Journal of Biotechnology and Natural Science
Vol. 4, No. 1, June 2024, pp. 38-46
E-ISSN 2808-2508

The Effect of *Calina Papaya* Leaves Ethanol Extract on the Red Blood Cell Profile of Wistar Rats Exposed to Cigarette Smoke

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ARTICLE INFO

Article history
Received: February 15th 2024
Revised: May 22nd 2024
Accepted: June 20th 2024

Keywords
Antioxidants
Cigarette smoke
Calina Papaya leaf
Red blood profile
Wistar rats

ABSTRACT

Cigarette smoke is a source of exogenous free radicals that can affect the blood. Calina Papaya leaves contain secondary metabolites that act as antioxidants. The purpose of the study was to analyze the antioxidant activity of ethanol extract of Calina Papaya leaves using DPPH method and to analyze the difference in red blood profile of wistar rats exposed to cigarette smoke after administration of Calina Papaya Leaves Ethanol Extract (CPLEE). The research method used 24 wistar rats with oral administration of CPLEE for 23 days and exposure to cigarette smoke for 15 days. There are 4 treatments, namely rats not exposed to cigarette smoke and given distilled water (K), rats exposed to cigarette smoke (KN), rats given CPLEE 100 mg/Kg BW then exposed to cigarette smoke (P1) and rats given CPLEE 200 mg/Kg BW then exposed to cigarette smoke (P2). Parameters observed were red blood profile (erythrocyte number, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW). Blood was collected through the orbital sinus and analyzed using a hematology analyzer. Data were analyzed using one way ANOVA and Duncan Multiple Range Test (P<0.05). The results showed the IC50 value of the extract was 1,194.1 ppm while ascorbic acid was 22.92 ppm. The number of erythrocytes, MCV, MCH and RDW showed significant differences between groups with the highest treatment in P1. The conclusion shows that the antioxidant activity of CPLEE is very weak compared to ascorbic acid but the red blood profile (erythrocytes, MCV, MCH, MCHC, RDW) in treatment P1 is higher than other treatments.

How to Cite: Ariesti, W. & Setiawan, H. (2024). The Effect of Calina Papaya Leaves Ethanol Extract on the Red Blood Cell Profile of Wistar Rats Exposed to Cigarette Smoke. Journal of Biotechnology and Natural Science, 4(1):38-46.

1. Introduction

Smoking is the cause of chronic diseases that can cause death, making it a major problem for public health (Prihatiningsih et al., 2020). According to the Global Adult Tobacco Survey (2021), the number of smokers in Indonesia in 2011 reached 60.3 million and increased in 2021 to 69.1 million. Smoking causes 225,700 people in Indonesia to die every year (WHO, 2020). Cigarette smoke is a source of exogenous free radicals with many chemical compounds such as tar, nicotine, carbon monoxide, phenol, carbon dioxide, ammonia, lead, formaldehyde, hydrogen cyanide and nitrosamines that can harm health. These chemicals can trigger the formation of free radicals that affect blood components such as hematocrit, hemoglobin, erythrocytes, platelets and others (Arif et al., 2013). Tar in cigarette smoke can damage bone marrow (where erythrocytes are produced), nicotine can narrow blood vessels so that blood pressure and viscosity increase, carbon monoxide can cause tissue hypoxia and death if levels in the air are very high (Kiriweno et al., 2021). The negative impact of free radicals in the body can be inhibited using antioxidants. Although able to produce its own antioxidants, the body tends to rely on exogenous antioxidants (external antioxidants) (Ganesha et al., 2020). Calina Papaya is a new papaya variety developed by researchers from the Bogor Agricultural Institute that is widely cultivated in Indonesia (Usmayani et al., 2015).

Papaya leaves contain secondary metabolite compounds that are thought to act as antioxidants such as flavonoids, alkaloids, steroids, saponins and tannins (Febrianti et al., 2016). Secondary metabolites that have the highest percentage in Calina Papaya leaves are flavonoids of 17.4633 mg/g or 1.7463% (Bangun et al., 2021). Wijayanti et al. (2016) stated that giving binahong leaf extract as much as 90 mg/body weight can increase the number of erythrocytes compared to the control treatment. The flavonoid content in binahong leaf extract acts as an antioxidant that can maintain and maintain the integrity of the erythrocyte membrane. The flavonoid group in Calina Papaya leaves that has the highest antioxidant activity is kaempferol 3-(2G-rhamnosylrutinoside) which works by helping the regeneration process of reduced glutathione (GSH) which is an important endogenous antioxidant in erythrocytes. GSH protects the erythrocyte membrane by removing reactive oxygen compounds and preventing oxidative stress (Nugroho et al., 2017). Therefore, it is important to conduct this research to determine the effectiveness of ethanol extract of Calina Papaya leaves (*Carica papaya* L.) on the red blood profile of rats (*Rattus norvegicus* Berkenhout, 1769) wistar strain exposed to cigarette smoke.

2. Methods

2.1 Location

The research was conducted from December 2022 to June 2023 at the Laboratory of Animal Structure and Physiology, Faculty of Applied Science and Technology, Universitas Ahmad Dahlan Yogyakarta. This research was also conducted based on procedures approved by the UAD Ethics Committee with number 012212200. Calina Papaya (*Carica papaya* L.) leaves were obtained from the Calina Papaya plantation in East Imogiri, Bantul, Yogyakarta.

2.2 Tools and Materials

2.2.1 Tools

The tools used in this study are Agrowindo brand oven, Ohauss brand digital scales, Ohauss brand pocket scales, Kern brand analytical scales, Philips brand blender, 50 mL and 100 mL measuring cups, Iwaki Pyrex brand, container vessels, test tube Iwaki Pyrex brand, erlenmeyer size 100 and 250 mL Iwaki Pyrex brand, drop pipette, 1 mL measuring pipette Iwaki Pyrex brand, rat cage, rat food and drink, smoking chamber, smoking pump Aquaman brand, hematocrit tube Marienfeld brand, Vaculab brand EDTA tube, Heidolph brand rotary evaporator, 1 mL oral sonde, 4 L dark glass bottle, Memmert brand waterbath, Thermoscientific brand spectrophotometer, distillation apparatus, Iwaki Pyrex brand glass funnel, 60 mesh sifter, Dragon Med brand micropipette, 22 1 mL syringe OneMed brand, 15 mL conical tube OneMed brand, stirring rod, Hematology analyzer Sysmex KX-21, hot plate, cooling box, cooling gel, killing bottle, micropipette tip, tube rack, green propipette, spatula, lighter and knife.

2.2.1 Materials

The materials used in the study were male wistar rats aged ± 3 months with a body weight of 150-200 g, Calina Papaya leaves 7kg, distilled water, 96% ethanol, 10% ether, 70% alcohol, Dji SamSoe brand clove cigarettes, RatBio brand rat feed, rat drink, filter paper, masks, Sensi brand gloves, aluminum foil, labels and tissues.

2.3 Preparation of Ethanol Extract of Calina Papaya Leaves

This study used the third Calina Papaya leaves from the shoots characterized by light green color and no holes. Metabolic activity in young papaya leaves (leaf position 1-4 strands from the shoot) is higher than old papaya leaves which correlates with increased flavonoid and alkaloid levels (Nilna et al., 2021). A total of 7 kg of Calina Papaya leaves were washed and dried using an oven at 40℃, then mashed using a blender. Simplisia was macerated using 96% ethanol and then filtered. The resulting extract was then evaporated using a rotary evaporator (speed 100 rpm) to evaporate the solvent in the extract. After that, the extract was waterbath at 45℃ until a blackish green semi-solid thick extract was obtained.

2.4 Antioxidant Activity Test with 1,1-diphenyl-2-pycrilhydrazil (DPPH)

Calina Papaya leaf extract was weighed as much as 1 mg then added 1 ml of methanol (as a stock solution with a concentration of 1000 µg/ml of each extract), then made a concentration series of 0 µg/ml, 250 µg/ml, 500 µg/ml and 1000 µg/ml. Stock solution of each extract was taken 0.05 ml and added 1 ml of DPPH solution in methanol (20 µg/ml) and 0.45 ml of 50 mM Tris-HCl Buffer (pH 7.5). The mixture was allowed to stand in a dark place at room temperature for 30 minutes then the absorbance of the mixture was measured using a spectrophotometer with a wavelength of 517 nm. DPPH radical inhibition activity was calculated in % as follows:

$$\% = (1 - A_{sample}/A_{blanko}) \times 100$$

Notes:
A sample (absorbance value of the sample)
A blanko (blank absorbance value)

2.5 Test Animal Treatments

The study used 24 male wistar rats (± 3 months old, weighing 150-200 g) which were divided into 4 treatment groups consisting of 6 individu as replicates and acclimatized for 5-7 days. The treatment consisted of control group (K) not exposed to cigarette smoke and given distilled water, negative control group (KN) given exposure to cigarette smoke, Group P1 and P2 were given ethanol extract of Calina Papaya leaves at doses of 100 mg/Kg BW and 200 mg/Kg BW respectively using a 1 ml oral sonde for 23 days and then exposed to cigarette smoke in the amount of 3 cigarettes per day for 15 days (day 9 to day 23).

The process of exposure to cigarette smoke in mice is done by first inserting them in the smoking chamber. The cigarette is attached to the coupler which is connected to the smoking pump then the cigarette is burned and the smoking pump is turned on. If the smoking chamber has been filled with a lot of smoke, then the electric current in the smoking pump is stopped. After the smoke in the smoking chamber decreases, the smoking pump is turned on again. This can be repeated 2-3x in the exposure of one cigarette. Combustion residue attached to the coupler, must be cleaned by taking and disposing.

2.6 Observation of Red Blood Profile

Rats were anesthetized using 10% ether on the 24th day then 2 ml of blood was taken from the orbital sinus using a hematocrit tube and inserted into an EDTA tube so that the blood would not coagulate and then placed in a cooling box to avoid damage to blood samples. Red blood profile analysis was performed using the Hematology Analyzer.

2.7 Data Analysis

All observed data were then analyzed using the one-way ANOVA test ($P<0.05$) and continued with the Duncan Multiple Range Test to see differences between treatments.

3. Results and Discussion

3.1 Antioxidant Activity of Ethanol Extract of Calina Papaya Leaves

Antioxidant activity testing on ethanol extract of Calina Papaya leaves using the 1,1-diphenyl-2-pycrilhydrazil (DPPH) method. The method uses the IC_{50} value to determination of the concentration of antioxidants that provide silencing of DPPH by 50% (Purwanti et al., 2019). Wahdaningsih (2022) states that a substance is classified as having very high antioxidant effectiveness if the IC_{50} value <50 ppm, considered strong if the IC_{50} value is in the range of 50-100 ppm, moderate if the IC_{50} is in the range of 101-150 ppm, weak if the IC_{50} is in the range of 151-200 ppm and classified as very weak if the IC_{50} is >200 ppm. If the IC_{50} value obtained is >200 ppm, the compound is considered less effective although it still has the possibility as an antioxidant.

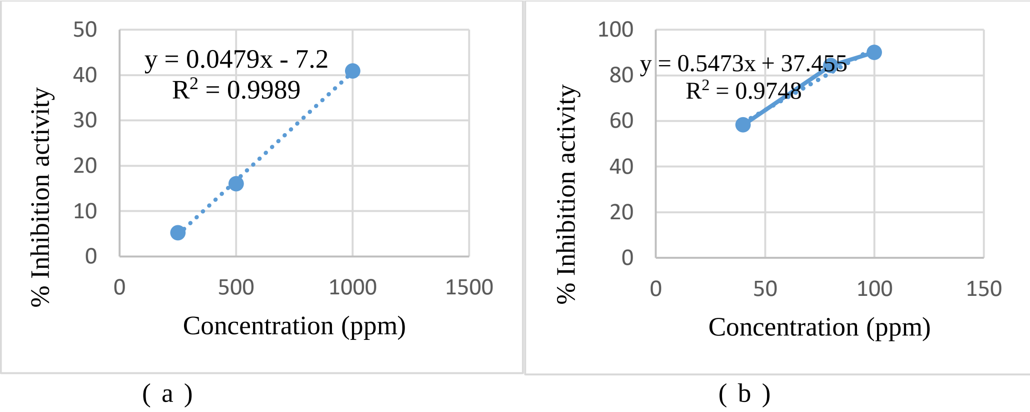

Figure 1. Comparison of % inhibition activity of ethanol extract of Calina Papaya leaves with ascorbic acid. Notes: a (% inhibition activity of ethanol extract of Calina Papaya leaves), b (inhibition activity of ascorbic acid), and ppm (parts per million).

After obtaining the linear regression equation of each sample shown in Figure 1, calculations can be made to obtain IC_{50}. Based on the antioxidant activity test results, the IC_{50} values of Calina Papaya leaf extract and ascorbic acid samples were 1,194.1 ppm and 22.92 ppm, respectively. The smaller the IC_{50} value, the higher the antioxidant activity. Therefore, it can be concluded that the antioxidant activity of ethanol extract of Calina Papaya leaves is very weak, while the antioxidant activity of ascorbic acid is very strong. Although the substances contained in the ethanol extract of Calina Papaya leaves are less active, they have the potential as antioxidants. This could be due to the high bioactivity of antioxidant compounds in papaya leaves allowing them to be effectively absorbed and utilized by the body even in low concentrations (Okoko & Diepreye, 2012).

Ascorbic acid has the ability as a very strong antioxidant because it is a single compound that generally reacts more easily with DPPH than a mixture of compounds. Single compounds have an advantage in reactivity because they are more focused and consistent in providing electrons or hydrogen to DPPH. Ascorbic acid donates electrons to free radicals by forming dehydroascorbic acid so that it can protect cells from oxidative damage (Afriani et al., 2014).

The antioxidant activity of ethanol extract of Calina Papaya leaves is classified as very weak may be due to the polarity of the solvent used in this case, ethanol. Methanol has a higher polarity than ethanol so it has a better ability to attract polar compounds including several types of antioxidant compounds. This is due to differences in chemical structure and intermolecular interactions in solvents (Widyawati et al., 2014). This is supported by Muaja et al. (2017) that the antioxidant activity of methanol extract of soyogik leaves is stronger than ethanol extract of soyogik leaves with IC_{50} values of 0.16 ppm and 38.01 ppm, respectively.

The content of secondary metabolites in papaya leaves that are thought to act as antioxidants such as flavonoids, saponins, steroids, tannins and saponins (Febrianti et al., 2016). Secondary metabolites with high concentrations in the ethanol extract of Calina Papaya leaves is flavonoids amounting to 17.4633 mg/g or 1.7463% (Bangun et al., 2021). Research conducted by Nugroho et al. (2017) showed that there are 7 types of flavonoids found in papaya leaves, namely quercetin 3-(2G-rhamnosyl rutinoside), kaempferol 3-(2G-rhamnosyl rutinoside), quercetin 3-rutinoside, myricetin 3-rhamnoside, kaempferol 3-rutinoside, quercetin and kaempferol. Quantitative analysis of High-Performance Liquid Chromatography (HPLC) showed that kaempferol 3-(2Grhamnosyl rutinoside) had higher levels when compared to other flavonoids, which amounted to 7.23 mg/g. Another study mentioned that the compound the main flavonoids in papaya leaves are quercetin and kaempferol (Hidayati et al., 2020). The way flavonoids work as antioxidants can occur directly or indirectly. Flavonoids act directly through the mechanism of providing hydrogen ions so that they can neutralize the toxic effects of free radicals. Flavonoids act indirectly by increasing the expression of natural antioxidant genes through several mechanisms, such as the activation of nuclear factor erythroid 2 relates factor 2 (Nrf2). Consequently, there is an increase in genes involved in the production of endogenous antioxidant enzymes such as the Superoxide Dismutase (SOD) gene (Kusuma, 2015).

3.2 Red Blood Profile

Blood profile examination is very important to help monitor the presence of disorders and diseases in animals (Mayulu et al., 2012). The results of observations of red blood profile of Wistar rats include the number of erythrocytes, hemoglobin, hematocrit, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Meancorpuscular Hemoglobin Concentration (MCHC) and Red Cell Distribution width (RDW) (Table 1).

Table 1. Red blood profile of Wistar rats after administration of ethanol extract of Calina Papaya leaves

Parameter	K	KN	P1	P2
Erythrocytes ($\times 10^6/\mu L$)	9,25±0,31[b]	8,35±0,70[a]	9,14±0,51[b]	8,80±0,26[ab]
Hemoglobin (g/dL)	15,06±0,57[a]	14,30±1,24[a]	15,08±0,87[a]	14,94±0,66[a]
Hematocrit (%)	47,74±1,59[a]	45,02±3,56[a]	47,82±2,30[a]	47,30±1,96[a]
MCV (fL)	51,62±0,47[a]	53,92±0,77[b]	52,36±1,03[a]	53,74±1,18[b]
MCH (pg)	16,30±0,25[a]	17,12±0,39[c]	16,52±0,56[ab]	16,98±0,41[bc]
MCHC (g/dL)	31,54±0,32[a]	31,74±0,44[a]	31,50±0,50[a]	31,60±0,37[a]
RDW (fL)	28,36±0,66[a]	31,58±2,09[b]	28,94±1,09[a]	28,88±0,30[a]

Notes : K (rats not exposed to cigarette smoke and given distilled water), KN (rats exposed to cigarette smoke), P1 (rats given 100 mg/Kg BW dose of ethanol extract of Calina Papaya leaves then exposed to cigarette smoke), P2 (rats given 200 mg/Kg BW dose of ethanol extract of Calina Papaya leaves then exposed to cigarette smoke), MCV (Mean Corpuscular Volume), MCH (Mean Corpuscular Hemoglobin), MCHC (Mean Corpuscular Hemoglobin Concentration), RDW (Red Cell Distribution Width). [a-c] Differences in numbers followed by different letters in the same row indicate significant differences. Mean ± SD.

Based on one-way ANOVA test, the number of erythrocytes showed a significant difference between treatments ($P<0.05$). Test result Duncan test results showed the number of erythrocytes in the K and P1 treatments was higher than the other treatments. This proves that administration of ethanol extract of Calina Papaya leaves at a dose of 100 mg/Kg BW has a higher the number of erythrocytes is higher than other treatments. One way ANOVA test on MCV showed there was a significant difference between treatments ($P<0.05$). The results of Duncan's test showed that MCV in the KN and P2 treatments were higher than the other treatments. This shows that MCV levels in Wistar rats after exposure to cigarette smoke and the administration of ethanol extract of Calina Papaya leaves at a dose of 200 mg/Kg BW are higher than other treatments. One way ANOVA test on MCH showed there was a significant difference between treatments ($P<0.05$). Duncan's test results showed that MCH in the K and P1 treatments was lower than the other treatments. This indicates that MCH levels after administration of ethanol extract of Calina Papaya leaves at a dose of 100 mg/Kg BW are lower than other treatments. One way ANOVA test on RDW showed that there was a significant difference between treatments ($P<0.05$). Duncan's test results showed that RDW in treatments K, P1 and P2 were lower than KN. This shows that RDW after the administration of ethanol extract of Calina Papaya leaves in doses of 100 mg/Kg BW and 200 mg/Kg BW is lower than other treatments.

One way ANOVA test on hemoglobin, hematocrit and MCHC showed no significant difference between treatments (P<0.05). Duncan's test results showed no significant differences between treatments. This shows that the administration of ethanol extract of Calina Papaya leaves at doses of 100 mg/Kg BW and 200 mg/Kg BW did not have a significant impact on hemoglobin, hematocrit and MCHC levels.

Cigarette smoke has more than 4.000 chemical compounds including carbon monoxide, nicotine and other harmful compounds. Oxidant molecules in cigarette smoke can enter the bloodstream to produce Reactive Oxygen Species (ROS) through interaction with the enzyme Nicotinamide Adenine Dinucleotide Phosphate (NADPH). The increase in ROS causes oxidative stress because antioxidants and antioxidants in the body are not balanced (Ganesha et al., 2020). Oxidative stress causes lipid peroxidation which can damage and reduce erythrocyte function (Malenica et al., 2017). This is in accordance with the results of the study that the number of erythrocytes, hemoglobin and hematocrit in KN was lower than the other treatments.

The low number of erythrocytes, hemoglobin and hematocrit in KN is thought to be due to exposure to carbon monoxide in cigarette smoke (Inayatillah, 2014). Carbon monoxide has a higher affinity for hemoglobin than oxygen which results in oxidative stress so that the number of erythrocytes decreases due to hemolysis of erythrocytes and hemoglobin is liberated to the plasma then cannot perform its function properly so that there is a decrease in hemoglobin levels (Kiriweno et al., 2021). Cigarette smoke exposure also affects hematocrit levels so that hematocrit levels in KN are lower than other treatments. This is in accordance with the research of Etim et al. (2013) that the decrease in hematocrit (PCV) is due to the low number of erythrocytes so that blood viscosity is low.

The decrease in the number of erythrocytes, hemoglobin and hematocrit in P2 could be caused by too high levels of carpain that can cause hemolysis. Hemolysis occurs when the erythrocyte membrane is disrupted or damaged resulting in the release of hemoglobin and a decrease in the number of erythrocytes. Carpain can affect the structure and stability of the erythrocyte membrane by disrupting lipid and protein components in the membrane, causing membrane damage (Barron & Ibrahim, 2011).

The number of erythrocytes, hemoglobin and hematocrit in P1 is equivalent to K, which indicates that the administration of ethanol extract of Calina Papaya leaves at a dose of 100mg/Kg BW is able to increase the number of erythrocytes, hemoglobin and hematocrit after exposure to cigarette smoke. This is thought to be because flavonoid compounds in the ethanol extract of Calina Papaya leaves can increase the process of erythrocyte formation (erythropoiesis) in the spinal cord by preventing the oxyHb molecule from being oxidized to metHb so that hemoglobin continues to carry out its function of binding oxygen because it is in the form of oxyHb (Restuti et al., 2020). This is reinforced by Maidah & Hariani (2022) that flavonoid content acts as an antioxidant that can maintain and maintain the integrity of the erythrocyte membrane through an electron or hydrogen donor mechanism so as to stop the chain reaction of free radicals that damage the membrane. Nugroho et al. (2017) stated that the flavonoid group of papaya leaves that has higher antioxidant activity than other groups is kaempferol 3-(2G-rhamnosylrutinoside) which works by helping the regeneration process of reduced glutathione (GSH) to protect the erythrocyte membrane by removing compounds of reduced glutathione (GSH). Erythrocyte membrane by removing reactive oxygen compounds and preventing oxidative stress. Okoko & Diepreye (2012) stated that quercetin content in papaya leaves can activate the transcription factor nuclear factorerythroid 2-related factor 2 (Nrf2) resulting in an increase in superoxide dismutase (SOD) gene expression which in turn stimulates the endogenous antioxidant system in the body and protects blood cells from oxidative damage. Barzegar (2016) stated that myricetin content in papaya leaves can stop oxidation chain reactions by donating electrons from myricetin hydroxyl groups to free radicals or reactive oxygen species (ROS) so as to protect cells from oxidative damage.

In addition to containing secondary metabolite compounds, papaya leaves also contain protein (5-10%), fiber (11-13%), carbohydrates (72-78%), calcium (267-366 mg/100g), magnesium (29-37 mg/100g), iron (5.9-6.3 mg/100mg) and potassium (Nwofia et al., 2012). Iron in papaya leaves

plays a role in the formation of hemoglobin (Sashmita et al., 2020). Hemoglobin binds iron that is absorbed through intestinal mucosal cells and then bound by apoferritin into ferritin (Fe + apoferitin). This bond is broken in the blood serum and ferrous iron is transported in the form of transferrin (Fe bonds with proteins containing 3-4 mg Fe) which is stored in the bone marrow, liver and lymphatic system. Iron is also used to synthesize hemoglobin and the breakdown of erythrocytes (Kiriweno et al., 2021). Calcium in papaya leaves also acts to maintain red blood cell membrane potential through interactions with sensor proteins such as calmodulin which then interacts with other proteins to regulate membrane activity and function (Oktavia et al., 2019).
Observation of erythrocytes or erythrocyte index can be seen from MCV, MCH, MCHC and RDW (Malenica et al., 2017). The data in Table 1 shows that the MCV value in KN is higher than the other treatments, indicating a macrocytic picture, namely the average size of large erythrocytes. This condition can occur due to damage to the erythrocyte membrane which causes the cells to become larger than normal. This is because the chemical compounds in cigarette smoke cause damage to the erythrocyte membrane (Ardina and Vira, 2018). Increased MCH can be caused by anemiamegaloblastic (vitamin B12 deficiency). Lack of this vitamin can cause disruption of DNA formation which causes the erythrocytes to become larger. Mean corpuscular hemoglobin concentration (MCHC) is the average concentration of hemoglobin in each erythrocyte (Rosidah, 2020). The highest MCHC level was found in the KN group at 31.74 ± 0.44 g/dL and the lowest in the P1 group at 31.50 ± 0.50 g/dL.
Red cell distribution width (RDW) is the range of variation in erythrocyte size in a blood sample. The highest RDW was found in KN at 31.58 ± 2.09 fL. The higher the value of RDW, the more varied the size of red blood cells in the blood sample. This can occur in various health conditions such as anemia, oxidative stress, impaired red blood cell production and dehydration (Malenica et al., 2017). Another theory states that the increase in RDW is due to the mechanism of tissue hypoxia which causes a surge in erythropoietin production by the kidneys so that the speed of erythrocyte production and the diversity of erythrocyte size increase (Putri et al., 2021).
Based on the description above, the best treatment group is P1 because it shows results equivalent to K in all parameters (erythrocytes, hemoglobin, hematocrit, MCV, MCH, MCHC, RDW). There is a decrease in P2 presumably because the high dose is less effective in acting as an antioxidant in the red blood profile of Wistar rats exposed to cigarette smoke. Therefore, it can be said that the administration of ethanol extract of Calina Papaya leaves at a dose of 100mg/Kg BW can improve the red blood profile of Wistar rats exposed to cigarette smoke.

4. Conclusion

The conclusion of the study shows that the antioxidant activity of ethanol extract of Calina Papaya leaves (*Carica papaya* L.) is classified as very weak and there are differences in the red blood profile (erythrocytes, MCV, MCH, MCHC, RDW) of rats (*Rattus norvegicus* Berkenhout, 1769) wistar strain exposed to cigarette smoke after giving ethanol extract of Calina Papaya leaves (*Carica papaya* L.) with the highest treatment at a dose of 100 mg/Kg BW (P1) ($P<0,05$). While hemoglobin, hematocrit and MCHC levels did not show significant differences between treatments ($P>0.05$).

ACKNOWLEDGMENT

This research was funded by the Research and Community Service Institute (LPPM) of Universitas Ahmad Dahlan 2023. There is no conflict of interest whatsoever in this research.

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