Protective effect of *Phaleria macrocarpa* (Scheff.) Boerl extract on the testicular damage of streptozotocin and nicotinamide-induced type 2 diabetic rats

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**ABSTRACT**

There was an increase in the global prevalence of diabetes mellitus, especially type 2 DM, resulting in higher prevalence of diabetic complication. Persistent hyperglycemia in diabetes mellitus induces systemic oxidative stress in testis. Oxidative stress disrupts germinal cells and caused abnormalities in the male reproductive system. *Phaleria macrocarpa* (Scheff.) Boerl contains active substances which have antidiabetic properties and also reduced the severity of diabetic complication in recent studies. This research aimed to evaluate the protective effect of PM pericarp extract on the testicular oxidative marker and testicular histological changes of type 2 DM-induced by streptozotocin and nicotinamide. Twenty male albino (Sprague Dawley) rats were divided into four equal groups: normal control; diabetic; diabetic + vitamin E 100 mg/kg; diabetic + PM methanolic extract 250 mg/kg. Diabetes was induced by injection of streptozotocin 65 mg/kg and nicotinamide 230 mg/kg intraperitoneally. PM extract and vitamin E (as a comparison group) were administered to diabetic rats for six weeks and at the end of the study, subjects were sacrificed to obtain their testes for routine staining and malondialdehyde (MDA) analysis. Treatment with PM extracts and vitamin E significantly reduced testicular MDA level compared with an untreated diabetic group (p < 0.01). Treatment with PM extract significantly improved sperm count, spermatogenic score, seminiferous tubule epithelial thickness, Sertoli and Leydig cell’s number which is reduced in untreated diabetic groups. It was concluded that PM extracts improved testicular damage and testicular MDA level.

**INTRODUCTION**

International Diabetes Foundation estimated that global diabetic prevalence to increase from 366 million in 2011 to 552 million in 2030 (Whiting et al., 2011). Prevalence of diabetes mellitus (DM) among residence above 18 years aged in 2014 had reached 8.5% of the population. Among four types of diabetes, type 2 diabetes is the most prevalence (World Health Organization, 2018). Not only its prevalence continues to rise, but there is a shifting trend to the younger onset of diabetes. This condition happened due to a global increase of population, longer life expectancy, higher prevalence of western lifestyle and obesity (IDF, 2013). The increasing prevalence will be accompanied with complication in multiple organs, one of which is the male reproductive system.

A persistent hyperglycemic condition in diabetes is well known to increase reactive oxygen species (ROS) (Zatalia et al., 2013) which further induce tissue morphological changes involving cellular components of the testis, mostly seminiferous tubules (Alves et al., 2013). Akinola et al. (2015) reported that oxidative stress is one the most agent promoting testicular damage in a diabetic patient, mostly affected lipid membrane of the
germinal cell and sperm’s DNA and further lead to abnormality in sperm structure and function. Male fertility requires the production of large numbers of normal and mature spermatozoa through a complex process which is affected by glucose metabolism (Alves et al., 2013). There is a decline in the number of mature sperms in seminiferous tubule due to diabetes and also in sperm quality due to a lower level of testosterone (Parhizkar et al., 2013). Clinically, infertility occurred in 35% diabetic patient and this number tends to increase lately (IDF, 2013). Other abnormalities include sexual dysfunction, decreased in sexual libido and impotent. An animal study of diabetes showed changes in sexual behaviors as well as reduced reproductive organs weight and reduced sperm parameter such as sperm count and motility (Alves et al., 2013).

While standard treatment of diabetes only focused on hypoglycemic control, a novel treatment which prevents the complication of diabetes is still needed. Recent studies discover the antidiabetic and protective effect of some natural products, such as Phaleria macrocarpa (Schefl) Boerl (PM). Phaleria macrocarpa is an Indonesian endemic plant, it has been reported to have hypoglycemic activities as an inhibitor of enzyme α-glucosidase in diabetic rats (Ali et al., 2012). Previous study suggested that PM had hepatoprotective effect on diabetic rat (Triastuti et al., 2009a) as well as nephroprotective effect on diabetic rats since it was found to reduce renal hypertrophy and blood urea nitrogen level in diabetic rats (Triastuti et al., 2009b) and also reduce expressions of profibrotic factors contributing in diabetic nephropathy (Sulistyoningrum et al., 2013b). In the present study, protection effect of an extract of PM’s pericarp on testicular MDA level, sperm count and testicular histological parameters in type 2 diabetes caused by streptozotocin and nicotinamide was examined.

**MATERIAL AND METHODS**

**Design of the study**  
An experimental study (post-test only with control design) was conducted in Integrated Research Laboratory, Islamic University of Indonesia, Yogyakarta, Indonesia from August to December 2016.

**Plant material collection**  
Merapi Herbal Farma (Yogyakarta, Indonesia) supplied ripe fruits of *P. macrocarpa*. Specimens of the plant was identified by Faculty of Mathematics and Natural Sciences, Islamic University of Indonesia, Yogyakarta, Indonesia. The pericarp of the fruits was sliced, dried in 70°C and then grounded into powder. About 1500 g of the grounded material was added with 6.5 liter methanol for 24 h. The filtrate was collected using Whatman No. 1 filter paper and residu was re-extracted with methanol 6,5 liters for 24 h and this step was repeated until 2 times. The filtrate from 3 days methanol extraction was collected and evaporated by rotary evaporation. The extracts were kept in the fridge (4°C) from where aliquots were withdrawn for the test procedures.

**Animals and intervention**  
In accordance with the Festing formula (Charan & Khantaria, 2013), a total of 20 healthy male *Sprague Dawley* rats (*Rattus norvegicus*) aged 8-12 weeks and weighing 200 ± 25 grams were housed under identical group cages. The cages were occupied in laboratory conditions of 22-30°C temperature and 30% relative humidity and 12 h light-dark cycle. Animals had free access to a standard pellet diet and mineral water. Prior experiment, rats were adapted for 12 days.

**Experimental design**  
There were four intervention groups: the normal control group received only distilled water; diabetic control group; diabetic + PM pericarp methanol extract (PMM), and diabetic + vitamin E. In normal and diabetic control, distilled water was used as a supplement. PM-supplemented groups were given 250 mg/kg, the dose had been administered for renal protective in diabetic rats (Sulistyoningrum et al., 2013). *Phaleria macrocarpa* extract was weighted using electronic balance (Ohaus PA214, United States of America) and reconstituted in distilled water. Vitamin E (Dexta Medika, Indonesia) and was given in 100 mg/kg dose (Momeni et al., 2012). All of the working solutions were kept at 4°C. The working solutions were prepared once a week to prevent any deactivation of the active compound in the extract and to maintain the quality of the working solution. The rats in each group were force-fed with 2 ml working solution according to their treatment groups for six weeks, after which animals were sacrificed under light anesthesia. The epididymal specimen was taken for sperm analysis. Right testis was taken for MDA level analysis and the left testis was taken for routine staining.

**Induction of diabetes**  
After an overnight fasting, the diabetic condition was induced with a single intraperitoneal injection of Nicotinamide (Sigma Aldrich, United States of America) 230 mg/kg and 65 mg/kg streptozotocin (Nacalai Tesque, Japan) dissolved in citrate buffer 4.5 pH with 15 minutes interval. After 72 h of induction, hyperglycemia was confirmed by measuring fasting blood glucose level taken from tail capillary with a glucometer (GlucoDr AGM 2100, Korea). Rats with a fasting blood glucose level above 130 mg/dl were considered diabetic and employed for further studies (Sedigheh et al., 2011).

**Testicular malondialdehyde analysis**  
Testicular MDA levels were determined using the procedure of Ohkawa (Akondi et al., 2011). After thawing the tissues, each sample was weighed and homogenized in 0.15 M potassium chloride solution; about 0.4 mL of homogenate was mixed with 1.5 mL thiobarbituric acid, 1.5 mL acetic acid (pH 3.5), and 0.2 mL sodium dodecyl sulfate. A set of MDA standard was freshly prepared. After mixing, all samples and standards were heated at 95°C for 1 h and cooled under tap water. The absorbance was recorded at 532 nm and compared with those obtained from MDA standards. The results were expressed as nmol/mg tissue.

**Histological analysis**  
Left testis from each animal was removed and placed in 10% buffer formalin, cast in paraffin. Isolated organs were stained with Hematoxylin-Eosin and then examined for the morphology. Quantitative analysis of histological reproductive parameters was carried out by determination of the spermatogenesis score, a number of spermatozoa, Sertoli cells, Leydig cells and seminiferous tubules epithelial thickness were measured with Olympus CX22 and Optilab Viewer (Miconos, Yogyakarta,
Indonesia). Regarding spermatogenesis score, spermatogenesis in seminiferous epithelia were scored semiquantitatively into ten scales according to Johnsen score on spermatogenesis (1, no cells were found in the seminiferous epithelia; 2, only Sertoli cell were found in the epithelia; 3: only spermagonia in the epithelia; 4: spermatocytes less than 5, no spermatid and spermatooza; 5: many spermatocytes, no spermatid and spermatooza; 6: spermatid less than 10, no spermatooza; 7: many spermatids, no spermatooza; 8: spermatooza less than 10; 9: many spermatooza, unorganized spermatogenesis, sloughing seminiferous epithelia, closed lumen; 10: complete and well-organized spermatogenesis, open lumen (Iftikhar et al., 2014). About 25 seminiferous tubules were analyzed in the testicular sections of each rat.

Sperm analysis

Sperm count analysis was performed according to Parhizkar et al. (2013) with slight modification using the hemocytometer. A drop with 10 μl of caudal epididymal sperm solution was loaded under the cover slip on the hemocytometer and then examined in 400x magnification under Olympus CX 22 light microscope. Counting was only performed for sperm heads that were found within 4 × 4 squares (horizontally or vertically) and calculated using the formula below:

\[
\text{Sperm count} = \left( \frac{\text{total number of sperm in 5 squares}}{\text{(solution) \times 0.05 \times 100^6 \ (cells/ml)}} \right)
\]

Table 1: Characteristic of subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Weight prior treatment (gram)</th>
<th>Weight after treatment (gram)</th>
<th>Fasting glucose level prior treatment (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>220,76 ± 16,90</td>
<td>244,98 ± 12,90</td>
<td>88,20 ± 14,28</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>214,74 ± 15,81</td>
<td>208,98 ± 11,41</td>
<td>152,20 ± 14,02*</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>213,70 ± 11,79</td>
<td>227,18 ± 13,62</td>
<td>163,40 ± 12,69*</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>210,50 ± 15,01</td>
<td>213,94 ± 13,35</td>
<td>154,20 ± 24,68*</td>
</tr>
</tbody>
</table>

Group I: normal control (receive only distilled water); Group II: diabetic control, Group III: diabetic + *Phaleria macrocarpa* extract 250 mg/kg, Group IV: diabetic + vitamin E 100 mg/kg, *p < 0.05 compared with Group I (ANOVA followed by post hoc LSD).

Testicular MDA level of untreated diabetic rats was significantly higher than other groups (Figure 1), treatment with vitamin E and PM extract significantly reduced testicular MDA level (p < 0.05 compared with untreated diabetic groups). Group treated with vitamin E had no significant difference in testicular MDA level compared with normal group, while the group with PM extract still gave a higher MDA level compared with normal group (p < 0.05). Diabetic rats also showed a significant reduction in sperm count of epididymal suspension (Figure 2). The diabetic group received treatment with PM extracts and vitamin E had higher sperm count (p < 0.05 compared with the diabetic group, Figure 2), but still lower than normal group (p < 0.05).

Majority of seminiferous tubule in Group I were intact, prominent and had an optimal epithelial thickness (Figure 3a) and a small amount of interstitial component. In higher magnification, seminiferous epithelium showed complete spermatogenesis, consist of many spermatogenic cells ranging from immature (spermatagonia) to mature stadia (spermatooza) (Figure 4a) and Sertoli cells characterized by triangular shape, located in the basal compartment of seminiferous tubule and prominent nuclei.

In an untreated diabetic group, the majority of seminiferous tubule had a low epithelial thickness (Figure 3b). There was also abnormal changes in interstitial compartment, that showed a loose connection, and sparse distribution of seminiferous tubule. In higher magnification, there was a depletion in the epithelial spermatogenic cells and heterogeneity of spermatogenesis. The diabetic group received *Phaleria macrocarpa* extract showed many tubuli with variability in sizes and epithelial thickness, some sparse elements in the interstitial compartment (3c). The diabetic group received vitamin E showed many tubuli with variability in sizes and optimal epithelial thickness In higher magnification, both in vitamin E and PMM extract groups, all stages of spermatogenic cells were observed in the epithelium but not as optimum as normal control.

Diabetes group had lowest spermatogenesis score, also other histological parameters in seminiferous tubule, including epithelial thickness, Sertoli cell’s number (Table 2). The diabetic group received *Phaleria macrocarpa* extract and vitamin E showed improvement in spermatogenesis score, seminiferous tubules’ epithelial thickness, and Sertoli cell number. The diabetic group received PMM extract and vitamin E showed an improvement in histological parameter indicated by improved epithelial thickness and spermatogenesis score compared with an untreated diabetic group (p < 0.05, Table 2). Improvement also

Statistical analysis

Normally distributed data were expressed as mean ± SD and analyzed using one way ANOVA and Tukey HSD post hoc test for multiple comparisons. Skewed distributed data were analyzed using Kruskal Wallis and were further subjected to Mann-Whitney post hoc. All analyses were performed with IBM SPSS 15.00 version 21 and differences between means were accepted significant at p < 0.05.

Ethical clearance

Ethical approval for this study was obtained from Research Ethics Committee, Faculty of Medicine, Islamic University of Indonesia (Registration number 21/Ka.Kom.Et/70.KE.VI/2015).

RESULT AND DISCUSSION

Research for investigating the effect of *Phaleria macrocarpa* extract on the testicular complication of type 2 diabetic rats was conducted for 6 weeks. Induction of type 2 DM with an injection of STZ and NAD significantly elevated fasting glucose level (Table 1). During interventions, animal were in proper condition. There was no report of abnormality in appetite, the volume of feces and urine, except in untreated diabetic group, animals had a slower weight gain and an increase of urine volume which need cage cleaning more frequent.
can be reported on Sertoli cell’s number, which was responsible for structural support of spermatogenic cell, also in nourishment, the secretory cell produces androgen binding protein and inhibin. In Figure 4, Sertoli cells were characterized by triangular nuclei, located at the basal compartment of seminiferous tubule and apical segment elongated at luminal compartment (arrow). The diabetic group treated with vitamin E showed better improvement on spermatogenesis score and epithelial thickness than PM and the result is not significantly different with the normal group. On the contrary, there were no significant differences on spermatogonia cell count among 4 groups (p > 0.05, Table 2).

On interstitial compartment, the diabetic group had an accumulation of loose connective tissue in interstitial (Figure 3b) and reduced number of Leydig cells (Table 2). This cell is responsible for testosterone secretion and it is characterized by prominent nuclei, acidophilic cytoplasm and variable lipid droplet in the cytoplasm (Figure 5, arrow). The normal group had the most Leydig cell’s number, followed by vitamin E group, PMM extract group, and the untreated diabetic group had the less. Treatment with vitamin E and PMM extract improved Leydig cell’s number (p < 0.05, Table 2), but vitamin E showed better improvement in Leydig cell number and had no different with the normal group.

Induction of streptozotocin 65 mg/kg and nicotinamide 230 mg/kg intraperitoneally can increase blood glucose level above the normal level. Streptozotocin (STZ) is a selective toxic substance for pancreatic beta cells, which enter the cells via glucosa-2 transporter (GLUT-2) (Eleazu et al., 2013). Streptozotocin inhibits insulin secretion via ATP reduction and NAD depletion in cells’ DNA. Combined with nicotinamide 230 mg/kg, STZ will induce more stable and irreversible diabetic condition (Khaneshi et al., 2013). This combination also induces a clinical condition of diabetes without plasma insulin reduction (Alkhamees, 2013) and also induced diabetes complications (Ghasemi et al., 2014).
Fig. 3: Lower magnification of testicular tissue among groups. 100x magnification, Routine staining. Normal seminiferous tubule and lesser interstitial compartment in Group I (a). In Group II: seminiferous tubule were smaller and had plenty of interstitial component (b), Group III showed many tubuli with variability in size and epithelial thickness while in Group IV showed many tubules with intact epithelium similar to Group I. Group I: normal control (receive only distilled water); Group II: diabetic control, Group III: diabetic + *Phaleria macrocarpa* extract 250 mg/kg, Group IV: diabetic + vitamin E 100 mg/kg.

Fig. 4: Higher magnification of seminiferous tubules among groups. 400x magnification, Routine staining. Group I (a) had thick epithelial layer and optimal spermatogenesis (score 10) and man Sertolli cells (arrow). Group II (b) showed epithelial depletion and less spermatogenic cells observed, less mature stage of spermatogenic cells, Groups III and IV (c and d) showed thick epithelial layer and complete stages of spermatogenic cells but not yet as optimum as Group I. Group I: normal control (receive only distilled water); Group II: diabetic control; Group III: diabetic + *Phaleria macrocarpa* extract 250 mg/kg, Group IV: diabetic + vitamin E 100 mg/kg.

The untreated diabetic rat had high testicular MDA level indicating oxidative stress in testicular tissue followed by reducing sperm count. Testicular MDA level is an indicator for lipid peroxidation in spermatogenic cell’s membrane. This substance had a toxic effect on to the spermatozoa, an eventually will reduce spermatozoa’s cell membrane integrity and lead to lower quality of the sperm. Streptozotocin and diabetes condition caused marked increased of MDA level (Ramzy et al., 2014).

Diabetic group had the lowest seminiferous tubule epithelial thickness and spermatogenesis score. Consistent with this research, Ozdemir et al. (2009) reported that diabetic rats had low seminiferous tubule epithelial thickness due to
decreased spermatogenesis and spermatogenic cells’ apoptosis in seminiferous epithelial. ROS-induced apoptosis also occurred in interstitial compartment, affected Leydig cell so the number of Leydig cells decreased and there was an accumulation in loose connective tissue in the interstitium. STZ-induced diabetic rats also had atrophy in reproductive organs and decrease sperm parameters (Navarro-Casado et al., 2010).

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Spermatogenesis score</th>
<th>Epithelial thickness (µm)</th>
<th>Spermatogonia cell number</th>
<th>Sertoli cell number</th>
<th>Leydig cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5</td>
<td>8.56 ± 0.41a</td>
<td>100.46 ± 2.13a</td>
<td>616.2 ± 77.95</td>
<td>89.40 ± 4.27b</td>
<td>17.42 ± 0.52a</td>
</tr>
<tr>
<td>II</td>
<td>5</td>
<td>5.88 ± 0.26</td>
<td>81.43 ± 5.99</td>
<td>469.8 ± 105.23</td>
<td>61.80 ± 6.44</td>
<td>8.34 ± 0.59</td>
</tr>
<tr>
<td>III</td>
<td>5</td>
<td>8.06 ± 0.33a</td>
<td>89.66 ± 1.10e</td>
<td>502.4 ± 97.36</td>
<td>87.80 ± 4.48e</td>
<td>13.41 ± 0.44a</td>
</tr>
<tr>
<td>IV</td>
<td>5</td>
<td>8.32 ± 0.19a</td>
<td>94.73 ± 1.95e</td>
<td>531.2 ± 118.83</td>
<td>88.00 ± 5.58e</td>
<td>17.44 ± 0.39a</td>
</tr>
</tbody>
</table>

Figure 5: Leydig cells in the interstitial compartment (arrows).
nuclear antigen (PCNA) and Ki67 immunostaining will give a specific and representative result on proliferating spermatogonia. Further investigation in safety of Phaleria macrocarpa is needed before implementing this research to the clinical setting.

In this research, degree of improvements on sperm count, spermatogenesis score, seminiferous tubule epithelial thickness, Sertoli and Leydig cell’s number of PM treated group seems not as good as or similar to Vitamin E. However, PM treatment can still be considered as alternative drug since it has benefit on testicular tissue as well as other properties of diabetic condition such as antihyperglycemic, antioxidant and renal protective due to diabetic-related complication. Given orally, treatment of PM extract can be used as a better comprehensive treatment of diabetes and its complication compared with vitamin E alone.

CONCLUSION

In brief, it can be concluded that Phaleria macrocarpa extract improved testicular oxidative stress parameter, sperm count and histological changes of the testis, which characterized by spermatogenesis score and histological morphometric parameters. In type 2 diabetes mellitus induced by streptozotocin and nicotinamide, there was an increase of testicular MDA level and a decrease of a histological parameter of spermatogenesis. However, improvement of Phaleria macrocarpa extracts not yet give the equal result as vitamin E treated group nor normal control. One of possible future implementation of this research is the use of PM extract as a complementary treatment for a testicular complication of type 2 diabetes mellitus.

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CONFLICT OF INTERESTS

There are no conflict of interests.

REFERENCES

Mallick C, Bera TK, Ali KM, Chatterjee K, Ghosh D. Diabetes induced testicular disorders vis-a-vis semen quality and spermatogenesis score, seminiferous tubule epithelial thickness, nuclear antigen (PCNA) and Ki67 immunostaining will give a specific and representative result on proliferating spermatogonia. Further investigation in safety of Phaleria macrocarpa is needed before implementing this research to the clinical setting. In this research, degree of improvements on sperm count, spermatogenesis score, seminiferous tubule epithelial thickness, Sertoli and Leydig cell’s number of PM treated group seems not as good as or similar to Vitamin E. However, PM treatment can still be considered as alternative drug since it has benefit on testicular tissue as well as other properties of diabetic condition such as antihyperglycemic, antioxidant and renal protective due to diabetic-related complication. Given orally, treatment of PM extract can be used as a better comprehensive treatment of diabetes and its complication compared with vitamin E alone.

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