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CARDIO PROTECTIVE EFFECT OF ETHANOLIC EXTRACT VERNONIA AMYGDALINA DELILE ON RATS INDUCED L-NAME

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Abstract

This study aimed to analyse the effects of ethanol extract of African leaves (Vernonia Amygdalina Del.) on the levels plasma renin, IL-6 and cardiac histopathology in L-NAME-induced rats. This research stage includes the preparation of ethanol extract of African leaves, standardization of extracts and testing the effectiveness of African leaves on rat heart damage. Data were analysed using one-way ANOVA with $p<0.05$. testing the cardioprotective effectiveness of ethanol extract of African leaves in hypertension model rats and then examining heart histopathology using HE staining and analysing parameters, namely plasma renin and IL-6 levels. This study used 6 groups, each group consisted of 5 male rats, namely the normal group, negative control by administering L-NAME 40 mg/kgBB, positive control by administering L-NAME plus lisinopril 2.5 mg/kgBB, EEDA 100 mg/kgBB, EEDA 300 mg/kgBB and EEDA 500 mg/kgBB. L-NAME responds to heart damage such as degeneration and necrosis of heart cells. In the negative group, plasma renin levels were 40.60 ± 9.98 ng/mL while the EEDA group with a dose of 500 mg/kgBB experienced the highest decrease in plasma renin compared to other EEDA groups and positive controls, namely 13.80 ± 1.30 ng/mL. The positive control group obtained plasma renin levels of 14.20 ±1.79 ng/mL. In the negative group, IL-6 levels were 4.80 ± 1.30 pg/mL while the group given EEDA, the group with a dose of 500 mg/kgBB experienced a decrease in IL-6, namely 1.52 ±0.96 ng/mL. The positive control group experienced a decrease in IL-6 levels of 0.77 ± 0.20 ng/mL. The result can be concluded that EEDA reduces plasma renin, IL-6 levels, improves the condition of cardiac cell degeneration and necrosis.

Keywords**:** *Antioxidant, EEDA, Hypertension, Heart, Cardioprotective, L-NAME*

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INTRODUCTION

Hypertension is defined as high blood pressure with a systolic pressure of more than equal to 140 mmHg and a diastolic pressure of more than equal to 90 mmHg on two measurements with an interval of five minutes in a state of sufficient rest or calm. Hypertension can increase the risk of ischemic heart disease, stroke, other cardiovascular diseases, chronic kidney disease, and dementia (Zhou et al., 2021). In 2019, based on data from the World Health Organization (WHO), there were 1.13 billion people worldwide suffering from hypertension. Adults with hypertension are reported to be 46% less aware of their condition (Riaz et al., 2023). Based on WHO predictions, in 2025 adults in the world will suffer from hypertension around 29% of the total world population. Hypertension is still one of the highest causes of morbidity worldwide (Hsu and Tain, 2020). The main goal of hypertension therapy is to achieve and maintain target blood pressure. Apart from using medication, lifestyle modifications are carried out, namely reducing weight, adjusting diet, and limiting salt consumption and physical activity (Muhadi, 2016). Vernonia amygdalina Delile. has many pharmacological effects including antidiabetic, nephroprotective, antioxidant, antihyperlipidemic, immunological, anthelmintic, anti-obesity, hepatoprotective, and anti-inflammatory effects. Vernonia amygdalina Delile. is a plant that is often found in Africa and also in tropical areas such as Indonesia. Based on previous research, it was reported that the ethanol extract of Vernonia amygdalina is also known to have higher inotropy than digoxin in rat hearts. African leaves contain compounds such as saponins, flavonoids, sesquiterpene lactones, and steroid glycosides. Flavonoids are essential compounds that are found in many plants, including African leaves, and have many pharmacological activities that have been tested in vitro, in vivo, and clinically in humans. Its pharmacological activities include inhibition of nuclear factor kappa-b (Nf-Kb), antioxidant, antiplatelet, antithrombotic, and ACEI (Syahputra et al., 2020).

Inhibition of Renin-Angiotensin-Aldosterone System (RAAS) activation helps lower blood pressure and reduces blood pressure fluctuations, thereby reducing the risk of cardiovascular events (Liu et al., 2020). Increased levels of pro-inflammatory serum cytokines such as Interleukin 6 (IL-6) in hypertensive patients affect increasing blood pressure values. Increased levels of pro-inflammatory serum cytokines such as Interleukin 6 (IL-6) in hypertensive patients affect increasing blood pressure values. In addition, IL-6 determines the hypertensive response to angiotensin II (Tanase et al., 2019). The overall effects of L-NAME are endothelial dysfunction, remodeling, and hypertrophy culminating in hypertension.

Based on the description above, researchers are interested in analyzing the effectiveness and effect of administering ethanol extract of African leaves (Vernonia amygdalina Delile) on levels of plasma renin, IL-6, and cardiac histopathological features in rats induced by L-NAME.

METHOD

Materials and Methods Materials

The materials used were Vernonia amygdalina Delile were collected from the Faculty of Pharmacy, Universitas Sumatera Utara, Indonesia (coordinates 3°33'36.5"N, 98°39'12.5"E) and identified under Medanese herbarium (MEDA), Laboratoirum Taxonomy of Plants, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Sumatera Utara, Medan (779/MEDA/2023), Buffer formalin, CMC Na (Sigma), Ethanol (BrataChem), L-NAME (Merck), Lisinopril 10 mg, Methanol (BrataChem), NaCl, Water pro- injection (Sigma Aldrich), Hematoxylin and Eosin (Sigma Aldrich). The tools used were glassware, hot plate (Fisons), desiccator, cage, label paper, pH paper, filter paper, Whatman No.42 filter paper, analytical balance (Boeco), light microscope (Boecp), infusion pot, plastic pot, oral probe, spatula, set of surgical tools, thermometer, scales, ELISA kits of Renin and IL-6 were purchased from Abclonal (China). The animals used were male Wistar rats aged 3-4 months (weight of 150-250 g) obtained from the Faculty of Pharmacy, Universitas Sumatera Utara.

Before the experiment began, the Wistar rats were kept for two weeks in a good cage to adapt to their environment and approved by the Animal Research Ethics Committees (AREC) Universitas Sumatera Utara with approval number 0437/KEPH-FMIPA/2024.

Extract Preparation

Vernonia amygdalina was collected in May 2022. The plant was identified at Herbarium Medanese, Universitas Sumatera Utara. The leaves was dried at room temperature and made into powder with a motor-driven grinder. The powder (1300 g) was macerated at room temperature (about 27°C) in 4 L of ethanol for 72 h and filtered. The filtrate was concentrated in a rotary evaporator at 70°C under reduced pressure and 38 g of a green residue was obtained.

Identification of Alkaloid Compounds

Weighed 0.5 g of powder, added 1 ml of 2N hydrochloric acid and 9 ml of water, heated over a water bath for 2 minutes, cooled, and then filtered. Take 3 drops of the filtrate, then add 2 drops of Mayer's reagent to produce a white or yellowish-white precipitate. Take 3 drops of the filtrate, then add 2 drops of Dragendorff reagent to produce an orange-red precipitate. Take 3 drops of the filtrate, then add 2 drops of Bouchard reagent to produce a brown-to-blackish precipitate (Izzazi dkk., 2020).

Identification of Flavonoid Compounds

10 g of extract sample was dissolved in 95% ethanol into the solution then 0.1 g of magnesium powder and 10.0 ml of concentrated HCl were added. The presence of flavonoids is indicated by the appearance of a yellow, purple or pink color in the solution (De Silva et al., 2017).

Identification of Saponin Compounds

0.5 g of sample was put into a test tube, then added 5 mL of water and 1 drop of HCl then shaken for 20 seconds and observed the changes that occurred. If foam forms that do not disappear for 20 minutes, this indicates the presence of saponin in the sample (Kurang & Adang, 2018).

Identification of Steroid Compounds

Extract samples were dissolved in 2 mL of chloroform. A few drops of anhydrous acetic acid and concentrated sulfuric acid were added to the solution. The presence of steroids is indicated by the appearance of a pink, red or green-blue color in the solution (De Silva et al., 2017).

Identification of Glycoside Compounds

3g of sample was put into a test tube, then 2N hydrochloric acid was added, then 25 ml of 0.4 M lead (II) acetate was added, shaken, and left for 5 minutes, then 2 ml of concentrated sulfuric acid was added through the tube wall, then 5 drops of Molisch reagent. A purple ring will form indicating the presence of sugar.

Testing of Cardioprotective Effects of African Leaves Treatment Design

Rats were divided into six groups, each consisting of 5 male rats: 1 (normal group), Group 2 (negative group) rats induced with L-NAME at a dose of 40mg/kgBW from days 1 to 30 orally, Group 3 (positive) rats were induced with L-NAME at a dose of 40 mg/kg BW from days 1 to 30 then given lisinopril at a dose of 2.5 mg/kg BW orally from days 31 to 60, Group 4 namely rats were induced with L-NAME at a dose of 40 mg/kg BW from day 1 to 30 then given EEDA dose of 100 mg/kg BW from day 31 to 60 orally, Group 5, namely rats induced by L-NAME at a dose of 40 mg/kg BW from day 1 to 30 then given EEDA dose of 300mg/kg BW from day 2 31 to 60, Group 6, namely rats were induced with L-NAME at a dose of 40 mg/kgBW from days 1 to 30 and then given EEDA at a dose of 500 mg/kg BW from days 31 to 60. On day 31, rats given L- NAME (negative control) were sacrificed, then on day 61 rats treated with EEDA and positive control were sacrificed. Next, blood samples were taken from the hearts of rats to measure levels of plasma Renin and IL-6, using a microplate reader at a wavelength of 450 nm. Rat hearts were taken and cleaned of fat and then prepared for histopathology using H and E staining.

Enzyme-Linked Immunosorbent Assay (ELISA) Method

Measurement of plasma renin levels and measurement of interleukin-6 levels were carried out using the ELISA method, namely by preparing the material, washing the plate with wash buffer. 2 times, 100 µl of standard, sample and zero control were added into each well, closed and incubated for 90 minutes at 37 oC. Wash the plate with wash buffer 2 times, add 100 µl of biotin solution into each well and incubate for 60 minutes at 37 oC. Wash the plate with wash buffer 3 times then add 100 µl of SABC solution into the well and incubate for 30 minutes at 37 oC protected from light. Wash the plate using wash buffer 5 times, add 90 µl of TMB substrate to each well and incubate for 15-30 minutes at 37 oC, protected from light. Observe the color change (some of the solution in the well will change to blue according to the concentration). Add 50 µl of stop solution to each well and observe the color change which appears yellow. The absorbance was read with a microplate reader at a wavelength of 450 nm and the levels were calculated.

Heart Histopathological Examination using Hematoxylin and Eosin (HE) Staining Method Preparation of Paraffin Blocks

The steps for making paraffin blocks are that the heart sample is soaked in 10% formalin buffer solution then a dehydration process is carried out with graded alcohol, starting with 70% alcohol, then successively 80% alcohol, 95% alcohol, and absolute alcohol. Each process takes 30 minutes to 1 hour.

The next stage is washing using xylol solution, namely xylol 1, xylol 2, and xylol 3 each for 1-2 hours. The planting process involves immersing the sample in a mixture of xylol and liquid paraffin at a temperature of 60-70oC, with a xylol:paraffin ratio of 3:1, 1:1, and 1:3 respectively for 2 hours. Then printing is carried out and allowed to freeze, then the paraffin blocks are cut using a microtome with a slice thickness of 5-7 µm.

Hematoxylin and Eosin (HE) Staining

Cardiac histopathological examination was carried out on all rat heart samples. HE staining begins with deparaffinization by placing the preparation in a series of xylol solutions I, II, III. The next stage is fixation by placing the preparation

in a 96% alcohol solution. Then washed with running water and soaked in distilled water. The preparations were soaked in hematoxylin for 5 minutes and then washed with running water for 3 minutes. Then the preparation was dipped in 1% acid alcohol solution for 1-2 dips and washed again with running water for 3 minutes.

After that, the preparations were stained using 1% eosin and washed again with running water for 3 minutes. Then the dehydration process was carried out with graded alcohol (80, 95% alcohol and absolute alcohol) for 3 minutes and clearing using xylol. The preparations were mounted and covered with a cover glass. The preparations are observed under a microscope to see cell or tissue morphology including damage with a magnification of 10x40.

Statistical Analysis

Data were analyzed using ANOVA (Analysis of Variance) with a confidence level of 95% and continued with the Post Hoc Tukey test to test whether there was a significant difference in average values between the treatment groups. If the probability () is greater than 0.05 then there is no significant difference between the means of each group.

RESULT AND DISCUSSION

Characterization of African leaves Results

Characterization of African leaves Simplicia includes water content, water-soluble essence content, ethanol-soluble essence content, total ash content, and acid-insoluble ash content. The results of the characterization can be shown in Table 1. The results of determining the water content of the Simplicia were 3.99 %, which meets the requirements for the water content of the Simplicia. The requirement for water content in Simplicia is no more than 10% (Kemenkes RI, 2017, 1985). The purpose of determining water content is to determine the water residue after the drying process. Water content that is too high can cause the growth of microbes that will reduce the stability of the extract.

Parameters	Results (%)
Water content	3,99
Water-soluble essence content	28,08
Ethanol-soluble essence content	22,28
Total ash content	10,35
Acid-insoluble ash content	0,47

Table 1. Simplicia characterization results

Determination of water-soluble essence content and ethanol-soluble essence content obtained results of 28.08 % and 22.28 % with the condition of not less than 18.8 % and not less than 11.8 %, this shows that the African leaf simplicia used has been meets the requirements of the Indonesian Herbal Pharmacopoeia (2017). Determination of water-soluble essence content and ethanol-soluble essence content aims to estimate the amount of active compounds that are polar and non-polar. The results obtained from determining the total ash content are 10.35% with the condition that it is not more than 11.5 % (Kemenkes RI, 2017). Ash content testing aims to determine the internal and external mineral content found in simplicia. The higher the ash content obtained, the higher the mineral content contained in the sample. The acid-insoluble ash content indicates the presence of mineral or metal contamination that is insoluble in acid. The high levels indicated the presence of soil or sand, and the metal elements silver, lead, and mercury. The results obtained in determining the acid insoluble ash content were 0.47 %, where the results met the requirements. The requirement for determining the acid-insoluble ash content is no more than 0.7 %.

Plasma Renin Level Measurement Results

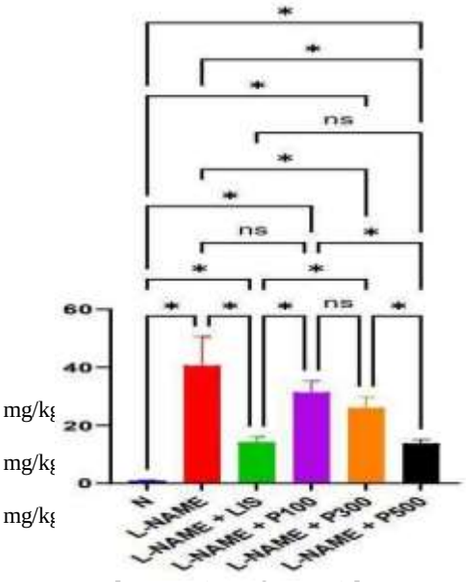
Plasma renin levels can be seen in Table 4 and Figure 2, in the group with addition EEDA, the group with a dose of 500 mg/kg BW experienced a decrease in plasma renin, namely 13.80 ± 1.30 ng/mL, while in the EEDA 100 mg/kg BW and EEDA groups 300 mg/kg BW also experienced a decrease in plasma renin, namely 31.39 ± 3.97 ng/mL and 26.00 ± 3.80 ng/mL. The positive control group was given lisinopril at a dose of 2.5 mg/kg BW and experienced a decrease in plasma renin levels, namely 14.20 ± 1.79 ng/mL. EEDA 100 mg/kgBW, 300 mg/kgBW, and EEDA 500 mg/kgBW had significant differences in plasma renin levels with the normal group. The EEDA 300 mg/kgBW and EEDA 500 mg/kg BW groups had significant differences in plasma renin levels with negative controls, while EEDA 100 mg/kgBW did not have significant differences in plasma renin levels with negative controls.

The EEDA 500 mg/kg BW group did not have a significant difference in plasma renin levels with the positive control, while EEDA 100 mg/kgBW and 300 mg/kg BW had a significant difference in plasma renin levels with the positive control. EEDA 500 mg/kgBB has a significant difference in plasma renin levels with EEDA 100 mg/kgBB and 300 mg/kgBB, while EEDA 100 mg/kgBB has no significant difference in plasma renin levels with EEDA 300 mg/kgBB. Therefore,

administering EEDA at a dose of 500 mg/kg BW can reduce plasma renin levels almost the same as lisinopril but there is still a significant difference from normal.

Group	Avarage plasma renin levels ± SD (ng/mL)
Normal	0,90 ± 0,22
Negative Control (L-NAME)	40,60 ± 9,98
Positive Control (L-NAME + Lisinopril)	14,20 ± 1,79
L-NAME + EEDA 100 mg/kgBW	31,39 ± 3,97
L-NAME + EEDA 300 mg/kgBW	26,00 ± 3,80
L-NAME + EEDA 500 mg/kgBW	13,80 ± 1,30

Table 2. Plasma Renin Level Measurement Results



* (significant) p ≤ 0,05

Figure 1. Graph of Renin Plasma levels

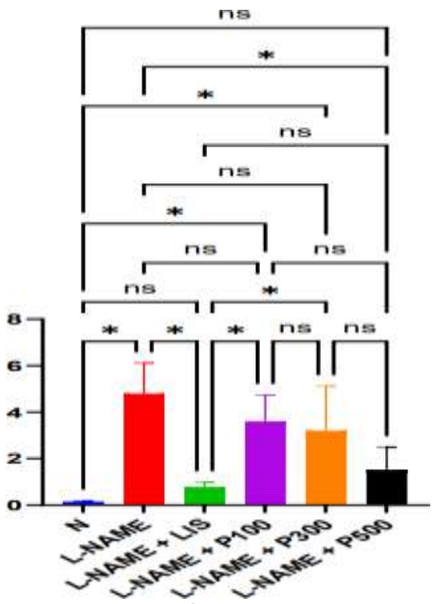
Renin is an initial component of the renin angiotensin aldosterone system (RAAS) containing 340 amino acid residues (37kDa) and is released from the juxtaglomerular cells of the kidney. Inhibition of RAAS activation helps lower blood pressure and reduces blood pressure fluctuations, thereby reducing the risk of cardiovascular events (Liu et al., 2020). In other research, it is known that saponins have a role in inhibiting the production of renin released by granular cells. Saponin in kidney samples shows a renoprotective effect through intrarenal RAAS inhibition. Long-term exposure to saponin results in the accumulation of saponin in blood vessel walls which has the potential to enter the afferent arteriole granular cells as a site for renin production. EEDA can reduce plasma renin, which plays a role in preventing hypertension. Therefore, the use of EEDA is able to reduce plasma renin and can be efficacious as an antihypertensive.

IL-6 Level Measurement Results

Based on Table 6 and Figure 4 shows that the EEDA 500 mg/kgBW group had a significant difference in IL-6 levels with the negative control, while EEDA 100 mg/kgBW and 300 mg/kgBW did not have a significant difference in IL-6 levels with the negative control. The EEDA 500 mg/kgBW group did not have a significant difference in IL-6 levels with the positive control, while EEDA 100 mg/kgBW and 300 mg/kgBW had a significant difference in IL-6 levels with the positive control. EEDA 500 mg/kgBW did not have a significant difference in IL-6 levels with EEDA 100 mg/kgBW and 300 mg/kgBW. EEDA 100 mg/kgBW did not have a significant difference in IL-6 levels with EEDA 300 mg/kgBW. Therefore, administering EEDA at a dose of 500 mg/kgBW is the right choice and can be used to reduce IL-6 levels.

Group	Avarage IL-6 levels ± SD (ng/mL)
Normal	0,15 ± 0,04$^{\#}$
Negative Control (L-NAME)	4,80 ± 1,30$^{*\ o}$
Positive Control (L-NAME + Lisinopril)	0,77 ± 0,20$^{\#}$
L-NAME + EEDA 100	3,60 ± 1,14$^{*\ o}$
L-NAME + EEDA 300	3,20 ± 1,92$^{*\ o}$
L-NAME + EEDA 500	1,52 ± 0,96$^{\#}$

Table 3. Level Measurement Results



Description: ns (not significant) p > 0,05

* (significant) p ≤ 0,05

Figure 2. Graph of IL-6 levels

L-NAME addition significantly increased proinflammatory cytokine markers such as IL-6. Elevated IL-6 in hypertension has been associated with an increased risk of endothelial myocardial infarction. Luteolin exhibits potent anti-inflammatory properties by inhibiting the synthesis and activity of pro-inflammatory cytokines such as IL-6. Vernodalin and vernonioside limit the production of proinflammatory cytokines including IL-6 through modulating the NF-κB signaling pathway (Farombi and Owoeye, 2011; Adesegun et al., 2008). Therefore, the use of EEDA can reduce IL-6 and can have anti-inflammatory and antihypertensive properties.

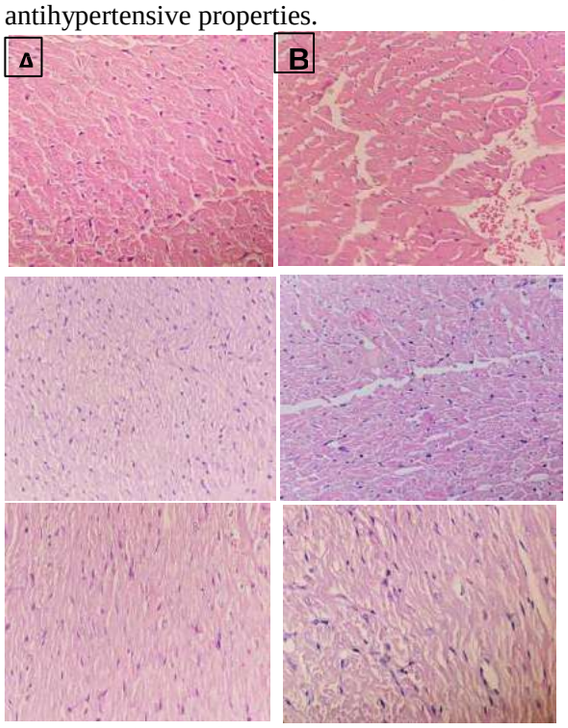


Figure 3. Histopathological images of the heart in various treatments. (A) Normal (B) Areas of degeneration, necrosis, and bleeding, damage >50 %-75 % or more (C-F) Areas of degeneration and necrosis, damage >25 %-50 %.

Using lisinopril as a positive control is because to the research of Maneesai et al., (2021) lisinopril can prevent L-NAME-induced hypertension, improve left ventricular and vascular function, suppress oxidative stress and the Ang II / AT1R / NOX2 / NF-kB pathway. Lisinopril has been shown to have other beneficial effects, such as antioxidant, anti-inflammatory, and cardiovascular protection. Lisinopril is one of the ACEi groups and its mechanism of action is included in the RAAS system and is related to renin.

Flavonoids are essential compounds that are found in many plants, including African leaves, and have many pharmacological activities that have been tested in vitro, in vivo, and clinically in humans. Its pharmacological activities include inhibition of nuclear factor kappa-b (Nf-Kb), antioxidant, antiplatelet, antithrombotic, ACEI (Syahputra et al., 2022). According to Atangwho et al. 2013, V.amygdalina is known to have a high level of antioxidants because it contains luteolin which is an active compound from the flavonoid group. Flavonoids in African leaves have potential as antioxidants which have an important role in reducing oxidative stress and can reduce damage caused by free radicals.

CONCLUSION

Based on the results of the research that has been carried out, it can be concluded that EEDA decreased plasma renin which was increased by L-NAME and decreased IL-6 which was increased by L-NAME in male Wistar rats so EEDA has the potential to reduce histopathological damage to the heart including degeneration and necrosis.

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