

Oral Administration of Ethyl Acetate Extract of Sanrego (*Lunasia amara Blanco*) Wood Increased the Number of Leydig Cells and Testosterone Levels in Old Male Wistar Rats (*Rattus norvegicus*)

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Abstract: Background: In older men, a decrease in various hormones such as testosterone occurs. It results in andropause. A decrease in testosterone levels due to aging is associated with a decrease in the number of Leydig cells. The efforts to increase the number of Leydig cells and testosterone levels is an Anti-Aging Medicine step. Ethyl acetate extract of Sanrego (*Lunasia amara Blanco*) wood contains alkaloids, flavonoids, tannins, steroids, antioxidants, terpenoids, polyphenols, and steroids. The aim of this study was to prove that oral administration of ethyl acetate extract of Sanrego (*Lunasia amara Blanco*) wood increase the number of Leydig cells and testosterone levels in old male Wistar rats (*Rattus norvegicus*). Methods: This study was true experimental research using randomized posttest-only control group design. Subjects were 36 rats (*Rattus norvegicus*), Wistar strain, male, aged 18 months and weighing 275 grams. Rats were divided into two groups, the control group was given aquabidest 2 ml as a placebo using intragastric forced-feeding once a day and the treatment group was given ethyl acetate extract of Sanrego Wood of 16.5 mg/275gr.BW of rats dissolved in 2 ml aquabidest using intragastric forced-feeding once a day. After 21 days of treatment, blood was drawn on the right or left medial canthus sinus orbitalis for the examination of testosterone levels (ELISA method) and testes were taken for examination of Leydig cell numbers (histopathological method). Results: The results showed that the average number of Leydig cells in the control group was 18.17 ± 6.69 cells/field of view and the treatment group was 31.63 ± 10.20 cells/field of view with a p -value <0.001 . The testosterone levels in the control group were 12.72 ± 0.55 nmol/L and the treatment group was 18.22 ± 0.63 nmol/L with a p -value <0.001 . Conclusion: It can be concluded that the oral administration of ethyl acetate extract of Sanrego (*Lunasia amara Blanco*) wood increased the number of Leydig cells and testosterone levels in old male Wistar rats (*Rattus norvegicus*).

Keywords: Sanrego wood ethyl acetate extract, Leydig cells, male Wistar rats

1. Introduction

Advancing age remains an unsolved problem because the aging process is always associated with the emergence of various kinds of degenerative diseases and disabilities. Chronological aging does not necessarily mean to experience helplessness. By growing old, humans in general have the impression of sickness, which underlies various efforts to stay young. However, these efforts are often superficial.

To overcome this challenge, advances in medicine have led to better understanding of human aging process so that these processes can be inhibited, prevented and reversed to its original structure and function. New branch of medicine that focus in this area is called the Anti-Aging Medicine. It treats the underlying causes of aging and aims at alleviating any age-related ailment. Its goal is to extend the healthy lifespan of humans having youthful characteristics. (Pangkahila, 2011).

There are several theories of aging, one of which is the theory of hormones (Jin, 2010). This theory is based on the role of various hormones in the function of organs. As a person ages, the body is only able to produce fewer hormones, disrupting various physiological function. Hormone replacement therapy (HRT) is proven to help restoring the body's hormonal function so that it can slow

down or even reverse the aging process (Jin, 2010; Pangkahila, 2011; Samaras et al., 2014).

Testosterone is a steroid hormone which function as anabolic (growth) and androgenic (maturation of sexual organs) factors (Almaman, 2018). The level of testosterone decrease at the age of 35 years and various diseases will arise when the testosterone levels are inadequate, such as loss of muscle mass, increased fat mass, heart disease, osteoporosis, arthritis, hypogonadism, depression, mood disorders, erectile dysfunction, loss of libido, etc (Pangkahila and Wong, 2015).

In men, more than 95% of androgen hormones are produced in the testes by Leydig cells. Decreased levels of testosterone due to aging are associated with a decrease in the number and activity of Leydig cells. Research shows that aging decreases the ability of Leydig cells to respond to LH (Midzak et al., 2009). Other studies suggest that the number of Leydig cells decreases due to aging due to increased oxidative stress that cause a damage on Leydig cells (Chen et al., 2009).

Decrease in testosterone, the number of Leydig cells have been widely associated with the aging process (Pangkahila, 2011). Thus, efforts to prevent the decline in testosterone and the number of Leydig cells caused by aging can be used as an Anti-Aging Medicine. Supplementation of natural ingredients which have the effect of inhibiting the decrease in testosterone and the number of Leydig cells can prevent

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the appearance of phenotypes and symptoms of aging, especially in men. HRT become increasingly popular (Pangkahila, 2017); however, it causes several side effects (Agarwal et al., 2018). In men, testosterone replacement therapy causes polycythemia, peripheral edema, heart and liver dysfunction, prostate cancer, infertility and others (Osterberg et al., 2014).

Several medicinal plants have been studied to increase testosterone levels in old experimental animals. *Eurycoma longifolia* (Tambi et al., 2012), *Pimpinella alpine molk* (Usmiati and Yuliani, 2010), *Eurycoma longifolia* root (Taufiqurrachman, 2012; Ibrahim, 2016), and *Euchresta horsfieldii* seed (Silalahi, 2016) have been scientifically proven to increase testosterone levels in old rats.

Sanrego wood emerges for its androgenic activity. It has been reported for its aphrodisiac activity on Wistar rats to improve climbing and coitus stages (Katno and Haryanti, 2009). Sanrego wood contains steroids, phenolics, saponins, alkaloids and coumarin (Hasnaeni and Aminah, 2019), calcium oxalate, formic acid, steroids and glucosides (Kanto and Haryanti, 2009; Zubair and Subehan, 2010). Research comparing the effects of *Pimpinella purwatjan Molkenb*, *Piper retrofractum L*, *Talinum paniculatum Gaertn*, *Litsea cubeba L* and Sanrego (*Lunasia amara*) showed that Sanrego has the highest aphrodisiac activity (Rahmawati, 2012).

Apart from the evidence showing the aphrodisiac effect of Sanrego wood extract, there is no evidence that shows its Anti-Aging property, especially those that related to Leydig cells and testosterone. The aim of this study was to prove that oral administration of ethyl acetate extract of Sanrego (*Lunasia amara Blanco*) wood increase the number of Leydig cells and testosterone levels in old male Wistar rats (*Rattus norvegicus*). The dose used in this study was based on the results of preliminary research that has been done. The 16.5 mg / 275grBB dose was a dose that effectively increase the number of Leydig cells and testosterone levels, so this dose was used in this study (Gomes, 2020).

2. Methods

This study was true experimental research using randomized posttest-only control group design. Subjects were 36 rats (*Rattus norvegicus*), Wistar strain, male, aged 18 months and weighing 275 grams. Rats were divided into two groups, the control group was given aquabidest 2 ml as a placebo using intragastric forced-feeding once a day and the treatment group was given ethyl acetate extract of Sanrego Wood of 16.5 mg/275gr.BW of rats dissolved in 2 ml aquabidest using intragastric forced-feeding once a day. After 21 days of treatment, blood was drawn on the right or left *medial canthus sinus orbitalis* for the examination of testosterone levels (ELISA method) and testes were taken for examination of Leydig cell numbers (histopathological method).

3. Results

The phytoconstituent analysis revealed that qualitatively that ethyl acetate extract of Sanrego (*Lunasia amara*) wood contains alkaloids, flavonoids, tannins, steroids,

triterpenoids, polyphenols, and antioxidants. The results of quantitative examination were shown in Table 1.

Table 1: Phytochemical screening of Sanrego (*Lunasia amara*) wood

Screening	Presence	Amount
Alkaloids	(+)	None
Flavonoids	(+)	5354.85 mg/100g
Tannins	(+)	1245.74 mg/100g
Steroids	(+)	2.70% w/w
Triterpenoid	(+)	None
Polyphenolic	(+)	1388.95 mg/100g
Saponins	(-)	(-)
Antioxidant capacity	(+)	3956.17 mg/L
IC50%	(+)	341.21 ppm

(+) = presence, (-) = absence, None= not measured

The *in vivo* results showed that the average number of Leydig cells in the control group was 18.17 ± 6.69 cells/field of view and the treatment group was 31.63 ± 10.20 cells/field of view with a p-value <0.001. The testosterone levels in the control group were 12.72 ± 0.55 nmol/L and the treatment group was 18.22 ± 0.63 nmol/L with a p-value <0.001 (Table 2, Figure 1 and 2).

Table 2: Comparison of Leydig Cells and Testosterone Levels

Variable	Group	n	Mean±SD	p
Leydig cells (cells/field of view)	Control	18	18.17±6.69	<0.001
	Treatment	18	31.63±10.20	
Testosterone (nmol/L)	Control	18	12.72±0.55	<0.001
	Treatment	18	18.22±0.63	

n= number of samples; SD= standard deviation; p= Significance

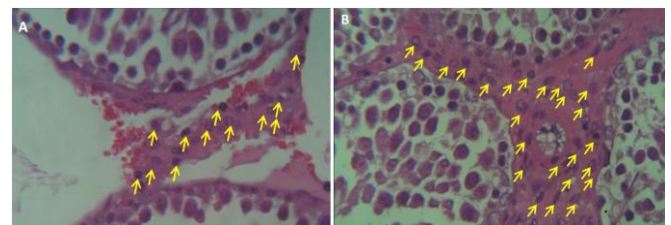


Figure 1: Histology of Leydig Cells in Interstitium. (A) Control Group and (B) Treatment Group. The number of Leydig cells in the treatment group (B) was higher than the control group (A). The arrows indicate Leydig cells. (H&E, 400x)

Figure 2: Graphical Comparison of (A) Leydig Cell Numbers and (B) Testosterone Levels, between group.

4. Discussion

Effects of Sanrego Wood Extract on Leydig Cell Numbers

The results of this study indicated that oral administration of Sanrego wood extract increase the number of Leydig cells in old male Wistar rats. Old rats have a decline number of Leydig cells as a result of increased oxidative stress that damages Leydig cells and decreases the capacity of Leydig cell progenitors to differentiate (Chen et al., 2009). Lower Leydig cells is a symptom of aging, so an effort to increase the number of Leydig cells in older organisms is one of Anti-Aging Medicine's efforts.

The mechanism of increasing Leydig cell number by Sanrego wood extract might be due to its phytochemical content. Alkaloids have previously been shown to increase total Leydig cell and its nucleus size (Vyas and Raval, 2016). Other studies have shown that alkaloids increase the number of Leydig cells (Chen et al., 2018). The molecular mechanism underlying these effects is that alkaloids activate the extracellular signal-regulated kinases $\frac{1}{2}$ pathway (ERK1/2) and the Akt that participates in the development of Leydig cells (Yamashita et al., 2011; Chen et al., 2018).

Flavonoid effect on Leydig cells are mediated by the hypothalamus-pituitary-testis axis, the inhibitory effect of 5-alpha reductase enzymes, and inhibition of aromatase which all have an impact on increasing testosterone levels (Azarneoshan et al., 2009; Ye et al., 2009; Zohre et al., 2015). Testosterone increases the number of Leydig cells and induce cell proliferation by activating proliferating cell nuclear antigen (PCNA) and cyclin D3 (Akingbemi et al., 2014).

Tannins can activate mitogen activated protein kinase (MAPK) (Yesudas et al., 2012), which can induce the proliferation and differentiation of Leydig cells; thus, improve the number of Leydig cells in the interstitial tubules (Sherrill et al., 2010).

Steroids are able to increase the number of Leydig cells which decreases due to aging (Widhiantara, 2010). The mechanism involved is through cAMP activation which then activates the Cyclic AMP response element binding protein (CREB). Activated CREB then induces steroidogenic acute regulatory protein (StAR) and GATA binding factor 4 (GATA-4) which then initiates testosterone synthesis (Yu et al., 2018).

Effects of Sanrego Wood Extract on Testosterone Levels

In this study, administration of ethyl acetate extracts of Sanrego (*Lunasia amara*) wood also can increase testosterone levels. This result could be due to the effect of Sanrego wood extract which increased the number of Leydig cells. Because Leydig cells are the main testosterone-producing cells, this also has a direct impact on increasing testosterone levels (Pangkahila, 2019).

Additionally, alkaloids can increase testosterone secretion by Leydig cell resulting on increase serum testosterone levels (Vyas and Raval, 2016). Research have shown that the increase in testosterone due to alkaloids is due to increased activity of CYP11A1 and CYP17A1 (Chen et al., 2018). Although alkaloids do not increase serum LH levels, a significant increase in LHCGR activates ERK1/2 (Chen et al., 2018).

Flavonoids can increase testosterone levels, although do not affect LH and FSH. Flavonoids can inhibit the enzyme 5-alpha reductase, which converts testosterone to dihydrotestosterone and increases testosterone levels (Azarneoshan et al., 2009). It also competitively binds to aromatase, reduces enzymatic expression, inhibits the conversion of testosterone to estrogen and consequently increases testosterone levels (Ye et al., 2009).

Tannins seem to be able to increase testosterone levels via induction of Leydig cells proliferation due to MAPK activation (Yesudas et al., 2012). And until now there has been no research that shows the direct effects of tannin on the activity of testosterone synthesis in Leydig cells or the adrenal cortex.

Steroids can bind to androgen receptors (AR) and cause physiological effects similar to natural testosterone produced endogenously. Steroids can activate CREB and StAR and GATA-4 which then initiates testosterone synthesis (Yu et al., 2018).

Sanrego Wood Extract and Anti-Aging Medicine

In older men, various hormones including testosterone is decline. These results in loss of libido, erectile dysfunction, depression, decreased cognitive abilities, lethargy, osteoporosis, and loss of muscle mass and strength. These symptoms are collectively known as the andropause, or Androgen Deficiency of the Aging Male (ADAM), or Partial Androgen Deficiency in the Aging Male (PADAM). This syndrome tends to worsen as advancing age (Pangkahila, 2011; McBride et al., 2016). In men, more than 95% of androgen hormones are produced in the testes by Leydig cells. Decreased levels of testosterone due to aging are also associated with a decrease in the number and activity of Leydig cells. Research showed that aging decreases the ability of Leydig cells to respond to LH so that testosterone production also decreases (Midzak et al., 2009). Other studies suggest that the number of Leydig cells decreases due to aging because it increases oxidative stress that damages Leydig cells, as well as decreasing the capacity of Leydig cell progenitors to differentiate (Chen et al., 2009).

Decrease in the number of Leydig cells and testosterone levels have been widely associated with the aging process (Pangkahila, 2011). Hence, efforts to prevent the decrease in testosterone and the number of Leydig cells caused by aging can be used as an Anti-Aging Medicine (AAM) modality. The results of this study showed that oral administration of ethyl acetate extract of Sanrego (*Lunasia amara Blanco*) wood increased the number of Leydig cells and testosterone levels in old male Wistar rats (*Rattus norvegicus*). Therefore, the administration of Sanrego wood extract is an AAM effort.

There have been several medicinal plants that have been studied at the Anti-Aging Medicine Program, Udayana University to increase testosterone levels in old experimental animals. Ethanol extract *Trigonella foenum graecum* seed has been proven to increase testosterone in old rats and contains a total phenol of 25.19 mg/100g, flavonoids of 1608.43 mg/100g, tannins of 4689.03 mg/100g, antioxidant capacity of 107.5 mg/L, and phytotestosterone of 7.09 pg/g (Herliane, 2018). In this study, Sanrego wood ethyl acetate extract contains total phenol of 1388.95 mg/100g, flavonoids of 5354.85 mg/100g, tannins of 1245.74 mg/100g, antioxidant capacity of 3956.17 mg/L and IC50 of 341.21 ppm. Sanrego wood extract contains phenols, flavonoids, antioxidants, and steroids / phytotestosterone higher than that of *Trigonella foenum graecum* seed extract.

5. Conclusion

It can be concluded that the oral administration of ethyl acetate extract of Sanrego (*Lunasia amara Blanco*) wood increased the number of Leydig cells and testosterone levels in old male Wistar rats (*Rattus norvegicus*). For suggestion, it is necessary to compare the effectiveness of Sanrego wood extract with other compounds such as extract of *Eurycoma longifolia*, *Pimpinella alpina molk*, or *Trigonella foenum graecum* simultaneously to find out which natural compound are the best in preventing male aging. Clinical research in humans is also prerequisite to study the Anti-Aging potential Sanreo wood extract in elderly.

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