Indonesian J. Pharm. Vol. 28 No. 1 : 10 – 18
ISSN-p : 2338-9427
DOI: 10.14499/indonesianjpharm28iss1pp10

Research Article

HEPATOPROTECTIVE ACTIVITY OF ETHYL ACETATE FRACTION OF SENGGUGU'S ROOT BARK (*Clerodendrum serratum* L. Moon) ON RATS INDUCED BY CARBON TETRACHLORIDE

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Submitted: 20-10-2016
Revised: 10-12-2016
Accepted: 7-01-2017

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ABSTRACT

Our previous study showed that ethyl acetate fraction of *Clerodendrum serratum* L. Moon (EAFCS) root bark had *in vitro* antioxidant activity. The aim of this study was to evaluate hepatoprotective effect of EAFCR in rats induced by carbon tetrachloride (CCl_4). Thirty-six rats were randomly divided into 6 groups. Group 1 as control was given 0.5% Na-CMC. Group 2 as positive control were given silymarin at a dose of 100mg/Kg.BW. Group 3 as negative control were induced by CCl_4. Group 4-6 as treatment groups were induced CCl4 and given EAFCR at a dose of 25; 50 and 100mg/Kg BW, respectively. Biochemical and oxidative stress parameters in liver were determined. The results showed that serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and bilirubin were significantly lower, whereas the total protein was significantly higher after pretreatment with EAFCR at the dose of 100mg/Kg.BW ($p<0.05$). Moreover, malondialdehyde (MDA) was significantly lower, whereas glutathione peroxidase (GPx) and catalase (CAT) were significantly higher after pretreatment with EAFCS at the dose of 100mg/Kg.BW ($p<0.05$). In conclusion, EAFCR has potent hepatoprotective activity due to its antioxidant properties of the active compounds contained in this plant.

Key words: *Clerodendrum serratum*, antioxidant, hepatoprotective effect, carbon tetrachloride

INTRODUCTION

Liver is one of the vital organs in the body that plays an essential role in regulating various physiological processes. It plays a pivotal role in metabolism and distribution of nutrients as well as detoxification of toxic metabolites and xenobiotics (Chiang, 2014). It is also involved the maintenance, performance and regulating homeostasis of the body. Moreover, the liver is involved with almost all the biochemical pathways to growth, fight against the disease, nutrient supply, energy provision and reproduction (Ward and Daily, 1999). Therefore, take care of a healthy liver is important for the overall prosperity of anybody.

Liver disease is a major cause of illness and death worldwide regardless of age, sex, region and race. According to European Association for the Study of the Liver (EASL), approximately 29 million people in the European Union still suffer from a chronic liver condition (Blachier *et al.,* 2013). In the US, liver disease is reported as the second leading cause of mortality amongst all digestive diseases (Everhart and Ruhl, 2009). In Indonesia in 2007, based on the Basic Health Research, the prevalence of clinical hepatitis varied from 0.2 and 0.9% with an average of 0.6% (National Institute of Health Research and Development, 2009). Liver disease is caused by various toxicants such as certain drugs, carbon tetrachloride, thioacetamide, and arsenic. It is also caused by chronic alcohol consumption, viral or microbial infections and autoimmune disorders (Adewusi and Afolayan, 2012; Jannu *et al.,* 2012).

In spite the remarkable advance in conventional medicine, liver disease is

challenging not only for clinician but also researchers in drug discovery and development. Currently, effective drugs that stimulate liver function that can protect the liver from injury or regenerate liver cells are not available in the market (Chattopadhyay, 2003). Therefore, the availability of alternative drugs to treat liver diseases is needed. Some Indonesian medicinal plants have been used traditionally to treat liver diseases for a long time. Some of them were proven to have hepatoprotective activity such as *Curcuma longa* (Baxia *et al.,* 2013; Singh *et al.,* 2012; Somchit *et al.,* 2005), *Curcuma xanthorrhiza* (Devaraj *et al.,* 2014; 2010), *Zingiber officinale* (Bardi *et al.,* 2013; Lebda *et al.,* 2013; Atta *et al.,* 2010), *Phyllanthus niruri* (Harish and Shivanandappa, 2006; Chatterjee *et al.,* 2006) and Andrographis paniculate (Nagalekshmi *et al.,* 2011; Rajalakshmi *et al.,* 2012).

Clerodendrum serratum L. Moon, with local name *senggugu*, is one of medicinal plants that has been used traditionally in India, China, Thailand, Korea, Japan and Indonesia to treat several illness such as syphilis, typhoid, cancer, jaundice, hypertension, asthma, bronchitis, and cough (Shrivastava and Patel, 2007; Heyne, 1950). *Senggugu* has been reported to possess several biological activities including anticancer, antinociceptic, anti-inflammatory, antipyretic (Narayanan *et al*., 1999; Wahyono, 2001), antifertility (Julaeha *et al*., 2013), mucolytic (Wahyono, 1998), tracheospasmolytic (Wahyono, 2004), antioxidant and antibacterial (Prasad *et al*., 2012).

Our previous study showed that ethyl acetate fraction of *C. serratum* L. Moon (EAFCS) root bark had *in vitro* antioxidant activity to scavenge DPPH radical with IC_{50} of 30.968±0.686μg/mL (Nasrudin, 2015). Furthermore, the hepatoprotective activity of the ethanol extract of C. *serratum* root has also been reported (Vidya *et al*., 2007). In this study, we reported that the hepatoprotective activity of the EAFCS root bark in rats induced by carbon tetrachloride (CCl_4). Moreover, the effect of the fraction on oxidative stress status of rats was also reported.

MATERIALS AND METHODS

Plant materials

Root bark of plant used in this study was obtained from several population of *C. serratum* growing in Imogiri Subdistrict, Bantul District, Yogyakarta. The species was authenticated by the Departement of Pharmaceutical Biology Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta. A voucher specimen had been deposited at the same department.

Extract preparation

The *C. serratum* root barks were air-dried at the room temperature (25-30°C). The dried root barks were then chopped in order to obtain small size and pulverized by using Retsch Muhle brand machine before extraction at the Laboratory of Pharmaceutical Biology, Universitas Gadjah Mada. Powdered plant material was graded and extracted by maceration method using *n*-hexane and ethyl acetate. The extract of ethyl acetate was then filtered and the filtrate was evaporated to dryness using a rotary vacuum evaporator to obtain ethyl acetate fractions for *in vivo* hepatoprotective activity testing.

Experimental animals

Male albino Wistar rats with 150-200g BW and aged 12 weeks were obtained from the Faculty of Pharmacy, Universitas Gadjah Mada. Rats were maintained under standard animal husbandry conditions (25±2°C, with humidity 40-70%, 12h dark/12h light cycle) and allowed access to standard laboratory food and water *ad libitum*. The protocol of this study has been approved by the Research Ethics Committee, the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta (No. 457/KEC-LPPT/IV/2016).

In vivo hepatoprotective activity testing

Hepatoprotective activity test was performed according to the previous study conducted by Gomes *et al.* (2011). Thirty-six rats were randomly divided into 6 groups of 6 rats in each group. Group 1 as normal control was given 0.5% Na-CMC. Group 2 as negative control were induced by CCl_4. Group 3 as positive control was induced by CCl_4 after given

silymarin at a dose of 100mg/Kg.BW in 0.5% Na-CMC orally. Group 4-6 as treatment groups were induced by CCl_4 after given ethyl acetate extract fraction at dose of 25; 50 and 100mg/Kg BW in 0.5% Na-CMC orally, respectively. The silymarin and ethyl acetate extract fraction were given once daily for 7 days. The CCl_4 induction was performed intraperitoneally at dose of 1mL/Kg.BW on day 8th. On day 9th rats were anesthetized with ketamine 60mg/Kg.BW intramuscularly and blood sample was drawn by retro-orbital puncture. The blood samples were placed at room temperature for 30min and then centrifuged (2000g, 10min) to separate the serum. The serum samples were collected and stored in vacutainer vials at -4°C temperature until analysis. At the end of the experiment, rats were sacrificed under deep anesthesia by inhalation of diethyl ether and then the abdominal incision was made. Livers were removed from the rats and immediately washed with salt (0.9% sodium chloride) in cold conditions. The liver tissues were dried and weighed as much as 100 mg (10%) for analysis of oxidative stress parameters (Pareek *et al.*, 2013).

Estimation of biochemical parameters

Liver blood tests were conducted to assess liver functions including the level of SGOT (Serum Glutamic Oxaloacetic Transaminase), SGPT (Serum Glutamic Pyruvic Transaminase), ALP (Alkaline Phosphatase), bilirubin, and total protein. All parameters were measured using test kit for blood biochemistry at Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta.

Estimation of oxidative stress parameters

Liver organs were homogenized using homogenizer for preparing of the sample by adding 500mL of 50mM Tris buffer (pH 7.4) containing 1mM EDTA and 10µg/mL leupeptin. The homogenates were centrifuged at 4°C temperature at 10.000rpm for 10min to obtain supernatants. The supernatants were used for analysis of oxidative stress parameters Including MDA (malondialdehyde), GSH (glutathione) and CAT (catalase) (Pareek *et al.*, 2013).

Liver MDA levels were measured according to Singh *et al.* (2008). As much as 0.5mL of liver supernatant was added 2.0mL of cold HCl (0.25N) containing 15% TCA, TBA 0.38%, and 0.5% BHT. The mixture was heated at 80°C for one hour. Once cooled, the mixture was centrifuged at 3500 rpm for 10min. The absorbance of the supernatant was measured at 532nm. Tetraethoxypropane (ETP) was used as the standard solution.

The activity of GPx enzyme was determined as follows: 200µL of 10mM reduced glutathione (GSH) and 200µL of glutathione reductase enzyme (2.4 units) were incubated at 37°C for 10min and added by 200µL of 1.5nM NADPH. The mixture was incubated again at 37°C for 3min, followed by addition of 200µL of 1.5nM H_2O_2. The absorbance was measured by using spectrophotometer at 340nm. The enzyme activity was calculated based on following formula:

$$M\ unit\ of\ GSH\text{-}Px = \frac{Abs \times V_t \times 2 \times 1000 \times 1/mg\ Protein}{6{,}22 \times V_s}$$

Abs = absorbance changes; Vt = total volume (mL); 6.22 = extrinsic coefficient of NADPH; 2 = 2mol of GSH that equivalent to 1mol of NADPH; 1000 = change into a milli unit, and Vs = sample volume.

The activity of CAT enzyme was measured according to Aebi *et al.* (1984) and Jayakumar *et al.* (2006). A total of 0.5mL of the liver supernatant was added into 2.0mL of 50mM potassium phosphate buffer (pH 7.0) containing 10mM of H_2O_2. The absorbance was measured by using spectrophotometer at 240nm and recorded every 15s for one minute. The enzyme activity was calculated using slope data from the curve of absorbance of the sample solution (SL) and the blank solution (SLb) based on following formula:

$$\text{CAT activity (U/mg)} = \frac{(SL - SLb)}{0.436} \times \frac{2.5}{0.5}$$

Statistical analysis

Data were presented as mean ± standard deviation (SD). The different in biochemical and oxidative stress parameters in each group were analyzed by using Mann-Whitney test. A p-value <0.05 was considered as significantly different.

RESULTS AND DISCUSSION

Estimation of biochemical parameters

This study was conducted to evaluate the hepatoprotective of EAFCS root bark on CCl_4 induced hepatotoxic in male albino Wistar rats. CCl_4 is widely used for experimental induction of liver damage due to it is one of the best models to describe the cellular mechanisms of oxidative damage (Basu, 2003). Hepatocellular damage is thought through reactive CCl_4 metabolites. CCl_4 is metabolized by cytochrome P_{450} in the endoplasmic reticulum and mitochondria resulting trichloromethyl radicals and then trichloromethyl-peroxyl radicals (Shenoy *et al.,* 2001). These radicals subsequently attack the cellular macromolecules such as proteins or fats resulting in lipid peroxidation and cell necrosis (Zhou *et al.,* 2013).

Table I shows the effect of EAFCS root barks on liver functions of rats after CCl_4 induction. The liver enzymes (SGOT, SGPT, and ALP) and serum bilirubin concentration of EAFCS group at the dose of 100mg/Kg.BW and silymarin at the dose of 100mg/Kg.BW was significantly lower than those of CCl_4 group at a dose of 1mL/Kg.BW, whereas their serum total protein concentrations were significantly higher ($p<0.05$). However, they were still significantly higher than those of Na-CMC group as normal control ($p<0.05$), whereas their serum total proteins were not significantly different ($p>0.05$). The results indicated that induction with CCl_4 at dose 1mL/Kg.BW caused hepatic damage of the rats and pretreatment with EAFCS at the dose of 100mg/Kg.BW protected hepatic damage.

The results obtained in this study were in agreement with the results of previous studies using extracts of different parts of *C. serratum* as samples. Vidya *et al.* (2007) reported the hepatoprotective activity of ursolic acid isolated from of *C. serratum* roots at the dose of 10mg/Kg.BW was more significant than its ethanolic extract at the dose of 20mg/Kg.BW against CCl_4 induced hepatotoxic in male Wistar rats. Another study showed that alcoholic and aqueous extracts of *C. serratum* leave at the dose of 200mg/Kg. BW had hepatoprotective activity against rifampicin induced hepatotoxic in male and female Swiss albino mice (Agrawal *et al.,* 2013).

Estimation of oxidative stress parameters

Liver injury due to CCl_4 induction in rats is caused by the formation of trichloromethyl-peroxyl radicals which initiates lipid per-oxidation (Shenoy *et al.,* 2001). The hepato-protective medicinal plants act through various mechanisms including reduced in lipid peroxidation and increase in glutathione level (Kumar *et al.,* 2013). In this study, the effect of the ethyl acetate fraction of *C. serratum* L. Moon root bark on oxidative stress status of rats induced by CCl_4 was also evaluated (Table II). The MDA level of the liver of rats induced CCl_4 was significantly higher than that of normal rats not induced CCl_4 ($p<0.05$). Malondialdehyde is one of the final products of lipid peroxidation process. It is commonly used as a marker of oxidative stress. The increase of MDA levels after CCl_4 induction indicated the occurrence of liver injury due to oxidative stress. Furthermore, pretreatment of the EAFCS root barks significantly reduced the MDA level in dose dependent manner ($p<0.05$). It was indicated that the EAFCS root barks could protect the increasing in lipid peroxidation process in the liver due to CCl_4 induction. Hence it may be possible that the mechanism of hepatoprotection of the fraction is due to its antioxidant effect.

In contrast, the GPx and CAT activities of liver of rats induced CCl_4 were significantly lower than that of normal rats not induced CCl_4 ($p<0.05$). Glutathione peroxidase and CAT are antioxidant enzymes that found in many mammalian cells, including red blood cells. It was well reported that low activity of these enzymes may render the liver tissue susceptible to lipid peroxidation damage (Recknagel *et al.*,

Table I. The effect EAFCR root bark on liver functions of rats after CCl4 induction

Group of treatment*	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	BRN (mg/dL)	TP (g/dL)
Na-CMC (1.0mL/Kg.BW)	215.06± 188.16	96.46± 192.28	206.11± 116.89	0.75± 0.11	7.29± 0.42
CCl_4 (1.0mL/Kg.BW)	716.13± 116.56	644.72± 137.57	761.26± 312.80	1.06± 0.14	5.85± 0.51
Silymarin (100mg/Kg.BW)	315.12± 190.80[a]	303.66± 137.84[a]	255.08± 90.92[a]	0.68± 0.10[a]	7.05± 0.76[a,b]
EAFCR (25mg/Kg.BW)	690.05± 263.17	665.22± 169.85	564.38± 176.58	0.91± 0.07	6.14± 0.43
EAFCR (50mg/Kg.BW)	838.42± 184.42	656.70± 184.59	426.67± 82.34	0.79± 0.06	5.95± 0.45
EAFCR (100mg/Kg.BW)	528.34± 154.37[a]	275.71± 146.23[a]	277.70± 41.28[a]	0.72± 0.08[a]	7.10± 0.33[a,b]

EAFCR: ethyl acetate fraction of *C. serratum* root barks; *Data was presented as mean ± SD from 5 rats of each group (n = 5); SGOT: serum glutamic oxaloacetic; SGPT: serum glutamic pyruvic transaminase; ALP: alkaline phosphatase; BRN: biliubin; TP: total protein. [a]significantly different compared to CCl_4 and Na-CMC groups ($p < 0.05$); [b]not significantly different compared to Na-CMC group ($p > 0.05$)

Table II. The effect of EAFCS root barks on stress oxidative status of rats after CCl_4 induction

Group of treatment	MDA (nmol/g)	GPx (U/mg)	CAT (U/mL)
Na-CMC (1.0mL/Kg.BW)	2.75±0.19	72.91±0.92	6.61±0.23
CCl_4 (1.0mL/Kg.BW)	9.15±0.47	11.35±0.32	1.66±0.22
Silymarin (100mg/Kg.BW)	3.68±0.38[a]	57.83±0.85[a]	5.46±0.27[a]
EAFCS (25mg/Kg.BW)	7.53±0.24[a]	15.98±0.39[a]	2.43±0.09[a]
EAFCS (50mg/Kg.BW)	5.35±0.23[a]	48.71±2.23[a]	4.33±0.15[a]
EAFCS (100mg/Kg.BW)	3.39±0.14[a]	58.70±0.51[a]	5.59±0.32[a]

EAFCS: ethyl acetate fraction of *C. serratum* root barks; *Data was presented as mean ± SD from 5 rats of each group (n = 5); MDA: malondialdehide; GPx: glutathione peroxidase; CAT: catalase; [a]significantly different compared to CCl_4 and Na-CMC groups ($p < 0.05$).

1989). The decrease of GPx and CAT after CCl_4 induction also indicated the occurrence of liver damage. It the present study we demonstrated the effectiveness of the ethyl acetate fraction of EAFCS barks in the recovering reduced GPx and CAT levels and to prevent tissue disorders and injuries.

The decrease in the level of lipid peroxidation and the increase in GPx and CAT levels might be due to the potent antioxidant activity of *C. serratum* L. Moon. Studies concerning *in vitro* and *in vivo* antioxidant activity of *C. serratum* L. Moon have been previously reported. The ethyl acetate fraction of roots bark of this plant has been proven to possess antioxidant activity through the DPPH free radical scavenging activity with an IC_{50} value of 30.968±0.686µg/mL (Nasrudin, 2015). Bhujbal *et al.* (2009) reported that ethanolic extract of the *C. serratum* Linn roots possessed antioxidant activity through the DPPH free radical scavenging activity, reducing power assay and scavenging of hydrogen peroxide. The IC_{50} value of the ethanolic extract compared with ascorbic acid as control through the DPPH free radical scavenging activity were 175 and 137µg/mL, respectively. Acharya and Patel (2016) investigated *in vitro* antioxidant

activity of methanolic extract and ethyl acetate, *n*-butanolic as well as aqueous fractions of *C. serratum* roots using DPPH and ABTS radical scavenging test. The result showed that DPPH and ABTS radical scavenging effects of ethyl acetate fractions of *C. serratum* roots were found to be significantly higher (IC_{50} value 12.52±2.21μg/mL and 18.12±1.76μg/mL) compared to others fractions. Furthermore, *in vivo* antioxidant activity study on Wistar albino rats showed that water extract of *C. serratum* leaves increased the antioxidant capacity of blood and had an inhibitory effect on the basal lipid peroxidation of liver and kidney (Rajlakshmi *et al*., 2003).

Phytochemical studies of *C. serratum* have been conducted by some authors. Phytochemically this plant contains more than 35 compounds consisting of different chemical classes of compounds dominated by saponins (triterpenoids and sterols), phenolics, flavonoids, and carbohydrates (Murade *et al.,* 2015). Phenolics, saponins, and flavonoids have been shown to possess antioxidant properties (Scalbert *et al.,* 2005; Francis *et al.,* 2002; Pietta, 2000). These antioxidant compounds scavenge free radicals such as peroxide, hydroperoxide of lipid hydroxyl and thus inhibit the oxidative mechanisms that lead to cells injury (Subramanion *et al.,* 2011). Further study to isolate of active compounds from *C. serratum* responsible for this potent antioxidant is being carried out.

CONCLUSION

This study proves that ethyl acetate extract fraction of *C. serratum* L. Moon root barks has potent hepatoprotective activity in rats induced by CCl_4. The hepatoprotective effect is probably due to its antioxidant properties of the active compounds contained in this plant. Further study is needed to identify and characterize the active compound(s) responsible for its hepatoprotective effect.

ACKNOWLEDGEMENT

Authors would like to thank the Directorate General of Science & Technology Resource and Higher Education, Ministry of Research, Technology and Higher Education of the Republic of Indonesia for the financial support through BPPDN (*Beasiswa Pendidikan Pascasarjana Dalam Negeri*).

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