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ORIGINAL ARTICLE

Aging induced testicular damage: analyzing the ameliorative potential of *Mucuna pruriens* seed extract

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Abstract

Mucuna pruriens Linn. (*M. pruriens*), a leguminous plant, was used extensively in Ayurveda, to treat male-related infertility. Previous studies have demonstrated antioxidant, androgenic, aphrodisiac, and spermatogenic properties of *M. pruriens* seed extract. Surprisingly, the biological activities of *M. pruriens* on aging-induced pathological changes in the testis microenvironment have never been explored and the present study was focused on the testing therapeutic efficacy of *M. pruriens* on aged rat testis. Male Wistar albino rats were grouped as; adult (3 months), aged (24 months), aged + *M. pruriens* and adult + *M. pruriens* ($N = 6$/group). The extract was administrated at a dose of 200 mg/kg body weight (dosage determined in our previous study) daily by gavage for 60 days. The total and free testosterone, FSH and LH levels were considerably increased in aged + *M. pruriens*. The diameter & volume of the seminiferous tubules, the height & volume of the epithelium, and the number of Leydig cells number were significantly decreased in aged rat testis, concomitantly connective tissue proportion was increased compared to adult rats. The seminiferous epithelium indicates significant rejuvenation or restoration of spermatogenic cells in aged + *M. pruriens* rat testis. The highlighting observations in aged + *M. pruriens* was increased in the following parameters i.e., tubular diameter (25%), number of tubules (35%), epithelial height (25%) & volume (20%), and number of Leydig cells (35%) when compared to untreated aged rat testis. The TNFα, NF-κB, cytochrome c, Caspase-9, Caspase-3, Bcl-2, Bax, PARP iNOS, and inflammatory and apoptotic factors were downregulated in aged + *M. pruriens*. *M. pruriens* was able to restore spermatogenesis and enhance the activity of Sertoli cells and Leydig cells and improve the pituitary–gonadal axis in aged rat testis and observations indicate the therapeutic activity of *M. pruriens* in aged rat testis.

Keywords Aging · *Mucuna pruriens* · Testis · Spermatogenesis · Histology · Inflammation

Introduction

Aging is the progressive reduction or loss of tissue and organ function over time (Flatt 2012). At the cellular level, oxidative-stress-induced cell senescence associated with a pro-inflammatory state may contribute to age-associated tissue and organ impairment (Fabian and Flatt 2011). In males, aging results in testicular dysfunction and shows a gradual decline in fertility (Gunes et al. 2016; Dominguezet al. 2011; Frungieri et al. 2021). Reports show that the chance of parenthood declines after 38.4 years in males. (Klonoff-Cohen and Natarajan 2004; Gunes et al. 2016). There is a growing interest in age-related changes in male fertility as more couples wish to have a child in their late reproductive years (Roustaei et al. 2019). The major categories of disorder associated with the aging of the male reproductive system comprise deviations in the testicular tissue microenvironment (Huang et al. 2018) and exhibit severe histological alterations (Paoli et al. 2019; Mularoni et al. 2020), a decrease of the steroidogenic and spermatogenic dysregulation (Beattie et al. 2015; Pataky et al. 2021) and erectile dysfunction and dysregulation of associated muscles (Prakash et al. 2018, 2020).

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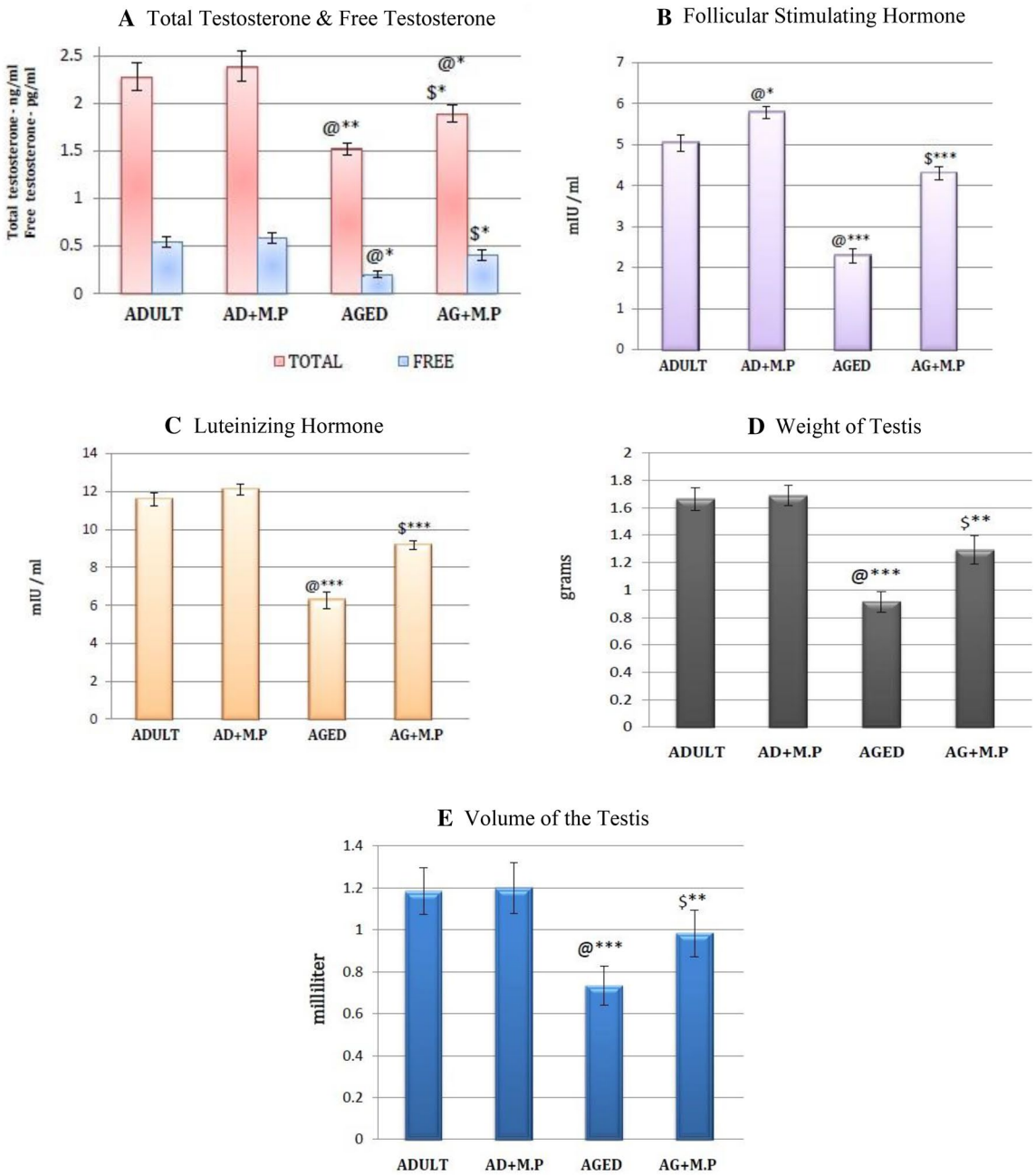


Studies indicated that the reproductive health of aging males presents versatile and complex pathobiology. Understanding this complexity would enhance the prospects of finding novel and specific therapeutic targets and suitable therapies. There is increasing research interest in controlling the onset or delaying aging by anti-aging therapies/drugs and implementing such approaches in clinical practice (Vaiserman and Lushchak et al. 2017). Herbal plants are considered a therapeutic strategy among several remedies because of their promising therapeutic potential with no or lesser side effects (D'Cruz et al. 2010). It has been estimated that about 80% of developing countries still rely on traditional herbal medicines as their therapeutic strategy and primary health care (Martins Ekor 2013).

Mucuna pruriens Linn. (*M. pruriens*), a leguminous plant, is used in Indian traditional medicine to improve fertility and treat Parkinson's disease (Sathiyanarayanan and Arulmozhi 2007; Rima et al. 2023). This plant was used extensively in traditional Ayurveda, which was practiced

◂**Fig. 1** **A** Illustrates total and free testosterone levels in the serum of control and various experimental groups. The values are expressed in ng/ml (total T) & pg/ml (free T) and presented as mean ± SEM ($n = 6$). Estimation indicated significantly reduced testosterone levels in aged rats, whereas testosterone levels were upregulated in aged + *M. pruriens* (AG + MP) rats. There was an increase in adult rats administrated with *M. pruriens* (AD + MP). @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$. **B** Illustrates follicular stimulating hormone (FSH) levels in serum samples from control and various experimental groups. The values are expressed in mIU/ml and presented as mean ± SEM ($n = 6$). Estimation indicated significantly reduced FSH levels in aged rats, whereas FSH level was upregulated in aged + *M. pruriens* (AG + MP) rats. There was an increase in adult rats administrated with *M. pruriens* (AD + MP). @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **C** Illustrates luteinizing hormone (LH) level in the serum of control and various experimental groups. The values are expressed in mIU/ml and presented as mean ± SEM ($n = 6$). Estimated values indicate significantly reduced LH levels in aged rats, whereas FSH level was upregulated in Aged + *M. pruriens* (AG + MP) rats. @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$. **D** This shows the weight of the testis taken from adult and various experimental groups. The values are expressed in grams and presented as mean ± SEM ($n = 6$). The assessment indicated significantly reduced weight in aged rats. Whereas the weight of the testis was increased in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **E** This shows the volume of testis in adult and other experimental groups. The values are expressed in milliliters and presented as mean ± SEM ($n = 6$). The assessment indicated a significantly reduced volume in aged rats. The testis volume increased in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

since the Vedic period, i.e., 1500–1000 BC, to treat male infertility and neurological disorders (Lampariello et al. 2012). However, without proper scientific validation.

M. pruriens comprises more than 200 indigenous drug formulations, and all the parts of the plant show medicinal properties and are rich in biomolecules and bioactive components (Pandey 1998, 1999). *M. pruriens* is rich in alkaloids, including prurieninine, prurienine and prurienidine (Misra et al. 2004). The seeds of *M. pruriens* contain l-3,4-dihydroxyphenylalanine (Singh et al. 2018), methionine, tyrosine, lysine, glycine, aspartic acid, glutamic acid, leucine and serine, along with globulins and albumins (Pant and Joshi 1970). In addition, triterpenes and sterols (β-sitosterol, ursolic acid, etc.) were found in the root and seeds of *M. pruriens*. Besides, fatty acids, carbohydrates, and related compounds such as oleic acid, linoleic acid, and palmitic acid (Adebowale et al. 2005; Dhale and Sanskruti (2010); Reuben-kalu et al. 2021).

Research is being carried out in our laboratory to examine the therapeutic potential of ethanolic extract of *M. pruriens* seed on male infertility, and impotence and results were promising. However, experimental investigation of the biological activities of *M. pruriens* on aging-induced pathological changes in the testis microenvironment has never been explored. Furthermore, in our earlier studies, it has been observed that the extract has antioxidant, aphrodisiac, spermatogenic potentials and improved erectile function in the aged rat (Suresh et al. 2009, 2013; Suresh and Prakash 2011, 2012; Prakash et al. 2018; Murugesan et al. 2022). These observations have been the motivating factors to test the therapeutic potential of this plant extract on aging-induced pathobiological changes in testis. Wistar albino rats were used as an experimental animal model. Our previous study showed a dosage and time-dependent effect of the *M. pruriens* extract in normal rats (Suresh et al. 2009). Previous studies have demonstrated antioxidant, androgenic, aphrodisiac, and spermatogenic properties of *M. pruriens* seed extract. Surprisingly, the biological activities of *M. pruriens* on aging-induced pathological changes in the testis microenvironment have never been explored and the present study was focused on the testing therapeutic efficacy of *M. pruriens* on aged rat testis. Therefore, the current experimental study aimed to analyze the therapeutic efficacy of the extract in aged rat testis for long-term exposure that lasts for sixty days and data were collected using histological and histometric parameters. Moreover, there is no previous experimental investigation on the biological activities of *M. pruriens* on aging-induced pathological changes in the testis microenvironment.

Materials and methods

Animals and experimental design

Male Wistar albino rats (*Rattus norvegicus*) were randomly divided into the following groups; adult (3 months), aged (24 months), aged + *M. pruriens* and adult + *M. pruriens* ($N = 6$ per group). The 24 months of aged rats are equivalent to 60 years in humans (Sengupta 2013). The study was approved by the institutional animal ethics committee (IAEC No: 04/02/2011). Animals were maintained under controlled conditions, at a room temperature of 23 ± 2 °C, humidity (50 ± 5%), and in a 14:10 light/dark cycle in the Central Animal House Facility, Dr.ALM.PGIBMS, University of Madras. The animals were fed with a standard rat pellet diet and drinking water ad libitum. The quarantine procedures and animal maintenance were carried out according to the recommendations of the CPCSEA [Committee for the Purpose of Control and Supervision of Experiments on Animals], guidelines for laboratory animal facility in India [2003].

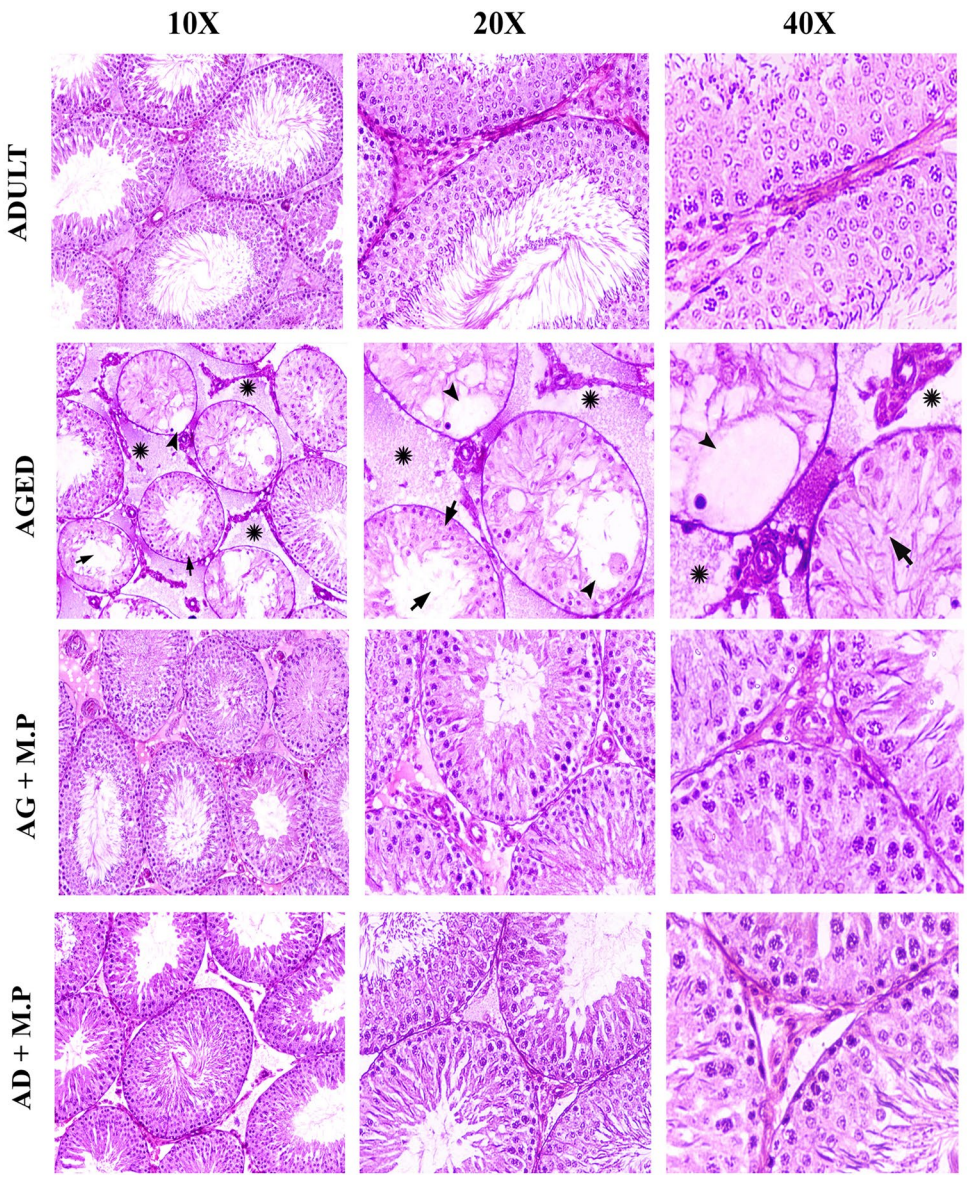


Fig. 2 Representative H&E-stained testis images from various experimental groups taken at various magnifications are aligned for comparison. The adult rat testis shows normal histoarchitecture seminiferous epithelium with typical spermatogenic stages, Sertoli cells, and characteristic interstitial tissue with capillaries and Leydig cells (ADULT). The images from aged rat testis demonstrate incomplete or arrested spermatogenesis, depleted seminiferous epithelium, hypertrophied interstitial tissue, thin-walled and rounded appearance of the tubules. Besides, vacuoles in the Sertoli cells and seminiferous epithelium. Depleted germ cells and hyalinization (AGED). The aged rats + *M. pruriens* illustrate restoration or rejuvenated seminiferous epithelium. The pathological changes seen in the aged testis were considerably reduced, and the histology image comes close to the adult testis image (AG + MP). The adult rat treated with *M. pruriens* also showed normal histoarchitecture and no toxic manifestation was seen after 60 days of extract administration (AD + MP)

Extract preparations, phytochemical screening and dosage

The ethanolic extract of *M. pruriens* seed was used in the present study. Details of the extract preparation are described elsewhere (Suresh et al. 2009; Prakash et al. 2018). Briefly, the seeds of *M. pruriens* were procured locally after authentication, and the voucher specimen (herbarium voucher no. 6907) was deposited in the Department of Plant Biology and Plant Biotechnology (The Presidency College, Chennai, India). After washing the seed using tap water followed by distilled water. The seeds were allowed to dry in the shade for two weeks. Then, the seeds were crushed into coarse powder and transferred into a container, and ethanol was added as a solvent until the coarse particles of the seed were completely soaked. The container was gently shaken every one-hour interval for 72 h (until the solvent became colorless) and the filtrate was lyophilized (Harborne 1973), and the yield was around 20–22% per Kg of seed and the extract was subjected to phytochemical and GC–MS analyses.

The extract was administered at a dose of 200 mg/kg body weight by gavage for sixty days (Suresh et al. 2009), and our studies also confirmed the effectiveness of this dosage (Suresh et al. 2013; Suresh and Prakash 2011, 2012; Prakash et al. 2018).

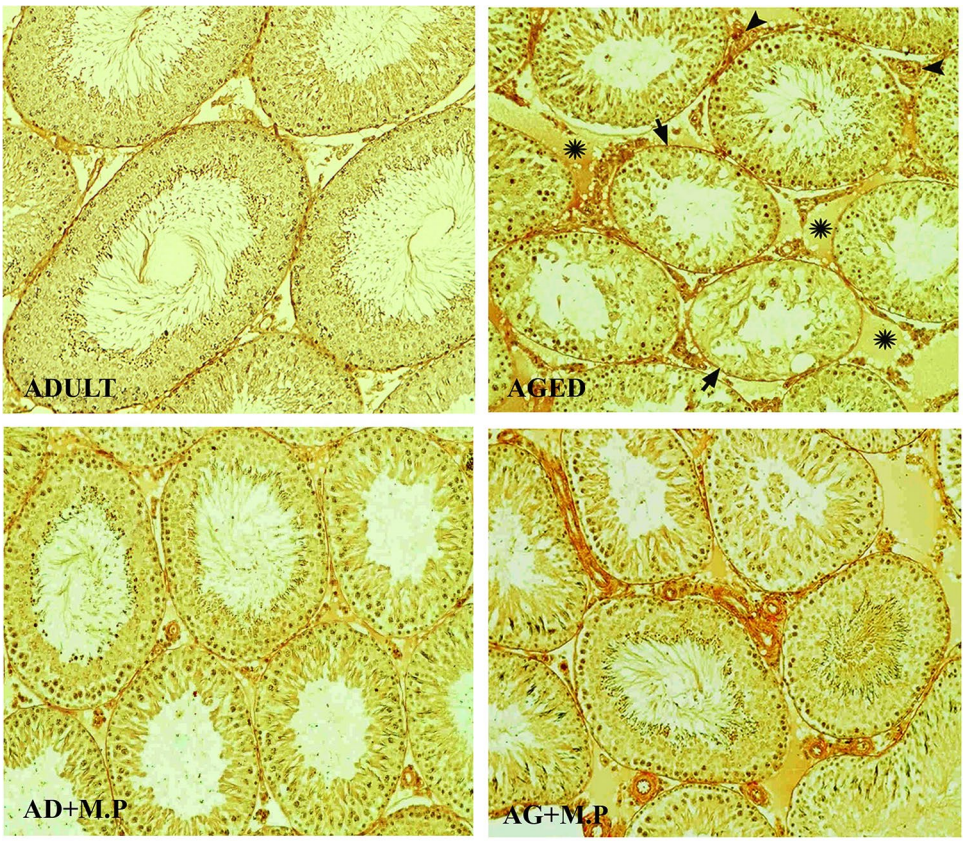


Fig. 3 Representative Van Gieson's trichrome histology images of testis from various experimental groups taken at various magnifications are aligned for comparison. The adult rat testis shows normal histoarchitecture seminiferous epithelium, and the interstitial tissue shows a normal appearance without hypertrophic changes (ADULT). The images from aged rat testis demonstrate dysregulation of spermatogenesis, depleted seminiferous epithelium, hypertrophied interstitial tissue with increased collagen deposition (RED colour) and fibrotic change (AGED). The aged rats + *M. pruriens* illustrate restoration or rejuvenated seminiferous epithelium. However, collagen deposition and hypertrophic changes persisted but without further accretion. *M. pruriens* extract was able to rectify testicular damage (AG + MP). The adult rat treated with *M. pruriens* also showed normal interstitial histoarchitecture (AD + MP)

Estimation of hormone level

Blood was collected from the retro-orbital venous plexus. The serum was used for estimating total and free testosterone (T), follicular stimulating hormone (FSH), and luteinizing hormone (LH) by the radioimmunoassay.

Histological analysis

Animals were killed by administering an overdose of anesthesia (Sodium Pentothal-i.p.) and cardiac perfusion was done using freshly prepared Bouin's fluid. The tissues were processed for paraffin embedding, and sections were taken at 5 µm thickness. The sections were stained using Harris hematoxylin and eosin (H&E) and Van Gieson's trichrome (VG). The stained sections were observed under a brightfield microscope, and the images were documented (Accu-scope, USA). The apoptotic nucleus in testis sections was analyzed using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Roche, Germany), as per manufacturer protocol.

Histometric analysis of testis

The H & E-stained sections were used to perform stereological analysis by the point count method, superimposing lattices (121 intersections). As outlined by Elias and Hyde (1980), the conventional stereological principles and accepted morphometric procedures were used to obtain quantitative information; details of our procedure have been described elsewhere (Prakash et al. 2008). The following histometric parameters were quantified. (i) The diameter, numerical and volume density of the seminiferous tubules, (ii) The height and volume of the seminiferous epithelium, (iii) The volume density of the connective tissue stroma and (iv) the number of Leydig cells. All the histometric results were expressed in relative values.

Estimation of inflammatory and apoptotic proteins in testis by western blot

To perform immunoblotting and biochemical parameters, animals were killed by administering an overdose of

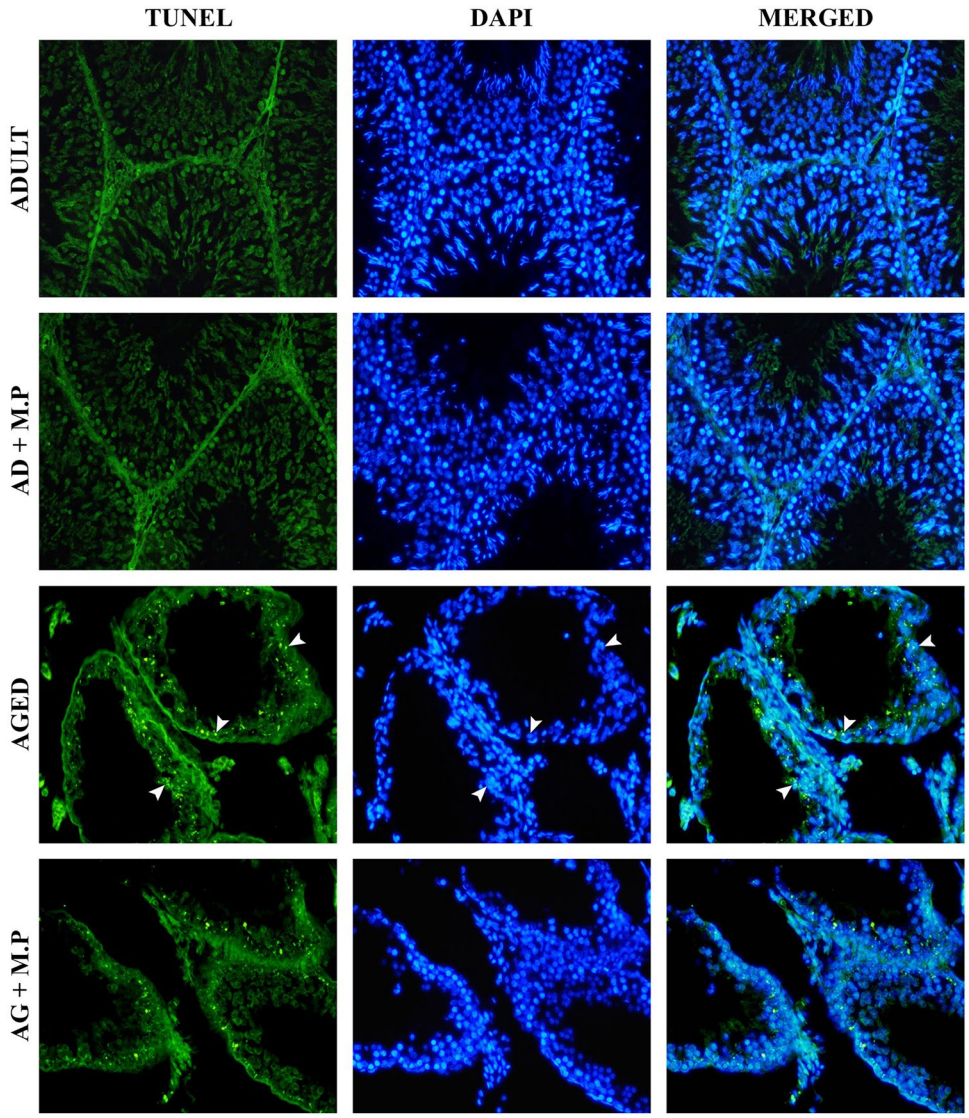


Fig. 4 Fluorescence images of testis from various experimental groups demonstrating *in-situ* detection of apoptotic cells by TUNEL stain in the aligned for comparison. Arrow-heads showing TUNEL-positive cells with bright green fluorescence. The images from aged rat testis demonstrate increased apoptotic cells (AGED). The aged rats + *M. pruriens* illustrate fewer apoptotic nuclei (AG + MP)

anesthesia (Sodium Pentothal-i.p.), at the end of the experimental period (60 days), harvested testicles were processed for western blot and biochemical analyses. The following protein markers of inflammation and apoptosis were analyzed by western blot, tumor necrosis factor-α (TNFα), nuclear factor Kappa B (NF-κB) cytochrome c, Caspase-9, Caspase-3, B-cell lymphoma-2 (Bcl-2), BCL2 associated X (Bax), Poly (ADP-ribose) polymerase (PARP) and inducible nitric oxide synthase (iNOS).

Estimation of antioxidants level and lipid peroxidation (LPO) in testis

The enzymatic antioxidants were estimated as follows, the superoxide dismutase (SOD) activity in the tissue homogenate was estimated using the method of Marklund and Marklund (1974). The activity of catalase (CAT) was estimated by the method of Sinha (1972). Estimation of glutathione reductase (GR) (NADPH: oxidized glutathione oxidoreductase—EC. 1.6.4.2.) activity was estimated in the testicular tissue homogenates by the method of Mize and Langdon (1962). The glutathione-*S*-transferase (GST) activity was assayed by the method of Habig et al. (1974). Estimation of activity of glutathione peroxidase (GPx)/ (Glutathione: Hydrogen peroxide oxidoreductase) in the tissues done by the methods of Rotruck et al. (1973) and Beutler et al. (1963).

The non-enzymatic antioxidants were estimated as follows, ascorbic acid (Vitamin C) content in the testicular tissue samples was determined according to the method of

Omaye et al. (1979). α-tocopherol (Vitamin E) content in the testicular tissue homogenate was estimated as detailed by Desai (1984). The reduced glutathione (GSH) content in the tissue homogenates was estimated by the method of Ellman (1959) with little modification (Beutler et al. 1963). The lipid peroxidation (LPO) level in testicular tissue was estimated by the method of Ohkawa (1979). The thiobarbituric acid reactive substance was expressed in nmol of malondialdehyde (MDA) formed / min /mg protein.

Estimation of total reactive oxygen species (ROS), superoxide anion, and hydroxyl radicals in testis

The total ROS was demonstrated in situ in testis cryosections using a dichlorofluorescein (DCFH) probe (Mundy et al. 1997; Suresh et al. 2011). In the presence of ROS, the non-fluorescent DCFH is rapidly oxidized to a highly fluorescent 2′,7′-dichlorofluorescein (DCF), and these sections were observed using a fluorescence microscope (Nikon Corporation, Japan). The superoxide anion presence was demonstrated in situ in testis cryosections using a dihydroethidium (DHE) probe (Bivalacqua et al. 2013). The superoxide anion ($O2\bullet^{-}$) presence oxidized the DHE to red fluorescent ethidium bromide. The sections were observed using a fluorescence microscope (Nikon Corporation, Japan).

The biochemical estimation of superoxide anion in the tissue homogenate was measured based on the reduction of *p*-Iodonitrotetrazolium (Podczasy and Wei 1988). Upon reduction by superoxide anion, a soluble reddish product with an absorption maximum of 505 nm was noted. The hydroxyl radicals were measured according to the method described by Puntarulo and Cederbaum (1988). The hydroxyl radical in the homogenate was assayed by measuring the generation of formaldehyde from dimethyl sulfoxide. The hydrogen peroxides present in the testicular tissues were assayed by the method of Pick and Keisari (1981). Horseradish peroxide converts hydrogen peroxide into water and oxygen. This causes the oxidation of phenol red which forms an adduct with dextrose that has a maximum absorbance at 610 nm. The absorbance was read against a reagent blank on a spectrophotometer.

The data collected ($N = 6$/parameter) were statistically analyzed using SPSS for Windows (SPSS, Version 16.0 Inc., Chicago, IL). The data were subjected to One-Way ANOVA, and the significance was determined using a "Tukey's post hoc" test with $P < 0.05$.

Results

Estimation of hormone level

The total and free testosterone, FSH and LH levels were considerably decreased in aged and aged + *M. pruriens* groups compared to adult rats. However, the aged + *M. pruriens* showed significantly increased total and free testosterone, FSH and LH levels compared with untreated aged. The total and free testosterone, FSH and LH levels were increased in adult + *M. pruriens* animals compared to adult control (Fig. 1A–C).

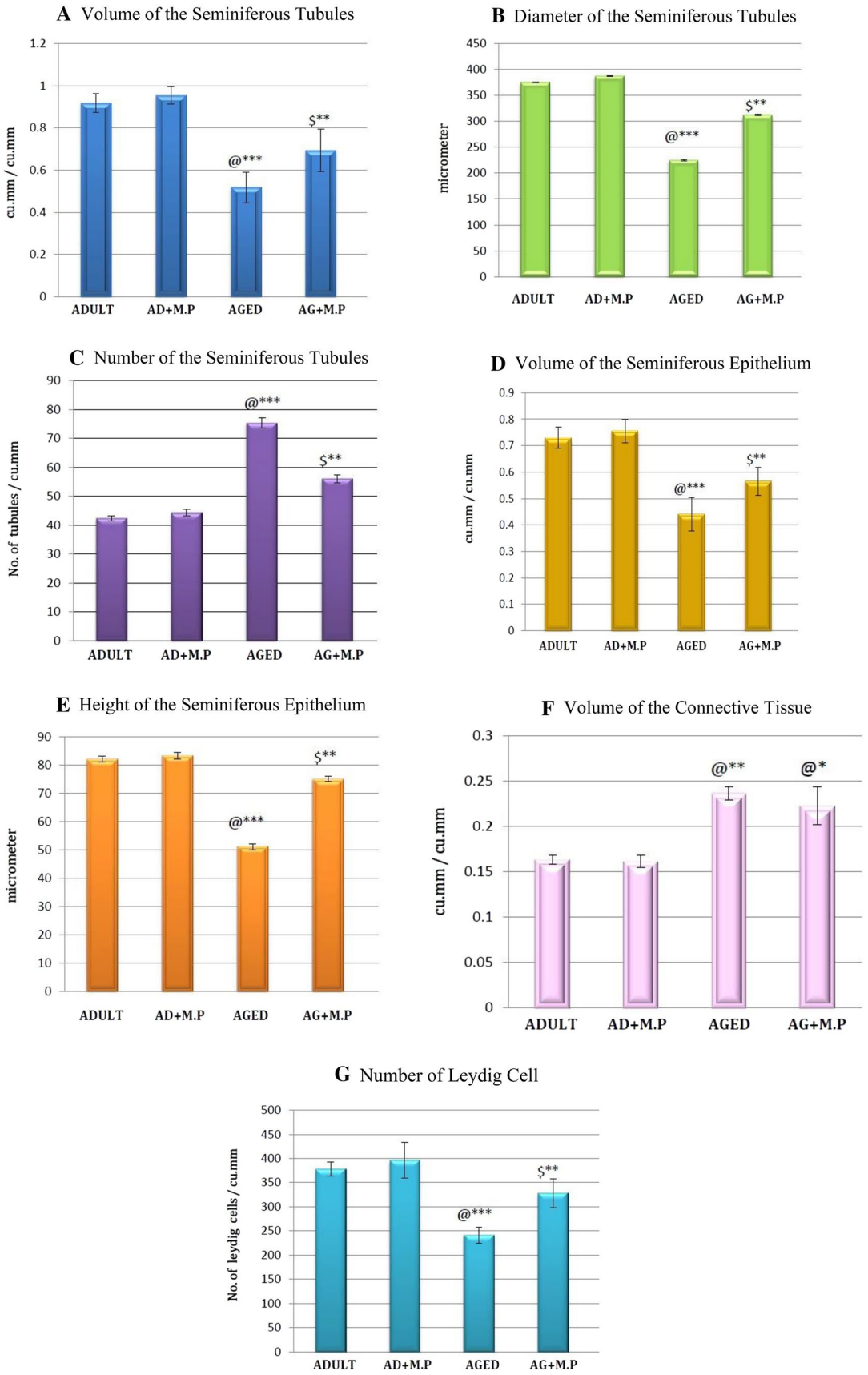
Gross measurements

Morphological analysis showed a significant reduction in the weight and volume of the aged rat testis compared to adult control, indicating severe testicular dysfunction. However, there was a significant improvement in testicular weight (Fig. 1D) and volume (Fig. 1E) in aged + *M. pruriens* group compared to untreated aged rats.

Histological study

Histological analysis using H&E-stained sections revealed normal histo-architecture and spermatogenesis in adult rat testis. The aged rat testis showed degenerative changes and dysregulation of spermatogenesis with vacuolation in the epithelium, sloughing of cells, reduced spermatid and sperm cell number. Multinucleated gait cells were seen in the tubules of aged rat testis, indicating severe dysregulation of spermatogenesis. Interstitial tissue was hypertrophied and increased in proportion. Besides, the blood capillaries in the interstitium showed wall thickening and inflammatory or vasculitis-like appearance in aged rat testis (Fig. 2).

The result from VG-stained sections taken from aged rat testis indicates increased interstitial deposition of collagen and fibrotic changes around the seminiferous tubules and the capillary wall (Fig. 3). The fluorescence images of testis from various experimental groups illustrate the in-situ localization of apoptotic nuclei by TUNEL stain. The images from aged rat testis demonstrate increased apoptotic nuclei. The aged rats + *M. pruriens* illustrate fewer apoptotic nuclei (AG + MP). (Fig. 4). All these above pathological changes were significantly reduced in aged + *M. pruriens* groups. However, collagen deposition and tubular wall thickening were not reduced significantly


A Volume of the Seminiferous Tubules
cu.mm / cu.mm
1.2
1
0.8
0.6
0.4
0.2
0
@***
$**
ADULT
AD+M.P
AGED
AG+M.P
B Diameter of the Seminiferous Tubules
micrometer
450
400
350
300
250
200
150
100
50
0
@***
$**
ADULT
AD+M.P
AGED
AG+M.P
C Number of the Seminiferous Tubules
No. of tubules / cu.mm
90
80
70
60
50
40
30
20
10
0
@***
$**
ADULT
AD+M.P
AGED
AG+M.P
D Volume of the Seminiferous Epithelium
cu.mm / cu.mm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
@***
$**
ADULT
AD+M.P
AGED
AG+M.P
E Height of the Seminiferous Epithelium
micrometer
90
80
70
60
50
40
30
20
10
0
@***
$**
ADULT
AD+M.P
AGED
AG+M.P
F Volume of the Connective Tissue
cu.mm / cu.mm
0.3
0.25
0.2
0.15
0.1
0.05
0
@**
@*
ADULT
AD+M.P
AGED
AG+M.P
G Number of Leydig Cell
No. of leydig cells / cu.mm
500
450
400
350
300
250
200
150
100
50
0
@***
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ADULT
AD+M.P
AGED
AG+M.P

◂**Fig. 5** **A** Histometric values of seminiferous tubular volume in adult and other experimental groups. The values are expressed in cubic millimeter / cubic millimeter (mm^3/mm^3) and presented as mean ± SEM ($n = 6$). The assessment indicated significantly reduced tubular volume density in aged rats. The tubular volume increased in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **B** Histometric assessment of seminiferous tubular diameter in adult and other experimental groups. The values are expressed in micrometers (μm) and presented as mean ± SEM ($n = 6$). The assessment indicated a significantly reduced average tubular diameter (axial ratio) in aged rats. The tubular volume increased in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **C** Histometric values of seminiferous tubular number in adult and other experimental groups. The values are expressed in the number of tubules per cubic millimeter and presented as mean ± SEM ($n = 6$). The assessment indicated a significantly increased average tubular number per unit area in aged rats. The tubular number was reduced in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). The tubular number increased due to the reduction in their diameter. AG + MP indicates that the diameter of the tubules was on the smaller side but in the regaining process. @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **D** Histometric values of seminiferous epithelial volume in adult and other experimental groups. The values are expressed in the number of tubules per cubic millimeter and presented as mean ± SEM ($n = 6$). The observed data indicated a significantly reduced epithelial volume proportion in aged rats. The seminiferous epithelial volume was increased in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). @ Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **E** This shows the histometric assessment of seminiferous epithelial height in adult and other experimental groups. The values are expressed in micrometers (μm) and presented as mean ± SEM ($n = 6$). The assessment indicated a significantly reduced volume proportion in aged rats. The tubular volume was increased in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). @ Control compared with other experimental groups; $—AGED compared to AG + MP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **F** Histometric analysis of connective proportion in adult and other experimental groups. The values are expressed in the cubic millimeter/ cubic millimeter (mm^3/mm^3) and presented as mean ± SEM ($n = 6$). The assessment indicated a significantly increased connective tissue proportion in aged rats due to hypertrophic changes with deposition collagen (seen in histology). The connective tissue was reduced in aged + *M. pruriens* (AG + MP) rats compared to untreated aged (AGED). Though data showed connective tissue proportion on the higher side in AG + MP than in adults but indicated a mild reduction or maybe under retrieval process and importantly, without further accretion. @ Control compared with other experimental groups; $—AGED compared to AG + MP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **G** Histometric values of Leydig cell number in adult and other experimental groups. The values are expressed in the number of Leydig cells per cubic millimeter and presented as mean ± SEM ($n = 6$). The assessment indicated a significantly reduced average Leydig cell number per unit area in aged rats. The Leydig cell number was reduced in aged + *M. pruriens* (AG + MP) rats than in untreated aged (AGED). @ Control compared with other experimental groups; $—AGED compared to AG + MP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

in aged + *M. pruriens*. But a considerable improvement was seen in the seminiferous epithelium and spermatogenesis in the presence of extract.

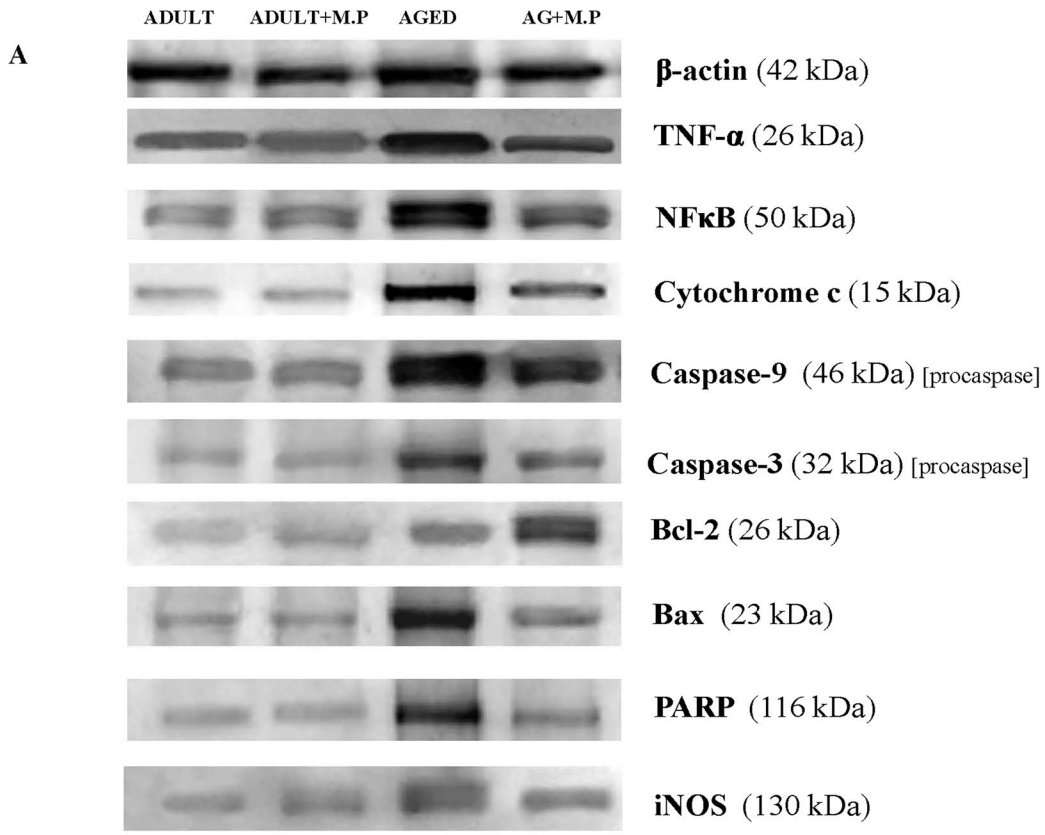
Testicular histometry

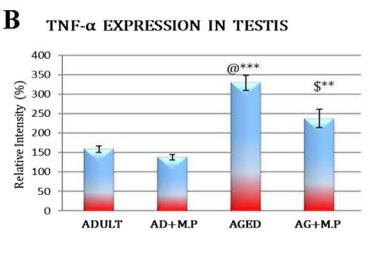
Histometric analysis of aged rat testis indicated severe depletion in the volume of the epithelial component, reduction in the epithelial height, and reduction in tubular diameter; as a result, more tubules (cross-section profiles) per unit area were seen in the aged rat testis than in adult testis. The volume of the connective tissue component in the aged testis was significantly increased. The number of Leydig cells estimated per unit area in the aged rat testis was reduced compared to adult rat testis.

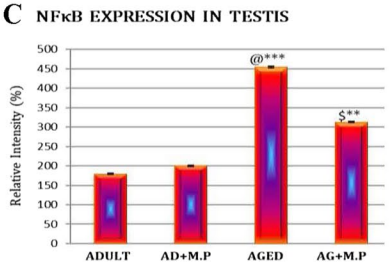
The quantitative data from seminiferous epithelium indicate significant rejuvenation or restoration of spermatogenic cells in aged + *M. pruriens* rat testis. The highlighting observations in this group were; increased tubular diameter, increased interstitial component, and Leydig cell number were increased compared to aged rat testis (Fig. 5A–G).

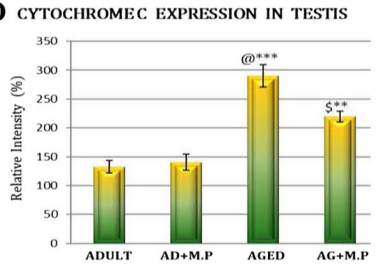
Inflammatory and apoptotic proteins in testis

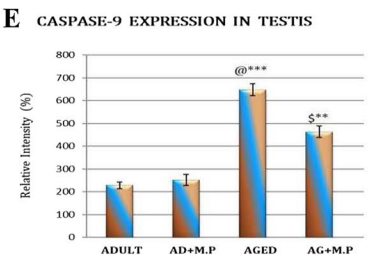
Western blot detection revealed the relative intensity of activated TNF-α expression was significantly increased ($p < 0.001$) in the aged rat when compared to the adult control. The intensity of TNF-α protein expression was decreased significantly in the aged rat treated with *M. pruriens* ($p < 0.01$). The relative intensity of NFκB protein expression was significantly increased ($p < 0.001$) in the aged rat when compared to the adult control. The NFκB protein expression was decreased significantly in the aged rat treated with *M. pruriens* ($p < 0.01$). The cytochrome-c expression was significantly increased ($p < 0.001$) in the aged rat when compared to the adult control. The relative intensity of cytochrome-c protein expression was decreased in the aged rat treated with *M. pruriens* ($p < 0.01$). Estimation of caspase-9, caspase 3, Bcl-2, Bax and PARP expressions showed significantly increased levels in the aged rat ($p < 0.001$) when compared to adult control. These protein expressions were decreased significantly ($p < 0.01$) in the aged rat treated with *M. pruriens*. The estimation of iNOS level in aged testis showed, a significantly increased ($p < 0.001$) than other groups. The iNOS protein expression was decreased significantly ($p < 0.01$) in the aged rat treated with *M. pruriens*. (Fig. 6A–J).

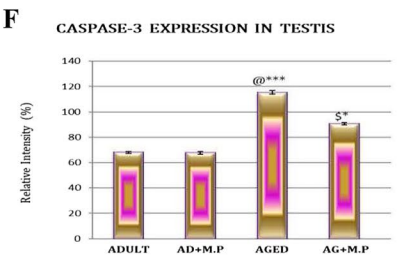

A
ADULT
ADULT+M.P
AGED
AG+M.P
β-actin (42 kDa)
TNF-α (26 kDa)
NFκB (50 kDa)
Cytochrome c (15 kDa)
Caspase-9 (46 kDa) [procaspase]
Caspase-3 (32 kDa) [procaspase]
Bcl-2 (26 kDa)
Bax (23 kDa)
PARP (116 kDa)
iNOS (130 kDa)

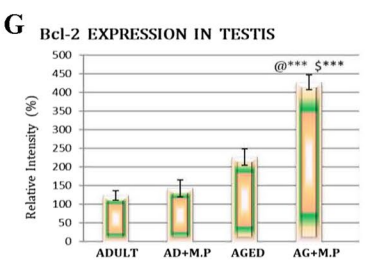

B
TNF-α EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P

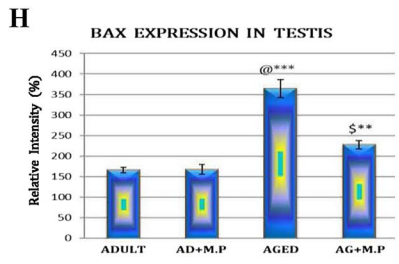

C
NFκB EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P

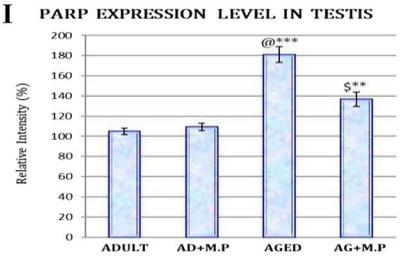

D
CYTOCHROMEC EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P

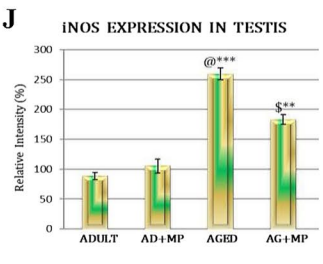

E
CASPASE-9 EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P


F
CASPASE-3 EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P


G
Bcl-2 EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P


H
BAX EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P


I
PARP EXPRESSION LEVEL IN TESTIS
Relative Intensity (%)
ADULT
AD+M.P
AGED
AG+M.P


J
iNOS EXPRESSION IN TESTIS
Relative Intensity (%)
ADULT
AD+MP
AGED
AG+MP

◂**Fig. 6** Images of western-blot analysis of the inflammatory proteins in various experimental groups. Western-blot bands are represented as images and the corresponding histograms represent the relative intensity of protein expression. Showing the relative intensities (%) of (TNFα), nuclear factor Kappa B (NF-κB) cytochrome c, Caspase-9, Caspase-3, B-cell lymphoma-2 (Bcl-2), BCL2 associated X (Bax), Poly (ADP-ribose) polymerase (PARP) and inducible nitric oxide synthase (iNOS) protein expression in testis of control and various experimental groups. The values are expressed as mean ± SEM. @—Control compared with other experimental groups; $—AGED compared to AG + MP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The values are expressed as mean ± SEM

Antioxidant level, lipid peroxidation (LPO) and ROS level in testis

The SOD level was significantly reduced in untreated aged groups. Similarly, the CAT level was also reduced significantly in aged rat testis. The GR, GST and GPx activities were depleted in aged groups when compared with the control. These enzyme levels were restored to the average level in the aged + *M.pruriens* group rat testis. Further, antioxidants viz. vitamin C, vitamin E, and glutathione were also found to decrease in aged rat testis. These antioxidant levels were increased in aged + *M.pruriens* rat testis. Relative values of antioxidants level in various experimental groups were presented in Fig. 7A–H.

The ROS levels were significantly increased in aged rat testis demonstrated by enhanced DHE fluorescence, indicating augmented generation of superoxide anion radicals (Fig. 8A, B) and hydroxyl anion levels in aged rat testis (Fig. 8C, D) than other experimental groups. Concomitantly the MDA levels were significantly increased in aged rat testis than the control, indicating increased LPO. The LPO and free radicals were significantly reduced in the aged + *M. pruriens* group testis. (Fig. 8E).

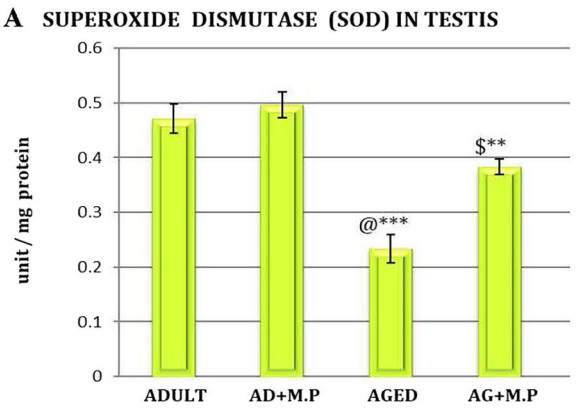
Discussion

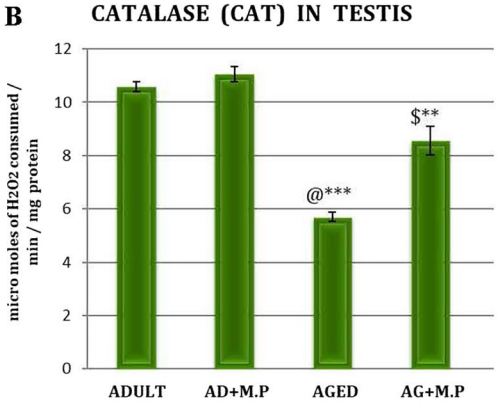
The gross testicular size and morphology indicate the well-being of the seminiferous epithelium. During the development and growth of the testis, the tubules elongated and convoluted in compartments and got filled with epithelial cells, resulting in increased size and weight of the testis (Prakash et al. 2008). As the aging process commences, the gross testicular morphology and weight reduction indicate diminished functionality gross testicular atrophy (Matzkin et al. 2019) and a significant reduction in volume indicates the internal cellular perturbations in aging. The aged + *M. pruriens* rats showed a remarkably increased testicular weight and volume.

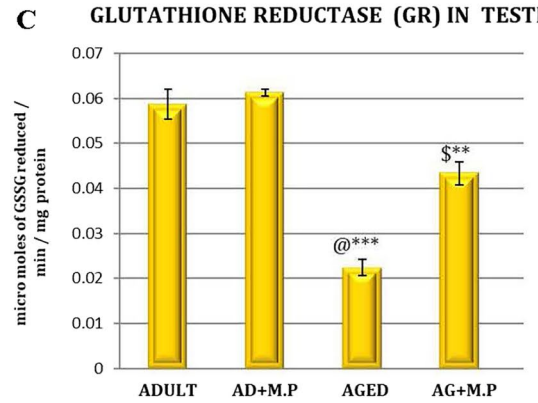
Histological analysis of aged rat testis revealed various grades of pathological changes, such as; distorted seminiferous tubules, many tubules showing only Sertoli cells, appeared like 'Sertoli cell-only syndrome' and incomplete or arrest of spermatogenesis. The seminiferous epithelial height was reduced, tubules were devoid of spermatozoa and obliterated, collagen deposition was increased in the interstitial tissue and the thickened basal membrane with fibrotic changes. Besides, Sertoli cells showed vacuolations, the appearance of multinucleated spermatogenic cells with a fragmented nucleus and shrunken cellular morphology. Vacuolation in the seminiferous epithelium and the disappearance or loss of germ cells and hyalinization.

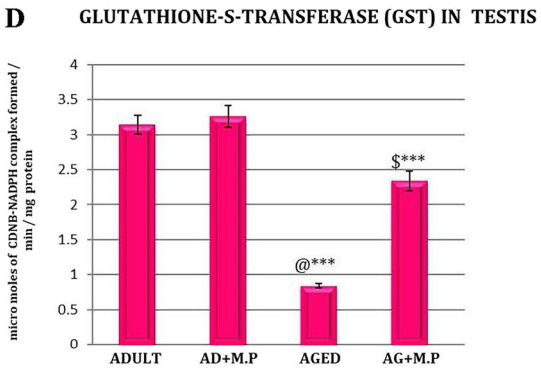
The alterations in histoarchitecture seen in the aged rat were progressive due to the continuous insult through cellular oxidative stress induced by free radicals during aerobic metabolism resulting in the peroxidation and oxidation of lipids and proteins (Phaniendra et al. 2015). The oxidative damage initiated by reactive oxygen species could be a major contributor to functional decline, a characteristic feature of the aging process (Nguyen-Powanda and Robaire 2020). With aging, the fluidity of the cell membrane decreases as a result of lipid peroxidation. This leads to damage to Sertoli–Sertoli and Sertoli-germ cell interactions and compromised membrane functionally under oxidative stress (Yang et al. 2020). Under normal circumstances, Sertoli cells, which form the blood-testis barrier, separate the germ cells from antigens exposure or antibody attack and have the endurance for a long duration in pathological and some demanding conditions (Prakash and Kamakshi 2014; Santiago et al. 2019). The microenvironment deterioration affects tight junction proteins that may cause a breakage in the blood-testis barrier leading to catastrophic changes in the testis (Matzkin et al. 2016; Yang et al. 2020).

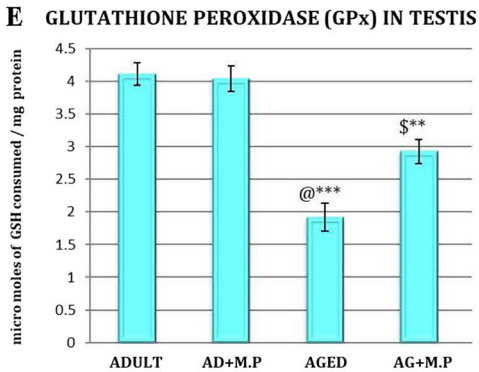
The aged rat administered with *M. pruriens* showed considerable recovery from all the pathological features observed in untreated aged rat testis. The restoration of seminiferous epithelium after *M. pruriens* treatment indicates the potential of the extract to protect the germinal epithelial cells from oxidative stress. The remedial effect could be influenced by the flavonoids and phenolic compounds in the extract that might exhibit high antioxidant and free radical scavenging activities (Suresh et al. 2009). The results from the present study demonstrate depleted antioxidants (both enzymatic and non-enzymatic) and increased LPO and free radicals. The *M. pruriens* seed extract contains a rich source of antioxidants and scavenging free radical activity (Kanazawa and Sakakibara 2000). These activities might enhance the testicular cells' antioxidant system to overcome oxidative injury and rejuvenate the spermatogenic process. Suppression of reactive oxygen species and simultaneously boosting free radicals scavenging before interacting with the plasma membrane has revived Sertoli cell structure and function. Thereby able to support and stabilize the germ cell population in

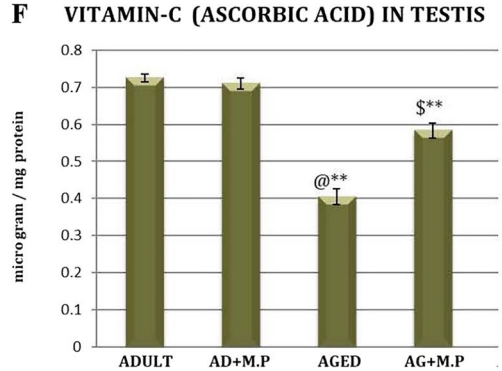

A SUPEROXIDE DISMUTASE (SOD) IN TESTIS
unit / mg protein
0.6
0.5
0.4
0.3
0.2
0.1
0
$**
@***
ADULT
AD+M.P
AGED
AG+M.P

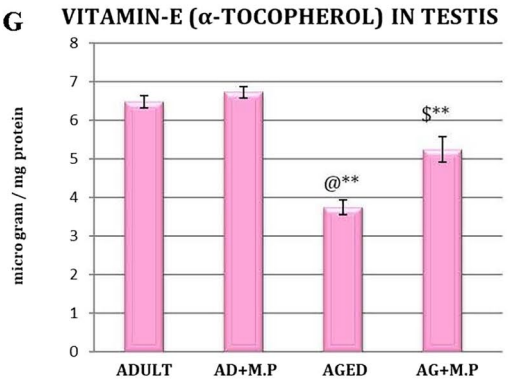

B CATALASE (CAT) IN TESTIS
micro moles of H2O2 consumed / min / mg protein
12
10
8
6
4
2
0
$**
@***
ADULT
AD+M.P
AGED
AG+M.P

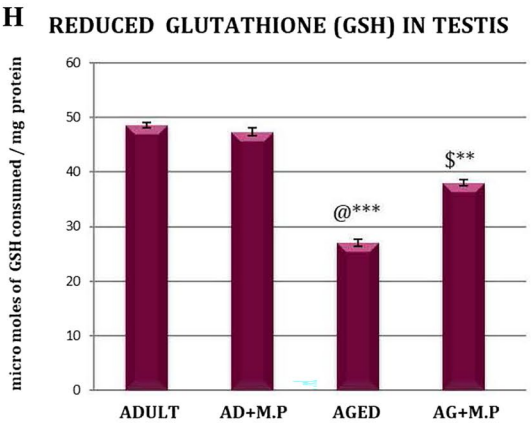

C GLUTATHIONE REDUCTASE (GR) IN TESTIS
micro moles of GSSG reduced / min / mg protein
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
$**
@***
ADULT
AD+M.P
AGED
AG+M.P


D GLUTATHIONE-S-TRANSFERASE (GST) IN TESTIS
micro moles of CDNB-NADPH complex formed / min / mg protein
4
3.5
3
2.5
2
1.5
1
0.5
0
$***
@***
ADULT
AD+M.P
AGED
AG+M.P


E GLUTATHIONE PEROXIDASE (GPx) IN TESTIS
micro moles of GSH consumed / mg protein
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
$**
@***
ADULT
AD+M.P
AGED
AG+M.P


F VITAMIN-C (ASCORBIC ACID) IN TESTIS
micro gram / mg protein
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
$**
@**
ADULT
AD+M.P
AGED
AG+M.P


G VITAMIN-E (α-TOCOPHEROL) IN TESTIS
micro gram / mg protein
8
7
6
5
4
3
2
1
0
$**
@**
ADULT
AD+M.P
AGED
AG+M.P


H REDUCED GLUTATHIONE (GSH) IN TESTIS
micro moles of GSH consumed / mg protein
60
50
40
30
20
10
0
$**
@***
ADULT
AD+M.P
AGED
AG+M.P

◂**Fig. 7** **A** shows superoxide dismutase (SOD) levels in control and other experimental groups. The values are expressed in units/mg of protein and presented as mean ± SEM ($n = 6$). Data indicate a significantly reduced level of SOD in aged animal testis when compared to other groups. **B** illustrates catalase levels in the testis of control and various experimental groups. The values are expressed in µmoles of H2O2 consumed/min/mg protein and presented as mean ± SEM ($n = 6$). Data indicate significantly reduced levels of catalase in aged groups testis when compared to other groups. **C** shows glutathione reductase (GR) levels in control and other experimental groups. The values are expressed in µmoles of GSSG reduced/min/mg protein and presented as mean ± SEM ($n = 6$). Observation indicates a significantly reduced level of GR in aged animal testis when compared to adult and aged + *M. pruriens*. **D** illustrates glutathione-*S*-transferase (GST) level in the testis of control and various experimental groups. The values are expressed in µmoles of CDNB-NADPH complex formed/min/mg protein and presented as mean ± SEM ($n = 6$). Data indicate a significantly reduced level of GST in aged rat testis when compared to other experimental groups. **E** shows glutathione peroxidase (GPx) levels in control and other experimental groups. The values are expressed in µmoles of GSH consumed/mg protein and presented as mean ± SEM ($n = 6$). Data indicate a significantly reduced level of GPx in aged rat testis when compared to adult and aged + *M. pruriens*. **(F)** illustrates vitamin-C levels in the testis of control and various experimental groups. The values are expressed in µgram/mg protein and presented as mean ± SEM ($n = 6$). The relative comparison of data indicates a significantly reduced level of vitamin C in aged rat testis when compared to adult and aged + *M. pruriens*. **G** Shows vitamin-E (α-tocopherol) levels in control and other experimental groups. The values are expressed in µgram/mg protein and presented as mean ± SEM ($n = 6$). Data indicate a significantly reduced level of α-tocopherol in aged rat testis when compared to adult and aged + *M. pruriens.* **H** illustrates reduced glutathione (GSH) levels in the testis of control and various experimental groups. The values are expressed in µmoles of GSH consumed/mg protein and presented as mean ± SEM ($n = 6$). The relative comparison of data from different experimental groups indicates significantly reduced levels of GSH and other antioxidants in aged rat testis. The overall level of antioxidants was increased in aged animals' testis after administration of *M. pruriens* seed extract indicating its ability to reduce oxidative stress and improve cellular well-being. Data were analyzed by one-way ANOVA and multiple comparisons between groups were performed by Tukey's post hoc test. @—Control compared with other experimental groups; $—AGED compared to AG + MP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

aged rats treated with the extract. Furthermore, it has been shown that the hydrogen sharing ability of *M. pruriens* can quench the reactive oxygen species (Lampariello et al. 2012; Pathania et al. 2020). They reduce membrane damage by impeding self-propagating LPO reactions.

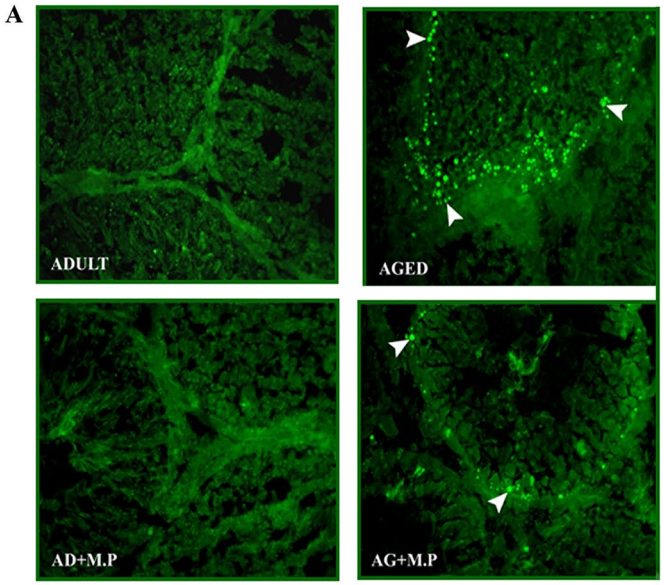
The SOD, CAT, GST, GPx, GSH, GR, vitamins C and E form the principal defense systems against oxygen-free radicals in the biological system (Fang et al. 2002). Thus, the action of bioactive and bioavailable rich components in *M. pruriens* rectified the antioxidant system in aged rat testis and was able to combat oxidative stress-induced apoptosis (Marouani et al. 2017).

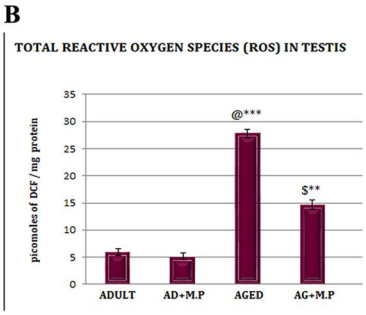
The aged rats showed remarkable depletion of total and free testosterone, FSH and LH. The depleted testosterone could have affected the dysregulation of cytokines in aging (Mohamad et al. 2019) and ensuing pathological changes. Concomitantly, the *M. pruriens* administrated aged rat showed restoration of testosterone, FSH, and LH levels and offered protection to aged rat testis. The increased testosterone level in *M. pruriens* treated aged rats might be through its potential to increase androgen biosynthesis (Shukla et al. 2009) by activating the pituitary–testicular axis (Misra et al. 2004). Further, the ability of the *M. pruriens* to reduce oxidative stress (Suresh et al. 2009) might reduce Leydig cell damage and the Phyto-androgenic influence of the *M. pruriens* extract on the androgen receptor (AR) (Prakash et al. 2018) distributed in nuclei of Sertoli cells, Leydig cells, and peritubular myoid cells (Javier et al. 2001) could have also rectified the perturbed testosterone level and improved histo-architecture.

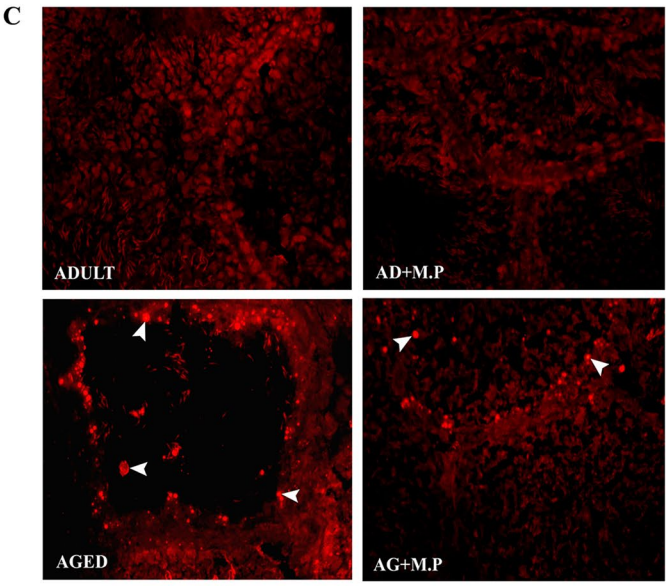
Another striking observation in the histology of aged rat testis was the perfectly round tubules in cross-section, unlike adult and aged + *M. pruriens* groups in which the seminiferous tubules showed slight undulation in their wall. Maybe because as the mass degenerated and sloughed, epithelial cells flushed out from various regions of the tubular system towards the rete testis and ultimately block it and creating back pressure in the tubules (Johnson 2015) and making the tubules take more of a round shape in cross-section. Besides, once set in, the backpressure scenario could add up to the degenerative process of aging. Interestingly, the aged + *M. pruriens* rat testis demonstrated an undulated tubular margin and showed a typical multicellular epithelium of the testis. Furthermore, reduces oxidative stress and reorganizes the membrane integrity (Suresh et al. 2013). *M. pruriens* seed extract has been shown to increase androgen levels (Prakash et al. 2001). It might stimulate the gonadotropin-releasing hormone (GnRH) secreting centers of the brain, i.e., the hypothalamus and forebrain (Shukla et al. 2009). The GnRH acts on the anterior pituitary to upregulate the FSH and LH levels, resulting in increased testosterone secretion, thereby able to exhibit spermatogenic potential (Singh et al. 2013).

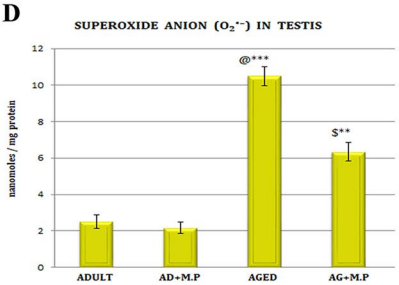
Van Gieson's stain for demonstrating collagen deposition

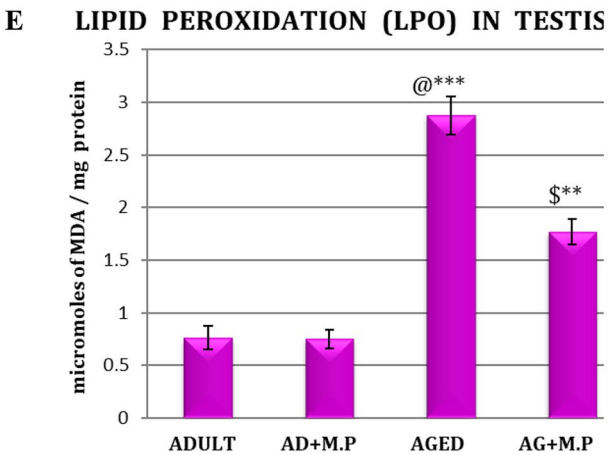
In the seminiferous tubule, the basement membrane is composed of three basic components viz., (i) the basal lamina, comprised of a thin, sheet-like extracellular matrix (ECM), (ii) a thin collagen layer, and (iii) a layer of peritubular myoid cells. ECM plays an important role in regulating Sertoli cell function by affecting its morphology, differentiation and migration. Van Gieson's staining showed increased collagen deposition around the tubules and in the interstitium. The dysregulated collagen in the matrix and basement membrane in aged rat testis could have led to Sertoli cell dysfunction and affected epithelium to slough (Siu et al. 2003; Bhanmeechao et al. 2018). The changes in interstitium could


A
ADULT
AGED
AD+M.P
AG+M.P


B
TOTAL REACTIVE OXYGEN SPECIES (ROS) IN TESTIS
picomoles of DCF / mg protein
@***
$**
ADULT
AD+M.P
AGED
AG+M.P


C
ADULT
AD+M.P
AGED
AG+M.P


D
SUPEROXIDE ANION ($O_2^{\bullet-}$) IN TESTIS
nanomoles / mg protein
@***
$**
ADULT
AD+M.P
AGED
AG+M.P


E
LIPID PEROXIDATION (LPO) IN TESTIS
micromoles of MDA / mg protein
@***
$**
ADULT
AD+M.P
AGED
AG+M.P

◂**Fig. 8** **A** Fluorescence microscope image of *in-situ* localization of total ROS in the testicular tissue sections demonstrated by 2'-7'dichlorofluorescin diacetate (DCFH-DA) staining. The aged rat testis showed an increased generation of total ROS (arrowheads ROS in germ cells) when compared to the adult. The aged + *M.pruriens* showed reduced ROS levels when compared to the aged rat (200×). **B** Illustrates relative values of total reactive oxygen species in the testis of various experimental groups. The values are expressed in picomoles of DCF per mg protein and presented as mean ± SEM ($n = 6$). @—Control compared with other experimental groups; $—AGED compared to AG + MP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **C** Fluorescence microscope image of *in-situ* localization of superoxide anion in the testicular tissue sections demonstrated by dihydroethidium (DHEA) staining. The aged rat testis showed increased superoxide anion (arrowheads) in germ cells of the aged rat testis when compared to the adult. The testis sections from aged + *M. pruriens* showed reduced superoxide anion levels when compared to the aged rat (200×). **D** Illustrates the relative values of superoxide anion in the testis in various experimental groups. The values are expressed in nanomoles per mg protein presented as mean ± SEM ($n = 6$). @—Control compared with other experimental groups; $—AGED compared to AG + M.P. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. **E** Illustrates lipid peroxidation (LPO) level in the testis of control and various experimental groups. The values are expressed in µmoles of MDA formed /mg protein and presented as mean ± SEM ($n = 6$). Significantly increased LPO was seen in aged rat testis. @—Control compared with other experimental groups; $—AGED compared to AG + MP. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The observation showed that administration of *M. pruriens* to aged animals was able to reduce ROS levels and improved enzymatic and non-enzymatic antioxidant levels resulting in reduced LPO and damage to cell membranes

have affected the vasculature and tubular function in aged rat testis, but no sign of inflammatory or granulomatous reaction apart from vasculitis-like lesions around the capillaries. Indicate that the aged rat testis interstitium still exhibits the immune privilege and immunosuppressive potential that is seen in normal and in a variety of pathological conditions (Itoh et al. 1999; Hedger 2011).

In *M. pruriens* treated aged rat testes showed fibrotic deposition as seen in untreated aged rat testis. Since the fibrotic accumulation has happened through ages before treatment, it was sustained and not reduced significantly during the treatment period, however, without further accretion. Improving vasculature is an important potential target for managing or even treating endocrine disorders and comorbidities related to aging (Stucker et al. 2021). Thus, improved communication between these structures and an effective nutrient transfer might bring in therapeutic influence in the extract-treated aged rats. Probably by controlling oxidative stress (Suresh et al. 2010) and exhibiting anti-inflammatory activity (Swain et al. 2008; Avoseh et al. 2020).

TUNEL staining of apoptotic nuclei

The TUNEL staining demonstrated numerous apoptotic nuclei in the seminiferous epithelium of aged rat testis. These pathological changes may result from increased free radical production, oxidative stress and diminished antioxidant level in the aged rat testis. The apoptotic process can be triggered by increased oxidative stress (Liguori et al. 2018) and inflammatory reactions (Cudicini et al. 1997) in aging. The administration of *M. pruriens* in aged rats showed considerably reduced apoptotic cells in the seminiferous epithelium than untreated aged rats. The downregulation of apoptosis and protection from the cell death pathway might be brought about by free radical scavenging, antioxidant potential and anti-inflammatory properties of *M. pruriens* (Suresh et al. 2011; Suresh et al. 2013; Avoseh et al. 2020).

Histometric analyses

The histometric parameters such as the diameter of the seminiferous tubules, the height of the seminiferous epithelium, the number of Leydig cells, volume of seminiferous tubule and seminiferous epithelium were significantly decreased in aged rat testis. As a pathological manifestation, the volume of connective tissue proportion was increased significantly in the aged rat testis compared to adult rats. Besides, an increase in the number of seminiferous tubules per unit area was observed in aged rat testis due to tubular shrinkage (reduced diameter vide supra) and thus, able to bring in more tubules per unit area in the aged. There was a positive relationship between the seminiferous tubule diameter and spermatogenic activity (Hikim et al. 1989; Kalwar et al. 2019). As the cellular components of the seminiferous epithelium degrade, leaving only the Sertoli cells with an empty lumen. We observed the volume of the tubule and the epithelium to be significantly reduced in the aged rat testis.

Inflammatory and apoptotic proteins in testis

Aging is caused by several factors which are categorized as 'the biological theories of aging. Among these theories of aging, the oxidative stress theory, the free radical theory and the mitochondrial theory are the most widely accepted harmful biological reactions leading to pathological changes and aging of organisms (Matzkin et al. 2021). Our study indicates an elevation in oxidative stress and a concomitant increase in inflammatory and apoptotic proteins in aged rat testis. TNF-α plays an important role in apoptosis and modulates spermatogenesis and an increase in TNF-α results in massive germ cell loss (Theas 2018). TNF-α has also been shown to affect Sertoli cell tight junction assembly and testicular basement membrane (Siu et al. 2003; Chen et al. 2017). NFκB, a key player in the early and late stages of inflammation, is activated by TNF-α, concomitantly they can induce apoptosis (Khandelwa et al. 2011) and it was also established that this can be inhibited by antioxidants (Machado et al. 2021). Cytochrome c complexes are components of the electron transport chain, located in the inner

mitochondrial membrane. The release of cytochrome c into the cytoplasm binds to apoptosis protease-activating factor 1 (Apaf-1) (Xiong et al. 2014), a powerful activator of apoptosis and facilitates the reduction or removal of damaged germ cells (Liu et al. 2006). Though precise molecular mechanisms controlling cytochrome c release from mitochondria in the presence of a pro-apoptotic stimulus are not clear. This may be possibly due to the loss of membrane potential as a result of dysregulated through pores. The cytochrome c in the cytoplasm promotes the activation of procaspase 9 and the ensuing proteolytic activation of effector caspases (Abrishamdar et al. 2022). The mitochondrial pathway of apoptosis is modulated by pro-survival proteins such as Bcl-2, whereas pro-apoptotic members including Bax proteins stimulate it. Activated Bax can induce outer mitochondrial membrane and cytochrome c release from mitochondria to cytosol (Heimlich 2004). Thus, activated caspases, Bcl-2 and Bax modulate death stimulus (Galluzzi et al. 2018). iNOS is a major downstream mediator of inflammation and apoptosis in various cell types including testis (Coştur et al. 2012) The up-regulation of iNOS leads to excessive NO production for prolonged periods and accounts for oxyradical-mediated damage through peroxynitrite formation by reacting with $O2\bullet^{-}$ (Weinstein et al. 2000; Fujisawa et al. 2000) causing cell death (Bonfoco et al. 1995). The inflammatory and pro-apoptotic proteins were downregulated in aged + *M.pruriens.* Indicating the ability of the extract to modulate these protein expressions, by reducing oxidative stress (Prakash et al. 2018) and could directly suppress inflammatory responses (Rachsee et al. 2021).

Conclusion

The *M. pruriens* treated aged rats showed a significant improvement in all the antioxidants levels, downregulation of inflammatory proteins, and reduce apoptotic process. Indicating the ability of the herbal extract to restore spermatogenic activity as well as the well-being of Sertoli cells and Leydig cells. Methodically organized in-depth studies are mandatory in understanding the versatile pathobiology of the reproductive health of the aging male. Thereby it would enhance the prospects of therapeutic agents and it would be possible to alleviate premature or aging-dependent infertility. The results from this experiment are encouraging to perform similar experiments focusing on protein and molecular aspects.

Author contributions PS: Conceived the study idea, methodology design, acquiring funding for the research, consolidating data, and writing the manuscript. KGM: Performed the experimental, data collection and statistical analysis. GL: Performed the experiment and consolidated data. MZIK: Performed the experiment and consolidated data. All authors participated in interpreting the results, reviewing drafts of the manuscript, and approving the final version of the manuscript submitted for publication. In addition, all authors read and approved the final manuscript.

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Data availability We declare that materials described in the manuscript, including all relevant raw data, can be available to any scientist wishing to use them for non-commercial purposes after the submission of the final report to the funding agency and with the consent of the funding agency.

Declarations

Conflict of interest None of the authors has any financial/commercial conflicts of interest to declare.

Statement of ethics The study was approved by the Institutional Animal Ethical Committee (IAEC), Dr. ALMPGIBMS, University of Madras (IAEC Approval No. 04/02/2011). The quarantine procedures and animal maintenance were according to the recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India, and guidelines for laboratory animal facility in India. The manuscript does not contain data from any person. Data presented were from animal studies (approved by the Institutional Animal Ethical Committee (IAEC), Dr. ALM PGIBMS, University of Madras, Taramani—details given above) All the authors have given their consent for publishing.

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