Clove (*Syzygium aromaticum*) Effect on Carbon Tetrachloride-Induced Rat: Comparison of Malondialdehyde Level of Liver and Blood Plasma

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Introduction: Studies suggested antioxidant properties of the content of *Syzygium aromaticum* (clove). The study was conducted to obtain better understanding about the effect of clove on concentration of oxidative stress marker malondialdehyde (MDA) in liver and blood plasma of rat initially induced by carbon tetrachloride (CCl₄); and whether blood plasma MDA level might represent liver condition.

Methods: Experimental research was done using 20 Wistar rats classified into 5 treatment groups: (1) CCl₄- and clove-positive treatment after 3 days of clove treatment, (2) one day after, (3) alpha-tocopherol as positive control, (4) CCl₄ only as negative control, and (5) normal control. Wills method was used for MDA concentration measurement. Results: Liver MDA concentration were 0.0262 ± 0.0010 for group 1, 0.0214 ± 0.0047 group 2, 0 for group 3, 0.0077 ± 0.0094 group 4, and 0.0039 ± 0.0009 control group in nmol/mg protein (p = 0.001), whereas in the blood plasma it was 29.6032 ± 6.8021 for group 1, 26.1103 ± 3.6920 for group 2, 1.1612 ± 0.3555 for group 3, 1.4585 ± 1.4747 for group 4, and 2.4217 ± 1.2382 control group in nmol/mL (p = 0.001).

Conclusion: Contrary to study in the past, no antioxidant properties were observed in treatment with dose 200 mg clove/kg body weight of rat. Such treatment increased MDA concentration and enhanced CCl₄-induced damage in a time-dependent fashion. Strong correlation between MDA concentration in the liver and blood plasma (R² = 0.97; p = 0.003) suggested blood plasma utilisation to represent hepatic MDA concentration or damage.

Keywords: Blood Plasma, CCl₄, Malondialdehyde, Oxidative Stress, Rat Liver, *Syzygium aromaticum*.

1. INTRODUCTION

*Syzygium aromaticum* (clove) is a plant native to east Indonesia¹ and rich in phenolic compounds, namely eugenol and eugenol derivatives.²,³ This plant is highly beneficial from different point of views. It has antioxidant, antimicrobial, antiviral and antinociceptive activity.²,³ The application of this plant can lead to pain relief,² free radicals scavenging activity,³,⁴ and even down regulation of lipogenic and adipogenic gene expression.⁶ This potent bacterial growth inhibitor deserves special attention due to its antioxidant properties standing out in comparison to other vegetables, spices and fruits.²,⁷ There is a high correlation between polyphenol content and the antioxidant properties against oxidative stress.²,³

Oxidative stress, which is the imbalance between antioxidant and prooxidant levels in favor of prooxidants,² can be induced by carbon tetrachloride (CCl₄). That condition threatens human life and leads to effort to find antioxidant alternative. The damage itself is actually caused by trichloromethyl radical formed by reaction between cytochrome P450 with that halomethane. Furthermore, a more reactive radical, namely trichloromethylperoxy radical can be produced from the previous free radical.⁸ Lipid peroxidation is one of the possible consequences of this adverse activity. Secondary to lipid peroxidation, malondialdehyde (MDA) is formed, which has been used as a biomarker to assess the antioxidant activity of a substance in tissue with oxidative stress-induced damage.³ Several studies used certain tissue only or duration of the treatment only as independent variable. Unidirectional flow of blood in the liver makes heart become the end destination of blood flow from the liver. There is possible hematogenous spread of chemical substance, including free radical, to the heart following its prior major biotransformation. However, blood in the heart should contain less harmful material to be pumped to lungs and other parts of the body in normal
calculated by using Federer formula as follows:

\[ t(n - 1) \geq 15 \]

where \( t \) is the number of treatment types and \( n \) is the sample required for each treatment.

In order to do this research, there are 10 kinds of treatment that were designated to make the MDA concentration comparison clear. For each type of material, there are 5 treatment types that will be used: (1) CCl4- and clove-positive treatment after three days of clove treatment, (2) CCl4- and clove-negative treatment after one day of clove treatment, (3) CCl4- and alpha-tocopherol-positive treatment as positive control, (4) CCl4- and clove-positive and clove-negative treatment as negative control, and (5) both CCl4- and clove-negative and clove-positive treatment as normal control. Since there were liver and blood plasma required for this research, total treatment types are 10. The calculation is as follows:

\[ (t - 1)(n - 1) \geq 15 \]
\[ 9(n - 1) \geq 15 \]
\[ 9n - 9 \geq 15 \]

Minimum value of \( n \) should be greater than or equal to four. By having \( n \) equal to 4, the required sample types will be 40, that need 20 preserved blood plasma samples and 20 preserved livers with prior specific treatment.

Clove extract and CCl4 were prepared prior to any treatment to the rat. CCl4 dose that was used was 200 mg/kg body weight, whereas the CCl4 dose was 0.55 mg/g body weight. One hand, 40 g of dried clove was crushed to become smoother and mixed with 1 L of water for 5 days. Every 24 hours, the mixture was stirred with glass stirring rod before being kept in cooler temperature as cool as 4 °C. Finally in the mixture, the concentration of clove was 40 mg/mL. On the other hand, CCl4 was mixed into palm oil afterwards based on the required dose. By having that standard, a rat that weighed 200 g received 110 mg CCl4. The density of the CCl4 is 1.59 g/mL. This meant that there was 0.11 g in 0.069 mL CCl4 solution. In order to do so, to create 50 mL solution to be used, 3.45 mL CCl4 was diluted in palm oil until the volume reaches 50 mL.

Treatment was done to rats followed by liver and blood plasma with the prepared material. There were 5 types of treatment. The treatment groups are (1) CCl4- and clove both-negative treatment as normal control, (2) CCl4-positive and clove-negative treatment as negative control, (3) CCl4- and alpha-tocopherol both-positive treatment as positive control, (4) CCl4- and clove-positive treatment after one day of clove treatment, and (5) CCl4- and clove-positive treatment after three days of clove treatment. The rats included in the last 4 aforementioned groups that underwent initial CCl4 treatment on day 0, followed by certain procedure, namely the ones with additional 3 days clove administration, those with only 1 day clove administration afterwards, those with alpha-tocopherol on the following day and those with immediate extraction of liver and blood plasma on the next day. The administration of prepared material used intubation method. Extraction was done to all 4 groups on the day after the last treatment. On the other hand, rats in normal control group proceeded directly to the extraction phase with no prior treatment. Rat was killed by neck dislocation method. Plasma and liver was extracted immediately. The tissue extraction started from thoracic region by using scissors. Furthermore, the tissues were put onto scale to know the weight. Blood plasma was taken from the apex of the rat heart. Intact rat heart apical region was cut and the blood is taken. Further process was done to separate the plasma from other components of the blood.

Following the extraction of liver and blood plasma, homogenate could be obtained by adding phosphate buffered saline (PBS) solution 1 mL per 100 mg of preserved tissue and the tissue was also crushed and blended to become smoother by using micropestle. The homogenate was mixed by using vortex and centrifuged for 10 minutes by applying 3000 rpm speed and 4 °C temperature. Supernatant was taken away from the homogenate to the tube. Parafilm covered the opening of the tube and the tube was put into –84 °C freezer afterwards.

Protein and MDA concentrations were measured in this study using spectrophotometry method. The former utilized BSA standard solution set into 5 different concentrations ranging from 100 mg/mL to 500 mg/mL. Then, absorbance test was conducted to compare the protein concentration the standard solutions and
sample by setting the wave length to 280 nm. On the other hand, MDA measurement utilized Wills method involving the mixing of several substances. Firstly, 5 standard solutions were prepared each of them having different initial MDA volume ranging from 5 μL to 80 μL. MDA used in standard solution 2 was twice as many as the one in standard solution 1. Similarly, MDA used for standard solution 3 was also twice as many as the one in standard solution 2, and so on. Additional blank tube was also prepared for this measurement with no MDA added at all. After all standards, blank and samples were prepared, distilled water was added to each of them so that the total volume would be 400 μL. For samples, the amount of water and added homogenate or blood plasma determined the dilution factor of the sample, which was important for further data analysis. 200 μL of TCA 20% was added to each mixture. Afterwards, each mixture was homogenized by using vortex and followed by centrifugation with 6000 rpm for 5 minutes. Supernatant obtained from the latter was obtained. Final addition of 400 μL of TBA 0.67% was required in this procedure. Mixing of the mixture adequately was important prior to heating for 10 minutes at certain temperature about 100 °C. Parafilm was utilized as cover of the tubes during the heating process. Running water cooled the tubes down after the heat. At last, spectrophotometer was used for absorbance reading with wave length of 530 nm.

The data was collected and analyzed by using Shapiro-Wilk method. If the data is normally distributed, ANOVA test and Pearson correlation will be selected to know the clove effect as an antioxidant and the effect of treatment duration respectively. In contrast, nonparametric test should be used if the data do not meet the assumption of the parametric test. In the case of abnormal data distribution, Kruskal-Wallis test and Spearman correlation should be selected.

Much effort has been made by the researcher to avoid any ethical issue. In order to do so, the researcher made an effort to receive the ethics agreement from Medical Research Unit of Faculty of Medicine Universitas Indonesia to receive the ethical permission, with No. 255/UN2.F1/ETIK/2016.

3. RESULT

After following the steps of laboratory work, the data was obtained from rat liver, see Table I and Figure 1. On the other side, similar increase of the concentration was observed in rat blood plasma, see Table II and Figure 2. By comparing the results of both tissues, scattergraph was made showing the mean value distribution, see Figure 3. The mean result of each group was compared to the normal control to obtain the ratio, see Figure 4.

Following the collection of the essential data for the analysis, the data were statistically analyzed. First, normality test was compulsory to determine the type of data. The data distribution of this research was proven to be normal. Thus, ANOVA test and Pearson correlation were selected.

### Table I. Mean liver MDA concentrations.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>+/- Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver day 3</td>
<td>0.028168612</td>
<td>0.001074686</td>
</tr>
<tr>
<td>Liver day 1</td>
<td>0.021301113</td>
<td>0.004792708</td>
</tr>
<tr>
<td>Liver positive control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Liver negative control</td>
<td>0.007730426</td>
<td>0.009498904</td>
</tr>
<tr>
<td>Liver normal control</td>
<td>0.0003895081</td>
<td>0.000920386</td>
</tr>
</tbody>
</table>

### Table II. Mean blood plasma MDA concentrations classified according to each group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>+/- Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma day 3</td>
<td>29.6032356</td>
<td>6.8020752</td>
</tr>
<tr>
<td>Plasma day 1</td>
<td>26.1103627</td>
<td>3.6920692</td>
</tr>
<tr>
<td>Plasma positive control</td>
<td>1.1612042</td>
<td>0.35555782</td>
</tr>
<tr>
<td>Plasma negative control</td>
<td>1.4585666</td>
<td>1.47474918</td>
</tr>
<tr>
<td>Plasma normal control</td>
<td>2.4217318</td>
<td>1.23819041</td>
</tr>
</tbody>
</table>

4. DISCUSSION

MDA is a by-product of lipid peroxidation in oxidative stress damage. In humans, the acceptable amount of eugenol consumption established by World Health Organization (WHO) is 2.5 mg/kg body weight per day. Post hoc test revealed that on the third day after clove administration, the MDA concentration was proven to be significantly different from the other liver groups, namely Plasma Positive Control (tocopherol), Plasma Negative Control (CCl₄), and Plasma Normal Control groups; except Liver Day 1 group. This also applied to the Plasma Day 3 group that there was significant difference between that group and other plasma groups, except MDA concentration of Plasma Day 1 group.
The increase of MDA is correlated with hepatocyte apoptosis possible antioxidant activity resulting from clove administration. With carbon tetrachloride. The result was against the theory of result of both rat liver and blood plasma that had been treated treatment, MDA concentration was much higher than the day-1 compared to negative control. After day 3 of the clove extract in both liver and plasma, in general MDA concentration increased in both liver and blood plasma.

As a result, Syzygium aromaticum (clove) administration with the dose utilized in this research increased MDA concentration of carbon tetrachloride-induced rat with oxidative stress both liver and plasma. After day 1 and day 3 treatment using clove extract in both liver and plasma, in general MDA concentration increased compared to negative control. After day 3 of the clove extract treatment, MDA concentration was much higher than the day-1 result of both rat liver and blood plasma that had been treated with carbon tetrachloride. The result was against the theory of possible antioxidant activity resulting from clove administration. The increase of MDA is correlated with hepatocyte apoptosis index.

Although the measurement result value of both tissues cannot be compared due to the difference of the unit, there was a strong correlation between the MDA concentration in the liver and blood plasma \((R = 0.97; p = 0.003)\). This approves that blood plasma MDA concentration can represent hepatic MDA concentration due to hepatic damage.

In human setting, eugenol is used widely as topical anesthetic to enhance the deep inhalation of kretek smoke containing hazardous substances, including nicotine, carbon monoxide and tar. Kretek is the clove/tobacco cigarette most Indonesians smoke. According to Centers for Disease Control (CDC), among 33 Indonesian types of kretek, all of them contained eugenol, specifically 11 of them contained eugenol only, 3 of them contained eugenol and anethole, 9 of them contained eugenol and coumarin, and 10 of them contained all three substances eugenol, anethole, and coumarin. Anethol and coumarin are possible carcinogens, with adverse effect exerted to the liver. There are to date no basis of deriving the acceptable daily dose of eugenol intake alone for human. In Indonesia, British American Tobacco (BAT) allowed 4 mg eugenol to be contained in every cigarette in Indonesia alone due to the cigarette consumption and usage figures in that country, although in 1960 there was a consensus that the maximum content of eugenol in a cigarette is as much as 2 mg. Supposing that the weight of a kretek-smoking man is 50 kg and he is smoking 10 cigarettes daily, his eugenol intake would be 0.8 mg/kg human body weight. That intake is still within the acceptable range established by WHO.

By interpreting the result of this study, the determined dose did not lead to the expected antioxidant effect. The clove dose that was used in this study was 200 mg/kg body weight of rat. The clove dose used in this research is equivalent to human dose of 32.25 mg/kg body weight per day, which contains up to 4.72 mg of eugenol for every kg human body weight. It is almost twice higher than the daily intake limit in human suggested by WHO. The unfavorable damage as interpreted from the result occurred probably due to the high eugenol content, although previous study in the past stated that acute toxicity might occur in rat following administration of 25 times fold higher dose compared to the dose in this study. That study utilized similar clove extract has shown that the dose of 100 mg clove extract per kg body weight of rat was safe and beneficial resulting in antioxidant effect. Moreover, that study also concluded that acute toxicity might occur with the administration as much as 5 g/kg body weight of rat and 2.5 g/kg body weight may induce toxicity in chronic setting of repeated administration for 28 days, whereas our study revealed that acute administration of clove extract as low as 200 mg/kg body weight of rat has resulted in adverse effect on the rat.

The dose used in this study contains up to 29.3 mg of eugenol for every kg body weight of the rat. This is significantly higher than the eugenol dose of 11 mg/kg body weight of rat (LD50), which was proven to be lethal to 50% of the rats exposed with eugenol. However, such fatal outcome was less expected because this study used ingestion as the route of clove extract administration rather than through inhalation. In addition, it might also be due to the type of extract used which is the water-based one.

**5. CONCLUSION**

This research is contradicted with study in the past, no antioxidant properties were observed in treatment with dose 200 mg clove/kg body weight of rat. Such treatment increased MDA concentration and enhanced CCl4-induced damage in a time-dependent fashion. Strong correlation between MDA concentration in the liver and blood plasma \((R = 0.97; p = 0.003)\) suggested blood plasma utilization to represent hepatic MDA concentration or damage.

**References and Notes**


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