Science and Technology Indonesia
e-ISSN:2580-4391 *p*-ISSN:2580-4405
Vol. 8, No. 4, October 2023

Research Paper

Antioxidant Activity Test of the Ethyl Acetate Fraction of Beluntas Leaves (*Pluchea indica L.*) in White Male Rats Wistar Strain Induced Carbon Tetra Chloride

Fitrya[1], Vitri Agustiarini[1*], Adelia Indriyani[1], Herlina[1], Dwi Hardestyariki[2], Eka Febri Zulissetiana[3]

[1]*Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Sriwijaya University South Sumatera, 30862, Indonesia*
[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, Sriwijaya University South Sumatera, 30862, Indonesia*
[3]*Department of Physiology, Faculty of Medicine, Sriwijaya University South Sumatera, 30862, Indonesia*
***Corresponding author**: vitriagustiarini@mipa.unsri.ac.id

Abstract
Beluntas leaves contain flavonoids which have antioxidant effects. The purpose of this research was to determine the flavonoid total content, the effect of ethyl acetate fraction of beluntas on Malondialdehyde (MDA) levels, catalase enzyme activity, macroscopic pathology, histopathology description of white male rats wistar strain induced by CCl_4 and of determining the best dose antioxidant. In this study, the in vivo tests used TBARS methods which consisted of six groups that are normal control, positive control (vitamin C 10 mg/kgBW), negative control (Na-CMC 1%), and treatment groups were given ethyl acetate fraction of beluntas leaves at doses 25 mg/kgBW, 50 mg/kgBW and 100 mg/kgBW. The total flavonoid content of the ethyl acetate fraction of beluntas leaves was 153 mgQE/g. The average results of MDA levels normal, positive, negative, doses fraction groups of 25, 50, and 100 mg/kgBW, respectively, were 1.155; 0.471; 1.892; 1.218; 0.826 and 0.512 nmol/mL. The average results of catalase enzyme activity of normal, positive, negative, doses fraction groups 25, 50, and 100 mg/kgBW, respectively, were 22.131; 40.489; 3.103; 14.145; 32.176, and 39.814 U/mL. The macroscopic and histopathology results show an improvement after being given the ethyl acetate fraction of beluntas leaves at doses of 25, 50, and 100 mg/kgBW compared to the negative control. The results showed that the ethyl acetate fraction of beluntas leaves reduce MDA levels and increases catalase enzyme activity. Ethyl acetate fraction of beluntas leaves at 100 mg/kgBW had the most potent antioxidant activity compared to doses of 25 and 50 mg/kgBB which were shown by Duncan's statistical analysis that there was no significant difference with positive control.

Keywords
Beluntas Leaves, Total Flavonoid, Ethyl Acetate Fraction, Antioxidant, In Vivo Test

Received: 28 May 2023, Accepted: 4 August 2023
https://doi.org/10.26554/sti.2023.8.4.599-606

1. INTRODUCTION

Free radicals are an unstable and reactive molecule with one or more unpaired electrons (Martemucci et al., 2022). Oxidative stress or free radicals are a fundamental mechanism of neurologic disease (Sarma et al., 2010). Reactive oxygen species (ROS) can damage DNA, lipids, and proteins, which can cause neurodegenerative diseases (Khan et al., 2018). The body can protect against the harmful effect of oxygen and nitrogen species using exogenous and endogenous antioxidants such as the enzyme catalases, polyphenols, and glutathione peroxidase (Patro et al., 2016). The antioxidant can be used to repair mitochondrial cell damage with mechanisms inhibition of ROS formation, include removed of O_2 and binding of metal ions needed for the catalysis of ROS, re-generation, and up-regulation of endogenous antioxidants (Khan et al., 2018). Antioxidants can be classified into natural and synthetic, depending on yheir origin. Natural antioxidants are usually found in vegetables, fruits, and spices, while synthetic antioxidants are exogenous and endogenous antioxidants (Ayoka et al., 2022). One plant that contains natural antioxidants is the beluntas plant (*Pluchea indica L.*) (Muchtaromah and Mustikasari, 2019).

Beluntas leaves are often used as an Indonesian traditional medicine to eliminate body odor, increase appetite, overcome diarrhea, joint pain, back pain, and irregular menstruation, and also help some digestion problem (Mahasuari et al., 2020). Beluntas are potential as their antioxidant and blood glucose-lowering potential (Widyawati et al., 2015). Beluntas leaves have a pharmacological effect as antioxidants, antifibrosis, antimicrobial, anti-inflammatory, and anti-cholesterol (Muchtaromah and Mustikasari, 2019). Beluntas leaves contain alkaloids, hydroquinone, sterols, tannins, and flavonols, such as myricetin, quercetin, and kaempferol. Phenolic and flavonoids have antioxidant activity (Hoda et al., 2019). The greater the

flavonoid content, the greater the antioxidant activity (Rahman et al., 2021).

Beluntas leaves which have antioxidants, come from the class of flavonoid compounds. Flavonoids, as antioxidants, reduce free radicals and block radical chain reactions (Zheng et al., 2022). Based on the Ultraviolet-Visible spectrum, it can be assumed that the flavonoid compounds contained in beluntas leaves are the type of flavonol, which can be seen from the long-range waves between (band II) 250-280 nm and (a band I) 350-385 nm (Koirewoa et al., 2012). Flavonol substituting the hydroxyl groups at the 3-position reduce the activity and increases flavonoid active radical scavenging capacity of flavonoids (Sarma et al., 2010).

Flavonoid is a polar compound because it has –OH groups that form hydrogen bonds. However, several types of flavonoids, such as isoflavones, flavones, flavanones, aurons, chalcones, anthocyanins, and flavonols, are less polar compounds. Flavonoid extraction is generally carried out using a polar solvent such as ethanol because most flavonoid compounds are polar such as flavonoid glycosides and aglycones. However, some less polar flavonoids should be extracted using ethyl acetate. Therefore, flavonols that are less polar are extracted using ethyl acetate solvent (Khoddami et al., 2013).

The research results on the uses of P. indica for managing diabetic liver injury (Nopparat et al., 2020). *Pluchea indica* leaf Ethanol extract 50 and 100 mg/kgBW for two weeks repair liver damage induced by STZ hyperglycemia through modulation of inflammatory response and oxidative stress by can lower hepatic apoptosis and improvement of hepatic with inhibiting TNF-α, NF-kB p65, TGF-β1, IL-6 and PKC (protein kinase C). The antioxidant activity of beluntas leaf extract method DPPH (2.2-diphenyl-1-picrylhydrazil) is very strong, with an IC_{50} value of 21 ppm (Sugiaman et al., 2021).

This research evaluates the antioxidant activity with various doses of ethyl acetate fraction of beluntas leaves (*Pluchea indica L.*) against male Wistar rats induced by CCl_4.

2. EXPERIMENTAL SECTION

2.1 Material

The materials used in the research consisted of beluntas leaves (*Pluchea indica L.*) obtained from Palembang which identified at Andalas University with No. 030/K-ID/ANDA/11/2017, ethyl acetate (PT. Bratachem, Palembang), n-hexane (PT. Bratachem, Palembang), aquadest (PT. Bratachem, Palembang), olive oil (Organic®), carbon tetrachloride (CCl_4), vitamin C, Sodium Carboxyl Methyl Cellulose (Na-CMC), NaCl 0.9%, 1.1.3.3-tetra ethoxy propane (TEP) (Sigma Aldrich®, Singapura), Trichloroacetic Acid (TCA) 20% (Merck & Co, Singapura), Thiobarbituric Acid (TBA) (Merck &Co, Singapura), Hematoxylin-Eosin stain, mg powder, sulfuric acid 2N, sodium hydroxide 10%, hydrochloric acid 10%, ethanol 70%, $FeCl_3$ 1%, chloroform, concentrated sulfuric acid anhydride, acetic acid anhydride, Mayer's reagent (Merck & Co®, Readington), Wagner's reagent (Merck & Co®, Readington), Dragendorf's reagent (Merck & Co®, Readington), Liebermann-Burchard reagent, cotton, and filter paper (Whatman®, South Jakarta).

2.2 Methods

2.2.1 Preparation of Extract Beluntas Leaves

Beluntas leaves are extracted using the maceration method. Beluntas leaf simplicia as much as 1 kg was macerated with 96% ethanol at a ratio of 1:10, then put into a dark container. Maceration was carried out for 3 × 24 hours while stirring occasionally. The maserate obtained is then filtered and residue was again mixed twice with 96% ethanol for 2×24 hours. All filtrate results were concentrated using rotary evaporators at 65 oC until a thick extract was obtained.

2.2.2 Fractionation

Fractionation using the liquid-liquid partition method with n-hexane, ethyl acetate and ethanol-water as solvents. The condensed ethanol extract of beluntas leaves was dissolved in 96% ethanol, then fractionated with n-hexane with a ratio of 2:1 (ethanolic liquid extract of beluntas leaves: n-hexane), replicated three times. This stage produces two layers, namely n-hexane (top) and ethanol (bottom). The two layers formed were separated, and the ethanol layer was taken. In the second stage, the ethanol residue was fractionated again with ethyl acetate and water in a ratio of 1:1:1 (ethanol: ethyl acetate: water fraction) replicated three times. In the second stage, the ethyl acetate fraction and the ethanol fraction were obtained, then they were separated and the ethyl acetate fraction was taken. The ethyl acetate fraction was concentrated with rotary evaporators at 65 degrees so that the viscous ethyl acetate fraction was produced and then the results were weighed.

2.2.3 Total Flavonoid Content Assay

Total flavonoid ware analyzed using aluminum chloride colorimetric. Quercetin was used a calibration curve. 10 mg quercetin was dissolved in 96% ethanol and diluted to 20; 30; 40; 50 and 60 ppm. As much 20 mg of the sample of the ethyl acetate fraction of beluntas leaves and dissolved in 10 mL of ethanol pa, then centrifuged to obtain a concentration of 2000 ppm. 0.5 mL of sample was added with 0.1 mL of 10% aluminum (III) chloride, 0.1 mL of 1 M sodium acetate, and 2.8 mL of distilled water. The solution was incubated for 30 minutes. The absorbance was measured using a UV-Vis spectrophotometer at the maximum wavelength obtained based on the determination of the wavelength of the quercetin standard solution.

2.2.4 Experimental Design

The test animals used were white male rats of Wistar strain. Based on Ferderer's dosage calculations, the number of rats needs 30 white male rats with four rats for each other group. One rat was added to each group to ensure sufficient samples, so 30 rats were needed. Test animals were randomly selected and divided into six treatment groups. Testing with these animals has received ethical clearance from the Ahmad Dahlan University Research Ethics Committee with number 022211807.

The test animal groups were given different treatments Table 1.

2.2.5 Measurement of Malondialdehyde (MDA)

Malondialdehyde (MDA) levels were measured when all groups after fasted for eight hours. The preparations were given to rats orally from day 1 to day 6 and CCl_4 was induced at 41 mL/kgBW orally on the seventh day. Rats that have been induced with CCl_4 was blood collection carried out on the eighth day through a medial *canthus of the orbital sinus* from each test animal. Blood is taken as much as two mL and stored in a tube vacutainer EDTA.

Measurement rate MDAs done centrifuged the blood sample for 10 minutes at 3000 rpm at 4°C so that the rats blood plasma and blood cells were separated. 0.5 ml of blood plasma was added to 2.5 ml of 20% trichloroacetate (TCA) solution, then centrifuged at 3000 rpm 4°C for 10 minutes to obtain protein-free plasma. Add 1.5 ml of the supernatant formed, put it in another test tube, and add 1.5 ml of 0.67% thiobarbituric acid (TBA) solution. The solution is heated at 95-100 C, then cooled in an ice-filled vessel. The filtrate results were measured at a wavelength of 530 nm with a UV-Vis spectrophotometer. MDA levels were measured using the TEP standard curve regression equation.

2.2.6 Determination of Catalase Enzyme Activity

Catalase enzyme activity was measured based on the amount of hydrogen peroxide reduction. The test solution was prepared by mixing 1 ml of liver homogenate with 5 mL of 0.05 M phosphate buffer with pH 7, vortexing until homogeneous, then adding 4 mL H_2O_2 0.2 M and incubating for 30 seconds. The standard solution used is H_2O_2 with a concentration of 0.04; 0.08; 0.12; 0.2; and 0.4 M. A total of 1 mL of the test solution was put into a test tube and added 2 mL of $K_2Cr_2O_7$ 5%. The test tube was heated in boiling water for 10 minutes and then cooled. The absorbance of the test solution was read at a wavelength of 570-610 nm. The absorbance read is equivalent to the concentration of H_2O_2 the remaining. Catalase enzyme activity is calculated in the following:

$$\text{Catalase enzyme(U/mL)} = \frac{2.303}{t} \times \left[\log \frac{S'}{S-M} \times \frac{VT}{Vs}\right]$$

Information :

t = incubation time (minute)

S' = absorbance H_2O_2 0.2 M

S = sample absorbance

M = blanko absorbance

2.2.7 Prepartion of Macroscopic Hepar

Livers of the test animals were taken and washed using 0.9% physiological NaCl, then weighed and observed for the color and surface of the liver organs. Observations were recorded and compared between each test group.

2.2.8 Prepartion of Hepar Histopathology

Histopathological preparations of the liver were performed with Hemooxylin-Eosin (HE) staining. Samples of the liver organs of the test animals were taken and immersed in formalin buffer solution (NBF). Organ samples were scaled with thin slices to be stored in a tissue cassette and fixed in an NBF solution. The fixed sample was then dehydrated and cleared with a solution of 70% alcohol, 80% alcohol, 90% alcohol, absolute alcohol, toluene, and paraffin, gradually within one day. The organ samples were blocked by embedding cells with liquid paraffin poured, then cooled. Blocks that have cooled, sectioning using a microtome with a thickness of ± 4-5 microns. After that, it was stained with Hematoxylin-Eosin and mounting media.

2.2.9 Data Analysis

Data on MDA levels and catalase enzyme activity were analyzed with SPSS 25.0^{TM} with the normality test Shapiro-Wilk and correlation with correlation test Pearsons, if the resulting data is typically distributed (p value> 0.05), then the data testing can be continued using one way ANOVA and test Post Hoc Duncan.

3. RESULTS AND DISCUSSION

3.1 Extraction and Fractionation of Beluntas Leaves

The results of the thick extract obtained as much as 6.13 grams of 920 grams of simplistic powder with a yield percentage of 11.535%. The fractionation results of 100 grams of ethanol extract of beluntas leaves to produce ethanol-water, n-hexane, and ethyl fraction were 26.64; 11.92; dan 17.38 grams with a yield percentage of 26.64%; 11.92%; dan 17.38%.

3.2 Total Flavonoid Content

Flavonoid compounds have free radical scavenging activity by donating their free electron pairs to radical molecul (Saraf and Balamurugan, 2018). The result of calculating the total flavonoid content of 1 gram of the ethyl acetate fraction of beluntas leaves is 153 mgQE/g fraction with a percentage of 15.3%. These results are supported by Sugiaman et al. (2021), which showed that in 1 g of ethanol extract of beluntas leaves there are 19.44 mg of flavonoids had antioxidants activity with an IC_{50} value was 21.53 ppm. The ethyl acetate fraction of beluntas leaves showed positive results for flavonoids which were indicated by a change in color to orange after the addition of concentrated HCl and Mg powder. The color change occurs because the flavonoid compounds are oxidized by Mg^{2+} by forming complexes with magnesium ions. The polyhydroxy of flavanones will be reduced by magnesium metal in hydrochloric acid in ethanol solution to form red, yellow, benzopyrilium salts or flavilium salts.

3.3 Measurement of MDA and Catalase Enzyme

MDA levels were measured using a UV-Vis spectrophotometer with a wavelength of 530 nm and the TEP standard curve

Table 1. Treatment Group Antioxidant Activity Test

Test Animal Group	Treatment of Test Animals
Normal	Na CMC suspension 1% p.o
Positive	Vitamin C 10 mg/kgBW p.o + CCl_4 1 mL/kgBW p.o
Negative	Na CMC Suspension 1% p.o + CCl_4 1 mL/kgBW p.o
Test Treatment I	SFEADB 25 mg/kgBW + CCl_4 1 mL/kgBW p.o
Test Treatment II	SFEADB 50 mg/kgBW + CCl_4 1 mL/kgBW p.o
Test Treatment III	SFEADB100 mg/kgBB + CCl_4 1 mL/kgBW p.o

Table 2. Result in Observation Macroscopic Liver

Group Treatment	Hepar Color	Heart Surface	Average Body Weight ± SD (gram)	Average Liver Weight ± SD (gram)	Index Organ ± SD
Normal Control	Red Brown	Fine	246.848 ± 34.340	9.563 ± 1.495	3.871 ± 0.259
Positive Control	Red Brown	Fine	222.576 ± 5.470	10.28 ± 1.580	4.609 ± 0.589
Negative Control	Red Pale	Freckles	190.69 ± 15.831	5.99 ± 0.269	3.148 ± 0.127
Dose 25 mg/kgBW	Red Brown	Fine	231 ± 62.031	9.843 ± 3.880	3.995 ± 0.673
Dose 50 mg/kgBW	Red Brown	Fine	181.244 ± 8.237	7.79 ± 0.248	4.301 ± 0.135
Dose 100 mg/kgBW	Red Brown	Fine	204.813 ± 24.130	8.086 ± 0.935	3.963 ± 0.391

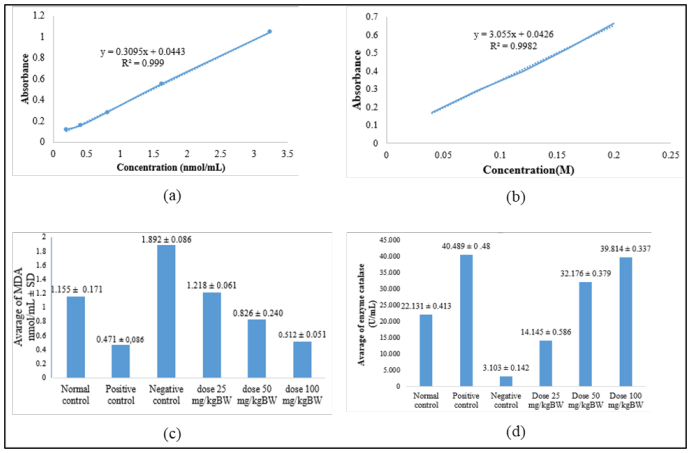

Figure 1. Antioxidant Activity In Vivo Result (a) TEP Standard Curve Graph (b) H_2O_2 Standard Curve Graph (c) The Average of MDA Levels and (d) The Average of Catalase Enzyme Activity

linear regression equation. The linear regression equation resulting from the standard TEP curve is y = 0.3095x + 0.0443 dengan nilai R^2 = 0.999. The TEP standard curve is shown in Figure 1(a). Determination of the maximum wavelength using a UV-Vis spectrophotometer aims to determine the absorption in determining the catalase enzyme's activity from rat liver tissue. The results of determining the maximum wavelength H_2O_2 obtained from this study amounted to 585. The linear regression equation resulting hydrogen peroxide curve is y = 0.3055x + 0.0426 with R^2 = 0.982 The H_2O_2 standard curve is shown in Figure 1(b).

Figure 1 (c and d) showed that the negative, positive, and ethyl acetate fractions of beluntas leaves groups had been induced by CCl_4. The results of measuring MDA levels in the normal group showed 1.155 nmol/mL. This value is the expected average MDA level in normal rats which will be compared with the treatment group. The normal group showed MDA has formed in the body because it is a normal process that exhibits an essential role in maintaining normal cellular homeostasis. Under these normal physiological conditions, cellular antioxidant defense mechanisms involve various enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase to regulate ROS production (Bhatti et al., 2022).

Measuring MDA levels in the negative control group induced by CCl_4 yielded a value of 1.892 nmol/mL. It shows an increase in MDA levels in the negative control compared to the normal control. The increase in MDA levels proves that CCl_4 induction as a toxic agent causes oxidative stress in the negative group. The results measuring MDA levels in the positive group given vitamin C showed significantly different results from the negative control. It was indicated by the average MDA level of the positive control group for vitamin C of 0.471 nmol/mL. These results showed a decrease in MDA levels in the positive control group compared to the normal and negative control groups. It follows Popovic et al. (2015), who stated that vitamin C as an exogenous antioxidant could reduce serum MDA levels and suppress the occurrence of lipid peroxide.

The measurement results for the treatment group that was given the ethyl acetate fraction of beluntas leaves with various of 25 mg/kgBW, 50 mg/kgBW and 100 mg/kgBW showed a decrease in MDA levels as indicated by the value of each dose of

Table 3. Score Level in Histological Images of Hepatic Cell

Group Treatment	Hydropic Degeneration	Fatty Degeneration	Necrosis
Normal Control	0	0	0
Negative Control	2	3	5
Positive Control	0	0	1
Dosage 25 mg/kgBW	1	3	3
Dosage 50 mg/kgBW	0	3	3
Dosage 100 mg/kgBW	1	0	2

1.218 nmol/mL; 0.826 nmol/mL and 0.512 nmol/mL. The results of reducing MDA levels at a dose of 25 mg/kgBW were close to those of the normal control group whereas at a dose of 100 mg/ kgBW they were close to the levels of the positive control group. The ethyl acetate fraction of beluntas leaves at 100 mg/kgBW is the most effective dose. Parwata et al. (2018) showed that the water extracts of *Gyrinops versteegii* leaves with a dose of 100 mg/kgBW in experienced oxidative stress lowering MDA levels with a mean of 5.56 nmol/mL. That showed that the antioxidant in beluntas leaves at 100 mg/KgBW is more potent than the antioxidant activity of *Gyrinops versteegii leaves*. It proves that the active flavonoids in beluntas leaves function as antioxidants can destroy and damage oxidants (Nirwana and Jamilah, 2021).

Based on the results of the normality test with Shapiro-Wilk, it was shown that data on rat plasma MDA levels are normally distributed with a value (p>0.05). Results of ANOVA analysis One Way to Test Homogeneity Variances A Significance value of 0.078 (p>0.05) was obtained, which indicated that the data was homogeneous. Further data analysis with test post hoc by method Duncan. Based on the test results, it was found that the negative control was significantly different from the normal, positive, and treatment groups. In contrast, the positive control group was not significantly different from the 100 mg/kgBW treatment group, and the normal group was not significantly different from the 25 mg/kgBW treatment group. It shows that the dose of 100 mg/kgBW has relatively strong antioxidant activity and the dose of 25 mg/kgBW has relatively weak antioxidant activity.

The normal control group has an average catalase enzyme activity of 22.131 U/mL. Vitamin C used as the positive control has the best scavenging activity with a value of catalase enzyme of 40.489 U/mL. The average yield of the catalase enzyme in negative control given CCl_4 experienced a decrease in catalase enzyme activity from the normal group as indicated with a value of 3.103 U/mL. Administration of CCl_4 caused induction of oxidative damage and DNA fragmentation with caused an increase in urea, uric, acid plasma and creatinine levels, lipid peroxidation and protein carbonyl. Meanwhile, Glutathione levels, glutathione transferase, catalase, superoxide dismutase and peroxidase activities significantly decreased (Makni et al., 2012).

The average results of each test treatment group with doses of 25 mg/kgBW, 50 mg/kgBW, and 100 mg/kgBW showed a value of 14.145 U/mL, 32.176U/mL, and 39.814 U/mL. A dose of 100 mg/kgBW showed a greater value of the catalase enzyme than doses of 25 mg/kgBW and 50 kgBW, having closer results to the positive control. It is because beluntas leaves contain flavonoid compounds that act as antioxidants. Flavonoids as antioxidant activity by reducing free radicals transferring single electrons. Flavonoids can form chelate or bind metal ions to prevent oxidation so that free radicals are not formed (Vo et al., 2019). Flavonoids played key roles in inhibiting free radical generating enzymes such as myeloperoxidase, xanthine oxidase, lipoxygenase, microsomal monooxygenase, and NADPH oxidase (Hritcu et al., 2017).

Based on data analysis from test normality using Shapiro-Wilkit, the catalase enzyme activity levels data were normally distributed with a value (p> 0.05). ANOVA results were obtained, indicating that the data was homogenous further data analysis using test post hoc by method Duncan. The analysis showed that the positive control group did not differ significantly from the 100 mg/kg BW treatment group. In contrast, there were significant differences between the positive control group and the normal control group, the negative control group, the 25 mg/kgBW, and the 50 mg/ kgBW treatment group. It shows that the ethyl acetate fraction of beluntas leaves at 100 mg/ kgBW is the best dose as an antioxidant. Researchers use Pearson's correlation analysis to determine the relationship between plasma levels and catalase enzyme activity. Correlation results obtained a 0.000 (P <0.05) with a correlation coefficient of -0.950. This value indicates a negative correlation between MDA levels and catalase enzyme activity, which is inversely proportional, so the lower the MDA level, the higher the catalase enzyme activity in rat livers. The value of the correlation coefficient in the study indicates a perfect correlation.

3.4 Macrosopic of Rat Liver

Macroscopic observation of the liver aims to determine the condition of the rat's liver macroscopically, such as the color, weight, and surface of the liver. The results of macroscopic observations of rat liver organs can be seen in Table 2 and Figure 2.

Observing the liver in the positive control group and the test treatment showed the same results as the normal control group. The liver was brownish red, and the liver surface was smooth. In the treatment group with a dose of 100 mg/kg

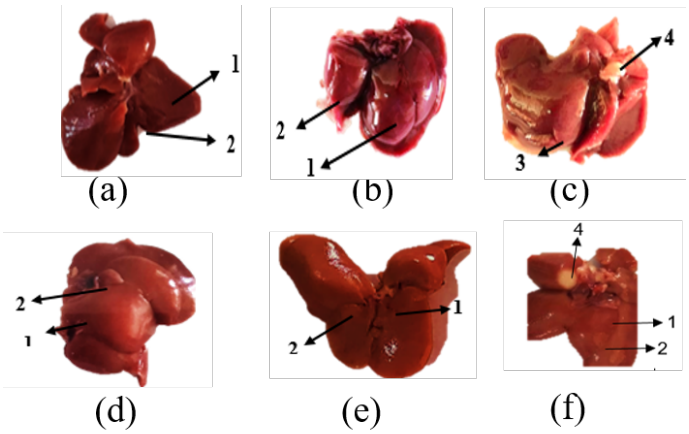

Figure 2. Macroscopic Liver (a) Normal Control (b) Positive Control (c) Negative Control (d) Test Treatment 1 (Dose 25 mg/kg BW) (e) Test Treatment II (Dose 50 mg/kg BW) and (f) Dose Treatment III (Dose 100 mg/kg BW). Note : 1= Brown-red; 2=Smooth; 3=Pale; and 4=Nodule

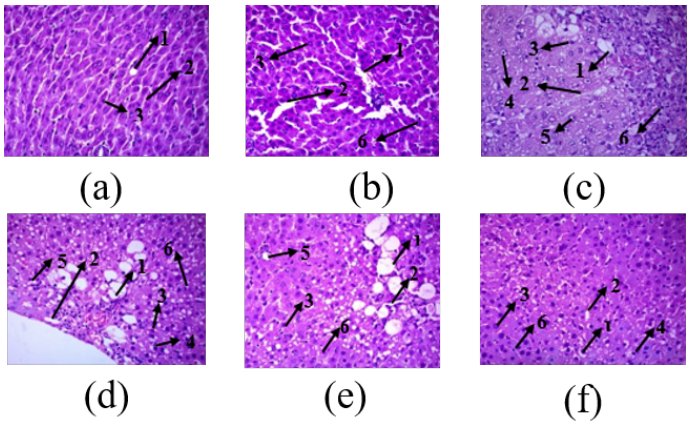

Figure 3. Macroscopic Liver. (a) Normal Control; (b) Positive Control (c) Negative Control; (d) Test Treatment 1 (Dose 25 mg/kg BW); (e) Test Treatment II (Dose 50 mg/kg BW); and (f) Dose Treatment III (Dose 100 mg/kg BW). Note : 1= Central Vein; 2=Sinusoid; 3=Normal Hepatocytes; 4=Hydropic Degeneration; 5=Fatty Degeneration; and 6=Necrosis

BW, there were nodules on the surface of the liver, but there was no change in color, and the surface of the liver remained smooth. The existence of protrusions (nodules) is suspected because there is still an inflammatory reaction from giving CCl_4 to rats. Based on this, administering the ethyl acetate fraction of beluntas leaves gives a macroscopic picture of rat liver according to normal liver criteria. So, it can ward off free radicals as an exogenous antioxidant.

The observations of negative control liver organs that were given CCl_4 showed different results to the positive control with a pale red liver appearance and a mottled liver surface. The change in the liver to a pale color is caused by the release of free fatty acids from visceral fat (Westbrook et al., 2016). Excess free fatty acid will increase the amount of free fatty acid transferred to the liver via portal vein drainage. Due to the abundance of free fatty acids in the liver, inflammatory cytokines will be released by visceral fat via the portal vein. It was caused by the administration of CCl_4, which are toxic, causing fatty liver (Khan et al., 2018).

The average liver index of rats showed an increase in liver index compared to the normal control group. Correlation statistical analysis of rat body weight and liver weight data showed a correlation coefficient of 0.817 and a significance of 0.000 ($P<0.05$). The correlation coefficient value in the Pearson correlation value ranges from 0.81 to 1, indicating a perfect correlation between rat body and liver weight. In contrast, a positive P value <0.05 indicates a relationship between body weight and rat liver weight. which is directly proportional, meaning that the heavier the rat's body weight, the greater the liver weight.

3.5 Histopathology of rat liver

Microscopic observation was carried out by observing the histop athological structure of the rat liver to see any damage to the liver tissue of the rats given CCl_4 and the ethyl acetate fraction of beluntas leaves. The result of histopathological observations of each group can be seen in Table 3 and Figure 3.

Figure 3 shows the differences in the normal control, negative control, positive control, and test treatment group. The normal control group showed a picture of hepatocytes arranged in a radial, polygonal shape with a homogeneous cytoplasm, the nucleus was in the middle, the granules were evenly distributed, and the sinusoids were arranged in an orderly manner. The positive control group showed liver damage in score 1 of necrosis, indicating mild necrosis criteria.

The results of microscopic observations on the negative control showed liver damage characterized by hydropic degeneration with a score of 2, fatty degeneration with a score of 3 (severe), and necrosis with a score of 5 (massive necrosis). This reversible hydropic degeneration arises because cells cannot maintain ion and fluid homeostasis, causing dependent ion pumps in the plasma membrane to lose their function (Yehya et al., 2019). This occurs due to a disturbance in the balance between triglyceride synthesis and VLDL secretion. Necrosis also occurs, which is marked by liver cell death. Necrosis begins with a liver inflammation reaction in the form of swelling of hepatocytes and tissue death. Necrosis is an advanced stage of degeneration because too much material must be reabsorbed by hepatocyte cells, causing cell death and rupturing the plasma membrane. This is caused if fat is deposited in large amounts resulting in the death of liver cells (Fahmi et al., 2019).

The histopathological picture in the treatment group, given the ethyl acetate fraction of beluntas leaves at doses of 25 mg/kgBW, 50 mg/kgBW, and 100 mg/kgBW showed a difference in the rate of liver improvement at each dose. Administra-

tion with low doses shows a higher damage level than with high doses. The results showed that at a dose of 100 mg/kgBW, it has the lowest level of damage in the absence of fat degeneration but still has hydropic degeneration damage with category one and necrosis category two (mild).

Histopathological results showed that administering the ethyl acetate fraction of beluntas leaves at 25 mg/kgBW and 50 mg/kgBW could not protect against liver damage. In contrast, at a dose of 100 mg/kgBW, it could only improve the parameters of fatty degeneration. Based on this, it showed that the ethyl acetate fraction of beluntas leaves at a dose of 100 mg/KgBW which has a total flavonoid of 153 mgQE/g, did not give the maximum effect as an antioxidant because the histopathological results still showed mild hydropic degeneration and mild necrosis.

4. CONCLUSION

The total flavonoid content of ethyl acetate fraction of beluntas leaves is 153 mgQE/g with a percentage of 15.3%. Ethyl acetate fraction of beluntas leaves dose 25 mg/kgBW, 50 mg/kgBW, and 100 mg/kgBW affected decreasing MDA levels and increasing catalase enzyme. The best dosage of the ethyl acetate fraction of beluntas leaves is 100 mg/kgBB because there has been a decrease in MDA levels and an increase in the catalase enzyme, which was not significantly different from the positive control group (P<0,05). The ethyl acetate fraction of beluntas leaves (*Pluchea indica L.*) at 100 mg/kg BW has provided a macroscopic picture according to normal day criteria. However, histopathology has not provided maximum improvement in the liver by induced CCl_4. Furthermore, studies are needed to isolate and identify individual flavonoid compounds to obtain better antioxidant activity.

5. ACKNOWLEDGMENT

The author gratefully acknowledged the Laboratory Departement of the Pharmacy University of Sriwijaya that has facilities for this research and the research/publication of this article was funded by DIPA of public service agency of sriwijaya university 2022. SP DIPA-023.17.2.677515/2022, On Desember 13, 2021. In accordance with the Rector's Decree Number: 0110/UN9.3.1/SK/2022, on April 28, 2022.

REFERENCES

Ayoka, T. O., B. O. Ezema, C. N. Eze, and C. O. Nnadi (2022). Antioxidants for the Prevention and Treatment of Non-communicable Diseases. *Journal of Exploratory Research in Pharmacology*, **7**(3); 178–188

Bhatti, J. S., A. Sehrawat, J. Mishra, I. S. Sidhu, U. Navik, N. Khullar, S. Kumar, G. K. Bhatti, and P. H. Reddy (2022). Oxidative Stress in the Pathophysiology of Type 2 Diabetes and Related Complications: Current Therapeutics Strategies and Future Perspectives. *Free Radical Biology and Medicine*, **184**; 114–134

Fahmi, U., I. Andriani, S. Salmah, T. H. Hatta, S. B. A. Omar, and D. K. Sari (2019). Histopathology of Liver and Intestine of Pangkilan Bare Fish (Oryzias Matanensis) Polluted by Nickel and Iron in Lake Matano, South Sulawesi. *Earth Environ*, **370**(1); 012078

Hoda, M., S. Hemaiswarya, and M. Doble (2019). Role Of Phenolic Phytochemicals In Diabetes Management. *Role of Phenolic Phytochemicals in Diabetes Management*; 123–143

Hritcu, L., R. Ionita, P. A. Postu, G. K. Gupta, H. Turkez, T. C. Lima, C. U. S. Carvalho, and D. P. de Sousa (2017). Antidepressant Flavonoids and Their Relationship with Oxidative Stress. *Oxidative Medicine and Cellular Longevity*, **2017**; 5762172

Khan, F., V. K. Garg, A. K. Singh, and T. Kumar (2018). Role of Free Radicals and Certain Antioxidants in the Management of Huntington's Disease: A Review. *Journal of Analytical and Pharmaceutical Research*, **7**; 386–392

Khoddami, A., M. A. Wilkes, and T. H. Roberts (2013). Techniques for Analysis of Plant Phenolic Compounds. *Molecules*, **18**(2); 2328–2375

Koirewoa, Y. A., F. Fatimawali, and W. Wiyono (2012). Isolasi dan Identifikasi Senyawa Flavonoid dalam Daun Beluntas (Pluchea indica L.). *Pharmacon*, **1**(1); 47–52

Mahasuari, N. P. S., N. Paramita, and A. Putra (2020). Effect of Methanol Concentration as a Solvent on Total Phenolic and Flavonoid Content of Beluntas Leaf Extract (Pulchea indica L.). *Journal of Pharmaceutical Science and Application*, **2**(2); 77

Makni, M., Y. Chtourou, E. Garoui, T. Boudawara, and H. Fetoui (2012). Carbon Tetrachloride-induced Nephrotoxicity and DNA Damage in Rats: Protective Role of Vanillin. *Human & Experimental Toxicology*, **31**(8); 844–852

Martemucci, G., C. Costagliola, M. Mariano, L. D'andrea, P. Napolitano, and A. G. D'Alessandro (2022). Free Radical Properties, Source and Targets, Antioxidant Consumption and Health. *Oxygen*, **2**(2); 48–78

Muchtaromah, B. and W. Mustikasari (2019). The Correlation of Pegagan and Beluntas Leaf Extract Co-Treatment on Liver Histological Alteration and Circulating Transaminase Enzyme Level. *IOP Conf Ser Earth and Environmental Sciences*, **276**(1); 012020

Nirwana, A. M. and J. Jamilah (2021). Length and weight of small intestine and digestion rate of quail, with the addition of beluntas leaf flour (Pluchea indica L.) to the ration. *IOP Conf. Series: Earth and Environmental Science*, **788**(1); 012073

Nopparat, J., A. Nualla Ong, and A. Phongdara (2020). Treatment with Pluchea indica (L.) Less. Leaf Ethanol Extract Alleviates Liver Injury in Multiple Low-dose Streptozotocin-induced Diabetic BALB/c Mice. *Experimental and Therapeutic Medicine*, **20**(2); 1385–1396

Parwata, A., P. Manuaba, and S. Yasa (2018). The Potency of Flavonoid Compounds in Water Extract *Gyrinops versteegii* Leaves as Natural Antioxidants Sources. *Biomedical and Pharmacology Journal*, **11**(3); 1501–1511

Patro, G., S. K. Bhattamisra, B. K. Mohanty, and H. B. Sahoo

(2016). In Vitro and in Vivo Antioxidant Evaluation and Estimation of Total Phenolic, Flavonoidal Content of Mimosa Pudica L. *Pharmacognosy Research*, **8**(1); 22

Popovic, L. M., N. R. Mitic, D. Miric, B. Bisevac, M. Miric, and B. Popovic (2015). Influence of Vitamin C Supplementation on Oxidative Stress and Neutrophil Inflammatory Response in Acute and Regular Exercise. *Oxidative Medicine and Cellular Longevity*, **2015**

Rahman, N. F., N. Nursamsiar, M. Megawati, H. Handayani, and C. A. Suares (2021). Total Phenolic and Flavonoid Contents and Antioxidant Activity of Kembang Bulan Leaves (Tithonia diversifolia (Hemsley) A. Gray). *Indonesian Journal of Pharmaceutical Science and Technology*, **1**(1); 57–65

Saraf, R. A. and J. Balamurugan (2018). The Role of Mass Media in Health Care Development: A Review Article. *Journal of Advanced Research in Journalism and Mass Communication*, **5**(1&2); 39–43

Sarma, A. D., A. R. Mallick, and A. Ghosh (2010). Free Radicals and Their Role in Different Clinical Conditions: an Overview. *International Journal of Pharma Sciences and Research*, **1**(3); 185–192

Sugiaman, V. K., N. Q. Nisyah, N. Anisa, and N. Pranata (2021). Pluchea indica Extract as a Potential Source of Nutrition for Accelerate Wound Healing. *Systematic Reviews in Pharmacy*, **12**(2); 570–573

Vo, Q. V., P. C. Nam, N. M. Thong, N. T. Trung, C. T. D. Phan, and A. Mechler (2019). Antioxidant Motifs in Flavonoids: O–H versus C–H Bond Dissociation. *ACS Omega*, **4**(5); 8935–8942

Westbrook, R. H., G. Dusheiko, and C. Williamson (2016). Pregnancy and Liver Disease. *Journal of Hepatology*, **64**(4); 933–945

Widyawati, P. S., T. D. W. Budianta, D. I. Gunawan, and R. S. Wongso (2015). Evaluation Antidiabetic Activity of Various Leaf Extracts of Pluchea Indica Less. *International Journal of Pharmacognosy and Phytochemical Research*, **7**(3); 597–603

Yehya, A. H., M. Asif, G. Kaur, L. E. Hassan, F. S. Al Suede, A. M. A. Majid, and C. E. Oon (2019). Toxicological Studies of Orthosiphon Stamineus (Misai Kucing) Standardized Ethanol Extract in Combination with Gemcitabine in Athymic Nude Mice Model. *Journal of Advanced Research*, **15**; 59–68

Zheng, Y. Z., G. Deng, and Y. C. Zhang (2022). Multiple Free Radical Scavenging Reactions of Flavonoids. *Dyes and Pigments*, **198**; 109877

