

The Potency of Kaffir Lime Leaf (*Citrus Hystrix*) Ethanol Extract Against Lead Acetate Toxicity on Spermatozoa Quality of Mice (*Mus musculus*)

Azarine Ariqoh Arysanti Bahyshidqi¹, Safira Nur Izzah¹, Sofia Zahra Kamila¹, Reny I'tishom¹

¹ Faculty of Medicine, Universitas Airlangga, East Java, Indonesia

Abstract

Lead is a heavy metal which can affect male reproductive system. The accumulation of lead in the body becomes a precursor to the formation of ROS, which is able to damage spermatozoa. The toxicity of lead can be prevented by antioxidants. Kaffir lime leaves (*Citrus hystrix*) with their flavonoid content have the potential to be the source of antioxidants. The purpose of this study is to analyze the antioxidant effect of kaffir lime leaves on the quality of spermatozoa of the mice that have been exposed to lead acetate. **Methods:** The literature study was conducted using narrative review technique by collecting related articles using specified keywords. **Result:** It is found that kaffir lime leaves contain *hesperetin*, which is the dominant flavonoid in Citrus group. The antioxidant activity of kaffir lime leaf is able to neutralize lead-induced ROS, as well as to stimulate the formation of enzymatic antioxidants. **Conclusions:** Ethanol extract of kaffir lime leaves can improve the quality of spermatozoa of the mice that have been exposed to lead acetate.

Keywords: Antioxidant, *Citrus hystrix* leaves, Lead acetate, Male mice, Sperm quality

Introduction

Lead (*Pb*), as a pollutant substance, can be found in several places in Indonesia. One of the sources is the industrial fumes of used battery smelters, which do not have residual particulate controlling facilities. Citing from the website of Ministry of Environment and Forestry, based on the research conducted by P3KLL and PSTNT-BATAN on 2011, the *Pb* level in ambient air of used battery smelter industrial area, such as Parung Panjang, district of Bogor; Cinangka, district of Bogor and Manis Jaya, district of Tangerang are 17,503 $\mu\text{g}/\text{m}^3$, 5,186 $\mu\text{g}/\text{m}^3$, and 4,142 $\mu\text{g}/\text{m}^3$, respectively. These numbers have exceeded the standard on the Government regulations of Indonesia or *PP* number 41/1999 about

Air Pollution Controlling, which stated that the standard is 2 $\mu\text{g}/\text{m}^3$ in an average of 24 hours. This will affect the people who live near the factory. Lead accumulation in the body can decrease the number of spermatozoa and cause male infertility (Panggabean *et al.*, 2008). Lead is classified as an oxidant which becomes the precursor of ROS (Reactive Oxygen Species) formation. ROS can attack nitrogenous bases, such as purine and pyrimidine. It can also directly attack DNA, which is the collection of nitrogenous bases. It will activate *caspase* enzymes, which cause apoptosis of spermatozoa as well as degradation of Spermatozoa DNA (Hayati *et al.*, 2005). Antioxidant is a substance which has the characteristic of *chelating*. This will enable antioxidants to overcome lead poisoning (Offor *et al.*, 2017). Antioxidants can be found in nature which, in this case, is in a plant. Wulandari (2017) has found that kaffir lime leaf (*Citrus hystrix*) is one of the producers of antioxidants. It contains phenol, flavonoid, terpenoid and citronellal. Flavonoid is one of the natural antioxidants. It has *chelating* ability; which makes it able to bind metal and form a flavonoid-metal complex. This ability comes

Correspondence Author :

Dr. Reny I'tishom,

M.Si, ritishom@fk.unair.ac.id

Faculty of Medicine, Universitas Airlangga, Jl. Mayjen

Prof. Dr. Moestopo No.47 Surabaya, East Java,

Indonesia.

from the carbonyl and hydroxyl group which attach to its flavonol ring structure (Al-Megrin *et al.*, 2019). Based on the background above, an analysis of the potency of kaffir lime leaf ethanol extract against lead toxicity on spermatozoa quality of mice is needed. The purpose of this study is to analyze the effect of antioxidant in kaffir lime leaf against the spermatozoa quality of the mice that have been exposed to lead acetate. The benefit of this study is that it can serve as a reference for readers regarding how to utilize kaffir lime leaf ethanol extract in order to maintain the spermatozoa quality of which has been exposed to lead acetate.

Material and Methods

This article was made by using literature study with narrative review technique. Narrative review is a comprehensive, critical, and objective analysis from recent knowledge on a certain topic. It is an important process of research in building the theoretical framework in order to identify patterns and trends in literature on many kinds of researches (Baker, 2016). The data used in this study originated from journals which have been published in both national and international journal platforms on the internet. The search engines used to find the journals are Pubmed, Science Direct, Clinical Key, NCBI and Google Scholar. The process of journal searching was focused on two different purposes; namely searching for reference and comparative journals. Reference journals are used to provide explanations related to the problem and strengthening the argument. Meanwhile, comparative journals are used to compare previous researches which are related to the topic of 'antioxidants in other herbs that are used to increase the quality of spermatozoa in mice.' Several keywords used in the search of comparative journals are citrus hystrix leaf, sperm quality, antioxidant, male mice, and lead acetate. Boolean technique with the word "AND" was also used, for example: Citrus hystrix leaf AND Sperm quality. The inclusion criteria for this study are: it has to be in English and available in full text. The abstract and the full text of the comparative journals obtained were also analyzed. Journal analysis was conducted by classifying the author, year, sample population, study model and result. The result obtained from both reference and comparative journals were then written in narrative review.

Result and Discussion

Lead enters the body through inhalation. The size, shape, and phase of the particle will most likely affect its entrance to the body (Schlosser *et al.*, 2010). Lead exposure can result in the decrease of spermatozoa quality, which can be seen in the amount of abnormal morphology of spermatozoa, motility, as well as the concentration of spermatozoa.

In the research by Li *et al.*, 2017, it is stated that on ICR-CD1 mice that received lead in their water; the percentage of non motile and non progressive spermatozoa increased, while the amount of motile spermatozoa decreased. The percentage of abnormal spermatozoa increased after the administration of lead acetate.

Other studies have proven that a group of mice (*Mus musculus*) exposed to lead acetate (with a dosage of 100 mg/kg) has shown an increase in concentration of lipid peroxidation (LPO) and significant decrease in GSH level. Also, there are no appearance of primary spermatocyte, secondary spermatocyte, spermatid and spermatozoa in seminiferous tubule. This means that no spermatogenesis processes occur. On histopathology examination of seminiferous tubules, degeneration of germinal cell, absence of spermatozoa, vacuolization of testicular tissue were found; while on histopathology examination of epididymis, no spermatozoa was found. This shows the reduction of the amount of spermatozoa. Spermatozoa morphology of a mice group exposed to lead acetate is significantly poor (Rao *et al.*, 2016).

ROS is also called free radical since it contains at least one unpaired valence electron and takes electron from other compounds - which in turn will cause oxidation (Durackova, 2014). ROS consists of superoxide ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2), proxy radicals ($\cdot\text{R}\text{OO}$), and hydroxyl ($\cdot\text{OH}$). Lead acetate causes an increase in ROS by inhibiting the work of antioxidant enzymes. This happens because lead has a high affinity towards the sulfhydryl group of enzyme, such as superoxide dismutase and glutathione peroxidase. Moreover, lead acetate also competes with essential ions in the body that have a role as cofactor for important reactions, such as the activity of superoxide dismutase and catalase enzyme (Sudjarwo *et al.*, 2019). It is known that an increase in ROS can affect spermatozoa through several mechanisms, such

as lipid peroxidation, protein modification and damage to spermatozoa DNA. Lipid peroxidation can cause damage or disturbance to the structure of spermatozoa, transport function, ion and metabolite regulation, as well as signal transduction of receptor. This will decrease intracellular ATP, and later cause acrosomal damage as well as decrease membrane fluidity and permeability. This series of events will decrease spermatozoa motility (Sutanto *et al.*, 2017). Plasma membrane of spermatozoa is susceptible to damage caused by oxidative stress, since plasma membrane mainly consists of Polyunsaturated Fatty Acids (PUFA) that contains double bonds such as DHA or Docosahexaenoic Acid (Drevet *et al.*, 2019). The molecule of this membrane is reactive to oxygen radicals and will produce highly reactive lipid aldehyde, such as malondialdehyde. This will result in protein modification and DNA damage.

Depending on the type of ROS, protein can be converted through thiol oxidation, tyrosine nitration, sulfonation or glutathionylation. The most common protein modification is thiol oxidation. It targets the enzyme of glycolysis and krebs cycle, and later decrease ATP production efficiency; targets α -tubulin, which damages the microtubules polymerization; and targets protamine, which affects the remodeling of chromatin during spermiogenesis (O'Flaherty & Matsushita, 2017). The increase in ROS can also damage spermatozoa DNA integrity and cause apoptosis of germinal epithelial cells. Furthermore, it affects the amount and morphology of the spermatozoa. Oxidative stress causes decondensation of nuclear, which increases the susceptibility of spermatozoa DNA that is destroyed by free radical (Bashandy, 2007). On the other hand, it also causes spermatozoa DNA fragmentation by modifying bases such as 8-OH-guanine and 8-OH-20-deoxyguanosine (Drevet *et al.*, 2019).

II. Antioxidant of kaffir lime leaf

(*Citrus hystrix*)

Antioxidant is a compound which is needed by the body to absorb or neutralize free radicals that damage normal cells, lipids and protein. This compound has a molecular structure which enables it to give its electron to free radical molecules and break the chain reaction of free radicals. Natural antioxidant compound can be found in several forms such as vitamin C, vitamin E,

carotenoid, phenolic and polyphenolic compound which belong to the flavonoid group, derivative of cinnamic acid, coumarin, tocopherol, or other polyfunctional organic acids. The members of flavonoid group which have antioxidant activity are flavones, flavonols, flavanones, isoflavones, catechins, flavonols, and chalcone. Natural antioxidant compound has -OH group and double bond ($>C=C<$). These groups can give one of its hydrogen molecules so that ROS will become stable and non reactive free radical can be formed (Murray *et al.*, 2009).

Kaffir lime leaf (*Citrus hystrix*) has the potential to be a source of natural antioxidant. According to Fidrianny *et al.*, 2016, kaffir lime leaves ethanol extract have antioxidant activity that can be seen by the number of total phenolic compound, which is 3,66 g GAE/100 g; total flavonoid compound, which is 4,46 g QE/100 g; and carotenoid compound, which is 0,36 g BE/100 g. On another study conducted by Butryee *et al.*, 2009, it is found that the main flavonoid compounds of kaffir lime leaves are hesperetin, peonidin, myricetin, cyanidin, and isorhamnetin in the amount of 365 ± 48 , 206 ± 47 , 179 ± 18 , 123 ± 7 , and 113 ± 22 mg/100 g dry weight, respectively. Based on the result, hesperetin is known as the dominant flavonoid compound of kaffir lime leaves. Hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone, $C_{16}H_{14}O_6$) is a natural flavanone and an aglycone of hesperidin which dominantly found in citrus group (Samie *et al.*, 2018) and has three hydroxy groups located in the position of 3', 5- and 7. It also has additional methoxy substituent in the position of 4' (PubChem, 2020). Flavanone is a type of flavonoid which has a C ring and saturated double bond between the position of 2 and 3 (Panche *et al.*, 2016). Hesperetin uses its antioxidant properties in two ways; which are direct radical scavenging and cellular defense augmentation. Direct radical scavenging plays a part in the protection of DNA, protein, and tissue against the damage caused by intrinsic factor (oncogene) or extrinsic factor (radiation, inflammation, toxin) (Parhiz *et al.*, 2014). Meanwhile, cellular defense augmentation plays a part in the inhibition of cell damage by increasing the regulation of Nrf2 and ERK 1/2 gene expression (Elavarasan *et al.*, 2012; Martinez *et al.*, 2009). Activation of Nrf2 and ERK 1/2 can increase the regulation of HO-1 expression, which later will decrease intracellular prooxidant and increase bilirubin as internal antioxidant. HO-1 expression increases the level of CO

by its anti apoptotic and anti inflammatory activity; as well as increases guanylyl cyclase level inside the cell (Chen *et al.*, 2010). This shows that hesperetin is able to neutralize ROS. Other literature mentioned that the administration hesperetin in a diabetic rat group induced with STZ shows a decrease in MDA and carbonyl protein level as parameter of oxidative stres, with an increase in GSH, SOD, catalase and Gpx level - which are antioxidant enzymes – if compared to the diabetic group that did not receive administration of hesperetin. Other than that, Hesperetin administration in a diabetic rat group resulted in a significant increase of amount,

motility and viability of spermatozoa compared to the diabetic rat group that did not receive administration of hesperetin. Histological analysis shows that administration of hesperetin in a diabetic rat group can increase the MSTD (Mean Seminiferous Tubule Diameter) and MTBS (Mean Testicular Biopsy Score) when compared to the diabetic rat group that did not receive administration - which result shows a decrease in amount, motility and viability of spermatozoa and damage of seminiferous tubules (Samie *et al.*, 2018). In table 1, analysis of the previous researches about the effect of antioxidant to spermatozoa quality can be seen.

Table 1. Analysis of comparative journals

No	Title	Author	Sample	Intervention Group	Result	Extract Content
1.	Effects of Red Dragon Fruit (Hylocereus polyrhizus) Skin Extract on Lead Acetate Toxicity in the Morphology of Balb/C Mice (Mus musculus) Spermatozoa	(Raharjo et al., 2017)	25 of male mice	NC group were given nothing. PC group were given 8mg/kg BW of lead acetate. T1 group, T2 group and T3 group each were given 250 mg/kg BW, 500 mg/kg BW and 1000 mg/kg BW of extract, respectively and 8mg/kg BW of lead acetate. The lead and extract given were intraperitoneally. The study conducted for 35 days.	a. Lead decreases the number of normal morphology of spermatozoa b. The extract increases the number of normal morphology of spermatozoa in the mice that have been exposed to lead, but the result of the number is always below the control group.	Phenolic, flavonoid and polyphenols
2.	Ferulago angulata extract ameliorates epididymal sperm toxicity in mice induced by lead and diazinon	(Naderi et al., 2017)	80 of male NMRI mice	C group were given nothing. T1 group were given 0,5% of lead acetate and T2 were given 400 mg/kg BW of extract. T3 were given both extract and lead as much as T1 and T2 group . The lead and extract given were orally. The study conducted for 6 weeks.	a. Lead decreases the number of normal morphology, viability, motility, as well as the number of spermatozoa. b. The extract decreases the number of spermatozoa, but it increases the number of normal morphology of spermatozoa, as well as viability and motility of spermatozoa in the mice that have been exposed to lead. However, the result of the number is always below the control group.	9-octade cenoic acid, methyl ester, (E)-, carvacrol, thymol, 1, 2, 3-benzenetriol (pyrogallol)

Cont... Table 1. Analysis of comparative journals

3.	Protective effects of hydro-alcoholic extract of <i>Quercus brantii</i> against lead-induced oxidative stress in the reproductive system of male mice	(Soleimanzadeh et al., 2018)	42 of male NMRI mice	C group were given nothing, while NC group were given 0,2 ml of distilled water. T3 and T4 group were given 500mg/kgBW and 1000mg/kgBW of extract, respectively. While T5 and T6 group each were given 1000 ppm/kg BW of lead and additional 500 mg/kg BW and 1000 mg/kg BW of extract, respectively. The lead and extract given were orally. The study conducted for 35 days.	<p>a. Lead decreases the number of normal morphology, viability, motility, as well as the number of spermatozoa</p> <p>b. The extract increases the number of spermatozoa, normal morphology of spermatozoa, as well as viability and motility of spermatozoa in the mice that have been exposed to lead. However, the result of the number is always below the control group.</p>	Phenol, tannin, gallic acid, p-coumaric acid, ellagic acid, rutin, epicatechin and quercetin
4.	Carob (<i>Ceratonia siliqua</i> L.) fruit hydro-alcoholic extract alleviates reproductive toxicity of lead in male mice: Evidence on sperm parameters, sex hormones, oxidative stress biomarkers and expression of Nrf2 and iNOS	(Soleimanzadeh et al., 2020)	42 of male NMRI Mice	C group were given nothing, while NC group were given 0,2 ml of distilled water. T3 and T4 group were given 500mg/kgBW and 1000mg/kgBW of extract, respectively. While T5 and T6 group each were given 1000 ppm/kg BW of lead and additional 500 mg/kg BW and 1000 mg/kg BW of extract, respectively. The lead and extract given were orally. The study conducted for 35 days.	<p>a. Lead decreases the number of normal morphology, viability, motility, as well as the number of spermatozoa</p> <p>b. The extract increases the number of spermatozoa, normal morphology of spermatozoa, as well as viability and motility of spermatozoa in the mice that have been exposed to lead. However, the result of the number is always below the control group.</p>	Phenol, flavonoid, gallic acid, caffeic acid, chlorogenic acid, rutin, coumaric acid, quercetin, cinnamic acid and apigenin

Note: C : Control, NC : Negative Control, PC: Positive Control, T1 : Treatment Group 1, T2 : Treatment Group 2, T3 : Treatment Group 3 , T4 :Treatment Group 4, T5 : Treatment Group 5, T6 :Treatment Group 6, Ext : Extract, PbAc : Lead Acetate, Pb : Lead.

In table 1, it can be seen that all studies on the exposure of lead acetate in mice resulted in a decrease of the amount of spermatozoa. In each of those studies, the level of lead used for the experiment is different; however, each of the extract above has the ability to increase some or all of the quality parameters of spermatozoa -

which are the amount, viability, normal morphology, and motility. However, the numbers are always below the ones in the normal spermatozoa/control group. Each of the extracts above contains phenolic and flavonoid as in the ethanol extract of kaffir lime leaf, although it belongs to a different type of flavonoid. From the studies

above, it can be concluded that flavonoid can improve the quality of spermatozoa.

III. Result Potential

Prediction of the result is that kaffir lime leaf ethanol extract can increase the quality of spermatozoa by using its flavonoid properties, which can serve as additional antioxidant to prevent the decrease of spermatozoa quality due to lead exposure.

Conclusion

Kaffir lime leaf ethanol extract can increase the quality of spermatozoa (amount, normal morphology, motility and viability) since it contains antioxidant compound, namely Hesperetin; which is classified as flavonoid.

Acknowledgement : The authors would like to thank the Rector of Universitas Airlangga, Directorate of Students Affairs of Universitas Airlangga, as well as the authors' supervisor; Dr. Reny I'tishom, M.Si for kindly provided all of the facilities and assistances needed in order to complete this article.

Ethical Clearance - None

Source of Funding- Indonesian Ministry of Education and Culture (Kemdikbud)

Conflict of Interest - Nil

References

1. Al-Megrin WA, Alkhuriji AF, Yousef AOS, Metwally DM, Habotta OA, Kassab RB, Moneim AEA, El-Khadragy MF. Antagonistic Efficacy of Luteolin against Lead Acetate Exposure-Associated with Hepatotoxicity is Mediated via Antioxidant, Anti-Inflammatory, and Anti-Apoptotic Activities. *Antioxidants (Basel)*. 2019; 9(1):10.
2. Baker JD. The purpose, process and methods of writing a literature review: Editorial. *Association of Operating Room Nurses. AORN Journal*. 2016; 103(3):265-269.
3. Bashandy AES. Effect of Fixed Oil Nigella Sativa on Male Fertility in Normal and Hyperlipidemic Rats. *International Journal of Pharmacology*. 2007; 3(1):27-33.
4. Butryee C, Sungpuag P, Chitchumroonchokchai C. Effect of processing on the flavonoid content and antioxidant capacity of Citrus hystrix leaves. *International Journal of Food Science and Nutrition*. 2009;60(S2):162-174.
5. Chen MC, Ye YY, Ji G, Liu JW. Hesperidin upregulates heme oxygenase-1 to attenuate hydrogen peroxide-induced cell damage in hepatic L02 cells. *Journal of Agricultural and Food Chemistry*. 2010; 58:3330-3335.
6. Drevet JR, Aitken RJ. Oxidative damage to sperm DNA: Attack and defense. *Advances in Experimental Medicine and Biology*. 2019; 1166:107-117.
7. Durackova Z. Free radicals and antioxidants for non-experts. *Systems Biology of Free Radicals and Antioxidants*. Springer Verlag. Berlin. 2014.
8. Elavarasan J, Velusamy P, Ganesan T, Ramakrishnan SK, Rajasekaran D, Periandavan K. Hesperidin-mediated expression of Nrf2 and upregulation of antioxidant status in senescent rat heart. *Journal of Pharmacy and Pharmacology*. 2012; 64:1472-1482.
9. Fidrianny I, Amaliah A, Sukrasno. Antioxidant activities evaluation of citrus leaves extracts from West Java-Indonesia using DPPH and FRAP assays. *International Journal of Pharmacognosy and Phytochemical Research*. 2016; 8(4):611-618.
10. Hayati A, Rahmaninta DA dan Pidada IB. Spermatozoa motility and morphological recovery process in mice (*Mus musculus*) after the induction of 2- methoxymethanol. *Folia Medica Indonesiana*. 2005; 41(2): 9095.
11. KLHK Miliki Desain Lanskap Mitigasi Pencemaran Timbal (Pb) di Kawasan Industri. Kementerian Lingkungan Hidup dan Kehutanan 2019; 22.25 Available from: ppid.menlhk.go.id/siaran_pers/browse/2049. Accessed September 26th 2020.
12. Li C, Zhao K, Zhang H, Liu L, Xiong F, Wang K, Chen B. Lead exposure reduce sperm quality and DNA integrity in mice. *Environmental Toxicology*. 2017; 33(5):594-602.
13. Martinez MC, Fernandez SP, Loscalzo LM, Wasowski C, Paladini AC, Marder M, Medina JH, Viola H. Hesperidin, a flavonoid glycoside with sedative effect, decreases brain pERK 1/2 level in mice. *Pharmacology, Biochemistry, and Behaviour*. 2009; 92:291-296.
14. Murray RK, Granner DK, Rodwell VW. *Biokimia*

- Harper. 27th ed. EGC Medical Publisher. Indonesia. 2009.
15. Naderi N, Souri M, Esfahani MHN., Hajian M, Vash NT. Ferulago angulata extract ameliorates epididymal sperm toxicity in mice induced by lead and diazinon. *Andrology*. 2020; 8(3):706-718.
 16. PubChem Compound Summary for CID 72281, Hesperetin. National Center for Biotechnology Information 2020; 19.30. Available from: <https://pubchem.ncbi.nlm.nih.gov/compound/Hesperetin>. Accessed September 27th 2020.
 17. O'Flaherty C, Matsushita-Fournier D. Reactive oxygen species and protein modifications in spermatozoa. *Biology of Reproduction*. 2017; 97(4):577-585.
 18. Offor SJ, Mbagwu HOC, Orisakwe OE. Lead induced hepato-renal damage in male albino rats and effects of activated charcoal. *Frontiers in Pharmacology*. 2017; 8:1-10.
 19. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. *Journal of Nutritional Science*. 2016; 5(e47):1-15.
 20. Panggabean PCT, Sylvia S, July I. Efek pajanan timbal terhadap infertilitas pria. *Maranatha Journal of Medicine and Health*. 2008; 8:87-93.
 21. Patrick L. Lead toxicity part II: the role of free radical damage and the use of antioxidants in the pathology and treatment of lead toxicity. *Alternative Medicine Review*. 2006; 11:114-127.
 22. Raharjo R., Sudjarwo SA, I'tishom R. Effects of Red Dragon Fruit (*Hylocereus polyrhizus*) Skin Extract on Lead Acetate Toxicity in the Morphology of Balb/c Mice (*Mus musculus*) Spermatozoa. *Folia Medica Indonesiana*. 2017; 53(4):237-241.
 23. Rao F, Zhai Y, Sun F. Punicalagin mollifies lead acetate-induced oxidative imbalance in male reproductive system. *International Journal of Molecular Science*. 2016; 17(8):1269.
 24. Samie A, Sedaghat R, Baluchnejadmojarad T, Roghani M. 2018. Hesperetin, a citrus flavonoid, attenuates testicular damage in diabetic rats via inhibition of oxidative stress, inflammation, and apoptosis. *Life Science*. 210: 132-139.
 25. Schlosser P, Asgharian B, Medinsky M. Inhalation Exposure and Absorption of Toxicants. *Comprehensive Toxicology*. 2010; 1:75-109.
 26. Soleimanzadeh A, Kian M., Moradi S, Malekifard F. Protective effects of hydro-alcoholic extract of *Quercus brantii* against lead-induced oxidative stress in the reproductive system of male mice. *Avicenna Journal of Phytomedicine*. 2018; 8(5):448-456.
 27. Soleimanzadeh A., Kian M., Moradi S, Mahmoudi S. Carob (*Ceratonia siliqua* L.) fruit hydro-alcoholic extract alleviates reproductive toxicity of lead in male mice: Evidence on sperm parameters, sex hormones, oxidative stress biomarkers and expression of Nrf2 and iNOS. *Avicenna Journal of Phytomedicine*. 2020; 10(1):35-49.
 28. Sudjarwo SA, Eraiko K, Sudjarwo GW, Koerniasari. The potency of chitosan-Pinus merkusii extract nanoparticle as the antioxidant and anticaspase 3 on lead acetate-induced nephrotoxicity in rat. *Journal of Advance Pharmaceutical Technology & Research*. 2019; 10(1):27-32.
 29. Sutanto, EB, Nasihun TR, Isradji, I, Sutanto, LB. The effect of vitamin C and E combination on sperm quality and cement 8-OHdG level of smoke exposed rats. *World Nutrition Journal*. 2017; 1(1): 28-33.
 30. Wulandari WY, Darmadji, P, Kurniawati L. Antioxidant Properties of Kaffir Lime Oil as Affected by Hydrodistillation Process. *Proceedings ICTESS. Universitas Slamet Riyadi*. 2017; 1(1):246.