In vivo Fecundity Evaluation of *Phaleria macrocarpa* Extract Supplementation in Male Adult Rats

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ABSTRACT

"Mahkota Dewa" fruit (*Phaleria macrocarpa* [Scheff.] Boerl.), is a traditional Indonesian plant-based remedy that has been used traditionally for generations in treating multiple illness and diseases. This investigation intends to assess the fecundity effects of *P. macrocarpa* supplementation in adult male rats through hormonal, physical and histological changes. Sixty male Sprague Dawley rats were randomly distributed into two by five experimental design with two supplementation periods (3 and 7 weeks) allotted to 5 different doses of extract (0, 24, 48, 240 mg PM aqueous extract/ kg bw and 80 mg of commercial PM product/ kg bw). The mean sperm count (455 cells/ml), body weight (301 g), histological assessment of spermatogonia cells (87 cells), and thickness of seminiferous tubule layer (79 µm) significantly increased ($P < 0.05$) in rats treated with 240 mg/ kg dose. However, there were no changes in both physical appearances of testes (size and volume) and testosterone hormone levels among the treatment groups. Our findings indicated that supplementation of *P. macrocarpa* significantly increased the fecundity of rats and the effect was dose and time-dependent. The study suggested that *P. macrocarpa* offered an attractive and alternative potential for improving the fertility in men.

Keywords: Fertility, herbal remedy, male, *Phaleria macrocarpa*, spermatogenesis, testosterone

INTRODUCTION

Andropause is an age-related decline of sexual hormones in men. Sex hormones, especially testosterone, are responsible in basic sexual differentiation, brain development, regenerative organs and different frameworks in the perinatal period. Thus, hormonal confusion may trigger

actuate irreversible changes in conceptive organs or capacity at developed ages. Testosterone level increases during puberty and is kept at high level during the adult age. Testosterone starts to reduce progressively with age starting at 35 (Oyebode et al., 2016) and if continued for a long period, results in andropause associated with slower sexual responses, fewer living sperm, low libido, age-related diseases such as atherosclerosis, high blood pressure, diabetes mellitus, loss of muscle or bone mass and prone to fatigue (Kaufman & Vermeulen, 2005). Five million men experience the ill effects from low testosterone levels but only 5% of them are being treated (Csatari, 2015). One-third of male Malaysians are facing impotency problems, while only a limited number of patients are currently being treated (Badarudin, 2017). Testosterone replacement therapy is the current treatment applied but causes various side effects to patients. Thus, plant-based medications are preferred as an alternative to treat andropause in a safer and more effective way.

A recent African government report shows that 80% of the world population utilizes plant-based remedies for treating various medical healthcare problems (World Health Organization [WHO], 2016). Herbal plants have been used traditionally for generations by the indigenous peoples although threatened by the introduction of synthetic medicines. Andropause caused by diseases or aging has increased dramatically and becoming a global concern. One of the famous medical plants which exhibits excellent prospects for development is *Phaleria macrocarpa* [Scheff.] Boerl.).

Indonesian plant-based remedies with *P. macrocarpa* fruits have been used traditionally for generations in treating multiple lifestyle health problems (Benzie & Wachtel-Galor, 2011) and diseases (Altaf et al., 2013). They have been proven effective as anti-cancer (Trilaksana et al., 2017), and anti-cholesterol (Chong et al., 2011) and remedies in treating diabetes mellitus (Harmanto, 2003). They have anti-microbial, anti-inflammatory, and antioxidant properties (Randhir et al., 2004) as well as improving sexual libido (Parhizkar et al., 2013).

Phaleria macrocarpa is claimed to induce testosterone production in the body associated with sexual strength improvement and libido behaviour in men by the rural people. Unfortunately, there are very few published data regarding the potential and estimation of *P. macrocarpa* in improving fertility in males. There is a need for a series of research to exploit this potential medicinal plant as an alluring alternative option to the synthetic hormonal medications currently in use for improving infertility in male. Thus, a study was outlined to evaluate the medicinal effects of the fruits on male fecundity in adult rats.

MATERIALS AND METHODS

Aqueous Extraction of *Phaleria macrocarpa*

The red colour fruit of *P. macrocarpa* (diameter of 3 cm, Registration no. SK1929/11) was washed, seed disposed, cut into thin slices then oven dried for 24 hours at 65°C. 250 ±1 g of the dried fruit cuts were drenched in 4 litres of water and heated until the water became half of the original

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amount. Then, the mixture was sifted and the filtrate was centrifuged at 3000 rpm (503 × *g*) for 30 minutes to separate the particles. The supernatant was filtered again and freeze-dried to obtain the powder extract. The powder extract was weighed and frozen at -20°C for later use. The yield obtained was approximately 13%. The extraction process was repeated until about 3 kg of dried fruit slices were extracted.

Experimental Design

Sixty adult male Sprague Dawley rats (5 to 7 weeks, weighing 200-250 g) were randomly distributed into two by five experimental design with two supplementation periods (3 and 7 weeks) allotted to 5 different doses of extract (0, 24, 48, 240 mg PM aqueous extract/kg bw and 80 mg of commercial PM product/kg bw as standard positive control). The extracts were given using oral gavage at the volume of 0.2 mL daily. The rats were maintained under standard laboratory conditions in a well-ventilated room at room temperature of 28° ± 1°C with 70-80 % humidity and constant programmed 12 hours light-dark cycle inside the faculty animal house, Universiti Putra Malaysia, Serdang, Malaysia. All the rats were fed with commercial rat pellet once a day in the morning and water was given *ad libitum*. They were kept in 20 cages with three rats per cage and they were checked to be free from disease and malformation. The experimental rats were allowed to acclimatize for one week before the treatments started and the bedding was changed weekly. All animals were approved by the institutional animal care committee.

Body Weight

The body weights of the experimental rats were measured weekly by utilizing electronic balance (Mettler Toledo, Malaysia) throughout the time frame.

Cauda Epididymal Sperm Collection

The rats were sacrificed by using diethyl ether overdose. The sperm was collected from the cauda epididymis of the rats by excising the cauda epididymis and mincing it into pieces on petri-dishes in the 1 mL 0.1M phosphate buffered saline (PBS). The spermatozoa were allowed to flow into the buffer. After that, the sperm suspension was left at the room temperature for 30 ± 0.1 minutes to allow debris to settle down. 1 ml of the top portion of the suspension was collected into a new clean tube with cap and centrifuged at 500 rpm (14 × *g*) for 5 minutes. Then, the supernatant was thrown away. The sperm pellet was reconstituted with 1 ml of 0.1M Phosphate buffered saline (PBS) and subjected to sperm count analysis. A drop (10 µl) of sperm solution was loaded on the hemocytometer and viewed under ×400 magnification under the light microscope. The counting of sperm number was done by calculating in 4 × 4 squares (horizontally or vertically) (Figure 1). Sperm numbers were calculated by the formula in equation [1]:

$$\text{Sperm cell count} = \text{Total number of spermatozoa in 5 squares} \times 50{,}000 \times 100 \text{ (cells/ ml)} \quad [1]$$

Plasma Testosterone Analysis

Blood sample of 3 mL was collected from each rat via retro-orbital sinus/ peri-orbital bleeding procedures 24 hours after the last treatment for each treatment period. The plasma testosterone levels were evaluated through radioimmunoassay technique utilizing the kit TESTO-CTK (DiaSorin Diagnostics GmbH, USA). The analysis was carried out using COBRA II auto-gamma analyzer.

Testis Tissue Histology Study

The testes together with epididymis were pulled out gently, removed and encapsulated. The testes specimen was cleaned 3 times with 0.9% normal saline and fixed in 10% formalin for 2-3 days. The lateral and longitudinal section of testes were cut in order to observe the internal cell arrangement. The specimens were later fixed in 10% formalin solution for 72 hours. Then, they were dehydrated in various levels of ethanol, cleared with xylene, and embedded in paraffin wax for sectioning. The sections were cut (5 μm thick), mounted and stained with hematoxylin and eosin.

The histopathological changes data were examined by counting the spermatogonia (SG) cell numbers and layer thickness of seminiferous tubules (ST) in testes.

Statistical Analysis

Data were subjected to two- way analysis of variance (ANOVA) using SPSS software version 21. All the mean differences were compared using Duncan multiple range test (DMR) after a significant F-test at $P < 0.05$ and $P < 0.01$.

RESULTS

Body Weight

The body weight in the study was significantly increased ($P < 0.05$) by the dose of *P. macrocarpa* supplementation treatment given, duration of the supplementation, and the experimental animals (Table 1).

Testosterone Concentration

The mean testosterone concentration was neither affected ($P > 0.05$) by the dose nor the period of *P. macrocarpa* supplementation (Table 2).

Table 1

Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on body weight (mean ± SE) (g) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total mean
	0	24	48	240	80	
3	210 ± 7.78[a] (2.83)	268 ± 10.86[cd] (2.64)	258 ± 14.22[bc] (1.64)	291 ± 10.44[cd] (2.67)	261 ± 11.80[bc] (2.01)	258 ± 2.02[X] (4.98)
7	229 ± 8.60[ab] (2.40)	300 ± 12.25[d] (3.96)	305 ± 11.86[d] (3.82)	305 ± 10.44[d] (3.04)	271 ± 10.02[cd] (3.22)	282 ± 1.32[Y] (4.02)

( )   Average daily gain (g/ day)
±   Standard Error of Mean
XY   Means within the same column with different superscripts are significantly different (p<0.05)
abcde   Means within the same row with different superscripts are significantly different (p<0.05)

Table 2
Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on the testosterone concentration (mean ± SE) (ng/ ml) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total Mean
	0	24	48	240	80	
3	0.91 ± 0.07^a	1.52 ± 0.52^a	1.49 ± 0.40^a	1.21 ± 0.25^a	0.86 ± 0.27^a	1.20 ± 0.15^X
7	1.14 ± 0.48^a	0.75 ± 0.07^a	1.35 ± 0.49^a	1.18 ± 0.35^a	1.16 ± 0.38^a	1.12 ± 0.16^X

±   Standard Error of Mean
XY   Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde   Means within the same row with different superscripts are significantly different ($p<0.05$)

Epididymal Sperm Count

The sperm count in the study was significantly increased ($P < 0.05$) by the treatment given. The duration of the supplementation (Table 3) did not show significant effect. The total mean sperm count was the highest ($P < 0.05$) in rats treated with 240 mg/kg, followed by the group supplemented with 48 mg/kg, 24 mg/kg dose and untreated rats. In term of total mean sperm count, there was a positive change ($P < 0.05$) among the treatments but no obvious difference between treatments for the dosage of 48 and 80 mg/kg. The commercial product had an effect and the value was equivalent to 42.5 mg/kg PM extract.

Table 3
Effects of various doses of P.macrocarpa *fruit extracts supplemented at different periods on the sperm count (mean ± SE) (million cells/ ml) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total Mean
	0	24	48	240	80	
3	223 ± 33.81^{ab}	304 ± 13.13^{bc}	370 ± 13.42^{cd}	433 ± 16.72^{de}	353 ± 24.79^{cd}	336 ± 15.88^X
7	162 ± 30.13^a	308 ± 13.64^{bc}	417 ± 47.45^{de}	478 ± 22.57^e	398 ± 45.44^{de}	353 ± 24.91^X

±   Standard Error of Mean
XY   Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde   Means within the same row with different superscripts are significantly different ($p<0.05$)

Testis Histological Study

The mean of SG cells counts and the thickness of ST layer of the treatment groups were significantly higher compared to the control group (Tables 4 and 5). The total mean count of SG cells and the thickness of ST layer were higher ($P < 0.05$) with the increase in *P. macrocarpa* dose. The increase of SG cells was dose and time dependent, but the thickness of ST was only dose-dependent. The commercial product had an effect and the value was equivalent to 45 mg/kg PM extract. A substantial increase in SG cells and ST thickness was observed between control and treatment rats (Figures 1-3).

Table 4

Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on the SG cell number (mean ± SE) (cells) of adult rats*

Period (weeks)	Dose of *P.macrocarpa* extract (mg/ kg bw)					Total Mean
	0	24	48	240	80	
3	36 ± 1.27^a	52 ± 0.47^b	69 ± 1.31^c	83 ± 1.93^d	55 ± 0.98^b	59 ± 3.02^X
7	33 ± 1.58^a	55 ± 0.93^b	71 ± 1.80^c	90 ± 3.08^e	83 ± 2.25^d	66 ± 3.92^Y

±   Standard Error of Mean
XY   Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde   Means within the same row with different superscripts are significantly different ($p<0.05$)

Table 5

Effects of various doses of P. macrocarpa *fruit extracts supplemented at different periods on the thickness of ST (mean ± SE) (µm) of adult rats*

Period (weeks)	Dose of *P. macrocarpa* extract (mg/ kg bw)					Total mean
	0	24	48	240	80	
3	34.22 ± 0.72^a	51.37 ± 1.57^b	60.17 ± 2.73^c	79.61 ± 2.26^e	58.11 ± 4.11^c	56.69 ± 2.92^X
7	36.88 ± 0.63^a	46.34 ± 0.68^b	62.60 ± 1.34^c	78.39 ± 2.69^{de}	72.66 ± 1.76^d	59.37 ± 2.98^X

±   Standard Error of Mean
XY   Means within the same column with different superscripts are significantly different ($p<0.05$)
abcde   Means within the same row with different superscripts are significantly different ($p<0.05$)
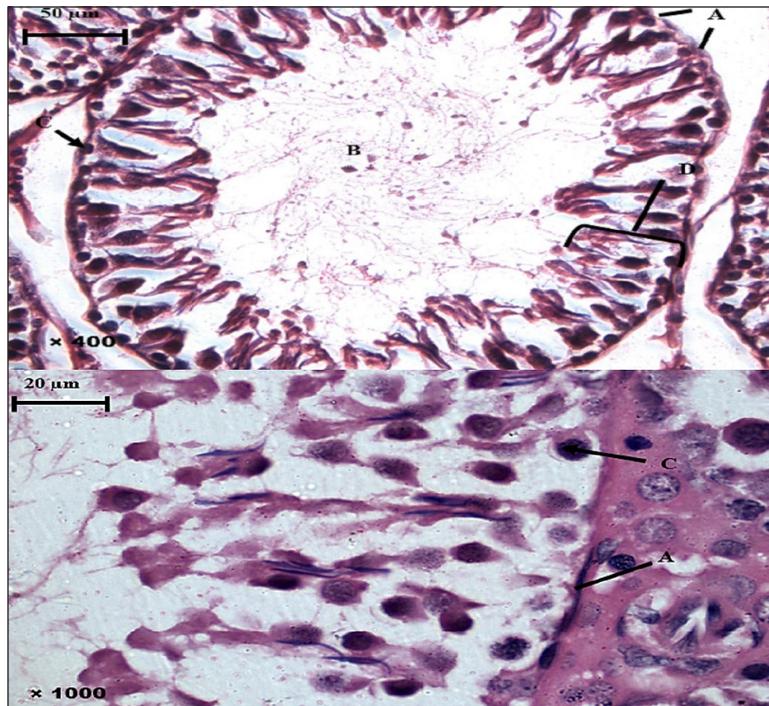
Figure 1. The thickness of ST layer and SG cells observed in control rats (untreated): (A) Basement membrane; (B) Lumen; (C) Spermatogonia cell; (D) Thickness of seminiferous tubule
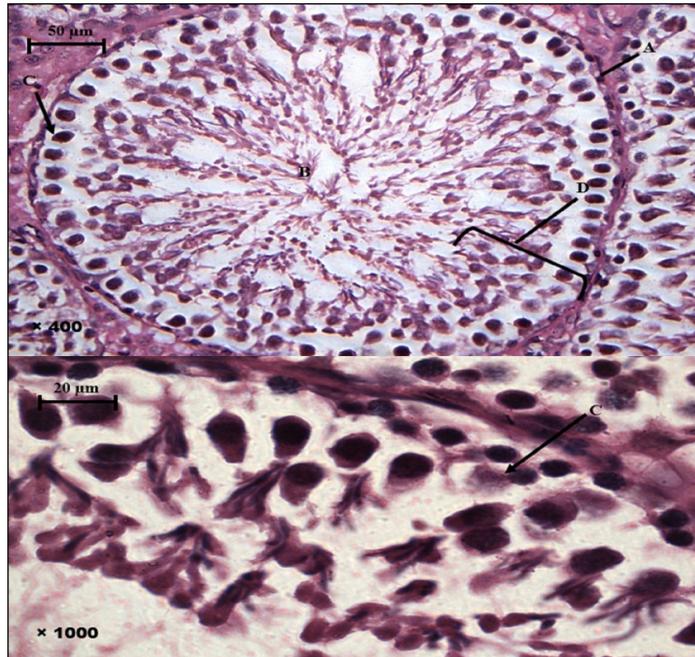
Figure 2. A substantial increase in SG cells number and thickness of ST layer was remarked in treated rats with PM extract (3 weeks): (A) Basement membrane; (B) Lumen; (C) Spermatogonia cell; (D) Thickness of seminiferous tubule
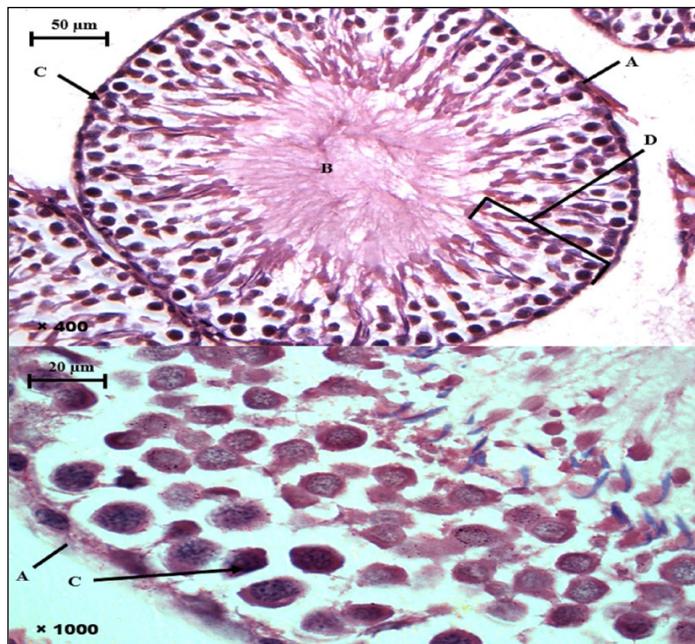

Figure 3. A substantial increase in SG cells number and thickness of ST layer was noticed in treated rats with PM extract (7 weeks): (A) Basement membrane; (B) Lumen; (C) Spermatogonia cell; (D) Thickness of seminiferous tubule

DISCUSSION

After the supplementation, the average daily gain of mature male rats increased at a positive slope and higher than the control group. The initial body weight of the rats was 200-250 g. Body weight changes were the first indication of the onset of the supplementation effect (Shapses et al., 2004). The increase in body weight of treated rats could be due to androgenic properties of *P. macrocarpa*. Testosterone has anabolic effects that mainly acts on the skeletal muscle which may account for part of the body weight (Carson & Manolagas, 2015). Testosterone also has a direct anabolic action on muscle, not to be confused with the skeletal muscle as it is not able to convert testosterone to DHT. This leads to the increase in nitrogen retention through the synthesis of protein resulting in the growth rate increase (Yoshioka et al., 2006). Previous studies by other researchers showed that testosterone levels corresponded positively with lean body mass, body weight and muscle mass (Gates et al., 2013). Thus, *P. macrocarpa* which contains saponin has the stimulatory effect on testosterone hormone (Djannah, 2009). Besides, body weight gain may be due to caloric intake because daily feed intake of the rats was not controlled in the present study. Hence this could increase body fat deposition. Previous research reported that a significant increase in body weight was mainly due to the increase of the percentage of body fat (Chantler et al., 2016). Thus, the increase of average daily gain was mostly caused by the increase of body fat.

Although the body weight of the treated rats increased significantly, the plasma testosterone levels tested were found in a pulsatile rhythm with doses. This may be due to insufficient supplementation duration to deliver any obvious effects in increasing the testosterone concentration in this study. Perhaps, longer period of supplementation would produce more significant anabolic effects. However, there was a slight increase in total mean testosterone concentration over the study periods. Testosterone hormone was released into the spermatic vein plasma in pulsatile pattern and demonstrated in these experimental rats. Besides, the duration of the testosterone secretion was about 15 to 90 minutes. Thus, the level of testosterone was fluctuating or varying among the rats during the blood collection causing the pulsatile pattern. Rats treated with 48 mg/kg produced the highest mean testosterone concentration as compared with all the other doses used. This may be caused by the fluctuation sequence of testosterone secretion. Previous research conducted on pre-pubertal and pubertal boys and girls (Mitamura et al., 2000) as well as removal of testosterone in men (Veldhuis et al., 2010) showed that serum testosterone peaked in the early morning and decreased to minimum value in the late evening. Thus, this diurnal rhythm of testosterone secretion may also apply to rats. Such inconsistency in plasma testosterone concentration has also been reported by most researchers. Besides, the formation and degradation of testosterone in the blood are constant. Luteinizing hormone (LH) and follicular

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stimulating hormone increase on short days and decreases on long days, thus responsible for the cycle of plasma concentration of testosterone (Jones et al., 2012). Any decline in the testosterone levels in the blood stream is regulated by the LH hormone secreted by anterior pituitary as it initiates the synthesis and secretion of testosterone to elevate the level back to original (Jones et al., 2012). Other studies have shown that testosterone level is positively regulated by libido. The male rats' testosterone level may increase if they are together with female rats. Female rats would increase the male rats' libido and when they are sexually active, the hormone level increases (Parhizkar et al., 2013). Change in serum testosterone levels demonstrates either an immediate effect of the medication at Leydig cell level or an indirect effect by blocking the hormonal milieu at hypothalamo-pituitary axis (Jones et al., 2012). A different result stated that *P. macrocarpa* extract enhanced the serum testosterone level significantly and also increased frequency of mounting but not latency of tested rats (Parhizkar et al., 2013).

Fecundity is commonly linked to sperm production. The study focused on sperm count production as the quality of a functional testes. The quality of testes was measured using sperm count, SG cells count and ST layer thickness. Following *P. macrocarpa* supplementation, the mean sperm count was improved in mature male rats. *Phaleria macrocarpa* has been known as a sexual tonic among rural people and is used for the treatment of impotence and infertility. The researcher documented that

P. macrocarpa fruit consisting of alkaloid, saponin and flavonoid (Noorehan & Piakong, 2016) can impact the microanatomic arrangement of testis cell in male mouse. Studies have shown that saponin in plants improves aphrodisiac properties since it has stimulatory impact in androgen secretion (Tang et al., 2017) which can upgrade sex charisma. Therefore, saponin has the potential to enhance the secretion of male primary testosterone hormone. The increase in the sperm production and quality is due to the expansion in testosterone hormone in the testicular tissue. Testosterone is critical in the development of sperms. In addition, it is the primary hormone in charge of spermatogenesis and spermiogenesis in ST (O'Donnell et al., 2017). A similar study done reported that supplementation *P. macrocarpa* increased sperm count (Abdul Razak et al., 2017).

Spermatogenesis cycle begins with the division of primary germ cells, producing a number of cells known as SG, follows by the production of primary spermatocytes. Primary spermatocytes are further divided into two secondary spermatocytes and young spermatozoa. The spermatozoa undergo maturation and develop into sperm cells. Thus, the increase of sperm production in this study was mainly linked to the histopathological changes. There was a quantitative increase in the SG cells number in testes for all treatment groups when compared to the normal control group as shown from the testicular histopathological study of the experimental rats (Tables 4 and 5). Testosterone is the basis of sex drive

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in males and females. A higher dose of *P. macrocarpa* had resulted in an increased sperm cell (Table 3) and a slight increase in testosterone level (Table 2) which triggered some effects and increased the SG cell number in the testes. Testosterone's main function in males is to facilitate sperm production.

The histopathological study expressed an improvement in the thickness of ST layer in the testes of treated rats. Eighty percent of testicular mass comprised seminiferous tubules. Thus, the morphological measurements of these ST are imperative in the evaluation of testis tissues (Hsieh et al., 2009). The small differences between the mean standard errors indicated that differences in the diameter of these 50 different ST collected were constant. The data were adequate for the estimation of average ST in treatment animals which is in agreement with Parhizkar et al. (2014). They proved that testosterone was imperative for spermatogenesis. The higher the testosterone level, the larger the diameter thickness of the ST layer. The current study result was in agreement with a previous study that reported similar effects of hexane and aqueous extract of *P. macrocarpa* leading to obvious enhancement of male animals' fertility by expanding the wall of thickness in ST (Parhizkar et al., 2015).

The testicles of typical rodents are framed by ST encompassed by tunica albuginea. Every tubule is covered by external stratified adventitial cells and inner basement film. There are interstitial connective tissues in between the tubules.

The ST are uniformly fit and lined by regularly arranged rows of SG cells of various phases of development. Following treatment with *P. macrocarpa*, both the quantities of SG cells and the thickness of ST layer were positively increased. The blood-testicles barrier is potentially a vital aspect while considering the conceptive and mutagenic impact of medications and environmental synthetics (Liu et al., 2009). Thus, the present study‑reported that aqueous extraction of *P. macrocarpa* was able to pass through the blood-testicles boundary and get access to the germ cells in the ST.

CONCLUSION

Phaleria macrocarpa possesses a potential value as an alternative for improving sexual strength by increasing the sperm count, SG cell‑count, ST layer thickness, and body weight in mature male rats. Thus, it is suggested that *P. macrocarpa* is able to improve the fecundity in men.

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