Journal of Natural Sciences Research www.iiste.org
ISSN 2224-3186 (Paper) ISSN 2225-0921 (Online)
Vol.4, No.17, 2014

The Activities of Pumelo Fruit Juice (Citrus maxima var nambangan), Vitamin C and Lycopene against Hepatotoxicity Ochratoxin Exposure Prevention on Induced Mice (Mus musculus)

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ABSTRACT

The aim of this study was to know the potency of pumelo fruit juice *(Citrus maxima var nambangan)*, vitamin C, lycopene, and combination of vitamin C and lycopene, as antioxydant compound on hepatotoxicity of ochratoxin A (OTA) on induced mice (*Mus musculus).*

Thirty-five male mice (*Mus musculus)* strain of balb/c, with age between two and three months were divided randomly into seven groups of treatments (n=5), including K0, Kl, K2, P1, P2, P3, P4. Each from respectively group for control group was feed only with Olive Oil adjuvant (KO), CMCNa (K1), ochratoxin A (K2), pumelo fruit juice (*citrus maxima var nambangan)* dose of 0,5 ml/30 gram BW/day (PI), vitamin C dose of 5,85 µg (P2), lycopene dose of 0,1025 µg (P3), and the combination of vitamin C dose of 5,85 µg /30 g BW mice and lycopene dose of 0,1025 µg/30 g BW (P4). The experiment was conducted for three weeks periode, which is K0, Kl, K2 groups were subjected with compound of treatment for a week period started at second week, whereas P1, P2, P3, and P4 groups were subjected with compound of antioxidant treatment for two weeks period started on second week, and subjected with ochratoxin A dose 1 mg/kg BW/day for a week in the beginning of the third week. All the experimentation mice were sacrifice for data collection on the 21st of days.

The statistical analisis result was analyzed with Kruskal Wallis test which continued with Mann-White showed there was a significant different ($p<0.05$) between groups of treatment. It has been proved that all groups subjected to antioxidant (PI, P2, P3, dan P4) significantly ($p<0,05$) were able to prevent the damage of liver cell after the ochratoxin A exposure, which was indicate on the descending of necrotic liver cell.

This research also proved there was no significant different ($p>0.05$) the biologic potency of Pummelo fruit juice compare with vitamin C, lycopene and the combination of lycopene and vitamin C on inhibited free radical reactivity due to OTA exposure on mice' liver.

Keywords: Pumelo Fruit *(Citrus maxima var nambangan),* Ochratoxin, Hepatotoxicity

INTRODUCTION

Ochratoxin (OTA) is a mycotoxin produced by a type of fungus. Mycotoxins are commonly found as contaminants in foodstuffs and processed foods, among others, on grains such as corn, wheat, rice, beans, soybeans, coffee, cocoa, spices, wine and fruits (Papachristou and Markaki, 2004). It was proof that yellowish discoloration on rice might occur due to the ochratoxin contamination, which produced by *Aspergillus ochracius* and *Penicillium verucosum* (Guzev et al, 2008).

Currently ochratoxin metabolisms in mammalian bodies are not yet fully recognize. Two organs that are acquinted to have an important role in the biotransformation ochratoxin, which are kidney and liver, all at once negatively affected in cases of ochratoxin poisoning. It was report that, in case of ochratoxin poisoning, the highest mycotoxin compounds found accumulated in blood, kidney and liver (Ringot et al., 2006).

Ochratoxin dimetabolism in the liver produce reactive metabolites. This change catalyzed by P - 450 enzymes by means of oxidizes ochratoxin through the chain reaction of hidroklasi substrate. This chain reaction will produce $O_2^{\bullet-}$ (superoxide) and H_2O_2. $O_2^{\bullet-}$ is very dangerous when it is concurrent with H_2O_2 because these might formed hydroxyl radicals ($OH^{\bullet}$). Besides, $OH^{\bullet}$ could reacted with transition metals such as Fe^{2+} and Cu^{2+} through the Fenton reaction (Halliwell and Gutteridge, 2007). Cell damage caused by free radicals may occur through lipid peroxidation of cell membranes or organelles.

Lipid peroxidation in cell membranes will lead to increasing permeability membrane, resulting in passive mitochondrial swelling which will exacerbate the cell damage (Hayati, 2011). Mitochondrial membrane damage may result in decreasing ATP production, which resulted in disruption of the cell permeability membrane and Na^- K pump; it leads to the accumulation of water in the cells and organelles (hydropic degeneration). The retrogressive cells process, which is reversible, may turn into irreversible and triggers the death of cell in necrosis manner, if there is an increase accumulation of intracellular calcium ions, which enter the cells through disruption of the permeability membrane cell (Gavin, 2007).

The increasing of intracellular Ca^{++} causes the activation of a number of enzymes such as phospholipase, protease, and endonuclease, which will cause damage to the DNA in the nucleus cell. The increasing of protease enzymes may also cause damage to the mitochondria which is characterized by the formation of the porous on mitochondria called *mitochondrial permeability* transition (MPT) that will result the death of cell in apoptotic manner (Widodo, 2003; Sudiana, 2008).

Basically free radicals such as, $O2^{\bullet -}$, H_2O_2 and $OH^{\bullet}$ can be formed in the normal body's metabolic processes, but this can be mitigated by antioxidant enzymes such as catalase body, *superoxide dismutase* (SOD), glutathione and other antioxidant compounds that derived from food material, such as vitamin C, vitamin E, and selenium. Continous induction of free radicals and the increasing number of free radicals that comes from outside the body may cause balance disorders of antioxidant defenses, resulting in oxidative stress.

The addition of antioxidant supplements that come from outside the body is one of the effective ways in reducing oxidative stress. One of the foods that contain many antioxidants is pomelo fruit in which recognized containing vitamin C and lycopene. Pomelo fruit *(Citrus maxima)* has been proven containing vitamin C, and lycopene, which has antioxidant effects. Lycopene is known to work as an antioxidant by capturing free radicals *(Scavenger antioxydant)* and break the chain peroxidation that triggered by free radical (chain breaking antioxydant), while vitamin C acts as an antioxidant by capturing free radicals.

The content of antioxidants vitamin C and lycopene in Pomelo fruit are quite high. Pomelo fruit pulpy *(Citrus maxima)* is containing 43 mg of vitamin C and 350 μ*g* of lycopene per 100 grams of the pulpy (Maulida and Zulkarnain, 2010).

Research on the impact of renal ochratoxin toxicity have carried out excessively, however, the impact of ochratoxin against liver damage (hepatotoxicity) is still very limited. As a tropical country with abundant biodiversity resources, this study aims to determine the potential of Pomelo fruit juice *(Citrus maxima var nambangan)* as local fruit varieties, as antioxidants in preventing damage and function of hepatocyte cells caused by exposure of ochratoxin.

Problem Formulation

Based on the background of the problem, following is the problem formulation: Is the provision of Pomelo fruit juice *(Citrus maxima var nambangan)*, vitamin C and lycopene may decrease the score of necrotic liver cells of mice that caused by ochratoxin exposure?

Research Objectives

- Understand the effect of Pomelo fruit juice provision (*Citrus maxima var nambangan),* vitamin C and lycopene in preventing liver damage due to ochratoxin exposure.
- Describe the effect of Pomelo fruit juice provision (*Citrus maxima var nambangan),* vitamin C and lycopene to the degree of liver damage due to ochratoxin exposure.

Research Methods

This kind of research is the experimental research laboratories, by using a design *Randomized post test only control group design.* Samples and treatments cultivated under controlled conditions and in order, which is the influence of treatments, are more believable.

Experimental Units

Experimental units were used in this study were (*Mus musculus)* strain male BALB / C adult, between the ages of two to three months and weigh between 20 to 30 grams . Experimental animals obtained from experimental animal care unit Veterinaria Farma Center (PUSVETMA) Surabaya.

Based on the results of a calculation, the amount of replication or the replication consist of at least four experimental animals in each group. This study used 35 male animals where there are five animals in each treatment group.

Operational Definition of Variables

Necrotic score hepatocyte cells in this study is the data that assessed by means of semiquantitative scoring method according to Isaac (2006) which has been modified, with the following model :

Score

0 (zero) : if there are no necrotic hepatocyte cells.
1 (one) : if the number of necrotic cells is less than 25 % of the field of view (FW) at 1000-x magnification.
2 (two) : if the number of necrotic cells between 26-50 % of the field of view (FW) at 1000-x magnification.
3 (three) : if the number of necrotic cells between between 51-75 % of the field of view (FW) at

1000x magnification.

4 (four) : if the number of necrotic cells more than 75 % of the field of view (FW) at 1000-x magnification.

Materials research which needed and used in this study were pomelo fruit juice that is standardized which is pomelo fruit juice (*citrus maxima var nambangari).* It is standardized that contain vitamin C at 416.50 µg/ml and lycopene was 7.60 µg/ml. Materials for the manufacture of animal models is needed in this study were male mice, chaff, standard feed, drinking water, HCL, ether, alcohol 70 %, and formalin.

Decision Procedure and Data Collection

The data analyzed were take from:

Results of histopathological examination of liver tissue by means of necrotic scoring of hepatocyte cells according to the method of Isaac (2006) on 10 field of view as well as counting the number of hepatocytes cells that undergo apostosis by staining cytochemistry on five field of view.

Data Analysis

Toward the obtained data, conducted statistical analysis using SPSS. 18. The method used to determine the normality of the variables distribution was to use a test for normality. Further testing on the data using ANOVA test when it was obtain the normal variable distribution data, while when the variable distribution obtained is not normall, the test will conducted using *Kruskal Wallis.*

Research Implementation

Animal Preparation Experiments

This study was conduct over three weeks, consisting of the first week on adaptation and the two-second week on treatments. Mice were adapted for one week before conducted the treatment, maintained, and shared by the group as it has shown in experimental design. This study obtained seven treatment groups, each consisting of five mice.

Research Results

In this study, necrotic hepatocyte cell score data is the data that assessed by means of semiquantitative scoring method according to Isaac (2006), which has modified. The degree of liver cell damage score in this study determined by the amount of the death of hepatocytes cell with scoring models as follows:

Score

0 (zero) : if there are no necrotic hepatocyte cells.

1 (one) : if the number of necrotic cells is less than 25 % of the field of view (FW) at 1000-x magnification.

2 (two) : if the number of necrotic cells between 26-50 % of the field of view (FW) at 1000-x magnification.

3 (three) : if the number of necrotic cells between between 51-75 % of the field of view (FW) at 1000x magnification.

4 (four) : if the number of necrotic cells more than 75 % of the field of view (FW) at 1000-x magnification.

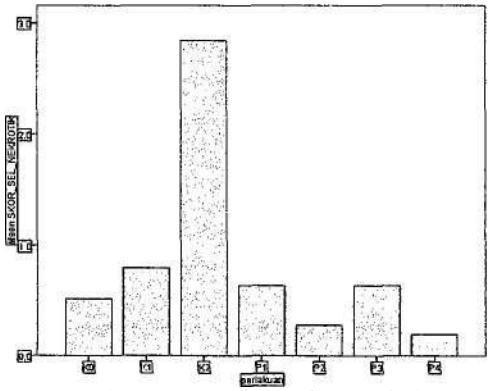
Statistical analysis by *Kruskal Wallis* test that the effect of pomelo fruit juice (*Citrus maxima var nambangan),* vitamin C, lycopene, and a combination of vitamin C and lycopene, to hepatocyte cells score of necrotic mice that exposed by ochtratoxin A, proving that there is a very real difference ($p < 0.01$) among treatments. The average and standard deviation of necrotic hepatocyte cells scores of mice in all treatment as we could see in the table 5.3 and figure 5.2.

In the table and figure 5.3 it is show us that, provision of the pomelo fruit juice (PI) was proved significantly ($p < 0.05$) to decreasing the scores level of necrotic hepatocyte cells in mice that exposed by ochratoxin. The benefits of pomelo fruit juice (PI) in decreasing scores of hepatocyte cells on necrotic mice that exposed by ochtratoxin A in this study, was not significantly different ($p > 0.05$) with vitamin C (P2), lycopene (P3), and a combination of vitamins C and lycopene (P4).

Table 5.3 Average ± SD hepatocyte necrotic cells score in all treatment

Treatments	Average ± Standard Deviation
K_o *(Olive Oil)*	0.52 [a]± 0.83
Ki (CMCNa)	0.80 [a]± 0.67
K_2 (Ochratoxin A)	2.80 [b]±0.45
P_1 (SBJB)	0.64 [a]± 0.29
P_2 (Vitamin C)	0.28 [a]± 0.52
P_3 (Lycopene)	0.64 [a]± 1.04
P_4 (Vitamin C + Lycopene)	0.20 [a] ±0.14

Different superscripts in the same column indicate the significant differences at the level of $\alpha = 0.05$ ($p < 0.05$)



Gambar 5.2 Average Bars Diagram for hnecrotic epatocyte cells score on all treatments. K0: *Olive oil,* Kl: CMCNa, K2: Ochratoxin A, PI: Pomelo Fruit Juice, P2: Vitamin C, P3: Lycopene, P4: Vitamin C+lycopene.

The average score of necrotic hepatocyte cells of mice in the group that given pomelo fruit juice (*Citrus maxima var nambangan),* vitamin C, lycopene, and a combination of vitamin C and lycopene, are respectively recalled 0.64 ± 0:29, 0:28 ± 0:52, 1:04 ± 0.64 , and 0:20 ± 0:14. It was significantly ($p < 0.05$) proved to be lower when compared to the group given only ochratoxin A (K2) where the average score of necrotic hepatocytes cells reached 2.80 ± 0:45. Meanwhile, the average score of necrotic hepatocyte cells of mice in the group given pomelo fruit juice (*Citrus maxima var nambangan),* (PI), vitamin C (P2), lycopene (P3), and a combination of vitamin C and lycopene (P4), not significantly different ($p > 0.05$) compared with all the negative control group who received adjuvant Olive Oil (K0) and the adjuvant CMCNa (Kl).

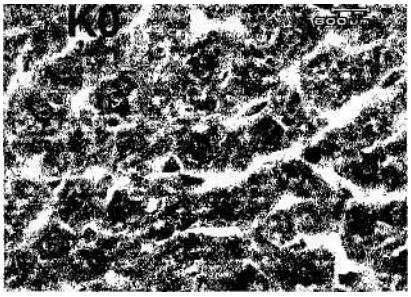


Figure 5.3 Hepatocyte cells overview on K0 group (provision of *Olive oil).* (HE coloring; 1000x magnified, OlympusCX41; DP 12 Olympus micro digital camera 2 megapixel)

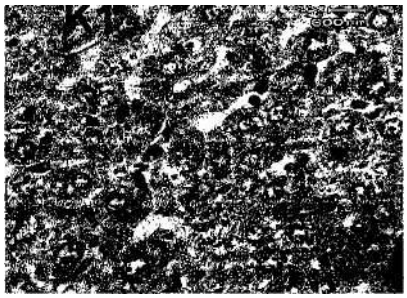


Figure 5.4 Hepatocyte cells overview on K1 group (provision of *CMCna).* (HE coloring; 1000x magnified, OlympusCX41; DP 12 Olympus micro digital camera 2 megapixel)

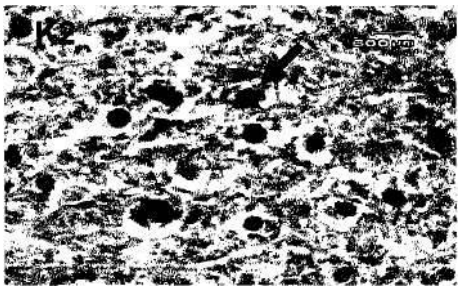


Figure 5.5 Amount of necrotic cells overview with percentage more than 75% (score 4) occurring on the group that given the ochratoxin A (K2). (HE coloring; 1000x magnified, OlympusCX41; DP 12 Olympus micro digital camera 2 megapixel)

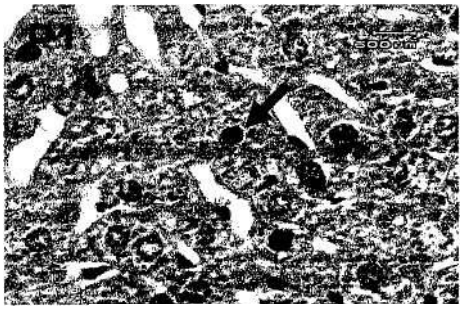


Figure 5.6 Hepatocyte cells overview on P1 group (provision of *pomelo fruit juice)).* Necrotic Hepatocyte cells score are less than 25% (score 1) (HE coloring; 1000x magnified, OlympusCX41; DP 12 Olympus micro digital camera 2 megapixel)

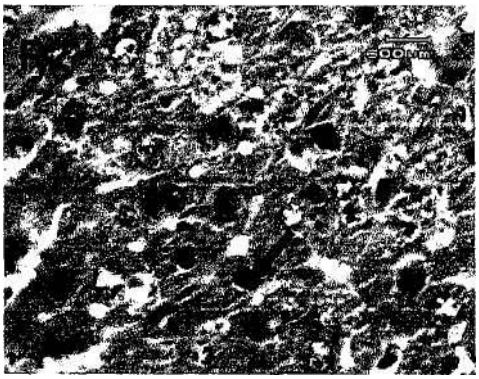


Figure 5.7 Hepatocyte cells overview on group of P2 (Vitamin C provision). Necrotic hepatocyte cells score are less than 25 % (score 1). (HE coloring; 1000x magnified, OlympusCX41; DP 12 Olympus micro digital camera 2 megapixel)

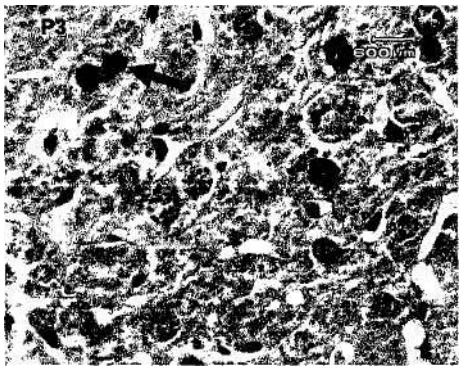


Figure 5.8 Hepatocyte cells overview on group of P3 (Lycopene provision). Necrotic hepatocyte cells score are less than 25 % (score 1). (HE coloring; 1000x magnified, OlympusCX41; DP 12 Olympus micro digital camera 2 megapixel)

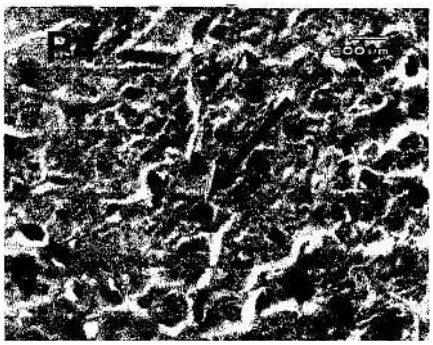


Figure 5.9 Hepatocyte cells overview on group of P4 (Vitamin C + Lycopene Provision). Necrotic hepatocyte cells score are less than 25 % (score 1). (HE coloring; 1000x magnified, OlympusCX41; DP 12 Olympus micro digital camera 2 megapixel)

Discussion

Biological potential of pomelo fruit *(Citrus maxima var nambangan)* in protecting against various hepatocyte cell damage, believed to be linked to the antioxidant compounds contained therein , among others, lycopene and vitamin C (Astawan, 2009). Vitamin C content of pomelo fruit *(Citrus maxima var nambangan)* in this study was 416, 50 ug / ml, and licopene content was 7.60 µg / ml.

Lycopene content in pomelo pulp is high enough, which is 350 micrograms per 100 grams of the pulps (Astawan, 2009), whereas the content of vitamin C in pomelo fruit is 43 milligrams per 100 grams of the pulps (Waljono, 2006). According to Tsai et al., (2007) on the antioxidant that containing on pomelo fruit (Citrus grandis (L.) Osbeck) with red pulps is higher than the white pulps. Pomelo (Citrus grandis (L.) Osbeck) with red colored pulps has a phenol content of 8.3 mg / mL, and vitamin C and tocopherols , respectively for 472 and 0:35 mg / mL.

Lycopene and vitamin C, which are contained in pomelo (Citrus maxima var nambangan), is believed to be the two very potent antioxidants in preventing oxidative stress in liver cells caused by chratoxin exposure (Waljono, 2006).

Lycopene is a powerful antioxidant, which is roled as a scavenger antioxydant, lycopene is able to bind superoxide radical ($O2^{\bullet-}$), which is formed during ochratoxin exposure (Mackinnon et al., 2011). The effectiveness of lycopene in capturing the superoxide radical is 100 times more powerful than vitamin E, as well as, or 12,500 times more powerful than gluthation (Mascio et al., 1989). Lycopene reported to have the ability to prevent the peroxidation of LDL (low-density lipoprotein) and lower levels of oxidized LDL (ox - LDL), so it could potentially useful to prevent the development of cardiovascular diseases (Giovannicci, 2005).

Vitamin C has reducing characteristics (reducing agent) as well as vitamin E as an antioxidant and works by capturing superoxide radicals, as well as muffle the activity of hydrogen peroxide, hypochlorite, hydroxyl radicals and peroxyl radicals. Vitamin C effectively inhibit lipid peroxide initiated by peroxyl radicals that can protect membrane damage (Padayatty, 2003).

The role of antioxidants in preventing damage to the hepatocyte cell membrane during the event of lipid peroxidation can occur through several mechanisms, among others, by means of binding the metal ions, capture free radicals and inhibit peroxidase enzymes. The workings of antioxidants in inhibiting peroxidation generally involves more than one mechanism at a time (Mokbel and Suganuma, 2006).

Natural antioxidant obtained through the consumption of fruits, such as pomelo fruit (*Citrus maxima var nambangan)* generally provide better results, when compared with the processed antioxidants or synthetic antioxidants. Antioxidant obtained by consuming natural fruit or vegetable can provide more optimal results, because in most cases the natural sources of antioxidants, some compounds may work synergistically with each other (Mokbel and Suganuma, 2006; Singh et al., 2011).

Based on the assumption that the damage of the hepatocyte cells by exposure ochratoxin A (OTA) is due to oxidative stress via lipid peroxidation of cell membranes, the proision of antioxidants, is expected to be used as an effective hepatoprotective compound (Mokbel and Suganuma, 2009).

The results of this study demonstrate that the provision of pomelo fruit juice (*Citrus maxima var nambangan);* can prevent necrosis on hepatocytes cells caused by exposure ochratoxin A (OTA). The degree of hepatocyte necrosis of the cells in the group that given only ochratoxin A (OTA) or the positive control group was 2.80 ± 0:45, while in the exposed group, but given the pomelo fruit (*Citrus maxima var nambangan),* the degree of hepatocyte necrosis cells smaller and significantly different ($p < 0.05$), ie 0.64 ± 0:29 .

These results are the same with the previous studies reported by Kundusen et al (2011), which proves that the fruit extract of *Citrus maxima Merr.,* with doses of 200 and 400 mg/kg BW/day, for 15 days may prevent the damage on white mouse hepatocyte cells that induced by high doses of paracetamol. Histopathological picture of the liver in the form of hydropic degeneration and necrosis fokalis dropped dramatically in the group given the extract of fruit of *Citrus maxima Merr.,* compared to the control group. Hepatoprotective function of *Citrus maxima Merr extracts.,* believed to be associated with a high content of antioxidants, which can prevent cell damage caused by oxidative stress induced by high doses of parecetamol.

Histopathological lesions overview hepatocyte cells post exposure with ochratoxin A in this study according to research conducted by (Kundusen et al, 2011), where the research using paracetamol as an inductor of damage, which is the dominant lesion are hydropic degeneration and necrosis.

Omar, et al, (2012) proved that vitamin C 100 mg / kg BW/day for four weeks as an antioxidant is effective to protect cells of white mouse hepatocytes from oxidative damage due to provision of CP (cisplatin), which is anti-cancer drugs that has broad spectrum.

Like vitamin C, lycopene is an antioxidant compound from the class of carotenoids that effective in protecting cells hepatocyte from due to oxidative stress.

In this study, the potential hepatoprotective of pomelo fruit juice (*Citrus maxima var nambangan)* are known to have lycopene content by 7, 60 µg/ml proved to be not significantly different ($p > 0.05$) with the combined provision of the lycopene compound or vitamin C and lycopene which are derived from the finished

product.

Shivashangari, et al., (2006) reported that provision of lycopene at a dose of 10 mg/kg/day for six days orally, might prevent Lipodosis (melemak) degeneration and centralis necrosis cells in the white mouse hepatocytes induced by D-Galactosamine/Lipopolysaccharide (D-GalN/LPS). According Shivashangari, et al., (2006), lycopene may prevent melemak degeneration through its role in inhibiting the activation of phospholipase A2, as well as preventing cell necrosis through its activity in maintaining homeostasis of calcium ions.

It was also report that, in addition to act as an antioxidant, potential hepatoproktetif of lycopene could derived from its ability to prevent cell damage induced by ischemia. Patel, et al., (2011) proved that provisiom of lycopene at a dose of 10 mg/kg BB may prevent the damage to hepatocytes cells on middle lobe and in the left manipulated due to ischemia by clamping the hepatic artery and the left branch of the hepatic portal vein for 45 minutes.

It was show that the provision of lycopene might improve the status of other antioxidants in the body, as reported by Sulityowati (2006). It was proof that the provision of lycopene at a dose of 0.36; 0.72 and 1.08 mg/head/day, might increase the levels of vitamin C, vitamin E and Glutathione in the blood plasma of white mouse that fed a high cholesterol diet.

Conclusions and Conclusion Recommendations

Based on these results, it could be concluded that:
The provision of pomelo fruit juice (*Citrus maxima var nambangan),* vitamin C and lycopene may reduce the levels of *Malondyaldehid* (MDA) mice liver tissue that caused by ochratoxin exposure.

Suggestion

Need to do the further research to:

1. Do the further research concerning potential antioxidant of pomelo fruit juice (*Citrus maxima var nambangan)* against ochratoxin toxicity in mice liver with different doses (chronic).
2. Do the further research concerning potential antioxidant of pomelo fruit juice (*Citrus maxima var nambangan)* against ochratoxin toxicity in mice liver by measuring the levels of SOD, GSH and catalase.
3. Do the further research concerning potential antioxidant of pomelo fruit juice (*Citrus maxima var nambangan)* against ochratoxin toxicity in mice by measuring the levels of free radicals directly.
4. Do the further research concerning potential antioxidant of pomelo fruit juice (*Citrus maxima var nambangan)* against ochratoxin toxicity in mice by measuring the expression of important proteins in inducing the death of cell in apoptotic manner.

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