutes)	**Treatment**
60	Xylene I
60	Xylene II
60	Xylene III

3.7.4: Albumin slides preparation

3.7.4.1: Preparation of albumin

- Two egg whites were added to 1200mL of deionized water.
- Stir for 5 minutes with a magnetic stirrer.
- 4mL of ammonium hydroxide was added into the solution.
- For 5 minutes stirred again.
- Then passed through a low –grade filter (coffee filter).
- Finally, the slides were coated with a thin layer of freshly prepared albumin and were allowed to dry for an overnight period.

3.7.5: Microtomy

After embedding the tissues in paraffin, they were mounted on wooden blocks with melted wax. Five micrometer thin sections were cut with the help of microtome Tissue filled long ribbons were stretched before being fixed in clean albumenized glass slides that have been previously prepared. These slides were stored on a Fischer slide warmer set to 60^{o}C. After that, glass slides were placed in an incubator for a night to finish stretching.

3.7.6: Staining

Slides were stained in the following grades:

Table 3: Sequential tissue slide staining treatments	
Time(minutes)	Treatment
3	Xylene I
3	Xylene II
3	70% ethanol
3	90% ethanol
3	100% ethanol
3	Washing with distilled water
8	Hematoxylin
8	Washing with distilled water
8	Acidified Alcohal
8	Washing with distilled water
8	Bluing water
8	Washing with distilled water
8	90% ethanol
8	Eosin
8	Washing with distilled water
8	90% ethanol
8	100% ethanol I
8	100% ethanol II
8	100% ethanol III
8	Xylene I
8	Xylene II

Then, on each slide, add 3 drops of DPX per mounting.

3.7.7: Light Microscopic Study

Tissue section (5 μm thickness) were observed under a light microscope at 100X magnifications (Nikon,187842, Japan). All experimental groups slides were examined. The testicular luminal and tubular diameter, and epithelial height was calculated using Fiji Image J software.

3.8: Hormonal Analysis

3.8.1: Quantitative Determination of Testosterone Concentration

Commercially available Enzyme Immunoassay Kit (Perkin Elmer Inc, USA) was utilized to measure the EDTA plasma testosterone level in the Sprague Dawley rats.

3.8.1.1: Principle of the Assay

The Enzyme Linked Immunosorbent Assay of Testosterone is based on competitive binding principle between Testosterone-HRP conjugate and Testosterone in a test sample for a constant value of rabbit anti-Testosterone. The incubation of goat anti-rabbit IgG coated wells with standard solutions, control, treated samples, testosterone -HRP conjugates and rabbit anti testosterone for 60 minutes at 37 ºC was carried out. During this period, a specific amount of HRP-labeled Testosterone competitively binds with endogenous testosterone in the standard solutions, control, and treated samples for a specific number of binding sites of Testosterone antibody. As the concentration of testosterone in the material rises, the amount of Testosterone peroxidase conjugates immunologically attached to the well gradually decreases. The wells are subsequently washed, and unbound Testosterone peroxidase conjugate is eliminated. Addition of TMB reagent into the wells and incubation results in a blue color. Addition of 2N HCl stops the reaction and absorbance of sample mixture is assessed at 450 nm of wavelength spectrophotometrically. The intensity of color is directly proportional to the enzyme's concentration and inversely proportional to unlabeled Testosterone concentration in the wells. A standard curve is plotted between standard's concentration and. Testosterone concentration values in unknown samples were calculated comparing the values with that of standard curve.

3.8.1.2: Procedure of Assay

Add 25 µl of standards, controls, and specimens into the wells. 50 µl of rabbit anti-Testosterone reagent was poured into the wells and mixed it properly for 30 seconds. 100 µl of Testosterone-HRP Conjugate Reagent was also added into the wells and incubated for 60 minutes at 37 ºC. Subsequently, washing procedure was carried out five times and flicked with washing Buffer (1X). 100 µl of TMB Substrate was added into the wells and incubated at 20 ºC for 20 minutes. Addition of 100µl of stop solution stopped the reaction. The blue color of

samples in the wells turned to yellow color. The absorbance was noted at 450 nm within 5 minutes using spectrophotometer (680xR, Bio-Rad, Tokyo, Japan)

3.9: Semen Analysis

3.9.1: Sperm Count

10 µl of semen was collected from cauda epididymis diluted with normal saline in 1: 300. The counting was carried out using Improved Neubauer Chamber ($0.1mm^2$) in large five squares at 400X magnification viewed under a light Microscope (Olympus CX 41 attached with Tucsen USB 2.0 H series Camera) (Anous *et al.*, 2017; Lorenzetti *et al.*, 2012; Mohamed *et al.*, 2012).Sperm concentration was assessed using formulae:

Concentration/ml =(Count in 5 squares) (Dilution Factor) (0.05×10^6)

3.9.2: Sperm Viability

Sperm viability was examined using eosin-nigrosin staining. Briefly 10 µl of diluted semen was poured onto a glass slide; mixed with 3µl of 1% Eosin solution and 2 µl of 10% Nigrosin solution. The slide was observed under 40x objective lense; live and dead spermatozoa were counted; and results were presented in form of percentages of viable and non-viable cells(Ngaha Njila *et al.*, 2019).

3.9.3: Sperm Morphology

Sperm morphology was assessed under the light microscope at 400X magnification. Different head and tail abnormalities were observed and sperms were categorized into abnormal and normal spermatozoa according to slight modifications in the existing protocols (Kapourchali *et al.*, 2020; Yelumalai *et al.*, 2019)

.3.9.4: Sperm Motility

Sperm motility was assessesed immediately after rats' disections.10 µl of diluted sperm suspension was poured onto a glass slide and examined with a light microscope at 100X magnification. Sperms were categorized into two categories: motile and non motile. Results were expressed as percentages of motile and non-motile spermatozoa (Uzunhisarcikli *et al.*, 2007).

3.10: Statistical Analysis

All the computed data was analyzed using one-way analysis of variance (ANOVA) carried out utilizing GraphPad Prism Software. Post-hoc Tukey' test was performed for multiple comparison between various groups. Mean ±SEM were used to present all the findings. The significant level was set to $P<0.05$. All the results are expressed in table forms.

4: Results

4.1: Characterization Of LM@AgNPs

4.1.1: UV-Visible Spectroscopy

Mixing $AgNO_3$ and aqueous extract of LM results in the formation of LM@AgNPs. The formation of LM@AgNPs was confirmed through UV- Spectroscopy. AgNO3 solution displayed a maximum absorbance peak at 296.77 nm. In Figure 3A, Absence of any peak from 350 nm to 700 nm indicated zero formation of silver nanoparticles without reducing agents. LM UV spectrum has shown two distinct peaks at 240 nm and 267 nm (Figure 3B) These peaks correspond to the phytochemicals present in the plant. While LM mediated silver nitrate solution is showing a peak at 415 nm (Figure 3C) serving as an indicator of the formation of LM@AgNPs. It also shows the ability of phytochemicals present in LM to reduce silver from Ag^{+1} state to Ag^{0} state. Several factors including initial concentration of $AgNO_3$ and plant extract, stirring time, and temperature affect the yield of LM@AgNPs.

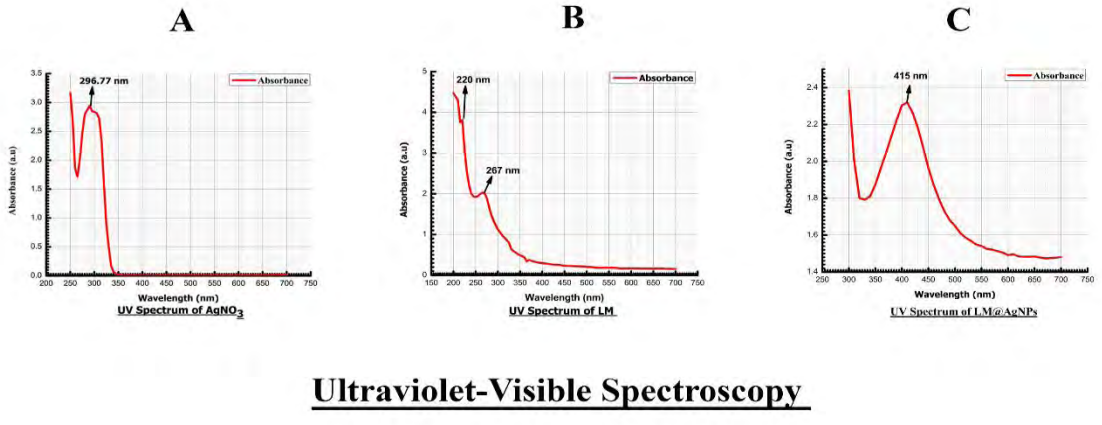

Figure 3: Comparative analysis of Silver Nitrate, LM, and LM@AgNPs through UV-Spectroscopy

4.1.2: Fourier Transform Infrared Spectroscopy

Fourier Transform Infrared Spectroscopy (FTIR) spectrum of LM demonstrated peaks at wavenumber: 3279 cm^{-1}, 3036 cm^{-1}, 2851 cm^{-1} ,1730 cm^{-1} ,1605 cm^{-1} ,1431 cm^{-1} ,1360 cm^{-1} ,1319 cm^{-1} ,1227 cm^{-1} ,1040 cm^{-1} ,742 cm^{-1} ,610 cm^{-1}. On the other hand, the newly biosynthesized LM@AgNPs demonstrated peaks at 3740 cm^{-1} ,3463 cm^{-1}, 2917 cm^{-1}, 2856 cm^{-1}, 2367 cm^{-1} 2067 cm^{-1} ,1739 cm^{-1}, 1620 cm^{-1} ,1385 cm^{-1} , 1115 cm^{-1} ,864 cm^{-1} , 750 cm^{-1} , and 642 cm^{-1} wavenumbers.. A characteristic peak at 3740 cm^{-1} and 3463 cm^{-1} represents the presence of free hydroxyl group and -OH stretching with intermolecular bounded forces respectively. The specific peaks at 2917cm^{-1} represents C-H bond and 2856 cm^{-1} represents -CH_3 . A sharp peak at 2367 cm^{-1} signifies the presence of the carboxylic acid group. It indicates the presence of flavonoids present in the plant extract. A peak at 1739 cm^{-1} corresponds to the ester group and 1620 cm^{-1} represents the bio fabrication of C=C on silver. The sharp peak at 1385 cm^{-1} represents the C-O functional group and 1115 cm^{-1} peak identifies Alkyl amine group. A minor groove at 864 cm^{-1} represents the presence of aromatic compounds. So, the chief functional groups which are involved in the bio reduction of silver are carbonyl, hydroxyl, carboxylic, and aromatic groups. These results have been in close association with all these previous findings. (Ahmad *et al.*, 2020; Lingegowda *et al.*, 2012).

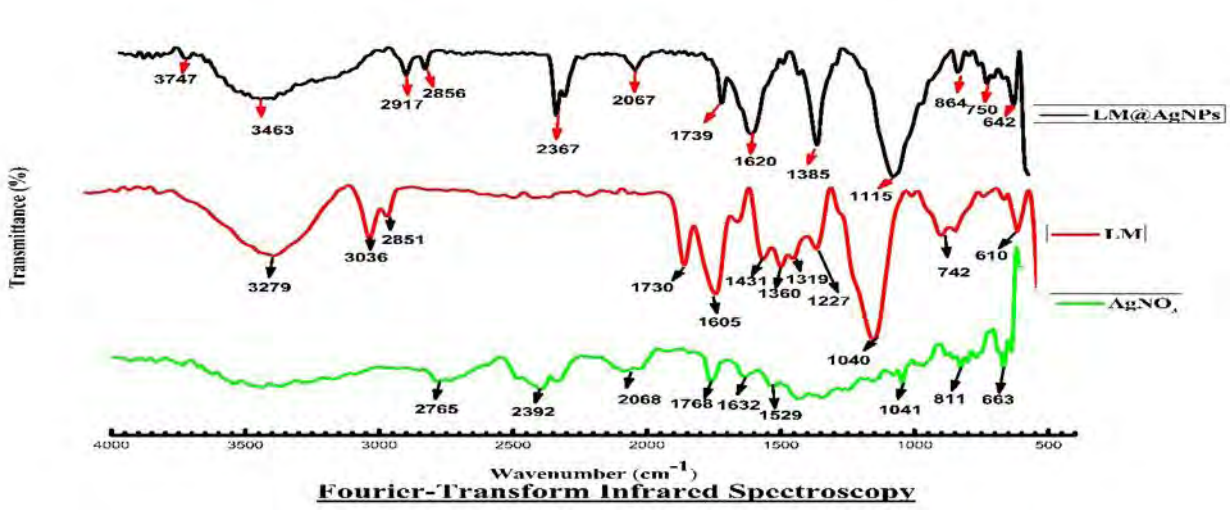

Figure 4 Comparative analysis of Silver Nitrate, LM, and LM@AgNPs through Fourier Transform Infrared Spectroscopy

4.1.3: Powder X-Ray Diffraction

In PXRD pattern four different peaks were observed at 2θ values of 27.46, 37.84, 44.03, and 64.27 degrees in correspondence with (111), (111), (200), and (220) planes respectively. Peaks corresponding to these planes indicate face centered cubic structure of nanoparticles and it is in accordance with the face centered cubic structure reference from Joint Committee of Powder Diffraction Standard card number-087-0720. These results are in alignment with previous studies (Ravichandran *et al.*, 2019).The crystallite size of LM@AgNPs is 15.57 nm which is calculated using Debye Scherrer equation, $D = K\lambda / \beta Cos\theta$ (Shaaban *et al.*, 2021). Thus, the newly fabricated LM@AgNPs crystals are Face Centered Cubic with crystallite size 15.57nm.

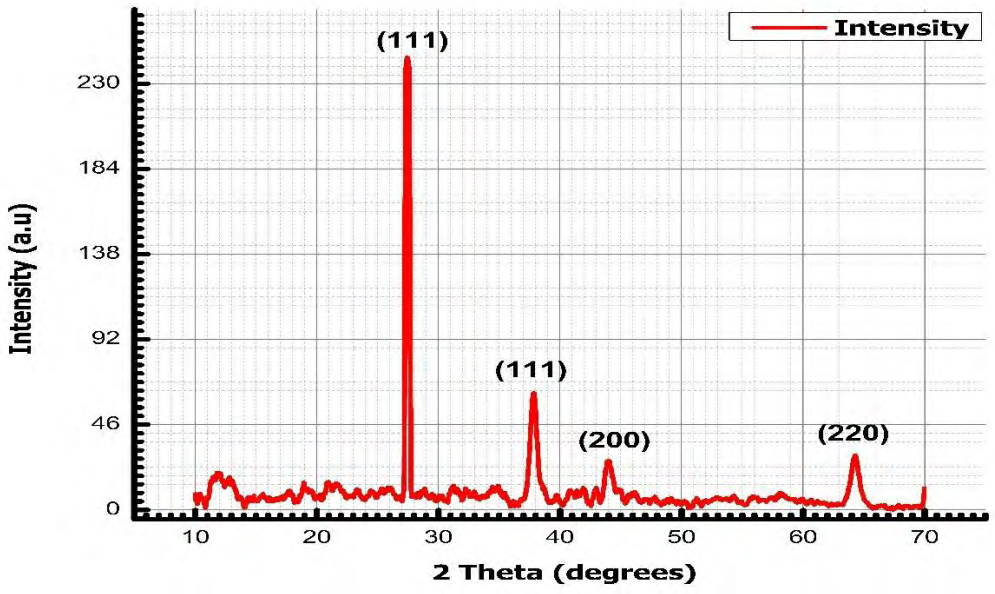

Figure 5:Powder X Ray Diffraction pattern of LM@AgNPs

4.1.4: Scanning Electron Microscopy

Scanning electron microscopy (SEM) was carried out to explore the morphology of plant-based silver nanoparticles. The SEM images showed the crystalline shape of silver nanoparticles. The average grain size of silver nanoparticles was measured using Fiji Image J software. The average grain size of LM@AgNPs is 21.126 nm.

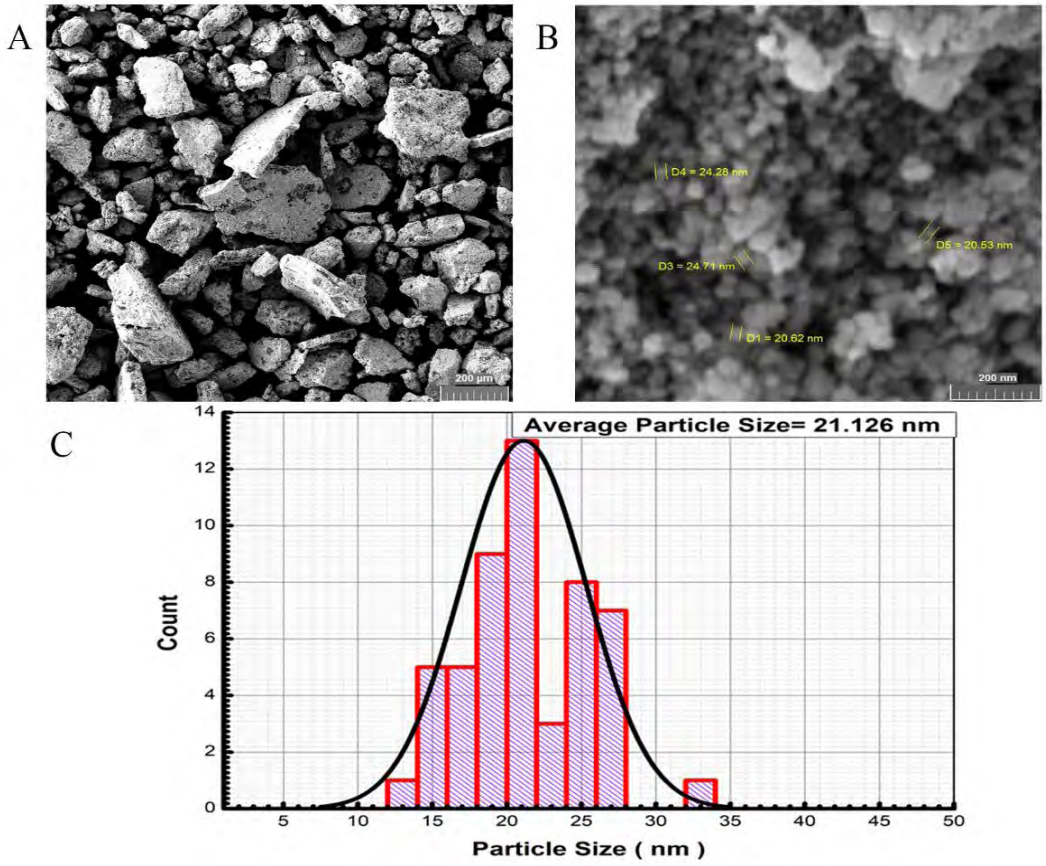

Figure 6: Scanning Electron Microscopy images of LM@AgNPs crystals at different magnifications (A and B) with particle Size distribution analyzed using Fiji image J (C)

4.1.5: Energy Dispersive X Ray Spectroscopy

Figure 7 displays the energy dispersive x-ray (EDX) micrographs of LM@AgNPs. The elemental composition of LM@AgNPs precipitates was assessed using SEM equipped with an EDX detector. Owing to surface plasmon resonance, the EDX analysis has depicted a strong signal at 3.1 keV of the silver region. The spectral signals of carbon oxygen, potassium and sodium were also present due to extra organic moieties adsorbed on the surface of LM@AgNPs from LM.

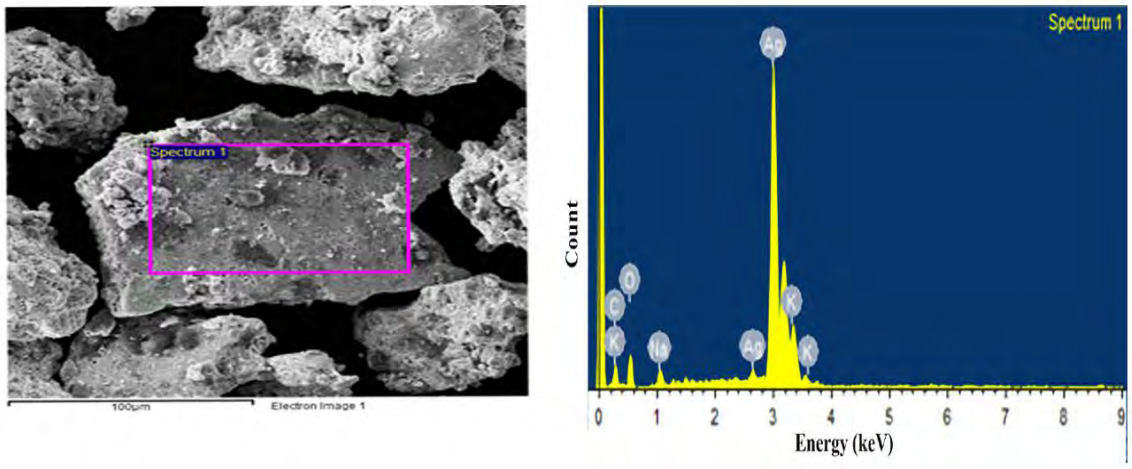

Figure 7: Energy Dispersive X-Ray micrograph showing elemental composition of LM@AgNPs

4.2: Weight Parameters

Table 4 represents body weight gain and change in reproductive organs weight after 28 days of continuous dosage exposure across various groups. The mean ± SEM values of all parameters are insignificant. There is non-significant change in body weight gain across different experimental groups. Reproductive organs' weight did not change significantly across non- treatment and treatment groups

Table 4: Effect of drugs treatment on body weight and reproductive organ weights in experimental groups of adult Sprague Dawley rats

	Treatment (µg/g per day)			
Parameters	**Control**	**LM**	**LM@AgNPs**	**P Value**
Initial Body Weight	135.8 ± 3.84	143.6 ± 4.29	153.6 ± 2.31	
Final Body Weight	197.2 ± 4.55	204.6 ±3.48	210.4 ±6.25	
Body Weight Gain (g)	61.40 ± 4.68	61.00 ± 1.70	56.80 ± 5.11	0.690
Testes Weight (g)	1.07 ± 0.02	1.21 ± 0.08	1.14 ± 0.03	0.179
Epididymis Weight (g)	0.54 ± 0.04	0.59 ± 0.04	0.51 ± 0.02	0.326
Seminal Vesicles Weight (g)	0.99 ± 0.09	1.19 ± 0.12	0.86 ± 0.06	0.079
Prostate Weight (g)	0.62 ± 0.03	0.66 ± 0.01	0.58 ± 0.02	0.459

Values are expressed as Mean ± SEM.
Statistical Analysis: Analysis of Variance (One Way ANOVA followed by Tukey's Multiple Comparison Test)
"a" represents comparison of control with respective treatment groups while "b" represents comparison of LM vs LM@AgNPs

4.3:Histology

4.3.1: Morphological Changes in Testes

Testicular histological examination showed intact semniferous tubular structure in control and treated groups.All the tubules are covered with a thick layer of tunica albugenia. Arrangement of all tubules were similar in control and treated groups. Lumen of LM@AgNPs treated semniferous tubules were completely filled with spermatozoa. Most of the lumens in control and LM treated groups were empty showing the least number of spermatozoa in comparison to that of LM@AgNPs. The morphological changes in testes are shown in Figure 8.

4.3.2: Morphometric changes in Testes

The morphometric analysis of seminiferous tubules (n=375; n=125 per group with randomly chosen n=25/animal) was carried out using Fiji image J. The methodology to carry out the analysis is shown in Figure 9. Changes in tubular diameter were observed in all experimental groups. The tubular diameter increased significantly after administration of LM ($P \leq 0.05$) and LM@AgNPs ($P \leq 0.0001$) in comparison to that of control. Moreover, a significant increase in LM@AgNPs ($P \leq 0.0001$) in tubular diameter is seen in comparison to LM. In addition to tubular diameter, LM@AgNPs increased lumen diameter and epithelium height and tubular area significantly as tabulated in table 5.

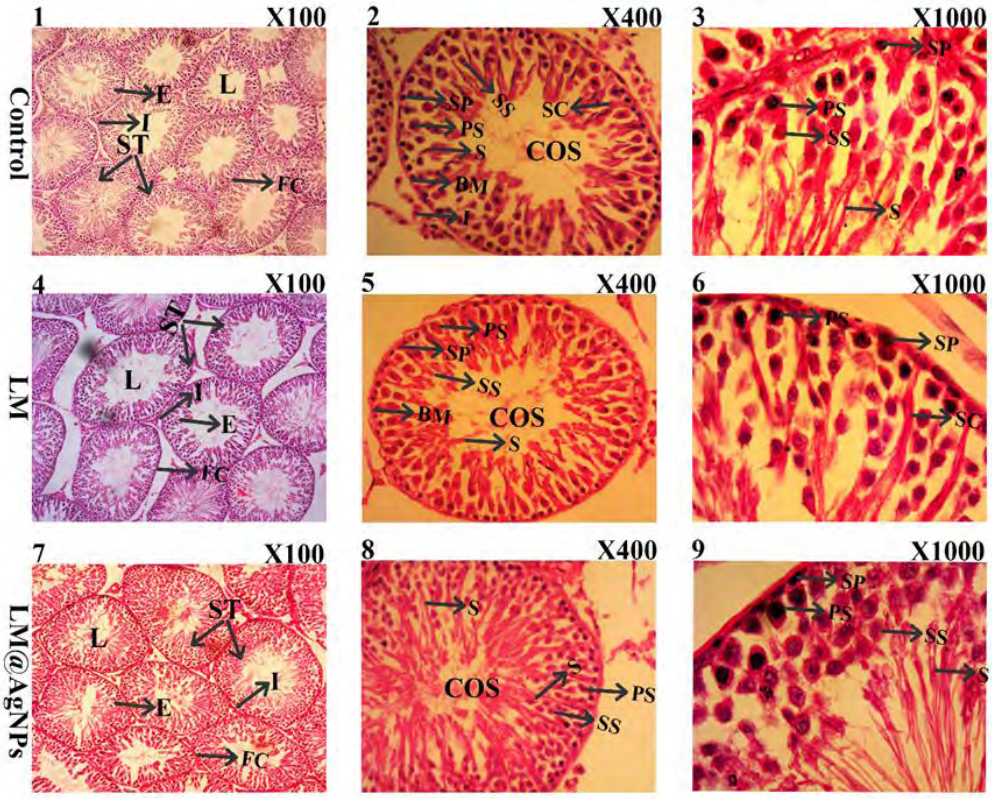

Figure 8: Photomicrographs of seminiferous tubules in control and experimental groups of adult male Sprague Dawley rats

Description

Picture1,2,3**;** 4,5,6**;** 7,8,9 represents testicular histology of control, LM, and LM@AgNPs groups at 40X,100X, and 1000X magnifications respectively. The control group is showing compact arrangement of STs, normal epithelia, and lumens with low number of spermatids. LM treated group is depicting compact STs with normal epithelium and lumens with low spermatid counts. LM@AgNPs supplemented group is demonstrating normal compact arrangement of STs, epithelium, and lumens filled with spermatozoa

Abbreviations

ST: Seminiferous tubule **L:** Lumen **I:** Interstitial space **E:** Epithelium **FC:** Fibrous Connective tissue **COS:** Clump of Spermatozoa **PS:** Primary Spermatocyte **SS:** Secondary spermatocyte **S:** Spermatids **BM:** Basal Membrane **SP:** Spermatogonia **SC:** Sertoli Cells

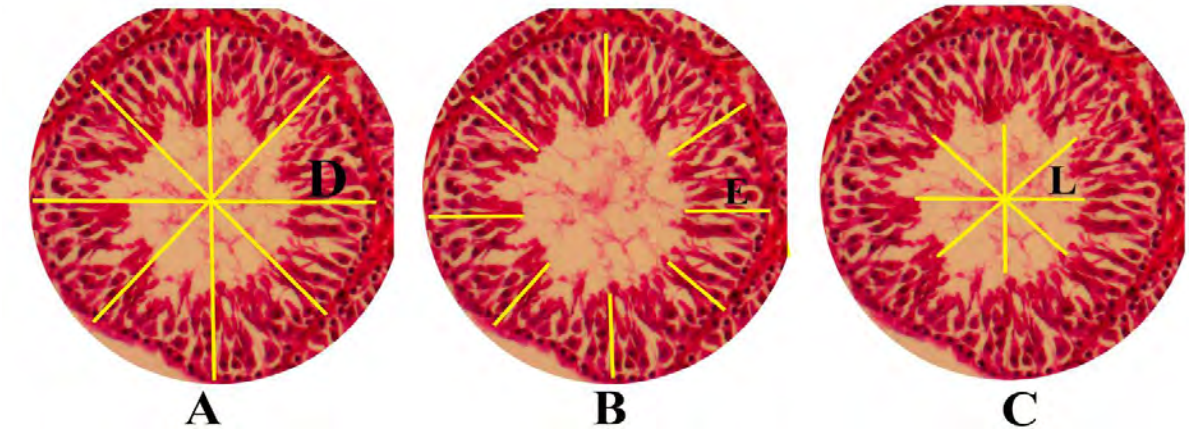

Figure 9: Photomicrographs of seminiferous tubules demonstrating the methodology to perform morphometric analysis

Picture A: D Represents Tubular diameter **Picture B:** E Represents Epithelium Height **Picture C:** L Represents Lumen Diameter

Table 5: Effect of drugs treatment on seminiferous tubules' parameters in experimental groups of adult Sprague Dawley rats

Parameters	Treatment (µg/g per day)			P Value
	Control	LM	LM@AgNPs	
Tubular Diameter (µm)	99.50 ± 0.94	103.10 ± 1.24 a^{*}	110.00 ± 0.87 a^{****} b^{****}	<0.0001
Lumen Diameter (µm)	44.95 ±0.56	45.22 ± 0.66	49.56 ± 0.68 a^{****} b^{****}	<0.0001
Epithelial Hight (µm)	23.48 ± 0.24	24.81 ± 0.28 a^{**}	26.02 ± 0.34 a^{****} b^{**}	<0.0001
Tubular Area (μm^2)	7862 ± 121.70	8513 ± 188.60 a^{**}	9662 ± 152.80 a^{****} b^{****}	<0.0001

Values are expressed as Mean ± SEM.
Statistical Analysis: Analysis of Variance (One Way ANOVA followed by Tukey's Multiple Comparison Test)
P value ≤ 0.05 is represented as **"*"**; P value ≤ 0.01 is represented as "**"; P value ≤ 0.001 is represented as "***"; P value ≤ 0.0001 is represented as "****".
"a" represents comparison of control with respective treatment groups while "b" represents comparison of LM vs LM@AgNPs

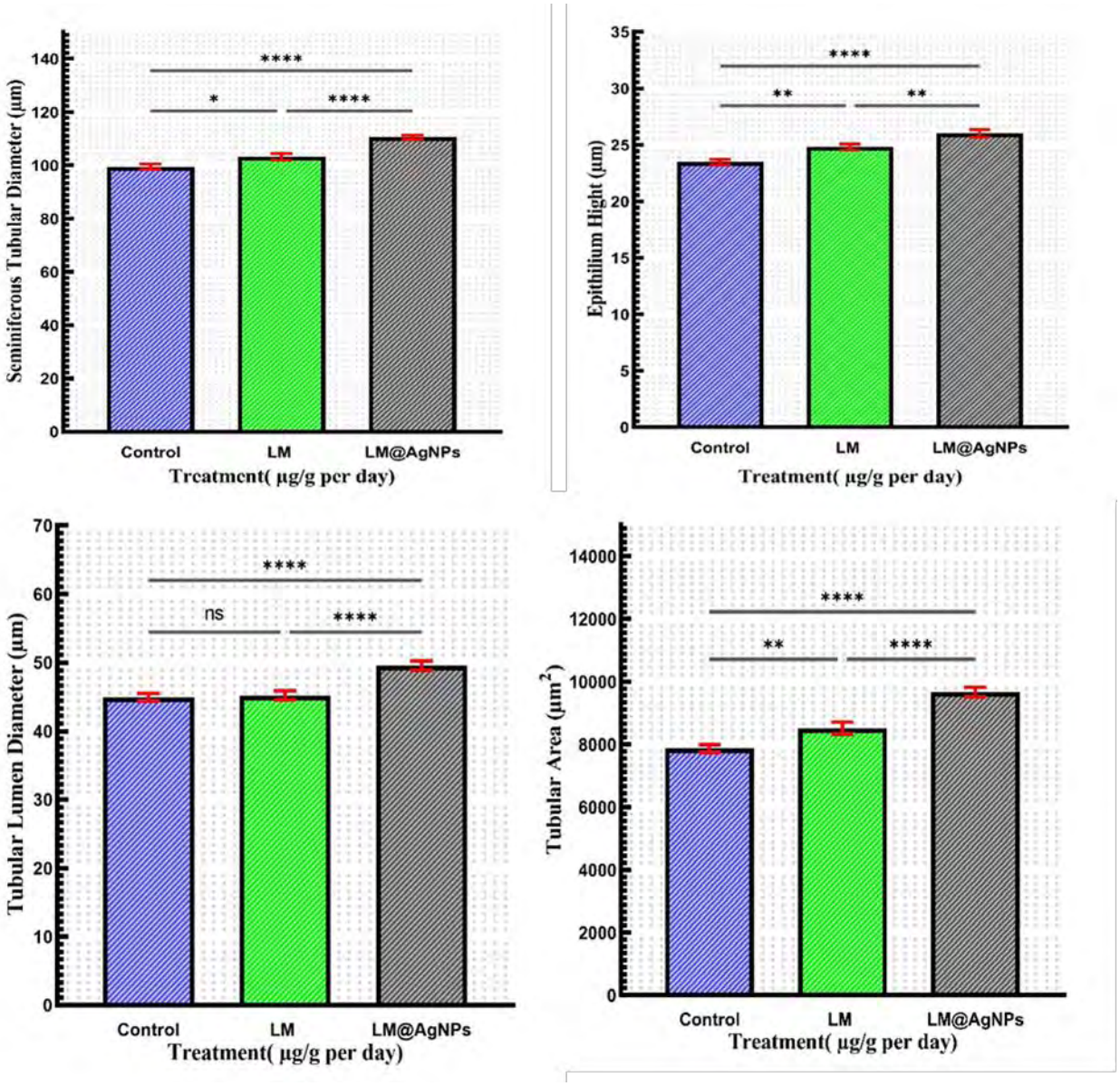

Figure 10: Effect of drugs treatment on seminiferous tubules' parameters in experimental groups of adult male Sprague Dawley rats

4.4: Hormonal Analysis

4.4.1: Quantitative Determination of Testosterone Concentration

Effect of drugs on concentration of plasma testosterone (ng/ml) in adult male Sprague Dawley rats following 28 days treatment is tabulated in table 6. The results of ELISA have shown that LM@AgNPs have significantly impacted free testosterone level of blood plasma**.** After application of LM@AgNPs the highest increase in testosterone level was observed in comparison to those of control ($P \leq 0.001$) and LM ($P \leq 0.01$). A non-significant increase in LM treatment group was observed while comparing it with testosterone level of the control.

Table 6: Effect of drugs treatment on plasma Testosterone concentration in experimental groups of adult male Sprague Dawley rats

	Treatment ((µg/g per day)			
	Control	**LM**	**LM@AgNPs**	**P value**
Testosterone Concentration(ng/ml)	1.04 ± 0.13	4.92 ± 0.88 a*	10.18 ± 1.43 a*** b**	0.0003

Values are expressed as Mean ± SEM
Statistical Analysis: Analysis of Variance (One way ANOVA followed by Tukey's multiple comparisons test)
P value ≤ 0.05 is represented as **"*"**; P value ≤ 0.01 is represented as "**"; P value ≤ 0.001 is represented as "***"; P value ≤ 0.0001 is represented as "****".
"a" represents comparison of control with respective treatment groups while "b" represents comparison of LM vs LM@AgNPs

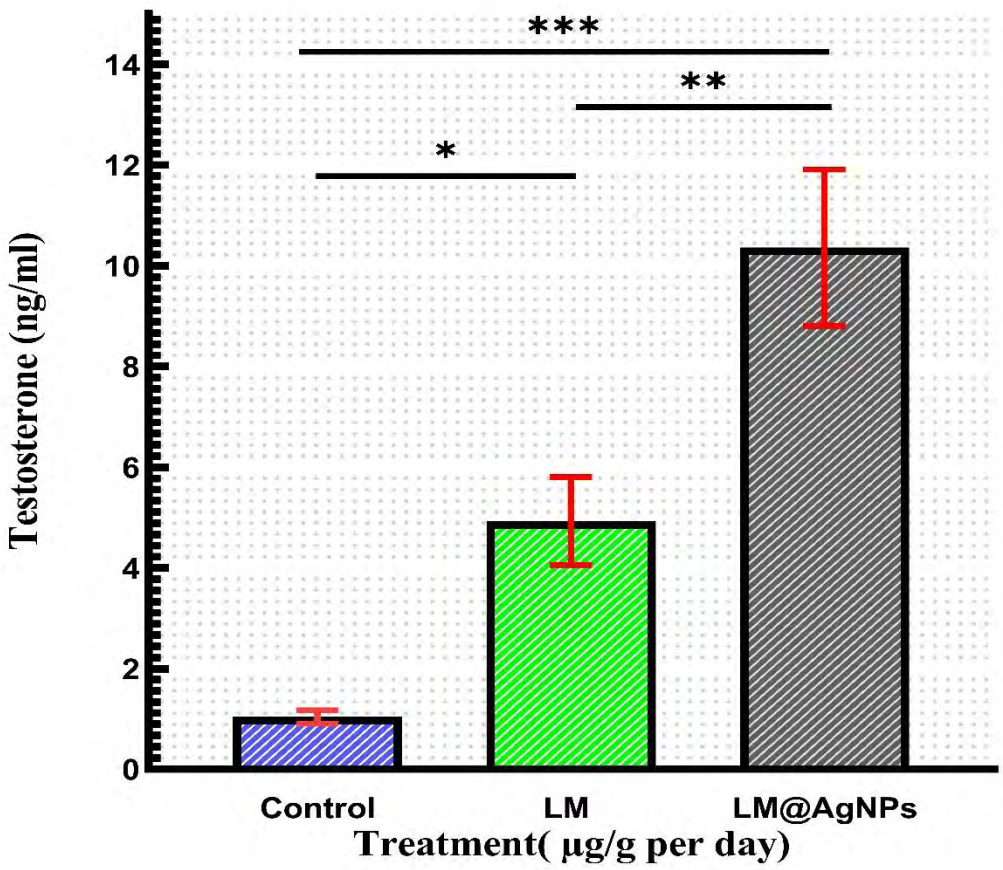

Figure 11: Effect of drugs treatment on plasma Testosterone concentration in experimental groups of adult male Sprague Dawley rats

4.5: Semen analysis

All the results of semen analysis are tabulated in Table 7

4.5.1: Sperm Count

Epididymal sperm count was increased after treatment with LM and LM@AgNPs in adult male Sprague Dawley rats. A significant improvement in sperm count was seen in LM ($P \leq 0.01$) and LM@AgNPs ($P \leq 0.0001$) when compared with that of control. The comparison of LM and LM@AgNPs proved the later more effective agent in increasing the count significantly ($P \leq 0.0001$).

4.5.2: Sperm Viability

Sperm viability get affected due to the treatment of LM and LM@AgNPs. After treatment with LM@AgNPs, a statistically significant increment in the percentage of live spermatozoa was observed in comparison to those of control ($P \leq 0.05$) and LM treated group ($P \leq 0.0001$). Moreover, in contrast to control, significant reduction ($P \leq 0.01$) in live spermatozoa was seen in LM treated group.

4.5.3: Sperm Morphology

Sperm morphology was also influenced after administration of LM and LM@AgNPs. Spermatozoa were characterized into two categories: abnormal and normal. The percentage of abnormal spermatozoa were significantly the highest ($P \leq 0.01$) in LM treated group when compared with control and LM@AgNPs. However, LM@AgNPs improved sperm morphology significantly ($P \leq 0.001$) in comparison to that of control.

4.5.4: Sperm Motility

Sperms are categorized into motile and non-motile spermatozoa. The motility of spermatozoa is impacted with treatment of LM mainly. LM caused a significant reduction ($P \leq 0.001$) in the percentage of motile spermatozoa when compared with control and LM@AgNPs. On the other hand, LM@ AgNPs have the highest percentage of motile spermatozoa but it is non-significant in comparison to that of control.

Table 7: Effect of drugs treatment on semen parameters in experimental groups of adult male Sprague Dawley rats

Parameters		Treatment (µg/g per day) Control	LM	LM@AgNPs	P Value
Sperm Concentration (millions/ml)		162.60 ± 8.35	205.80 ± 3.37 a^{**}	353.40 ± 8.51 a^{****} b^{****}	<0.0001
Sperm Viability	**Viable Cells (%)**	83.40 ± 2.23	70.00 ± 2.21 a^{**}	93.40 ± 1.44 a^{*} b^{****}	<0.0001
	Dead Cells (%)	16.60 ± 2.23	30.00 ± 2.21 a^{**}	6.60 ± 1.44 a^{*} b^{****}	<0.0001
Sperm Morphology	**Normal (%)**	67.67 ± 1.89	57.80 ± 1.41 a^{**}	80.67 ± 1.94 a^{***} b^{****}	<0.0001
	Abnormal (%)	32.33 ± 1.89	42.20 ± 1.41 a^{**}	19.33 ± 1.94 a^{***} b^{****}	<0.0001
Sperm Motility	**Motile (%)**	91.54 ± 0.96	73.76 ± 4.29 a^{***}	98.10 ± 0.34 a^{ns} b^{****}	<0.0001
	Non-Motile (%)	8.46 ± 0.96	26.24 ± 4.29 a^{***}	1.90 ± 0.34 a^{ns} b^{****}	<0.0001

Values are expressed as Mean ± SEM.
Statistical Analysis: Analysis of Variance (One Way ANOVA followed by Tukey's Multiple Comparison Test)
P value ≤ 0.05 is represented as **"*"**; P value ≤ 0.01 is represented as "**"; P value ≤ 0.001 is represented as "***"; P value ≤ 0.0001 is represented as "****".
"a" represents comparison of control with respective treatment groups while "b" represents comparison of LM vs LM@AgNPs

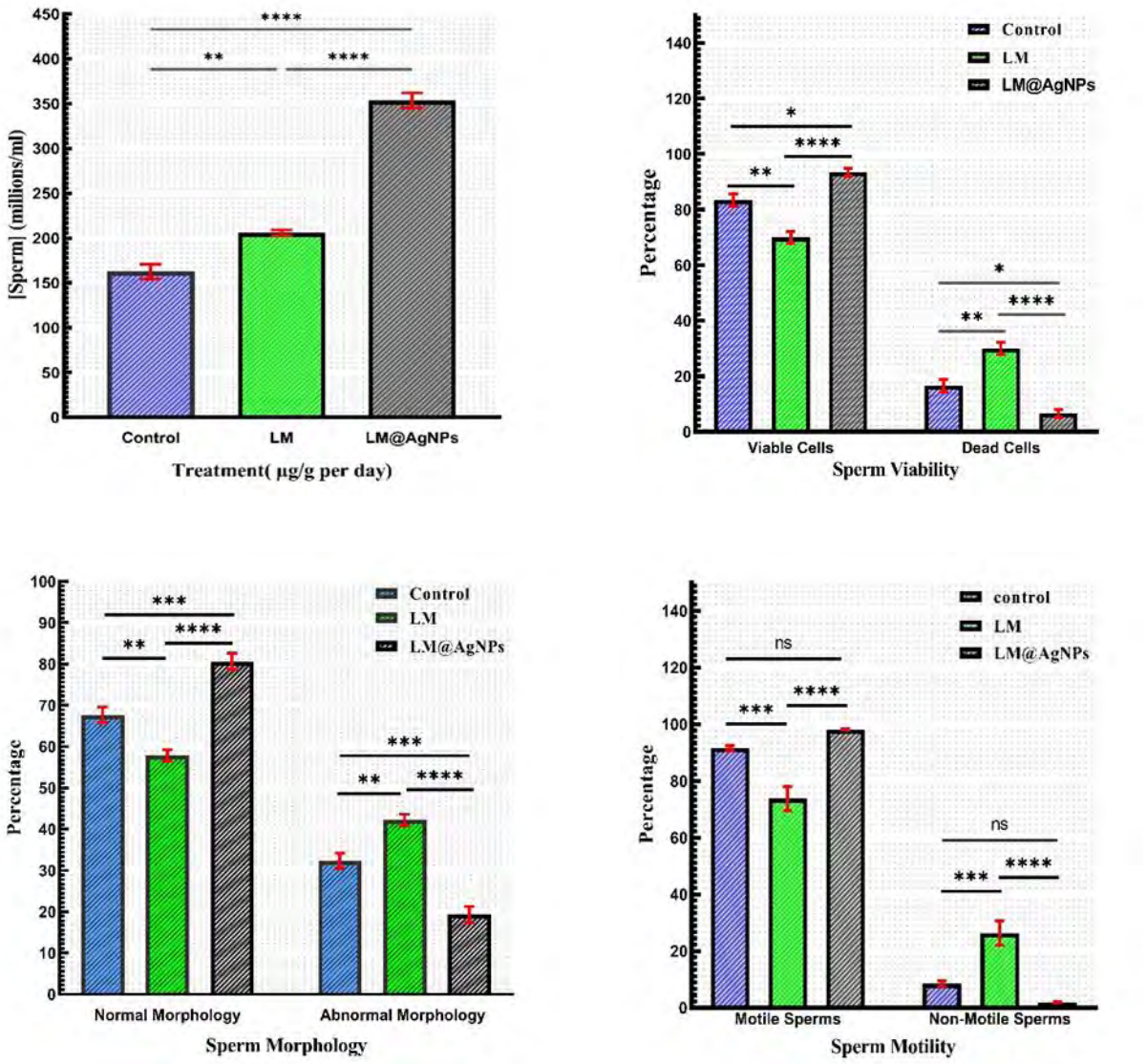

Figure 12: Effect of drugs treatment on semen parameters in experimental groups of adult male Sprague Dawley rats

5: Discussion

Decrease in sperm count and prevalence of male infertility throughout world has become an issue of global concern. The documentation of potential risk factors and cures need further attention to deal in idiopathic cases of male infertiliy (Okonofua *et al.*, 2022).The decline in semen quality has become an issue of grave perturbation for the males of Asia, Europe, Africa and North America (Dissanayake *et al.*, 2019).Scientists have developed various chemical agents to treat infertility which induces serious complications e.g. Clomiphene Citrate, a potential therapeutic agent in the reproductive disorders, is associated with serious mood disorders,visual disturbance and gynacomastia (Herzog *et al.*, 2020). So there is a dire need to synthesize and test various biological material based agents with targetted response in the reproductive system to treat male infertility. *Lepedium meyeni* based green synthesized nanoparticles provide us with this opportunity to treat the male infertility disorders with minimum side effects. *Lepedium meyeni* based silver nanoparticles have the ability to cross blood testes barriers (Arisha *et al.*, 2019; Jia *et al.*, 2020) and blood brain barrier (Rudi *et al.*, 2021) inducing maximum potential of biological compounds present on the surface of LM@AgNPs.

Present findings demonstrated that LM@AgNPs administration have non significant impact on body and organ weights.The results also demonstrates an increase in seminiferous tubular diameter, lumen diameter, epithilium height, tubular area after application of LM@AgNPs. Increase in the values of these parameters is an indicator of increased spermatogeneis as evident in the previous studies of (Tripathi *et al.*, 2015). Moreover increase in spermatozoa concentration in the lumen of tubules also proves the effectiveness of LM@AgNps as a potential agent to treat male infertility problems.This is due to the fact that LM@AgNPs has potential to cross the blood testes barriers with enhancing the efficacy of phytocompounds present on its surface in testicular tissues (Abu-Taweel *et al.*, 2021).

In the current findings, plasma testosterone level increased significantly after treatment with LM@AgNPs in comparison to that of control and LM treated groups. Moreover, LM did not change plasma testosterone level significantly which is in accordance with studies of (C. Gonzales *et al.*, 2006; G. F. Gonzales *et al.*, 2005) . LM biocomponents are unable to cross the blood brain barrier and blood testes barriers due to their large particle sizes (Yu *et al.*, 2021).Nanoscale LM@AgNPs have advantage to cross these barriers very easily with increased

efficacy of phytocomponents. Moreover, plant based saponins,flavonoids,and steroids play important role in increasing testosterone concentrations (El-Tantawy *et al.*, 2007; JianFeng *et al.*, 2012). LM is rich in phytochemicles such as steroids, flavonoids, and saponin which may act as a active agents in boosting testosterone concentration. The specific phytochemical responsible for increase in testosterone concentration after administration of LM and LM@AgNPs is still unknown and it further needs to be investigated.

Moreover, LM@AgNPs improved semen parameters as compared to LM trated group proving it more effective agent in reproductive tract of adult male Sprague Dawley rats. LM@ AgNps improved the sperm count due to the presence of biological active functional groups fabricated on the surface of metalic silver. It has been reported in the previous studies that bioactive components such as alkaloids (Derbak *et al.*, 2023), phenols (Inoue *et al.*, 2016), and flavonoids (Ye *et al.*, 2020) has improved the sperm count. The phytochemical analysis of LM, FTIR of LM, and FTIR of LM@AgNPs indicate the presence of the above mentioned bioactive components in dosages of different drugs. However, it is hard to mention which specific component improves sperm count both in LM and LM@AgNPs treatment groups. Moreover, It has been documented that LM enhance sperm production through affecting the stages of mitosis including initial stages IX- XIV (Tafuri *et al.*, 2021). The current result of increment in sperm count after application of LM is in accordance with previous studies (Gasco *et al.*, 2007) where it has been reported that LM increases sperm count in vas deferens and in contrast with (C. Gonzales *et al.*, 2006) where no effect has been reported on the parametre. LM@AgNPs is a novel agent formed from bioreduction of silver showed greater efficacy for sperm count in comparison to the administration of LM.

Present findings also suggests that LM@AgNPs improved sperm viability and motility in comparison to those of control.In contrast, sperm viability and motility decreased due to the application of LM. In the present studies, it has been found that LM demonstrated cytotoxic impacts on prostate and seminal vesicles. The secretory epithilium of the organs decreased resulting in reduced secretion of the glands. So, sperm motility and viability decreased in LM treated group. In contrast LM@AgNPs improved the secretion of both glands by increasing secretory epithilium area resulting in improvement of sperm viability and motility in comparison to that of control and LM treated groups. Sperm morphology adversly affected due to the suplementation of LM suspension. FTIR analysis of LM and LM@AgNPs has revealed

presence and absence of glucosinolates in both drugs respectively. In previous studies, it has been demonstrated that LM derived glucosinolates have adverse impacts on sperm morphology with antifertility properties (Gonzales-Daga *et al.*, 2020; Leiva-Revilla *et al.*, 2012). So glucosinolates may be a contributing factor for sperm abnormalities and its absence shows sperm morphology improvement which needs further investigations.

Finally, it can be concluded that LM@AgNPs are potential therapeutic agent in reproductive system of adult male Sprague Dawley rats. Moreover, the specific phytochemicals responsible for improvement of histological and hormonal profile in male reproductive system of rats need further investigations. Similarly, the biochemical components responsible for semen profile improvement with their specific roles at molecular levels need to be elucidated.

6: Conclusion

Addressing the issue of male infertility is crucial across the globe.The above discussion highlights the potential of *Lepedium meyeni* based green synthesized silver nanoparticles,a promising agent, in treating the male infertility with minimal side effects.The findings suggest: LM@AgNPs have multiple advantages over chemically synthesized drugs which are associated with serious complication. LM@AgNPs demonstrated its safety potential in terms of weight parameters and improved the histological profile including the key indicator of fertility i.e. morphometric changes in seminiferous tubular parameters. In addition to histological profile, significant increase in plasma testosterone level was also observed. LM@AgNPs improved the semen parametres of testicular tissues reinforcing their potential as an active drug for reproductive system. So, LM@AgNPs have the potential of demonstrating ameliorative effects on the reproductive systems of adult male sprague dawley rats.

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