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Research Article

EFFECT OF RED BETEL (*Piper crocatum* Ruiz and Pav.) ETHANOL EXTRACT AGAINST CARBON TETRACHLORIDE INDUCE HEPATIC INJURY IN RATS

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ABSTRACT:

Hepatic disorder is one of a big problem in health system especially cirrhosis. Based on WHO, the prevalence of cirrhosis is 1,3% and 18th caused death about 800.000 cases. Herbal medicines are widely used in treatment and prevention of liver diseases. One of the plants used is red betel. The purpose of this study is to determine the hepatoprotective activity of ethanol extract of red betel, the reduced levels of ALT, AST, and ALP, and the description of changes in hepatocytes. The ethanol extract of red betel was founded by maceration. The hepatoprotective test was divided into two groups: the curative and preventive test. In the curative test, a rat that had been induced with CCl_4 given ethanol extract 600 mg/kg bw each day for 15 days. In the preventive test, rats were given ethanol extract 600 mg/kg bw each day and CCl_4 each 4 hours for 45 days. During giving the extract, ALT, AST, and ALP were measured and the hepatocytes were observed and calculated at five visual fields. Phytochemical screening showed that bioactive compounds contained in red betel leaves are alkaloids, flavonoids, saponin, tannins, glycosides, steroid/triterpenoid. Results indicate that red betel ethanol extract has a hepatoprotective effect on CCl_4 induced hepatotoxicity in rats.

Keywords: *CCl_4, Hepatoprotective, Rats, Red betel.*

INTRODUCTION

Liver disease is a big problem in the health system, and the use of conventional medicine for the treatment of liver diseases are sometimes inadequate and cause serious side effects [1]. Based on WHO (2004), the prevalence of liver cirrhosis is 1.3% and it is the 18th leading cause of death with 800,000 cases. In the United States in 2009, chronic liver disease and cirrhosis were responsible for about 30.444 cases of deaths [2]. Liver cells contain enzymes, some of which are important for the diagnostics of liver damage. Enzymes that can be used is alanine aminotransferase (ALT) and aspartate aminotransferase (AST). These enzymes are specific indicators for determining liver cell damage. Increased activity of these enzymes shows damage of the liver cells [3].

Damage of liver cell can be caused by chemotherapy drugs, carbon tetrachloride (CCl_4), tiosetamid (TAA), high doses or long terms drug use like paracetamol and excessive alcohol consumption [4]. CCl4 is xenobiotics used to induce lipid peroxidation and liver damage [5]. Giving CCl4 in high doses will damage the endoplasmic reticulum, accumulate lipids, damage the protein synthesis, disrupt the process of oxidation, lose weight, and long-term provision of CCl4 can lead centrilobular necrosis and degeneration of fat in the liver [6].

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Hepatoprotective are compounds that have the effect to maintain and treat liver damage[7]. Most herbs can be used as a hepatoprotective because it can protect and enhance the regeneration of liver cells. One empirically medicinal plants used in traditional medicine is red betel (Piper crocatum Ruiz & Pav.) [3, 8].

The ethanol extract of red betel is able to reduce the activity of ALT significantly in male rats induced by paracetamol [9]. This present study aims to investigate hepatoprotective effect of red betel, the reduced levels of ALT, AST, and ALP, and the description of changes in hepatocytes.

EXPERIMENTAL

plant preparation

The leaves of Red betel were collected from Brastagi. The sample was identified by Herbarium Bogoriense, Bidang Botani Pusat Penelitian Biologi, Lembaga Ilmu Pengetahuan Indonesia (LIPI), Bogor. The leaves were cleaned, dried at 50-60^{o}C and powdered. the powder was extracted with ethanol by maceration method. The extract was concentrated by a rotary evaporator.

Identification the active compounds of red betel leaves

Identification was done on alkaloids, flavonoid, glycoside, saponin, tannin, and steroid/triterpenoid.

Animals

Male Wister albino rats of weighing about 160-200 g acclimatized for seven days to adjust to the environment [10]. The rats that had been acclimatized were measured ALT, AST, and ALP to the initial value (H0). The CCl4 solution was given orally every four days with a dose of 1 ml/kg BW for 45 days in olivarum oleum (50%). ALT, AST, ALP from the serum of rats were measured on On day 15, 30 and 45.

Evaluation of hepatoprotective activity

The hepatoprotective test was divided into two groups: the curative and preventive test.

curative hepatoprotective test

In the curative test, rats were divided into 3 groups. The first group served as baseline. Necrosis rats in the second group received Na CMC 0.5% suspension as a negative control. Group III necrosis rats were treated with ethanol extract of red betel at a dose of 600 mg/kg BW. ALT, AST, and ALP were observed on day 5,10 and 15. Histopathological studies of liver were performed on day 16 to observe liver repairment.

preventive hepatoprotective test

Measurement of ALT, AST, and ALP before the treatment is the initial value. In the preventive test, rats were divided into 3 groups. Group I was healthy rats were given CCl4 every four days with a dose of 1 ml/kg BW for 45 days as a control hepatotoxic. Group II is healthy rats were given daily Na.CMC and CCl4 every four days with a dose of 1 ml/kg BW for 45 days as a negative control. Group III is healthy rats were given a daily dose of red betel ethanol extract at a dose of 600 mg/kg BW and CCl4 four days with a dose of 1 ml/kg BW for 45 days. Rats serum was taken for testing the activity of ALT, AST, and ALP on day 15, 30, and 45. The liver is taken on day 46 to observe the histopathology after administration of the extract.

examination of liver damage rats

macroscopic examination

Rats were sacrificed by cervical dislocation. Macroscopic examination of the liver was performed by observing the color and texture of the liver surface.

preparation of liver histology (tissue processing)

The samples were fixed in 10% buffered formalin solution at least 4 hours until hardened. Then put the pieces of tissue. The samples were put in the automatic tissue processor to be prepared and selected. Then the selected samples were immersed sequentially into 12 tubes containing various reagents as follow.

Tabel I. Reagent which was used to immerse the sample

Tube I, II	Tube III	Tube IV	Tube V, VI, VII	Tube VIII, IX, X	Tube XI. XII
Formalin 10% for 2 hours.	Alcohol 70% for an hour	Alcohol 96% for an hour	Alcohol absolute for an hour.	Xylene for 1.5 hours	Paraffin for 2 hours

Then Tissue stained with the Hematoxylin-eosin method before observed under a microscope with a magnifier (x100 & x400).

Measurement of degree of liver damage

Observations in the liver were done by counting the number of normal liver cells in five visual fields around the central vein.

Statistical analysis

ALT, AST, and ALP values were processed using SPSS 17.0 for windows by two-way analysis of variance (ANOVA). Calculation data hepatocytes were processed using One Way ANOVA statistical test.

RESULT AND DISCUSSION

Phytochemical screening

The result of phytochemical screening shown in Table 2.

Tabel II. Phytochemical screening simplicial powder and ethanol extract of red betel leaf

Examination	Simplicial	Extract
Alkaloid	+	+
Flavonoid	+	+
Saponin	+	+
Tanin	+	+
Glycoside	+	+
Steroid/Triterpenoid	+	+

Phytochemical screening show either simplicial or ethanol extract of red betel has alkaloid, flavonoid, saponin, tannin, glycoside, and steroid/ triterpenoid.

Curative hepatoprotective test

In the curative test, the ethanol extract is given after induction with CCl4 1 ml/kg BW every 4 hours for 45 days. The measurement of the activity of ALT, AST, ALP test can be seen in the table below.

Table III. Measurement ALT, AST, and ALP Activity in The Curative Test

Group	Treatment	ALT Activity (U/L)	P	AST Activity (U/L)	P	ALP Activity (U/L)	P
I	Normal	102,515 ± 5,336	0,000[b] 0,059[c]	179,260 ± 21,085	0,000[b] 0,073[c]	28,500 ± 3,505	0,000[b] 0,825[c]
II	Negative control	591,345 ± 40,060	0,000[a] 0,000[c]	727,340 ± 110,064	0,000[a] 0,009[c]	72,050 ± 7,911	0,000[a] 0,000[c]
III	Extract dose 600 mg/kg BW	134,940 ± 13,883	0,059[a] 0,000[b]	337,450 ± 57,835	0,073[a] 0,009[b]	27,500 ± 2,831	0,825[a] 0,000[b]

[a] significant difference with the normal group , [b] significant difference with the negative control group
[c] significant difference with the group of 600 mg/kg BW

Based on Table 4 above, the activity of ALT, AST, and ALP in group III with extract dose is 600 mg/kg BW did not differ significantly in the normal group (p> 0.05) and significantly different from the negative group (p < 0.05). This shows the effect of a decrease in ALT, AST, and ALP from the application of the extract. ALT is more accurate for liver function test than AST because ALT formed in the liver.

Red betel contains flavonoids. Flavonoid compounds suspected to able to repair damaged liver cells. These compounds react restore damaged liver cells. The hepatoprotection working mechanism by way of detoxifying toxic compounds, increasing the regeneration of liver cells, anti-inflammatory and as an immunomodulator [11]. Flavonoids as the anti-inflammatory mechanism are to restore the permeability and increase the resistance of capillary blood vessels [12].

Preventive hepatoprotective test

In the preventive test, the ethanol extract of red betel dose of 600 mg/kg BW given every day and CCl4 was given then on the fourth day 2 hours before the extract. Results of preventive tests can be seen in Table below.

Tabel IV. The Measurement of ALT, AST, ALP in preventive test

Group	Treatment	ALT Activity (U/L)	P	AST Activity (U/L)	P	ALP Activity (U/L)	P
I	Hepatotoxic	377.420 ± 86,763	0,263[b] 0,003[c]	262.755 ± 43,595	0,057[b] 0,003[c]	49.350 ± 9,585	0,587[b] 0,008[c]
II	Negative Control	470.040 ± 96,161	0,263[a] 0,012[c]	386.560 ± 70,682	0,057[a] 0,003[c]	38.300 ± 6,387	0,587[a] 0,004[c]
III	Extract dose 600 mg/kg BW	179.695 ± 22,778	0,003[a] 0,012[b]	101.100 ± 13,218	0,003[a] 0,003[b]	18.850 ± 2,378	0,008[a] 0,004[b]

[a] significant difference with the hepatotoxic group
[b] significant difference with the negative control group
[c] significant difference with the group of 600 mg/kg BW

In the group III with extract dose 600 mg/kg BW significantly different (p <0.05) with the negative control group and the group hepatotoxic. This proves that extract may protect liver cells from damage caused by CCl4. Flavonoids are supposed to influence inhibit liver damage by binding free radicals so, the impact is reduced. Free radicals will disrupt the membrane integrity of hepatocytes thus removing various enzymes from hepatocytes, such as AST and ALT [7].

Liver organ damage

Macroscopic liver organ

The difference between the normal liver organ, the negative group, the hepatotoxic, the curative dose group of 600 mg/kg BW and a preventive dose of 600 mg/kg BW group, which include color and texture of the CCl4 lead to fatty liver which is indicated by a color change from dark red heart becomes pale pink with white spots (Fig.1). This is due to physiological changes and macroscopic structure of the tissue [13]. CCl4 caused autooxidation Polyunsaturated Fatty Acids (PUFAs) which is in phospholipid

membranes and caused lipid peroxidation [14].

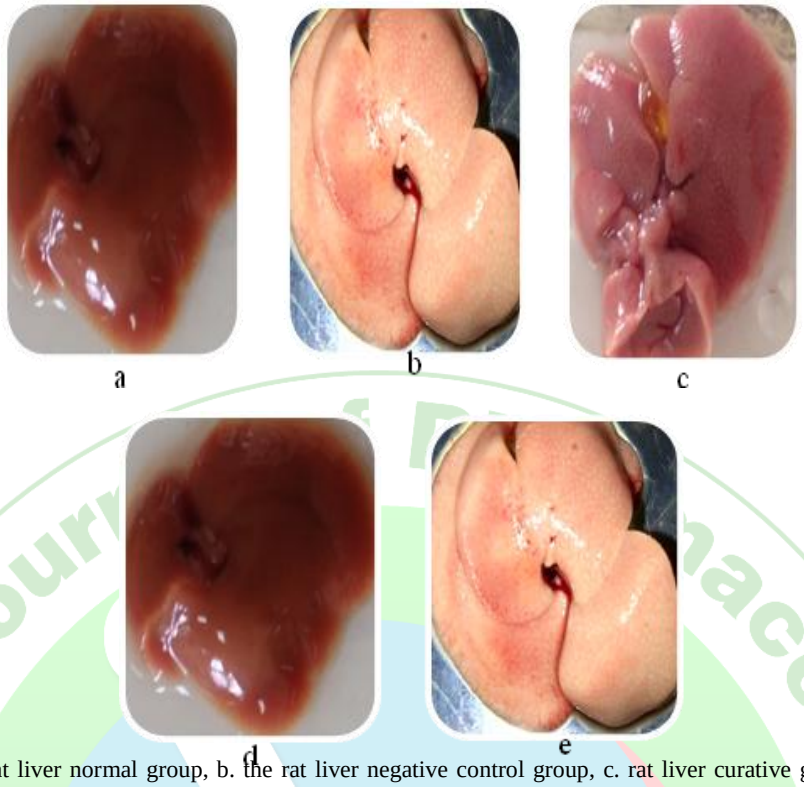

Figure I.a. the rat liver normal group, b. the rat liver negative control group, c. rat liver curative group EEDSM 600 mg/kg BW, d. groups of the rat liver hepatotoxic group, e. groups of rat liver preventive EEDSM 600 mg/kg BW

Microscopic liver organ

Histopathological study was observed on the 16th day after 24 hours with a given extract. The results of these observations are used to determine the degree of improvement of liver cells due to the red betel Extracts. Hepatocytes were counted with five visual fields. A number of hepatocyte in curative trials are presented in the table below.

Table V. Normal Hepatocytes Calculations in Curative Test

Group	Treatment	Number of Normal Hepatocytes	P
I	Normal	82,3567 ± 1,24478	0,009[b] 0,573[c]
II	Negative Control	51,6667 ± 7,04683	0.009[a] 0,031[c]
III	Extract Dose 600 mg/kg BW	75,2000 ± 4,21584	0,573[a] 0,031[b]

[a] significant difference with the normal group
[b] significant difference with the negative control group
[c] significant difference with the group of 600 mg/kg BW

The number of normal hepatocytes in the third group of 600 mg/kg BW is not much different from the number of hepatocytes in the normal group. There is no significant difference between the two groups shown in the value of significance ($p > 0.05$). Compared to the negative control group, the group with extract is significantly different from ($p < 0.05$). The activity of serum was lowered by ethanolic extract treatment.

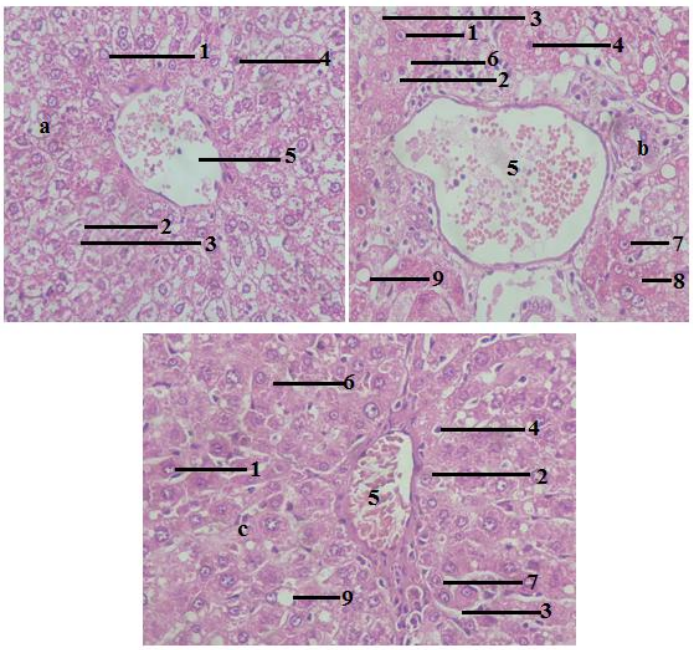

Figure II. Representative histological sections of the liver in Curative Testing. a. normal group. b. negative control group. c. Rats treated with 600 mg/kg BW red betel extract. 1. the cell nucleus, 2. cytoplasm, 3. sinusoid, 4. pyknotic cell, central venous 5., 6. macrovesicular hydropic degeneration, hydropic degeneration microvesicular 7., 8. karyolysis, 9. steatosis .negatif, EEDSM groups significantly different from the control group negative ($p < 0.05$).

In the normal group, there was no change in hepatocytes. In the negative groups, such damage hydropic degeneration, karyolysis pyknotic cells, fatty looks like clear vacuoles small to large evenly distributed throughout the lobules. Hydropic degeneration can occur due to disruption of sodium-potassium pump in the regulation of entry and exit of ions [15], causing cell swelling or degeneration turbid [16]. In the group extract dose of 600 mg/kg BW microscopic observation showed slightly steatosis, macrovesicle hydropic degeneration, hydropic degeneration microvesicle which is sublethal changes. The damage that occurs in liver tissue in a group of 600 mg/kg BW is not as severe in the negative group. This proves that EEDSM effect repairs on damaged liver cells. In addition cessation of time CCl4 giving thought to trigger a recovery effect. According to Price and Wilson (1984), a cell disruption due to certain stimuli will bring adaptation mechanism when the stimulation stops or can be a backlash recovery [17]. Reaction recovery or regeneration of cells allows the cells can be returned as before. This is due to the effects of flavonoids contained in red betel. The flavonoid compound is antihepatotoxic. These compounds are most contribute restore damaged liver cells compared alkaloids and saponins (fig. 3) [13].

Table VI. The result of the calculation of normal hepatocytes (Means ± SE) of five field of vision on preventive test

Group	Treatment	Number of Normal Hepatocytes	P
I	Hepatotoxicity	45,0000 ± 8,14453	0,528[b] 0,018[c]
II	Negative control	51,6667 ± 7,04683	0,528[a] 0,043[c]
III	extract dose 600 mg/kg bb	77,2000 ± 5,71781	0,018[a] 0,043[b]

[a] significant difference with the hepatotoxicity group, [b] significant difference with the negative control group
[c] significant difference with the group of 600 mg/kg BW

In the group EEDSM 600 mg/kg BW (77.2000 ± 5.71781) there are significant differences with a hepatotoxic group (45.0000 ± 8.14453) and negative group (51.6667 ± 7.04683) to the number of normal hepatocytes. Whereas in the group hepatotoxic and negative group, this difference was not significant. This proves that extract of red betel is able to reduce damage caused by CCl4. this is because the red betel has antioxidant compounds such as flavonoids, alkaloids, and saponins. These secondary metabolites worked as a scavenger that reduces free radicals thereby lowering oxidative stress such as peroxide-oxidized lipids and DNA. If oxidative stress is prevented, then the apoptosis and necrosis of liver cells will not occur (Fig 3).

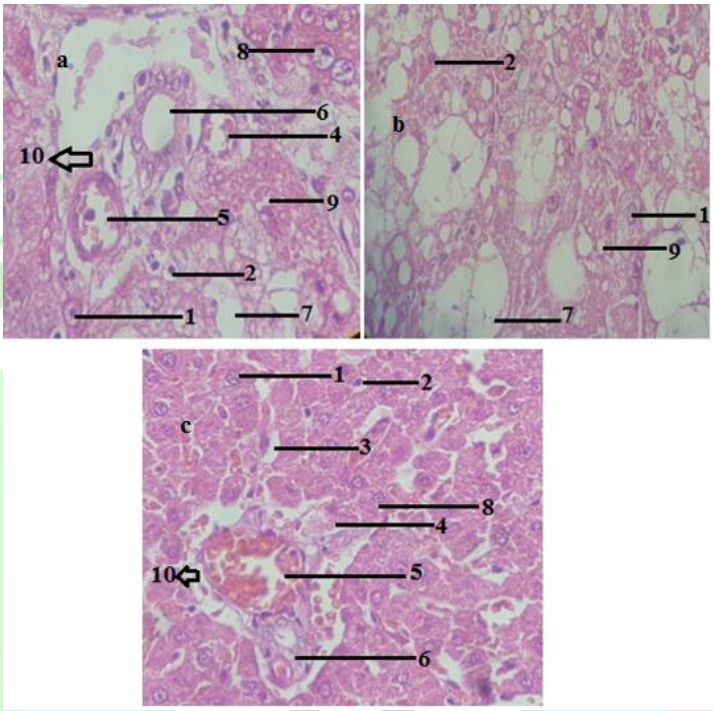

Figure III. Representative histological sections of the liver in Preventive Testing. a. negative control group. b. hepatotoxic group. c. Rats treated with 600 mg/kg BW red betel extract. 1. the nucleus, 2. pyknotic cell 3. sinusoid, 4. Hepatic artery, 5. Portal vein, 6. Bile ducts, 7. macrovesicular fatty, 8. hydropic degeneration microvesicular, 9. Microvesicular fatty, 10. Portal triad.

On histopathological observations is seen a significant difference between the negative control group and the group hepatotoxic. In the group of hepatotoxic and negative groups seen fatty cells is still prevalent in the entire lobe of the liver, while the fatty cells in a group EEDSM dose of 600 mg/kg BW are lower. This indicates that a very strong CCl4 attack the liver causing severe damage to liver cells. However, with the provision of EEDSM 600 mg/kg BW, generating a positive image in the process of liver cell protection. These fatty cells caused by CCl4 disrupt mitochondria resulting in a decrease in calcium as well as in the endoplasmic reticulum, but instead, there was an increase of calcium in the cytosol [18]. This observation complemented the results that the ethanolic extract of Red Betel is able to against hepatotoxicity induced by CCl4 in rats as an antioxidant.

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