

RESEARCH ARTICLE

Propolis Extracted Using CMCE Decreases TNF- α and C-Reactive Protein level of CCl₄- Induced Liver Damage in Rats

Arief Adi Saputro¹, Taufiqurrachman Nasihun², Atina Husaana³

¹ Master Program of Biomedical Sciences, Faculty of Medicine, Universitas Islam Sultan Agung

² Department of Biochemistry, Faculty of Medicine, Universitas Islam Sultan Agung

³ Department of Biochemistry, Faculty of Medicine, Universitas Islam Sultan Agung

*corresponding author : ariefadi_s_dr@yahoo.com

ABSTRACT

Introduction: Carbon tetrachloride (CCl₄) is a hepatotoxic substance inducing inflammation in liver cells. Inflammation is marked by increase in proinflammatory cytokines level such as tumor necrosis factor alpha (TNF- α), and C-reactive protein (CRP) produced by hepatocytes. Propolis extracted using continuous multi-stage countercurrent Extraction (CMCE propolis extract) contains active balsamic, caffeic acid phenetyl ester (CAPE), and flavonoids capable of affecting pro-inflammatory cytokines. **Objective:** to investigate the effect of CMCE propolis extract on TNF- α and CRP levels in CCl₄-induced liver damage.

Methods: In this study using a post test only control group design, 28 male Wistar rats were divided into four groups. The control group (C-G) was given CCl₄ 0.4 ml/200 g on day 14. The P3-G, P7-G, P14-G groups were treated with 3.6 mg/200 g, 7.2 mg/200 g, 14.4 mg/200 g CMCE propolis extract for 14 days and given CCl₄ 0.4 ml/g on day 14. The TNF- α and CRP levels were assessed using the ELISA kit one-way ANOVA and Post Hoc test (LSD) were applied for the statistical analysis.

Results: TNF- α level in P14-G group (3.26 \pm 0.93), P7-G (6.17 \pm 0.78), and P3-G (10.4 \pm 1.34) were significantly lower than that of C-G (17.29 \pm 1.56) (p <0.05). CRP levels in P14-G group (0.61 \pm 0.09), P7-G (1.38 \pm 0.48), and P3-G (12.01 \pm 0.65) were significantly lower than those of C-G (2.95 \pm 0.668) (p <0.05). The greatest reduction in TNF- α & CRP level was found in the group given propolis at a dose of 14.4 mg/200 g.

Conclusion: The administration of CMCE propolis extract reduces TNF- α & CRP levels in CCl₄-induced liver damage.

Keywords: Propolis extract (CMCE method), TNF- α levels, CRP levels

ABSTRAK

Pendahuluan: Carbon tetrachloride (CCl₄) merupakan salah satu senyawa hepatotoksik yang dapat menyebabkan inflamasi. Inflamasi ditandai oleh peningkatan kadar sitokin proinflamator TNF- α dan CRP yang diproduksi oleh hepatosit. Ekstrak CMCE propolis mengandung zat aktif balsamic, senyawa *caffeic acid phenetyl ester* (CAPE) dan flavonoid yang berpengaruh terhadap sitokin proinflamasi.

Tujuan: Membuktikan pengaruh pemberian ekstrak CMCE propolis terhadap penurunan kadar TNF- α dan CRP pada tikus jantan wistar yang diinduksi CCl₄.

Metode: Penelitian eksperimental dengan desain *post test only control group design*, melibatkan 28 tikus jantan wistar, dibagi menjadi empat kelompok. Kelompok kontrol (C-G), diberikan 0,4 ml/200 g CCl₄ pada hari ke-14. Kelompok P3-G, P7-G, P14-G, masing-masing mendapatkan ekstrak CMCE propolis sebanyak 3,6 mg/200 g, 7,2 mg/200 g, 14,4 mg/g selama 14 hari dan diberikan CCl₄ pada hari ke-14 sebanyak 0,4 ml/g. Pemeriksaan kadar TNF- α dan CRP menggunakan metode ELISA. Analisis statistik yang digunakan adalah *One Way Anova* dilanjutkan dengan *Post Hoc LSD*.

Hasil: Analisa statistik Post Hoc menunjukkan bahwa kadar TNF- α kelompok P14-G (3,26 \pm 0,93), P7-G (6,17 \pm 0,78), dan P3-G (10,4 \pm 1,34) lebih rendah bermakna dibanding C-G (17,29 \pm 1,56), p <0,05. Demikian pula dengan analisa statistik *Post Hoc* pada kadar CRP yang menunjukkan bahwa kelompok P14-G (0,61 \pm 0,09), P7-G (1,38 \pm 0,48), dan P3-G (12,01 \pm 0,65) mempunyai kadar CRP lebih rendah bermakna dibanding C-G (2,95 \pm 0,668), p <0,05.

Kesimpulan: hasil penelitian ini menunjukkan bahwa ekstrak CMCE propolis dapat menurunkan kadar TNF- α & CRP. Penurunan kadar TNF- α & CRP paling banyak ditemukan pada kelompok yang diberikan ekstrak CMCE propolis dengan dosis 14,4 mg/g.

Kata Kunci: Ekstrak propolis (Metode CMCE), Kadar TNF- α , Kadar CRP

INTRODUCTION

Liver is an important organ in substances synthesis, storage, metabolism and detoxification (Herwana, Marwoto and Setiabudy, 2011). Free radicals, both endogenous and exogenous, play an

important role in the progression of liver oxidative stress, and thus induced liver damage. Oxidative stress is characterized by an imbalance between oxidants and antioxidants. The imbalance can be due to increased levels of oxidants or decreased levels of antioxidants.

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Therefore, to prevent free radical-induced organ damage, antioxidant supplementation is needed (Tatukude, Loho and Lintong, 2014). Propolis, one of the widely source of antioxidant, has been assumed to suppress free radicals. However, it remains unclear whether propolis can suppress the inflammatory response characterized by increased levels of tumor necrosis factor alpha (TNF- α) and C-reactive protein (CRP) in carbon tetrachloride (CCl₄) - induced liver damage.

Liver disease remains eminent global health problem, especially in developing countries, causing an increase in morbidity and mortality. It affects approximately 10% of the population (Arief et al., 2014). The World's Health Organization reported that liver disease ranked eighth in the leading causes of death in Indonesia with a death rate of 3.37% accounting for 55.860 deaths (WHO (World Health Organization), 2018). Southeast Asia regions, an estimated 100 million people and 30 million people were living with chronic hepatitis B and chronic hepatitis C respectively. Hepatitis B is responsible for nearly 1.4 million new cases and 300,000 deaths. Meanwhile, Hepatitis C caused about 500,000 new cases and 160,000 deaths. The National Basic Health Research reported that the prevalence of hepatitis surface antigen (HBsAg) was 7.2%. This data is lower than that of 2007, i.e., 9.4% of the general population (Kementerian Kesehatan RI, 2013). An estimated 18 million people and 3 million people had Hepatitis B and Hepatitis C, respectively (Alamudi, Hadi and Kumalasari, 2018). About 50% of those people are likely to develop chronic liver disease and 10% are likely to develop liver fibrosis which can lead to liver cancer. Together, these data indicate that 1,050,000 patients are at a greater risk to develop liver cancer (Aeni, Kurniawan and Sinaga, 2018).

Previous studies on the benefits of propolis supplementation in mice showed that propolis supplementation at a dose of 200 mg/kg mice for 14 days reduces cytosol leakage of the lactate dehydrogenase (LDH) enzyme, decreases lipid peroxides and maintains reduced glutathione (rGSH) in CCl₄-induced liver damage (Bhadauria, 2012). Other studies indicated that extracts of propolis have been shown to be effective in the treatment of dengue hemorrhagic fever patients indicated by laboratory findings, the clinical condition of the patient, and length of treatment (Soroy et al., 2014). Meanwhile, research on the effect of propolis on brain injury showed that propolis extract decreases TNF- α expression, apoptosis and necrosis of traumatic brain injury in rats (Dewi, Ali and Purnomo, 2016). Another study using rat liver cell showed that the administration of propolis extract at a dose of 100

mg/kg BW in thioacetamide-induced hepatotoxicity cause a significant reduction in CRP levels (Ahmed et al., 2012).

The "superblend" propolis is extracted through four stages of extraction known as the continuous multi stage countercurrent extraction (CMCE) to increase the final extraction results (15% of the bee-derived propolis and maintain its concentration of flavonoid and phenol. It is verified by oxygen radical absorbance capacity (ORAC) assay showing the value of antioxidants of 21,921 (Soroy et al., 2014). The propolis extract serving as an effective immunomodulatory has been shown to boost the immune system quickly and help the body to slow down the effects of degenerative diseases. In long-term, it can reduce free radicals in the human body to prevent cell damage (Soroy et al., 2014). The most important components of propolis are flavonoids and folic acid, including caffeic acid phenethyl ester (CAPE) making up nearly 50% of the total propolis composition. Flavonoids are one of the largest natural phenol groups which include many pigments and are found 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 inhibits T-cell proliferation. Flavonoids will inhibit pro-inflammatory cytokines such as TNF- α , IL-1 α and IL-2. Proinflammatory cytokines, IL-1, IL-6 and TNF- α are responsible for metabolic changes in the body as well as pathogen attacks (Soroy et al., 2014). CAPE and flavonoids contained in propolis extract have a major effect on proinflammatory cytokines, so further research is needed to determine the effect of propolis extract on TNF- α and CRP levels in CCl₄-induced liver damage.

METHODS

This research was an experimental research with a posttest only control group design. This research was conducted at the Laboratory of the Center for Food and Nutrition Studies (PSPG), Gadjah Mada University, Yogyakarta. A total of 28 male wistar rats (*Rattus novogicus*) aged 8 weeks, weighing 180-200 g were divided into 4 groups. Control (CG) was given 0.4 ml/200 g CCl₄ on day 14. P3-G, G-P7, P14-G were given propolis extracts at the dose of 3.6 mg/200 g, 7.2 mg/200 g, 14.4 mg/200 g respectively and followed by 0.4 ml/gr CCl₄ on day 14. At the end of the study, blood samples were collected for the evaluation of levels of TNF- α and CRP using the ELISA kit. This study has received approval from the bioethics committee of Faculty of Medicine, Sultan Agung Islamic University Semarang (No. 270/V/2019/Bioethic Committee).

Preparation of CMCE Propolis Extract

This study used a commercially available propolis (HDI, Indonesia) extracted using continuous multi-stage countercurrent extraction (CMCE). Multistage extraction is developed from a single stage extraction. Raffinate (R) resulted in the first stage is mixed with fresh solvent in the second stage, and the raffinate of the second stage is mixed with a fresh solvent in the third stage. The extract (E) from the first stage is combined with the extract from the second and third stage. The final result is the extract (E1 + E2 + E3) and raffinate R3. The composition of the various components in the flow E and R is in equilibrium, so that E and R are located on the equilibrium curve: E1 equilibrium with R1, E2 equilibrium with R2 and E3 equilibrium with R3

Measurement of TNF- α Level

The serum samples were taken from 28 Wistar rats. The reagent used was RayBio® Rat TNF- α ELISA kit including wash buffer, RDIF, dilution, substrate solution and standard TNF- α . For the standard preparation of TNF- α Control Kit, 1.0 ml of deionized water was added then centrifuged. The microplate was prepared

for the sample and a series of standard solutions was made. Fifty milliliters diluents RD 1-63 were mounted into all wells. After that, 50 μ l of standard, control and samples were added to each well, then incubated for 2 hours at room temperature. While waiting for incubation, a 2 ml wash buffer was prepared. After 2 hours incubation, washing was done 5 times. After washing, 100 μ l of the TNF- α conjugate was mounted into each well, then incubated again for 2 hours at room temperature. After incubation, washing was repeated again for 5 times. After the second 2 hours incubation, 100 μ l of substrate was added, then incubated for 30 minutes at room temperature, and kept away from light. A 100 μ l of stop solution was then added and mixed. The results were read with a 150 nm ELISA Reader, correction of 540 nm/570 nm.

Measurement of CRP Level

The CRP levels were determined using ELISA kit. Twenty eight samples of serum of Wistar rats were used. The reagent used was BD™ ELISA Rat CRP ELISA Kit. The principle of CRP examination is that the sample was reacted with antibodies containing specific antibodies to CRP, then horseradish peroxidase

Table 1. The results of the statistical analysis of the measurement of TNF α and CRP levels

Variables	Groups				P-value
	C-G	P3-G	P7-G	P14-G	
	N=5 $\bar{x} \pm SD$	N=5 $\bar{x} \pm SD$	N=5 $\bar{x} \pm SD$	N=5 $\bar{x} \pm SD$	
TNF-α levels (pg/ml)	17.29 \pm 1.56	10.40 \pm 1.34	6.17 \pm 0.78	3.26 \pm 0.93	
Saphiro Wilk					> 0.05
Levene's Test					0.257
One way ANOVA					0.000
CRP levels (pg/ml)	2.95 \pm 0.68	2.01 \pm 0.65	1.38 \pm 0.48	0.61 \pm 0.09	
Saphiro Wilk					> 0.05
Levene's Test					0.079
One way ANOVA					0.000

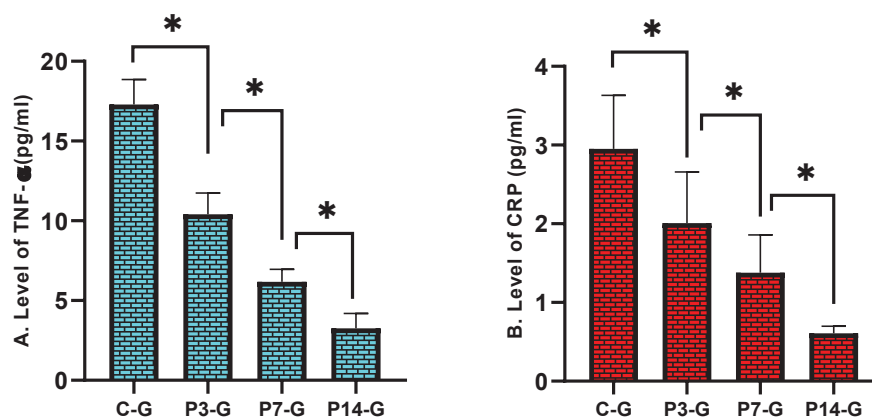


Figure 1. A. Level of TNF- α and B. Level of CRP in each group. Post Hoc analysis: * p<0.05

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(HRP) and Avidin were added. The color change will only occur in wells containing CRP. Color changes were measured with an ELISA reader at a wavelength of 450 nm. The CRP concentration was determined by comparing the absorbance of the sample against a standard curve

Statistical analysis

Data on TNF- α levels and CRP levels were analyzed using one way ANOVA followed by Post Hoc test (LSD). P values less than 0.05 were considered as statistically significant.

RESULTS

Male wistar rats were given CMCE propolis extract for 14 days followed by CCl₄ on day 14th. The results of statistical analysis on levels of TNF- α and CRP as seen on Table 1.

In this study, TNF- α and CRP levels in P14-G were found to be lower compared to those of control group as shown in Table 1. One-way ANOVA statistical analysis on TNF- α and CRP levels showed that there was a significant difference between groups ($p < 0.05$). To determine which group had a significant difference, a post hoc test was performed.

TNF- α Levels

Post hoc test (LSD) showed that the levels of TNF- α in P3-G, P7-G, and P14-G were significantly lower than that of CG ($p < 0.05$). TNF- α level in P14-G was significantly lower than that of P 3-G and P 7-G ($p < 0.05$). Likewise, TNF- α level in P7-G was significantly lower than that of P3-G ($p < 0.05$). The lowest level of TNF- α was found in the group administrated with CMCE propolis extract at a dose of 14.4 mg (Fig 1A).

CRP Levels

Post Hoc test (LSD) showed that CRP levels in P3-G, P7-G, and P14-G were significantly lower than of CG ($p < 0.05$). The CRP level at P14-G was significantly lower than that of P3-G and P7-G ($p < 0.05$). Likewise, the level of CRP at P7-G was significantly lower than that of P3-G ($p < 0.05$). These results showed that the lowest CRP levels was found in the group administered with CMCE propolis extract at a dose of 14.4 mg (Fig 1B).

DISCUSSION

In this present study, the CMCE propolis extract showed a protective effect against CCl₄-induced liver damage. This hepatoprotective effect of CMCE propolis

extract was associated with a polyphenol caffeic acid phenethyl ester (CAPE) compound which can act as an antioxidant (Akbari and Hestianah, 2013). Propolis extract compounds have potent antioxidant potential (Damayanti, Fitri and Dalhar, 2016). The extract can significantly reduce serum transaminase activity which increases due to liver damage and plays a role in increasing the activity of antioxidant enzymes such as liver cytosolic superoxide dismutase, catalase, and glutathione peroxidase in CCl₄-intoxicated in mice and a significant decrease and decreasing MDA production (Lee et al., 2003).

The results of this study showed that the administration of CMCE propolis extract at doses of 3.6, 7.2, 14.4 mg/200 g BW significantly reduces TNF- α levels. The finding of this study supports previous studies stating that administration of propolis extract prevent the increase in TNF- α and CRP levels in CCl₄-induced hepato-renal injury. In addition, the administration of propolis maintain activity of catalase, adenosinetriphosphatase, glucose-6-phosphatase, acid, and alkaline phosphate in normal conditions (Bhadauria, 2012). Furthermore, propolis has been shown to stimulate the production of cytokines such as IL-6 and TNF- α in mice (Mustafiah, Fatmawati and Yusuf, 2011). This is likely due to caffeic acid phenethyl ester (CAPE) in propolis extract having anti-inflammatory activity by inhibiting the release of arachidonic acid from cell membranes, suppressing the activity of the enzymes cyclooxygenase-1 (COX-1) and cyclooxygenase -2 (COX-2) (Prasetyo, Suparyanti and Guntur, 2013). The administration of CAPE at doses of 1.5 and 10 μ M can inhibit activity of deoxyribonucleic acid (DNA) binding, transcription of nuclear factor kappa-B (NF κ B), and activator protein-1 (AP-1), without affecting the degradation of the NF κ B inhibiting protein located in the cytoplasm. Thus, propolis has immunomodulatory and anti-inflammatory properties (Bhadauria, 2012).

Propolis extract, bee product, rich in flavonoids and having antioxidant property, has been shown to prevent free radical-induced cell injury (Soroy et al., 2014). Propolis extract also functions in detoxification because the various ingredients in propolis can clean pollutants and toxins in the body so that cell metabolism can resume optimally (Krisnansari, Sulisty and Ati, 2014). Beberapa penelitian juga telah melaporkan bahwa ekstrak propolis yang mengandung CAPE menunjukkan pengaruh inhibisi pada produksi sitokin proinflamasi (interleukin (IL)-1 β , TNF- α , dan MCP). Several studies have also reported that propolis extract containing CAPE has an inhibitory effect on the

production of proinflammatory cytokines interleukin (IL)-1 β , TNF- α , and MCP. Propolis extracted using CMCE method resulted in higher concentration of flavonoids and phenols. Propolis extract as an effective immunomodulator is able to boost the immune system quickly and to help the body to slow down the effects of degenerative diseases. The decrease in TNF- α levels occurs through suppression of the antigen-antibody complex in the inflammatory cell infiltration mechanism leading to reduced free radicals activity and the Matrix Metalloproteinase (MMP) enzyme resulted in decreased inflammatory reaction (Soroy et al., 2014). Propolis also has a neuroprotective effect via its antioxidant, anti-inflammatory and immunomodulatory properties (Annis, Purnomo and Balafif, 2017).

The administration of propolis extract at the dose of 3.6, 7.2, 14.4 mg/200 g BW also significantly reduced C-Reactive Protein (CRP) level. This finding is in line with previous studies stating that the administration of 0.054 g and 0.108 g of propolis decrease IL-6 levels and CCl₄-induced liver damage (Krisnansari, Sulistyono and Atri, 2014). Inflammation will increase CRP levels. C-Reactive Protein is an acute phase protein that is present in normal serum, although in a very small amounts. In certain circumstances with an inflammatory reaction or tissue damage (necrosis), either caused by an infectious disease or not due to infection. CRP is a biomarker acting as an acute phase protein in the inflammation process (Delfanti et al., 2018). CRP is a marker of inflammation produced and released by the liver under the stimulation of cytokines such as IL6, Interleukin 1 (IL-1), and tumor necrosis factor α (TNF- α). The synthesis of CRP in the liver occurs very rapidly after a single stimulation, the serum concentration increases above 5 mg/L for 6-8 hours and reaches a peak after about 24-48 hours (Ingle and Patel, 2011). Serum of CRP is produced by hepatocytes and was influenced by proinflammatory cytokines interleukin (IL)-6 (Bhadauria, 2012). Flavonoids have a lot of effect on the activation of the immune system, in which flavonoids are antioxidants contained in propolis (Prasetyo, Suparyanti and Guntur, 2013). Another study stated that propolis can reduce prostaglandins, leukotrin, proinflammatory cytokines (TNF- α , IL-6, IL-1, IL-10 and IL-8) (Lee et al., 2003). The content of propolis extract (CMCE), namely CAPE (caffeic acid phenethyl ester) has anti-inflammatory activity by inhibiting the release of arachidonic acid from cell membranes, suppressing the activity of COX-1 and COX-2 enzymes. The propolis extracted using CMCE contains flavonoids and CAPE which will inhibit IL-6 levels leading to reduce CRP levels (Bhadauria, 2012).

CONCLUSION

The study showed that administration of CMCE propolis at the dose of 3.6, 7.2, and 14.4 mg/200 g BW reduces levels of TNF- α & CRP levels in CCl₄-induced liver damage. The greatest reduction in TNF- α & CRP levels was found in the group given propolis at a dose of 14.4 mg/g.

CONFLICT OF INTEREST

There is no conflict of interest in this publication

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