CMCE Propolis Extract Improve Caspase 3 Expressions of The Hepatocytes and IL-6 Levels in Rats Exposed with CCl4

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ABSTRACT

Introduction: Liver plays an important role in the metabolic processes possibly exposed to the toxic materials. One of those hepatotoxic substances is carbon tetrachloride. The propolis extract contains the balsamic active substances, CAPE compounds and flavonoids. Flavonoids can prevent from the apoptosis and reduce the inflammation. Objective: To validate propolis extract’s (CMCE method) ability in improving caspase 3 expressions of hepatocytes and IL-6 levels in Wistar male rats induced with carbon tetrachloride.

Methods: An experimental study with a post-test only control group design applied to the study. The research subjects were 28 male Wistar rats, divided into four groups. The negative control group (CN-G) was only injected with CCl4, while the experimental groups were administered with the CMCE propolis at the dosages of 3.6 mg/200g (CM3-G), 7.2 mg/200g (CM7-G), and 14.4 mg/200g (CM14-G). The CMCE propolis extract was administered for 14 days and on day 14, the CCl4 was then given. The caspase 3 expression of hepatocytes was measured using HE liver cell preparations, while the IL-6 levels were measured using ELISA method.

Results: The results of Mann-Whitney U statistical analysis showed that the hepatocytes’ caspase 3 expressions in CM14-G (2.77 ± 0.531), CM7-G (3.14 ± 0.378), and CM3-G (4.22 ± 0.690) were lower than those in CN-G (5.43 ± 0.535), p<0.05. Meanwhile, the Post Hoc LSD analysis Results showed that IL-6 levels in CM14-G (55.032 ± 9.336), CM7-G (78.362 ± 8.313), and CM3-G (114.975 ± 10.359) were lower than those in CN-G (180.301 ± 5.428), p<0.05.

Conclusion: The administration of propolis extract (CMCE method) improve the caspase 3 expressions of hepatocytes and IL-6 levels in Wistar male rats induced with carbon tetrachloride.

Keywords: Propolis extract (CMCE method), caspase 3 expression in hepatocytes, IL-6 levels.

INTRODUCTION

Liver is an essential organ for human body. As a metabolism organ, liver has function in synthesis, storage, metabolism and detoxification processes (Yenny et al., 2011). Free radicals underlying various cells and tissues damages, including liver, in oxidative stress. Thus, to prevent the damages caused by free radicals to the body, additional (exogenous) antioxidants from outside of the body is greatly required (Tjok Istri Anom S and Wibawa, 2012). Propolis is believed containing antioxidant able to suppress free radicals. However, it is still lacking of evidence that the administration of propolis able to improve the hepatocytes’ damages caused by the apoptosis and reducing interleukin-6.
The main components of propolis are flavonoid and phenolic acid, including Caffeic acid phenetyl ester (CAPE), in which almost 50% of all propolis compositions contain CAPE. Flavonoid is the biggest natural phenolic group covering many pigments generally contained in all plants (Mihai et al., 2011). CAPE significantly inhibits the production of cytokines and lymphokines, including TNF-α, IL-2, IL-10, IL-12, and IFN, and the proliferation of T-cells. Pro-inflammatory cytokines, IL-1, IL-6, and TNF-α are responsible for changes in the body's metabolic and pathogenic attacks (Soroy et al., 2014). Since CAPE and flavonoid contained in the propolis extract have a significant effect in decreasing the pro-inflammatory cytokines, this research aims at validating the effect of propolis administrations in improving caspase 3 expressions of hepatocytes and IL-6 levels in Wistar male rats induced with carbon tetrachloride.

METHODS

This experimental research used a “Post Test Only Control Group Design”. Twenty eight Wistar male rats aged 8 weeks, weighed 180-200 g were used, and then divided into four groups. Negative control group (CN-G) was administered with CCl4 at the dosage of 0.4 ml/200 gr in day 14, while the experimental groups were administered with propolis extract at the dosages of 3.6 mg/200 gr (CM3-G), 7.2 mg/200 gr (CM7-G), 14.4 mg/gr (CM14-G) for 14 days, and then administered with CCl4 in day 14 at the dosage of significantly reduced caspase-3 expression (Harahap, Irfannuddin and Murti, 2018).
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0.4 ml/gr. This research was conducted at the Food and Nutrition Research Center Laboratory (known as PSPG/Laboratorium Pusat Studi Pangan dan Gizi) of Universitas Gajah Mada Yogyakarta and Sultan Agung Islamic Hospital Semarang. This research was conducted after obtaining permit from the Bioethics Committee of Medical Faculty, Universitas Islam Sultan Agung Semarang, No. 270/V/2019/Komisi bioetik.

Propolis Extract and Dosage
The propolis used in this research was the propolis extract obtained from High Desert Indonesia. The propolis ethanol extract was made using the CMCE (Continuous Multi-stage Countercurrent Extraction) technique. The multistage extraction is the development of one stage extraction. The rafinat secreted from the first stage was mixed with the fresh solvent in the second stage and then was mixed with the fresh solvent in the third stage. The extract obtained from the first stage was combined with the extract obtained from the second and the third stage. The final results were the extracts (E1+E2+E3) and Rafinat R3. The composition of components in E and R flows had been balanced that E and R were located in the balance curve: E1 was balanced with R1, E2 was balanced with R2, and E3 was balanced with R3. The propolis extract was then diluted with aquadest 1:10. The volume of propolis extract was orally administered as much as 1 ml. The volume permitted to be administered based on the normal volume of rat's stomach was 3-5 ml. Dosage I with the calculation of dosage conversion in rats (Body Mass = 200 grams), was 200 mg x 0.018 = 3.6 mg/200g rat. Dosage II with the calculation of dosage conversion in rats was 400 mg x 0.018 = 7.2 mg/200g rat. Dosage III with the calculation of dosage conversion in rats was 800 mg x 0.018 = 14.4 mg/200g rat.

Preparation of Liver’s Specimens and Caspase 3 Expression Examination
Liver apoptotic examination began by making the liver specimen preparation. The liver specimen preparation making stages are explained as follows. Tissue fixation stage began by entirely cleaning the liver using PBS 1x and then put in the fixative for 1 hour. Furthermore, the liver was cut into the size of 1 x 1 cm. The specimen was once again immersed into the fixative for < 24 hours. The specimen was then repeatedly cleaned using alcohol 50% without holding and pressing the specimen. If stored for > 24 hours, the specimen should be immersed in alcohol 70% and then cleaned once again using alcohol 70%. Fixation aimed at minimalizing or stopping the tissue's auto-catalyst process. The next step was making the paraffin block. The specimen was dehydrated in alcohol 85% for 1-2 hours, alcohol 96% for 1-2 hours, and alcohol 100% for 2-3 hours. The specimen was cleared using xylol:alcohol 100% = 1:3 for 1 hour, xylol:alcohol 100% = 2:2 for 1 hour, xylol:alcohol 100% = 3:1 for 1 hour, pure xylol I for 1 hour, and pure xylol II for 1 hour. Specimen infiltration was made in the oven using xylol:paraffin 1:1 (45-500C) for 1 hour, paraffin I (65-700C) for 1 hour, and paraffin II (65-700C) for 1 hour. This process aimed at cleaning the tissues from the alcohol remains to ease the attachment using the mounting medium. The block was made using paper. The specimen was put into the paper box, added with liquid paraffin, and then labeled. The paraffin was then cooled using cold water. This paraffin blocking aimed at simplifying the tissue cutting using microtome with the thickness of 5-10 micron. The next was paraffin block cutting. The well- prepared paraffin block was then sliced using the rotary microtome. The liver tissue slices were in the thickness of 4 µm. The mounting was then performed in the object glass/slide using gelatin 5%. The staining was next performed. After sliced, the paraffin block was then put on top of the object glass/slide. Since the occurring layer was still covered, the paraffin should be removed by immersing the layer in the dehydration solution (alcohol, xylol). Still in the dehydration series, staining was made in order that the observed tissues looked clear. Haematoxilin eosin was the solution used.

The hepatocytes underwent apoptosis was observed using an Olympus microscope and the slide blot was pictured with the circular magnification of 400x. The cells experiencing apoptosis was then observed based on the characteristics of shrinkage cells, condensation-experiencing nucleus, and formed apoptotic bodies.

IL-6 Level Measurement
IL-6 level measurement used the ELISA method. The samples used were 28 serum samples of wistar male rats. The IL-6 examination principle was that the sample was reacted with antibody containing specific antibody against IL-6, then Horseradish Peroxidase (HRP) was added and Avidin was incubated for 1 hour in 37°C. The IL-6 level was measured using spectrophotometer with the wave length of 450 nm.

Statistical Analysis
The data analysis on the caspase 3 expression of hepatocytes was analyzed using Kruskal Wallis
Table 1. Average IL-6 levels and caspase 3 expressions of hepatocytes in each Group

| Variable          | CN-G       | CM3-G      | CM7-G      | CM14-G     | P       |
|-------------------|------------|------------|------------|------------|---------|
|                   | N=5, χ(± SD) | N=5, χ(± SD) | N=5, χ(± SD) | N=5, χ(± SD) |        |
| Caspase 3 expression (%) | 5.43 ±0.535 | 4.22 ±0.690 | 3.14 ±0.378 | 2.77 ±0.531 | 0.001*  |
| IL-6 Level (pg/ml) | 180.301 ±5.428 | 114.975 ±10.359 | 78.362 ±8.313 | 55.032 ±9.336 | 0.000** |

Note: * Kruskal Wallis; ** One Way Anova

Figure 1. Caspase 3 expression in group CN-G; CM3-G; CM7-G; and CM14-G. The brown color indicated the caspase 3 expression expression. positive

Figure 2. A. Caspase 3 Expression in Liver, and B. IL-6 Concentration. Post Hoc Analysis: * p<0.05; ns: not significant
continued with Mann-Whitney U. Meanwhile, the IL-6 level was analyzed using the One Way Anova test and then continued with the Post Hoc LSD. The statistical analysis result was considered significant if the p-value was <0.05.

RESULT
This research used propolis extract daily administered for 14 days, and in day 14, the CCl4 was administered to the Wistar male rats. The average IL-6 levels and caspase 3 expressions of hepatocytes were presented in the following table 1.

The results of this research showed that the average value caspase 3 expressions of hepatocytes in CN-G was the highest, respectively followed by that in CM3-G and CM7-G, while the lowest was that in CM14-G. Since the caspase 3 expression hepatocytes data were not normally distributed, Kruskal Wallis test was then performed to see the differences between groups. The analysis result showed that caspase 3 expression between groups was significantly different with p<0.05.

On the other hand, the highest level of IL-6 level found in CN-G, respectively followed by that in CM3-G and CM7-G, while the lowest was that in CM14-G. As the caspase 3 expression of hepatocytes data were normally distributed, one way anova test was then performed. The analysis result showed that the IL-6 level between groups was significantly different with p<0.05. To know the group which had a significant difference, each was then examined using Mann-Whitney U and Post Hoc test as explained below.

Caspase 3 Expression of Hepatocytes
Mann-Whitney U test result showed that caspase 3 expression in CM7-G and CM14-G was significantly lower than that in CN-G with p<0.05. Meanwhile, the caspase3 expression in CM3-G was lower, yet not significantly different than that in CN-G with p>0.05. Similarly, the caspase 3 expression in CM14-G was lower than that in CM7-G with p>0.05 (figure 1, 2A).

This result showed that propolis extract administration with the dosages of 3.6 mg/200g, 7.2 mg/200g, and 14.4 mg/200g reduced the caspase 3 expression of hepatocytes due to the CCl4 injection. The propolis extract with the dosages of 7.2 mg/200g and 14.4 mg/200g showed better results than that with the dosage of 3.6 mg/200g.

IL-6 Level
Post Hoc test result showed that the average IL-6 level in CM3-G, CM7-G, and CM14-G was significantly lower than that in CN-G with p<0.05. The average IL-6 level in CM14-G was significantly lower than that in CM7-G and CM3-G with p<0.05. Similarly, the IL-6 level in CM7-G was lower than that in C3-G with p<0.05 (figure 3). The Post Hoc LSD test result in IL-6 level showed that the propolis extract at the dosages of 3.6 mg/200g, 7.2 mg/200g, and 14.4 mg/200g could reduce the IL-6 level in the Wistar male rats induced with carbon tetrachloride (figure 1).

DISCUSSION
The results of this research showed that the administration of CCl4 may cause hepatocytes damages mediated by apoptosis. It was shown by the high IL-6 level as the proinflammatory marker and caspase3 expression as apoptosis marker in the negative control group and was significantly different with that in the propolis group. Moreover, the administration of propolis extract in this research could significantly reduce the IL-6 level and caspase 3 expression after injected with the CCl4.

This research result also showed that the caspase 3 expressions of hepatocytes after the administration of propolis at the dosages of 7.2 mg/200g and 14.4 mg/200g experienced a significant reduction. The result of this research was supported by the previous studies stating that the administration of propolis affected the caspase 3 expressions in WiDr cell cultures (Anandani, Kusnanto and Purwanto, 2018) as the propolis had a high flavonoid content functioning as neuroprotector. The results of research conducted by Dewi, on rats with focal cerebral ischemia showed that flavonoids could significantly reduce the caspase-3 expression (Dewi, Ali and Purnomo, 2016). It explained that the caspase 3 decreasing expression was as the reflection of cells' decreasing apoptosis (Hidayat et al., 2011). The propolis extracts could not significantly reduce the hepatocytes' caspase 3 expressions at the dosage of 3.6 mg/200g, yet there was a difference with the result of positive control. It was proven that the lipid peroxidation and hepatocytes' damages could be significantly suppressed by the antioxidant supplementations, such as flavonoid, silymarin, or vitamin E (Soroy et al., 2014). Propolis extract is bee product containing high active composition of flavonoid functioning as antioxidant. Propolis has antioxidant function able to prevent damage from free radicals compounds. Propolis extract also has the function as the toxic neutralizer since propolis has various contents to possibly clean the pollutants and toxic in the body. Thus, the metabolism of cells can work optimally (Krisnansari, Sulistyoo and Kusdaryanto, 2014). Some researchers have also reported that the propolis extract containing CAPE showed the inhibition influence in the production

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of pro-inflammatory cytokines (interleukin (IL)-1β, TNF-α, and MCP). Propolis contains neuroprotective components through the properties of antioxidant, anti-inflammatory and immunomodulator (Krisnansari, Sulistyo and Kusdaryanto, 2014).

The result of this research showed that IL-6 level in the administration of propolis extract at the dosages of 3.6 mg/200g, 7.2 mg/200g and 14.4 mg/200g experienced a significant reduction. The result of this research also was also in accordance with the previous research stating that the administration of propolis at the dosages of 0.054 g and 0.108 g showed the hepatoprotective activity to the liver damages induced with CCI4 shown by the reduction of IL-6 and SOD levels and hepatocytes’ damage percentage (Krisnansari, Sulistyo and Kusdaryanto, 2014). This activity was due to the existence of phenolic compounds contained in propolis in the form of flavonoid which can cover the cell structures that the body has the defense against the microorganisms. Flavonoid greatly influences the development of immune system in which flavonoid is an antioxidant contained in the propolis. The other research mentioned that propolis could decrease the prostaglandins, leukotrienes, proinflammatory cytokines (TNF-α, IL-6, IL-1, IL-10, and IL-8) (Lee et al., 2017). Propolis with its various benefits may increase the macrophage activity mediated by the stimulation of cytokines production, such as IL-6 and TNF-α in the rats. The content of propolis extract, that is, CAPE (Caffeic Acid Phenethyl Ester), has the anti-inflammatory activity by inhibiting the arachidonate acid release from the cell membrane and suppressing the COX-1 and COX-2 enzyme activity. Besides, CAPE (1.5 and 10 µM) may also inhibit the deoxyribonucleic acid (DNA) binding activity, NFkB transcription, and activator of protein-1 (AP-1) without affecting protein degradation as the NFkB inhibitor located in the cytoplasm. Thus, propolis has an activity as immunomodulator and anti-inflammatory (Bhadauria, 2012).

CONCLUSION

The administration of Propolis extract (CMCE method) possibly decreased the hepatocytes’ caspase 3 expressions and IL-6 levels in the Wistar male rats inducted with carbon tetrachloride.

CONFLICT OF INTEREST

There is no conflict of interest in this publication.

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