E-ISSN 2549-8703 I P-ISSN 2302-7282

BIOTROPIKA *Journal of Tropical Biology*

https://biotropika.ub.ac.id/

Vol. 10 | No. 1 | 2022 | DOI: 10.21776/ub.biotropika.2022.010.01.04

POTENTIAL OF COMBINATION *Marsilea crenata* AND *Curcuma xanthorriza* TO IMPROVE SPERM QUALITY OF MALE MICE EXPOSED BY MONOSODIUM GLUTAMATE

POTENSI KOMBINASI Marsilea crenata DAN Curcuma xanthorriza DALAM MEMPERBAIKI KUALITAS SPERMA MENCIT JANTAN YANG TERPAPAR MONOSODIUM GLUTAMAT

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Received : October, 26 2021

Accepted : February, 21 2022

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ABSTRACT

This study aims to determine the potential of water clover (M. crenata), curcuma (C. xanthorriza), and the combination of both extracts in order to improve the sperm quality of mice after MSG administration. This study used 35 Balb/C male mice (3 months old, 25-30 grams body weight (BW)). The animals randomly divided into 7 treatment groups, namely K0 (the group given MSG 4 mg/gBW), K1 (the group given MSG 4 mg/gBW and M. crenata 0.09 mg/gBW), K3 (the group given MSG 4 mg/gBW and C. xanthorriza 0.2 mg/gBW), K4 (the group that was given MSG 4 mg/gBW and a combination of M. crenata extract 0.045 mg/gBW and C. xanthorriza 0.1 mg/gBW), K5 (the group given M. crenata 0.09 mg/gBW), and K6 (the group given C. xanthorriza 0.2 mg/gBW). MSG and all extracts are given orally and daily for 30 days. The observed parameters were sperm's motility, viability, and concentration. The data are analyzed using SPSS for windows with a One-way ANOVA test (p≤0.05) and Tukey HSD test. The administration of water clover, curcuma, and the combination of both extracts can significantly improve the sperm quality in mice exposed by MSG. The administration of single and combination extract can improve the motility, viability, and concentration of the sperm in treatment groups with the extract. In conclusion, the combination of water clover and curcuma ethanol extract significantly improved sperm quality.

Keywords: *curcuma, monosodium glutamate, sperm quality, water clover*

ABSTRAK

Penelitian ini bertujuan untuk mengetahui potensi ekstrak semanggi air (M.crenata), temulawak (C.xanthorriza), dan kombinasi keduanya dalam memperbaiki kualitas sperma mencit setelah pemberian MSG. Penelitian ini menggunakan 35 ekor mencit Balb/C jantan umur 3 bulan dengan berat badan 25 - 35 gram. Mencit dibagi menjadi 7 kelompok perlakuan yaitu K0 (kelompok kontrol), K1 (kelompok yang diberi MSG 4 mg/gBB), K2 (kelompok yang diberi MSG 4 mg/gBB dan M.crenata 0,09 mg/gBB), K3 (kelompok yang diberi MSG 4 mg/gBB dan C. xanthorriza 0,2 mg/gBB), K4 (kelompok yang diberi MSG 4mg/gBB dan kombinasi ekstrak M. crenata 0,045 mg/gBB dan C.xanthorriza 0,1 mg/gBB), K5 (kelompok yang diberi M. crenata 0,09mg/gBB), dan K6 (kelompok yang diberi C. xanthorriza 0,2 mg/gBB). Semua perlakuan diberikan secara peroral selama 30 hari. Parameter kualitas sperma yang diamati meliputi motilitas, viabilitas, dan konsentrasi sperma. Data hasil penelitian dianalisis dengan one-way ANOVA (p≤0,05) dan uji beda Tukey HSD. Pemberian MSG dosis 4mg/gBB dapat menurunkan kualitas sperma, seperti penurunan motilitas sperma, viabilitas spermatozoa, dan konsentrasi sperma. Pemberian ekstrak tunggal maupun kombinasinya mampu meningkatkan motilitas, viabilitas, dan konsentrasi sperma kelompok perlakuan secara signifikan jika dibandingkan dengan Kelompok K1. Kesimpulan menunjukkan bahwa kombinasi ekstrak etanol semanggi air dan temulawak meningkatkan kualitas sperma secara signifikan.

Kata Kunci: *kualitas sperma, monosodium glutamat, semanggi air, temulawak*

How to cite:
Firstiantono, A. 2022. Combination of *Marsilea crenata* and *Curcuma xanthorriza* to improve sperm quality of male mice exposed by monosodium glutamate. *Journal of Tropical Biology* 10 (1): 33-39.

INTRODUCTION

Monosodium Glutamate (MSG) is an additive substance that is usually found in food packaging, fast food, and kitchens. MSG is commonly used to increase the "umami" taste and stimulate appetite [1]. Excessive administration of MSG can cause direct and indirect effects on the body and reproductive system [2]. The direct effect occurs in the testes, while the indirect effect is related to the *Hypothalamus-Pituitary-Gonadal* (HPG) Axis. MSG that enters orally into the body will be metabolized into sodium (Na) and glutamate (Glu) in the oral cavity, then absorbed in the small intestine. The glutamate will diffuse throughout the

body via the bloodstream and binding to NMDA (N-Methyl-D-Aspartate), Kainate (Ka), and Metabotropic Glutamate (mGlu) receptors [1].

Excessive glutamate concentration leads to excessive activation of glutamate receptors, increasing the number of intracellular Ca^{2+} ions that causes excessive Reactive Oxygen Species (ROS) production in the Tricarboxylic Acid (TCA) cycle in mitochondria. High concentrations of ROS will generate lipid peroxidation, which leads to membrane damage, DNA alteration in Leydig cells, and disruption of spermatogenesis hormone secretion [3].

MSG administer also causes excitotoxic effects, such as Hypothalamus-Pituitary-Axis pathway disruption. The hypothalamus is a homeostatic regulatory center that regulates the secretion of hormones, including gonads hormones. High levels of MSG can cause damage to the arcuate nucleus and ventromedial nucleus in the hypothalamus. It leads to the decreasing of GnRH (Gonadotropin-Releasing Hormone) secretion that affects the anterior pituitary in secreting gonadotropin hormones, such as FSH and LH [4]. Both hormones play an important role in the spermatogenesis process [5].

Negative effects of MSG consumption can be observed on the characteristics of the spermatozoa. The observable characteristics of the sperm are such as amount decreasing, motility, viability, and abnormality of sperm morphology. Moreover, the use of MSG also affects on oxidative stress in the testes, which causes a decrease in Superoxide Dismutase (SOD) and Glutathione reduction (GSH) levels. Previous studies have shown that giving a dose of MSG 6 mg/BW for 45 days in male rats can increase testicular MDA levels [1].

Marsilea crenata is an aquatic plant that is often used as a vegetable. This plant is proven to be useful as anti neuroinflammation, anticholesterol, antiosteoporosis, and so on. Active compounds such as quercetin, vitamin A, vitamin C, and zinc in this plant can act as antioxidants. The administration of *M. crenata* extract showed an increase in LH and testosterone hormones in mice administered by MSG. In addition, there was also a decrease in MDA levels and an increase in spermatogonia, spermatocytes, spermatids, and Leydig cells [3].

This plant has many benefits, such as antihepatitic, anticarcinogenic, antimicrobial, anti-inflammatory, and much more. The composition of an important compound in *Curcuma xanthorriza* is curcumin which can act as an antioxidant [6]. Previous studies demonstrated that the administration of curcumin could increase sperm count and repair histological structures in mice exposed to MSG [7].

M. crenata and *C. xanthorriza* are plants that have chemical components that can act as a source of antioxidants. Research on the potential of *M. crenata* and *C. xanthorriza* extract combination in improving the quality of sperm administered with MSG has not been widely carried out. Considering the importance of maintaining sperm quality in the health reproduction field, this research needs to be done.

METHODS

Extract preparation. *M. crenata* was obtained from the local farmers in Surabaya. *C. xanthorriza* was obtained from Materia Medica, Batu, Malang, Indonesia. Each powdered sample of as much as 1 kg was extracted with 5 L of 70% ethanol solvent at room temperature for 24 hours. Samples were filtered and evaporated using a rotary evaporator after 24 hours of maceration. So that ±150 grams each of *M. crenata* and *C. xanthorriza* ethanol extract was obtained and stored at 4^{o}C for further analysis [8].

Experimental design. Thirty-five male BALB/c mice aged 12 weeks were obtained from Malang Murine Farm, Singosari, Indonesia. All The experimental animals were randomly divided into seven groups, namely K0, K1, K2, K3, K4, K5, and K6. K0 was the group that was given standard food without MSG and extracts. K1 was the group treated with MSG at 4 mg/gBW dose. K2 was the group treated with MSG at 4mg/gBW dose and a single extract of *M. crenata* at 0.09mg/gBW dose. K3 was the group treated with MSG at 4 mg/gBW dose and a single extract of *C. xanthorriza* at 0.2 mg/gBW dose. K4 was the group treated with MSG at 4 mg/gBW dose, and the extracts combination at 0.045 mg/gBW dose of *M. crenata* and 0.1 mg/gBW dose of *C. xanthorriza* (1:1). K5 was the group given *M. crenata* at 0.09 mg/gBW dose. K6 was the group given *C. xanthorriza* at 0.2 mg/gBW dose. The study procedure was proved by the Animal Care and Use Committee of Brawijaya University (Legal number: 033-KEP-UB).

Mice were acclimatized for seven days. Then, MSG and extract were given orally every day for 30 days. On the 31st day, mice were sacrificed, and epididymis organs were isolated. The cauda epididymis was quickly sucked and diluted with warm 0.1 M PBS 1.5 ml pH 7.4 solution at 34-37°C to collect the semen.

Sperm motility. Semen liquid was taken 10 µl and dropped onto object-glass. Sperm movement was observed using a light microscope with 100X magnification in 5 fields of view (each repetition). The percentage calculation of the sperm individual motility was assessed by progressive motility sperm [3].

Sperm viability. A total of 10 µl of liquid semen was taken, dripped onto the object-glass, and stained with 1% eosin and 5% nigrosin on the object-glass. The mixture was homogenized thoroughly with a toothpick. The smear preparation was performed by using a cover glass. Counting the live and dead sperm on smears was carried out using a microscope with 400X magnification. Live sperm will look transparent, while the dead sperm will be colored by eosin-nigrosin (Figure 1). Each treatment was repeated three times (until the counted sperm cells counted was at least 200 cells). The sperm viability is calculated according to the following formula [3]:

$$Viability\ (\%) = \frac{total\ living\ sperm}{total\ sperm}\ x\ 100\%$$

Sperm concentration. The semen was diluted 10x by adding 10 µl of semen with 100 µl of fixative solution (normal saline). Then, the cement solution was put in the hemocytometer chamber. The sperm counting was carried out in 5 small boxes with three replications for each treatment group. The number of counted sperm in 5 fields of view in the small box is used to calculate the sperm concentration using the following formula [3] :

$$Concentration = n\ x\ k\ x\ DF\ x\ 10^4$$

n = total counted sperm on 5 small squares
k = total small squares counted (5)
DF = dilution factor (10)
10^4 = haemocytometer chamber volume

Data analysis. The sperm motility, viability, and spermatozoa concentration data were analyzed using One-way ANOVA with SPSS software ($p \leq 0,05$) and Tukey HSD test.

RESULTS AND DISCUSSION

The respective administration of a single *M. crenata* ethanol extract, a single *C. xanthorriza* ethanol extract, and a combination ethanol extract between *M. crenata* and *C. xanthorriza* (1:1) for 30 days could improve the sperm quality of mice administered by MSG (K2, K3, and K4). These results can be seen in Table 1.

Sperm motility. The percentage of sperm motility in the K0 group was approximately 70.40 ± 9.66% (Table 1). A highly significant reduction in the sperm motility of approximately 50 ± 13.69% was confirmed in the K1 group exposed to MSG. The administration of *M. crenata* extract, *C. xanthorriza*, and the combination in the treatment group was able to significantly increase the percentage of sperm motility when compared to the group that was not treated with the extract (K1). The administration of a single extract of *M. crenata* and *C. xanthorriza* could significantly increase sperm motility when compared to the MSG-exposed group (71.2% and 73.4%, respectively). The percentage of normal mice motility is preferably not less than 70% [9].

Table 1. The effects of MSG and extracts treatment on semen quality of the mice

Treatment groups	Sperm Quality Parameter ± SD		
	Motility (%)	Viability (%)	Sperm concentration (x 10^6 cells/ml)
K0	70.40 ± 9.66^{ab}	43.52±13.15^{bc}	30.3±10.93^{ab}
K1	50 ± 13.69^{b}	27.6±9.86^{c}	12.5±6.6^{b}
K2	70.6 ± 7.54^{ab}	52.4±10.74^{ab}	26.45±13.05^{ab}
K3	71.8 ± 13.46^{ab}	65.2±5.93^{a}	23.25±6.1 ^{ab}
K4	69.8 ± 9.68^{ab}	71.2±6.3^{a}	28±3.5^{ab}
K5	71.2± 6.30^{ab}	63.94± 12.46^{a}	24.95 ± 6.41^{ab}
K6	73.4 ± 16.28^{a}	62 ± 7.10^{ab}	39.20 ± 13.36^{a}

There was a significant difference between the group given a single extract of *C. xanthorriza* and the K1 group. This result corresponds with other studies which stated that the administration of MSG in various doses, such as 4 mg/gBW for 35 days [[10]], and 2 g/kgBW for 15 days [11], was able to cause a decrease in sperm motility of mice by 50% and 19.59%.

Administration of MSG at 4 mg/gBW dose for 21 days could increase testicular MDA production caused by lipid peroxidation [12]. MSG exposure can stimulate lipid peroxidation in the testes, indicating oxidative stress due to the formation of ROS and decreased antioxidants in the body. The increase in ROS production will be followed by PARP-1 activation. PARP-1 will catalyze the hydrolysis of NAD^+ into nicotinamide and PAR. The result of this was a reduction in the supply of NAD^+ so that the production of ATP as a source of energy for sperm cells decreased, which caused cell death [13]. Lack of ATP synthesis by mitochondria could result in reduced sperm motility [14].

M. crenata contains antioxidant compounds such as zinc, quercetin, vitamin A, and vitamin C. The activity of zinc and vitamin C is thought to be a factor in increasing sperm motility and is supported by the presence of quercetin [3]. Quercetin act as an exogenous antioxidant that could prevent lipid peroxidation in sperm cell membranes caused by ROS [15]. The decrease in free radicals could cause the mitochondrial function to increase so that ATP production also increased [16].

C. xanthorriza contained curcumin compounds which act as an antioxidant [17]. Curcumin could act as an exogenous antioxidant and induce the synthesis of endogenous antioxidants as indicated by a significant increase of glutathione peroxidase

(GPx) and superoxide dismutase (SOD) [18]. The improving amount of exogenous and endogenous antioxidants in the body can improve sperm quality.

Some contents such as zinc and vitamin C found in *M. crenata* were thought to play a role in increasing sperm motility. This was because zinc had the ability to increase the availability of energy in sperm by regulating energy utilization through the ATP system and regulation of phospholipids [19]. Previous research stated that MSG exposure could reduce testosterone and LH in rats. However, the presence of curcumin as a treatment could prevent its effect from occurring. The administration of curcumin was able to increase LH and testosterone significantly. Therefore, the spermatogenesis process could be improved, and the MSG toxic effects on the testes could be reduced [7].

Sperm viability. MSG exposure could reduce sperm viability by 15.92% compared to controls (Table 4). Excess concentration of MSG that entered the body could increase the production of Ca^{2+} in mitochondria [20]. The excess accumulation of ROS could also cause lipid peroxidation in spermatogonia, spermatid, and sperm cells. This caused a decrease in sperm fluidity and a loss of sperm membrane integrity [21]. Previous studies had shown that giving MSG at 2 g/kgBW dose daily could reduce sperm viability by 37% [22].

The administration of *M. crenata* extract, *C. xanthorriza*, and the combination in the MSG treatment group, as well as a single extract of *M. crenata* and *C. xanthorriza*, could significantly increase sperm viability when compared to the group that was not treated with herbal extracts (K1). The results showed that the highest sperm viability level was in the K4 group at 71.2%, followed by the K3 group at 65.2%, and the K5 group at 63.94%. The administration of a single extract of *M. crenata* and *C. xanthorriza* was able to significantly increase the viability of spermatozoa when compared to the group exposed to MSG. *M. crenata* had many antioxidant compounds such as flavonoids, phenolics, zinc, and vitamin C [2]. In addition, zinc also played a role in increasing sperm viability. This result was in line with previous research, which stated that zinc was able to act as an antioxidant and increase sperm membrane stability [23]. Exogenous antioxidants can protect sperm cell membranes from free radical reactivity by free radical scavenger mechanism so that the fluidity and integrity of the phospholipid membrane remain in good condition. The fluidity and high integrity of the sperm cell membrane could prevent pathogenic compounds from entering the cell. Therefore, sperm cells could have a longer and increased viability [21].

C. xanthorriza contains curcumin which is able to act as a good antioxidant. This was supported by previous research, which stated that curcumin acts as scavengers on free radicals [24]. Furthermore, curcumin also reduced oxidative stress and increased the motility, viability, and integrity of the plasma membrane [25].

The combination of *M. crenata* and *C. xanthorriza* extracts on the viability of the sperm of mice exposed to MSG showed a significant effect ($p \leq 0.05$). The presence of compounds that have a similar role as free radical scavengers can increase the viability of spermatozoa in mice exposed to MSG. MSG could lead to lipid peroxidation that affected the decreasing of membranes fluidity and integrity of spermatozoa. This condition caused the vitality or viability of spermatozoa to decrease. The presence of exogenous antioxidants could trigger an increase in the fluidity and integrity of the spermatozoa membrane. Based on the Tukey test, it was found that there was a significant difference between the viability of spermatozoa in the K4 group and the K1 group. It was also shown that the administration of a single *M. crenata* extract and a single *C. xanthorriza* extract had a significant difference with the K1 group. The results of this study indicated that the combination of extracts could improve sperm quality effectively. Treatments with extracts could increase levels of LH, testosterone, sperm quality and decrease levels of testes MDA [3]. Sperm maturation in the epididymis would also increase the capability of spermatozoa, such as the capacity to perform glycolysis, resulting in increased motility and viability of spermatozoa [26].

Sperm concentration. Oral administration of MSG at 4 mg/gBW dose for 30 days could reduce the sperm concentration of normal mice by 17.8 x 10^6 cells/ml (Table 4). This was in line with previous studies where MSG at a dose of 4 ml/kgBW for 28 days in adult rats could reduce sperm concentration from 88.07 x 10^6 cells/ml to 80.24 x 10^6 cells/ml [27]. Administration of MSG also lowers LH and testosterone concentration. Testosterone and LH played an important role in testicular function and spermatogenesis [7].

The excessive accumulation of MSG could trigger the formation of ROS, which resulted in a decrease in cell membrane fluidity and enzymes inactivation that caused cell death [28]. ROS formation was indicated by increases of Malondialdehyde (MDA) levels in testes and epididymis. Elevated ROS lead to DNA mutation or fragmentation, and protein oxidation that affected cell cycle irregularity, DNA repair or replication disorder, and gene mutations of cells and lead to apoptotic and necrotic that caused in reduced of sperm production in semen [1].

The administration of *M. crenata* extract, *C. xanthorriza*, and their combination could significantly increase sperm concentration ($p \leq 0.05$). The highest sperm concentration was in group K6, which was 39.20 x 10^6 cells/ml. These results represent an increase when compared to the K1 group. The administration of *M. crenata* extract in treated mice could significantly increase sperm concentration by 13.95 x 10^6 cells/ml compared to the K1 group.

The bioactive components found in *M. crenata* include flavonoids, phenolics, vitamin C, zinc, and carotene compounds [2]. These compounds can act as antioxidants by scavenging mechanisms. Therefore, ions or atoms from free radicals could be taken [3]. This action caused the free radicals to be inactive so it would not damage the lipid membrane and sperm DNA, thus preventing apoptosis and increasing sperm concentration [24]. *M. crenata* also had components such as vitamin A, which plays an important role in the process of spermatogenesis [2]. Vitamin A was thought to be able to increase sperm concentration because it could initiate meiotic division in germ cells [[29]]. In addition, the flavonoid derivative compound, quercetin, also act as an antioxidant and minimizes oxidative stress.

C. xanthorriza extract administered to treated mice could significantly increase sperm concentration ($p \leq 0.05$) by 10.75 x 10^6 cells/ml compared to group K1 mice. Based on the different Tukey HSD test, it also showed a significant difference of concentration between the experimental animals given *C. xanthorriza* and those that were only exposed to MSG. *C. xanthorriza* contained some bioactive components, such as curcumin, essential oils, saponins, alkaloids, flavonoids [2]. The majority of the active components contained in *C. xanthorriza* was curcumin [30]. Curcumin in *C. xanthorriza* could act as a protector against free radicals and also reduce oxidative stress. It was proven that the administration of *C. xanthorriza* extract had a preventive effect on the testes, which was characterized by an increase in spermatogonia, primary spermatocytes, and spermatids in the microscopic image score. Curcumin was also able to increase sperm concentration, testosterone levels, and SOD levels [24].

The *M. crenata* and *C. xanthorriza* extract and *C.xanthorriza* extract combination administered in experimental animals showed a significant effect ($p \leq 0.05$), indicated by an increase in sperm concentration of 16 x 10^6 cells/ml. This result was also supported by the Tukey HSD test, which showed a significant difference. *M. crenata* contained vitamin A that could increase sperm concentration by initiating meiosis division in germ cells [29]. In addition, the flavonoid-derived compound, quercetin, also act as an antioxidant and minimizes oxidative stress so that the spermatogenesis process could run optimally [3]. Curcumin in *C. xanthorriza* played a role in the process of increasing LH and testosterone hormones, so that it became the carrying capacity of the spermatogenesis process [7, 26]. The curcumin could role as an antioxidant that minimized the occurrence of oxidative stress caused by MSG as well. It could also protect sperm DNA from damage, thus the sperm concentration increases. Curcumin was able to significantly increase sperm motility and density, while also increasing FSH and testosterone, which played an important role in spermatogenesis [30].

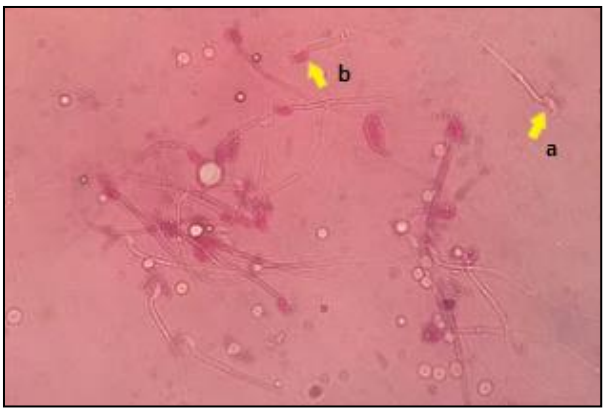

Figure 1. The difference between living and dead sperm, a) living sperm, b) dead sperm

CONCLUSION

The administration of ethanol extract of *Marsilea crenata*, *Curcuma xanthorriza*, and the combination of the two had a significant effect on improving sperm quality of mice exposed to MSG. This was indicated by an increase in motility, viability, and sperm concentration ($p \leq 0.05$).

ACKNOWLEDGMENT

This research was financially supported by the DPP/SPP Faculty of Mathematics and Natural Science (FMIPA) Universitas Brawijaya 2021.

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