



## HEPATOPROTECTIVE ACTIVITY EVALUATION OF DLBS1433 BIOACTIVE FRACTION FROM *PHALERIA MACROCARPA*

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### ABSTRACT

*Phaleria macrocarpa* is an Indonesian medicinal plant which has been known for its various uses. The present study aims to evaluate efficacy, antioxidant activity and cytotoxicity of DLBS1433, a bioactive fraction obtained from *P. macrocarpa*, as a hepatoprotective agent in ethanol-induced liver damage in *Rattus norvegicus*. Ethanol was administered to all rats (5 g/kg/day, 20% v/v) for 4 w and followed by treatment with DLBS1433-60 and DLBS1433-90 for 1 w, respectively. *Curcuma domestica* extract treatment group was used as a comparison. Blood biochemical parameters, which are the markers of liver function including alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin and gamma-glutamyl transferase (GGT), were examined to determine the hepatoprotective effect of DLBS1433. Liver histopathology examination was also conducted in this study. Ethanol administration for 4 w was able to increase blood biochemical parameters which are the markers of liver function. Ethanol administration followed by DLBS1433-60 treatment showed lower blood biochemical parameters and better liver histopathology results compared to DLBS1433-90 treatment group. Based on the results of the present study, it can be concluded that DLBS1433-60 was considered as a potential hepatoprotective agent as shown by the results of blood biochemical parameters and liver histopathological examination.

**KEYWORDS:** *hepatoprotective, liver, Phaleria macrocarpa, ethanol*



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## INTRODUCTION

It has been recognized that various chemicals, drugs, viruses and lifestyle habits may affect liver function by creating hepatic disturbances and causing cell damage which further lead to liver diseases, fatty liver, hepatitis and/or cirrhosis.<sup>1,2</sup> These potential damages are known to be complex mechanisms involving cellular metabolites that have direct cytotoxicity as well as formation of protein adduct with several hepatocyte protein.<sup>3,4</sup> Regarding various factors that may affect liver function, agents with hepatoprotective activity will be very useful to prevent and overcome hepatic disturbances. Previous studies have been done to investigate hepatoprotective activity of various plants.<sup>5-11</sup>

*Phaleria macrocarpa* (Scheff.) Boerl (Thymelaeaceae), commonly known as Crown of God, mahkota dewa and pau, is an Indonesian medicinal plant that originated from Papua Island. It is one of the most popular medicinal plants in Indonesia. *P. macrocarpa* grows throughout the year in tropical areas reaching a height of around 1-6 meter. It is a complete tree which stem, leaves, flower and also fruit can be differentiated. Fruit shape is eclipse with 3-cm diameter. The color of the fruit is green before ripening and red when the fruit is fully ripe.<sup>12</sup> This plant has been empirically used to control cancer, impotency, hemorrhoid, diabetes mellitus, allergy, kidney disorder and other diseases. Several studies regarding standardized fractions of *P. macrocarpa* flesh fruit have been done in our laboratory. The fractions have been scientifically studied for its anti-cancer activity<sup>13,14</sup> and for its effect to control premenstrual syndrome and/or primary dysmenorrhea.<sup>15,16</sup> This study is aimed to investigate the effect of DLBS1433, a bioactive fraction obtained from *P. macrocarpa*, for its effect as a hepatoprotective agent in ethanol-induced liver damage. As an attempt to evaluate the hepatoprotective activities of DLBS1433, a series of experiment were performed in animal model using *Rattus norvegicus* induced by ethanol which caused oxidative stress and further resulted in liver damage.

## MATERIALS AND METHODS

### Materials

DLBS1433 bioactive fraction was obtained from Dexa Laboratories of Biomolecular Sciences (DLBS), PT Dexa Medica (Cikarang, Indonesia). HepG2 human hepatocellular carcinoma cell line (ATCC® HB-8065™) was obtained from ATCC (Manassas, Virginia, USA). Penicillin, streptomycin, trypsin-EDTA, FCS and 2,2-diphenyl-1-picrylhydrazil (DPPH) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). Methanol was purchased from Merck (Darmstadt, Germany). Ascorbic acid, semi-automated blood biochemical analyzer Microlab 300 and spectrophotometer Hitachi U-2900 (Japan) were used in this study.

### *Phaleria macrocarpa* extraction process

*P. macrocarpa* fresh fruits were sliced, dried and weighed. The dried fruits were then poured into the extractor followed by water at temperatures of 60 and 90°C. The extracts were then filtrated and evaporated with low pressure to obtain concentrated extracts. The

concentrated extracts were collected immediately and dried using oven at a temperature of 60-70°C. Extracts that were extracted at temperatures of 60°C and 90°C were further named DLBS1433-60 and DLBS1433-90, respectively.

### *Curcuma domestica* extraction process

Fresh *Curcuma domestica* rhizome were sliced, dried and weighed. The dried rhizome was poured into the extractor followed by water at a temperature of 90°C. Maceration was then done for 2 h at a temperature of 90°C continued by filtration and evaporation with low pressure to obtain concentrated extract. The concentrated extract was dried using oven at a temperature of 60-70°C.

### HepG2 cell culture

FCS, L-glutamine, sodium bicarbonate, penicillin and streptomycin were added to Dulbecco's Modified Eagle Medium (DMEM). In this culture solution, at a temperature of 37°C and partial pressure of 5% CO<sub>2</sub>, HepG2 cells were multiplied and subcultured in 75-cm<sup>2</sup> culture flask (Falcon, BD). The multiplied HepG2 cells were separated using trypsin and suspended in fresh culture solution. These cells were put in 6-well plate (Falcon, BD). After reaching the confluent state, media solution with FCS 10% was collected and changed into media solution without FCS. All cells were treated with various concentrations of DLBS1433-60 and DLBS1433-90 to measure DLBS1433 cytotoxicity. The cells remaining on the plate were separated using 0.25% trypsin-EDTA and suspended in culture solution. Cell count measurement was conducted using hemocytometer.

### Animal

Male Wistar rats (*Rattus norvegicus*) obtained from Bandung, Indonesia were used in this study. All rats were 2 months old with body weight range of 200-250 g. Animal were housed individually in polysulfone cage and maintained under standard condition (12 h light-dark cycle). Before the study, all rats were acclimatized for 2 w. All procedures in this experiment were carried out in accordance to the guide for the care and use of laboratory animals and standard operating procedure at Dexa Laboratories of Biomolecular Sciences (DLBS).

### Experiment

In this experiment, rats were divided into 11 groups. The groups were consisted of normal control group, ethanol 20% control group, DLBS1433-60 groups at doses of 37.5 mg/kg; 50mg/kg; 75mg/kg body weight, DLBS1433-90 groups at doses of 37.5mg/kg; 50 mg/kg; 75mg/kg body weight, and *Curcuma domestica* (turmeric) extract groups at doses of 37.5 mg/kg; 50 mg/kg; 75 mg/kg body weight as positive control groups. Ethanol (5 g/kg/day, 20% v/v) was administered to all rats for 4 w and followed by DLBS1433-60, DLBS1433-90 and *Curcuma domestica* extract treatment for 1 w. At the end of the study, rats were anesthetized with ketamine and xylazine at doses of 80 mg/kg body weight and 10 mg/kg body weight, respectively. Blood were collected and the rats were euthanized under anesthesia. Liver were collected for histopathology examination.

**Analysis of blood biochemical parameters**

At the end of experiment, blood plasma was used for blood biochemical parameters measurement, including alanine transaminase (ALT), aspartate transaminase (AST), total bilirubin and gamma-glutamyl transferase (GGT), using blood chemistry analyzer Microlab 300.

**Histopathology analysis**

All liver samples were collected and fixed in 10% buffered formalin and processed with routine staining using hematoxylin eosin to prepare histopathology slides for liver damage examination. Histopathology examination was carried out according to the standard method.<sup>17</sup> Pathological changes of fatty liver and hepatocytes degeneration were graded as follows<sup>17</sup>:

- Level 0 (normal): Normal liver morphology, hepatocytes had centrally-placed round nucleus and homogenous cytoplasm, flat endothelial cells around central vein and sinusoid.
- Level 1 (mild degree): Some hepatocytes rows around central vein demonstrated hepatic cells degeneration, necrosis (loss of nucleus), endothelial cells showed less injury around central vein and less fat vacuoles in hepatocytes.
- Level 2 (moderate degree): Hepatocytes rows around central vein is swelling, intracytoplasmic vacuolar degeneration in centrilobular, midzonal and periportal area of endothelial cells around central vein with more injury compared to level 1 and increased fat vacuoles in hepatocytes as compared to level 1.

- Level 3 (severe degree): three to four hepatocytes rows around central vein showed hepatocytes degeneration and necrosis (loss of nucleus), cells degeneration including centrilobular, midzonal and periportal areas (diffused intracytoplasmic vacuolar degeneration), endothelial lining of central vein exhibited more cell injury, as well as increased fat vacuoles in hepatocytes as compared to level 2. Focal necrosis and bile duct proliferation were also marked.

**DPPH radical scavenging activity**

DLBS1433 was evaluated for free radical scavenging activity using DPPH assay. DLBS1433-60 at different concentrations were respectively mixed with 0.3 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol. Fifty milliliters of stock solution at a concentration of 500 ppm was prepared by dissolving ascorbic acid in methanol which was used as a standard for antioxidant activity comparison. Serial concentrations were prepared in separate test tubes using the formula of serial dilution  $M_1V_1=M_2V_2$ . One milliliter of DPPH was added to all samples. After incubation for 10 minutes at room temperature in dark condition, the absorbance of DLBS1433-60 and DPPH mixtures were measured using spectrophotometer (Hitachi U-2900) at 517 nm. The absorbance differences between test solutions and control were calculated and expressed as percentage radical scavenging activity or percentage of DPPH inhibition. The percentage of DPPH inhibition was calculated using the following formula:

$$\% \text{ Inhibition of DPPH} = (\text{Ac}-\text{As}/\text{Ac}) \times 100$$

As = Absorbance of the test solution

Ac = Absorbance of the control

**STATISTICAL ANALYSIS**

Data was expressed as mean  $\pm$  S.E.M. The results were analyzed statistically by one-way analysis of variance (ANOVA) followed by *post hoc* test for multiple comparisons (Tukey's HSD or Games-Howell test) using SPSS(R) 20 for statistic software. All statistical tests were done at a significance level of 5%.

**RESULTS****Changes in HepG2 cell numbers**

Results of this experiment in HepG2 cells line showed that DLBS1433 did not cause decreased cell number at doses up to 150  $\mu\text{g}/\text{ml}$ . All treatment groups exhibited no significant difference compared to normal group. This result indicated that DLBS1433-60 and DLBS1433-90 did not cause cellular death, as shown in Fig. 1. This finding was confirmed by the absence of the signs of cellular death which was indicated by changes in cells morphology after treatment (Fig. 2).

**Changes in animal blood biochemical parameters**

The results of ethanol group treatment exhibited significant increases of AST, ALT, total bilirubin and GGT levels compared to normal group. DLBS1433-60

treatment groups showed lower and better liver function parameters compared to those of DLBS1433-90 treatment group.

**Histopathology**

In histopathology examination, the normal group showed normal liver morphology. Hepatocytes constantly showed a centrally-placed round nuclei and homogenous cytoplasm with flat endothelial cells around central vein and sinusoid were clearly visible. When rats were treated by ethanol to induce liver damage, hepatocytes cytoplasm did not show homogeneity, while endothelial nucleus cells around central vein were severely damaged. Histopathology examination was done for DLBS1433-60 treatment groups and the results were compared to normal group. DLBS1433-60 treatment groups were further used in histopathology examination due to improved blood biochemical parameters results related to liver function. In other groups where ethanol treatment for 4 weeks was continued by DLBS1433-60 treatment at doses of 50 mg/kg and 75 mg/kg, respectively, the results exhibited an improved histopathology condition. The results of DLBS1433-60 treatment at doses of 50 mg/kg and 75 mg/kg also showed similar histopathology condition as those of *Curcuma domestica* extract at the same doses, respectively (Fig. 3).

**Antioxidant activity**

In addition to histopathology examination, various concentrations of DLBS1433-60 were measured for antioxidant activity. The results showed that DLBS1433-

60 exhibited the lowest free radical scavenging activity at a concentration of 10 ppm (6.85%), and the highest activity at a concentration of 100 ppm (76.8%) (Table II).

**Table I**  
**Blood biochemical parameters from different groups**

Groups	Doses (mg/kg)	Blood chemistry			
		ALT (U/L)	AST (U/L)	Total Billirubin	Gamma GT
Normal		31.15 ± 1.78	54.38 ± 3.96	3.66 ± 0.1	54.83 ± 11.17
Ethanol 20%		133.26 ± 13.19**	135.58 ± 18.15**	16.74 ± 0.48**	104.39 ± 14.62**
DLBS1433-60	37.5	95.5 ± 5.9*	92.69 ± 3.72	5.34 ± 1.25*	70.83 ± 2.52*
	50	85.8 ± 12.44*	94.87 ± 9.86	6.23 ± 0.59*	61.51 ± 11.09*
	75	44.78 ± 3.32*	60.64 ± 4.45*	3.19 ± 0.19*	61.45 ± 12.83*
DLBS1433-90	37.5	135.07 ± 10.52	96.24 ± 3.69	7.29 ± 0.05*	89.23 ± 3.45
	50	112.44 ± 11.52	85.32 ± 8.14	6.8 ± 0.1*	91 ± 1.41
	75	102.86 ± 12.52	98.52 ± 4.12	6.14 ± 0.13*	80.57 ± 1.06
<i>Curcuma domestica</i>	37.5	62.24 ± 13.69*	59.26 ± 4.44*	4.39 ± 0.83*	43.5 ± 8.21*
	50	80.66 ± 14.14*	92.38 ± 5.32*	4.03 ± 1.14*	42.58 ± 5.73*
	75	49.51 ± 9.52*	59.41 ± 3.91*	2.94 ± 0.64*	49.53 ± 2.94*

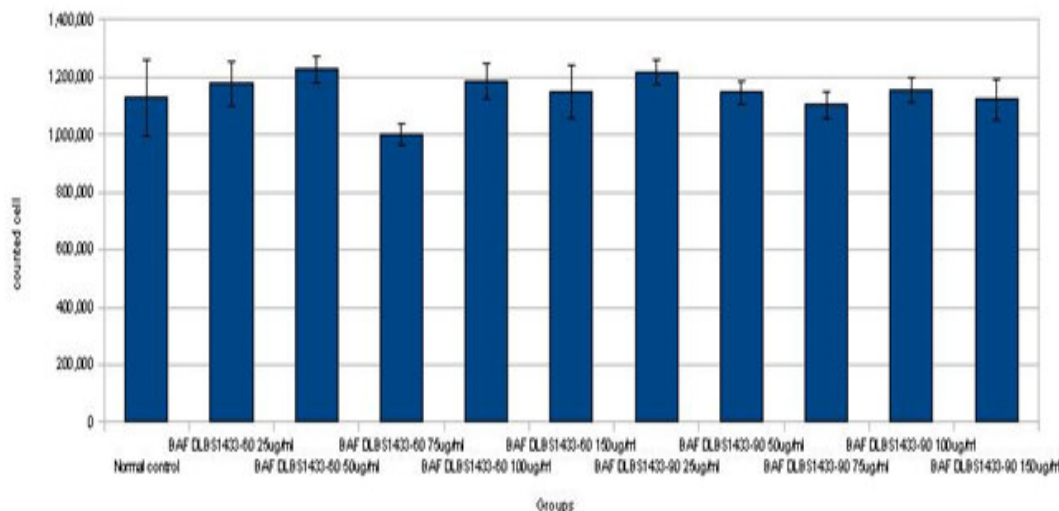
All values are expressed as mean ± SEM

\* p<0.05 indicating significant difference compared to ethanol group

\*\* p<0.05 indicating significant difference compared to normal group, treatment group (DLBS1433-60 and DLBS1433-90) and *Curcuma domestica* treatment group

**Table II**  
**Percentage of DPPH free radical-scavenging activity of DLBS1433-60 and ascorbic acid**

Group	Concentration (in ppm)	DPPH free radical-scavenging activity (%)
BAF DLBS1433-60	10	6.89±0.06
	20	39.57±0.04
	30	42.33±0.04
	40	47.74±0.04
	50	54.51±0.06
	60	66.2±0.05
	70	71.45±0.06
	80	76.25±0.06
	90	76.57±0.02
	100	76.81±0.02
Ascorbic acid	100	77.29±0.11



**Figure 1**  
**HepG2 cells count**



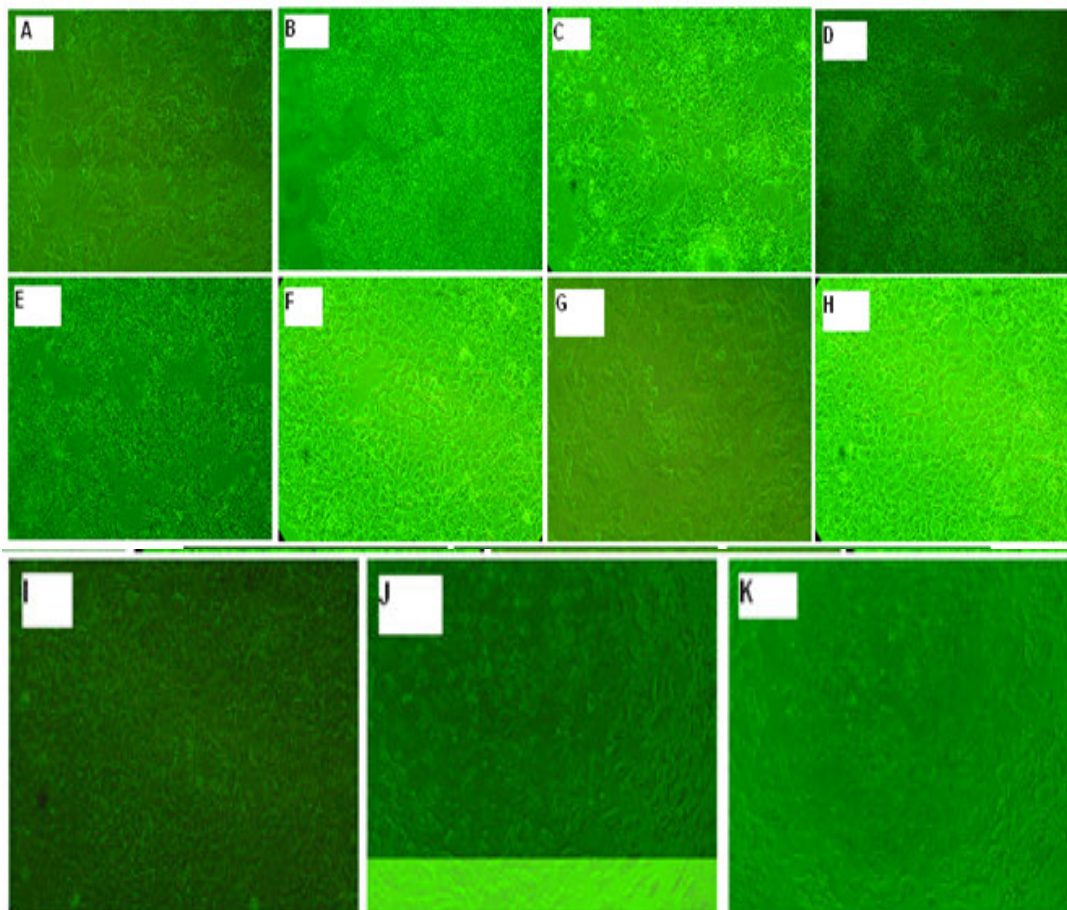


Figure 2

HepG2 cells morphology: A. Normal group; B. DLBS1433-60 25 µg/ml; C. DLBS1433-60 50 µg/ml; D. DLBS1433-60 75 µg/ml; E. DLBS1433-60 100 µg/ml; F. DLBS1433-60 150 µg/ml; G. DLBS1433-90 25 µg/ml; H. DLBS1433-90 50 µg/ml; I. DLBS1433-90 75 µg/ml; J. DLBS1433-90 100 µg/ml; K. DLBS1433-90 150 µg/ml.

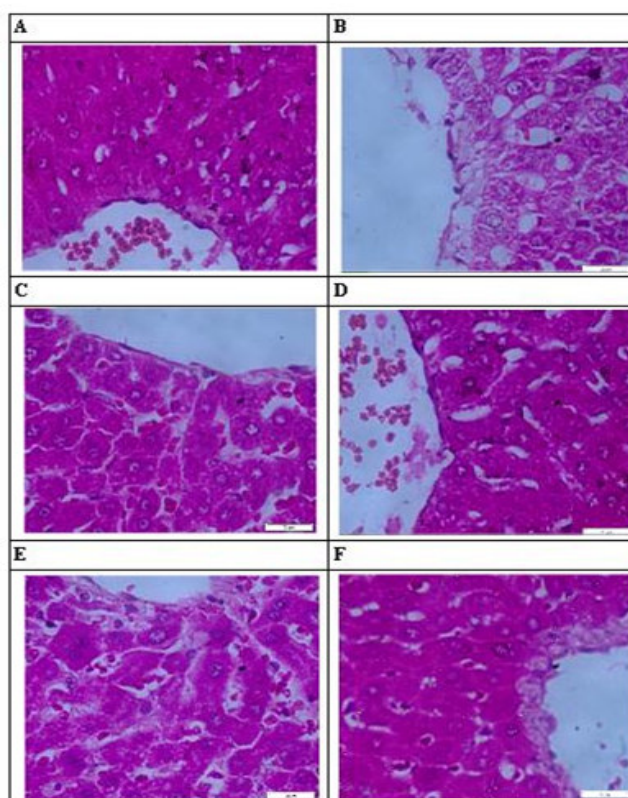


Figure 3

Liver histopathology: A. Normal group; B. Ethanol 20%; C. Curcuma domestica 50 mg/kg; D. Curcuma domestica 75 mg/kg; E. DLBS1433-60 50 mg/kg, F. DLBS1433-60 75 mg/kg (Magnification 100x)

## DISCUSSION

*Phaleria macrocarpa* is a native Indonesian plant which is known to possess activities against various diseases and disorders. Various extracts and bioactive fractions of this plant have been scientifically investigated for several pharmacological activities to control cancer<sup>13,14</sup> and endometriosis-related pain.<sup>15,16</sup> Ethanol-induced liver damage in rats was used as animal model in this experiment. Induction using ethanol was resulted in liver damage as exhibited by deterioration of blood biochemical parameters by 72.60%, 59.89%, 78.14% and 47.48% for ALT, AST, GGT and total bilirubin, respectively, compared to normal group. In addition to blood biochemical parameters, liver damage was also found in histopathology examination of ethanol 20% group. Treatments with DLBS1433-60 and *Curcuma domestica* extract at doses of 37.5 mg/kg, 50 mg/kg and 75 mg/kg showed improved blood biochemical parameters as well as liver histopathology conditions compared to DLBS1433-90. *Curcuma domestica* was used in this study due to its pharmacology activities that has been scientifically revealed as antioxidant, anti-inflammatory<sup>18</sup> and hepatoprotective agents.<sup>19-21</sup> The results of the present study were supported by previous study in our laboratory, which investigated *in vitro* hepatoprotective activity of DLBS1433 bioactive fraction (Proliverenol).<sup>22</sup> The previous *in vitro* study clearly showed that DLBS1433-60 repressed ALT expression at mRNA level. It also decreased protein leakage from cytoplasm through upregulation of APX1 that repressed DNA fragmentation and downregulation of NF- $\kappa$ B, TNF- $\alpha$  and caspase-8.<sup>22</sup> ALT which also known as serum glutamate-pyruvate transaminase (SGPT) and

AST which also known as serum glutamate-oxaloacetate transaminase (SGOT) are enzymes released by liver in responses to liver damage, fatty liver and/or oxidative stress. Increased levels of these enzymes are associated with parenchymal cell damage in the liver. ALT is a more specific marker for liver inflammation, while AST are less specific for liver damage. Antioxidant activity evaluation of DLBS1433-60 was also conducted in this study. Among various concentrations of DLBS1433-60, maximum antioxidant activity was showed by DLBS1433-60 at a concentration of 100 ppm. This result showed a comparable antioxidant activity to ascorbic acid as the comparison at a concentration range of 80-100 ppm. The evaluations of antioxidant activity of *P. macrocarpa* were previously done on several studies.<sup>12,23</sup> In conclusion, our study has clearly demonstrated an effective hepatoprotective effect of DLBS1433-60 at a dose of 75 mg/kg. DLBS1433-60 also exhibited antioxidant activity, with maximum activity at a concentration of 100 ppm.

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## CONFLICT OF INTEREST

Conflict of interest declared none.

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