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Hepatoprotective and antioxidant activity of pentagamavunon-0 against carbon tetrachloride-induced hepatic injury in rats

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ABSTRACT

Objective: To investigate the hepatoprotective and antioxidant activity of pentagamavunon-0(PGV-0) against CCl_4-induced hepatic injury in rats. **Methods:** The groups of animals were administered with PGV-0 at the doses 2.5, 5, 10, and 20 mg/kg b.w., *p.o.* once in a day for 6 days and at day 7 the animals were administrated with carbon tetrachloride (CCl_4) (20%, 2 mL/kg b.w. in liquid paraffin (*i.p.*). The effect of PGV-0 on serum transaminase (SGPT), alkaline phosphates (ALP) and total bilirubin were determined in CCl_4-induced hepatotoxicity in rats. Further, the effects of PGV-0 on glutathione (GSH) content, catalase (CAT) and NO free radical scavenging activity also were investigated. **Results:** The results demonstrated that PGV-0 significantly reduced the activity of SGPT, serum ALP and total bilirubin in CCl_4 induced rat hepatotoxicity. PGV-0 has effect on the antioxidant and free radical defense system. It prevented the depletion level of GSH and decrease activity of CAT in CCl_4-induced liver injury in rats. PGV-0 also demonstrated the free radical scavenger effects on NO free radical scavenging activity with ES value of 32.32 μM. **Conclusion:** All of our findings suggests that PGV-0 could protect the liver cells from CCl_4-induced liver damages and the mechanism may through the antioxidative effect of PGV-0 to prevent the accumulation of free radicals and protect the liver damage.

1. Introduction

Liver play an important role in regulation of physiological processes mainly for carbohydrate, protein and fat metabolism, secretion of bile, storage of vitamins and xenobiotics or drug detoxification. In other hand, xenobiotics or drug can damage the liver cells. Almost all of the hepatotoxic chemicals damage liver cells mainly by inducing lipid peroxidation and other oxidative liver damages in liver[1,2]. Currently there are not many available drugs for treatment of liver disorders, which makes a consideration to develop new drugs with less side effect.

Curcumin (diferuloylmethane), a yellow pigment present in the food flavour spice turmeric, has been shown to have a wide spectrum of biological actions[3,4]. Curcumin exerted hepatoprotective effects of liver injury in various animal models against toxic effects of agent such as carbon tetrachloride[5,6], endotoxin[7] and thioacetamide[8]. Our groups has been synthesized pentagamavunon-0 (PGV-0), the derivatives of curcumin which has the biological activities higher than curcumin. PGV-0 has demonstrated the biological activities such as antioxidant, antiinflammatory and inhibit cyclooxygenase enzyme[9]. In addition, the derivatives did not significantly shown toxic effects on acute and subchronic toxicity testing.

Carbon tetrachloride (CCl_4) proves highly useful as an experimental model for the study of certain hepatotoxic effects for screening the anti-hepatotoxic/hepatoprotective activity of drugs or new compound in experimental model, because the CCl_4-induced hepatotoxicity is regarded and similar as and analogue of liver injury caused by variety of hepatotoxins in human[10,11]. It has been accepted that CCl_4-induced heptotoxicity results from its hepatotoxic metabolite and radical such as trichloromethyl free radical

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($^{\bullet}CCl_3$) and trichloromethylperoxy free radical ($CCl_3O_2^{\bullet}$). This free radicals can react with sulfhydryl groups, such as glutathione (GSH) and thiol-groups in the side chain of protein. Both trichloromethyl and its peroxy radical are also capable covalently binds to proteins or lipids, and then initiates the lipid peroxidation in the cellular membrane and liver damage, which eventually leads to the various liver pathological processes of diseases[11,12]. Therefore, one of the terapeutic strategy against hepatotoxicity and liver injury is to find the antioxidant compounds that are capable to protect liver injury through free radical scavenging generated by CCl_4.

There has been growing interest in the analysis of PGV-0 conducted by intense research into their potential benefits to human health. One of their main activities in this regard is their antioxidant activity, which enables them to attenuate the development of tumor and inflammatory disease. PGV-0 have the antioxidant activity more potent than curcumin[9]. Antioxidant plays an important role in inhibiting and scavenging free radicals, thus providing protection to humans against infection and degenerative diseases. According to the important role of that antioxidant activity of PGV-0, the present study was carried out to evaluate the antioxidant and hepatoprotective activity of PGV-0 against CCl_4-induced liver damage in rats.

2. Materials and methods

2.1. Materials

PGV-0 was obtained from Curcumin Research Center, Faculty of Pharmacy, Gadjah Mada University. CCl_4, glutathione, and 5,5′-dithio bis-2-nitrobenzoic acid (DTNB), H_2O_2, sodium nitroprusside (SNP), sulphanilamide and N-naphthyletylenediamine dihydrochloride were purchased from E. Merck, Darmstadt, Germany. Serum glutamate pyruvate transaminase (SGPT), serum alkaline phosphatase (SALP), total bilirubin were analyzed using reagent kits (DiaSys Diagnostic, Holzheim, Germany). All other reagents were of analytical grade.

2.2. Animals

Studies were carried out using male Wistar albino rats (180-200 g). Rats were obtained from the animal house, Faculty of Pharmacy Gadjah Mada University, Yogyakarta, Indonesia. The animals were grouped and housed in polyacrylic cages (38 cm × 23 cm × 10 cm) with not more than five animals per cage and maintained under standard laboratory conditions [temperature (25±3) ℃] with dark and light cycle (12/12 h) and allowed free access to standard pellet diet and reverse osmosis water *ad libitum*. Rats were acclimatized to laboratory condition for 1 week before commencement of experiment. All procedures and protocol described were conducted in accordance with Guideline for Care and Use of Laboratory Animals of Institutional Animal Ethics Commitee of Gadjah Mada University.

2.3. Hepatoprotective study

Thirty five healthy Wistar albino rats were divided into 7 groups each containing 5 animals. Group 1 (normal) administered with vehicle (CMC Na 0.5%, *p.o.*) for 6 days. Group 2 (PGV-0 only) administered with PGV-0, 20 mg/kg, *p.o.* for six days. Group 3 (hepatotoxic group) administered with vehicle (CMC Na 0.5 %, *p.o.*) for six days and on day 7 treated with CCl_4, 2.0 mL/kg, *i.p.* Groups 4-7 administered with PGV-0 at a dose of 2.5, 5, 10, and 20 mg/kg, *p.o.*, respectively for six days and on day 7 treated with CCl_4, 2.0 mL/kg, *i.p.* After 48 hours of CCl_4 administration, the blood was collected through retro-orbital plexus for determination of SGPT, ALP and total bilirubin. Finally, animals were sacrificed and liver quickly removed and excised, rinsed in ice cold normal saline followed by 0.15 M Tris-HCl (pH 7.4) blotted dry and weighed. A 10 % w/v of homogenate was prepared in 0.15 M Tris-HCl buffer. A part of homogenate after precipitating proteins with trichloroacetic acid (TCA) was used for estimation of glutathione by the method of Ellman[13]. The rest of the homogenate was centrifuged at 15 000 rpm for 15 min at 4 ℃. The supernatant thus obtained was used for the estimation of catalase (CAT) activities was measured by the method of Aebi[14].

2.4. Estimation of glutathione (GSH)

The procedure to estimate the reduced GSH level followed to the method as described by Ellman [13]. The homogenate (in 0.1 M phosphate buffer, pH 7.4) was added with equal volume of 20% TCA containing 1 mM EDTA to precipitate the tissue proteins. The mixture was allowed to stand for 5 min prior to centrifugation for 10 min at 200 rpm. The supernatant (200 μL) was then transferred to a new set of test tubes and added 1.8 mL of the Ellman′s reagent (DTNB, 0.1 mM) was prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes make upto the volume of 2 mL. After completion of the total reaction, solutions were measured at 412 nm against blank. Absorbance values were compared with a standard curve generated from standard curve from known GSH. The glutathione level in liver was calculated as micromoL/g liver.

2.5. Estimation of CAT

CAT activity was measured by the method of Aebi[14]. Supernatant (0.1 mL) was added to cuvette containing 1.9 mL of 50 mM phosphate buffer (pH 7.0). Reaction was started by the addition of 1.0 mL of freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as

specific activity as unit/mg protein or total activity as unit. mg liver.

2.6. Assay of NO radical scavenging activity

Various concentrations of PGV-0 and SNP (10 mM, final concentration) in phosphate buffered saline in a final volume of 2 mL were incubated at 25 ℃ for 150 min. A control experiment without tested compounds but with the equivalent amount of vehicles was conducted in an identical manner of control. After incubation, 1.0 mL samples of reaction mixtures containing nitrite were removed and diluted with 1.0 mL of Griess reagent (1% sulphanilamide and 0.1% N-naphthyletylenediamine dihydrochloride in 2% H_3PO_4). The absorbance of these solutions were measured at 540 nm against the corresponding blank solutions. The effective scavenging (ES) value is the concentration of sample required to scavenge 50% of the NO radicals. Addition of varied concentrations of PGV-0 into the reaction mixture affected an increase in total absorbance upon treatment with Griess reagent. It indicated that the PGV-0 interfered with the absorbance value of nitrite detection. Therefore, in these experiments we had to exclude this interference by subtracting their absorbance at each concentration.

2.7. Statistical analysis

The data are presented as the mean±SEM. The statistical significance of differences between the groups were assessed with a one-way ANOVA, followed by Bonferroni *post-hoc* test analysis using rel 13.0 software SPSS (Chicago, IL, USA). * $P<0.05$; ** $P<0.01$; and *** $P<0.001$ considered statistically significant.

3. Results

3.1. Effect of PGV-0 on SGPT, ALP and total bilirubin

The result demonstrtated in Table 1 showed the effect of PGV-0 on SGPT, ALP and total bilirubin. The result demonstrated that SGPT, SALP and total bilirubin were found to be significantly increased in rats treated with CCl_4 when compared with the normal group ($P<0.001$). The administration of PGV-0 for 6 days significantly decreased the activity of serum transaminase in a dose-dependent manner, SALP and total bilirubin in CCl_4-induced liver damage in rats compared to that of hepatotoxic group (CCl_4 treatment) ($P<0.01$, $P<0.001$).

3.2. GSH level in liver tissues

The result in Table 2 showed the effect of PGV-0 on glutathione content in the liver. The content of GSH level in liver in hepatotoxic group (CCl_4 treatment) was significantly depleted compared to normal group ($P<0.001$). The administration of PGV-0 at doses 2.5-20 mg/kg was significant dose-dependent to restore the GSH content of

Table 1
Effect of administration of PGV-0 on serum GPT level, ALP and total bilirubin against CCl_4-induced liver damage in rats.

Treatment/group	SGPT (IU/L)		ALP (IU/L)		Total bilirubin (mg/dL)	
	Pre-treatment	Post- treatment	Pre-treatment	Post- treatment	Pre-treatment	Post-treatment
Normal(CMC Na, 0.5%)	64.20±3.04	62.80±2.08	331.40±14.44	337.20±22.16	0.388±0.013	0.372±0.014
PGV-0 only(20 mg/kg)	57.40±2.98	95.40±6.68	479.40±31.39	637.40±30.88	0.290±0.010	0.400±0.017
Hepatotoxic(CCl_4, 2.0 mL/kg)	69.20±1.07	982.00±57.10$^{###}$	330.80±18.90	1 627.20±43.75$^{###}$	0.406±0.016	0.612±0.030$^{###}$
PGV-0(2.5 mg/kg) + CCl_4	71.40±2.87	906.00±29.30	612.40±15.76	1 127.60±68.43**	0.388±0.016	0.552±0.043
PGV-0(5 mg/kg) + CCl_4	74.75±3.53	803.60±56.39	446.00±23.76	1 091.60±93.23**	0.314±0.022	0.584±0.017
PGV-0(10 mg/kg) + CCl_4	61.50±1.55	581.50±65.43***	523.00±42.04	791.50±59.39***	0.330±0.017	0.480±0.018*
PGV-0(20 mg/kg) + CCl_4	65.00±2.19	203.20±9.82***	316.80±29.69	510.40±14.60***	0.392±0.015	0.322±0.016***

Data were expressed as mean±SEM. $^{###}$ $P<0.001$ significantly different to normal group. * $P<0.05$; ** $P<0.01$; and *** $P<0.001$ significantly different to hepatotoxic group (CCl_4 treatment).

Table 2
Effect of administration of PGV-0 on catalase and glutathione content against CCl_4-induced liver damage in rats.

Treatment/group	CAT		Glutathione content(μ mol/g liver)
	Specific activity (unit/mg protein)	Total activity (unit. mg liver)	
Normal (CMC Na, 0.5%)	18.72±0.97	63.80±3.35	1.99±0.11
PGV-0 only (20 mg/kg)	22.52±0.98	113.29±4.63	2.23±0.08
Hepatotoxic (CCl_4, 2.0 mL/kg)	5.72±0.51$^{###}$	23.06±1.16$^{###}$	0.76±0.11$^{###}$
PGV-0 (2.5 mg/kg) + CCl_4	6.09±0.77	35.84±3.59	1.40±0.07**
PGV-0 (5 mg/kg) + CCl_4	9.21±0.10*	55.60±0.58***	1.38±0.14**
PGV-0 (10 mg/kg) + CCl_4	10.67±0.39**	68.19±2.68***	1.85±0.05***
PGV-0 (20 mg/kg) + CCl_4	14.65±0.77***	88.63±4.51***	1.89±0.10***

Data were expressed as mean±SEM. $^{###}$ $P<0.001$ significantly different to normal group. * $P<0.05$; ** $P<0.01$; and *** $P<0.001$ significantly different to hepatotoxic group (CCl_4 treatment).

liver (P<0.01, P<0.001) against CCl_4-induced GSH depletion. PGV-0 (10 and 20 mg/kg) administration for 6 days was able to reverse of GSH to normal level (P<0.001). Therefore, PGV-0 is capable to protect the depletion of GSH content against CCl_4-induced liver damaged.

3.3. Catalase activity in liver tissues

The result demonstrated that CAT activity in liver of CCl_4 treatment group was significantly lower than normal group (Table 2) (P<0.001). The administration of PGV-0 significant dose-dependent increased CAT activity compared to CCl_4 treatment group (specific activity, P<0.01, P<0.001 and total activity, P<0.001). The administration PGV-0, 10 and 20 mg/kg completely restored the CAT activity to the normal level. The results suggest that PGV-0 capable to protect the reduction of CAT against CCl_4-induced liver damaged.

3.4. In vitro NO free radical scavenging activity

The effect of PGV-0 on NO free radical scavenging activity is presented in Table 3. In order to know the mechanisms of hepatoprotective of PGV-0, it is necessary to investigate the antioxidant effect of PGV-0. The result demonstrated the dose-dependent radical scavenging activity of NO free radical upon decomposition of sodium nitroprusside. The result suggest that PGV-0 is capable to scavenge NO free radical (ES_{50}=32.34 μM).

Table 3
The effects of PGV-0 on the accumulation of nitrite upon decomposition of sodium nitroprusside (10 mM).

Group/treatment	% Free radical scavenger
Control (DMSO)	-
PGV-0, 5 μM	3.23±4.08
PGV-0, 10 μM	11.73± 0.47
PGV-0, 20 μM	40.08±3.20
PGV-0, 30 μM	46.51±1.13
PGV-0, 40 μM	60.91±1.95

Data were expressed as mean±SEM (n=5).

4. Discussion

In the assessment of liver damage by hepatotoxin, CCl_4 is one of the most commonly used in the experimental study of liver diseases[10,11]. The administration of CCl_4 induced hepatotoxic undergoes enzymatic activation, majorly by CYP2E1, into membrane of endoplasmic reticulum. These processes are followed by chlorometylation and lipid peroxidation of endoplasmic reticulum membrane[11,12]. The elevated levels of serum enzymes such as SGPT and SGOT are indicative of cellular leakages and loss of functional integrity of membrane cell of liver. The membrane damage or necrosis releases the enzyme from the liver into circulation. The serum level of SGPT and SGOT are largely used for determination of liver damage. However, the level of SGPT is more specific to the liver, and is thus a better parameter for detecting liver injury[15]. Administration of CCl_4 significantly raises the serum levels of SGPT. The results demontrated that PGV-0 at doses 10 and 20 mg/kg significantly decreased the activity of SGPT levels, which may be a consequence of the protection of membrane cell from trichloromethyl free radical and trichloromethylperoxy free radical as well as repair of the liver tissue damage caused by CCl_4. In the other hand, activity of serum ALP and bilirubin also related to the function of hepatic cell. Our results using CCl_4-induced hepatotoxicity model in rats demonstrated that PGV-0 significantly decrease the activity of serum ALP and total bilirubin levels at the dose-dependent manner. The stabilization of SGPT, ALP and total bilirubin level by PGV-0 is an indicator of the improvement of the functional status of the liver. This findings were supported by our previous result that HGV-1, an analogue curcumin also demonstrated the hepatoprotective effect against CCl_4-induced liver injury[16]. GSH is an intracellular reductant and plays major role in catalysis, metabolism and transport. GSH plays a protective role in tissue by detoxification of xenobiotics and essential to maintain structural and functions integrity of the cell. It protects cells against free radicals, peroxides and other toxic compounds. GSH is a naturally occurring substance that is abundant in many living cells. It is widely known that a deficiency of GSH within living organisms can lead to tissue disorder and injury[17]. In the present study, administration of hepatotoxin CCl_4 significantly induced the depletion of GSH in the liver and caused imbalance of GSH/GSSH. Interaction of the reduced form of GSH with free radical readily converted to oxidized form GSSH. The present study showed the administration of PGV-0 prevent depletion of GSH in CCl_4-treated rats. The administration of PGV-0 during severe liver damage condition has elevated the GSH levels, which in turn helps in maintaining the liver tissue damage. This results indicates the additional antioxidant property of PGV-0. The results are consistent with the previous studies that some hepatoprotective compounds has a capability of enhancing GSH level to preserve GSH level[18,19].

CAT is a one of the key component of the antioxidant defense system. CAT decomposes hydrogen peroxide and protects the tissue from highly reactive hydroxyl radical. Therefore, the reduction of these protective mechanisms results in enhanced sensitivity to free radical-induced cellular damage. The accumulation of some free radical may results in a number of deleterious effects and alterations in the biological activity of cellular macromolecules. Administration of PGV-0 increases the activities of catalase in CCl_4-induced liver damage rats to prevent the accumulation of free radicals and protects the CCl_4-induced liver damage.

Several lines of evidences reported that curcumin is exhibit a strong antioxidant activity. It is a potent scavenger of a variety of ROS including superoxide anion radicals, hydroxyl radicals and nitrogen dioxide radicals[3,20]. PGV-0 is one of curcumin analogue that may have a similar action as hepatoprotective. In order to clarify the mode of action of PGV-0, the in vitro NO radical scavenger was carried out. The compound that possesses NO scavenging activity inhibited nitrite formation by competing with oxygen to react with NO. This lead to the reduction of nitrite concentration in the medium assay. PGV-0 effectively reduced the generation of NO radicals and scavenged NO radicals in a dose dependent manner. PGV-0 exhibited strong NO radical scavenging with low ES_{50} values (32.34 μM). Sardjiman[9] reported that change β-diketo to cyclopentanones having a C5 linker and the 4-hydroxyphenyl group confers potent antioxidant activity, which is much enhanced by one, or two, methoxy susbstituents ortho to the hydroxy group such like PGV-0. Therefore, PGV-0 may act as hepatoprotective in CCl_4-treated by scavenger free radical and reactive oxygen species formed during CCl_4 metabolism.

In conclusion, the results of the present study demonstrate that PGV-0 up to 20 mg/kg has a potent hepatoprotective action upon carbon tetrachloride-induced hepatic damage in rats. Our results show that the hepatoprotective effects of PGV-0 may be due to its antioxidant and free radical scavenging properties.

Conflict of interest statement

We declare that we have no conflict of interest.

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