Potential of Leaves of Water Mimosa (*Neptunia plena*) in Increasing Spermatogenesis Activity of Male Rat

Rusmiati¹, S G Sari¹, C R F Budyarti²

¹Faculty of Mathematics and Natural Sciences, Biology Department, Lambung Mangkurat University, Banjarbaru

²Students of Biology Department, Faculty of Mathematics and Natural Sciences, University of Lambung Mangkurat, Banjarbaru

*Corresponding author: rusmiati39@ymail.com

**Abstract.** *Neptunia plena* is one of a plant that includes water mimosa. The leaves of this plant contain steroids, saponins, flavonoids, and alkaloids that may increase testosterone hormone. One function of the testosterone is to increase the activity of spermatogenesis. The purpose of this study was to obtain scientific evidence about the potential of *N. plena* leaves in increasing the spermatogenesis activity of male rats to get natural fertility enhancer ingredients. This study used a Completely Randomized Design (CRD) with a negative control treatment (Na-CMC 0.5%), and the groups were treated with ethanol extracts of *N. plena* leaves each at a dose of 87.5; 175; and 350 mg kg⁻¹ body weight rat. Male rat was treated orally 2 mL 200-250 g⁻¹ body weight for 14 days. On day 15, all rats were sacrificed by neck dislocation, dissected, testicular organs were taken, weighed, and microanatomy preparations made using paraffin and Hematoxylin-Eosin staining. The parameter can be observed from the spermatogenesis activity, such as testicles weight, testicles tubules seminiferous diameter, and the number of spermatogenic cell layers. The result showed that *N. plena* leaves had the potential to increase the spermatogenesis activity of male rats.

1. Introduction

Water mimosa (*Neptunia* spp) is one of the floras that grows and adapts well in swamps. The morphological characteristics of *Neptunia* spp are like the *puteri malu* (*Mimosa pudica*). The striking difference between the two plants is that the *puteri malu* plants have thorny stems, purplish-pink flowers, and grow on land, while *Neptunia* spp are hollow and non-thorny stems, yellow flowers and grow in damp places, by the river or in swamps. The term water mimosa refers to the species of *Neptunia oleracea* and *Neptunia plena* [1]. For most people in South Kalimantan, the two types of plants are known as weeds because of their rapid development, and their foul stems play a role in the silting of swamps (inhibiting drainage), reducing O₂ in the waters. *Neptunia oleracea*, in Hulu Sungai Utara, is referred to as *susupan bini*, whereas *Neptunia plena* is referred to as *susupan laki* or *susupan gunung*.

One of the water mimosas examined was *N. plena*. This plant is herbaceous, perennial, terrestrial, or semi-aquatic, upright stems, and rarely spread [2]. *Neptunia plena* leaf is believed by a group of
people in the Pal Batu Village, Paminggit District, Hulu Sungai Utara Regency to be able to increase male libido (sexual arousal) although the empirical data has not been strengthened by scientific data. [3] found that N. plena leaves contain steroids, saponins, flavonoids, and alkaloids. According to [4], these compounds have the potential to be aphrodisiac because they can increase libido (sexual arousal). The increased libido after the administration of the compound allegedly because the increase of testosterone level. Hence, the plant is potential to increase the testosterone. Besides its function to increase libido, testosterone also increases spermatogenesis activity in the testicular seminiferous tubules, which stimulates the growth of spermatogonia, development of spermatocytes into spermatids [5], as well as the differentiation of spermatids into spermatozoa [6]. Therefore, it is necessary to discuss the testicular seminiferous tubules as the site of spermatogenesis.

An increase in spermatogenesis activity means that it will increase the number of spermatozoa, then this plant also has the potential to increase fertility. This is interesting to study, considering there is no information about the potential of this plant as a fertility enhancer. If this is applied to humans, it helps infertility couples in their efforts to have children. Male infertility is an issue that needs important attention and treatment because it can save the family [7]. The problem is whether the extract of N. plena leaves has the potential to increase the activity of spermatogenic cells in the testicular seminiferous tubules.

The purpose of this study was to obtain scientific evidence about the potential of N. plena leaves in increasing the spermatogenesis activity of male rats. The results of this study are expected to contribute to improve commercial value of N. plena, which has only been known as weeding and silting of swamps.

2. Research Methods
This research was conducted in the Biology and Chemistry Laboratory of FMIPA ULM Banjarbaru, with the following stages.

2.1. Sampling
N. Plena leaf samples were obtained from Mandar Sari Village, Kertak Hanyar District, Banjar Regency, South Kalimantan.

2.2. Extract making
Young N. plena leaves were washed thoroughly with running water and then dried in the open air. After drying, the leaves blended with a blender, and filtered until a homogeneous powder was obtained. Every 25 g of N. plena leaf powder was extracted with 200 mL of methanol p.a. using a soxhlet at 40-50 °C. The extract was concentrated using a rotary evaporator at a heating temperature of 40 °C [8], and then waterbathed at 40-45 °C until a thick extract with constant weight was obtained.

2.3. The extract making of ethanol suspension of N. plena leaves
Suspension was made by mixing extract of ethanol of N. plena leaves and suspending agent – Na-CMC 0.5%.

2.4. Grouping and treatment of test animals
This study used a Completely Randomized Design (CRD), which was divided into four groups. Each group was repeated five times. The treatments were as follows:
- The group given 0.5% Na-CMC as a negative control (KO)
- The group was given ethanol extract of N. plena leaves 87.5 mg kg⁻¹ bw rat (P1)
- The group was given ethanol extract of N. plena leaves 175 mg kg⁻¹ bw rat (P2)
- The group was given ethanol extract of N. plena leaves 350 mg kg⁻¹ bw rat (P3)

Dosage based on preliminary studies (orientation) using the mice.

The treatment was given to rats given orally once a day in the morning as much as 2 mL 200-250 g bb every day for 14 days. On the 15th day, all rats were killed by neck-dislocation, operated, testicles
organs were taken, weighed, and made into microanatomy preparation using paraffin method and Haematoxilyn-Eosin coloring of 4µ thickness. The testicles preparation was observed under the microscope using 10x40 magnification and documented [9]. The observation was conducted on tubulus seminiferous which was roundly cut and randomly taken.

The calculation was done on three tubuli and for each tubulus four data were taken – upper right, upper left, lower right, and lower left parts, then the average of cell number was calculated. The observed parameters cover the amount of spermatogenic cell layer (spermatogonium, spermatocyte, spermatid, and spermatozoa). The spermatozoa measurement on lumen tubulus following Johnsen in Wardo [10], as follow: "Score 3: if the amount of spermatozoa is full, Score 2: if the amount of spermatozoa is half full, Score 1: if the amount of spermatozoa is a third full or less, until there was no more spermatozoa found in lumen tubulus. The diameter measurement of tubulus seminiferous testis under the microscope was equipped with micrometer of 10x40 magnification. The measurement was conducted by measuring the distance of two opposite points. Those two points were on the limit between membrane basalis. The result of measurement was stated in micrometer unit (µm). The number of measured tubulus was three tubuli of each microanatomy preparation.

2.5. Data analysis
The data collected was quantitative and qualitative form. The quantitative data covered the measurement result of testicles weigh, the amount of spermatogenic cell layer (starts from lamina basalis to lumen, covering spermatogonium, spermatocyte, spermatid and spermatozoa), and the measurement result of tubulus seminiferous diameter on tubuli seminiferi testicles and presented in table. The qualitative data covered the observation of spermatogenic cells and presented in photos.

The quantitative data (testicles weigh and tubulus diameter) were measured statistically using one-way ANOVA with α = 0.05 and continued with Duncan’s Multiple Range Test (DMRT). The quantitative data (spermatogonium cell, spermatocyte cell, spermatide cell, and spermatozoa cell were measured using nonparametric test of Kruskal Wallis, continued with Dunnet test.

3. Results and Discussion

3.1. Results

3.1.1. Analysis of testis weight and diameter of seminiferous tubules.
The mean and standard deviation of testicular weight and diameter of seminiferous tubules of rats treated every day for 14 days is presented in table 1.

| No | Treatment | Mean and standard deviation of testis weight (g) | Mean and standard deviation of seminiferous tubules diameter (µm) |
|----|-----------|--------------------------------------------------|---------------------------------------------------------------|
| 1. | Control (Na-CMC 0.5%) | 1.2750 ± 0.0300 | 281.360 ± 0.5478 |
| 2. | Treatment 1 extract of N. plena leaves 87.5 mg kg⁻¹ bw rat | 1.3275 ± 0.1887 | 351.185 ± 0.5584 |
| 3. | Treatment 2 extract of N. plena leaves 175 mg kg⁻¹ bw rat | 1.3300 ± 0.9737 | 367.800 ± 0.3061 |
| 4. | Treatment 3 extract of N. plena leaves 350 mg kg⁻¹ bw rat | 1.3475 ± 0.0492 | 379.940 ± 0.6284 |

Note: the same letter is not significantly different in the 5% DMRT.

Table 1: The mean and standard deviation of testicular weight and diameter of seminiferous tubules of rats treated with ethanol extract of N. plena leaves every day for 14 days
From table 1, there was no significant difference between the control and all treatment groups on testicular weight. In the diameter of the seminiferous tubules, there was a significant difference between control and treatment groups 3 (high dose), but there were no significant differences between the three treatment groups.

3.1.2. Analysis of the number of spermatogenic cell layer of the testicular seminiferous tubules.
The mean and standard deviation number of spermatogenic cell layers of the rat testicles seminiferous tubules given N. plena ethanol extract every day for 14 days can be seen in table 2.

| No | Treatment                                      | Number of spermatogenic cell layers |
|----|-----------------------------------------------|------------------------------------|
|    |                                               | Spermatogonia | Spermatocytes | Spermatids | Spermatozoa |
| 1. | Control (Na-CMC 0.5%)                         | 1.0 ± 0.000  | 3.10 ± 0.210 | 4.83± 0.308 | 2.20± 0.258 |
| 2. | Treatment 1 extract of N. plena leaves 87.5 mg kg⁻¹ bw rats | 1.02 ± 0.040 | 3.69 ± 0.194 | 4.87 ± 0.250 | 2.39 ± 0.183 |
| 3. | Treatment 2 extract of N. plena leaves 175 mg kg⁻¹ bw rats | 1.12 ± 0.144 | 3.83 ± 0.070 | 5.8 ± 0.000 | 3.8 ± 0.000 |
| 4. | Treatment 3 extract of N. plena leaves 350 mg kg⁻¹ bw rats | 1.18 ± 0.000 | 4.25 ± 0.243 | 5.8 ± 0.000 | 3.8 ± 0.000 |

Note: the same letter is not significantly different in the 5% DMRT.

Table 2. The mean and standard deviation number of spermatogenic cell layers of the testicular seminiferous of rats given N. plena ethanol extract every day for 14 days

Based on table 2, it appears that the layers of spermatogonia and spermatids were not significantly different in all treatments. In the spermatocyte layer, the control treatments were significantly different from all treatment groups, but Treatments 2 and 3 were not significantly different, both were different from Treatment 4. In the spermatozoa layer, it was found that the control was not different from Treatment 1, but both were different from Treatment 2 and 3.

3.1.3. Overview of Tubular Micro-Anatomical Testicular seminiferous tubules.
A description of the microanatomy structure of the rat testicular seminiferous tubules after administration of N. plena leaves ethanol extract for 14 days can be seen in Figure 1.
Remark: A = Control, B = Treatment with extract of ethanol of *N. plena* leaves, C = Treatment with extract of ethanol of *N. plena* leaves D = Treatment with extract of ethanol of *N. plena* leaves. A and B: Spermatogenic cells which arrange tubulus seminiferous are completely arranged: 1 layer of spermatogonium, 1-2 spermatocyte layers, some layers of spermatid, and lumen with spermatozoa. C and D: spermatogenic cells are completely arranged, but more condensed, more compact with full lumen of spermatozoa.

3.2. Discussion

The results showed that there was a tendency for the average weight of the testes and diameter of the tubules (Table 1) treated with *N. plena* leaf extract was higher than the control although statistically there is no significant difference between control and treatment (on the weight of the testes), and the only real difference is between Control and Treatment 3 (highest dose) (on the diameter of the seminiferous tubules).

Based on Table 2, it was found that *N. plena* leaf extract only affected the number of layers of spermatocyte cells and spermatozoa cells. *N. plena* leaf extract, which was not so influential in this study, was allegedly due to the administration of the extract for only 14 days, thus causing the active compound contained in the extract of the plant to have not been so influential on the testes.

[3] found that *N. plena* leaves contain steroids, saponins, flavonoids, and alkaloids. According to [4], these compounds have the potential as aphrodisiac ingredients (libido enhancers) because they can increase the body's testosterone content. Flavonoid compounds function to increase dehydroepiandrosterone (DHEA) levels. By the increment of DHEA, testosterone in the body will also
increase. The central action of saponins is to increase levels of LH and FSH, increase androgen production through direct and indirect channels. Saponins (steroid glycosides) play a role in the biosynthesis of DHEA (dehydroepiandrosterone), thereby increasing testosterone levels in the body. Saponins are bounded to enzymes that stimulate the synthesis of testosterone, thus increasing the production of testosterone [11]. The central action of the alkaloid increases testicular cholesterol content through steroidogenesis, which will increase DHEA (dehydroepiandrosterone), which in turn will increase testosterone [4].

[3] found that N. plena has a higher fat content than N. oleracea. Plant fat is called phytosterol, which is a derivative of a steroid compound, as a basic ingredient of the testosterone. Sterol compounds (a form of steroids in plants) that have a cholesterol-like structure can be converted into pregnenolone through a series of biosynthetic processes that can be converted into testosterone [12]. Through these various mechanisms, the active compound in the leaves of N. plena increases testosterone, which subsequently increases the activity of spermatogenesis. This is consistent with the opinion [13], which states that testosterone, together with FSH, LH function in spermatogenesis, sperm maturation, and increased fructose excretion by the seminal vesicles as the primary nutrients of sperm.

The function of testosterone in the seminiferous tubules, among others, is needed to stimulate the growth of spermatogonia, the development of spermatocytes into spermatids [5], [14], for the differentiation of spermatids into spermatozoa [6] Testosterone and FSH have an essential role in the process of spermatogenesis. FSH stimulates Sertoli cells to synthesize ABP, which functions to bind testosterone secreted by Leydig cells to be transported to the lumen of the seminiferous tubules used in the process of spermatogenesis [14].

From the result of the research it was found that the extract of N. plena leaves did not influence the number of the layers of spermatogonium cells. This was caused by the fact that there were two kinds of spermatogonium cells, which was active in splitting mitosis and those cells which make differentiation to become spermatocyte. It was suspected that N. plena leaf extract did not affect cells that actively carry out mitosis but affects cells that differentiate into spermatocytes. As a result, the number of spermatocyte cell layers increases compared to controls. However, the proliferation of spermatocytes to spermatids is inhibited because testosterone levels are still low; as a result, the number of spermatid cells is not significantly different from control. This was consistent with the opinion [12] which states that the function of testosterone in the seminiferous tubules, among others, is needed for the development of spermatocytes into spermatids. The differentiation of spermatids into spermatozoa was not affected by low testosterone levels so that an increasing number of spermatozoa is found in the seminiferous tubule lumen.

The effect of N. plena extract on the arrangement of spermatogenic cells after the treatment can be seen in figure 2. From the qualitative observation by comparing testicle tubulus seminiferous microanatomy structure, it can be found out that control group (figure 2A), spermatogenic cells were completely arranged with associated cells to lumen based on its development, such as 1-layer of spermatogonium, 1-2 layer of spermatocyte, some layers of spermatid and lumen with spermatozoa. This was not far different with the description of tubulus seminiferous given with Treatment 2 extract (figure 2B). the Treatment 2 and 3 (figure 2C and 2D), spermatogenic cells can be seen arranged more densed, with lumen full of spermatozoa. This means that N. plena leaf extract could increase the number of spermatogenic cells better when compared to controls.

The parameters of increased spermatogenesis activity were the increase of testicular weight, the increase of the diameter of the testicular seminiferous tubules, and the increasing number of spermatogenic cells. The function of the testes is to produce testosterone and spermatogenic cells. Since the testosterone levels were still low, then only a portion of spermatogenic cells was affected due to the administration of N. plena leaf extract. Consequently, the weight of the testes was not affected. Other allegations due to lack of testosterone and FSH cause spermatogenic cells not to develop. This was thought to cause the seminiferous tubule diameter not to develop.
The results of spermatogenesis analysis showed that only a portion of spermatogenic cells showed differences between treatments due to the administration of *N. plena* leaf extract. However, treatment of group 3 had the potential to increase spermatogenesis activity better than groups 1 and 2. Thus, *N. plena* leaves had the potential to increase the spermatogenesis activity of male rats, so they can be used to increase fertility. In order to get maximum results, further research is needed with a longer extract administration time, for example, one cycle of spermatogenesis.

4. Conclusion

*Neptunia plena* leaves have the potential to increase the spermatogenesis activity of male rats.

References

[1] Departement of Agriculture and Fisheries, Biosecurity Queensland 2016 *Water mimosa (Neptunia oleracea or Neptunia plena)* (Queensland Government)

[2] Departement of Agriculture and Fisheries Biosecurity Queensland 2016 *Water Mimosa (Neptunia oleracea), Dead and Awake (Neptunia plena)* (Queensland Government)

[3] Rusmiati dan S G Sari 2018 *Karacteristik Morfologi, Profil Gizi, dan Golongan Senyawa Afrodisiak Tumbuhan Mimosa Air (Neptunia oleracea dan Neptunia plena) di Lahan Rawa Kabupaten Banjar* Penelitian Didanai oleh DIPA-PNBP FMIPA UNLAM Semester Genap TA 2017/2018

[4] Yakubu M T and A Akanji 2010 *Effect of aqueous extract of Massularia acuminate stem on sexual behavior of male Wistar rats* Hindawi Publishing Corporation Evidence-based Complementary and Alternative Medicine 10 pp

[5] Norris D O 1980 *Vertebrate Endocrinology* (Philadelphia: Lea & Febioger Co)

[6] Hafez E S 1976 *Human Semen and fertility Regulation in Men* (The CV Mosby Company)

[7] Sumapraja S 1999 *Infertilitas dalam Prawiroharjo, S. Ilmu Kandungan. Edisi 2*, Jakarta. Yayasan Bina Pustaka Sarwono Prawirohardjo, p. 497-521.

[8] Harborne JB *Phytochemical methods 2nd edition* (London: Chapman and Hall) 1984 DOI: 10.1007/978-94-009-5570-7 Print ISBN: 978-94-010-8956-222 Trease GE Evans WC Pharmacognosy

[9] Suntoro S H 1983 *Metode Pewarnaan* (Jakarta: Penerbit Bratara Karya Aksara) p 48-77

[10] Wardoyo B P E 1990 *Pengaruh fraksi kloroform dan fraksi air buah Momordica charantia terhadap spermatozoa epididymis tikus* (Yogyakarta: Thesis Fakultas Farmasi UGM) p 53-102

[11] Gauthaman K and Ganesan Ap. *The hormonal effects of Tribulus terrestris and its role in the management of male erectile dysfunction: an evaluation using primates, rabbit, and rat Phytomedicine. [Internet]. 2008;15(1):44-54. Available from: Pubmed.*

[12] Weil, P.A. 2012. *Endocryn System Kinds in Biochemistry Harper Biokimia, Editor Murray*

[13] Jahromy MH, Moghadam AA, 2014. *Effect of sertraline on sperm motility, number and viability and its relation to blood levels of testosterone, FSH and LH in adult male mice* Advances in Sexual Medicine 4:17-24.

[14] Sherwood L 2013 *Introduction to Human Physiology*. Brooks/cole, Cengage Learning