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Administration of Bajakah (*Spatholobus littoralis* Hassk) Stem Ethanol Extract Increased the Number of Leydig Cells and Testosterone Levels in Male Wistar Rats (*Rattus Norvegicus*) with Excessive Swimming Activity

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Abstract: ***Background: Swimming activity has been shown to be associated with increased ROS production. ROS can cause aging because it triggers damage to cells, tissues, organs and then leads to death. Research has found that excessive swimming activity leads to apoptosis of Leydig cells and lowers testosterone levels. Bajakah (Spatholobus littoralis Hassk) stem contains flavonoids, polyphenols, tannins, steroids and antioxidants; hence, it can be used to prevent aging caused by excessive swimming. The purpose of this study was to prove that the administration of Bajakah stem ethanol extract increases the number of Leydig cells and testosterone levels in male Wistar rats with excessive swimming activity. Methods: This study was experimental research using a randomized posttest-only control group design. Subjects were 40 male Wistar rats, 3-4 months old, weighing 200 grams and healthy. The control group (20 rats) were given excessive swimming activity and 2 ml aquadest as a placebo, while the treatment group (20 rats) were given excessive swimming activity and Bajakah stem ethanol extract with a dose of 9 mg/200g BW of rats. After 21 days of treatment, testosterone levels were measured using the ELISA (KIT Bioassay Technology Laboratory) method and the number of Leydig cells was examined using the histopathological method with Hematoxylin-Eosin (HE) staining. Data analysis was performed using Independent T-test for the number of Leydig cells and the Mann-Whitney test for the testosterone levels. Results: The mean number of Leydig cells after 21 days of treatment in the control group was 14.6±3.17 cells/field of view, whereas in the treatment group was 38.4±7.50 cells/field of view (p <0.001). Testosterone level in the control group was 3.56 nmol/mL with a minimum value of 3.40 nmol/mL and a maximum value of 3.68 nmol/mL. In the treatment group, the median testosterone level was 6.31 nmol/mL with a minimum value of 4.81 nmol/mL and a maximum value of 6.54 nmol/mL (p <0.001). Conclusion: It can be concluded that the administration of bajakah (Spatholobus littoralis Hassk) stem ethanol extract increased the number of Leydig cells and testosterone levels in male Wistar rats (Rattus norvegicus) with excessive swimming activity***

Keywords: Bajakah stem ethanol extract, Leydig cells, testosterone, excessive swimming activity

1. Introduction

Anti-Aging Medicine (AAM) has been a focus of research in medicine because of its interesting perspective on the aging process. Based on AAM science, aging is considered a disease in general. If the causes of aging processes are well-understood, preventive steps can be taken to prevent, slow down and even reverse the aging process. Aging can be defined as a decrease in the physiological function of organs which then causes various pathological conditions and leads to death.[1]

Aging of the reproductive system includes a decrease in the number of Leydig cells and a decrease in testosterone levels. Research showed that the number of Leydig cells also decreases with age.[2] Serum testosterone levels have been shown to gradually decrease with aging. This age-related decline has been confirmed in several cross-sectional and longitudinal studies and it is known to be the result of dysfunction in the hypothalamus-pituitary-testis axis.[3] Aging is associated with decreasing total serum testosterone, increased levels of sex hormone-binding globulin (SHBG), and decreased free testosterone.[4] In a cross-sectional study, it was found that serum testosterone concentrations decrease by 0.4-2% per year after passing the age of 30.[5]

Reproductive system aging can be occurred both naturally (chronological aging) and accelerated by many factors (biological aging). One of the biological aging factors is excessive swimming.[6] Swimming activity has been shown to be related to the production of reactive oxygen species (ROS).[7] The main sources of endogenous ROS include mitochondria, NADPH oxidase (NOX), and xanthine oxidase (XO).[8-9] Study has shown that oxidative stress can cause damage to Leydig cells[10], through the induction of the intrinsic and extrinsic apoptotic pathway.[11] Leydig are testosterone-producing cells in the testes. Hence, decreasing testosterone is the result of a significant decrease in the ability of Leydig cells to produce testosterone or a decreasing number of Leydig cells.[10,12]

A decrease in testosterone has been associated with the aging process[1], so efforts to prevent a decrease in testosterone levels and prevent a decrease in the number of Leydig cells caused by excessive swimming activity can be categorized as an Anti-Aging Medicine effort. Bajakah (*Spatholobus littoralis* Hassk) stem extract contains flavonoids, phenol, tannins, and antioxidants. However, until now, scientific research on the Bajakah plant was limited to its qualitative content and its effect on the wound healing process. Its effects on the pathology of excessive swimming

have never been reported. This study aimed to prove that the administration of Bajakah stem ethanol extract increases the number of Leydig cells and testosterone levels in male Wistar rats with excessive swimming activity.

2. Methods

This study was experimental research using a randomized posttest-only control group design. This research was conducted at the Integrated Biomedical Laboratory Unit, Faculty of Medicine, Udayana University. The Bajakah stem ethanol extract was prepared and analyzed phytochemically at the Faculty of Agricultural Technology, Udayana University. Steroid level analysis was carried out at the Integrated Research and Testing Laboratory, Gadjah Mada University. The examination of the number of Leydig cells was carried out at the Pathology Diagnostic Center Laboratory, Denpasar. Subjects were 40 male Wistar rats, 3-4 months old, weighing 200 grams, and healthy. The control group (20 rats) were given excessive swimming activity and 2 ml aquadest as a placebo, while the treatment group (20 rats) were given excessive swimming activity and Bajakah stem ethanol extract with a dose of 9 mg/200g BW of rats. After 21 days of treatment, testosterone levels were measured using the ELISA (KIT Bioassay Technology Laboratory) method and the number of Leydig cells was examined using the histopathological method with Hematoxylin-Eosin (HE) staining. Data analysis was performed using *Independent T-test* for the number of Leydig cells and the *Mann-Whitney* test for the testosterone levels.

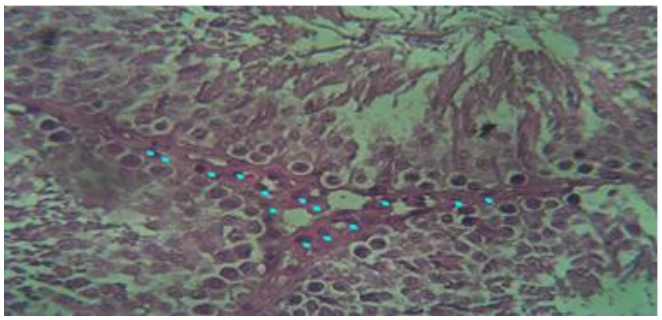
3. Results

The number of Leydig cells and testosterone levels were tested for normality by the *Shapiro-Wilk* test. Testosterone levels in the treatment group were not normally distributed ($p < 0.05$), while other data were normally distributed ($p > 0.05$). Because there is abnormal testosterone data, it was transformed using the square root method and tested again for normality. However, the testosterone data in the treatment group still had an abnormal distribution ($p < 0.05$). Furthermore, homogeneity was tested using *Levene's* test. The data variant for the number of Leydig cells was not homogeneous ($p < 0.05$), while the variant of the testosterone levels was homogeneous ($p > 0.05$) (Table 1).

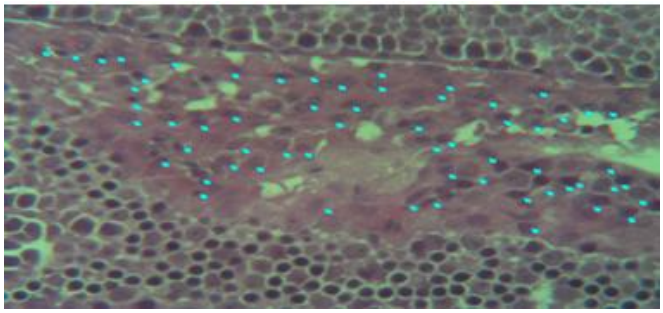
Table 1: Normality and Homogeneity Test

Variable	*Group*	*Normality*		*Homogeneity*	
		*p**	*Description*	*p***	*Description*
Leydig Cells (cells/field of view)	Control	0.103	Normal	0.005	Not Homogenous
	Treatment	0.133	Normal		
Testosterone (nmol/ml)	Control	0.758	Normal	0.153	Homogenous
	Treatment	0.000	Not Normal		
SQRT_ Testosterone	Control	0.744	Normal	Not calculated	
	Treatment	0.000	Not Normal		
Note: *p* = Shapiro-Wilk test, p**= Levene's test.*					

The mean number of Leydig cells after 21 days of treatment in the control group was 14.6±3.17 cells/field of view, whereas in the treatment group was 38.4±7.50 cells/field of view. Analysis using an independent *t*-test revealed that this difference was significant ($p < 0.001$). Testosterone level in the control group was 3.56 nmol/mL with a minimum value of 3.40 nmol/mL and a maximum value of 3.68 nmol/mL. In the treatment group, the median testosterone level was 6.31 nmol/mL with a minimum value of 4.81 nmol/mL and a maximum value of 6.54 nmol/mL. Analysis using *Mann-Whitney* test revealed that testosterone difference was significant ($p < 0.001$).



(a)



(b)

Figure 1: Histological examination of Leydig cells on testicular tissue stained with HE (magnification 400x). (A) Leydig cells of the control group, and (B) Leydig cells of the treatment group. It was apparent that the number of Leydig cells in the treatment group was higher than that of the control group.

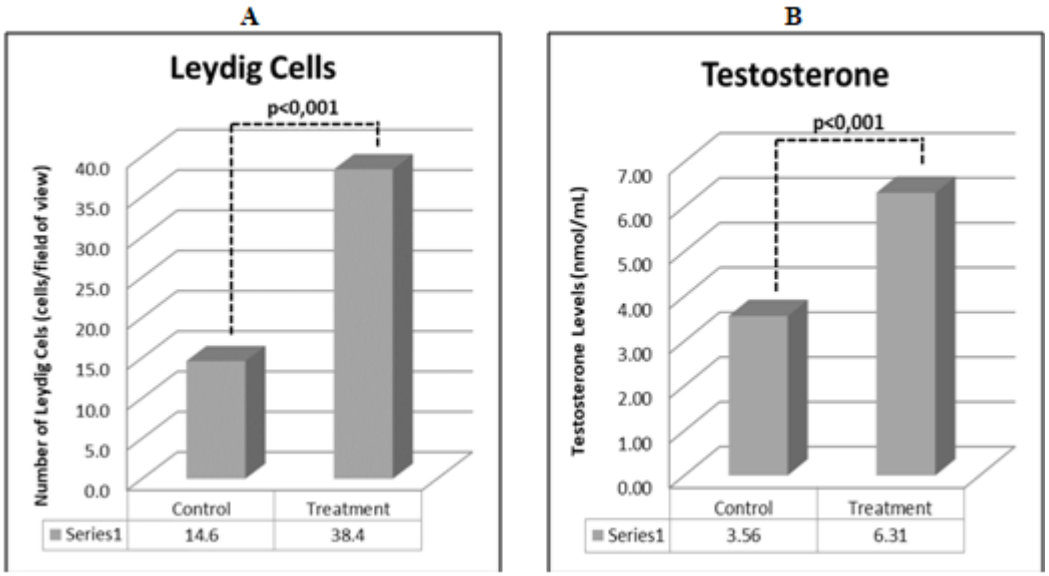


Figure 2: Graphical comparison of the: (A) Leydig cells number and (B) testosterone levels between the control and the treatment group

Next, Correlation analysis was performed using the Spearman nonparametric test because the testosterone data were abnormal. The correlation test results showed a strong relationship between the number of Leydig cells and testosterone levels (p <0.001; Table 2). These results further prove that the pathophysiological effect of the Bajakah stem extract on increasing testosterone levels is mediated and closely correlated with the increase in the number of Leydig cells.

Table 2: Correlation of Leydig Cell Number and Testosterone Levels

Variable	Coefficient correlation	p
Leydig Cells	0.719	<0,001
Testosterone		
Note: p= signifikansi *Spearman*		

4. Discussion

Effect of Bajakah Stem Ethanol Extract on Leydig Cells and Testosterone

The results of this study indicate that the administration of Bajakah stem ethanol extract increased the number of Leydig cells and testosterone levels in male Wistar rats with excessive swimming activity. Based on the above results, Bajakah stem ethanol extract can be used as an Anti-Aging Medicine, especially in aging caused by excessive swimming activity.

Bajakah stem ethanol extract contains active compounds and antioxidants that can prevent the pathological effects of free radicals that increase due to excessive swimming activity. The content of bioactive compounds in this extract includes flavonoids, saponins, steroids, terpenoids, tannins, and phenols.[13] The results of phytochemical examinations in this study showed the content of flavonoids, total phenols, tannins, antioxidants and steroids (Table 3).

Table 3: Phytoconstituent in Bajakah Stem Ethanol Extract compare to other extracts

No	Extract	Flavonoid (mg/100g QE)	Phenol (mg/100g GAE)	Antioxidant (mg/L)	Reference
1	Bajakah stem	79.739,70	14.952,12	63.141,06	This study
2	Soursop leaves	476,54	1500,95	10.981,13	14
3	Red ginger	2.311,93	14.597,05	52.300,05	15
4	Sanrego bark	5.354,85	1.388,95	3.956,17	16

In comparison to other extracts that have been proven to increase the number of Leydig cells, the extract in this study possesses a higher phytochemical component (Table 3). Additionally, the results of this study are similar to the results of research conducted previously using different active extract ingredients. Soursop leaves extract has been shown to increase the number of Leydig cells in diabetic mice.[14] In that study, the number of Leydig cells in the control group was 29.57±7.46 cells/field of view, while in the group treated with soursop leaf extract was 38.13±7.97 cells/field of view. When compared with that study, the treatment of excessive swimming activity in this study can reduce the number of Leydig cells lower than the induction of diabetes (14.5±2.60 vs 29.57±7.46 cells/field of view). Then the treatment of Bajakah stem extract and soursop leaf extract can both increase the number of Leydig cells to almost the same extent (38.4±7.50 vs 38.13±7.97 cells/field of view).

Besides, red ginger extract can inhibit the decrease in the number of Leydig cells in male Wistar rats exposed to cigarette smoke.[15] In that study, cigarette smoke in the control group caused the average number of Leydig cells to be 29.23±6.26 cells/field of view. This also showed that excessive swimming activity reduces the number of Leydig cells lower than exposure to cigarette smoke. After being given red ginger extract to the treatment group, the number of Leydig cells was 45.88±7.66 cells/field of view. When compared to the difference between the control group and the treatment group, red ginger in Suriati's (2018)[15] study had a Leydig cell difference of about 16.65 cells/field of

view, whereas in this study the difference between the Bajakah stem extract group and the control group was 23.8 cells/field of view. This indicates that the Bajakah stem extract is more effective than the red ginger extract.

Oral administration of sanrego bark extract has also been shown to increase the number of Leydig cells and testosterone levels in old male Wistar rats.[16] Those study used 18 months old rats (the human equivalent of 45 years), the mean number of Leydig cells in the control group was 18.17±6.69 cells/field of view. In this study, the average number of Leydig cells after excessive swimming activity treatment was 14.5±2.60 cells/field of view, indicating that excessive swimming activity can induce the aging of Leydig cells to reach a lower number compared to rats aged 18 months.

The physiological mechanisms of each active compound to increase the number of Leydig cells and testosterone levels in this study are as follows: (1) Phenol is a bioactive compound that can reduce the adverse effects of free radicals by inhibiting lipid peroxidation[17], thereby inhibiting the swimming-induced Leydig cells apoptosis and decreasing testosterone. (2) Flavonoids can transfer electrons to free radicals, bind to metal catalysts, activate enzymatic antioxidants, reduce α-tocopherol radicals, and inhibit oxidase[18], thus inhibiting Leydig cell damage and testosterone reduction. (3) Tannins can bind to metals, especially iron, and directly neutralize free radicals.[19] (4) Steroids can increase endogenous testosterone in animals, have anabolic and androgenic effects, through activation of endogenous testosterone production.[20]

In this study, the dose of Bajakah stem ethanol extract which was given to the rats was 9 mg/200g BW. The conversion ratio from rats weighing 200 g to humans weighing 70 kg is 56. If converted to humans, the dose of Bajakah stem ethanol extract becomes = 9 mg x 56 = 595 mg or 0.6 grams/70 kg BW for humans.

Bajakah Stem Ethanol Extract and Anti-Aging Medicine

A decrease in testosterone has been linked to the aging process[1], so efforts to prevent a decrease in testosterone levels and the number of Leydig cells caused by excessive swimming can be categorized as an Anti-Aging Medicine. In this study, it has been proven that Bajakah stem ethanol extract which contains flavonoids, total phenols, tannins, antioxidants, and steroids can increase testosterone levels. Hence, Bajakah stem ethanol extract can be used as an Anti-Aging Medicine. However, a lot of further research needs to be done including examining the effect of the Bajakah stem ethanol extract on the characteristics of aging in addition to testosterone levels, testing its toxic effects, and clinical trials in humans before it can finally be used by the public.

5. Conclusion

It can be concluded that the administration of bajakah (*Spatholobus littoralis* Hassk) stem ethanol extract increased the number of Leydig cells and testosterone levels in male Wistar rats (*Rattus norvegicus*) with excessive swimming activity. However, it is necessary to do further research with a more comprehensive molecular approach to understand the mechanism of action of the bajakah stem ethanol extract. Additionally, a toxicity test is required to ensure the safety of this extract.

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