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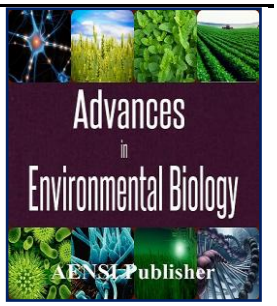

Plasma Testosterone and Testis Histological Features of Mice Treated with Averrhoa Bilimbi, Cosmos Caudatus and Pereskia Bleo Ethanolic Extract

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ABSTRACT

Averrhoa bilimbi (AB), *Cosmos caudatus* (CC) and *Pereskia bleo* (PB) are among the local plants in Malaysia enriched with plant secondary compounds scientifically proven to contain high value in medical pharmacology. This study involving these three selected plants was conducted to analyses the plasma testosterone concentration and testis histological features of mice supplemented with four different concentrations (50, 125, 500 and 1000 mg/kg b. wt) of AB, CC and PB ethanolic extract. There was a significant reduction in plasma testosterone level across all AB treatment groups (177.83±0.03pg/ml, 266.07±0.05pg/ml, 281.84±0.03pg/ml and 277.07±0.00 pg/ml, respectively) compared to control group (515.82±0.01pg/ml), in both 50 and 500mg/kg b. wt of CC (546.39±0.03pg/ml and 728.62±0.04, respectively), and in 50, 125 and 1000mg/kg b. wt of PB ethanolic extracts (375.84±0.05pg/ml, 865.96±0.03pg/ml and 473.15±0.01pg/ml, respectively). All concentrations of AB extract (206.77±5.51µm, 194.29±4.25µm, 202.92±3.34µm and 207.34±6.34µm, respectively) and 50 and 500 mg/kg b. wt of PB extract (212.30±5.57µm and 214.70±4.31µm, respectively) seem to reduce the diameter of seminiferous tubules, while comparing to the control (236.81±7.07µm). The lumen diameter in the seminiferous tubules also decreases in 125mg/kg b. wt of AB (70.14±3.09µm), 1000mg/kg b. wt of CC (73.47±2.80µm) and in the 50 and 125 mg/kg b. wt of PB extracts (72.65±3.99µm and 78.54±3.85µm, respectively). The spermatogenic cells in Figure1: C1, B2, C2, D2, E2, B3, C3 and E3 were packed and improved as compared to Figure 1A. The degeneration of spermatocyte and spermatogonia layer was also observed (Figure 1: B1, C1, D1, E1, D2, B3, C3, D3 and E3).

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INTRODUCTION

Malaysia is among one of the South East Asian country enriched with more than 120 species of traditional vegetables which are known to possess high medicinal values [1]. The plants include; Averrhoa *bilimbi* (AB), *Cosmos caudatus* (CC) and *Pereskia bleo* (PB) which are usually consumed as part of a meal, eaten either raw or taken after a blanching processes [2]. The nutritional studies showed that these plants are enriched with bioactive compounds and secondary metabolites including flavonoids, alkaloids, phenolics, saponins and many more are enriched with resourceful pharmacological activities [3, 4]. The pharmacological activities include anti-bacterial and anti-oxidant, anti-osteoporosis effect, anti-hypertensive and anti-diabetic activities [5, 6]. All the plants have showed anti-oxidative properties with radical scavenging activity which enables the plants to be taken as supplements to maintain wellbeing, especially on the reproductive system. This property also enables the plants to reduce oxidation by reactive oxygen species, especially in sperm production during spermatogenesis. Androgen like testosterone is a principal male sex hormone. It is an important hormone for the development of male sexual reproductive system where it regulates spermatogenesis and mating behaviour in males [7]. Testosterone, a steroid hormone, is secreted by interstitial (Leydig) cells as response to the stimulation of pituitary luteinizing hormone. Synthesis and secretion of testosterone in seminiferous tubules by leydig cells is sufficient enough to induce the differentiation of spermatogonia to spermatozoa [8]. In this present study, the different concentrations of AB, CC and PB ethanolic extract were used. The part of AB used was their fruits which it bearing is fleshy and juicy but it becomes acidic when it is ripen [9]. For both CC and PB, part of the plant used was their leaves. CC leaves have distinctive taste and odour [10] meanwhile leaves of

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PB are simple, spiral, glossy, succulent and has orange-coloured flowers [11]. The aim of this study is to emphasize on changes on plasma testosterone level by the different plants extract concentrations with the comparative testis histological features of mice treated with these selected ethanolic plants extract.

Materials and Procedures:

Ethanolic plants extract, experimental animals and procedure preparations:

Ethanolic extract of AB, CC and PB, experimental animals and experimental procedures preparations were conducted as outlined in [12]. The sliced fruits of AB, both leaves of CC and PB were subjected to cryogenic grinding with the addition of liquid nitrogen. The concentrations used in the experiment were 50, 125, 500 and 1000 mg/kg b.wt. The control group was given 0.9% saline. The treatments were administered through force-feeding using oral gavage. The experimental animals used were sexually matured male mice, *Mus musculus* strain ICR. Male mice were divided into 5 individuals per treatment group.

Plasma testosterone analysis:

Blood were collected using cardiac puncture and stored inside EDTA vacutainer. The blood samples were centrifuged for fifteen minutes at 2500xg, 4˚C within 30 minutes of collection. The plasma obtained were kept inside -80˚C freezer until assayed. The analyses were carried out by using enzyme-linked immunosorbent assay kit by Cloud-Clone Corp US, assembled by Uscn Life Science Incorporated, China.

Testis histological features:

Testis of each mouse including those of the control group were removed, trimmed off extraneous and fragments of testicular tissues and were fixed in 10% formalin. Fixed testis were embedded in paraffin wax and cut for histological study. The sections were observed using inverted microscope (Olympus BX41) with 20x magnification and visualized using Q-Capture Pro 6.0 software. The observations were done to analyse the diameter of seminiferous tubules and the lumen-; and width of spermatid-sperm, spermatocytes and spermatogonia layer.

Statistical analysis:

One-way Analysis of Variance (ANOVA) including descriptive Waller-Duncan post hoc test were conducted using SPSS software, Version 20.0. All data were presented by mean ± SEM. The difference in the mean values was considered significant at $p<0.05$.

RESULTS AND DISCUSSION

Plasma testosterone analysis:

The outcome of analysis for plasma testosterone level indicated that all three ethanolic extract of plant treatments with different concentrations have significant effect ($p\geq0.05$) on mice (Table 1). The extracts of AB in all concentrations have significantly reduced the plasma testosterone level as compared to control groups. Meanwhile, the groups of 50mg/kg, 500mg/kg b. wt CC and 125mg/kg b.wt PB extract have increased the mean concentration of plasma testosterone level when comparing to the control treatment.

Table 1: Plasma testosterone concentration (pg/ml) on mice treated with the different concentrations of the selected plant extract.

Treatment groups/ Concentrations	Plasma concentration testosterone, pg/ml			
	Control	AB	CC	PB
50 mg/kg b. wt	515.82±0.01^{a,b}	177.83±0.03^{d}	546.39±0.03^{a,b}	375.84±0.05^{b,c}
125 mg/kg b. wt		266.07±0.05^{c,d}	421.70±0.02^{b,c}	865.96±0.03^{a}
500 mg/kg b. wt		281.84±0.03^{c}	728.62±0.04^{a}	375.84±0.02^{b,c}
1000 mg/kg b. wt		266.07±0.00^{c,d}	421.70±0.01^{b,c}	473.15±0.01^{a,b}

Superscripts ^{c,d} within the row show significant difference ($p< 0.05$)

Table 2: Diameter of seminiferous tubules of mice supplemented with different concentrations of AB, CC and PB ethanolic extract.

Treatment groups/ Concentrations	Diameter of seminiferous tubules, µm			
	Control	AB	CC	PB
50 mg/kg b. wt	236.81±7.07^{b}	206.77±5.51^{a}	239.21±6.37^{b}	212.30±5.57^{a}
125 mg/kg b. wt		194.29±4.25^{a}	221.45±7.77^{b}	216.50±5.24^{a,b}
500 mg/kg b. wt		202.92±3.34^{a}	238.06±7.16^{b}	214.70±4.31^{a}
1000 mg/kg b. wt		207.34±6.34^{a}	231.28±6.22^{b}	218.07±4.30^{a,b}

Superscript ^{a} within the row show significant difference ($p< 0.05$).

Testis histological features:

In the histological features of mice testis, there are significant values in the parameters involved after the experimental animals were treated with the different concentrations of AB, CC and PB ethanolic extract. Table 2 shows the diameter of seminiferous tubules in mice treated with the given plants extract. Again, the extract of AB significantly reduced the diameter of seminiferous tubules in all concentrations as compared to the control. Meanwhile, extract of CC carries no significant value. The concentrations of 50 and 500 mg/kg b. wt of PB extract have shown significantly lower values in the seminiferous tubules diameter when compared to the

control group. In the comparisons among those three extracts, the extract of AB significantly reduced the seminiferous diameter as compared to all the concentrations of CC and also at 50 and 500 mg/kg b. wt of PB. The lumen diameter inside the seminiferous tubule is tabulated in the Table 3. The concentrations of 50 mg/kg b. wt of PB, 125mg/kg b. wt of AB and PB, 1000mg/kg b. wt of CC were found to have significant reductions in the lumen diameter as compared to the control group. At the lower concentrations of PB and 125mg/kg b. wt of AB, the lumen diameter has reduced significantly as compared to the CC extract. Table 4,5 and 6 shows the width of spermatid-sperm, spermatocytes and spermatogonia layers of mice testis that were treated with different concentrations of those selected plants. Table 4 showed significant increments in the mean of spermatid-sperm layer width for 125mg/kg b. wt and 1000mg/kg b. wt of PB extract when compared to control. In comparison among those three plants, 125mg/kg b. wt of PB extract also showed significant increase in the spermatid-sperm layer width as compared to AB and CC extracts, and increase the spermatid-sperm width significantly in 1000mg/kg b. wt as compared to AB extract. For the width of spermatocytes layer (Table 5), all concentrations of AB extract showed significant decrement as compared to the control. Meanwhile, the significant decrement of the width of spermatocytes layer was also found in 500mg/kg b. wt and 1000mg/kg b. wt of PB, and only at 500 mg/kg b. wt in CC ethanolic extract. In the comparisons with the control group, at 50mg/kg b. wt and 1000mg/kg b. wt of AB, 500 mg/kg b. wt and 1000mg/kg b. wt of CC and all concentrations of PB ethanolic extracts have significantly reduced the width of spermatogonia layer (Table 6). Figure 1 illustrates the effects of the ethanolic extracts of AB, CC and PB at four different concentrations and control group to the mice testis histological features. AB treatment in all concentrations, 50mg/kg b. wt and 500mg/kg b. wt of PB have degenerated the diameter of seminiferous tubules of mice testis. It is obviously clear that the lumen in Figure1: C1, E2, B3 and C3 have lessened as compared to Figure 1A. Greater abundance of spermatogenic cells are observed in those concentration groups when observed at 20x magnifications. The spermatogenic cells in Figure1: C1, B2, C2, D2, E2, B3, C3 and E3 were packed and improved as compared to Figure 1A. The degeneration of spermatocytes and spermatogonia layer width was also found in Figure 1: B1, C1, D1, E1, D2, B3, C3, D3 and E3. The appearance of Leydig cells are more in groups of higher treatment concentration and control group. In contrast, Sertoli cells are comparable in all concentration groups of mice testis features.

Table 3: Diameter of lumen of the seminiferous tubules of mice supplemented with different concentrations of AB, CC and PB ethanolic extract.

Treatment groups/ Concentrations	Diameter of lumen, µm			
	Control	AB	CC	PB
50 mg/kg b. wt	89.06±4.18^{b}	79.17±4.18^{a,b}	92.55±3.81^{b}	72.65±3.99^{a}
125 mg/kg b. wt	89.06±4.18^{c}	70.14±3.09^{a,b}	82.31±4.33^{b,c}	68.30±3.36^{a}
500 mg/kg b. wt	89.06±4.18	83.25±2.67	82.05±4.79	84.85±2.13
1000 mg/kg b. wt	89.06±4.18^{b}	81.65±3.56^{a,b}	73.47±2.80^{a}	78.54±3.85^{a,b}

Superscripts ^{a} within the row show significant difference ($p< 0.05$), for 125mg/kg b.wt concentration, superscripts ^{a,b} show significant difference ($p<0.05$).

Table 4: Width of spermatid-sperm layer of mice supplemented with different concentrations of AB, CC and PB ethanolic extract.

Treatment groups/ Concentrations	Width of spermatid-sperm layer, µm			
	Control	AB	CC	PB
50 mg/kg b. wt	27.84±1.37	26.94±1.10	28.28±1.63	31.17±1.43
125 mg/kg b. wt	27.84±1.37^{a}	26.08±1.21^{a}	26.31±1.46^{a}	36.23±1.61^{b}
500 mg/kg b. wt	27.84±1.37^{a,b}	23.17±0.87^{a}	33.25±2.30^{b}	29.98±1.51^{b}
1000 mg/kg b. wt	27.84±1.37^{a,b}	25.29±1.45^{a}	31.94±1.34^{b,c}	33.56±1.73^{c}

Superscripts ^{c} within the row show significant difference ($p < 0.05$), for 125mg/kg b.wt concentration, superscript ^{b} show significant difference ($p<0.05$).

Table 5: Width of spermatocytes layer of mice supplemented with different concentrations of AB, CC and PB ethanolic extract.

Treatment groups/ Concentrations	Width of spermatocytes layer, µm			
	Control	AB	CC	PB
50 mg/kg b. wt	27.17±0.96^{b,c}	21.82±1.20^{a}	29.01±1.55^{c}	24.30±0.94^{a,b}
125 mg/kg b. wt		21.61±1.02^{a}	24.05±1.10^{a,b}	23.58±1.20^{a,b}
500 mg/kg b. wt		21.48±0.81^{a}	21.23±1.05^{a}	20.44±1.15^{a}
1000 mg/kg b. wt		21.18±0.75^{a}	23.65±1.12^{a,b}	20.78±1.06^{a}

Superscripts ^{a} within the row show significant difference ($p< 0.05$)

Table 6: Width of spermatogonia layer of mice supplemented with different concentrations of AB, CC and PB ethanolic extract.

Treatment groups/ Concentrations	Width of spermatogonia layer, µm			
	Control	AB	CC	PB
50 mg/kg b. wt	15.56±0.61^{b}	11.05±0.52^{a}	16.46±0.83^{b}	12.23±0.49^{a}
125 mg/kg b. wt	15.56±0.61^{b}	13.68±0.56^{a,b}	14.93±0.89^{b}	11.48±0.45^{a}
500 mg/kg b. wt	15.56±0.61^{c}	15.21±0.59^{b,c}	12.99±0.86^{a,b}	10.78±0.59^{a}
1000 mg/kg b. wt	15.56±0.61^{c}	13.34±0.56^{b}	10.72±0.45^{a}	11.28±0.48^{a}

For 50 and 125 mg/kg b. wt concentrations, superscript ^{a} within the row show significant difference ($p< 0.05$), 500 and 1000 mg/kg b.wt concentration, superscripts ^{a,b} show significant difference ($p<0.05$).

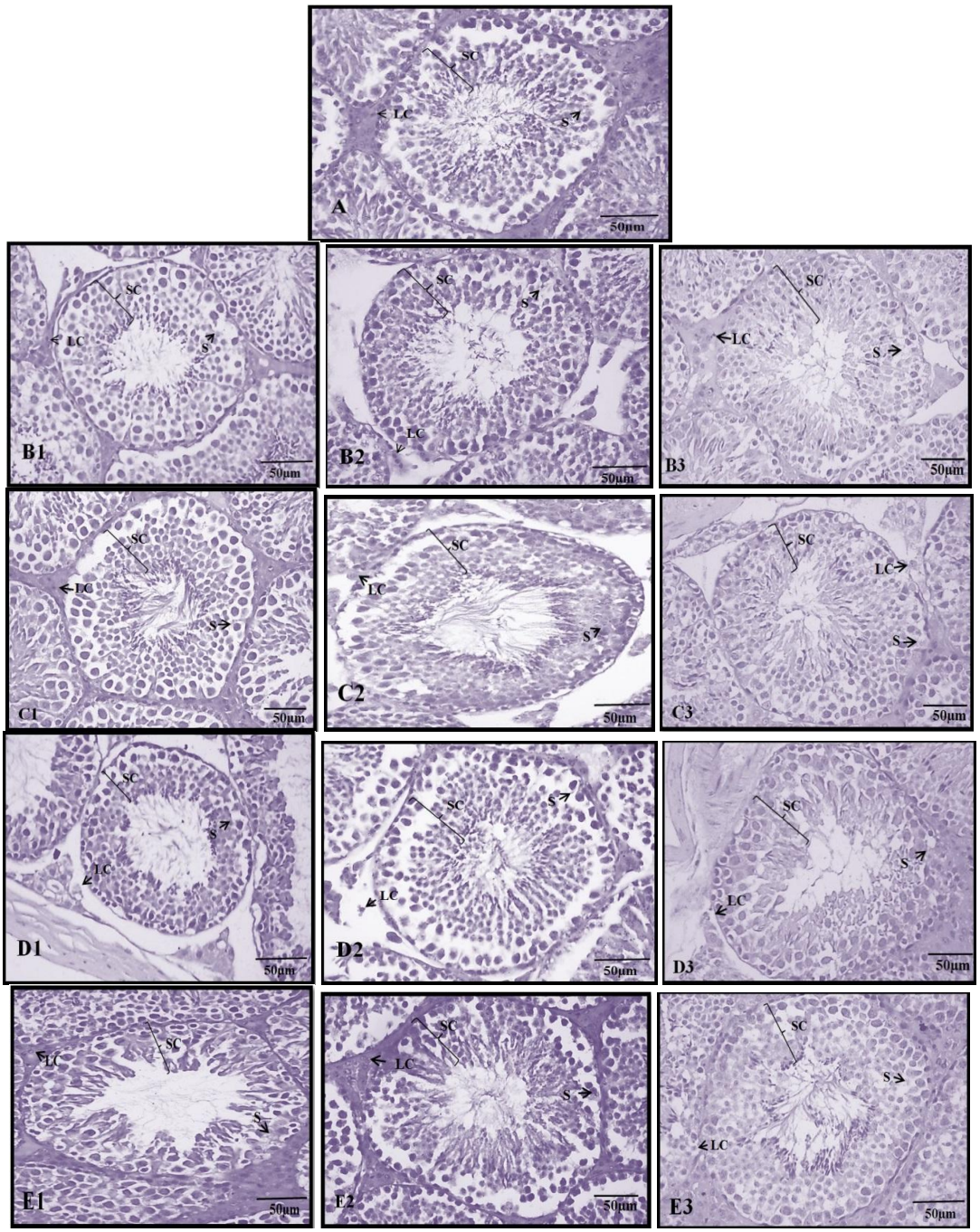

Fig. 1: Effect of AB, CC and PB ethanolic extracts on the testis histological features by haematoxylin and eosin staining (Scale bar: 50µm). A: control, B1: 50mg/kg b. wt AB, B2: 50mg/kg b. wt CC, B3: 50mg/kg b. wt PB, C1: 125mg/kg b. wt *AB*, C2: 125mg/kg b. wt CC, C3: 125mg/kg b. wt PB, D1: 500mg/kg b. wt AB, D2: 500mg/kg b. wt CC, D3: 500mg/kg b. wt PB, E1: 1000mg/kg b. wt AB, E2: 1000mg/kg b. wt CC, E3: 1000mg/kg b. wt PB, SC: spermatogenic cells, LC: Leydig cell, S: Sertoli cell.

Discussion:

In all of the concentrations under study, AB plants extract possessed detrimental effect to most of the test parameters as compared to the other two plants. Previous preliminary study was done by [13] on the antifertility activity of AB fruits juice and the result showed that the different dosages of AB fruits extract reduced the fertility rate in mice by 50% as compared to the control. Meanwhile, CC ethanolic extracts did increase most of the mean test parameter involved. Knowing that this plant is among those local plants that exhibit potent anti-oxidant properties [14], CC ethanolic extract is able to enhance sperm quality as reported in [12]. There are mixed outcomes for PB extract in all different concentrations for the test parameter analysis. Regardless no study reported on the effects of PB extract to male fertility. However the study on acute toxicity of PB on mice

was conducted and the outcome regarded this plant as safe in the experimental mice [15], which may suggest that this plant could aid in male fertility especially at 125/kg b.wt concentration. However, it is advised more experimental designs should be carried out to sanction this hypothesis. The presence of Leydig and Sertoli cells inside the seminiferous tubules of mice testis shows the relation of plasma testosterone and the internal structures of the seminiferous tubules. The interaction between Leydig and Sertoli cells are important for the normal intratesticular testosterone production [16]. The extract of AB has very low concentration of plasma testosterone as compared to control, CC and PB treatment groups. As testosterone is the hormone responsible in male reproductive system [17], the prolonged low on blood testosterone levels is able to cause spermatogenic deficiencies [8], which detected in width of spermatocyte and spermatogonia layers treated with different concentrations of AB extract. The deficiencies on the spermatogenic cells affected the quality of sperm produced and thus, attenuating the capability to reproduce. As AB, CC and PB are enriched with various plant secondary compounds with high medical values [5, 18, 19] this could be the reason that testosterone reduction in AB extract did not affect testis architecture severely.

Conclusion:

Consumption of these selected plants as an alternative medicine on male fertility can be considered with the right formulation, as they are able to either enhance or diminish sperm quality at spermatogenesis level and affect the synthesis of testosterone. More profound study is needed to be conducted as to further validate the outcome of this study especially on the molecular-cellular level.

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