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Original Article

In vivo study of the antioxidant test of ethanolic extract of *Chromolaena odorata* Linn. leaves

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A B S T R A C T

Background: *Chromolaena odorata* L. leaf was reported to contain phenolic group compounds, such as flavonoids. Flavonoid compounds have been reported to have antioxidant activity. Based on in vitro study, *Chromolaena odorata* L. leaves have potent antioxidant activity. However, in vivo, studies with dose variations have not been reported.

Purpose: This study evaluates the antioxidant activity with various doses of ethanolic extract of *Chromolaena odorata* L. leaves against male Wistar rats induced by paracetamol.

Methods: Flavonoid contents were measured spectrophotometrically based on the formation of a complex flavonoid-aluminum. Quercetin was used to make a calibration curve. In vivo test was used TBARS method carried out by measured malondialdehyde (MDA) level in male Wistar rats induced by paracetamol 2g/Kg BW. The test was carried out on extracts with doses of 125, 250, and 500 mg/Kg BW. Vitamin C with dose 6,5mg/Kg BW used as a positive control, and 1% of Na CMC used as the negative control. Histopathology assessment of liver used Hema-toxylin Eosin Stain.

Results: Ethanolic extract of *Chromolaena odorata* L. leaves contain flavonoid 126.459±0.163 mg/g extract as quercetin equivalent. Intoxication paracetamol on rats increased MDA serum level significantly different (p-value < 0.005) with normal control. Treatment of ascorbic acid and extracts decreased MDA serum level significantly different (p-value < 0.005) with control negative and improved the histological structure of hepatocytes.

Conclusion: Ethanolic extract of *Chromolaena odorata* Linn. dose 500 mg/Kg BW was the best treatment with exhibited 58.974% reduction of MDA serum level and better improve the histological structure hepatocytes than other doses.

INTRODUCTION

Degenerative diseases, such as diabetes, cardiovascular diseases, inflammation, cancer, aging, neurodegenerative diseases, and immunosuppression, are developed from the excess free radical molecules[1,2]. The primary role of free radical molecules causes of these diseases is related to lipid peroxidation[3]. Free radical molecules are formed by standard metabolic action and have been reported to be formed by radiation, bacterial and viral toxin, smoking, alcohol, and psychological or emotional stress[4].

Antioxidant compounds from medicinal plants play an essential role in slowing or preventing free radical molecules' oxidation. Phenolic compounds, alkaloids, organic sulfur compounds, α-tocopherol, and β-carotene are phytochemical compounds reported with antioxidant properties[5]. *Chromolaena odorata* Linn. is one of the tropical plants used as traditional medicines for diabetes in Indonesia's regions. Most of the *Chromolaena* genus contains the flavonoids group. The previous study had reported that about 40 flavonoids had been identified from the *Chromolaena* genus and some of these flavonoid groups have strong antioxidant properties[6,7].

In vitro, antioxidant activities of *Chromolaena odorata* L. leaves have been reported widely. Ethanolic extract of *Chromolaena odorata* L. leaves with concentration

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300µg/mL has 59,89±0,002% DPPH scavenging activity[8]. Ethanolic extract of *Chromolaena odorata* L. contains 7,39±0,40g chlorogenic acid in 100g of dry leaves showed the DPPH radical scavenging activity with IC_{50} of 72,23µg/mL and 35,61% antioxidant activity of β-Carotene bleaching[9]. Methanolic extract of *Chromolaena odorata* L. leaf has IC_{50} scavenging of DPPH radical at 82,18µg/mL[10]. Ethanolic extract of *Chromolaena odorata* L. leaves has the highest DPPH radical scavenging activity than n-hexane and ethyl acetate extract[11].

In vivo study using the TBARS method reported that ethanolic extract of *Chromolaena odorata* L. leaf has lower lipid peroxidation at 20mg/Kg BW compared with the control group. However, this value was not significantly different ($p>0,05$) statistically[12]. However, antioxidant in vivo studies use the TBARS method with dose variations that have not been reported. Currently, this research aims to evaluate the antioxidant activity with various doses of ethanolic extract of *Chromolaena odorata* L. leaves against male Wistar rats induced by paracetamol.

METHOD

Study Design

This is a true-experimental randomized pretest-posttest controlled trial.

Study Site

Pharmacological and Biological Pharmacy laboratories, Faculty of Mathematics and Natural Sciences, Sriwijaya University. Dyatnitalis anatomical pathology laboratory, Palembang

Materials

The dried leaves of *Chromolaena odorata* Linn., ethanol, aqua dest, methanol, Quercetin (Sigma-Aldrich®), Sodium acetate, $AlCl_3$, aquabidest, Paracetamol standard (Dexa Medica), thiobarbituric acid (TBA) (Sigma-Aldrich®), trichloro acetate (TCA) (Sigma-Aldrich®), tetra-ethoxy-propane (TEP) (Sigma-Aldrich®), Na CMC, Ascorbic acid standard (Dexa Medica Pharm. Industry). Hematoxylin Eosin stain, Buffered formalin, Paraffin wax.

Plant Extraction

The dried leaves of *Chromolaena odorata* Linn. obtained from Belitung, South Sumatera, Indonesia. The 2 Kg of dried leaves were ground into powdered form then soak with ethanol 96% (1:10). The maceration process was kept in the amber bottle at room temperature for 72 hours and shaken occasionally. The macerate filtered then evaporated with a rotary evaporator at 60°C until getting a thick extract.

Total Flavonoid Content Assay

Total flavonoid content was measured spectrophotometrically based on the formation of complex flavonoid-aluminium[13]. Quercetin was used to make a calibration curve. Quercetin was prepared by concentration 5, 10, 15, 20, and 25 µg/mL in methanol solution. The extract was prepared by concentration 1000 µg/mL in methanol solution. One milliliter of sample solution was added with 1 mL of $AlCl_3$ 10% solution and 1 mL of sodium acetate 5%. The sample solution was incubated for 30 minutes, then measured absorbance value at λ 438 nm. The total flavonoid content was expressed as quercetin equivalents in mg/g (QE mg/g) extract.

In Vivo Procedure

Animal Preparation

Male Wistar rats (150-200 g) were used in this study, and seventh days were acclimated at the laboratory facility. All animals were maintained with approved animal care operating procedures consistently.

Experimental procedure

In vivo, the antioxidant assay was measured with the thiobarbituric acid reactive substance (TBARS) method[14]. The rats were divided into six groups, each comprising six rats. Group 1 (normal control) had free access to food. Group 2 (Negative control) received 2 g/Kg BW of paracetamol suspension once a day for six days, orally. Group 3 (Positive control) received 6.5 mg/Kg BW of ascorbic acid solution (in aquabidest) once a day six days later after receiving the same treatment of negative control. Group 4-6 (extract treatment) were received 125, 250, and 500 mg/Kg BW, respectively, once a day for six days later after received the same treatment of negative control. Blood serum was collected on the seventh and 14th day at sinus orbitalis. The MDA serum levels were obtained spectrophotometrically at 532 nm. Percent reduction of MDA level calculated with equation (1).

$$\text{Percent reduction MDA level } (\%) = \frac{\text{MDA before treatment} - \text{MDA after treatment}}{\text{MDA before treatment}} x\ 100\ \% \quad (1)$$

Histopathology of Liver

The histopathological assessment used the Hematoxylin Eosin stain as described before[15]. Liver tissues in each group were collected in 10% neutral buffered formalin. These tissues were processed and embedded in paraffin wax. Sections were cut used microtome with 5µm of thickness and stained with hematoxylin and eosin (H&E). The sections were examined microscopically for the evaluation of histopathological changes.

Statistical Analysis

Results are expressed as mean ± standard deviation and analyzed using one-way ANOVA followed by Tukey's posthoc test and paired student T-test for serum MDA level

before and after treatment. The p values were two-tailed, and p < 0.05 was regarded as significant. Histopathological data were analyzed descriptively.

Ethical Consideration

The Health Research Review Committee approved this study of Mohammad Hoesin Central General Hospital and Faculty of Medicine Sriwijaya University (182/kepkrsmhfkunsri/2019).

RESULTS

Plant Extraction and Total Flavonoid Measurement

Chromolaena odorata L. leaves were extracted with 96% ethanol to produce a 321.79 g viscous extract. The extract yield was 16.08% of the dry weight. Measurement of the total flavonoid levels of the extract used a quercetin calibration curve. Calibration curve equation obtained y = 0.0188x - 0.0057 with a value of R^2 = 0.9998. The quercetin calibration curve is shown in Figure 1. Based on this equation, the total flavonoid of ethanolic extract of *Chromolaena odorata* L. leaves express as quercetin equivalent was 126.459±0.163 mg/g.

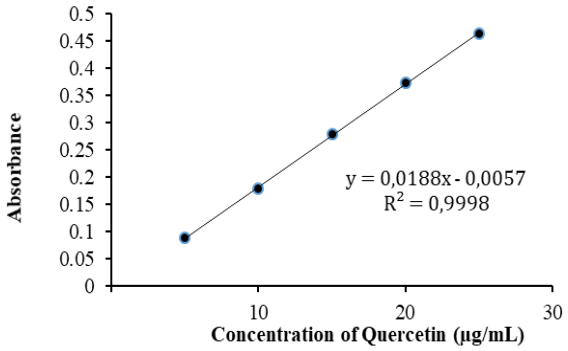

Figure 1. Quercetin Calibration Curve

In Vivo Test

Figure 2 shows that the negative, positive, and extract groups have been increased MDA levels after being intoxicated by 2g/Kg BW of paracetamol once a day for six days. In contrast, the standard/normal control group was not induced by paracetamol as a reference for normal MDA levels. ANOVA analysis shows significantly different (p-value < 0.05) in the MDA level of each group. Based on Tukey's posthoc analysis, there is known that negative control, positive control, and 500mg/Kg BW extract groups have a significantly different value (p-value < 0.05) of MDA levels with the normal control group. However, the MDA level on all group treatment had no significant difference (p-value > 0.05) with the negative control. It is indicated that paracetamol has been increased the MDA serum level of rats.

After the rats were treated for seven days, each group show a decrease in MDA levels, except for the normal and negative control groups. Figure 3 shows that the positive control group and each extract group have significantly different (p-value < 0,05) MDA levels from the negative control group. Table 1 shows the percent reduction value of MDA level after treatment.

Table 1. Percent Reduction in MDA Level

Group	MDA level after Paracetamol intoxication (nmol/mL)	MDA level after treatment (nmol/mL)	Percent Reduction (%)
Negative control	2.654±0.599	2.510±0.434	5.426
Positive control	3.516±0.874	1.120±0.131*	68.146
125 mg/Kg BW of extract	2.471±0.261	1.490±0.093*	39.701
250 mg/Kg BW of extract	2.364±0.407	1.224±0.176*	48.223
500 mg/Kg BW of extract	2.729±0.899	1.120±0.318*	58.974

(* = significantly different (p-value <0.05) of MDA before and after treatment with paired student T-test analysis)

A paired student T-test had used to analyze the differences of MDA level after paracetamol intoxication and MDA level after treatment. Table 1 found that a positive group and extract groups significantly reduce MDA level after treatment (p-value < 0.05). Positive control has the highest reduction of MDA level compared to other treatment groups. While in extract groups, 500 mg/Kg BW of ethanolic extract of *Chromolaena odorata* L. leaves was the best dose of an extract with 58.974% reduction of MDA level. Based on Tukey's posthoc analysis, all extract groups treatment had no significant difference (p-value > 0.05) in MDA level with the positive control group. It can be concluded that the ethanolic extract of *Chromolaena odorata* L. leaves has the same effectiveness as ascorbic acid.

Histopathology of Liver

There are differences in hepatocyte structure in the paracetamol-induced group of rats compared to the normal control. Paracetamol induction causes inflammation and necrosis of the hepatocyte cells (Figure 4)[15].

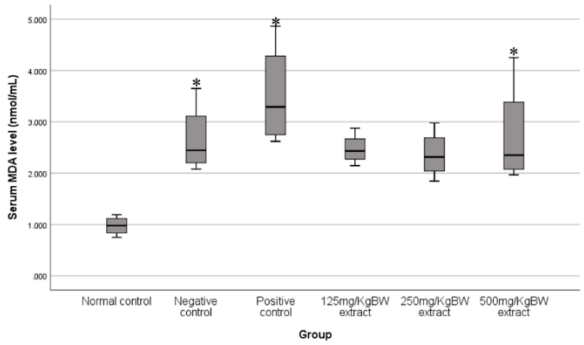

Figure 2. Serum MDA Level After Paracetamol Intoxication
(* = significantly different with normal control (p-value < 0.05))

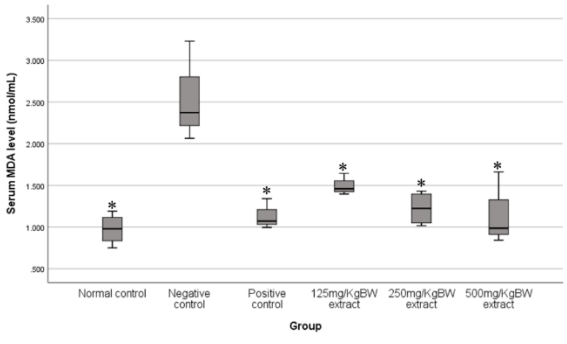

Figure 3. Serum MDA Level After Treatment
(* = significantly different with negative control (p-value < 0.001))

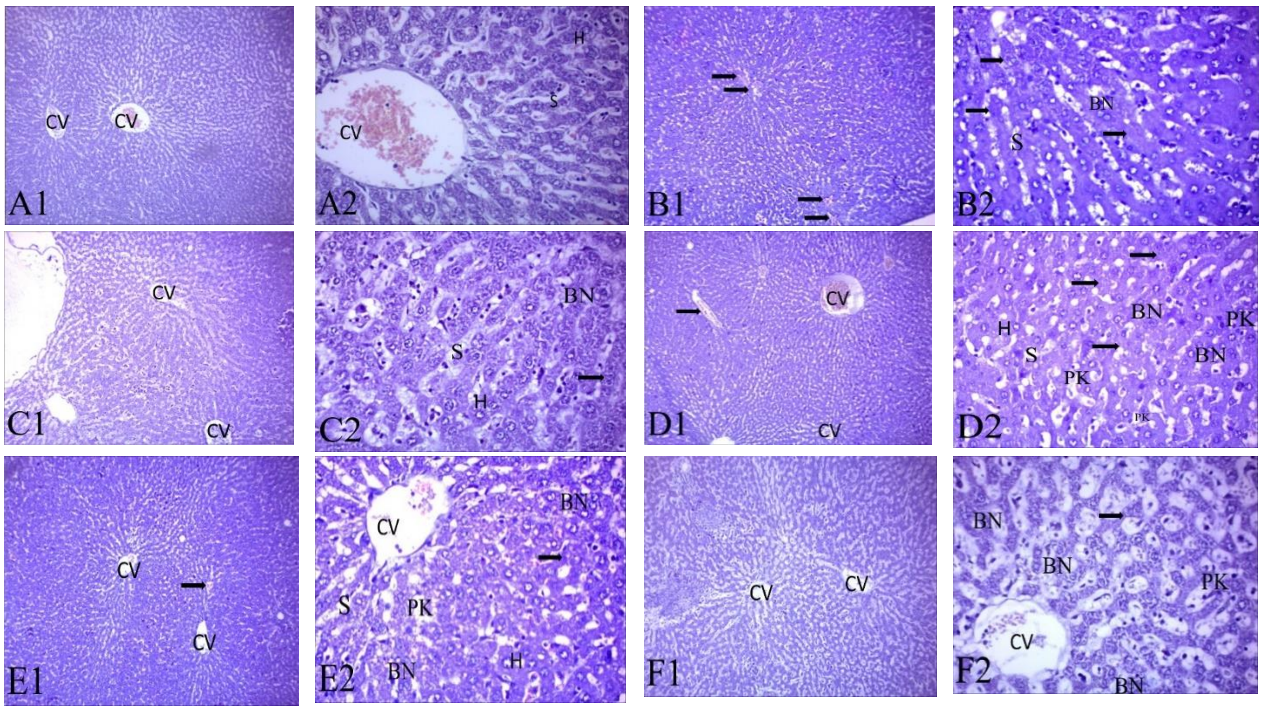

Figure 4. Photograph of rat liver section shows A). Normal control shows the normal histological structure of hepatocyte (H) and sinusoid (S), B). Negative control shows more inflammatory infiltration, necrosis (arrow), and binucleated cell (BN) of hepatocyte, C). Positive control shows less necrosis (arrow) and binucleated cell (BN) of hepatocyte, D). Extract dose I shows inflammatory infiltration, necrosis (arrow), pyknotic nuclei (PK), and binucleated cells (BN) of hepatocyte, E). Extract dose II shows necrosis (arrow), pyknotic nuclei (PK), and binucleated cells (BN) of hepatocyte, F). Extract dose III shows necrosis (arrow), pyknotic nuclei (PK), and binucleated cells (BN) of the hepatocyte. (1) magnification 40x, (2) magnification 400x

DISCUSSION

Chromolaena odorata Linn. is a member of the family Eupatoriae (Asteraceae)[7]. In Belitung, South Sumatera region, Indonesia, *Chromolaena odorata* Linn is known as "merdekaan," a popular folk medicine for diabetes treatment. Ethanolic extract of *Chromolaena odorata* Linn. leaves contain flavonoids, alkaloids, tannins, phenolics, saponins, steroids, and triterpenoids (data not shown). Some phenolic group compounds have been found in *Chromolaena odorata* L. leaf; there are p-coumaric acid, p-hydroxybenzoic acid, protocatechuic acid, ferulic acid, vanillic acid[16]. Odoratenin, isosakuranetin, and subscandenin, a new flavanone compound, in *Chromolaena odorata* L.leaf was found by Putri[6]. High phenolic content is beneficial as the phenolic compound quench primary oxidants or free radicals[17]. Balamurugan[9] was investigated that indirect ethanolic extraction of *Chromolaena odorata* L. leaves has the highest total phenolic compound and total antioxidant content compare to other solvents in sequential extraction. Ethanol is a useful solvent to extract phenolic and flavonoid compounds and is safe for human consumption[18]. Flavonoid and phenolic compounds have free radical scavenging activity by donating their free electron pairs to radical molecules[9]. This study was measured

the total flavonoid of ethanolic extract of *Chromolaena odorata* L. leaves; it has 126.459±0.163 mg QE/g extract.

Chromolaena odorata L. leaves have been shown as a potent antioxidant in various in vitro assays [8–11,13,16,19–24]. However, in vivo assays of antioxidant activity of this plant are limited. Uhegbu[12] was investigating the activity of *Chromolaena odorata* L., *Ageratum conyzoides* L, and their combination to reduce lipid peroxidation in rats. The evaluation shows that ethanolic extract of *Chromolaena odorata* L. leaf has lower lipid peroxidation (TBARS) at 20mg/Kg BW than the control group. However, this value was not significantly different (p-value > 0.005) statistically. To our knowledge, in vivo study with various doses of *Chromolaena odorata* L. leaves has not been evaluated. This study was aimed to evaluate the antioxidant activity in system organisms used various doses of the extract. We used Wistar strain male rats as the test animal. The study used six groups: normal control, negative control, positive control, 125 mg/Kg BW, 250 mg/Kg BW, and 500 mg/Kg BW of ethanolic extract *Chromolaena odorata* Linn. leaves. Except for normal control, the five groups received 2mg/Kg BW of Paracetamol once a day for six days to elevate the MDA serum level.

Paracetamol with toxic dose can significantly elevate Malone dialdehyde (MDA) level serum of rats. The elevation of Malone dialdehyde (MDA) level serum indicates of increase in lipid peroxidation[25]. Paracetamol had reported disturbing the balance between free radical molecules production and antioxidant protection, especially in the liver. N-acetyl-p-benzoquinone imine (NAPQI) formation by cytochrome P450 (CYP), a highly reactive toxic electrophile, is a toxic compound produced by paracetamol. When the rate of NAPQI formation exceeds the detoxification rate by glutathione, it is oxidized tissue macromolecules such as lipid or -SH group of proteins. Lipid peroxidation is an autocatalytic process, which is a common consequence of cell death[26]. MDA is one of the end products in the lipid peroxidation process. The increased MDA content might have resulted from an increase of free radical molecules due to stress due to Paracetamol intoxication[27].

Serum MDA level of negative control, positive control, 125 mg/Kg BW, 250 mg/Kg BW, and 500 mg/Kg BW of ethanolic extract of *Chromolaena odorata* Linn. leaves after paracetamol intoxication has significantly higher (p-value < 0.05) than normal control (Figure 2). The reduction of MDA level serum in the positive control group, 125 mg/Kg BW, 250 mg/Kg BW, and 500 mg/Kg BW of ethanolic extract of *Chromolaena odorata* Linn. leaves have significantly different (p-value < 0.05) with negative control (Figure 3). Ascorbic acid in the positive control group has the highest percent reduction in MDA level (68.146%). Besides that, 500mg/Kg BW of ethanolic extract has a 58.974% reduction of MDA level, higher than other doses. Ascorbic acid as a standard drug, flavonoid, and phenolic compound in the extract has antioxidant activity that inhibits the enzyme involved in free radical molecules formation[28]. Peluso[29] has been reported that flavonoids could interfere with drugs' bioavailability through competition with cytochrome P_{450} (CYP) enzymes. So, the metabolism of paracetamol into NAPQI form can be reduced.

Figure 4, A1-A2 liver sections of the normal control group showed the typical histological structure of hepatocytes with well-preserved cytoplasm and well-defined nucleus. The negative control group (Figure 4, B1-B2) showed disarrangement of normal hepatocytes, necrosis of cells, and inflammatory infiltration. There were binucleated cells and less necrosis of hepatocytes (Figure 4, C1-C2). Apart from necrosis of cells and binucleated cells, pyknotic nuclei were also found in extract treatment groups (Figure 4, D-F1-2). In our present study, there were histopathological changes in response to paracetamol. Indication of marked changes in the liver's overall histoarchitecture could be due to its toxic effects by the generation of radical molecules that damage the various membrane components of the cell—the necrotic condition in rats mostly characterized by pyknosis cytoplasm[30]. The formation of highly reactive radicals because of paracetamol intoxication can cause liver function failure[31]. Lipid peroxidation can disturb cellular membranes' integrity, leading to the leakage of cytoplasmic enzymes due to severe histopathological damages[32,33]. The phenolic compound, such as quercetin, was proven to possess hepatoprotective effect in an animal test against liver damage induced by lipid peroxidation[34]. Phenolic compounds prevent paracetamol-induced lipid peroxidation was due to antioxidant activities. *Chromolaena odorata* L. leaves have strong antioxidant properties and inhibit lipid peroxidation due to hepatoprotective effect in rats against liver damage induced by paracetamol.

CONCLUSIONS AND RECOMMENDATION

This study concludes that ethanolic extract of *Chromolaena odorata* Linn. leaves contain a flavonoid of 126.459±0.163 mg QE/g extract. Extract with a 500 mg/Kg BW dose was the best treatment with exhibited 58.974% reduction of MDA serum level. Ascorbic acid and all extract treatments showed improvement in the histological structure of hepatocytes. Therefore, the next study should be directed toward using extract rich flavonoids to reduce doses and increase the activity. The small extract dosage will be more acceptable to make dosage preparations for human usage.

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