

## RESEARCH ARTICLE

# The Influence of Continuous Multi Stage Countercurrent Extraction Process (CMCE) Propolis Extract Administration on Superoxide Dismutase Activity and Hepatocytes Fibrosis Degree (An Experimental Study on Male Wistar Rats Induced with Carbon Tetrachloride (CCl<sub>4</sub>))

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### ABSTRACT

**Introduction:** Continuous Multi Stage Countercurrent Extraction Process (CMCE) propolis extract containing Caffeic Acid Phenethyl Ester (CAPE) may inhibit the formation of lipid peroxidation. However, it is still unclear whether or not CMCE propolis is able to increase SOD activity and reduce acute hepatocytes damages induced by CCl<sub>4</sub>. **Objective:** To evaluate the influence of CMCE propolis extract administration in increasing the SOD activity and reducing the hepatocytes fibrosis of male Wistar rats induced with CCl<sub>4</sub>.

**Method:** an experimental research with a post-test only control group design. Twenty eight male Wistar rats were divided into four groups. Group C-N was only induced with CCl<sub>4</sub>, while group CM-3, CM-7, and CM-14 were induced with CCl<sub>4</sub> and received CMCE propolis extract respectively with 3.6 mg/200 g BW, 7.2 mg/200 g BW, and 14.4 mg/200 g BW. CMCE propolis was administered for 14 days and then CCl<sub>4</sub> was administered on the last day. SOD activity was measured using the colorimetric method, while the hepatocytes fibrosis degree was assessed using Image J Software method with NASH score modification.

**Results:** Post Hoc LSD analysis indicated that SOD activity in group CM-3 (51.67 ± 4.20), CM-7 (70.21 ± 6.26), CM-14 (80.85 ± 4.59) was lower than that in group C-N (23.70 ± 5.12) with P < 0.05. Meanwhile, the Mann-Whitney U test showed that the hepatocytes fibrosis degree in group CM-3 (0.85 ± 0.69), CM-7 (1.14 ± 0.37), and CM-14 (0.71 ± 0.48) was significantly lower than that in group C-N (2.28 ± 1.25) with p < 0.05.

**Conclusion:** The CMCE propolis extract administration was able to increase the SOD activity and reduce the hepatocytes fibrosis degree of male Wistar rats induced with CCl<sub>4</sub>.

**Keywords:** CMCE propolis extract, SOD enzyme activity, hepatocytes fibrosis degree

### ABSTRAK

**Latar Belakang:** Ekstrak propolis Continuous Multi Stage Countercurrent Extraction Process (CMCE) mengandung Caffeic Acid Phenethyl Ester (CAPE), dapat menghambat oksidasi lipid dan pembentukan lipid peroksida. Belum diketahui apakah CMCE propolis dapat meningkatkan aktifitas SOD dan mengurangi kerusakan hepatosit akut akibat induksi CCl<sub>4</sub>. **Tujuan:** Membuktikan pengaruh pemberian ekstrak CMCE propolis terhadap aktivitas enzim SOD dan derajat fibrosis hepatosit tikus jantan wistar yang diinduksi CCl<sub>4</sub>.

**Metode:** Penelitian eksperimental dengan posttest only control group design, sebanyak 28 ekor tikus jantan wistar, dibagi menjadi empat kelompok. Kelompok C-N, hanya diinduksi CCl<sub>4</sub>; kelompok CM-3, CM-7, CM-14, masing-masing diinduksi CCl<sub>4</sub> dan mendapatkan ekstrak CMCE propolis dengan dosis 3,6 mg/200 g BB, 7,2 mg/200 g BB dan 14,4 mg/200 g BB. Pemberian ekstrak CMCE propolis diberikan selama 14 hari, pada hari terakhir diberikan CCl<sub>4</sub>. Aktivitas SOD diukur dengan metode kolorimetri. Derajat fibrosis hepatosit diukur dengan metode Software Image J modifikasi skor NASH.

**Hasil:** Analisis Post Hoc LSD menunjukkan bahwa aktivitas SOD pada kelompok CM-3 (51,67±4,20), CM-7 (70,21±6,26) CM-14 (80,85±4,59) lebih kecil dari pada C-N (23,70±5,12), P<0,05. Uji Mann-Whitney U terhadap derajat fibrosis hepatosit menunjukkan bahwa CM-3 (0,85±0,69), CM-7 (1,14±0,37), dan CM-14 (0,71±0,48) lebih rendah bermakna dibanding C-N (2,28±1,25), p<0,05.

**Kesimpulan:** Pemberian ekstrak CMCE propolis mampu meningkatkan aktivitas SOD dan mengurangi derajat fibrosis hepatosit tikus jantan wistar yang diinduksi CCl<sub>4</sub>.

**Kata Kunci:** Ekstrak CMCE propolis, aktivitas SOD, derajat fibrosis hepatosit

### INTRODUCTION

Liver is one body organ which has an important role in the detoxification and metabolism systems

(Yenny et al., 2011). Liver disease is mostly caused by biochemical reactions of free radicals in cells (Kumar, Abbas AK and Fausto N, 2009). Excessive free radicals

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may cause oxidative stress due to the decreasing natural antioxidants of superoxide dismutase (SOD) which may result in various diseases and liver dysfunctions (Maulina, Meutia, 2013). Chronic hepatitis involves the liver progressive destruction and parenchymal regeneration process leading to fibrosis, cirrhosis, and even hepatoma (Widianto and Rosyidah, 2017). Liver fibrosis is the formation of connective tissues as the response of chronic injuries or free radicals characterized by the activation of hepatic stellate cells (HSC). Antioxidant is able to prevent liver damage (Linawati, 2018). Thus, the administration of antioxidants may prevent and decrease the liver fibrosis (Singal, Jampana and Weinman, 2011). Antioxidants contained in the CMCE propolis extract are believed suppressing the free radicals and increasing the SOD, yet further research is greatly necessary.

“Superblend” propolis is extracted with a four-stage process known as Continuous Multi Stage Countercurrent Extraction Process (CMCE) not only to increase the extraction result (15% from source) but also to maintain the flavonoid and phenolic content (Sabir, 2009). Propolis or bee glue is a natural product from honey bees which has a strong antioxidant activity to fight against oxidants and free radicals. The contained flavonoid may neutralize the antioxidants' bad effects. A strong antioxidant activity may increase the glucose-6-phosphate dehydrogenase (G6PD) expression higher than vitamin E and 4-6 times stronger than vitamin C and N-acetyl-cysteine (NAC) in fighting against the H<sub>2</sub>O<sub>2</sub> oxidant, O<sub>2</sub> free radicals, and reactive oxygen species (ROS) (Pasupuleti et al., 2017).

Superoxide dismutase (SOD) is body's natural defense system which neutralizes and accelerates the degradation of toxic free radical compounds to prevent from the cell damages (Ighodaro and Akinloye, 2018). The Caffeic Acid Phenethyl Ester (CAPE) in propolis extract has an anti-inflammatory activity. The other active compounds include the ester form of coumaric, prenylated p-coumaric, and diterpenic acid which have anti-bacterial and cytotoxic effects as well as the caffeoylquinic acid derivate which has immunomodulator and hepatoprotective effects (Wali et al., 2015). Those various compounds contained in the propolis extract may be used as hepatoprotector to maintain the antioxidant activity in chronic liver damage by inhibiting the hepatic stellate cells (HSC) activation, preventing hepatocyte apoptosis, and reducing liver fibrosis (Wali et al., 2015). Based on the results of previous studies, there were some benefits obtained from active flavonoid and CAPE contained in propolis extract. This research aims examining the

influence of CMCE propolis extract administration to the increasing superoxide dismutase (SOD) enzyme activity and hepatocytes fibrosis degree.

## METHODS

This experimental research was conducted using a post-test only control group research design. The research samples were 8-week male white Wistar rats weighed 180-200 grams randomly divided into four groups consisting of positive control group (N-G) and three treatment groups (CM-3, CM-7, and CM-14). All rats were induced with CCl<sub>4</sub>, while CM-3, CM-7, and CM-14 groups were administered with propolis extract for 14 days. At the end of the research, all rats were induced with 0.4 mg/200 gr of CCl<sub>4</sub>. On day 15, the rats' blood was taken using the colorimetric method to measure the activity of superoxide dismutase (SOD) continued with euthanasia to take the rats' liver organ and then the liver histological specimens were made and observed to examine the fibrosis degree using the Image J Software with NASH score modification. This research was approved by the Medical Faculty of UNISSULA with the Ethical Clearance No.215/III/2019/Komisi Bioetik.

## CMCE Propolis Extract

The propolis extracted using the CMCE method, resulted in flavonoid and phenolic with higher concentration than the original source. The Oxygen Radical Absorbance Capacity (ORAC) verification developed by Brunswick laboratory showed that CMCE propolis has stronger antioxidants. The propolis extracted using the CMCE process had succeeded purifying and eliminating 99.95% dirt, such as heavy metals and pesticides without chemical changes as well as maximally maintaining substances which provide benefits to the propolis extract (Sabir, 2009).

## Determining the Propolis Dosages

The propolis extract dosages used in this research were 200 mg, 400 mg, and 800 mg converted from the dosages for humans to rats weighed 200 g calculated using the formula of  $200 \times 0.018$  and resulted in the dosages of 3.6 mg/200 g, 7.2 mg/200 g and 14.4 mg/200 g administered for 14 days.

## SOD Examination

The superoxide dismutase (SOD) was measured using the BioVision Superoxide Dismutase (SOD) Activity Assay Kit method. Twenty eight male Wistar rats' blood samples were used in this research. The principle of SOD examination was performed with

**Table 1. Fibrosis score of liver cells measured by Image J Software with NASH modification**

| Stages  | Marker  | Score |
|---------|---|-------|
| Stage 0 | No fibrosis                                   | 0     |
|         | Zone 3 perisinusoidal fibrosis                |       |
| Stage 1 | Mild: 1a; Moderate: 1b; Portal/periportal: 1c | 1     |
| Stage 2 | Perisinusoidal and portal/periportal fibrosis | 2     |
| Stage 3 | Bridging fibrosis                             | 3     |
| Stage 4 | Cirrhosis                                     | 4     |

**Table 2. Statistical Analysis Results of SOD Enzyme Activity and Hepatocytes Fibrosis Degree**

| Variable                       | C-N<br>N=5 X(+SD) | CM-3<br>N=5 X(+SD) | CM-7<br>N=5 X(+SD) | CM-14<br>N=5 X(+SD) | P value                        |
|--------------------------------|-------------------|--------------------|--------------------|---------------------|--------------------------------|
| SOD Enzyme Activity (%)        | 23.70±5.13        | 51.67±4.20         | 70.22±6.27         | 80.85±4.59          | >0.05*<br>0.701**<br>0.000***  |
| Liver Cell Fibrosis Degree (%) | 2.286±1.2536      | 0.857±0.6901       | 1.143±0.3780       | 0.714±0.4880        | <0.05*<br>0.010**<br>0.039**** |

Note: \*Saphiro Wilk Test; \*\*Lavene Test; \*\*\*One Way Anova; \*\*\*\*Kruskal Wallis

the xanthine and xanthine oxidase reaction resulted in superoxide radical which possibly reduced the nitroblue tetrazolium (NBT) into purple formazan. The SOD inhibited the NBT reduction through its reaction with the superoxide radical producing O<sub>2</sub> and H<sub>2</sub>O. The SOD was measured using the colorimetric method with the wavelength of 450 nm.

### Liver Fibrosis Degree Examination

The investigation was conducted by analyzing the damaged hepatocytes fibrosis degree using the Sirius Red staining method. After administered for 14 days, the rats were anesthetized with chloroform (euthanasia) to take their liver organ and the specimen preparation was then made for the liver microscopic histopathology observation in which one liver tissue specimen was obtained from each rat. The examination was conducted using a microscope with 400x magnification. The measurement was conducted using the Image J Software with NASH score modification on the fibrosis score (table 1).

### Statistical Analysis

The data normality test was calculated using the Shapiro-Wilk, while the homogeneity of variance between groups was calculated using the Levene Test. The SOD activity test result showed that the data was normal and homogeneous that the difference between groups was then examined using One-way Anova and continued with Post-Hoc LSD test. The test results showed that the liver fibrosis degree was not normal and not homogeneous that the difference between groups

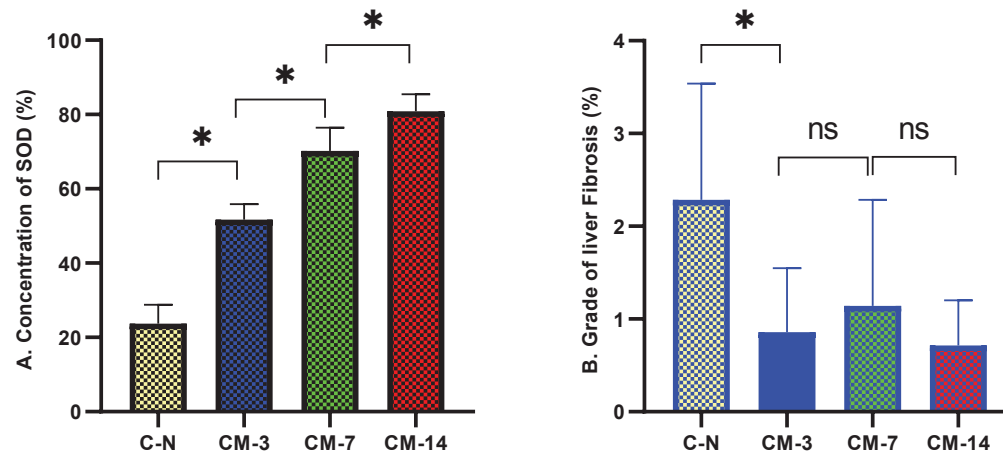
was then examined using Kruskal Wallis, while the discrimination test was conducted using the Mann-Whitney U. The statistical analysis was considered accepted if p<0.05.

### RESULTS

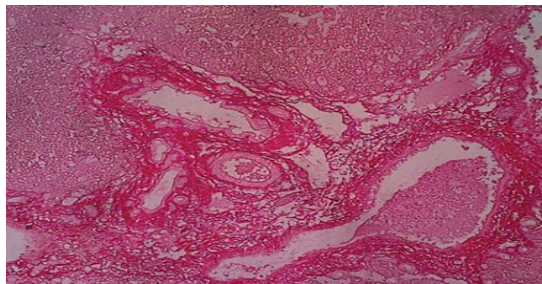
After CMCE propolis extract was continuously administered for 14 days, the CCl<sub>4</sub> was then inducted on day 14. Furthermore, on day 15th, the rats' blood samples were collected to examine the superoxide dismutase enzyme (SOD) and the hepatocytes fibrosis degree as shown in the table 2.

The research result showed that CM-14 group had the highest mean SOD activity, followed by CM-7 and CM-3 groups, while C-N group had the lowest mean. In contrast, CM-14 group had the lowest liver fibrosis degree, followed by CM-3 and CM-7 groups, while C-N group had the highest degree. To see the differences between groups, statistical test was done. One Way ANOVA statistical test was used because the SOD of the four groups was normally distributed. Analysis results showed that SOD activity of different groups were significant, p<0.05. On the other hand, Kruskal Wallis was used to calculate the hepatocytes fibrosis degree as the data were not normally distributed. Analysis results showed that there were significant differences between groups, p<0.05. To know which groups had the differences, Post Hoc LSD test was done regarding to SOD activity and Mann-Whitney U was done regarding the degree of liver fibrosis as described below.

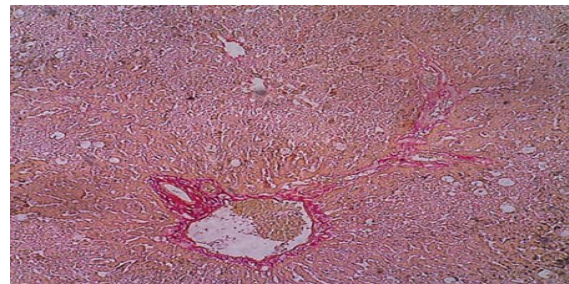




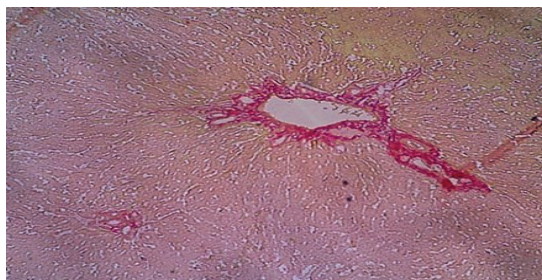
C. C-N



CM-3



CM-7



CM-14

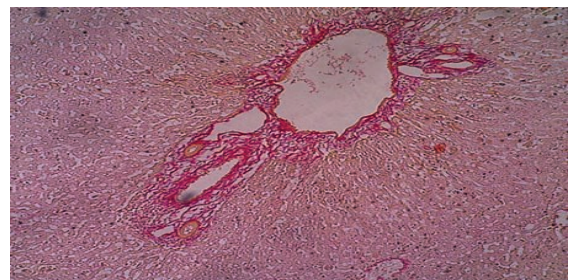


Figure 1. A SOD concentration in each group, B. liver fibrosis degree in each group. C. Histologic preparation with the Sirius Red staining for liver fibrosis degree in each group. Post Hoc/Mann Whitney Analysis: \* $p < 0.05$ ; ns: not significant

### SOD Activity

Post Hoc LSD analysis results showed that SOD activity in CM-14, CM-7, and CM-3 groups were significantly higher than C-N group,  $p < 0.05$ . Further, CM-14 group was significantly lower than CM-7 and CM-3 groups,  $p < 0.05$ . SOD activity in CM-T group was also significantly lower than CM groups,  $p < 0.05$  (Picture 1A).

### Liver Fibrosis Degree

Mann-Whitney U test stated that the liver fibrosis degree in group CM-3 and CM-14 was significantly lower than that in group C-N, each with  $p < 0.05$ . Although the liver fibrosis degree in group CM-7 was lower than that in C-N, the difference was not significant, with

$p > 0.05$ . On the other hand, the liver fibrosis degree in group CM-3, CM-7, and CM-14 was not insignificantly different, each with  $p > 0.05$  (Figure 1B and 1C). Based on the Mann-Whitney U test related to the liver fibrosis degree, it showed that the administration of CMCE propolis extract with the dosages of 3.6 mg/200 g and 14.4 mg/200 g could protect the liver from the CCl<sub>4</sub> induction.

### DISCUSSION

The research results showed that SOD enzyme activity on the administration of propolis extract with the dosages of 3.6 mg/200 g, 7.2 mg/200 g, and 14.4 mg/200 g significantly increased. The results of this research were supported by the previous studies mentioning that

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the administration of propolis supplementation with the dosage of 200 mg/kg for 14 days could increase the rats' SOD enzyme activity, glutathione oxidase, and liver catalase induced by CCl<sub>4</sub> with the dosage of 0.15 mL/kg for 12 weeks (Bhadauria, 2012). The increasing SOD enzyme activity was due to the presence of Caffeic Acid Phenethyl Ester (CAPE), one active component contained in the propolis extract produced from the bee hives and known for its benefits. CAPE is a flavonoid which may inhibit enzymes reactive to the formation of oxygen. Besides, polyphenol may also inhibit the xanthine oxidase enzyme which has an important role in the formation of superoxide anion free radicals (Rajoo et al., 2014).

The research results on the administration of propolis extract with the dosages of 3.6 mg/200 g and 14.4 mg/200 g significantly decreased the liver fibrosis degree. The results of this research were supported by the previous studies showing that the propolis supplementation with the dosages of 0.054 g and 0.108 g might suppress the SOD antioxidant activity. SOD protects the stellate hepatocyte cells from the injuries caused by the exposure of CCl<sub>4</sub> and fibrosis by decreasing the oxidative stress (Gandhi, 2012). The propolis extract with the dosage of 7.2 mg/200 g might not significantly decrease the liver fibrosis, yet different with the positive control results. The antioxidants contained in the propolis extract may inhibit the hepatic stellate cells (HSC) activation, prevent from the hepatocyte apoptosis, and decrease the experimented liver fibrosis. The propolis extract as the immunoregulator, may increase immunity and protect the liver cells from damages by suppressing the activity of kupffer cells in producing ROS and cytokine which worsens the inflammation condition.

## CONCLUSION

The administration of CMCE propolis extract with the dosages of 3.6 mg/200 g, 7.2 mg/200 g, and 14.4 mg/200 g significantly increased the superoxide dismutase (SOD) enzyme activity of male Wistar rats induced with the carbon tetrachloride (CCl<sub>4</sub>). The administration of CMCE propolis extract with the dosages of 3.6 mg/200 g and 14.4 mg/200 g significantly decreased the male Wistar male rats' liver fibrosis degree induced with the carbon tetrachloride (CCl<sub>4</sub>), while that with the dosage of 7.2 mg/200 g could not significantly decrease the liver fibrosis degree, yet different with the positive control results.

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## CONFLICT OF INTEREST

Authors declare there is no conflict of interest within this manuscript.

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