

ICOPMAP SPECIAL EDITION

RESEARCH ARTICLE

Antidiabetic and antioxidant activity of *Clitoria ternatea* flower extracts and fractions on blood glucose and MDA in rats induced by alloxan

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Keywords

Antidiabetic
Antioxidant
Clitoria ternatea
Malondialdehyde
Total flavonoid content

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Abstract

Background: *Clitoria ternatea*, rich in anthocyanins with antioxidant properties, can help prevent oxidative stress and reduce complications associated with diabetes mellitus (DM). **Objective:** To investigate the antidiabetic and antioxidant properties of the ethanol extract and ethyl acetate fraction of *Clitoria ternatea* in rats induced by Alloxan. **Method:** Ethanol extract from *Clitoria ternatea* was fractionated with ethyl acetate and then screened for phytochemicals using TLC. Antidiabetic and antioxidant activities were evaluated on alloxan-induced hyperglycemic rats at 150 mg/kgBW and 300 mg/kgBW, respectively. **Result:** The *Clitoria ternatea* flower's ethanol extract and ethyl acetate fraction had a flavonoid content of 68.003±0.366 mg QE/g and 78.767±0.262 mg QE/g, respectively. The ethyl acetate fraction, administered at 300 mg/kg BW, showed a 45.5986 mg/dl reduction in blood glucose levels, not significantly different from the positive control (acarbose ($p = 0.14$)). The ethyl acetate fraction showed a significant reduction of 76.5% in MDA levels, similar to quercetin as a positive control ($p = 0.275$). **Conclusion:** *Clitoria ternatea* showed antidiabetic and antioxidant properties in alloxan-induced rats.

Introduction

Diabetes Mellitus is a chronic non-communicable disease (NCD) that results from the body's inability to produce enough insulin. This is due to the pancreas being unable to produce sufficient quantities of the hormone insulin, which results in the body being unable to use insulin effectively (Jayaningrum, 2016). High blood sugar levels, known as hyperglycemia, can indicate diabetes mellitus caused by insufficient insulin production. This condition can result in long-term complications. Diabetes treatment typically aims to normalise blood glucose levels, prevent complications, and educate patients on managing their disease (Dipiro *et al.*, 2016).

The butterfly pea flower, which belongs to the Fabaceae family and is scientifically named *Clitoria*

ternatea, is one type of plant that thrives in Indonesia. Anthocyanins are water-soluble flavonoids that are present in *Clitoria ternatea* flowers. Anthocyanins found in *Clitoria ternatea* have unique characteristics with polyacylated anthocyanins in abundant quantities known as "*ternatin*". Ternatin is a polyacylated derivative of delphinidin 3,3',5'-triglucoside (Netravati *et al.*, 2022). *Clitoria ternatea* is one plant that can be used for anti-diabetic drugs. Still, most Indonesians do not realise it is an effective traditional medicine. Research has shown that anthocyanins have the potential to lower blood glucose levels by inhibiting α -amylase enzyme activity (Chu *et al.*, 2017). It is believed that antioxidants, like anthocyanins, can help combat oxidative stress and associated vascular complications in diabetes by reducing intracellular free radical production and enhancing the activity of defence enzymes (Prawitasari, 2019). Previous research showed

chloroform, ethyl acetate, and methanol extracts of *Clitoria ternatea* with the same dose of 300 mg / KgBW for 12 consecutive days. The results showed decreased blood glucose levels (Rajamanickam et al., 2015).

Methods

Preparation of simplicia

The fresh *Clitoria ternatea* flower was sorted and cleaned. It was then dried using a dehydrator at 40°C for 48 hours. The dried simplicia was weighed and ground into powder using a grinder. The powder was stored in an airtight container.

Characterisation of simplicia powder

Simplicia powder samples and viscous extracts are characterised, including macroscopic and microscopic tests, water-soluble extract content, ethanol-soluble extract content, water content, total ash content, acid-insoluble ash content, and drying shrink.

Phytochemical screening of simplicia powder

Analysing the *Clitoria ternatea* flower's simplicia powder involves conducting a phytochemical screening. This screening was done by employing various reagents to detect the presence of secondary metabolites. The flavonoid test used HCl reagents, magnesium powder, and amyl alcohol. Meanwhile, the alkaloid test involves using HCl and distilled water to separate the parts, mixed with Mayer, Dragendorf, and Wagner reagents. The tannin test uses a 5% FeCl₃ reagent, while the saponin test utilises distilled water and HCl reagents. The macerated steroid test separated the n-hexane results and added anhydrous acetic acid and concentrated sulfuric acid. Finally, the terpenoid test requires chloroform and adding anhydrous acetic acid and concentrated sulfuric acid.

Simplicia extraction and fractionation preparation

Simplicia powder was extracted using the maceration method with 96% ethanol solvent for 1x24 hours. A re-maceration of up to three repetitions was performed. The fibre was then dried using a rotary evaporator and water bath at 50°C. Fractionation was done using the liquid-liquid partition method with distilled water, n-hexane, and ethyl acetate solvents. The sample was dissolved using distilled water. Then, n-hexane was added to the water filtrate and separated with a funnel. The lower layer of water filtrate was repeatedly mixed with ethyl acetate solvent in a separate funnel for three replications. The upper layer after ethyl fractionation

was evaporated using a rotary evaporator and water bath at 50°C until a dry fraction was obtained.

KLT profile of extract and fraction of ethyl acetate of *Clitoria ternatea*

Ethyl acetate extracts and fractions were carried out using profile search and compound tests using the KLT method. The KLT plate labelled as GF254 contained silica and was used with chloroform: ethyl acetate eluent in ratios of 8.75:1.25. To obtain the phytochemical profiles, the KLT plates were viewed under visible light, UV light at 254 nm and 366 nm, and sprayed with H₂SO₄ universal spotting. Specific spots were used for testing compound classes, including Dragendroff alkaloids, tannins with FeCl₃, flavonoids with AlCl₃ and ammonia vapour, and terpenoids/steroids with Lieberman Burchard.

Measurement of total flavonoid levels

To experiment, 75 mg of extracts and fractions were placed in an Erlenmeyer flask and mixed with 50 ml of ethanol. The mixture was sonicated for 30 minutes using a sonicator. The solution was strained into a 50 ml measuring flask, and ethanol was added to reach the limit mark. Thereafter, 0.5 ml of the fraction test solution and extract were pipetted separately and mixed with 1.5 ml of ethanol p.a, 0.1 ml of 10% AlCl₃, 0.1 ml of 1 M acetate, and 2.8 ml of distilled water. The mixture was homogenised using sonication and allowed to stand for 58 minutes at room temperature. The absorption was measured at the maximum absorption wavelength, and the resulting absorbance value was used to calculate the test solution content using the linear regression equation "y = 0.0064x - 0.0355". For each analysis, three replications were made, and the total flavonoid levels were calculated using either the linear regression equation or the formula:

$$\% = \frac{C_p \times \frac{A_u}{A_p} \times V \times f}{W} \times 100$$

Notes:

C = quercetin equivalence concentration

Au = Absorbance of the test solution

Ap = Absorbance of quercetin solution

V = volume of the test solution before dilution

f = dilution factor of the test solution

W = weight of the test material

Preparation of experimental animals and testing

The experimental animals used in this study were male rats of the Wistar strain weighing 150-200 g and as many as 36 rats with six groups. Each group consisted of six white rats induced twice with alloxan 150 mg/kg body weight intraperitoneally. Before testing, rats were acclimatised for one week in cages by feeding, drinking, and using husks as ad libitum so that rats could adjust to their environment. Blood samples were taken through the orbital vein of the eyes.

Measurement of blood glucose levels

After blood serum was obtained, it was analysed using the GOD-PAP method using a UV-VIS spectrophotometer. The reagent consists of phosphate buffer, 4-aminoantipyrine, phenol, GOD (Glucose oxidase), and POD (Peroxidase). Exactly 10 μ m of blood serum is inserted into 1 ml of reagent. Then, the serum of the reagent was vortexed for 10 seconds. After vortexing, the samples were incubated for 15 minutes. After incubation, the samples were measured using a spectrophotometer. The absorbance of the samples was read using a visible spectrophotometer at a wavelength of 512.7 nm.

Measurement of blood plasma MDA levels

Blood plasma samples were analysed using the TBARS method. 0.5 ml of blood plasma was inserted into a vacutainer tube, and 1 ml TCA 20% and 2 ml TBA 0.67% were added. The tube was then heated in a bath at 100°C for 20 minutes. The sample was centrifuged at 3000 rpm for ten minutes and then cooled at room temperature. Measurements with a UV-visibility spectrophotometer followed TBARS measurement results at a wavelength of 533.1 nm, which were calculated using linear regression $y = 1.3337x - 0.0094$.

Data analysis

The data was normally distributed for the ANOVA test and had a homogeneous variance ($p \geq 0.05$).

Results

Samples of butterfly pea flower (*Clitoria ternatea*) were obtained from the Martani flower garden of Purwomartani village, Kalasan District, Sleman Regency, Yogyakarta, which had been determined at the Laboratory of Ecology and Biosystematics, Faculty of Science and Mathematics, Diponegoro University, Indonesia. After drying with a dehydrator for 48 hours, a total of 2.10 kg of simplicia powder was obtained.

Figure 1 shows that *Clitoria ternatea* simplicia had fragmented epidermis with stomata, calcium oxalate in prism form, vascular tissue, and trichomes with distinct curved lengths.

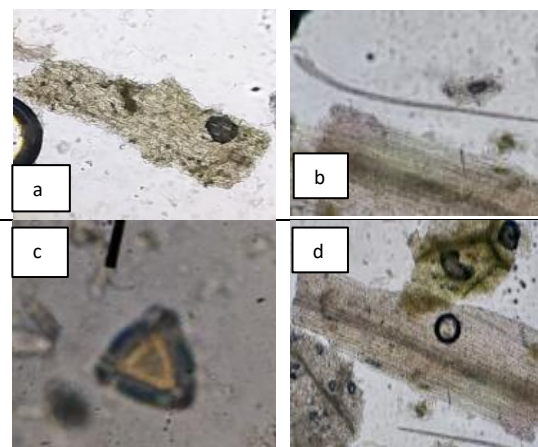


Figure 1: Fragment identifying of 100x magnification simplicia powder of *Clitoria ternatea* flower (a) epidermis; (b) trichomes; (c) calcium oxalate crystals; (d) vascular tissue

Farmakope Herbal Indonesia II (2008) states that simplicia must be characterised to ensure traditional medicines meet material standards for efficacy, safety, and quality. Table I indicates that simplicia from the *Clitoria ternatea* flowers met existing standards.

Table I: Results of characterisation of *Clitoria ternatea* simplicia

Characterisation	Result
Water-soluble extract content	41.66 \pm 2.0%
Ethanol-soluble extract content	34.33 \pm 1.15%
Water content	5.33 \pm 1.15%
Total ash content	5.95 \pm 1.33%
Acid-insoluble ash content	0.50 \pm 0.05%
Drying shrinkage	7.0 \pm 0.87%

According to the results of the phytochemical screening presented in Table II, *Clitoria ternatea* simplicia tested positive for alkaloids, flavonoids, steroids, tannins, and terpenoids. The extract's yield value was 20.05%, and the yield value of the ethyl acetate fraction was 2.08%. This means that 9 g of ethyl acetate fraction was obtained from 432 g of ethanol extract of *Clitoria ternatea*.

Table II: Phytochemical screening results of *Clitoria ternatea* simplicia

Phytochemicals	Reagent	Result
Alkaloid	Reagent Mayer	(-)
	Reagent Dragendorff	(+)
	Reagent Wagner	(+)
Flavonoid	Mg powder + HCl + Amyl alcohol	(+)
Saponin	Warm aquades + HCl 2N	(+)
Tanin	5% FeCl ₃	(+)
Terpenoid	Chloroform and Lieberman Burchard Extraction	(+)
Steroid	Extraction of N-hexane and Lieberman Burchard	(+)

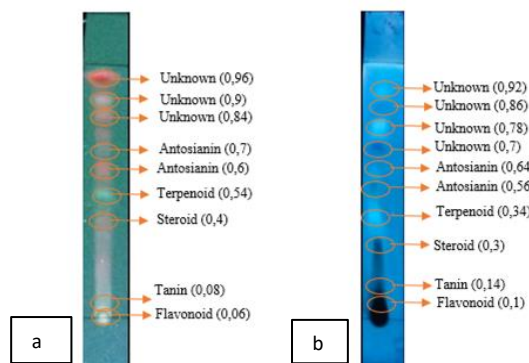


Figure 2: TLC profile (a) ethanol extract of *Clitoria ternatea*; (b) ethyl acetate fraction of *Clitoria ternatea*

The positive results of the compounds contained in *Clitoria ternatea* can be seen using a specific spraying reagent. Figure 2 (a) displays the R_f values of each flavonoid compound (R_f =0.06), tannins (R_f =0.08), steroids (R_f =0.4), terpenoids (R_f =0.54), and anthocyanins (R_f =0.6 & R_f =0.7) in *Clitoria ternatea* ethanol extract. Figure 2 (b) shows the R_f values of each flavonoid compound (R_f =0.1), tannins (R_f =0.14), steroids (R_f =0.3), terpenoids (R_f =0.3), and anthocyanins (R_f =0.56 & R_f =0.64) in the ethyl acetate fraction of *Clitoria ternatea*. The observations were made by comparing the samples' colours after applying specific spraying reagents and positive controls.

According to Farnsworth (1966), AlCl₃ spraying reagent turns yellow to indicate the presence of flavonoids, the FeCl₃ spraying reagent turns blackish-brown to indicate the presence of tannins, the ammonia vapour spraying reagent turns blue to indicate the presence of anthocyanins, Liebermann Burchard spraying reagent turns green to indicate the presence of steroids, and purplish-orange to indicate the presence of terpenoids.

Total flavonoid levels in ethanol extract and ethyl acetate fraction of *Clitoria ternatea* were expressed at mg QE/g, the equivalence of quercetin in every 1 g sample. Based on Table III, it was found that the ethanol extract and ethyl acetate fraction of *Clitoria ternatea* obtained were 68 mg QE/g and 78.77 mg QE/g.

Table III: Results of determining total flavonoid levels

Sample	Average levels (mg QE/g)	t-test (p < 0.05)
Ethanol extract of <i>Clitoria ternatea</i> 150 ppm	68.003 ± 0.366	0.000
Ethyl acetate of <i>Clitoria ternatea</i> 150 ppm	78.767 ± 0.262	

The graph in Figure 3 shows increased blood glucose on day ten after alloxane-induced in the P1-P5 group. On day 24 of treatment of the P2-P5 group, there was a decrease in blood glucose.

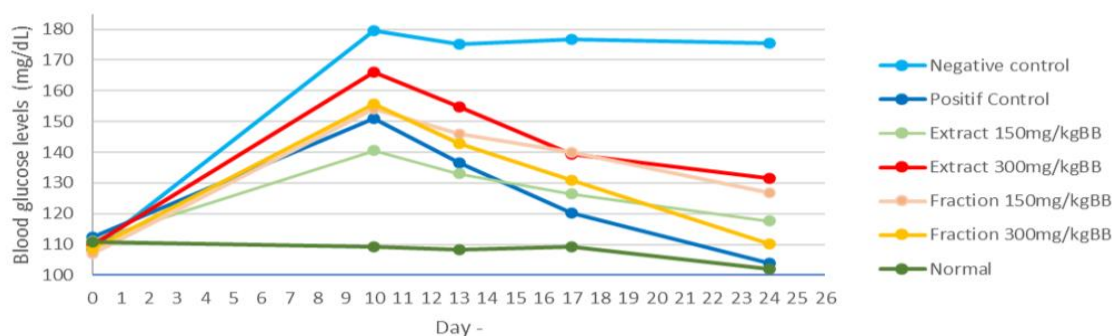


Figure 3: Blood glucose level measurement. Day 0 = Rat blood glucose levels before treatment; Day 10 = Blood glucose levels of hyperglycemic phase rats; Day 13 = Blood glucose levels after three days; Day 17 = Blood glucose levels after seven days; Day 24 = Rat blood glucose levels after fourteen days

Based on Table IV, dosing the ethyl acetate fraction at 300 mg/kg body weight is the most effective dose compared to other dose variations. The activity of the ethyl acetate fraction at 300 mg/kg BW produced was not significantly different from the positive control group (acarbose) with a value of ($p = 0.147$). The results of the Post Hoc LSD test showed that based on the test of the extract group of 150 mg/kg BW and 300 mg/kg BW, fractions of 150 and 300 mg/kg BW had a value ($p < 0.05$) to the negative control group which means that the three groups are different meaningfully. The results showed an increase in all four doses of the extract and the fraction in lowering blood glucose levels in alloxan-induced rats.

Table IV: Decrease in lowering blood glucose levels before and after treatment

Group	Δ Decreased blood glucose levels (mg/dL)	p-value
Normal control	7.3269 ^a ± 0.46	< 0.05
Negative control	4.097 ^a ± 1.67	
Positive control	47.0510 ^b ± 1.47	
Extract 150 mg/kg BW	22.9129 ^a ± 2.93	
Extract 300 mg/kg BW	34.5056 ^a ± 1.43	
Fraction 150 mg/kg BW	27.4560 ^a ± 1.15	
Fraction 300 mg/kg BW	45.5968 ^b ± 1.56	

The results of the Pearson correlation test in Table V showed a significant relationship between an increase in blood glucose levels and an increase in MDA levels ($p = 0.002$). They had a strong relationship category ($r = 0.605$). The type of relationship is positive.

Table V: Relationship of elevated blood glucose levels with MDA levels in alloxan-induced rats based on the Pearson correlation test

Variable	p-value	r-value	Note
Increase in blood glucose levels against alloxan-induced rat blood plasma MDA.	0.002	0.605	There are meaningful relationships, strong relationship categories, and positive relationship types.

Table VI shows a decrease in MDA levels in this study. The most significant decrease occurred in the ethyl acetate fraction 300 mg/kg BW. It showed results that were not significantly different from the positive control (quercetin) with a value of ($p = 0.275$) with a percentage decrease of 76.5%.

Table VI: MDA levels of blood plasma of rats

Group	MDA levels before induced by alloxan (ppm)		MDA levels after being induced by alloxan (ppm)		MDA percentage decrease (%)	One-way ANOVA test ($p < 0.05$)
	Day-0	Day-10	Day-10	Day-24		
Normal control	0.032±0.004	0.039±0.003	0.031±0.003	0.031±0.003	20.254 ^a ±8.165	
Negative control	0.045±0.020	0.262±0.048	0.211±0.043	0.211±0.043	19.300 ^a ±6.560	
Positive control	0.047±0.018	0.291±0.053	0.051±0.008	0.051±0.008	82.210 ^d ±1.474	
Extract 150 mg/kgBW	0.056±0.220	0.285±0.042	0.148±0.038	0.148±0.038	48.270 ^b ±9.384	0.002
Extract 300 mg/kgBW	0.053±0.024	0.280±0.041	0.152±0.038	0.152±0.038	53.612 ^{b,c} ±7.674	
Fraction 150 mg/kgBW	0.050±0.015	0.292±0.012	0.112±0.011	0.112±0.011	61.418 ^c ±8.697	
Fraction 300 mg/kgBW	0.037±0.006	0.301±0.049	0.069±0.010	0.069±0.010	76.476 ^d ±5.657	

Note: Day 0: MDA levels before alloxan-induced; Day 10: MDA levels after the mice were hyperglycemic and before the mice were treated; Day 24: MDA levels after 14 days of treatment; Different superscript letters showed a meaningful difference ($p < 0.05$), while the same superscript letters showed no difference using LSD's post-hoc test.

Discussion

Simplicia must be characterised to ensure traditional medicines meet material standards for efficacy, safety, and quality. In a previous study by Yumni *et al.* (2022), the ethyl acetate fraction yield value of bracelet flowers was only 0.96%. The difference in yield value can be attributed to the differences in the content of dissolved

compounds, as per the principle of like dissolve like, which suggests that a high yield value is directly proportional to the high compound content in a sample, as stated by Harborne (1987).

The ethanol extract and ethyl acetate fraction of *Clitoria ternatea* obtained were 68 mg QE/g and 78.77 mg QE/g. Based on Warsi & Puspitasari (2017), the high levels of total flavonoids in the ethyl acetate

fraction of *Clitoria ternatea* are due to further fractionation carried out on *Clitoria ternatea* ethanol extract using ethyl acetate solvent to isolate active compounds such as semipolar flavonoids.

Flavonoid compounds in *Clitoria ternateas* are thought to play an active role in antidiabetic and antioxidant activity. Other studies from Vifta and Advistasari (2018) have suggested a positive correlation between the strong correlation category between total flavonoid content and the antioxidant activity of *Clitoria ternatea* extract.

Antidiabetic effect testing was done on hyperglycemic rats using alloxane induction at 150 mg/kg BW on days zero and three. Swastini et al. (2018) state that alloxan damaged mice's pancreas, reducing insulin production. It can cause blood sugar to be unable to be converted into energy, resulting in high blood sugar levels. In this study, rats experienced increased blood sugar levels within ten days after alloxan induction.

The decrease in blood glucose levels in the extract treatment group and fraction occurred because the flower has inhibitory activity against the enzyme α -amylase. Another study by Chu et al. (2017) stated that research models using in vitro studies of starch hydrolysis on various types of flour resulted in that extract of butterfly pea (*Clitoria ternatea*) can provide inhibition of alpha-amylase enzymes and can provide a decrease in the amount of glucose released from various types of flour. Decreased blood glucose levels in rats are thought to result from anthocyanin compounds. Nizamutdinova et al. (2009) state that anthocyanins in *Clitoria ternateas* can reduce blood glucose levels by the mechanism of action. Inhibits free radicals to prevent oxidative stress that can cause pancreatic beta cell damage.

According to Diyah (2014), there is a direct relationship between the increase in glucose levels and MDA levels in alloxan-induced mice. Diabetic patients experience oxidative stress due to four primary sources: 1) auto-oxidation of glucose, 2) excessive production of ROS in mitochondria, 3) non-enzymatic glycation, and 4) polio pathways. High glucose levels in individuals with hyperglycemia can trigger non-enzymatic glycosylation processes in proteins by oxidising the aldehyde groups in glucose. Based on Hasim et al. (2020), Alloxan induction can cause an increase in ROS production through a chain reaction, producing a reduction product in the form of dialuric acid. Autooxidation of glucose from channelled acid can produce superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^*). The presence of hydroxyl radicals will cause lesions in pancreatic beta cells.

In addition, the presence of pancreatic beta cell damage will cause insulin secretion deficiency and disruption of homeostasis of glucose levels in the blood, causing hyperglycemia conditions. Priyanto et al. (2023) state that hyperglycemia can increase oxidative stress due to increased non-enzymatic glycosylation processes in proteins due to the oxidation of aldehyde groups in highly reactive glucose. Thus, resulting in the formation of advanced glycation end products (AGEs) and free radicals that can increase oxidative stress, this condition can increase oxidative stress markers such as malondialdehyde (MDA).

The ethyl acetate fraction with 300 mg/kg BW showed the most significant decrease. This is because the compound in *Clitoria ternatea*'s ethyl acetate fraction was simpler and classified based on polarity level. At the same time, ethanol extract is still a multi-compound compound. This result is also supported by the results on the test of total flavonoid levels of ethanol extract with ethyl acetate fraction of *Clitoria ternatea*, which shows that flavonoid levels in the ethyl acetate fraction produce higher flavonoid levels, the content of flavonoid compounds is thought to reduce the number of free radicals and prevent oxidative stress due to alloxan induction which is characterised by a decrease in MDA levels after giving the ethyl acetate fraction of *Clitoria ternatea*. Flavonoids are reducing compounds that can inhibit many oxidation reactions. Flavonoids have the ability as antioxidants because they can transfer electrons to free radical compounds, and free radicals can be stabilised. In addition, previous studies from Narayan et al. (1999) on anthocyanin activity in vitro stated that anthocyanins have enzymatic lipid peroxidation inhibitory activity in lipoxygenase enzymes and non-enzymatically.

Conclusion

Ethyl acetate fraction of butterfly pea (*Clitoria ternatea* L.) dose 150 and 300 mg/kgBW, ethanol extract dose 150 and 300 mg/kgBW have antidiabetic activity against lowering blood sugar levels and decreasing MDA levels in alloxan-induced male Wistar rats. The ethyl acetate fraction of butterfly pea (*Clitoria ternatea* L.) dose of 300 mg/kgBW was more effective in lowering blood sugar levels and alloxan-induced MDA of rats than the ethyl acetate fraction dose of 150 mg/kgBW, ethanol extract of 150 and 300 mg/kgBW.

Acknowledgement

The author would like to thank the Faculty of Medicine, Diponegoro University, Indonesia, which has funded and provided facilities for this research.

Source of funding

This study was funded by the Faculty of Medicine, Diponegoro University, Indonesia.

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