

## ALTERATION IN TESTICULAR MORPHOLOGY AND SPERM COUNT DUE TO GLYCOWITHANOLIDES TREATMENT DURING AGING

NILOFAR H SHAIKH, VIDYA M DESHMUKH, MADHURI V WALVEKAR\*

Department of Zoology, Shivaji University, Kolhapur - 416 001, Maharashtra, India. Email: madhuri\_walvekar@rediffmail.com

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### ABSTRACT

**Objective:** The purpose of this study was to evaluate protective effects of glycowithanolides (WSG) extracted from *Withania somnifera* leaves against altered testicular morphology and reduced sperm count during aging.

**Methods:** For this purpose, adult male mice (*Mus musculus*) of 6 months old, weighing 50-55 g were divided into following four groups viz. control Group (I), D-galactose treated Group (II), protective Group (III) and curative Group (IV). The effect of glycowithanolides (20 mg/kg body weight) was evaluated against aging. The body weight was recorded before and after treatment. Testes, epididymis, and seminal vesicle were collected, cleaned, and weighed and also were used for histopathological study. Epididymal sperm count was done.

**Results:** The current study demonstrated that male mice receiving D-galactose caused a significant decrease in sperm count, body weight, weight of testes, epididymis, and seminal vesicle as compared to control (Group I) which was significantly increased in both protective (III) curative Group (IV). Histological studies of the testes revealed anti-spermatogenic effects of D-galactose reflected by a significant decrease in number of spermatogonia, spermatocytes, spermatids and leydig cells in the mice treated with D-galactose. In case of epididymis and seminal vesicle also showed marked destructive morphological changes. Histological structure which was returning back to normal as that observed in the control group (I) mice.

**Conclusion:** Glycowithanolides enhance antioxidant system which consequently reduced oxidative stress in mice. The beneficial effect of glycowithanolides (WSG) is thus potentially reducing reproductive dysfunction and tissue damage.

**Keywords:** Glycowithanolides, D-galactose, Sperm count, Testes, Epididymis and seminal vesicle.

### INTRODUCTION

Infertility is a major clinical problem, affecting people medically and psychosocially. 15% of all couples in the US are infertile, and the male factor is responsible for 25% of these cases [1]. Free radicals contribute to the pathogenesis of male infertility. Free radicals are a group of highly reactive chemical molecules with one or more unpaired electrons that can oxidatively modify biomolecules they encounter. Reacting almost immediately with any substance in their surrounding area, they begin a chain reaction leading to cellular damage [2]. Superoxide anion, hydroxyl radical, and hydrogen peroxide are major reactive oxygen species (ROS) present in seminal plasma. Cells living under aerobic conditions require oxygen to support life; however, metabolites, like ROS, can modify cell functions and endanger cell survival [3]. Male germ cells at various stages of differentiation have the potential to generate ROS, and low physiologic levels are needed to regulate sperm capacitation, acrosome reaction, and sperm-oocyte fusion [4,5]. During oxidative stress (OS), the oxidation exceeds the antioxidant symptoms in the body secondary to a loss of the balance between them [6]. OS arises when excess free radicals overwhelm the antioxidant defense of the male reproductive tract, [7,8] damaging cells, tissues, and organs [8,9]. Seminal OS correlates negatively with sperm concentration, motility, and function-adversely affecting fusion events required for fertilization [7,9-13]. The polyunsaturated fatty acids of the sperm plasma membrane are susceptible to ROS damage as low concentrations of the scavenging enzymes are found in sperm cytoplasm [14].

ROS attack on the cell membrane leads to a chain of chemical reactions called lipid peroxidation, which reduces membrane fluidity and the activity of membrane enzymes and ion channels, resulting in the inhibition of normal cellular mechanisms required for fertilization. Several endogenous protective mechanisms have evolved to limit ROS effect and the damage caused by them [15]. However, when this protection is not complete or when the formation of ROS is excessive,

additional protective mechanisms of dietary antioxidants may be of a great importance. Therefore, many natural and synthetic agents possessing antioxidative properties have been proposed to prevent and treat infertility and reproductive hormonal imbalance induced by OS [16].

*Withania somnifera* commonly known as ashwagandha for examination of its antioxidative status. Bhattacharya *et al.*, in 1996 reported anticancer and anti-inflammatory effect of glycowithanolides, a major active constituent of *W. somnifera* [17]. *W. somnifera* significantly inhibited haloperidol or reserpine-induced catalepsy and provide hope for treatment of Parkinson's disease [18].

The aim of the present study was to throw a light on cytoprotective effects of glycowithanolides (WSG) as a model of powerful antioxidant against the adverse effects of aging on the histology of testes and accessory reproductive organs and sperm count.

### METHODS

#### Animals

Swiss albino male mice *Mus musculus* (Linn.) of age 6 months, weighing 50-55 g were used for present investigation. Animals were housed and maintained in the departmental animal house (Committee for the Purpose of Control and Supervision of Experiments on Animals [CPCSEA]) in separate cages under proper conditions. Experimental work was conducted in accordance with the guidelines set by CPCSEA, New Delhi, India and experimental protocols were approved by the Institutional Animal Ethics Committee. Mice were divided into following four groups of eight mice each.

- I. Control group: Mice were injected subcutaneously with 0.5 ml distilled water/day/animal for 20 days
- II. D-galactose treated group: Mice were injected subcutaneously with 5% D-galactose 0.5 ml/day/animal for 20 days [19,20]

- III. Protective group: Mice were injected subcutaneously with 0.5 ml of 5% D-galactose/day along with glycowithanolides 20 mg/kg body weight for 20 days [21]
- IV. Curative group: Mice were injected with 0.5 ml of 5% D-galactose for 20 days, and then to study recovery glycowithanolides was injected 20 mg/kg body weight for next 20 days.
1. Plant material: The plant was identified by taxonomist from Botany Department, Shivaji University, and Kolhapur. Fresh leaves of *W. somnifera* were collected from town hall garden Kolhapur.

#### Plant extraction

The major chemical constituents of this plant, glycowithanolides (WSG), are mainly localized in leaves, and their concentration usually ranges from 0.001% to 0.5% dry weight [22,23]. Glycowithanolides were extracted from leaves of *W. somnifera* plant [24]. Fresh leaves of *W. somnifera* were collected, separated, washed with distilled water, blotted properly, and kept in shade for drying. Dried leaves were crushed, powdered, and sieved. Then soaked in chloroform for 72 hrs to remove fatty material and separate the withanolides, the solution was filtered, and chloroform evaporated by evaporator, thick paste was obtained. It was stored in a glass bottle at 4°C and used as active ingredient for dose preparation.

Analysis of glycowithanolides was done by the following methods.

#### Thin layer chromatography (TLC)

##### Preparation of plates

Silica gel - G 20 g was macerated in 40 ml of ethanol. The slurry obtained was transferred to the applicator immediately and applied to the plates. The adjustable applicator was set at 0.25 mm. The edges of plates were trimmed of excess silica gel G. Residue dissolved in chloroform was applied with thin capillary, 0.5-1 cm from the bottom edge of the plate. The chromatographic chambers were prepared 20 minutes before the insertion of the plates. The chambers were lined on three sides Whatman No.3 Filter paper wetted with developing solvent. The plates were developed in a solvent mixture of chloroform:methanol (9:1 v/v). Detection of glycowithanolides on dry plates was made by exposing the plate to the iodine vapor.

##### Preparation of sample for gas chromatography mass spectroscopy (GCMS) analysis

GCMS analysis of this extract was performed using a Shimadzu GP 2010 system and GC interfaced to a MS equipped with an Elite-1 fused silica capillary column (60 m × 0.25 mm ID × 1 μm, composed of 100% dimethylpolysiloxane) for GCMS detection, an electron ionization system with ionization energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at a constant flow rate of 1 ml/minutes, and an injection volume of 2 μl was employed. Injector temperature 250°C; ion-source temperature 280°C. The oven temperature was programmed from 80°C (isothermal for 2 minutes), with an increase of 10°C/minutes to 380°C, then 5°C/minutes to 280°C, ending with a 9 minutes isothermal at 280°C. MS were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 650 Da. Total GC running time was 34 minutes the relative percentage amount of each component was calculated by comparing its average peak area to the total areas. Software adapted to handle MS and chromatograms was GCMS real time analysis.

The animals from respective groups were killed by cervical dislocation after 24 hrs of completion of treatment testes and accessory reproductive organs were dissected out, blotted and weighed on an electronic balance and used for the following parameters.

#### Determination of body weight of mice

Animals were weighed (in g) before starting of drug treatment and after completion of each drug treatment. The record of their body weight was maintained.

#### Determination of testes and accessory reproductive organs weight of mice

After the completion of treatment, testes and accessory reproductive organs were dissected out, thoroughly washed in ice cold saline (0.9% NaCl), dried with the help of blotting paper and wet weight of tissues (in mg) was recorded.

#### Histology

Age-related changes in the male reproductive organs were studied by histological method.

#### Sperm count [23]

The sperm count was carried out using hemocytometer.

#### Statistical analysis

All values are expressed as a mean±standard deviation. The statistical analysis was performed using Student's *t*-test. A value of \*\*\**p*<0.001 was considered statistically highly significant.

## RESULTS

#### Analysis of glycowithanolides

The TLC separation of *W. somnifera* leaf extract is shown in Fig. 1a. The TLC plate showed three distinct spots, in that the spot showing  $R_f$  value 0.65 was glycowithanolide [25]. Further, for confirmation, we have done GCMS with standard withanolides as a reference compound. Both our sample and standard compound obtain peak at same retention time 10.424<sup>th</sup> minutes as shown in Fig. 1b.

Table 1 shows the body weight of D-galactose treated aging induced mice was reduced as compared to the body weight of control group mice. The reduction in the body weight in D-galactose induced aged mice was significant (1:2, *p*<0.01). In protective group, body weight was slightly increased as compared to D-galactose treated group and no significant difference was observed in body weights of mice from the protective group (2:3, *p* - non-significant). The body weight of mice

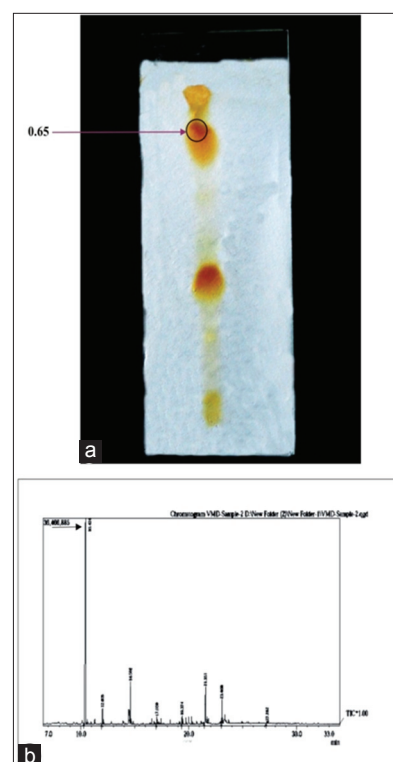


Fig. 1. (a) Thin layer chromatographic separation of glycowithanolides from extract, (b) analysis of glycowithanolides by gas chromatography mass spectroscopy

from the curative group was increased, and it was significant (2:4,  $p < 0.01$ ). When body weight from curative group and protective group were compared, slight increase in body weight in curative group was observed and it was almost significant as compared to protective group (3:4,  $p < 0.05$ ).

Table 2 shows the weight of testes, epididymis, and seminal vesicle in D-galactose induced aged mice group was decreased as compared to control group mice and the decrease was significant (1:2,  $p < 0.01$ ). In the protective group and curative group, weight of testes and weight of epididymis were increased significantly as compared to D-galactose treated group. Whereas in case of seminal vesicle, weight in both protective and curative group no significant increase was observed as compared to D-galactose treated group (2:3,  $p$  – nonsignificant; 2:4,  $p$  – nonsignificant). No significant increase in weight of testes, epididymis, and seminal vesicle was observed in the curative group as compared to protective group (3:4,  $p$  – nonsignificant).

**Changes in testes, epididymis and seminal vesicle (hematoxylin- eosin technique)**

The normal histological structure of testes, epididymis, and seminal vesicle stained with hematoxylin-eosin. Histological section of testes from control mice (Fig. 2a and b) revealed multiple rounded seminiferous tubules with regular outlines. They were undergoing spermatogenic cycle composed of spermatogonia, primary spermatocytes, secondary spermatocytes, round spermatids, and elongated spermatids consistent with general spermatogenesis. Sertoli cells are large tall slender cells having an irregular and columnar shape. The interstitial spaces between the tubules contained leydig cells and some blood capillaries. The light microscopic observation of testes from D-galactose treated group (Fig. 2c and d) showed seminiferous tubules with irregular outlines. The normal structure of testes was disturbed. All the cells in seminiferous tubules get disturbed, and their number was also decreased. Especially number of spermatocytes was decreased. Histological observation of testes from the protective group (Fig. 2e and f) showed reparation in structural integrity. Slightly remarkable recovery in structural damage was observed, as compared to D-galactose treated group. Seminiferous tubules appearing with regular lining, multiple layers of spermatogenic cells were observed. Sections of testes from the curative group (Fig. 2g and h) showed multiple seminiferous tubules with regular outlines and spermatogenic cells at different stages of spermatogenesis. Furthermore, enlarged congested blood vessels and cellular exudates in the interstitial cells were observed as compared to D-galactose treated aging accelerated group. Structure was returning to normal as that observed in control group mice testes sections.

**Table 1: Effect of glycowithanolides on body weight (in g) of D-galactose induced aged mice**

S. no.	Treatment group (n=8)	Body weight	Statistical significance
I	Control	46.875±2.748	1:2, $p < 0.01$
II	D-galactose treated	42.375±1.685	2:3, $p$ -non significant
III	Protective	43.875±2.356	2:4, $p < 0.01$
IV	Curative	47.5±2.267	3:4, $p < 0.05$

Values are mean±SD numbers in parenthesis denotes number of animals. \* $p < 0.05$ : Almost significant, \*\* $p < 0.01$ : Significant, SD: Standard deviation

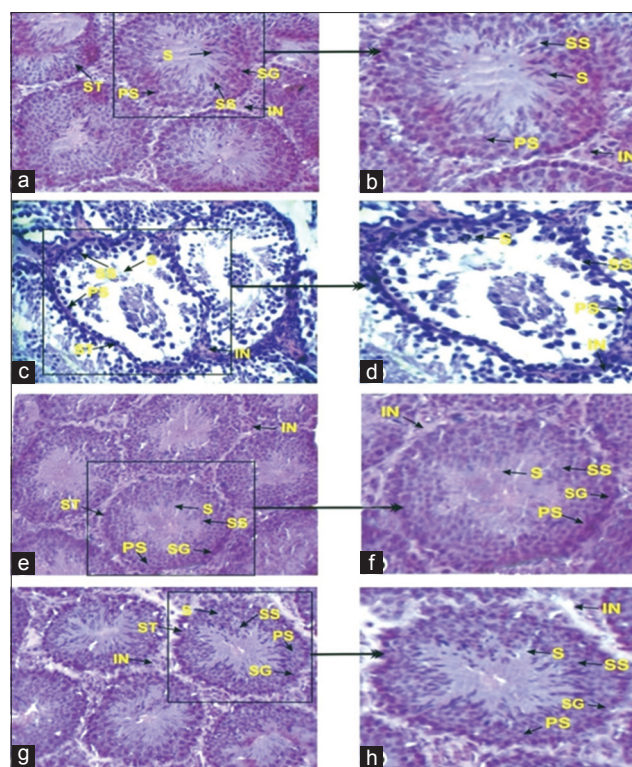
**Table 2: Effect of glycowithanolides on weight (in mg) of testes, epididymis and seminal vesicle in D-galactose induced aged mice**

Relative organs weight (in mg)							
S. no.	Treatment (n=8)	Testes	Statistical significance	Epididymis	Statistical significance	Seminal vesicle	Statistical significance
I	Control	95.125±4.823	1:2, $p < 0.01$	45.25±1.581	1:2, $p < 0.01$	40.5±3.625	1:2, $p < 0.01$
II	D-galactose	65.5±9.6362	2:3, $p < 0.05$	39.625±2.56	2:3, $p < 0.05$	34.375±4.274	2:3, $p$ -non significant
III	Protective	90±6.761	2:4, $p < 0.01$	43±1.511	2:4, $p < 0.01$	36.875±1.356	2:4, $p$ -non significant
IV	Curative	92.5±5.952	3:4, $p$ -non significant	45.375±2.065	3:4, $p$ -non significant	38.375±2.445	3:4, $p$ -non significant

Values are mean±SD numbers in parenthesis denotes number of animals. \*\* $p < 0.01$ : Significant, \* $p > 0.5$ : Non-significant. SD: Standard deviation

In case of epididymis, control group mice (Fig. 3a and b) showed normal structure. Epididymal epithelium enclosed a lumen containing spermatozoa. The interstitial space in between the epididymal tubules was filled with sparse stroma. The lining of the tubules showed pseudostratified epithelium. It consists of low basal cells and columnar cells. The tall columnar cells with long stereocilia. In D-galactose treated groups (Fig. 3c and d) epididymis lost the structural integrity, decreased the number of sperm from lumen and disappearance of the muscle layer as compare to control group. Structure of epididymis from the protective group (Fig. 3e and f) showed reparation in structural integrity. Slightly remarkable recovery in structural damage was observed, as compared to D-galactose treated group. Increase in staining intensity was observed. In epididymis from the curative group (Fig. 3g and h), considerable recovery and regeneration of tubules were observed. Epididymal tubules appearing with regular lining, pseudostratified epithelium, and stereocilia were observed. Structure was returning to unbroken intact structure as that observed in control group mice epididymis sections.

The light microscopy examination of the seminal vesicle of the control mice (Fig. 4a and b) had normal structure. The mucosa was folded,



**Fig. 2: (a and b) T. S. of testes of control group mice, (c and d) T. S. of testes of D-galactose treated mice, (e and f) T. S. of testes of protective group mice, (g and h): T. S. of testes of curative group mice stained with H & E, ×400, ×1000 respectively. ST: Seminiferous tubules, SG: Spermatogonia, PS: Primary spermatocytes, SS: Secondary spermatocytes, S: Sperms**

forming numerous irregular crypts. The epithelium is composed of cuboidal or columnar cells and irregular shaped basal cells. The lamina propria is rich in elastic fibers and forms continuous layer around the vesicle. In D-galactose treated groups (Fig. 4c and d) seminal vesicle lost the structural integrity, disappearance of the muscle layer, cuboidal cells as compare to control group. Structure of seminal vesicle from protective group (Fig. 4e and f) showed reparation in structural integrity. Slightly remarkable recovery in structural damage was observed, as compared to D-galactose treated group. In seminal vesicle from curative group (Fig. 4g and h), considerable recovery and regeneration of crypts were observed. The structural integrity was somewhat similar like seminal vesicle of the control group.

Table 3 shows the epididymal sperm count from D-galactose induced aged mice was reduced as compared to the control group (1:2, \*\*p<0.01). The epididymal sperm count from the protective group was slightly increased as compared to D-galactose induced aged mice group, but increase was not significant (2:3, p - nonsignificant). The epididymal sperm count from the curative group was again increased. The increase was significant as compared to D-galactose induced aged mice group (2:4, \*\*p<0.01). The epididymal sperm count from the curative group was significantly increased as compared to protective group (3:4, \*\*p<0.01).

**DISCUSSION**

In the present investigation, D-galactose was used to induce aging. Recent reports implicate the chronic administration of D-galactose in accelerating aging, influencing age-related cognitive decline in mice [26]. D-galactose is a physiological nutrient and a reducing sugar that reacts with free amines of amino acids in proteins to form advanced glycation end products through non-enzymatic glycation [27]. The body weight was significantly decreased in D-galactose treated group

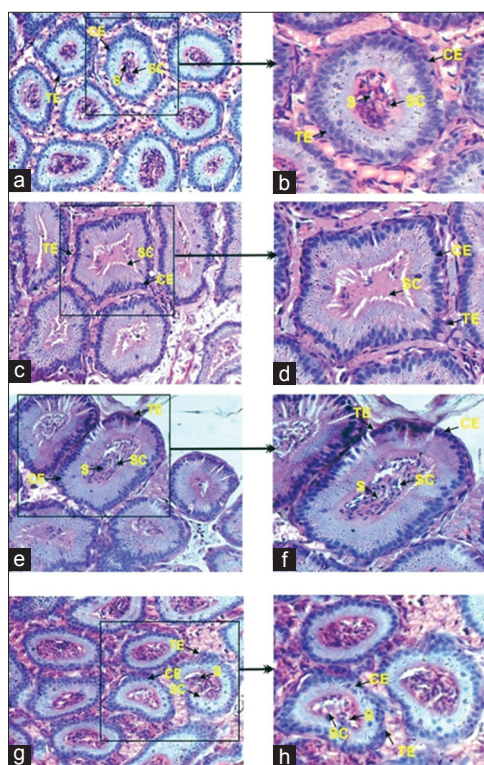
as compared to control, whereas, a significant increase in the curative group was observed as compared to D-galactose treated group. This decline in body weight during aging occurs primarily as a result of loss in lean tissue, including muscles mass and bone [28-31]. Longitudinal evidence suggests that during older age muscle mass decrease 3-6% per decade, [32] while bone loss occurs at a rate high as <1-2% per year [33]. In fact, aging related changes in body composition are mirrored by a gradual decline in physical activity in older age [34-37]. The reduction in the body weight in the rat feeding with 50% galactose diet [38]. The body weight goes on increasing up to adult age but due to D-galactose treatment the weight loss may be related to drop in the sex hormone testosterone.

Significant decrease in weight of all the reproductive organs i.e., testes, epididymis, and seminal vesicle was observed in D-galactose treated mice as compared to control, which was significantly increased in both protective and curative groups except seminal vesicle. Difference in weight of all the reproductive organs in protective and curative group was not significant. The decrease in testicular weight in D-galactose treated mice may be due to reduced tubule size, spermatogenic

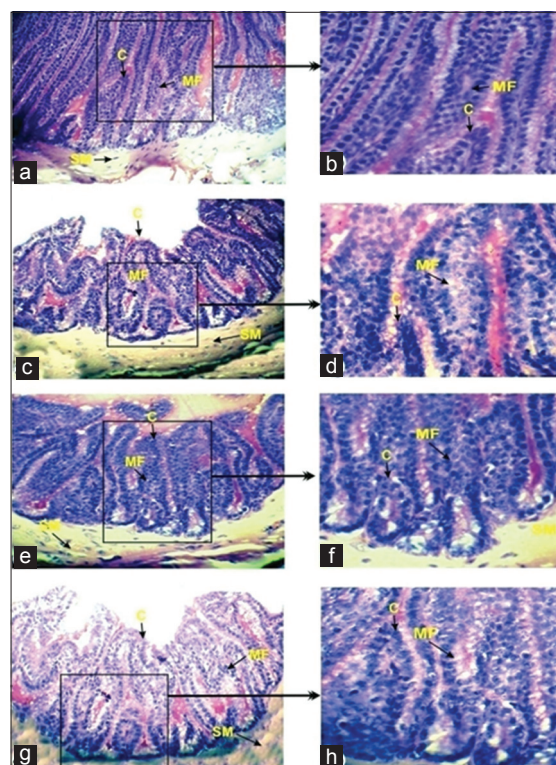
**Table 3: Effect of glycowithanolides on epididymal sperm count ( $\times 10^6$ /epididymis) in D-galactose induced aged mice**

S. no.	Treatment group (n=8)	Sperm count	Statistical significance
1	Control	6.786 $\pm$ 0.775	1:2, p<0.01
2	D-galactose treated	4.4575 $\pm$ 0.627	2:3, p-non significant
3	Protective	5.178 $\pm$ 0.767	2:4, p<0.01
4	Curative	6.421 $\pm$ 0.559	3:4, p<0.01

Values are mean $\pm$ SD numbers in parenthesis denotes number of animals. \*\*p<0.01: Significant, \*p>0.5: Non-significant. SD: Standard deviation



**Fig. 3: (a and b): T. S. of epididymis of control group mice, (c and d): T. S. of epididymis of D-galactose treated mice, (e and f): T. S. of epididymis of protective group mice, (g and h): T. S. of epididymis of curative group mice stained with H & E,  $\times 400$ ,  $\times 1000$  respectively. TE: Tubules of epididymis, CE: Columnar epithelium, SC: Stereocilia, S: Sperms**



**Fig. 4: (a and b): T. S. of seminal vesicle of control group mice, (c and d): T. S. of seminal vesicle of D-galactose treated mice, (e and f): T. S. of seminal vesicle of protective group mice, (g and h): T. S. of seminal vesicle of curative group mice stained with HE  $\times 400$ ,  $\times 1000$  respectively. MF: Mucosal fold, SM: Smooth muscle, C: Crypts**

arrest, and inhibition of steroid biosynthesis of leydig cells, a site of steroid biosynthesis [39,40]. The decrease in testis weight is related to spermatogenic failure, as suggested by the concomitant decrease in sperm concentration. The observed weight loss of accessory sex organs may be due to reduced bioavailability of sex hormones [41]. Similarly Chakraborty *et al.* reported that atrophic testes commonly observed in amiodarone-treated rat possibly for the reduction of spermatogenesis, which ultimately results in a decrease in testis weight since it is very vulnerable to OS [42].

Aging lead to a decrease in the size of testes which leads to alteration and dysfunction of these cell components. There was a progressive dysfunction of leydig cells in the D-galactose treated group. Present study demonstrated many changes in the components of mice testes with aging i.e., reduction in number of primary spermatocytes, secondary spermatocytes, and spermatozoa that may be due to reduced testosterone capacity per leydig cell with advancing in age which was increased after glycowithanolides treatment. In epididymis also decrease in spermatozoa from lumen was observed in aging mice, which was significantly increased in both protective and curative group. For its confirmation, we have done the sperm count/epididymis. There also we found same results. In seminal vesicles also significant recovery was observed after glycowithanolides treatment.

## CONCLUSION

Glycowithanolides administration contributed to normalize histological changes in the OS during aging in reproductive organs that may suggest its remarkable efficacy. In addition, such study may explore the importance of studying the efficacy of plant product in the reducing infertility caused by OS.

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