



# Effect of dietary pomegranate peel (*Punica granatum* L.) and Aloe vera gel (*Aloe barbadensis miller*) supplementation on testicular antioxidant biomarkers and spermatogenesis enzymes in mature V-Line rabbit bucks

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## Abstract

Rabbit meat is considered as an economic source of white meat, increasing its production is limited by the buck fertility, since one rabbit can be used to inseminate up to 15 female. The aim of the current study is to enhance male rabbit fertility by using dietary antioxidants including Aloe vera gel (AVG) and pomegranate peels (PP). In a 60 days experiment, 48 V-Line 5-month-old rabbit bucks of average body weight ( $2,300 \pm 20$ ) kg were allocated into four dietary treatments ( $n = 12/\text{group}$ ) as follow: CON (fed on control diet), ALOE (received AVG in drinking water; 500 mg/L drinking water), POM3 (fed on basal diet + 3% of pomegranate) and POM5 (fed on basal diet + 5% of pomegranate). Semen samples were collected at d30 and d60 of the experiment and used for analysis of semen quality. Sexual behaviour was reported in terms of latency to first mating and ejaculation interval. At the end of the experiment, six bucks were euthanized from each group, blood samples were collected and used for testosterone level determination and testicular tissue samples were collected and used for key antioxidant and spermatogenesis enzymes assessment, and testes histopathological evaluation. The UNIVARIATE procedures of SAS 9.4 were used to analyse the data, significance was declared at  $p \leq .05$ . PP supplementation improved percentage of progressive motile sperms while AVG negatively impacted it ( $p = .04$ ), sperm concentration and metabolically active sperm cells were the highest in PP and lowest in ALOE supplemented bucks ( $p = .01$  and  $.01$ ; respectively). Testicular alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) decreased in AVG supplemented group ( $p = .01$  and  $.02$ ; respectively). From our findings, AVG in its fresh form decreased fertility of rabbit bucks, while PP is potent fertility boosting for rabbit bucks.

## KEYWORDS

antioxidant, fresh Aloe Vera, pomegranate peels, rabbit bucks, spermatogenesis

## 1 | INTRODUCTION

The polyunsaturated lipid nature of rabbit sperm is associated with high rate of reactive oxygen species (ROS) production in testicular

tissue (FAO, 1994) resulting in poor semen quality and reduced fertility. Therefore, dietary manipulations are crucial, to provide natural antioxidants will sustain testicular antioxidant system, semen quality, and rabbit buck fertility.

Pomegranate and Aloe Vera were formerly reported to possess antioxidant activity, that is variable according to the type of extract and part of the plant used (Avdatek et al., 2018; Khalaf, Arafat, & Ghoneim, 2019; Zeweil, Elnagar, Zahran, Ahmed, & El-Gindy, 2013). Pomegranate peels were found to possess more potent antioxidant properties than pomegranate juice (Dkheil, Al-Quraishy, & Moneim, 2013; Zeweil et al., 2013). Additionally, Aloe vera gel (AVG) was proved for its antioxidant properties (Akinloye, Ugbaja, & Dosumu, 2019; Behmanesh, Najafzadehvarzi, & Poormoosavi, 2018). On the other side, pomegranate peels improved semen quality and sperm progressive motility of rabbit bucks reared under heat stress condition (Zeweil et al., 2013). Aloe Vera ethanolic extract improved sexual behaviour via increasing mounting and intromission frequencies in rat model at a rate of 100 to 300 mg/kg body weight, however, dose of 400 mg/kg body weight negatively affected sexual behaviour (Erhabor & Idu, 2017). However, it negatively impacted semen quality in goat bucks received 3%–4% AVG extract where it increased the level of sperm abnormality (Oyeyemi, Olukole, Adeoye, & Adejoke, 2011); therefore, species variation might regulate the effect of AVG on male sexual behaviour.

Sertoli cell plays a major role in spermatogenesis (Abel et al., 2008), testicular gamma-glutamyl transpeptidase (GGT) activity is indicator of sertoli cell activation (Sherins & Hodgen, 1976). Additionally, testicular alkaline phosphatase iso-enzyme (ALP) and lactate dehydrogenase (LDH) are involved in spermatogenesis and sperm maturation (Asgharzade, Rafieian, & Salimzadeh, 2015). Therefore, increase the level of these key enzymes is indicator of spermatogenic potential of rabbit bucks. Additionally, key antioxidant enzymes superoxide dismutase (SOD), glutathione-S-transferase (GST) and glutathione peroxidase (GPX) were affected by dietary antioxidants like pomegranate, where total oxidant status was low in 100 mg/kg lyophilized pomegranate extract supplemented rabbits (Avdatek et al., 2018) and AVG (Behmanesh et al., 2018) which increased GSH and decreased MDA at a dose of 400 mg/kg body weight in rat model.

However, AVG negatively impacted rat testicular tissue via nitric oxide-dependent apoptosis (Asgharzade et al., 2015). Therefore, the aim of the current study is to investigate the effect of AVG and PP on rabbit bucks semen quality, testicular enzymes and testicular antioxidant biomarkers. We hypothesize that PP and AVG could be used as potential natural antioxidants to enhance rabbit semen quality.

## 2 | MATERIALS AND METHODS

### 2.1 | Aloe vera gel and pomegranate peel preparation

Mature fresh leaves of *Aloe Vera* (*Aloe Barbadensis*) were obtained from El-Hossary garden plantations (Giza, Egypt). After rinsing the leaf with running tap water, the outer shell was removed with sharp knife and central solid gel was obtained and homogenized in blinder (Cat# 3420E17, Thomas Scientific) according to (Oyewopo, Oremosu, Akang, Noronha, & Okanlawon, 2011). This process was

repeated every day to be sure that the gel was freshly prepared. Dried pomegranate peels were purchased from local herbal store (Cairo, Egypt). Peels were then grinded with grinder (Jet Pulverizer) into fine powder then mixed with the diet.

### 2.2 | Experimental design, animals and diet

A 60 days experiment was conducted in rabbit husbandry of Faculty of Veterinary Medicine, Cairo University. Forty-eight V-Line 5-month-old rabbit bucks were blocked for their body weight ( $2,300 \pm 20$ ) kg and were randomly distributed into four groups ( $n = 12/\text{group}$ ), namely control (CON) which fed on the basal diet, Aloe vera gel group (ALOE) in drinking water (500 mg/L drinking water), group fed on diet containing 3% of pomegranate (POM3), and group fed on diet containing 5% of pomegranate (POM5). The inclusion rates of AVG and PP were similar to (Alshatwi & Subash-Babu, 2016) and Zeweil et al., 2013; respectively. Rabbit was housed individually in commercial cages ( $55 \times 60 \times 34$ ), equipped with automatic nipple drinkers. Diets were formulated to satisfy the nutrient requirements of rabbits as recommended by rabbit national research council (NRC, 1977). Diet ingredients and proximate chemical composition are presented in Table 1.

### 2.3 | Semen collection and evaluation

Bucks were trained for semen collection from d1 to d30 of the experiment, then after they have reached sexual maturity (6-month-old; Macari & Machado, 1978), semen was collected twice at day 30 and day 60. Semen was obtained from each buck between 8:00 and 10:00 a.m. Semen volume was recorded, semen pH was measured using pH probe (pH600, Milwakuue), sperm mass motility was assessed using light microscope with thermostatically controlled hot stage adjusted at 38–40°C (Olympus, USA), Mass activity of spermatozoa was scored (0–5) according to (David et al., 2015) as follows: 0 = no current, 1 = few slow current, 2 = many moderate waves, 3 = many sweeping waves, 4 = numerous vigorous waves and 5 = numerous rapid and vigorous waves.

Individual sperm motility was assessed using light microscope (Olympus; Seed et al., 1996). Sperm concentration was measured by counting the number of spermatozoa present on both sides of Neubauer haemocytometer slide (Marienfeld) according to (Seed et al., 1996). Assessment of live to dead sperm ratio and abnormal spermatozoa were performed as detailed in (Rodríguez-De Lara et al., 2008). Resazurin reduction test (RRT) was conducted to evaluate spermatozoa metabolic activity as detailed in (Glass et al., 1991).

### 2.4 | Behavioural measurements

Sexual activity of the bucks was evaluated through mating test, once a week from d30 to d60. On the test day, a receptive female

**TABLE 1** Ingredients and chemical composition of the experimental diets<sup>1</sup>

Ingredients	CON <sup>2</sup>	POM3 <sup>3</sup>	POM5 <sup>4</sup>
Berseem hay	30.00	27.00	25.00
Pomegranate peel	—	3.00	5.00
Barley grain	21.00	21.00	21.00
Yellow corn	5.00	5.00	5.00
Wheat bran	21.10	21.10	21.10
Soybean meal (44%)	17.50	17.50	17.50
Molasses	3.00	3.00	3.00
Lime stone	1.50	1.50	1.50
Sodium chloride	0.40	0.40	0.40
Premix <sup>4</sup>	0.30	0.30	0.30
DL-Methionine	0.20	0.20	0.20
Proximate analysis			
Moisture	9.40	9.40	9.50
Crude protein	17.50	17.40	17.43
Crude fibre	14.00	14.70	14.69
Ether extract	2.70	3.60	3.55
Total Ash	7.10	7.50	7.35
Nitrogen free extract	49.30	47.40	47.60

<sup>1</sup>Percentage, on air-dry basis.

<sup>2</sup>Control basal diet.

<sup>4</sup>Diet supplemented with 3% pomegranate peels.

<sup>5</sup>Diet supplemented with 5% pomegranate peels.

<sup>4</sup>Vitamin and mineral premix per kg diet contained IU/g for vitamins or minerals: A-4,000,000, D3-5000,000, E-16.7 g, K-0.67 g, B1-0.67 g, B2-2 g, B6-0.67 g, B12-0.004 g, B5-16.7 g, Pantothenic acid-6.67 g, Biotein-0.07 g, Folic acid-1.67 g, Choline chloride-400 g, Zn-23.3 g, Mn-10 g, Fe-25 g, Cu-1.67 g, I-0.25 g, Se-0.033 g, and Mg-133.4 g.

was introduced to the male 'cage for 10 min testing period for each buck (Erhabor & Idu, 2017). Latency to first mount (the time from introducing the female until the first mount with pelvic thrust and was expressed in seconds; Erhabor & Idu, 2017), ejaculation interval was measure as (interval between the first and second mating and was expressed in minute) according to Erhabor & Idu, 2017).

## 2.5 | Euthanasia and sampling

At the end of the experiment, six bucks from each group were euthanized and hot carcass and testicular weight were recorded. Detailed euthanasia protocol was mentioned in (Abdelatty, Badr, et al., 2020). Gonadosomatic index (GSI) was calculated as the (testis weight/ hot carcass weight) ×100. A piece from the middle of right testis was kept in liquid nitrogen for assessment of selected key testicular enzymes (ALP, LDH and GGT), key antioxidant parameters (MDA, SOD, GST and GPX). Another testicular specimen was fixed in 10% formalin for histopathological examination. Blood

samples were collected, and serum was separated for testosterone hormone level evaluation using ELISA kit (Cat No. 582701, Cayman Chemicals).

## 2.6 | Testicular enzymes and antioxidants determination

Key testicular enzymes involved in spermatogenesis including ALP, LDH and GGT were evaluated in testicular tissue using enzymatic kits (Spinreact) according to the manufacturer protocol. Key antioxidant biomarkers were measured in testicular tissue using HPLC (Agilent HP 1200 series) including MDA, SOD, GST and GPX, detail analysis protocol and HPLC condition were formerly reported in (Abdelatty et al., 2020).

## 2.7 | Histopathological examination of testicular tissue

Tissue samples were processed according to (Abdelatty, et al., 2020) using a microtome (Leica 2135). Tissue sections were stained with haematoxylin and eosin stain and examined using light microscope and photographed using Olympus XC30 camera (Olympus, USA).

## 2.8 | Statistical analysis

Shapiro–Wilks test was used to evaluate normality for all parameters, where Shapiro–Wilks *p*-value was >.05. The UNIVARIATE procedures of SAS 9.4 were used to identify the difference between treatments. Significance was declared at *p* ≤ .05, whereas tendencies were determined at .05 < *p* ≤ .10.

## 3 | RESULTS

Effect of AVG and PP on rabbit bucks semen characteristics is shown in Table 2. The percentage of progressively motile sperm was the highest in POM3 and POM5 groups regardless of the PP inclusion rate, while it was the lowest in ALOE group (*p* = .04). Sperm viability was the highest in POM5 group and the lowest in ALOE group (*p* = .01). Similar results were found for the sperm concentration where it was the highest in POM5 group and the lowest in ALOE group (*p* = .01). Metabolically active sperm cells detected by RRT test was the highest in POM5 group, followed by CON and POM3 groups, and it was the lowest in ALOE group (*p* = .01).

Effect of PP and AVG on male sexual behaviour and GSI is presented in Figure 1. The interval between two successive ejaculations was significantly increased in bucks received AVG (*p* = .01). None of the treatments alters latency to first mount or GSI. Figure 2. Represents the effect of AVG and PP on selected

Item	CON <sup>2</sup>	ALOE <sup>3</sup>	POM3 <sup>4</sup>	POM5 <sup>5</sup>	SEM	<i>p</i> -value
Volume, ml	0.71	0.68	0.76	0.72	0.07	.82
pH	7.40	7.46	7.39	7.25	0.12	.69
Mass motility	3.20	3.00	3.31	3.63	0.23	.27
Progressive motility,%	80.78 <sup>ab</sup>	77.88 <sup>b</sup>	83.85 <sup>a</sup>	85.67 <sup>a</sup>	1.87	.04
Sperm viability,%	78.47 <sup>bc</sup>	77.31 <sup>c</sup>	83.81 <sup>ab</sup>	86.34 <sup>a</sup>	2.01	.01
Sperm abnormality,%	10.75	9.86	10.41	11.01	0.54	.95
Sperm concentration, ×10 <sup>6</sup> /ml	300.31 <sup>bc</sup>	294.10 <sup>c</sup>	307.80 <sup>ab</sup>	309.5 <sup>a</sup>	2.82	.01
RRT	4.78 <sup>b</sup>	3.84 <sup>c</sup>	4.66 <sup>b</sup>	5.72 <sup>a</sup>	0.17	.01

<sup>1</sup>Values are least square mean, *n* = 12 samples/group.

<sup>2</sup>Control group received basal diet.

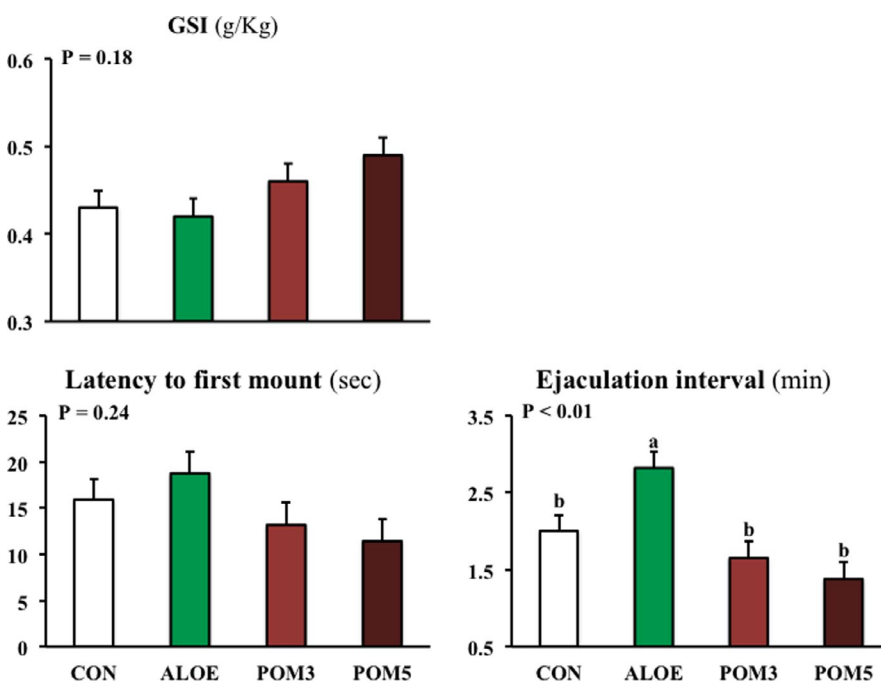
<sup>3</sup>Group fed on CON diet + Aloe vera gel in drinking water.

<sup>4</sup>Group fed on diet supplemented with 3% pomegranate peels.

<sup>5</sup>Group fed on diet supplemented with 5% pomegranate peels.

<sup>6</sup>Resazurin reduction test for identification of metabolic activity rate of sperm cells.

\*Different superscripts denote *p* ≤ .05 between treatments.



**FIGURE 1** Effect of Aloe Vera gel (AVG) and pomegranate peels (PP) on male sexual behaviour indices and gonado-somatic index (GSI) of rabbit bucks. Where CON = control group, ALOE = group received AVG in drinking water, POM3 = group fed on diet containing 3% PP, and POM5 = group fed on diet containing 5% PP

testicular enzymes and serum testosterone hormone levels. The level of testicular ALP and LDH was significantly decreased in AVG supplemented group (*p* = .01 and .02; respectively); however, none of the dietary treatments altered testicular GGT or serum testosterone levels. Serum testosterone level tended to decrease in ALEO group (*p* = .10).

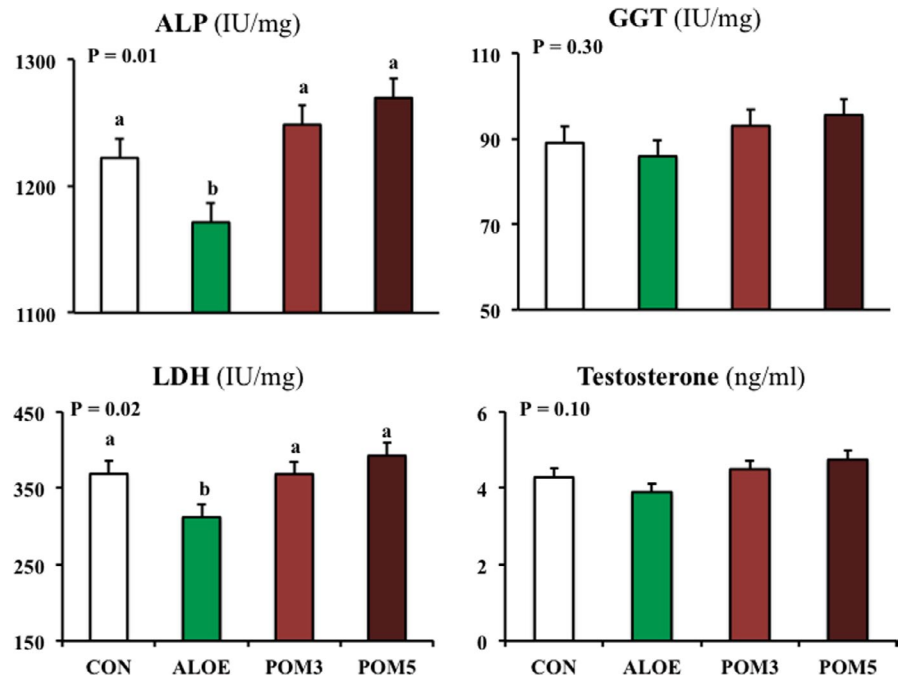
The effect of AVG and PP on testicular antioxidant enzymes is shown in Figure 3. There was no effect of the AVG or PP on the level of testicular GST, GPX and MDA. However, the level of SOD was the highest in POM5 group, and the lowest in ALEO group (*p* = .04). Histopathological changes of testicular tissue of rabbit bucks of all

groups were of normal structure; however, AVG fed bucks show interstitial oedema and congestion of testicular blood vessels as illustrated in Figure 4.

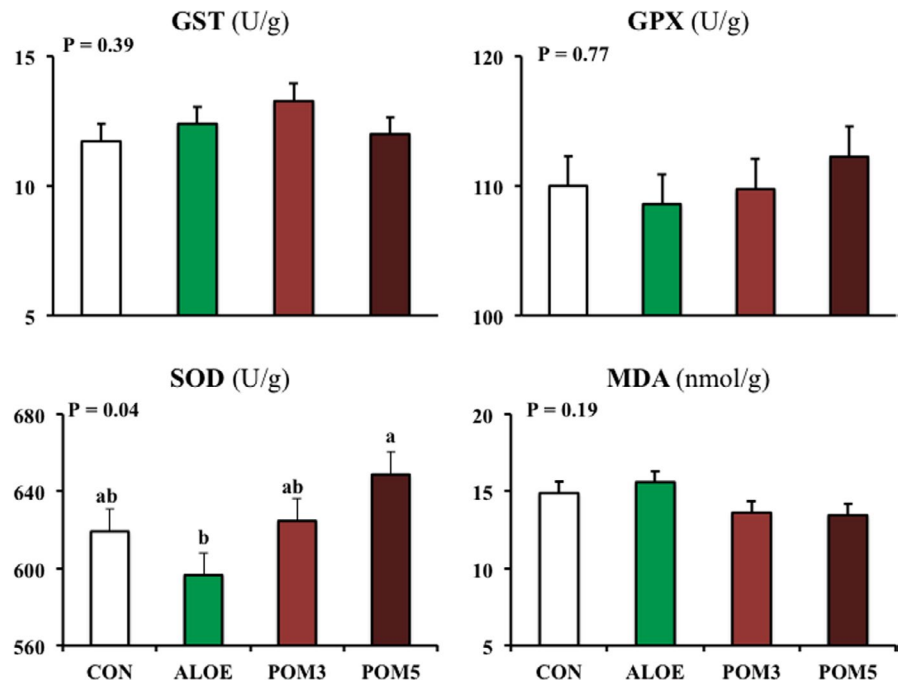
## 4 | DISCUSSION

The high level of unsaturated fat and abundance of ROS in testicular tissue rendered it highly vulnerable to oxidative stress, which affects spermatogenesis and male reproductive potential (Aitken & Roman, 2013). Aloe Vera and Pomegranate are well known for their

**FIGURE 2** Effect of Aloe Vera gel (AVG) and pomegranate peels (PP) on selected testicular enzymes and serum testosterone levels of rabbit bucks. Where CON = control group, ALOE = group received AVG in drinking water, POM3 = group fed on diet containing 3% PP, and POM5 = group fed on diet containing 5% PP. The selected enzymes were alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and gamma-glutamyl transpeptidase (GGT)



**FIGURE 3** Effect of Aloe Vera gel (AVG) and pomegranate peels (PP) on testicular antioxidant enzymes of rabbit bucks. Where CON = control group, ALOE = group received AVG in drinking water, POM3 = group fed on diet containing 3% PP and POM5 = group fed on diet containing 5% PP

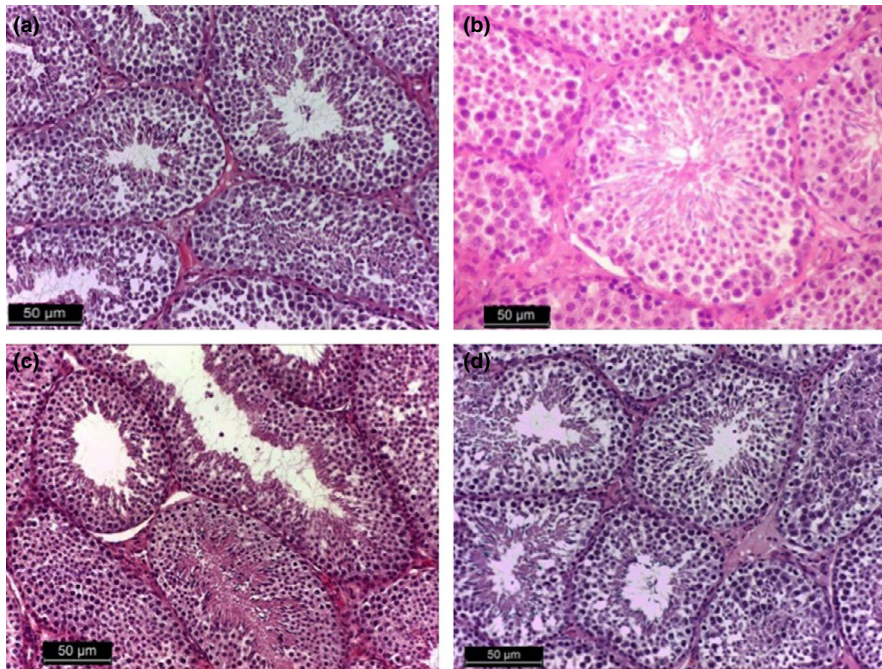


antioxidant, anti-inflammatory and immune-stimulant properties (Li et al., 2006; Oyeyemi et al., 2011).

In the current study, AVG and PP were used as antioxidants to enhance rabbit bucks fertility and semen quality. AVG increased ejaculation interval which is indicator of enhanced libido, this finding is similar to former report of Erhabor & Idu, 2017, who fed ethanolic extract of AVG to rat at dose range from 100 to 400 mg/kg body weight. Additionally, AVG supplementation negatively impacted sperm motility via decreasing its metabolic activity as noticed in RRT test results, similar observation was reported in west African dwarf buck (body weigh 11–15 kg) received 3%–4%

of fresh AVG (Oyeyemi et al., 2011). However, opposite positive effect of AVG ethanol extract was reported in mice and rat (Erhabor & Idu, 2017; Modaresi & Khodadadi, 2014), indicating that fresh AVG contains different 2<sup>nd</sup> metabolites that could negatively impact semen quality, possibly through its effect on spermatogenic enzyme LDH, where it is involved in glycolysis and ATP production that is essential for sperm motility (Odet et al., 2008). Unlike AVG, PP enhanced semen quality in rabbit bucks under heat stress condition (Zeweil et al., 2013) and enhanced properties of frozen rabbit semen (El-Seadawy et al., 2017). In a former in vitro study comparing the antimicrobial effect of AVG and pomegranate





**FIGURE 4** Effect of Aloe Vera gel (AVG) and pomegranate peels (PP) on testicular tissue morphology of rabbit bucks. Photomicrograph of testicular tissue of rabbit buck (Haematoxylin and eosin stain). Normal histological structure of the control group (a), interstitial oedema and congestion of testicular blood vessels in testicular tissue of aloe Vera fed bucks (b), normal histological structure of pomegranate peels fed groups at inclusion rate of 3% (c) and 5% (d)

on *Streptococcus mutans* (Subramaniam, Uma, Dwivedi, & Girish Babu, 2012), they noted that pomegranate has more potent antibacterial activity than AVG due to potent antioxidant action of pomegranate than AVG.

The increased testicular SOD activity by PP is similar to (Dkhil et al., 2013; Khalaf et al., 2019) where PP was more potent antioxidant than pomegranate juice. The potent antioxidant activity of PP explains its enhancement effect on semen quality. Contrary to PP, AVG did not enhance antioxidant status of testicular tissue. Nevertheless, it decreased testicular SOD. Reports about the effect of AVG on testicular tissue activity and spermatogenesis are contradicting, where it enhanced testicular antioxidant system under certain conditions like toxic chemicals exposure (e.g. Bisphenol A; a testicular toxic substance; Behmanesh et al., 2018) and exposure to radiation (Bala, Chugh, Bansal, Garg, & Koul, 2017). However, similar to our findings AVG negatively impact spermatogenesis under normal condition (Asgharzade et al., 2015; Oyeyemi et al., 2011), the negative impact of AVG on testicular tissue was modulated by the activation of inducible nitric oxide synthase (iNOS) and cell apoptosis of male germ cells (Asgharzade et al., 2015).

The negative effect of AVG on testicular tissue ALP and LDH confirms the impairment effect of AVG on semen quality reported in this study. iNOS increase in testicular tissue was associated with decrease in ALP (Atta et al., 2017) in rat model. In addition, iNOS elevation was associated with oedema and hyperemia in rat testes (Dokumacioglu et al., 2018). To the authors' knowledge, this is the first study to investigate the effect of AVG on sperm quality, testicular enzymes and testicular antioxidant biomarkers in rabbits.

## 5 | CONCLUSION

Pomegranate peels and Aloe vera gell were known for their antioxidant and anti-inflammatory effects in different animal species and different tissues. However, data are lacking on their effect on rabbit fertility and spermatogenic activity. To improve rabbit fertility under normal conditions, pomegranate peels are a potent stimulator of spermatogenesis and enhances male fertility in rabbits. However, fresh Aloe Vera gel negatively impacts semen quality and spermatogenesis. More studies on the effect of Aloe vera gel on Sertoli cell and Leydig cells apoptosis will explain the non-antioxidant dependent negative effect of aloe vera on male rabbit reproduction.

## 6 | STUDY LIMITATION

Extra group receiving both AVG and PP should have been tested to study the effect of interaction of AVG and PP on testicular tissue and semen quality of rabbit bucks. Additionally, antioxidant capacity of AVG and PP was not tested.

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## CONFLICT OF INTEREST

There is no conflict of interest to declare.

## ETHICAL APPROVAL

Institutional Animal Care and Use Committee (IACUC) of Cairo University approved the protocol of the experiment (IACUC), approval # Vet-CU 20022020138.

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