AL-KAUNIYAH: Jurnal Biologi, 18(1), 2025, 65-75
Website: http://journal.uinjkt.ac.id/index.php/kauniyah
P-ISSN: 1978-3736, E-ISSN: 2502-6720

ANTI-INFLAMMATORY EFFECTS OF VELVET BEAN LEAVES ETHANOLIC OINTMENT (*Mucuna pruriens* L. (DC.)) ON MICE LEUKOCYTES LEVEL

EFEK ANTIINFLAMASI SALEP ETANOL DAUN KACANG MIANG (*Mucuna pruriens* L. (DC.)) TERHADAP KUANTITAS LEUKOSIT MENCIT

Fadilaturahmah[1]*, Resti Rahayu[2], Putra Santoso[2]
[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Padang, West Sumatra 25132*
[2]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Andalas, Padang, West Sumatra 25166*
**Corresponding author:* fadilaturahmah@unp.ac.id

Submitted: 25 September 2023; Revised: 26 March 2024; Accepted: 27 July 2024

Abstract

Inflammation is a serious problem that needs to be treated. The use of steroid and non-steroidal drugs can treat inflammation well, but long-term use causes many side effects. So, it is necessary to use effective natural medicinal ingredients, one of which is velvet bean leaves (*Mucuna pruriens* L. (DC.)). This study aims to analyze the effect of velvet bean leaf ointment on the level of leukocyte components in mice experiencing inflammation. This research method was an experiment with a completely randomized design consisting of 6 treatment groups (normal, negative control, positive control (ketoconazole ointment)), velvet bean leaf ointment treatment at doses of 200, 400, and 600 mg/kgBW. The results showed that the use of velvet bean leaf ointment at doses of 200 and 400 mg/kgBW was able to significantly reduce the quantity of total leukocytes and monocytes in mice compared to the negative control ($P < 0.05$); ointment at a dose of 200 mg/kgBW was able to significantly reduce the quantity of granulocytes and lymphocytes in mice compared to the negative control ($P < 0.05$). The ability of velvet bean leaf ointment at a dose of 200 mg/kgBW is better in reducing the leukocyte quantity component compared to commercial drugs (ketoconazole ointment). Therefore, velvet bean leaf ointment at a dose of 200 mg/kgBW has great potential to be developed into an effective standardized anti-inflammatory drug.

Keywords: Granulocytes; Inflammation; Lymphocytes; Monocytes; Steroids

Abstrak

Inflamasi merupakan masalah serius yang perlu ditangani. Penggunaan obat steroid dan nonsteroid dapat mengatasi inflamasi dengan baik, namun penggunaan jangka panjang menimbulkan banyak efek samping. Maka dari itu perlu digunakan bahan obat alami yang efektif, salah satunya adalah daun kacang miang (Mucuna pruriens L. (DC.)). Penelitian ini bertujuan untuk menganalisis pengaruh salep daun kacang miang terhadap kadar komponen leukosit pada mencit yang mengalami inflamasi. Metode penelitian ini adalah eksperimen dengan rancangan acak lengkap yang terdiri dari 6 kelompok perlakuan (normal, kontrol negatif, kontrol positif (salep ketokonazol)), perlakuan salep daun kacang miang dosis 200, 400, dan 600 mg/kgBB. Hasil penelitian menunjukkan bahwa penggunaan salep daun kacang miang dosis 200 dan 400 mg/kgBB mampu menurunkan kuantitas total leukosit dan monosit mencit secara bermakna dibandingkan dengan kontrol negatif ($P < 0,05$); Salep daun kacang miang dosis 200 mg/kgBB mampu menurunkan jumlah granulosit dan limfosit pada mencit secara signifikan dibandingkan dengan kontrol negatif ($P < 0,05$). Kemampuan salep daun kacang miang dosis 200 mg/kgBB lebih baik dalam menurunkan komponen jumlah leukosit dibandingkan dengan obat komersial (salep ketokonazol). Oleh karena itu, salep daun kacang miang dosis 200 mg/kgBB memiliki potensi besar untuk dikembangkan menjadi obat antiinflamasi terstandar yang efektif.

***Kata Kunci**: Granulosit; Inflamasi; Limfosit; Monosit; Steroid*

Permalink/DOI: http://dx.doi.org/10.15408/kauniyah.v18i1.34979

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INTRODUCTION

Inflammation is the body's positive response to tissue damage to overcome infection, repair tissue damage, and restore tissue homeostasis (Hagemann et *al.*, 2013). However, if the inflammatory response occurs excessively it will also cause acute tissue damage. Inflammation causes many physical symptoms such as fever, pain, and edema as a result of vasodilation, increased vascular permeability, and the release of inflammatory mediators (Ferrero et *al.*, 2007).

Currently, steroid and non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen, aspirin, and diclofenac are very effective drugs used to reduce inflammation (Ghlichloo & Gerriets, 2023). However, long-term use causes many side effects such as stomach ulcers (Schellack & Fourie, 2015), inflammation of the digestive tract, acute renal failure (Harirforoosh *et al.*, 2013), cardiovascular toxicity (Olsen *et al.*, 2012), and liver toxicity (Sriuttha *et al.*, 2018). The high number of side effects from using NSAIDs has led to efforts to find alternative anti-inflammatory compounds derived from plants as potential natural and safer medicines (Ghasemian et *al.*, 2016).

One of the traditional medicinal ingredients used by the local community of Lintau, Tanah Datar Regency, West Sumatra as an anti-inflammatory is velvet bean leaves (*Mucuna pruriens* L. (DC.)). There is something unique about the use of this plant as medicine, velvet bean leaves are used by the community as an anti-inflammatory, even though the pod skin of the velvet bean is an allergen that can cause severe and acute inflammation (Fadilaturahmah *et al.*, 2023).

Research conducted by Bala *et al.* (2011) revealed that aerial extract of velvet bean was able to inhibit the volume of carrageenan-induced edema in mice's feet. Another research conducted by Fadilaturahmah *et al.* (2023), proved that 400 mg/kgBW velvet bean leaf extract was effective in treating inflammation in mice and had 30% higher anti-inflammatory power than diclofenac sodium (a commercial non-steroidal anti-inflammatory drug). Inflammatory conditions cause a significant increase in the leukocyte component. If the increase in leukocytes occurs continuously, it causes an increase in blood viscosity, resulting in a risk of stroke. Therefore, drugs are needed that can control and normalize leukocyte levels after inflammation. One natural ingredient that is thought to have this ability is velvet bean leaves. Velvet bean leaf extract can reduce the volume of edema significantly because it contains several compounds that have anti-inflammatory properties such as hexadecanoic *acid* (fatty acids), *geranylgeraniol* (diterpenoid), *geraniol* (monoterpenoid), *3-amino benzamide* (benzamide), *octadecanoic acid* (fatty acids) *and 4-hydroxycinnamic acid* (phenilpropanoid) (Fadilaturahmah *et al.*, 2023).

Studies regarding the effectiveness of velvet bean leaves as an anti-inflammatory are still very limited and have not been carried out extensively. Testing of the anti-inflammatory effects of velvet bean leaves has so far only been tested in the form of extracts that cannot be stored for long periods and require special temperatures for storage so as not to damage the active substances contained in the extract. However, its development into a standardized anti-inflammatory drug requires testing in other dosage forms that can be stored for long periods, such as ointments. Therefore, this study aims to analyze the anti-inflammatory effect of velvet bean leaf ointment on the leukocyte component of mice as an effort to produce standardized anti-inflammatory drugs that are effective, safe and affordable.

MATERIALS AND METHODS

The tools used in this research were filter paper, grinder, *rotary evaporator* (IKA RV 10), *hematology analyzer* (I need BM800), syringe sterile 1 cc (Onemed), mouse cage, and analytical balance (KERN ABS 220–4 Analytical Balance). Meanwhile, the material used in this research is velvet bean leaf ointment (*Mucuna pruriens* L(DC.)); mice (*Mus musculus*) DDY (body weight 20–30 grams); hair removal cream (*Veet),* ethanol 70%, NaCl solution 0.9%, *adeps lanae*, vaselin album, 2,4 *dinitrochlorobenze* (DNCB), acetone, *olive oil*, EDTA tube, and ketamine.

This research was carried out using an experimental method with a Completely Randomized (CRD) design consisting of 6 treatments and 5 replications. The types of treatment are as follows, N= not induced inflammation (normal); NC= inflammatory (DNCB) + ointment base (negative control); PC= inflammation (DNCB) + ointment ketoconazole (positive control); P1= inflammation (DNCB)

+ velvet bean leaf ointment 200 mg/kgBW; P2= inflammation (DNCB) + velvet bean leaf ointment 400 mg/kgBW; P3= inflammation (DNCB) + velvet bean leaf ointment 600 mg/kgBW. The treatment dose refers to Bala's research et al. (2011) and modified Fadilaturahmah et al. (2023).

Sampling and Extraction

Velvet bean leaf samples were collected in Tanjuang Bonai, Lintau, Tanah Datar Regency, West Sumatra. The leaves are air-dried at room temperature and then crushed using a grinder. The extraction process refers to Fadilaturahmah et *al.* (2023) used a maceration method with 70% ethanol solvent (the ratio of velvet bean leaf powder and ethanol was 1:6). Soaking is carried out for 72 hours, then the solution is filtered using a filter paper. After that, concentrate it using a rotary *evaporator* until a thick extract of velvet bean leaves is obtained.

Preparation of Velvet Bean Leaf's Ointment

Making ointment begins with the process of making the ointment base. The basic formula for this ointment refers to Agoes (2006), using A*deps* L*anae* 15 g and Vaselin Album 85 g. The ointment base is melted in a water bath at ±60 °C and stirred until homogeneous. The formulation for making velvet bean leaf extract ointment refers to Purwanto et *al.* (2021). The base of the ointment is melted and the extract of velvet bean leaves is added until it is homogeneous according to the measurements in Table 1.

Table 1. Velvet bean leaf extract ointment formulation for anti-inflammatory test

Material	Dose percentage (%)		
	200	400	600
Velvet bean leaf extract	0.6	1.2	1.8
Ointment base (Vaseline Album : Adeps Lanae (17:3))	99.4	98.8	98.2

Preparation of 2,4 Dinitrochlorobenzene (DNCB) Solution

2,4 dinitrochlorobenzene (DNCB) is a substance used to induce inflammation in the skin of mice. One gram of DNCB was weighed, and then dissolved in 50 mL of acetone and olive oil solution (1:3), to obtain a DNCB concentration of 2%. The procedure for making the DNCB solution is based on research conducted by Kwon et al. (2021).

Provision of Test Animals

The test animals used were 30 adult male white mice of the DDY strain with body weight criteria of ±25 g and an age of ±2.5 months which were obtained from the Baso Veterinary Center, West Sumatra. Mice were acclimatized in the laboratory for 1 week before treatment. Mice were given Rat-Bio animal feed and water optional. The procedures for handling and treating test animals have been approved by the Research and Ethics Committee of Andalas University (Approval Number: 512/UN.16.2/KEP-FK/2022).

Inflammatory Conditioning and Ointment Application in Mice

Inflammatory conditioning of mice is carried out on the back (dorsal). The mice's back hair was cut and smeared with cream (Veet) and clean the hair on the mice's backs using a spatula. Mice were left for 24 hours to avoid inflammation caused by hair loss (Pratiwi, 2016). After 24 hours and ensuring that there was no inflammation due to hair loss, the mice's backs were smeared with 200 µL of 2% DNCB every day for 4 days of induction. The DNCB dose used and the duration of inflammation induction refer to the Ku study et al. (2018). After being induced by DNCB for 4 days, the test ingredient ointment was smeared evenly on the mice's backs according to the type of treatment. The ointment is applied topically using cotton bud every day for 14 days. The duration of ointment application refers to Krishna and Sharma's research (2016) and Sardjono et al. (2017).

Quantity of Inflammatory Mouse Leukocyte Components Applied with Velvet Bean Leaf Extract Ointment

At the end of treatment, the mice were injected intraperitoneally using ketamine at a dose of 100 mg/kg BW as an anesthetic (Leung et al., 2021). Then, blood was isolated from the tails of the mice. After that, the mice had their vertebrae dislocated and dissected. Then, blood from the mice's hearts was sucked using a 1 mL syringe that had been rinsed with EDTA. The blood obtained was put into an EDTA tube to prevent coagulation. Blood hematology examination is carried out using an automatic machine hematology analyzer to determine the quantity of total leukocytes (white blood cell) which include lymphocytes, monocytes, and granulocytes. Whole blood sample (whole blood) as much as 500 µL was applied to the column analyzer and then the quantity of blood values is presented automatically on the monitor screen (Kakel, 2013).

Data Analysis

Data were analyzed using the SPSS version 21 program. Data on the quantity of leukocyte components were analyzed using Shapiro Wilk to find out the distribution of data and Levene Statistic Test to find out the homogeneity of the data. Normally and homogeneously distributed data were Analyzed using Analysis of Variance (ANOVA) one way with a confidence level of 95%. Results that show significant differences are tested with Duncan's Multiple Range Test (DNMRT).

RESULTS

Based on research that has been carried out, the results show that DNCB 2% can induce inflammation in the skin of mice. Inflammation of the skin of mice is characterized by redness, swelling, and scabbing. The inflammatory conditions on the skin of mice after being induced by 2% DNCB for 4 days are presented in Figure 1.

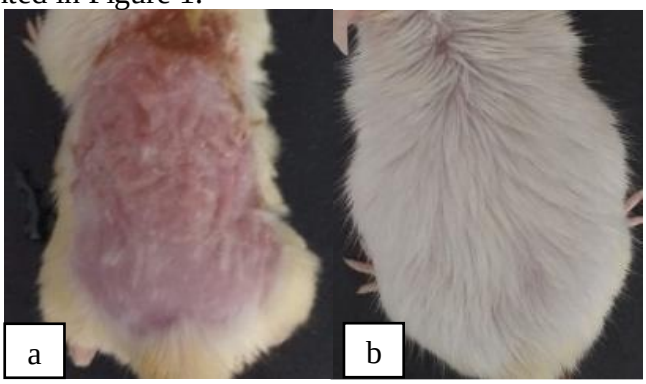

Figure 1. Skin condition of mice after being induced by DNCB 2% inflammation for 4 days, before DNCB induction (a) and after DNCB induction (b)

One of the body components that plays the most role in the inflammatory process is leukocytes. Inflammatory conditions involve the recruitment of leukocyte cells to the inflamed area to fight pathogens harmful to the body. Measuring the quantity of leukocyte components can describe the physiological condition of the blood when an inflammatory reaction occurs. The leukocyte component consists of neutrophils, eosinophils, and basophils which are included in the granulocyte group, and monocytes and lymphocytes which are classified as agranulocytes.

Total Leukocytes

Total leukocyte count results in Figure 2 showed that induction of inflammation using DNCB caused a significant increase in the number of total leukocytes (negative control 13.14 10^3/µL). Meanwhile, the induction of inflammation accompanied by the application of velvet bean leaf ointment at doses of 200 and 400 mg/kgBW showed leukocyte levels approaching normal conditions. This indicates that the use of velvet bean leaf ointment at doses of 200 and 400 mg/kgBW can normalize leukocyte levels in mice experiencing inflammation. The number of leukocytes in the normal treatment, velvet bean leaf ointment doses of 200 and 400 mg/kgBW were within the normal range, while the negative control, positive control, and velvet bean leaf ointment 600 mg/kgBW had leukocyte counts exceeding the normal range.

The total leukocyte count of the normal treatment, ointment doses of 200 and 400 mg/kgBW was significantly different (P <0.05) compared to the negative control treatment. This indicates that treatment with velvet bean leaf ointment at doses of 200 and 400 mg/kgBW has anti-inflammatory properties because it can reduce and normalize the number of leukocytes in mice experiencing inflammation. Apart from that, ointment doses of 200 and 400 mg/kgBW have better anti-inflammatory properties when compared to commercial ointment (ketoconazole).

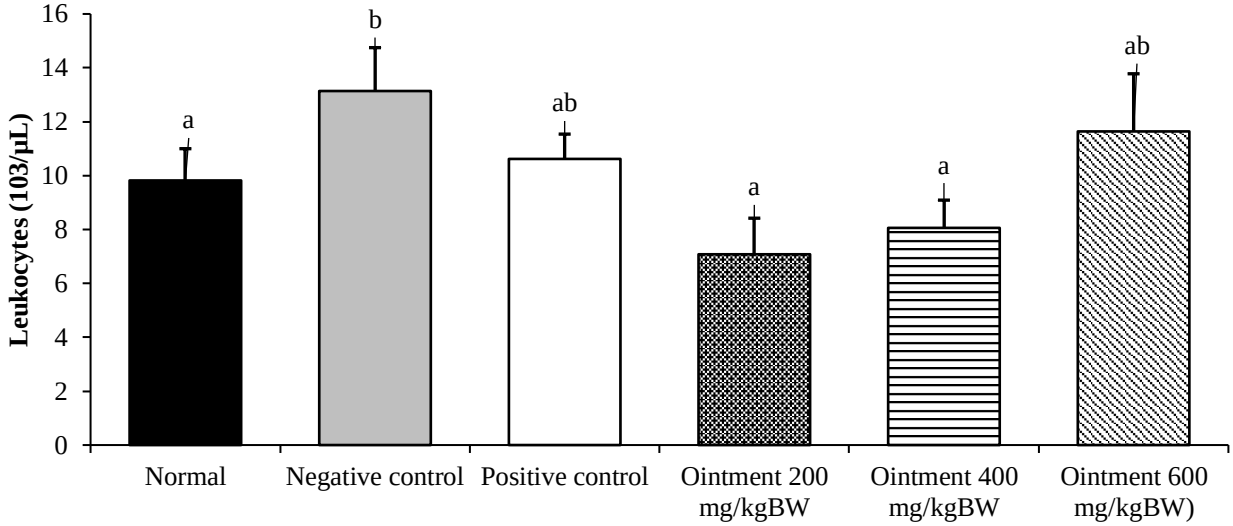

Figure 2. Quantity of total leukocytes in inflammatory mice after application of the test ointment for 14 days Note: different letters indicate significant differences (P <0.05) based on Duncan's test

Lymphocytes

One component of leukocytes that does not have granules (agranulocytes) is lymphocytes. Based on the calculation of the number of lymphocytes (Figure 3), the negative control treatment had the highest lymphocyte count (6.84 10^3/µL), while the lowest lymphocyte count was in the velvet bean leaf extract ointment treatment of 200 mg/kgBW (3.9 10^3/µL). The number of lymphocytes in all treatments was within the normal range.

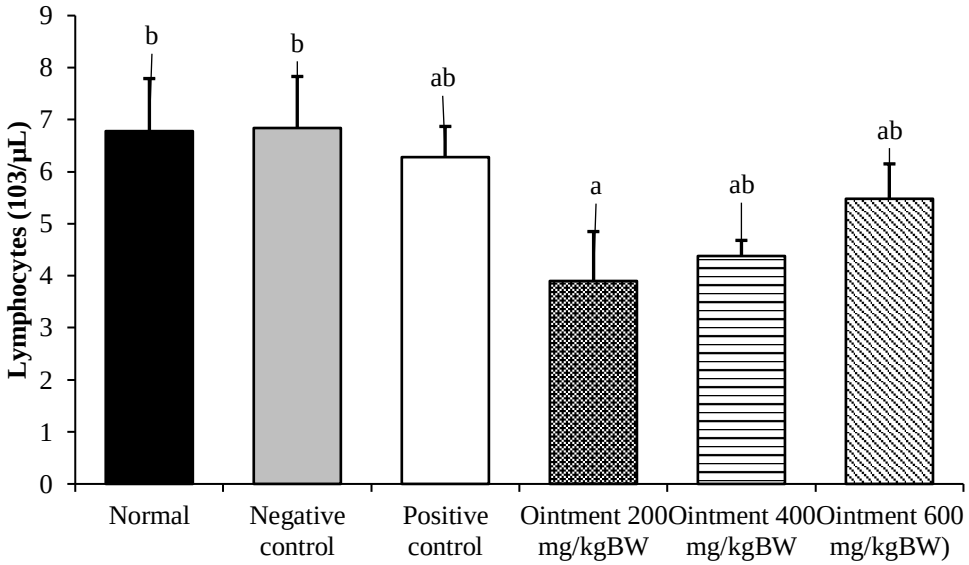

Figure 3. Quantity of inflammatory mouse Lymphocytes after application of the test ointment for 14 days. Note: different letters indicate significant differences (P <0.05) based on Duncan's test

Statistical analysis showed that the number of lymphocytes in the 200 mg/kgBW ointment treatment was significantly different (P <0.05) from the normal treatment and the negative control. However, the lymphocyte count for all types of treatment was within the normal range. This indicates that inflammatory conditions do not cause an increase in the number of lymphocytes in the blood. Lymphocytes play a role in specific body defense in forming antibodies. Meanwhile, the induction of inflammation in this study is thought to only involve non-specific body defenses so it does not significantly affect the quantity of lymphocytes.

Monocytes

Monocytes are a component of leukocytes that do not have granules (agranulocytes). Based on the calculation of the number of monocytes (Figure 4) results showed that the highest number of monocytes was in the negative control treatment (1.02 thousand/µL), while the lowest number was in the velvet bean leaf extract ointment treatment of 200 mg/kgBW (0.52 10^3/µL). The number of monocytes in all types of treatment was within the normal range.

Statistical analysis showed that the number of monocytes in normal treatment, ointment doses of 200 and 400 mg/kgBW was significantly different (P <0.05) compared to the negative control. This indicates that ointment treatment at doses of 200 and 400 mg/kgBW can reduce and normalize the number of monocytes in mice experiencing inflammation. The use of ointment doses of 200 and 400 mg/kg BW is better than commercial drugs (ketoconazole) in reducing the quantity of monocytes although not significantly different (P >0.05).

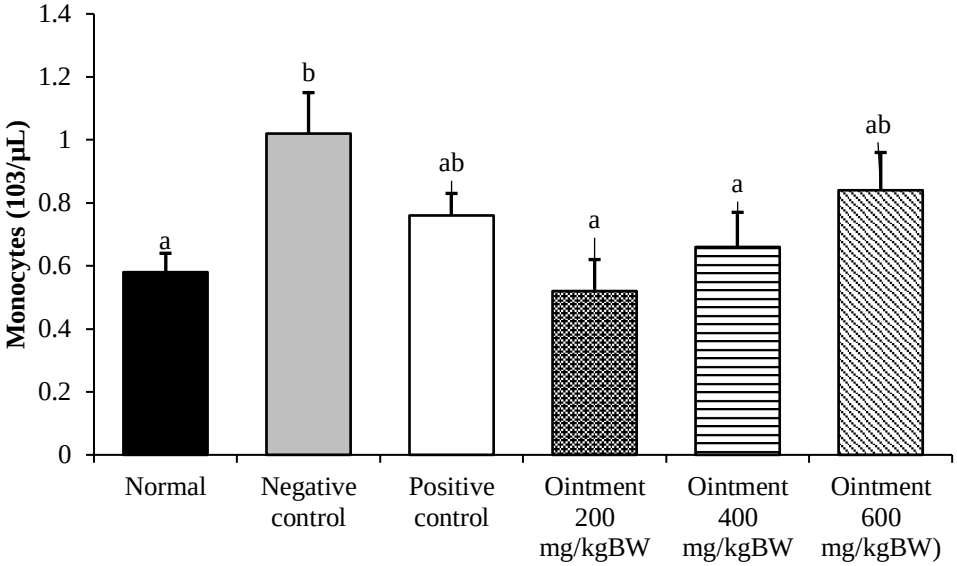

Figure 4. Quantity of inflammatory monocytes in mice after application of the test ointment for 14 days. Note: different letters indicate significant differences (P <0.05) based on Duncan's test

Granulocytes

The white blood cell components of granulocytes consist of eosinophils, basophils and neutrophils. Based on the calculation of the number of granulocytes (Figure 5), the negative control treatment had the highest number of granulocytes (5.38 10^3/µL), while the lowest number of granulocytes was in the normal treatment (2.46 10^3/µL). The number of granulocytes in normal treatment, positive control, 200 and 400 mg/kgBW ointment was within the normal range. Meanwhile, the negative control and 600 mg/kgBW ointment had granulocyte numbers exceeding the normal range.

Statistical analysis showed that the number of granulocytes in the normal treatment and 200 mg/kgBW ointment was significantly different (P <0.05) compared to the negative control treatment and 600 mg/kgBW ointment extract. This indicates that induction of inflammation causes a significant increase in granulocytes. Apart from that, the use of 200 mg/kgBW ointment was able to

reduce and normalize the number of granulocytes in mice experiencing inflammation. Ointment doses of 200 and 400 mg/kgBW have a better ability than commercial drugs in reducing and normalizing the quantity of granulocytes in mice experiencing inflammation. Figure 6 explain mechanism of velvet bean leaf ointment reducing leukocytes involved in inflammation.

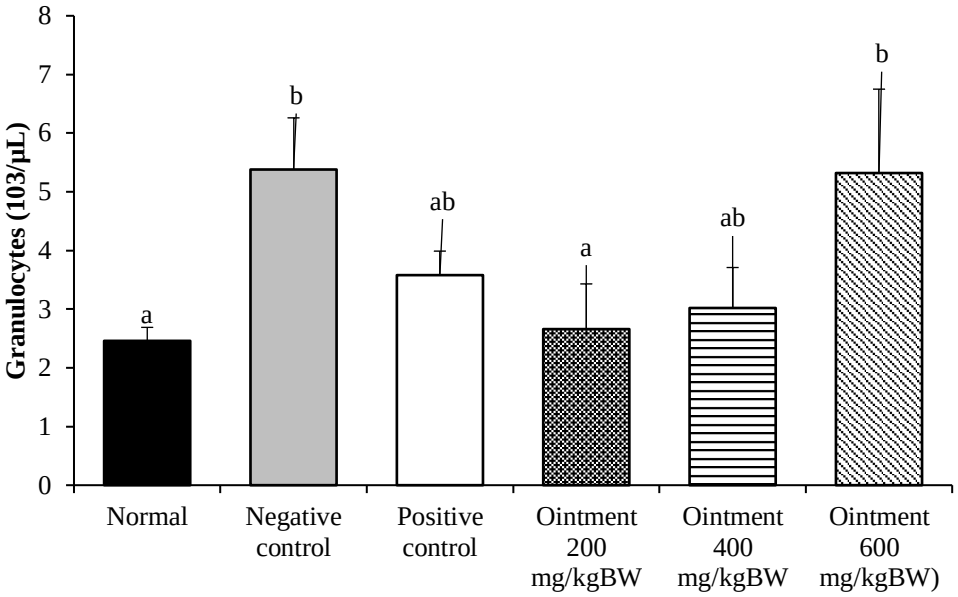

Figure 5. Quantity of inflammatory granulocytes in mice after application of the test ointment for 14 days. Note: different letters indicate significant differences (P <0.05) based on Duncan's test

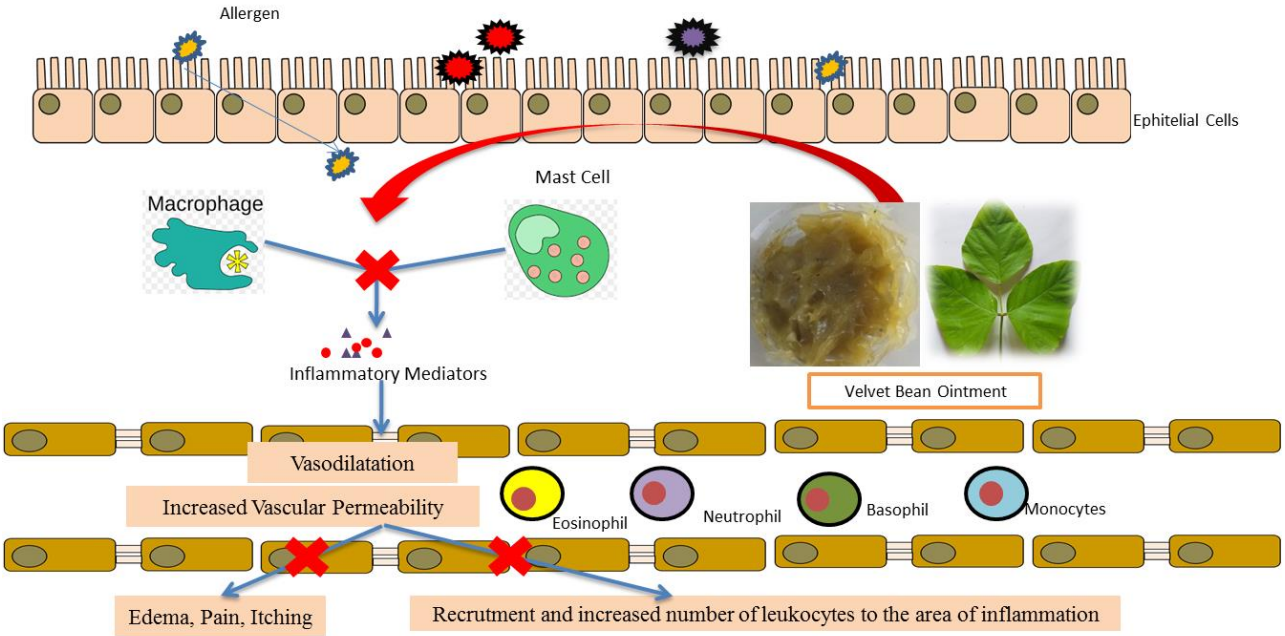

Figure 6. Mechanism of velvet bean leaf extract ointment in reducing the number of leukocytes

DISCUSSION

This research shows that the use of velvet bean leaf ointment on mice has a significant anti-inflammatory effect. Induction using DNCB causes the test animal's skin area to experience severe inflammation which is characterized by redness, swelling, and scabbing on the skin. Application of velvet bean leaf ointment was able to reduce and normalize the number of total leukocytes, granulocytes, and monocytes in mice which increased after induction of inflammation. Meanwhile, the number of lymphocytes in mice that experienced inflammation in this study did not increase. Lymphocytes play a role in specific body defense in forming antibodies. Meanwhile, the induction of

inflammation in this study is thought to only involve non-specific body defenses so it does not significantly affect the quantity of lymphocytes.

Previous research has proven that the compound 2,4-dinitrochlorobenzene (DNCB) is effective as an inflammation inductor (Heo et al., 2018; Bajgai et al., 2021). Application of DNCB to the skin area of the test animal causes inflammation which is characterized by several symptoms, namely a reddish rash; dry, scaly, and cracked skin; the skin thickens or swells (Tamagawa & Katoh, 2020; Chovatiya, 2023). This is in line with Kim's research et al. (2012) which revealed that DNCB induction in mice can significantly increase the number of leukocyte cells. According to Leick et al. (2014), certain chemicals can trigger an inflammatory response by recruiting leukocyte cells to sites of injury or infection. Therefore, the number of leukocytes tends to increase when the body experiences inflammation.

Study from De Siquueira et al. (2022) proved that induction of inflammation in mice caused a significant increase in leukocyte quantity. Foliar lectin application *Microgramma vacciniifolia* in mice that experience inflammation, the quantity of leukocyte components can be significantly reduced. Fadilaturahmah et al. (2023) proved that oral application of ethanol extract of velvet bean leaves to mice experiencing inflammation was able to significantly reduce the quantity of leukocytes and granulocytes.

Inflammatory reactions can be induced by various factors such as allergens, chemicals, microbes, or injury. This induction causes tissue damage which is recognized by macrophages and mast cells, so these cells produce inflammatory mediators such as cytokinins and prostaglandins. Inflammatory mediators induce vasodilation (widening of blood vessels) and increase blood vessel permeability. This allows migration to occur and an increase in the number of leukocytes that migrate to the injured area. Inflammatory conditions are characterized by pain, itching, and swelling in areas that have experienced tissue damage (Sousa et al., 2013; Chen et al., 2018).

The ability of velvet bean leaf extract ointment to reduce the number of leukocytes in inflammatory mice is thought to be related to the ointment's bioactive compounds which have anti-inflammatory properties. Fadilaturahmah Research et al. (2023) proved that the ethanol extract of velvet bean leaves contains several compounds that have anti-inflammatory properties, namely hexadecanoic acid, geranylgeraniol, geraniol, 3-amino benzamide, octadecanoic acid, and 4-hydroxycinnamic acid. Hexadecanoic acid is the compound with the highest percentage in velvet bean leaf extract and has properties as a prostaglandin inhibitor (an inflammatory mediator). If the formation of prostaglandins is inhibited, inflammatory reactions will not occur so that the number of leukocytes in the blood is within the normal range (Pubchem, 2023).

The mechanism of velvet bean leaf ointment in reducing the number of leukocytes involved in inflammation is presented in Figure 6. Research by Fadilaturahmah et al. (2023) proved that the bioactive compounds contained in velvet bean leaf ointment can prevent macrophage cells and mast cells from producing inflammatory mediators. According to Chen et al. (2018), if inflammatory mediators are not formed, vasodilation and increased blood vessel permeability will not occur. This prevents an increase in the number of leukocytes in the blood and inflammatory reactions such as edema, pain, and itching will not occur.

The leukocyte component consists of agranulocytes (lymphocytes and monocytes) and granulocytes (neutrophils, eosinophils, and basophils). Inflammation induction in this study only increased the number of total leukocytes and granulocytes significantly beyond normal levels. Meanwhile, the quantity of lymphocytes and monocytes is still within the normal range. Sonnenberg and Hepworth (2019) revealed that inflammation is a type of innate immune system (innate immunity) non-specific. The immune system consists of the non-specific immune system (innate immunity) and specific (adaptive immunity).

Leukocytes are a key component involved in inflammatory reactions. The types of leukocytes that play a role in the innate immune system are granulocytes (neutrophils, basophils, and eosinophils) and monocytes (Pezhman et al., 2021). Neutrophils contain proteolytic enzymes in their granules which function in the phagocytosis process. Meanwhile, eosinophils have a special role in allergic reactions. Basophils contain heparin and histamine which function as inflammatory mediators. In

tissues, basophil cells develop into mast cells which can release histamine, causing an inflammatory reaction. Monocytes are cells that have an important role in inflammatory mechanisms. Monocytes can migrate into tissues and develop into macrophages and dendritic cells. Macrophages can phagocytose foreign substances because they contain lysozyme and cytokines and can move amoeboid so that it can penetrate the capillaries to enter the tissue (Mauersberger et al., 2022).

The induction of inflammation in this study did not cause a significant increase in lymphocytes above normal levels. This is thought to be related to DNCB which is used as an inflammatory inductor only responding to the non-specific innate immune system. According to Pezhman et al. (2021), lymphocytes are a type of leukocyte that plays a role in the specific immune system (adaptive immunity). According to Moelyono et al. (2017), lymphocytes are components of leukocytes that have a role in the body's specific (adaptive) defense system. B lymphocytes have a role in humoral immunity, while T lymphocytes have a role in cellular immunity. Therefore, it can be interpreted that in this study all types of treatment only responded to the body's non-specific defense, namely the second line of defense which was characterized by inflammatory reactions and recruitment of leukocyte cells. According to Kim et al. (2021), when an inflammatory reaction occurs, one of the symptoms is an increase in the number of lymphocytes as the body's defense mechanism.

CONCLUSION

Velvet bean extract ointment (*Mucuna pruriens* L. (DC.)) dose of 200 mg/kgBW is effective as an anti-inflammatory because it can significantly reduce the number of total leukocytes, granulocytes, lymphocytes, and monocytes. The ability of velvet bean leaf ointment as an anti-inflammatory is better when compared to commercial ointments (ketoconazole). In further research, it is hoped that genetic studies will also be carried out on the cytokine levels of mice.

ACKNOWLEDGEMENTS

Thank you to the Ministry of Education, Culture, Research and Technology for the funds provided to facilitate research through the National Competitive Program (Contract No. 086/E5/PG.02.00.PT/2022).

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