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Research

Testicular Spermatogenic Activity of *Marsilea quadrifolia* Linn. against Monosodium Glutamate

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Abstract

Marsilea quadrifolia is an aquatic plant that contains high antioxidants level and could prevent cell damages caused by free radicals. The present study aimed to investigate the effect of *Marsilea quadrifolia* ethanol extract on luteinizing hormone (LH), testosterone levels, sperm quality, and testis histology of adult male rats induced by monosodium glutamate (MSG). This study randomly divided 30 adult male rats into five groups (n6). The LH and testosterone levels at this time were measured to know the differences between the MSG and control groups. After 15 days of receiving MSG, the rats were continuously treated with MSG and followed by the administration of extract until 30 days (day 30). Both hormones between the MSG and treatment groups were measured at this time. The LH and testosterone levels significantly increased (p0.05) after *Marsilea quadrifolia* administration at all doses. The higher dose of M. quadrifolia ethanol extract demonstrated a high decrease in MDA level in MSG-treated rat testis increase of spermatogonia, spermatocytes, spermatids, and Leydig cells number and increase of seminiferous tubular diameter and germinal epithelium thickness. The ethanol extract of *Marsilea quadrifolia* can improve the levels of LH, testosterone, sperm quality, number of testis morphometric, spermatogenic, and Leydig cells in MSG-treated male rats.

Keywords: Monosodium glutamate, spermatogenic, Infertility, sperm quality, testosterone.

INTRODUCTION

One year of regular, unprotected sexual activity without conception is considered infertile. Based on analysis, the reason of infertility in about 50% of affected couples is related to orcaused by male factors [1]. Infertility sufferers manage this condition individual often both modern medicineand traditional therapies derived from natural botanicals [2,3]. Since roughly 25% of current medications are derived from plant. Based, the use of medicinal plants in the treatment of illnesses and dysfunctions dates back several millennia and has greatly influenced the creation of medicines. Furthermore, up to 60% of people use herbal medications for medical purposes worldwide [4,5]. Today, many of the extracts, fractions or molecules that have been extracted from these plants are widely utilized to treat or cure many aspects of male infertility, including loss of orgasm, sperm abnormalities, erectile dysfunction, sexual asthenia, libido absence and relaxation and ejaculatory dysfunctions. Male infertility has a variety of causes but psychogenic and endocrine diseases, vascular injuries and drug usage are among the symptoms that infertile individuals experience [6]. Numerous in vitro, in vivo and clinical investigations have demonstrated the practical application of plants in enhancing male fertility metrics. The use of medicinal plants to treat male infertility symptoms such as libido problems, erectile dysfunction, ejaculatory disorders and sperm abnormalities, was the main emphasis of this review, along with theefficacy of phytomedicines as a therapeutic approach. Data from a few chosen papers was categorized based on the intended impact of a plant extract on male reproductive function as wellas the subject (human, rodent) that the extract was tested on to determine its possible activity. [7]

PLANT PROFILE

Water clover, or *Marsilea quadrifolia* L., is an aquatic plant that is widely distributed throughout Europe and is a member of the Marsileaceae family [8,9]. In Surabaya, Indonesia, the leaves of this plant are frequently consumed as vegetables. The herb has antineuroinflammation [10], anticholesterol [11], antiosteoporotic [12], and anti-aging properties for the skin [13]. Comparing *M. quadrifolia* to other species in the Marsileaceae family (such *Marsilea minuta* Linn), there are still few pharmacological research available. Additionally, it is uncertain what research has been done on *M. quadrifolia's* effects on the reproductive system. High levels of phytoestrogens, such as lignans, stilbene, coumestans, coumarin, dihydrochalcone, triterpenoids, and flavones, are found in M. quadrifolia [14,15]. Antioxidants such as flavonoids, genistein, daidzein isoflavones, and vitamin C are also abundant in M. quadrifolia [16,17]. *Marsilea quadrifolia's* flavonoids shield membranes from the harm that free radicals can do [18]. By speeding up lipid metabolism in the mitochondria, vitamin C lowers hydroxyl free radicals and is essential for the creation of L-carnitine, a substance that helps cells produce energy [19].

It is anticipated that M. quadrifolia will raise LH and testosterone levels and shield the Leydig cells from free radical damage brought on by MSG induction. Rats' genetic, biochemical, and behavioral traits are quite similar to those of humans, which is why they were chosen as a model for this study. Furthermore, rats are able to mimic a number of human illnesses. Therefore, the purpose of this study was to examine how *Marsilea quadrifolia* ethanol extract affected the levels of LH and testosterone, sperm quality parameters, and testis histology in adult male rats that were given MSG induction.

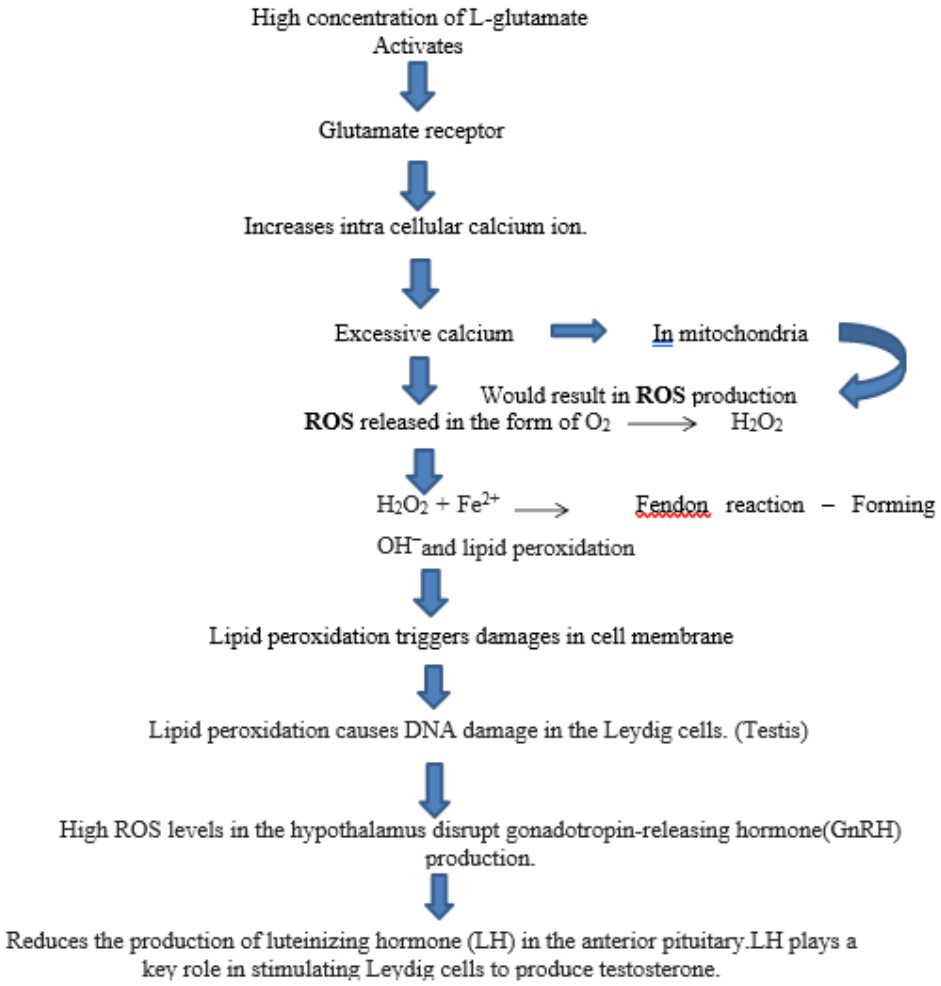
ETHICAL APPROVAL

The study was approved by the Institutional Animal Ethical Committee approved by CCSEA New Delhi [Proposal Number: AKCP/IAEC/26/24-25]

STUDY PERIOD

This study was carried out from July 2024 to November 2024 at the Laboratory of Animal Physiology and Department of Pharmacology, Arulmigu Kalasalingam College of Pharmacy, Krishnankoil, Virudhunagar, Tamilnadu.

CAUSTIVE AGENT: MONOSODIUM GLUTAMATE [20]



EXTRACT PREPARATION

Marsilea quadrifolia was obtained from the local farmers in Saptur, Madurai district. The plant was identified and authenticated by a Botanist, Professor Dr.Stephen, Department of Botany, American college, Madurai-625002, Tamilnadu. In addition, 10,000 g of *Marsilea quadrifolia* fresh leaves were collected and washed with tap water. The leaves were dried and pulverized into a fine powder. Moreover, 100 g of powdered samples were extracted with 3 L of 70% ethanol at room temperature for 24 h. Samples were filtered and evaporated using a rotary evaporator after 24 h of maceration. The ethanol extract of *Marsilea quadrifolia* was stored at 4°C for further analysis.

EXPERIMENTAL DESIGN

This experimental study used 30 adult male rats strain Wistar, aged 3-4 months and weighing 200-250 g. The rats were obtained from Bio cape labs, kaliyakavilai. All animals were kept in standard rat cages (32 cm × 28 cm) at controlled temperature (22±2°C), humidity level 50±5%, and under 12 h light and dark cycle. Each animal was kept in one cage. Animals were fed standard pellet chow and water *ad libitum*. The animals were acclimatized for 2 weeks before and during the study at the AKCP-Animal house.

This study randomly divided 30 adult male rats into five groups (n=6). The control group received 15 mL/200 g body weight (b.w.) distilled water orally for 30 days; the MSG group received 4 mg/g b.w. of MSG for 30 days; the STD, LOW, and HIGH groups orally received 4 mg/Kg b.w. of MSG for 30 days and extracts at different doses of 200, and 400 mg/Kg b.w., respectively, for 30 days; then, standard drug at a doses of mg/Kg b.w., respectively, for 30 days;

The rats were treated with MSG in this study for 15 days before giving with extract (day 0). The LH and testosterone levels at this time were measured to know the differences between the MSG and control groups. After 15 days of receiving MSG, the rats were continuously treated with MSG and followed by the administration of extract until 30 days (day 30). Both hormones between the MSG and treatment groups were measured at this time.

TESTOSTERONE AND LH SERUM ASSAY

PRINCIPLE

The DIAsource Testosterone ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA), based on the principle of competitive binding. The microtiter wells are coated with a monoclonal [mouse] antibody directed towards an unique antigenic site on the Testosterone molecule.

PROCEDURE

Blood was collected on days 0 and 30. The blood was collected in microtubes and incubated at 37°C for 6 h. The obtained clear sera were stored at −20°C. Testosterone and LH were measured using Rat Testosterone enzyme-linked immunosorbent assay (ELISA) Kit 96T and Rat LH ELISA Kit 96T Bioassay Technology Laboratory, Shanghai, China (catalogue No. E0259Ra) and Rat LH ELISA Kit 96T, Bioassay Technology Laboratory, Shanghai, China (catalogue No.E0179Ra).

SPERM QUALITY ANALYSIS

Animals were sacrificed by neck dislocation and dissected to isolate the epididymis organs at the end of the study (day 30). The cauda epididymis was exposed and incised on one side. Moreover, 1.5 mL of the sperm was quickly sucked and diluted with warm 0.1 M phosphate saline buffer at pH 7.4. Furthermore, 10 µL of sperm sample was assessed for motility, viability, morphology, and sperm concentration. The diluted semen was observed using a microscope with 100× and 400× magnification to observe sperm motility and abnormality. The sperm viability and abnormality were calculated using the following formula:

$$\text{Viability} = (\text{number of live sperm/total of sperm}) \times 100\%$$

$$\text{Abnormality} = (\text{number of abnormal sperm/total of sperm}) \times 100\%.$$

Sperm viability and morphology were determined using eosin nigrosine staining . Thus, 10 µL of diluted semen was added to eosin nigrosine, and a smear was then made. The smear was observed using a microscope with ×400 magnification. To evaluate the sperm concentration, 20 µL of semen was added into 980 µL of fixative solution (1:1 $NaHCO_3$ formalin), inserted into hemocytometer, and counted for spermatozoa concentration using a microscope with 400× magnification.

$$\text{Sperm count} = (\text{number of sperm in 5 little square box}) \times 5 \times 10 \times \text{dilution factor} \times 1000$$

HISTOLOGICAL EXAMINATION

Animals were sacrificed through neck dislocation and dissected to isolate the testis at the end of the study (day 30). The histological analysis of the testis was evaluated according to a previously described method. The testis was washed with phosphate-buffered saline and then fixed in 10% formaldehyde for 24 h. The tissue samples were dehydrated in an alcohol solution by follow-up routine methods and embedded in paraffin. Five-micrometer-thick section was stained with hematoxylin–eosin and histologically analyzed using an Olympus BX51 microscope (Olympus Corporation, Inc., New York, NY, USA). The germinal epithelium thickness and the diameter of the seminiferous tubules were investigated with 400× magnification. The assessment of spermatogonia, spermatocyte, spermatid, and Leydig cells was quantified in ten random microscopic fields in the interstitial compartment located between the three tubules.

DETERMINATION OF MALONDIALDEHYDE (MDA) LEVEL IN TESTIS

PRINCIPLE

This assay based on the reaction of malondialdehyde (MDA) with thiobarbituric acid, forming a MDA-TBA2 adduct that absorbs strongly at 532nm.

PROCEDURE

The MDA levels were measured using the thiobarbituric acid method. The testis was crushed and 0 g of the testis was then homogenated and added with 900 µL of potassium chloride solution, 200 µL of 0.67% trichloroacetic acid, 200 µL of 20% thiobarbituric acid, and 200 µL of hydrogen chloride. The samples were then incubated at 100°C for 15 min and centrifuged at 3000 rpm at 25°C for 10 min. The supernatant was taken to calculate the absorbance value using a spectrophotometer at a wavelength of 535 nm.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 16.0 for Windows (SPSS Inc., Armonk, NY, USA). Comparison between groups was analyzed using a one-way analysis of variance ($p<0.05$) and followed by Tukey's test. To determine the changes in hormone levels from days 1 to 30, paired sample t-tests were used. Data were represented as mean±standard deviation and $p<0.05$ was considered statistically significant.

RESULTS

PHARMACOLOGICAL STUDIES

Extract prevents monosodium glutamate adverse effects on the serum levels of reproductive hormones, sperm quality, and testis histology in male rats.

Table 1: Effects of extract on Body weigt,Testis weight,Epididymis and prostate weight rats exposed to monosodium glutamate and treated with extract (mean±standard deviation).

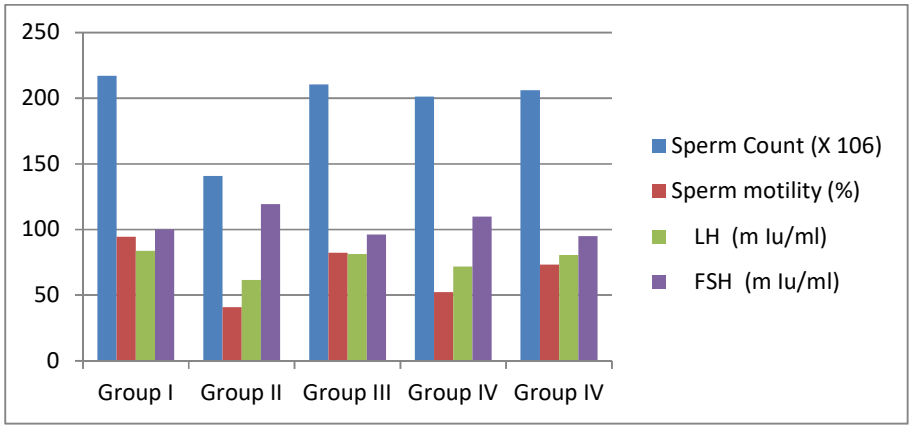
Groups	Body weight (g)		Testis weight (g)	Epididymis (g)	Prostate weight (g)
	Initial	Final			
Group I	174.82±0.02	186.26±0.04	1.40±0.02	0.485±0.02	0.620±0.10
Group II	179.23±0.04	220.22±3.02	0.80±0.10	0.295±0.15	0.452±0.10
Group III	175.29±0.08	190.24±5.07	1.30±0.04	0.432±0.12	0.592±0.02
Group IV	173.24±0.09	191.27±0.07	1.10±0.01	0.307±0.02	0.487±0.04
Group V	176.24±0.03	195.27±1.07	1.27±0.0	0.427±0.02	0.527±0.03

Different letters within the same column show a statistically significant ($p<0.05$)

The results also showed that the Body weight in the MSG group were significantly increased on day 30 compared with the control group ($p<0.05$; Table-1 All doses treatment groups demonstrated a significant increase in body weight on day 30. The results also showed that the Testis weight, Epididymis, Prostate weight in the MSG group were significantly decreased on day 30 compared with the control group ($p<0.05$; Table-2). All doses treatment groups demonstrated a significant increased in Testis weight, Epididymis, Prostate weight on day 30.

Table 2: Effects of extract on Sperm count, Sperm motility, and luteinizing hormone and FSH serum levels rats exposed to monosodium glutamate and treated with extract (mean±standard deviation)

Groups	Sperm Count (X 10^6)	Sperm motility (%)	LH (m Iu/ml)	FSH (m Iu/ml)
Group I	216.9±0.60	94.45±0.02	83.62±0.01	99.9±0.7
Group II	140.80±0.10	40.95±0.05	61.42±0.11	119.2±0.3
Group III	210.30±0.41	82.43±0.02	81.52±0.03	96.09±0.2
Group IV	201.11±0.10	52.37±0.12	71.87±0.14	109.9±0.1
Group V	206.07±0.10	73.47±0.03	80.52±0.23	95.02±0.1

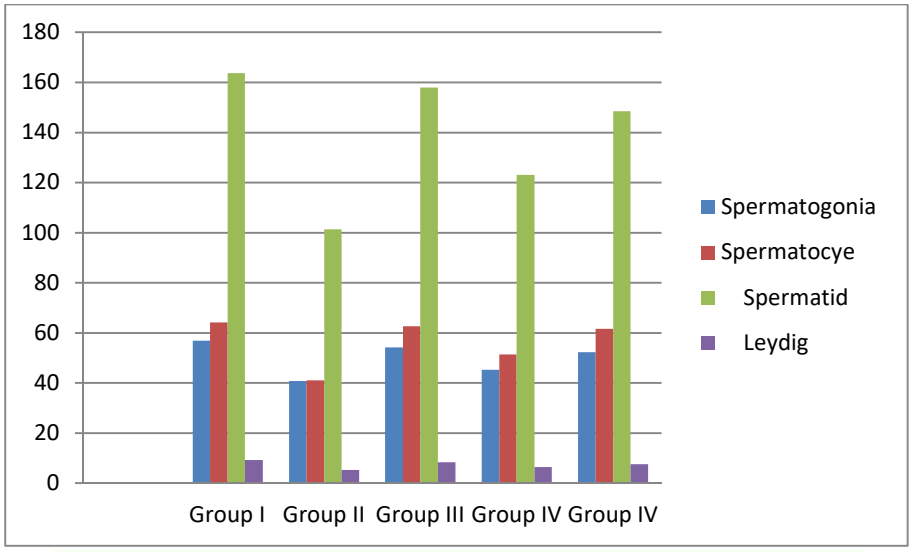


Different letters within the same column show a statistically significant ($p<0.05$).

The results also showed that the Sperm count, Sperm motility, luteinizing hormone in the MSG group were significantly decreased on day 30 compared with the control group ($p<0.05$; Table-1). All doses treatment groups demonstrated a significant increase in Sperm count, Sperm motility, luteinizing hormone on day 30. The results also showed that the **FSH** in the MSG group were significantly increased on day 30 compared with the control group ($p<0.05$; Table-2). All doses treatment groups demonstrated a significant decreased in **FSH** on day 30.

Table 3: Effect of extract on the number of spermatogenic cells in rats exposed to monosodium glutamate and treated with extract.

Groups	Spermatogonia	Spermatocyte	Spermatid	Leydig
Group I	56.9±1.00	64.23±0.20	163.62±0.01	9.12±0.15
Group II	40.80±0.07	41.03±0.15	101.32±0.11	5.22±0.56
Group III	54.25±0.16	62.63±0.13	158.02±0.03	8.29±0.32
Group IV	45.17±0.13	51.31±0.20	123.07±0.14	6.39±0.12
Group V	52.27±0.02	61.57±0.31	148.50±0.23	7.62±0.11



Different letters within the same column show a statistically significant ($p<0.05$)

The results also showed that the **number of spermatogenic cells** in the MSG group were significantly decreased on day 30 compared with the control group ($p<0.05$; Table-1). All doses treatment groups demonstrated a significant increase in **number of spermatogenic cells** on day 30.

Table 4: Effect of extract on the quality of semen in rats exposed to monosodium glutamate and treated with extract.

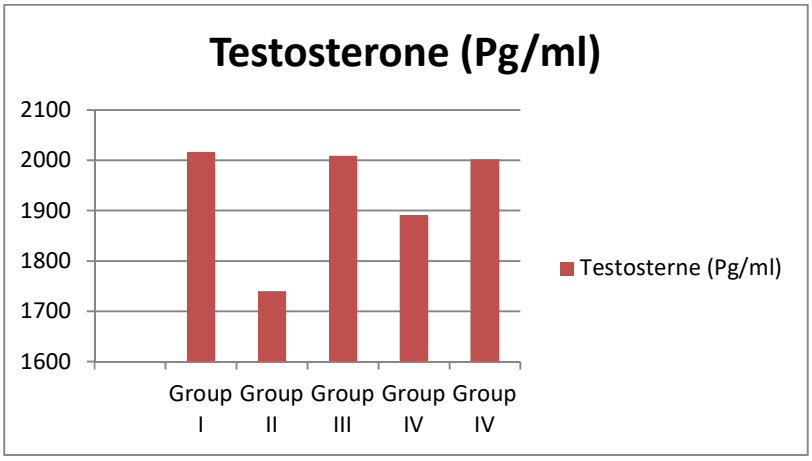
Groups	Viability (%)	Abnormalities (%)	Semen con (%)
Group I	67.09±0.40	10.03±0.20	63.02±0.11
Group II	32.82±0.07	4.01±0.52	42.03±0.21
Group III	62.05±0.06	9.30±0.23	58.04±0.23
Group IV	43.19±0.03	5.01±0.24	47.01±0.24
Group V	60.23±0.20	7.57±0.12	52.01±0.27

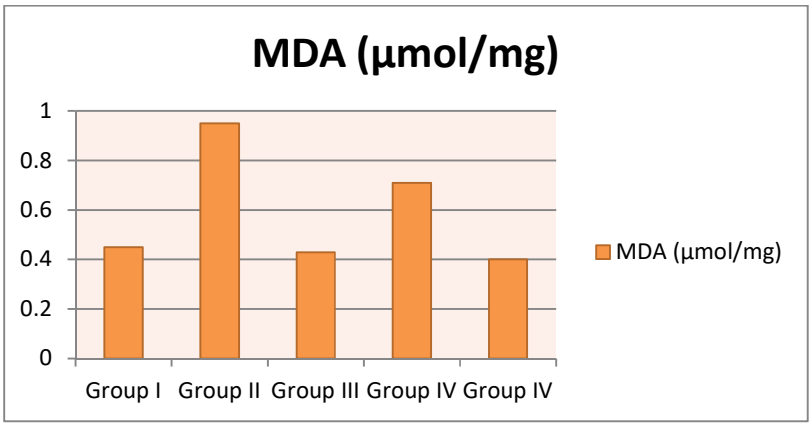
Different letters within the same column show a statistically significant ($p<0.05$)

The results also showed that the quality of semen in the MSG group were significantly decreased on day 30 compared with the control group ($p<0.05$; Table-1). All doses treatment groups demonstrated a significant increase in quality of semen on day 30.

Table 5: Effects of extract on, luteinizing hormone and MDA levels rats exposed to monosodium glutamate and treated with extract (mean ±standard deviation).

Groups	Testosterone (Pg/ml)	MDA (µmol/mg)
Group I	2016.7±0.67	0.45±0.3
Group II	1740.09±0.08	0.95±0.5
Group III	2009.30±0.01	0.43±0.2
Group IV	1891.11±0.23	0.71±0.2
Group V	2002.07±0.10	0.40±0.3

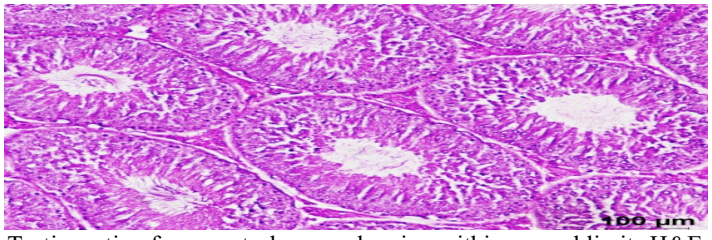




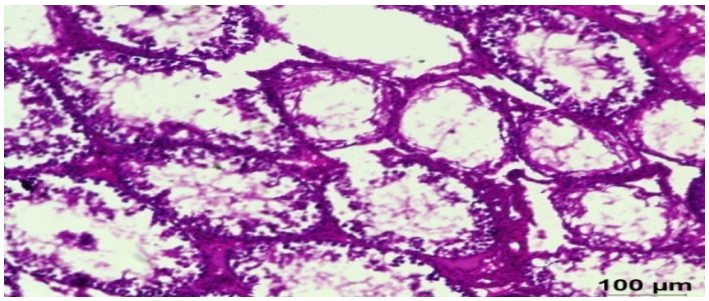
Different letters within the same column show a statistically significant ($p<0.05$)

The results also showed that the Testosterone in the MSG group were significantly decreased on day 30 compared with the control group ($p<0.05$; Table-1). All doses treatment groups demonstrated a significant increase in Testosterone on day 30.The results also showed that the MDA in the MSG group were significantly increased on day 30 compared with the control group ($p<0.05$; Table-2). All doses treatment groups demonstrated a significant decreased in MDA on day 30.

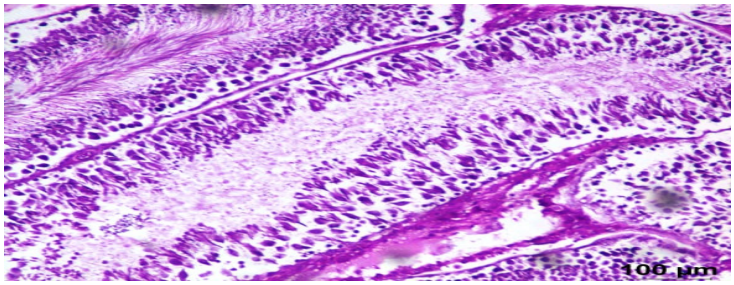
HISTOLOGY OF TESTIS



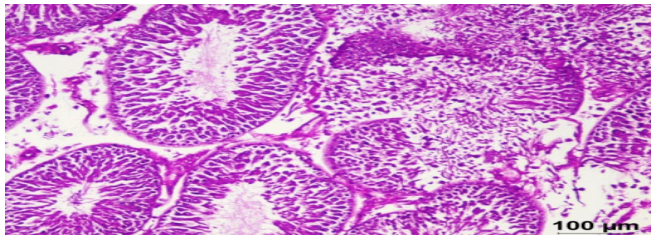
Testis section from control group showing within normal limits H&E



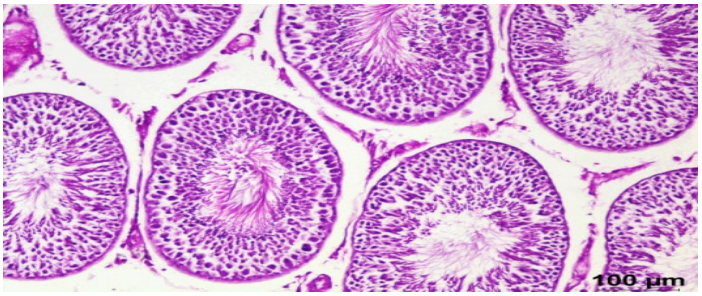
Testis section from Disease control group (MSG dose)
showing moderate Degeneration, testicular, multifocal (arrow).H&E



Testis section from Standard group clomiphene citrate dose
showing mild Degeneration, testicular, multifocal (arrow).H&E



Testis section from Treatment group low dose (EEMQ dose)
showing mild Degeneration, testicular, multifocal (arrow) H&E



Testis section from Treatment group High dose (EEMQ dose)
showing minimal Degeneration, testicular, multifocal (arrow).H&E

Fig 1: Histology of Testis after administration of EEMQ.

PHARMACOLOGICAL STUDIES

In -vivo pharmacological screening of EEMQ for Testicular Spermatogenic activity by inhibiting the adverse effects of monosodium glutamate. Treatment of EEMQ shows the improvement on the levels of LH, testosterone, sperm quality, and number of testis morphometric, spermatogenic and Leydig cells treated with MSG. The Testicular Spermatogenic potential of EEMQ may be due to the presence of the compounds Squalene, Heptatriacontane, Vitamin E**,** gamma. Sitosterol**,** Phytyl tetradecanoate, alpha.-Tocopherolquinone.

Protective Effects of Extract

The extract demonstrated protective effects against the adverse impact of MSG on reproductive health in male rats. It significantly improved body weight, testicular and prostate weights, sperm quality (count, motility), serum hormone levels (LH, FSH, testosterone), and spermatogenic cell count. Additionally, the extract reduced oxidative stress (MDA levels) and improved testicular histology.

Optimal Dose

The high dose of the extract (Group IV) generally showed the most significant protective effects across all parameters tested, suggesting that higher doses may be more effective in mitigating MSG-induced reproductive toxicity.

Oxidative Stress Reduction

The extract reduced oxidative stress, as evidenced by a significant decrease in malondialdehyde (MDA) levels, which suggests that the extract may have antioxidant properties.

Improvement in Semen Quality and Histology

The treatment groups, especially the high-dose group, showed marked improvement in semen quality (viability, abnormalities, concentration) and testicular histology.

Body and Organ Weights

Body Weight: The MSG group showed a significant increase in body weight compared to the control group after 30 days. All treatment groups demonstrated significant increases in body weight compared to the MSG group.

Testis, Epididymis, and Prostate Weights: The MSG group showed significant decreases in testis, epididymis, and prostate weights compared to the control group. All treatment groups showed significant improvements in these organ weights, with the extract treatment groups (especially the high dose) restoring the weights toward normal levels.

1. Count, Sperm Motility, LH, and FSH Levels

Sperm Count and Motility: The MSG group showed a significant decrease in sperm count ($140.80 \pm 0.10 \times 10^6$) and sperm motility (40.95%) compared to the control group. Treatment with the extract significantly increased sperm count and motility, with the high-dose group showing the most improvement (206.07×10^6 sperm count, 73.47% motility).

Luteinizing Hormone (LH): LH levels were significantly decreased in the MSG group compared to the control. All treatment groups showed significant improvements in LH levels, with the high-dose treatment group showing a near-normal recovery.

Follicle-Stimulating Hormone (FSH): FSH levels were significantly elevated in the MSG group. All treatment groups demonstrated a significant decrease in FSH levels, with the high-dose group showing the most improvement.

2. Spermatogenic Cell Count

The MSG group showed significant reductions in spermatogenic cells (spermatogonia, spermatocytes, spermatids, and Leydig cells). All extract treatment groups showed a significant increase in the number of spermatogenic cells, with the high-dose group showing the best recovery.

3. Semen Quality

Viability and Abnormalities: The MSG group exhibited significantly lower semen viability (32.82%) and higher abnormalities (4.01%). The treatment groups, particularly the high-dose group, showed significant improvements in semen viability (60.23%) and reduced abnormalities (7.57%)

Semen Concentration: The semen concentration in the MSG group was lower (42.03%) compared to the control group (63.02%). All treatment groups showed a significant improvement in semen concentration.

4. Testosterone and MDA Levels

Testosterone: The MSG group showed significantly lower testosterone levels (1740.09 pg/mL) compared to the control (2016.7 pg/mL). The extract treatment groups, especially the high-dose group, showed significant increases in testosterone levels.

Malondialdehyde (MDA): The MSG group had significantly higher MDA levels (0.95 µmol/mg), indicating increased oxidative stress. All treatment groups showed significant decreases in MDA levels, with the high-dose group showing the lowest MDA levels (0.40 µmol/mg).

5. Histology

Testis Histology: The control group showed normal testicular histology. The MSG group showed moderate degeneration of the testis. The extract treatment groups (low and high doses) showed varying degrees of testicular degeneration, from mild
to minimal, with the high-dose group showing the least damage.

S .no	Group No	Histomorphological observation
1.	Control	Within normal limits
2.	Disease control MSG	Degeneration, testicular, multifocal, moderate
3.	Standard clomiphene citrate	Degeneration, testicular, multifocal, mild
4.	Treatment plant extract low	Degeneration, testicular, multifocal, mild
5.	Treatment plant extract high	Degeneration, testicular, multifocal, minimal

CONCLUSION

The in vivo study demonstrated that the ethanol extract of *Marsilea quadrifolia* (EEMQ) exhibits significant dose-dependent testicular spermatogenic activity against MSG-induced reproductive toxicity in male rats. The extract improved luteinizing hormone (LH), testosterone levels, sperm quality, spermatogenic cell count, and testicular histology while reducing oxidative stress (MDA levels). These findings justify the traditional use of *M. quadrifolia* leaves in the treatment of male infertility and spermatogenesis-related disorders, and further isolation of bioactive compounds from this plant is warranted for therapeutic development.

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