

Research Article

HEPATOPROTECTIVE EFFECT OF GAMAVUTON-0 AGAINST D-GALACTOSAMINE/LIPOPOLYSACCHARIDE-INDUCED FULMINANT HEPATIC FAILURE

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ABSTRACT

The objective of this study is to determine the hepatoprotective effect of GVT-0 (one of curcumin analogues) against liver damage in rat-induced D-galactosamine (D-GalN)/lipopolysaccharide (LPS) as a model of fulminant hepatitis. In the study D-GalN/LPS elevated serum GPT activity that indicate a particular occurrence of liver damage due to depletion of UTP and UDP-glucuronic acid. Administration of GVT-0 (10 mg/kg) showed decreased enzyme activity of SGPT/SGOT but had no effect on serum ALP and total bilirubin levels, whereas at doses of 20 and 40 mg/kg, the protective effect of GVT-0 was decrease. The glutathione content in the D-GalN/LPS (0.76 ± 0.07) mol/g liver content was found lower than controls (0.90 ± 0.03) mol/g liver. Administration of GVT-0 dose of 10, 20 and 40 mg/kg restored glutathione content returned to normal levels. The results showed that treatment of GVT-0 showed no effect on TBARS and catalase activity. Treatment of D-GalN/LPS, indicating the trend of increased TNF- α , although statistically not significant, while the administration of GVT-0 showed a tendency to decrease the concentration of TNF- α . All findings of the results indicated that the GVT-0 mainly lower dose (10 mg/kg) showed hepatoprotective action in rat model of fulminant hepatitis induced by D-GalN/LPS. The results indicated that the mechanism of hepatoprotective effect of GVT-0 is not via antioxidant properties of GVT-0. However, further studies are necessary to explain the molecular mechanism of hepatoprotective effect of GVT-0.

Key words: Gamavuton-0, hepatoprotective, fulminant hepatitis, D-galactosamine/LPS

INTRODUCTION

Liver is an important organ that regulates many important metabolic functions including drug elimination and detoxification (Meyer and Kulkani, 2001). Some of drug or xenobiotics can damage the liver through the metabolism via P450. Liver cells can be damaged by microorganisms, hepatotoxins (chemicals or drugs), and certain pathological conditions. Exposure of mice to D-galactosamine (D-GalN) and lipopolysaccharide (LPS) are widely used for an animal model of fulminant hepatic failure. A low dose of LPS in combination with D-GalN has been shown to induce experimental liver injury which is similar to acute hepatic failure in the clinical setting (Nakama *et al.*, 2001). D-GalN/LPS-

induced fulminant hepatic failure in mice is a promising animal model for elucidating the mechanisms of clinical liver complaints and for evaluating the efficiency of hepatoprotective activity (Tiegs, 1994; Matsumoto *et al.*, 2004; Matsuda *et al.*, 2001) It was found that dysfunction of liver microcirculation, metabolic changes, direct cytotoxicity, and macrophages-mediated liver injury occurred in LPS-treated mice (Lee *et al.*, 1993). D-GalN, a specific hepatotoxic agent, was used to increase the sensitivity to the lethal effects of endotoxin.

It is known that Kupffer cells produce several kinds of biologically active mediators and seem to be the most important source of tumor necrosis factor (TNF)- α (Decker, 1990).

As LPS is a potent stimulator of TNF- α released from Kupffer cells, TNF- α plays a critical role in the pathogenesis of D-GalN/LPS-induced fulminant hepatic failure in mice (Mignon *et al.*, 1999). TNF- α induces apoptosis in hepatocytes and neutrophil transmigration, a critical step in the necrosis of hepatocytes, that occurs at a later stage in this liver injury (Tiegs *et al.*, 1989; Chosay *et al.*, 1997). The hepatic lesions in this model resemble those of human hepatitis because the up-regulation of TNF- α expression and hepatic apoptosis have been reported as pathogenic symptoms in human hepatitis (Galanos *et al.*, 1979).

Curcumin (diferuloylmethane) is a yellow pigment and the active constituents of turmeric. In animal studies turmeric demonstrated the hepatoprotective effects against variety of hepatotoxin such as carbon tetrachloride (CCl₄) (Deshpande *et al.*, 1998; Park *et al.*, 2000), galactosamine (Kiso *et al.*, 1983), acetaminophen (paracetamol) (Donatus *et al.*, 1990), and *Aspergillus aflatoxin* (Soni *et al.*, 1992). Our groups has been synthesized Gamavuton-0 (GVT-0), one of analog curcumin (Fig.1). GVT-0 has demonstrated the biological activities such as antioxidant, anti-inflammatory (Reksohadiprodjo *et al.*, 2004) and inhibit cyclooxygenase enzyme (Nurrochmad *et al.*, 1998). The derivatives also less effects of ulcer and did not significantly shown toxic effects on acute and subchronic toxicity testing (Soni *et al.*, 1992).

The study of potential effect of GVT-0 have been conducted related into their potential pharmacological effect in human. One of their main activities in this regard is their antioxidant activity (Resohadiprodjo *et al.*, 2004; Sardjiman *et al.*, 1997). Antioxidant plays an important role in inhibiting and scavenging free radicals, thus providing protection to humans against infection and degenerative diseases. Regarding to the important role of antioxidant activity of GVT-0, the present study was conducted to evaluate the hepatoprotective activity of GVT-0 against D-Galactosamine/LPS-induced liver damage in rats model fulminant hepatitis.

METHODOLOGY

Materials

Gamavuton-0 (GVT-0) was obtained from Curcumin Research Center, Faculty of Pharmacy, Universitas Gadjah Mada. D-Galactosamine (D-GalN), lipopolysaccharide from *Escherichia coli* O26:B6 (LPS), glutathione, and 5,5'-dithio bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chem., St Louis. Serum Glutamate Pyruvate Transaminase (SGPT), Serum Alkaline Phosphatase (SALP), total bilirubin were analyzed using reagent kits (DIALAB GmbH, Neudorf). Trichloroacetic acid (TCA), K₂HPO₄ p.a., KH₂PO₄ p.a., EDTA p.a., H₂O₂ p.a., acetic acid p.a., CMC Na (E Merck, Germany). 5,5'-dithio bis-2-nitrobenzoat (DTNB), glutathione standard, TBA (Sigma Chemical Co. St. Louis, MO). For determined serum TNF- α was used ELISA Kit (Boster Immunoleader Co. Ltd). All other reagents were of analytical grade.

Animals

Studies were carried out in male Wistar albino rats (180–200 g). Animals are obtained from Pre-clinical Research Laboratories Integrated Research and Testing (LPPT)-UGM, Yogyakarta. The animals were grouped and housed in polyacrylic cages (38 × 23 × 10 cm) with not more than five animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C) with dark and light cycle (12/12 h) and allowed free access to standard pellet diet (PT. Comfeed, Indonesia) and water ad libitum. The rats were acclimatized to laboratory condition for 1 week before commencement of experiment. All procedures described conducted in accordance with Guideline for Care and Use of Animals Laboratory of Faculty of Pharmacy, Gadjah Mada University.

Hepatoprotective study

Thirty male rats were randomly divided into 5 groups (6 rats each group): group I, normal; group II, hepatotoxic group of D-GalN (300 mg/kg) and LPS (1 µg/kg), while the group III-V for the treatment of three doses of GVT-0 (10, 20 and 40 mg/kg), orally once daily

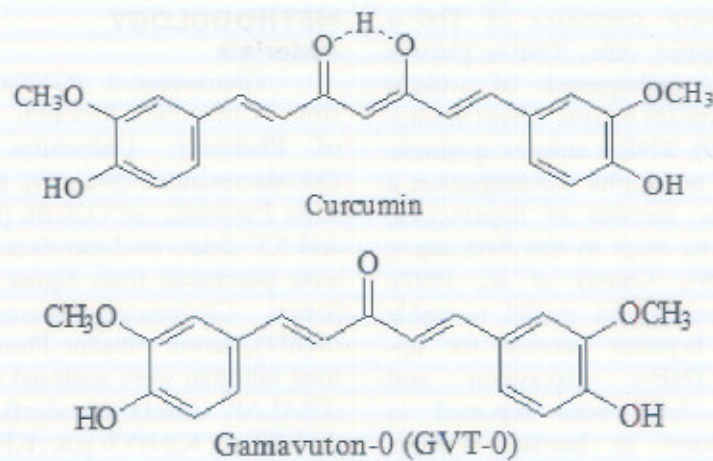


Figure 1. Structure of curcumin and its analog Gamavuton-0 (GVT-0)

Table I. The effect of administration of Gamavuton-0 (GVT-0) once daily for 6 days on serum GPT and GOT-induced by D-GalN/LPS. The activity of serum GPT and GOT described as Mean \pm SEM of 4-6 rats.

Group	Treatment	GPT-serum (U/l) (Mean \pm SEM)	GOT-serum (U/l) (Mean \pm SEM)
I.	Control CMC Na 0.5%	40.7 \pm 4.8	154.6 \pm 2.8
II.	Hepatotoxic, D-GalN/LPS	319.4 \pm 91.1 ^a	536.1 \pm 70.2 ^a
III.	GVT-0 (10 mg/kg, p.o) + D-GalN/LPS	213.0 \pm 21.6*	361.3 \pm 32.1*
IV.	GVT-0 (20 mg/kg, p.o) + D-GalN/LPS	369.0 \pm 71.2	478.5 \pm 37.0
V.	GVT-0 (40 mg/kg, p.o) + D-GalN/LPS	513.8 \pm 58.2*	537.6 \pm 60.1

*p<0.001, significantly different to normal control group. ^ap<0.05, significantly different to D-GalN/LPS group.

for 6 consecutive days. One hour after the last administration of test preparations, test animals (kel III-V) were given D-GalN (300 mg/kg) and LPS (1 μ g/kg). Twenty-four hours later blood sampling for determination of the activity of SGPT, SGOT, alkaline phosphatase (ALP), and serum bilirubin and serum TNF- α .

After collection of blood samples the rats were sacrificed and their livers 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 (1959). The rest of the homogenate was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant thus obtained was used for the estimation of catalase (CAT) activities was measured by the method of Aebi (1974).

Estimation of GSH

The procedure to estimate the reduced glutathione (GSH) level followed to the method as described by Ellman (1959). The homogenate (in 0.1 M phosphate buffer, pH 7.4) was added with equal volume of 20 % trichloroacetic acid (TBA) 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.

Table II. The effect of administration of Gamavuton-0 (GVT-0) once daily for 6 days on serum ALP and bilirubin-induced by D-GalN/LPS. The activity of ALP and bilirubin level described as Mean \pm SEM of 4-6 rats.

Group	Treatment	ALP (U/l) (Mean \pm SEM)	Bilirubin (mg/dl) (Mean \pm SEM)
I.	Control CMC Na 0.5%	572.9 \pm 37.1	0.3 \pm 0.03
II.	Hepatotoxic, D-GalN/LPS	753.9 \pm 49.3 ^a	0.4 \pm 0.04
III.	GVT-0 (10 mg/kg, p.o) + D-GalN/LPS	828.5 \pm 70.3	0.4 \pm 0.02
IV.	GVT-0 (20 mg/kg, p.o) + D-GalN/LPS	816.7 \pm 87.1	0.4 \pm 0.03
V.	GVT-0 (40 mg/kg, p.o) + D-GalN/LPS	836.5 \pm 84.9	0.3 \pm 0.02

^ap<0.001, significantly different to normal control group.

Table III. The effect of administration of Gamavuton-0 (GVT-0) once daily for 6 days on glutathione content and protein level-induced by D-GalN/LPS. The glutathione content and protein level described as Mean \pm SEM of 4-6 rats.

Group	Treatment	GSH (μ mol/g liver) (Mean \pm SEM)	Protein (mg/dl) (Mean \pm SEM)
I.	Control CMC Na 0.5%	0.90 \pm 0.03	23.34 \pm 3.31
II.	Hepatotoxic, D-GalN/LPS	0.76 \pm 0.07 ^a	17.54 \pm 2.07 ^a
III.	GVT-0 (10 mg/kg, p.o) + D-GalN/LPS	0.86 \pm 0.03*	15.78 \pm 1.36
IV.	GVT-0 (20 mg/kg, p.o) + D-GalN/LPS	0.87 \pm 0.03*	23.92 \pm 0.03*
V.	GVT-0 (40 mg/kg, p.o) + D-GalN/LPS	0.85 \pm 0.02*	25.48 \pm 1.99*

^ap<0.001, significantly different to normal control group. *p<0.05, significantly different to D-GalN/LPS group.

The supernatant (200 μ l) was then transferred to a new set of test tubes and added 1.8 mL of the Ellman's reagent (5, 5'-dithio bis-2-nitrobenzoic acid) (0.1 mM) was prepared in 0.3 M phosphate buffer with 1% of sodium citrate solution). Then all the test tubes make up to 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.

Lipid Peroxidation

Tissue TBARS determined according to the method of Ohkawa *et al* (1979). In 0.5 mL of homogenate was added 0.5 mL of saline and 1.0 mL 10% TCA, mixed well and centrifuged at 3000 rpm for 20 min. Take 1.0 mL of protein-free supernatant in a test tube and add

0.25 mL of thiobarbituric acid (TBA), then mixed and heated for 1 hour at 95°C. Reaction tube was cooled to room temperature and the absorbance was measured at 532 nm. The level of lipid peroxidation expressed as mol thiobarbituric acid reactive substances (TBARS)/mg protein.

Estimation of CAT

Catalase activity was measured by the method of Aebi (1974). 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 H₂O₂. The rate of decomposition of H₂O₂ was measured spectrophotometrically from changes in absorbance at 240 nm. Activity of catalase was expressed as units/mg protein.

Table IV. The effect of administration of Gamavuton-0 (GVT-0) once daily for 6 days on Catalase and TBARS-induced by D-GalN/LPS. The Catalase and TBARS described as Mean \pm SEM of 4-6 rats.

Group	Treatment	Catalase (U/mg liver)	TBARS (nmol/mg protein)
		(Mean \pm SEM)	(Mean \pm SEM)
I.	Control CMC Na 0.5%	18.84 \pm 2.07	0.050 \pm 0.011
II.	Hepatotoxic, D-GalN/LPS	14.44 \pm 7.13	0.040 \pm 0.005
III.	GVT-0 (10 mg/kg, p.o) + D-GalN/LPS	7.27 \pm 3.42*	0.053 \pm 0.009
IV.	GVT-0 (20 mg/kg, p.o) + D-GalN/LPS	21.94 \pm 4.62	0.030 \pm 0.004
V.	GVT-0 (40 mg/kg, p.o) + D-GalN/LPS	19.47 \pm 6.39	0.028 \pm 0.003

*p<0.001, significantly different to normal control group. *p<0.05, significantly different to D-GalN/LPS group.

Table V. The effect of administration of Gamavuton-0 (GVT-0) once daily for 6 days on TNF- α induced by D-GalN/LPS. The TNF- α described as Mean \pm SEM of 4-6 rats.

Group	Treatment	TNF- α (pg/mL) Purata \pm SEM
I.	Control CMC Na 0.5%	16,84 \pm 17,98
II.	Hepatotoxic, D-GalN/LPS	28,01 \pm 36,13
III.	GVT-0 (10 mg/kg, p.o) + D-GalN/LPS	17,14 \pm 21,23
IV.	GVT-0 (20 mg/kg, p.o) + D-GalN/LPS	26,51 \pm 22,45
V.	GVT-0 (40 mg/kg, p.o) + D-GalN/LPS	15,30 \pm 36,21

TNF- α assay

Blood was collected at 1 h after D-GalN/LPS administration. Serum TNF- α was determined at 1 h after D-GalN/LPS injection by an enzyme-linked immunosorbent assay kit according to the manufacturer's protocol (Booster Immunoleader Co. Ltd.).

Statistical analysis

The data are presented as the mean \pm S.E.M. The statistical significance of differences between the groups were assessed with a one-way ANOVA, followed by Bonferroni/Tuckey post-hoc test analysis using rel 15.0 software SPSS (Chicago, IL, USA).

RESULTS AND DISCUSSION

This research objective of this study is evaluate the hepatoprotective effect Gamavuton-0 (GVT-0) in male rats induced by D-GalN/LPS. Liver damage can be characterized by measuring the activity of GPT, ALP and serum bilirubin. Necrosis or damage

to liver cells can cause the release of these enzymes into the blood circulation. Changes in enzyme activity of GPT, GOT, ALP and total serum bilirubin described in table I-III. Activity of marker enzymes GPT and ALP has increased significantly in rats induced D-GalN/LPS compared to controls.

Increased activity of serum GPT indicate liver damage induced by D-GalN/LPS mainly depletion of UTP and UDP-glucuronic acid, whereas ALP activity increased due to increased synthesis in the presence of increasing biliary pressure (Kim *et al.*, 2009; Willianson *et al.*, 1996, Moss and buuterworth, 1974). While the administration of GVT-0 dose of 10 mg/kg showed decreased enzyme activity of GPT, GOT serum but not for ALP and bilirubin levels (Tables I and II). Decrease in enzyme activity of serum GPT and GOT indicated the protection of liver damaged as a model of hepatitis induced by D-GalN/LPS, whereas GVT-0 has no effect on secretory system of hepatic cellular.

The results showed that administration of GVT-0 at doses over 20 and 40 mg/kg the protective effect of diminishing GVT-0 to the increased activity of serum GPT and GOT in a mouse model of fulminant hepatitis induced by D-GalN/LPS. This results suggest that the protection doses of GVT-0 is 10 mg/kg and decreased with increasing dose. Reduced protective effect at doses over 10 mg/kg is still unexplained mechanism.

D-GalN-induced liver damage has similarities with drug-induced hepatitis in humans that characterized by parenchymal necrosis and inflammation (Jonker *et al.*, 1990; Kasravi *et al.*, 1996; Liehr *et al.*, 1978). Previous studies showed that D-GalN inhibits glucuronidation through rapid conversion into UDP-GalN and subsequently lead to depletion of UTP and UDP-glucuronic acid and reduced GSH content by pressing the synthesis of proteins in hepatocytes (Gregus *et al.*, 1988; Keppler *et al.*, 1970).

Study the effects of GVT-0 on the content of glutathione (GSH) liver are shown in Table III The content of glutathione in the D-GalN/LPS (0.76 ± 0.07) $\mu\text{mol/g}$ liver content was found lower than controls (0.90 ± 0.03) $\mu\text{mol/g}$ liver $p < 0.05$. Administration of GVT-0 dose of 10, 20 and 40 mg/kg increased glutathione content returned to normal levels starting dose of GVT-0 10 mg/kg. Glutathione distributed in many cells and plays an important role in the process of catalysis, metabolism and transport. GSH has an important role as a protective agent against electrophilic compounds, peroxides and free radicals that are toxic (Jollow, 1980). It has been widely known that GSH deficiency gives a hint of interference or damage. The results decline in GSH content is also correlated with higher levels of protein. Treatment of D-GalN/LPS also shown to reduce levels of protein and can be restored by treatment GVT-0 starting dose of 20 mg/kg. Decreased levels of this protein due to inhibition by GalN glucuronidation through rapid conversion into UDP-GalN and subsequent cause depletion of UTP and UDP-glucuronic acid and reduced GSH content by pressing the protein synthesis in hepatocytes (Gregus *et al.*, 1988; Keppler *et al.* 1970).

Malondialdehida (MDA) is a by product produced in the reaction of lipid peroxidation. The increasing in TBARS (MDA) showed indications of tissue damage mainly due to the cell membrane reactive compounds and free radicals. The results showed that treatment of D-GalN/LPS showed no increase in TBARS (Table 4). This suggests that the mechanism of liver damage is not through the formation of reactive compounds or radicals. The administration of GVT-0 also does not alter TBARS parameters, so the mechanism of GVT-0 protection against liver damage rather than on the inhibition of TBARS formation through an antioxidant mechanism. Besides, catalase is also a component of antioxidative system against reactive compounds and radicals. Decreased activity of this enzyme shows a number of cellular changes caused by the accumulation of superoxide radicals and hydrogen peroxide. The results showed that treatment of D-GalN/LPS and the administration of GVT-0 does not alter significantly the activity of catalase. This is according to previous reports that the mechanism of hepatic cell damage caused by D-GalN/LPS not through antioxidative mechanisms against the accumulation of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2).

It is known that Kupffer cells produce several mediators that are biologically active and it seems like one of the most important is tumor necrosis factor (TNF)- α (Decker and Keppler, 1974; Decker, 1990). LPS is a potent stimulator of TNF- α release from Kupffer cells, TNF- α plays an important role in the pathogenesis of D-GalN/LPS-induced fulminant hepatic failure and induces apoptosis in the early stages of liver damage (Mignon *et al.*, 1999). The results showed that treatment of D-GalN/LPS, indicating the trend of increased TNF- α , although not statistically significant, while the administration of GVT-0 showed a tendency to decrease the concentration of TNF- α . TNF- α plays an important role in damage to liver cells by caspase-8 and then stimulate caspase-3, which is a downstream cysteine proteinase, through multiple apoptotic pathways (Mignon *et al.*, 1999; Sakon *et al.*, 2003).

As a whole shows that GVT-0 at least in part involved in hepatoprotective such as GPT/GOT-serum and protection against the suppression of protein synthesis and GSH depletion in rats induced D-GalN/LPS. In some other aspects such as antioxidative systems such as catalase activity, lipid peroxidation and inflammatory pathways (TNF- α). Further research is needed to determine the mechanism of protection GVT-0 with a wide range of inductor of liver damage via different pathways. Our results also showed hepatoprotective effects of GVT-0 is not caused by the antioxidant properties of GVT-0. Further studies are necessary to explain in greater depth the molecular mechanism of hepatoprotective GVT-0.

CONCLUSION

GVT-0 mainly lower dose (10 mg/kg) showed hepatoprotective action in rat model of fulminant hepatitis induced by D-GalN/LPS. the mechanism of hepatoprotective effect of GVT-0 is not via antioxidant properties of GVT-0. However, further studies are necessary to explain the molecular mechanism of hepatoprotective effect of GVT-0.

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