

## Antioxidant Activity of Ethanol Extract of Puguntano Herb (*Picria fel-terrae* Lour.) and Effect on Superoxidase Dismutase and Lactate Dehydrogenase Levels in Doxorubicin-Induced Rats

Khairina Amalia<sup>1</sup>, Aminah Dalimunthe<sup>1,\*</sup>, Denny Satria<sup>2</sup>,  
Panal Sitorus<sup>2</sup> and Syukur Berkhat Waruwu<sup>3</sup>

<sup>1</sup>Departement of Pharmacology, Faculty of Pharmacy, Universitas Sumatera Utara,  
Medan 20155, Indonesia

<sup>2</sup>Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Sumatera Utara,  
Medan 20155, Indonesia

<sup>3</sup>Faculty of Pharmacy and Health Sciences, Universitas Sari Mutiara Indonesia,  
Medan 20123, Indonesia

(\*Corresponding author's e-mail: aminah@usu.ac.id)

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### Abstract

Antioxidants are chemicals that inhibit free radical oxidation. Puguntano plants have secondary metabolite compounds that have the potential to act as antioxidants. This study aims to determine the antioxidant activity of Puguntano *in vitro* and *in vivo*. Secondary metabolite examination was carried out using thin-layer chromatography. Antioxidant activity was tested using the ABTS and FRAP methods, and a complete analysis of phenols and flavonoids was also carried out. Examination of superoxide dismutase (SOD) and lactate dehydrogenase (LDH) levels in blood serum was carried out *in vivo* on male white rats induced by 10 mg/kg BW Doxorubicin using the Elisa Reader Kit. Ethanol extract test samples were carried out with dose groups of 400, 200, 100 and 50 mg/kg BW; Quercetin 50 mg/kg BW was used as a positive control. The results showed that the sample had total phenolics of  $20.22 \pm 0.96$  mg GAE/g and total flavonoids of  $1.11 \pm 0.06$  mg QE/g. The IC<sub>50</sub> value with ABTS was  $419.73 \pm 1.61$  µg/mL and FRAP  $36.77 \pm 0.71$  µg/mL. SOD and LDH levels increased and showed significant differences with negative controls ( $p < 0.05$ ). The 400 and 200 mg/kg BW groups significantly increased SOD levels ( $1.47 \pm 0.03$  and  $1.46 \pm 0.02$  U/mL) and LDH ( $8.02 \pm 0.01$  and  $7.90 \pm 0.04$  ng/mL). These results show the potential of this plant as an antioxidant that can be developed.

**Keywords:** Antioxidant, ABTS, FRAP, Doxorubicin, Lactate dehydrogenase, Superoxide dismutase

### Introduction

Antioxidants inhibit or stop the free radical oxidation process, which causes cell damage [1]. Most diseases caused by free radicals are the leading cause of tissue and cell damage, as demonstrated by the reactions of free radicals with proteins, fatty acids and even DNA [2]. The body is protected from reactive oxygen free radicals by antioxidants. By providing extra electrons to free radicals, antioxidants neutralize them and stop damage to the body. Free radicals are produced when other molecules undergo oxidation, which can be prevented or slowed by antioxidants [3,4].

One of the local Indonesian plants that grows widely in North Sumatra and is widely used as traditional medicine is the Puguntano plant (*Picria fel-terrae* Lour.). This plant belongs to the *Scrophulariaceae* family and is often used traditionally as a medicine for rheumatism, gout and diabetes. Several studies show the use of Puguntano as an anti-inflammatory, anthelmintic, antidiabetic, diuretic effect, cardioprotective effect, anti-breast cancer, immunomodulator, antipyretic, malaria, skin problems and colic medicine [5,6]. Puguntano has anti-ageing properties, relieves pain, and builds endurance. According to phytochemical literature, this plant contains secondary metabolite components such as flavonoids, tannins, saponins and phenylpropanoid glycosides. These chemicals have potential applications in human medicine related to oxidative stress and antioxidants [7,8]. In addition, the essential oils found in the fruit stems, roots and leaves of the Puguntano plant provide a fragrant aroma with a significant concentration of flavonoid compounds [9].

This research began with a phytochemical examination using thin-layer chromatography, which was carried out to obtain information on the secondary metabolite compounds contained therein. A complete analysis of phenols and flavonoids was also carried out. Antioxidant activity was carried out *in vitro* using the ABTS and FRAP methods. The IC<sub>50</sub> (Inhibitory Concentration) value indicates the level of antioxidant activity. This value indicates the concentration of the test substance, which has a capacity of 50 % for trapping free radicals [10]. Next, *in vivo*, experiments were carried out using male rats that were induced by doxorubicin by measuring the levels of superoxidase dismutase (SOD) and lactate dehydrogenase (LDH) in blood serum using the ELISA (Enzyme-Linked Immunosorbent Assay) technique [11,12].

## Materials and methods

### Sample collection

*Picria fel-terrae* Lour herb was collected from Suka Dame Village, Kutalimbaru District, Deli Serdang Regency, North Sumatra Province, Indonesia. Other materials used are Aluminum chloride (Smart lab), ammonium acetate (Smart lab), distilled water, dichloromethane, DPPH, ethyl acetate, methanol, neocuproine, chloride dihydrate, quercetin (TCI), sodium acetate, NA-CC, Doxorubicin HCl injection and SOD ELISA Kit (ABclonal).

### Extract preparation and phytochemical screening

A total of 500 g of Puguntano herb powder was extracted using 5 L of 70 % ethanol solvent using the maceration method. Soak for 6 h, stirring occasionally, then let sit again for 18 h. Then, the extract is filtered using filter paper until the first maceration is obtained. The dregs obtained were macerated with 2.5 L of 70 % ethanol solvent, and then the same procedure was carried out with 2 repetitions until the second and third macerations were obtained. The 3 maserates were collected and then evaporated using a rotary evaporator at 40 °C until a thick extract was obtained [13]. Secondary metabolite examination uses thin-layer chromatography to obtain information on the secondary metabolite compounds contained therein. One mg of Puguntano herb extract was dissolved in methanol and added to the stationary phase. The stationary phase used was a silica gel 60 F254 plate (Merck, Germany) measuring 10×20 cm<sup>2</sup>. Next, the plate is inserted into a chamber saturated with mobile phase vapour. The mobile phase used according to the examination carried out was sprayed with a spotting agent and heated in an oven at 100 °C for 5 min, and then the colour change that occurred was observed. Measure and record the distance of each spot from the spotting point. Determine the price of Rf [14].

### Measurement of total phenols

Folin reagent measures the sample's total phenol concentration (TPC). In summary, 100  $\mu$ L of extract at a concentration of 500  $\mu$ g per mL was mixed with 7.9 mL of distilled water and 0.5 mL of Folin-Ciocalteu reagent in a ratio of 1:10 volume to volume. The mixture was then swirled for 1 min. After mixing, 1.5 mL of a solution containing 20 % sodium bicarbonate in water was introduced. The resulting mixture was then left undisturbed for 90 min, occasionally agitated. The spectrophotometer quantified the degree of light absorption at a wavelength of 775 nm [14].

### Measurement of total flavonoids

A total of 2 mL of extract dissolved in methanol was mixed with 0.10 mL of 10 % aluminium chloride ( $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ ), 0.10 mL of sodium acetate (1M) and 2.80 mL of distilled water. The spectrophotometer measured the level of light absorption at a wavelength of 432 nm for 40 min [15].

### Antioxidant test using the ABTS method

The ABTS solution was prepared by weighing 7 mg of ABTS salt and 3.5 mg of potassium persulfate. Then, dissolve it in a 5 mL Aqua Pro Injection measuring flask and put it in a 25 mL measuring flask. Incubate for 12 - 16 h. After that, 1 mL was pipetted and put into a 10 mL measuring flask up to the limit mark and the maximum wavelength was measured with a UV-Vis spectrophotometer at 400 - 800 nm. A stock solution of 1000 ppm was prepared and made into 5 concentration series (50, 100, 150, 200 and 250). Then, the sample was pipetted and mixed with the ABTS solution in a brown bottle. Incubate and measure the absorbance at the maximum wavelength [14].

### Antioxidant test using the FRAP method

Five mg of extract was dissolved in 5 mL of 96 % ethanol. Then, 1 mL of the solution was pipetted and mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1 %  $\text{K}_3[\text{Fe}(\text{CN})_6]$ . The mixture was then incubated at 50 °C for 20 min. Following incubation, 1 mL of trichloroacetic acid (TCA) was introduced, and the mixture was subjected to centrifugation at a speed of 3,000 rpm for 10 min. After centrifugation, 1 mL of the uppermost layer was transferred into a test tube, adding 1 mL of distilled water and 0.5 mL of 0.1 %  $\text{FeCl}_3$ . The solution was allowed to sit for 10 min, after which the absorbance was quantified at a wavelength of 720 nm [13].

### Measurement SOD and LDH levels

This examination used male Wistar rats with an average body weight of 180 - 200 g obtained from the Faculty of Pharmacy, Universitas Sumatera Utara. All animal use procedures have received approval and guidelines from the Animal Research Ethics Committee, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, with letter number 0225/KEPH-FMIPA/2024. 35 rats were divided into 7 groups, each consisting of 5 rats with the following treatment:

Group I (Normal) only received food and drink.

Group II (Positive Control) received quercetin 50 mg/kg BW orally.

Group III (Normal Control) only received food and drink.

Group IV received an extract dose of 400 mg/kg BW orally.

Group V received an extract dose of 200 mg/kg BW orally.

Group VI received an extract dose of 100 mg/kg BW orally.

Group VII received an extract dose of 50 mg/kg BW orally.

Each received treatment for 8 days and intraperitoneal doxorubicin at a dose of 10 mg/kg BW on the 8<sup>th</sup> and 9<sup>th</sup> days (except group I). After treatment, on day 10, 3 mL of rat blood was taken through the heart and centrifuged at 1,000 rpm for 5 min. The serum obtained was checked for SOD (U/mL) and LDH (ng/ml) levels using the Elisa Reader Kit. Absorbance was measured at  $\lambda$  450 nm [16].

### Statistical analysis

Data are expressed as mean  $\pm$  SD and analyzed using the ANOVA test to determine treatment differences. If there are differences, we use the Post Hoc Tukey test to determine which variables differ. Based on the significance value,  $p < 0.05$  is considered significant. All statistics were analyzed using SPSS 26 software.

## Results and discussion

### Phytochemical screening

**Table 1** presents the screening results on the ethanol extract of the Puguntano herb, which was observed to contain flavonoids, glycosides, saponins, tannins and steroids.

**Table 1** Results of phytochemical examination of extracts using thin-layer chromatography.

Alkaloids	Flavonoids	Glycosides	Saponins	Tannins	Steroids
Rf = -	Rf 1 = 0.47 Rf 2 = 0.58 Rf 3 = 0.82	Rf 1 = 0.34 Rf 2 = 0.44 Rf 3 = 0.58 Rf 4 = 0.72	Rf 1 = 0.33 Rf 2 = 0.38 Rf 3 = 0.48 Rf 4 = 0.86 Rf 5 = 0.96	Rf = 0.70	Rf = 0.41

Note: Rf = Retention factor, - = Not available

Flavonoids get 3 points with Rf values of 1 = 0.47, Rf 2 = 0.58 and Rf 3 = 0.82. Glycosides get 4 points with Rf values of 1 = 0.34, Rf 2 = 0.44, Rf 3 = 0.58 and Rf 4 = 0.72. Saponin gets 5 points with Rf values of 1 = 0.33, Rf 2 = 0.38, Rf 3 = 0.48, Rf 4 = 0.86 and Rf 5 = 0.96. Tannin gets 1 point with a value of Rf = 0.70. Steroids get 1 point with a value of Rf = 0.41. The retention factor value (Rf) is a value or measurement determined based on the position of the spot on the solute in thin-layer chromatography. The resulting staining results on the TLC plate are then calculated as Rf values [17].

### Total phenols and total flavonoids

The total phenol and flavonoid content of the ethanol extract of Puguntano herb was high, as shown in **Table 2**.

**Table 2** Total phenol and total flavonoid contents of Puguntano herb extract.

Sample	Total phenol (mg GAE/g)	Total flavonoid (mg QE/g)
Puguntano herb extract	20.22 ± 0.96	1.11 ± 0.06

Note: Results are displayed as average and standard error of the mean with 3 repetitions.

In plants, phenol is the most abundant secondary metabolite. Plants can contain phenols, phenolic acids, tannins, lignin and flavonoids as phenolic compounds. A class of phenolic chemicals commonly found in nature is called flavonoids. Because flavonoids have double bonds (> C=C <) and -OH groups, they can absorb and neutralize free radicals. Flavonoids have antioxidant effects by inhibiting the production of reactive oxygen species (ROS) and by increasing the production of endogenous antioxidants such as catalase, SOD and GPx [18,19].

Combining aluminium chloride and quercetin molecules is the basis for calculating total flavonoid levels using the aluminium chloride (AlCl<sub>3</sub>) method [20,21]. The ethanol extract of the Puguntano herb has significant antioxidant activity, and the total phenolic content is higher than the total flavonoid content [22].

#### IC<sub>50</sub> value with ABTS and FRAP methods

Furthermore, **Table 3** shows that the Puguntano herb ethanol extract shows antioxidant levels using the ABTS method (419.73 ± 1.61 µg/mL) and FRAP (36.77 ± 0.71 µg/mL). The substance's ability to produce radical cations (ABTS<sup>+</sup>) is the basis for testing the ABTS method. Potassium persulfate reacts with ABTS solution to produce radical cations (ABTS<sup>+</sup>). Antioxidants that react with ABTS radical cations are measured using ABTS. The wavelength that absorbs the most radical cations (ABTS<sup>+</sup>) is 734 nm. The FRAP method also showed that the sample had a high antioxidant content. The IC<sub>50</sub> concentration of Puguntano herb ethanol extract samples increased along with the number of Fe<sup>2+</sup> complexes formed [23,24].

**Table 3** IC<sub>50</sub> value of Puguntano herb extracts.

Sample	IC <sub>50</sub> (µg/mL)	
	ABTS	FRAP
Puguntano herb extract	419.73 ± 1.61	36.77 ± 0.71

Note: Results are displayed as average and standard error of the mean with 3 repetitions.

#### Results of measurement SOD and LDH levels

SOD and LDH levels were examined in doxorubicin-induced rats. The results of checking SOD levels are as follows in **Table 4**.

The results of examining SOD levels showed that the normal group had the highest levels, while the NC + DOX group had the lowest; The EEHP groups 50 + DOX, 100 + DOX, 200 + DOX, 400 + DOX and Q + DOX are ordered from lowest to highest. The EEHP 400 + DOX and 200 + DOX groups experienced the highest increase. Meanwhile, the NC + DOX group, which had also been induced by doxorubicin, showed the lowest SOD levels, even lower than the EEHP 50 + DOX group; This shows that administration of EEHP has a more significant effect on increasing SOD levels. Superoxide dismutase is an endogenous antioxidant enzyme that can fight free radicals and prevent activity imbalances in the body. The enzyme superoxide dismutase is the first line of defence against the activation of reactive oxygen compounds. Cell damage can occur due to excess free radicals if their production exceeds the capacity of endogenous

antioxidants to fight them [1,25]. The SOD levels in the EEHP administration, when compared with the Q + DOX group, were indeed lower. Quercetin has high antioxidant activity because it is a pure compound. Quercetin is a flavonol of the polyphenolic flavonoid compounds found in almost every type of plant, and standard quercetin is a natural antioxidant with strong antioxidant activity [26].

**Table 4** Results of analysis of serum SOD and LDH levels.

Groups	Results $\pm$ SEM	
	SOD (U/mL)	LDH (ng/mL)
Normal	3.10 $\pm$ 0.03 <sup>bc</sup>	2.18 $\pm$ 0.01 <sup>b</sup>
Q + DOX	2.62 $\pm$ 0.27 <sup>ab</sup>	2.22 $\pm$ 0.01 <sup>b</sup>
NC + DOX	0.07 $\pm$ 0.01 <sup>ac</sup>	12.29 $\pm$ 0.03 <sup>ac</sup>
EEHP 400 + DOX	1.47 $\pm$ 0.03 <sup>abc</sup>	3.07 $\pm$ 0.06 <sup>abc</sup>
EEHP 200 + DOX	1.46 $\pm$ 0.02 <sup>abc</sup>	5.09 $\pm$ 0.05 <sup>abc</sup>
EEHP 100 + DOX	1.32 $\pm$ 0.01 <sup>abc</sup>	8.00 $\pm$ 0.05 <sup>abc</sup>
EEHP 50 + DOX	1.18 $\pm$ 0.01 <sup>abc</sup>	8.03 $\pm$ 0.02 <sup>abc</sup>

Note: Results are displayed as average and standard error mean. Abbreviations: Q = Quercetin, DOX = Doxorubicin, NC = Normal Control, EEHP = Puguntano herb ethanol extract, SE = Standard Error Mean, a = significantly different from the normal group ( $p < 0.05$ ), b = significantly different from the NC + DOX group ( $p < 0.05$ ), c = significantly different with the Q + DOX group ( $p < 0.05$ ).

Furthermore, the lowest LDH levels were in the normal group, and the highest was in the NC + DOX group, followed by the EEHP 50 + DOX, 100 + DOX, 200 + DOX, 400 + DOX and Q + DOX groups. The EEHP 50 + DOX group was still lower than NC + DOX; this means that giving EEHP reduces LDH levels in male rats induced by doxorubicin. The EEHP 400 + DOX group had the best LDH-reducing effect, although the reduction was still lower than the Q + DOX group. However, this shows that administration of quercetin and EEHP affected reducing LDH levels in male rats after being induced by doxorubicin. LDH is an essential enzyme of the anaerobic metabolic pathway and belongs to the oxidoreductase class [27]. The LDH test is used to detect tissue damage in the body. Elevated LDH is usually a sign of tissue or organ injury. Damaged cells will release LDH into the bloodstream, causing an increase in blood levels [28].

The SOD and LDH levels of the normal group were found to be significantly different ( $p < 0.05$ ) from the other groups; this shows that doxorubicin administration affects increasing LDH levels and decreasing SOD levels in male rats. An increase in LDH due to doxorubicin administration has also been reported [29]. Doxorubicin is an anthracycline used to treat cancer but can cause cardiotoxicity [30]. Doxorubicin induces cardiac damage through several pathways, such as decreased antioxidant effects, impaired mitochondrial function, increased lipid peroxidation and increased inflammatory response. Superoxide dismutase, which scavenges typically free radicals, may decrease its activity with doxorubicin [31-33]. Through various mechanisms that reduce ROS, lipid peroxidation, mitochondrial permeability and suppress apoptosis, dietary supplements containing flavonoids, such as luteolin, apigenin, hesperidin, anthocyanin and naringenin play an essential role in preventing cardiac toxicity due to free radicals caused by doxorubicin due to stress-oxidative [34].

Antioxidants have the primary function of stopping or breaking the chain reaction of free radicals in the body and neutralizing free radicals so that they can protect against detrimental effects caused by excessive oxidation. Therefore, if there is an increase in free radicals in the body, large amounts of endogenous antioxidants are needed [35]. Antioxidants in plant extracts can repair damaged cells, such as

Superoxide Dismutase and Lactate Dehydrogenase, by neutralizing free radicals that cause chronic disease or cancer. Antioxidants have been considered a promising therapy for preventing and treating oxidative stress conditions triggered by increased ROS [36].

## Conclusions

Based on TLC results, Puguntano herb ethanol extract contains flavonoids, glycosides, saponins, tannins and steroids. The total phenolic and flavonoid content is  $20.22 \pm 0.96$  and  $1.11 \pm 0.06$  mg GAE/g. Chemical compounds in plants, such as phenols, flavonoids and tannins, indicate the possibility of antioxidant activity. The antioxidant test results showed high antioxidant levels with an IC50 value using the ABTS method, namely  $419.73 \pm 1.61$   $\mu\text{g/mL}$  and the FRAP method, namely  $36.77 \pm 0.71$   $\mu\text{g/mL}$ . Administration of Puguntano herb extract also affected SOD and LDH levels in doxorubicin-induced rats. SOD levels were known to increase, while LDH levels decreased and were statistically significantly different when compared with normal controls ( $p < 0.05$ ). These results show the potential of this plant to be developed as an antioxidant.

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