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Anti-inflammatory effects of velvet bean (*Mucuna pruriens* L. (DC.), Fabaceae) leaf ethanolic extract against carrageenan in male mice

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ABSTRACT: Inflammation is an immune response that could lead to serious health problems. Some steroids and non-steroidal drugs are capable of relieving inflammatory reactions but may promote various detrimental side effects. Therefore, the search for effective but less side effects of natural antiinflammatory drugs are urgently needed. One of the medicinal plants that may have the potential as an anti-inflammatory is velvet beans (*Mucuna pruriens* L. (DC.)). This study aimed to investigate the effectiveness of velvet bean leaf ethanolic extract against inflammation in mice as an animal model and to elucidate the phytochemical constituents of velvet bean leaf extract regarding their anti-inflammatory properties by deploying gas chromatography-mass spectrophotometry (GCMS) analysis. This study used 40 male BALB/c mice with 4 treatment groups, namely negative control (Na-CMC 1%), positive control (diclofenac sodium 4.5 mg/kg BW), velvet bean leaf extract 200 mg/kg BW, and 400 mg/kg BW, respectively. The extract or diclofenac sodium was given orally to the mice 30 minutes upon intraplantar injection of carrageenan 1%. Subsequently, the volume of paw edema, area under curve (AUC) values of edema, and percent of anti-inflammatory power as well as leukocyte counts were determined. The results demonstrated that ethanolic extract of velvet bean leaf, particularly at the dose of 400 mg/kg BW exerted a substantial anti-inflammatory effect against carrageenan as indicated by the lowest edema volume, lowest AUC value, the highest anti-inflammatory power, and stronger suppression on leukocyte counts. In addition, the GC-MS analysis revealed some potent anti-inflammatory compounds namely hexadecanoic acid, geranylgeraniol, geraniol, 3-aminobenzamide, octadecanoic acid, and 4-hydroxycinnamic acid.

KEYWORDS: Carrageenan; edema; hexadecanoic acid; inflammation; velvet bean.

1. INTRODUCTION

Inflammation is the immune response to noxious stimuli such as pathogens, damaged cells, toxic compounds, or radiation [1]. Inflammation treatment includes two aspects, namely relieving pain and reducing pain to mitigate the subsequent tissue damage. Some steroids and non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to be effective in relieving the inflammatory reaction, however, they are also linked to various detrimental side effects particularly for a long-term use. For instances, systemic steroid drugs could decrease the synthesis of endogenous glucocorticoids, decrease general response of the body against infection, and promote osteoporosis, moon face, and hypertension. Likewise, non-steroidal anti-inflammatory drugs (NSAIDs) have been associated with digestive tract disorders, platelet malfunction, and inhibition of pregnancy [2]. Therefore, the exploration and development of natural anti-inflammatory drugs are urgently needed. The effective and less side effects natural based medicines can be derived from plant species [3]. It has been shown that various plants used as ethnomedicinal materials by various local tribes have unique phytochemical constituents thus a tract scientific investigation [4]. One of such plants is velvet bean (*Mucuna pruriens* L. (DC.), Fabaceae).

Despite the fruit skin is a potent allergen causing severe and acute inflammation, local people living in West Sumatra, Indonesia usually use velvet bean leaves as inflammatory reliever. The indicator of anti-inflammatory effect of velvet bean leaf has been suggested by such traditional practices. However, until recently, there is no scientific evidence to clarify it.

Velvet bean leaf has been reported to contain alkaloids, coumarins, flavonoids, methionines, tyrosines, and alkylamines that could elevate endogenous antioxidants in the body [5]. Another study has also showed that velvet bean seeds contain several bioactive compounds including glycosides, saponins, tannins, terpenoids, calcium, phosphorus, potassium, phytic acid, and L-DOPA [6]. Previous studies reported that velvet bean seed extract was effective as an anti-inflammatory, antibacterial, antivenom, and antioxidant [7,

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8]. The ethanol extract aerial part of velvet bean has been shown to be effective as an anti-inflammatory in rats. The extract significantly reduced carrageenan-induced paw edema in rats [9]. Taken together, these findings suggest the medicinal benefits of velvet bean as a potent natural drug. However, until recently, scientific information about the efficacy of velvet bean leaf extract particularly as an anti-inflammatory drug is still very limited. Hence, this study aimed to investigate the effectiveness of velvet bean leaf ethanolic extract against inflammation in mice as an animal model and to explore the phytochemical constituents of velvet bean leaf extract that may be associated with the anti-inflammatory properties.

2. RESULTS

2.1. Effect of velvet bean leaf extract on carrageenan-induced inflammation

In order to induce the edema, as an inflammatory response, mice paws were injected intraplantar with carrageenan. As depicted in the Figure 1, the paw edema was profoundly observed after 1 hour post injection in all groups of treatment. However, during 5 hours of visual observation, it was found that the magnitude of edema was lower in mice treated with velvet bean leaf extract (both dose of 200 and 400 mg/kg BW) as compared to negative control group (without any treatment).

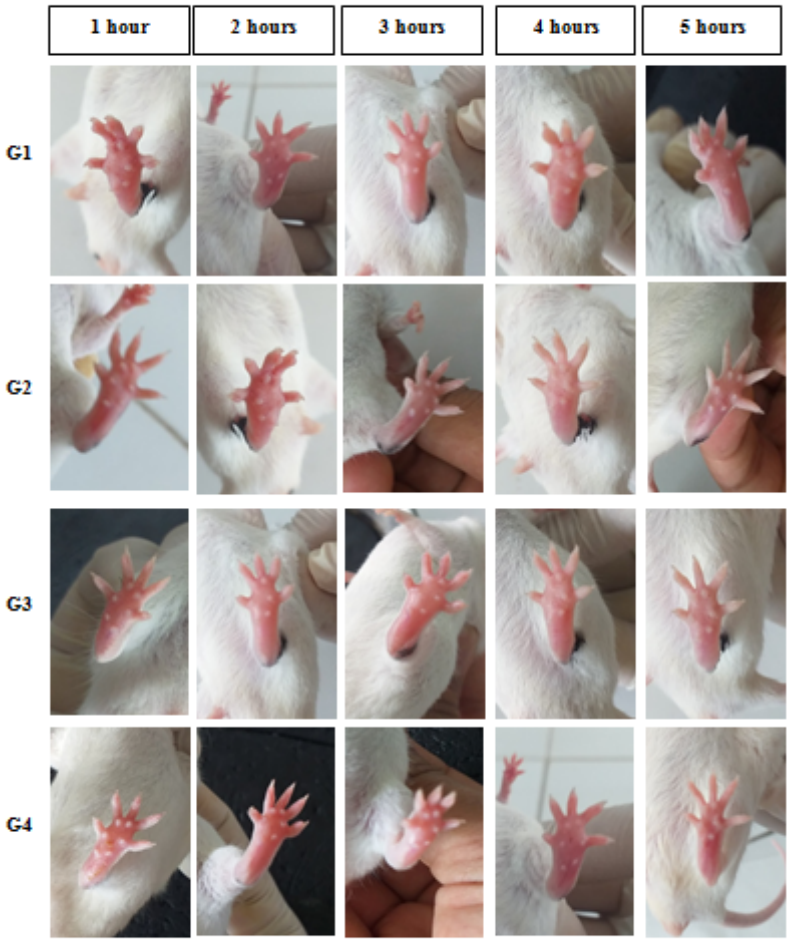

Figure 1. Representative photograph of paw edema of mice in each treatment group.

G1=Negative Control (without any treatment), G2=Positive Control, G3=Velvet bean leaf extract 200 mg/kg BW, G4= Velvet bean leaf extract 400 mg/kg BW.

In addition to visual observations, the volume of paw edema was also measured. As shown Figure 2, the edema volumes of mice paws in negative control group were sustained at higher levels at every time points of measurements (1-5 h). Otherwise, in groups treated with velvet bean leaf extract (200 mg/kg BW and 400 mg/kg BW) and diclofenac, the volume of edema just slightly increased until 3 hours post carrageenan

injection, but then turned back to a normal condition (non inflammatory state) at 4 h after injection. The edema volume remained statistically higher at the latest time of observation in negative control group as compared with all other group ($p<0.05$). Interestingly, the suppression of edema by velvet bean leaf extract (particularly at the dose of 400 mg/kg BW) outperformed the diclofenac (a positive control).

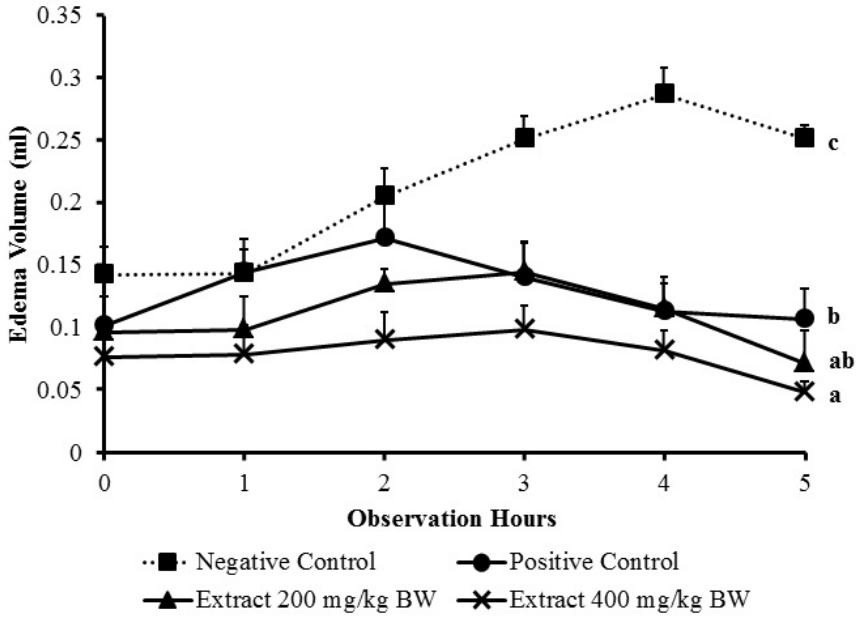

Figure 2. The effect of velvet bean leaf extract on paw edema volume of mice. The lower-case characters on graph indicate significant difference base on LSD test ($p<0.05$)

Furthermore, the AUC values (Figure 3A) derived from edema volumes also indicated a substantial reduction in mice groups treated with velvet bean extract as compared with non-treated group ($p<0.05$). Importantly, the AUC values were also lower in groups treated with velvet bean leaf extract as compared with those treated with diclofenac (positive control), even statistically was not significant. The anti-inflammatory force data (Figure 3B) exhibited a highest value (57.59%) in group treated with 400 mg/kg BW of velvet bean leaf extract that was significantly different as compared with other groups (46.16% in group treated with 200 mg/kg BW of velvet bean leaf extract and 34.43% in diclofenac-treated group, $p<0.05$).

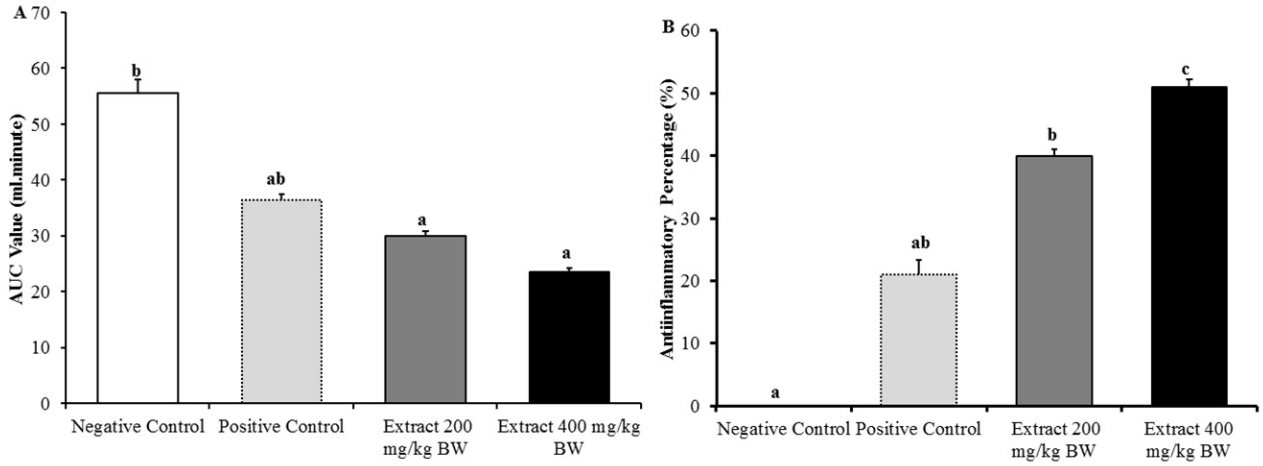

Figure 3. The value of AUC (Area Under Curve) and percentage of anti-inflammatory power of velvet bean leaf extract

2.2. Effect of velvet bean leaf extract on the leukocytes

In order to determine the modulatory effect of velvet bean leaf extract on leukocytes, the number of total leukocytes, lymphocytes, granulocytes, and monocytes were counted at three different time points (1,3,5, and 5 h post carrageenan injection) along with the edema measurement. The result is presented in Figure 4.

The data of total leukocytes count (Figure 4A) indicated that, in the negative control group, leukocyte numbers were sustained at highest level at any time points of measurements as compared with the other groups. Meanwhile, total leukocytes were significantly lower in groups treated with velvet bean leaf extract. The reduction in total leukocyte number was statistically significant between mice treated with 400 mg/kg BW of extract and those treated with diclofenac (positive control, $p<0.05$). The data of lymphocyte counts (Figure 4C) also depicted a similar pattern, showing that mice treated with velvet bean leaf extract, particularly at dose of 400 mg/kg BW, had a substantial reduction in lymphocytes at any time points of measurement as compared with other groups. Likewise, the granulocyte numbers (Figure 4B) and monocytes number (Figure 4D) at 1 hour after treatment were also significantly suppressed in mice treated with velvet bean leaf extract, especially at the dose of 400 mg/kg BW, as compared with negative control group (Figure 4C).

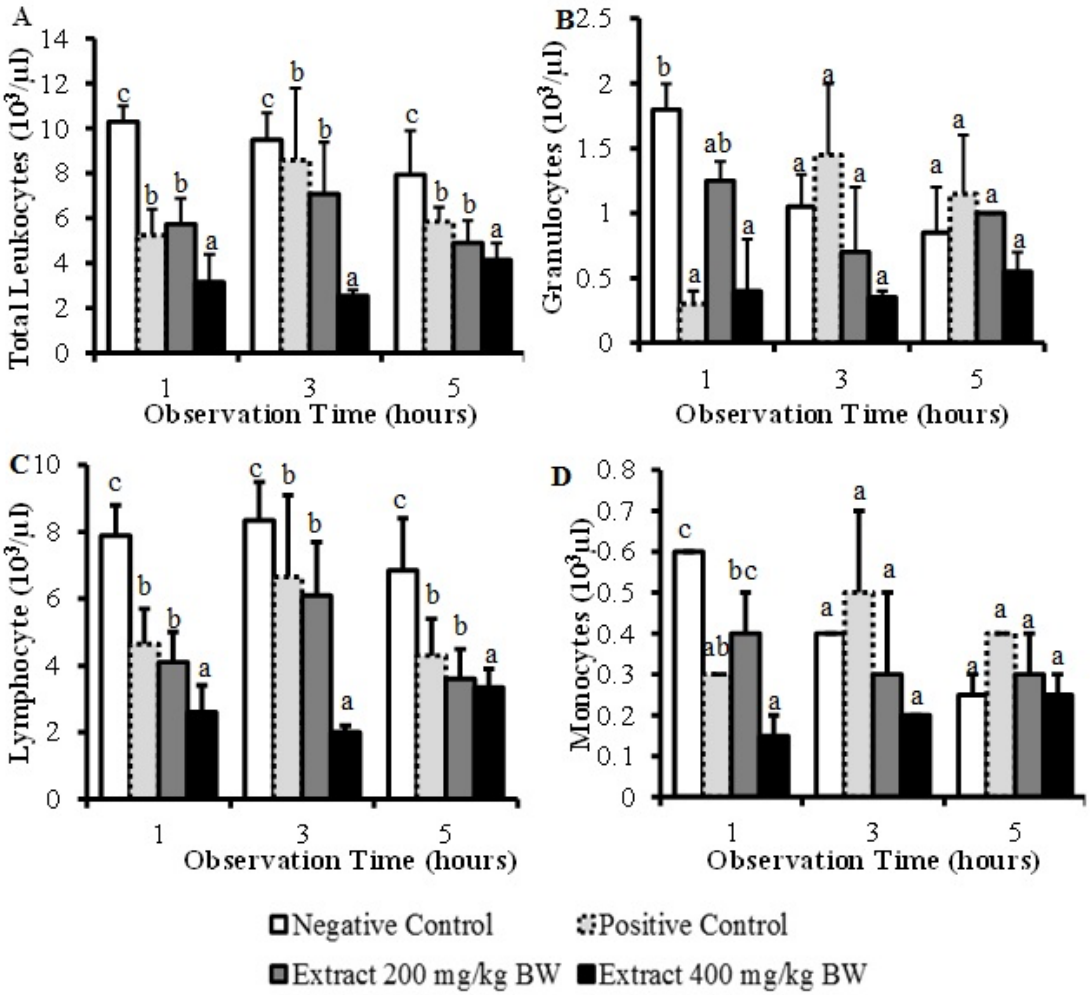

Figure 4. Effect of velvet bean leaf extract on leukocyte count of mice. Different lower case letters above the bars indicate the statistical significant based on LSD test ($p<0.05$)

2.3. Phytochemical constituents of velvet bean leaf extract

The GC-MS analysis revealed 170 phytochemical compounds detected in velvet bean leaf ethanolic extract. As depicted in chromatogram (Figure 5), among all detected compounds, there were 6 substances that markedly higher in their concentration (indicated by higher peaks in chromatogram) namely hexadecanoic acid, geranylgeraniol, geraniol, 3-aminobenzamide, octadecanoic acid, and 4-hydroxycinnamic acid. The predominant compounds are suggested to contribute in the suppression of inflammatory response (Table 1).

Table 1. The main phytochemical compounds of the velvet bean leaf ethanolic extract and their bioactivity

Compound Name	% Area	Group	Bioactivity
Hexadecanoic acid ($C_{16}H_{32}O_2$)	24.50	Fatty acids [10]	Prostaglandin inhibitors [10]
Geranylgeraniol ($C_{20}H_{34}O$)	18.66	Diterpenoids [10]	Antioxidant, antiinflammatory [11]
Geraniol ($C_{10}H_{18}O_2$)	9.84	Monoterpenoids [10]	Antioxidant and antiinflammatory [12]
3-Aminobenzamide ($C_7H_8N_2O$)	9.48	Benzamide [10]	Anti-inflammatory [13]
Octadecanoic acid ($C_{18}H_{36}O_2$)	7.30	Fatty acids [10]	Antiinflammatory [14] Antioxidant [15]
4-Hydroxycinnamic acid ($C_9H_8O_3$)	6.44	Phenylpropanoid [10]	Antioxidant [10]

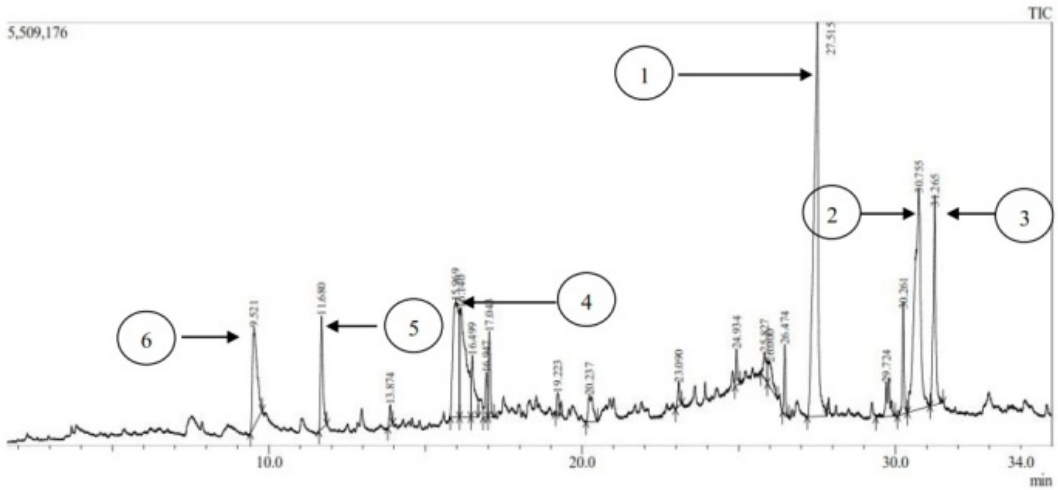

Figure 5. The GC-MS chromatogram of velvet bean leaf ethanolic extract. Numbers on particular peaks indicate the compounds that have been suggested as anti-inflammatory agents. (1) Hexadecanoid acid, (2) Geranylgeraniol, (3) Geraniol, (4) 3-Aminobenzamide, (5) Octadecanoic acid, (6) 4-Hydroxycinnamic acid

3. DISCUSSION

Our present study demonstrates a profound anti-inflammatory effect exerted by velvet bean leaf ethanolic extract in mice as an animal model. The oral administration of velvet bean leaf extract could markedly reduce edema, an inflammatory response, caused by carrageenan injection in mice paws. Moreover, velvet bean leaf extracts effectively modulated the leukocyte counts. In addition, the GC-MS analysis of phytochemical constituents of the extract revealed six potential compounds that may participate in suppressing the inflammatory responses.

A previous study in the carrageenan-induced paw edema of rats indicated that the ethanolic extract of aerial part of velvet bean at the doses of 200 and 400 mg/kg BW showed a significant inhibitory effect on the edema formation from one to five hours after oral administration with the highest inhibitory effect was found during the third hour of post-treatment [9]. In line with the previous report, our current study also indicated that ethanolic extract of velvet bean leaf administrated orally significantly suppressed inflammatory response at the doses of 200 and 400 mg/kg BW. However, the onset of inhibition was apparently observed at two hours after extract administration and the maximum inhibition was observed at four hours. It means that the leaf extract of velvet bean was slower than the aerial part extract in exerting its anti-inflammatory effect. This discrepancy might be due to the differences in the phytochemical constituents and concentrations of particular compounds between the leaf and aerial parts of the velvet bean. Since the aerial part of the plant include leaves, stem, flower, and fruits, thus its bioactive compounds in the extract could be higher and richer than in leaf extract. Unfortunately, in this present study, we did not include the phytochemical analysis of the aerial part of the velvet bean. Hence, further investigation is required to confirm the speculation.

Carrageenan-induced edema occurs via the mechanism involving the release of inflammatory mediators including prostaglandin 1 and prostaglandin 2 leading to an increase in vascular permeability [16, 17]. Thereafter, the subsequent vasodilation and increased vascular permeability promoted by prostaglandin could elevate blood flow and migration of phagocytic cells in the affected area thereby developing inflammation [18]. Likewise, in our present study, it was found that two hours after carrageenan injection, the inflammation in the paw of mice was profoundly observed particularly those without velvet bean leaf extract treatment. Otherwise, oral administration of velvet bean leaf extract in both lower and higher doses successfully counteracted the carrageenan-induced inflammation. This counteractive effect of velvet bean leaf extract against inflammation might be attributed to its phytochemical constituents. As revealed by phytochemical analysis, some compounds including hexadecanoic acid, geranylgeraniol, and geraniol were detected in the extract. İt has been previously reported that hexadecanoic acid is a potent prostaglandin inhibitor [10]. Moreover, geraniol is also capable of suppressing an enzyme namely cyclooxygenase-2 that plays a key role in the biosynthesis of prostaglandin [18]. Geranylgeraniol and 3-aminobenzamide are also suggested to participate in alleviating inflammation by counteracting prostaglandin action [10]. Therefore, the inhibitory effect of velvet bean leaf extract as found in this study could be mediated via the mechanisms involving the prevention of prostaglandin biosynthesis, release, and action. However, the level of prostaglandin and the response of its receptors toward the velvet bean leaf extract treatment was not determined in this study. Thus, the further experiment is required to clarify it.

In addition to its action as prostaglandin suppression, the velvet bean also might modulate the inflammatory response by inhibiting the nuclear factor kappa B (NF-kB), a key factor for the proinflammatory cytokine release [19]. Previous reports demonstrated that the velvet bean seed extract was effective in decreasing various proinflammatory cytokines including nitric oxide (NO), interleukin 1β, IL-6, and tumor necrosis factor α (TNF-α) to counteract inflammation [20, 21]. Moreover, our previous in silico simulation using molecular docking also suggested that several bioactive compounds detected in velvet bean leaf ethanolic extract (particularly geraniol, octadecanoic acid, benzyl acetate, artemin, and zedoarondiol) were capable of acting as potent NF-kB antagonists thereby preventing inflammatory response [22]. However, our present data could not provide the actual inhibitory actions of bioactive compounds in velvet bean leaf extract against NF-kB expression and proinflammatory cytokine release.

Upon proinflammatory cytokine release provoked by foreign substances like carrageenan and certain pathogens, the leukocytes will be recruited in the affected tissues. As a result, the number of leukocytes will markedly increase along with the inflammatory response [23]. Accordingly, as observed in our present study, the leukocyte counts including total leukocytes, granulocytes, and agranulocytes (lymphocytes and monocytes) were profoundly elevated in the circulatory system after carrageenan injection, indicating leukocytes recruitments. However, such elevations were effectively mitigated by velvet bean leaf ethanolic extract in a dose-dependent manner. İt has been shown by a previous report that under normal conditions (non-inflammatory state), the incorporation of velvet bean leaf in the diet did not affect the leukocyte number [24], suggesting its safety. Thus, the suppressive effect of velvet bean leaf extract on leukocyte counts, as observed by our present study, could be a subsequent implication of inflammatory inhibition.

Some limitations should be considered in our present study. Firstly, the cytokine levels including prostaglandin and histamines were not determined. As a result, it remains unknown whether the velvet bean leaf extract is capable of inhibiting proinflammatory cytokine release to alleviate inflammation. Secondly, the histological alterations in the inflammated paws of the mice also were not examines thus hinders essential revelation at the tissue level. Thirdly, the phytochemical analysis of the extract was performed solely by GC-MS technique that is specific for volatile compounds with a lower molecular weight. The future study deploying LC-MS analysis is absolutely required to explore the other potent anti-inflammatory compounds in velvet bean leaves. Moreover, the purification of the extract and further testings on its effectivity against inflammation are also needed.

4. CONCLUSION

The ethanol extract of velvet bean leaves, particularly at the dose of 400 mg/kg BW, exerted a substantial anti-inflammatory effect with the lowest edema volume, lowest AUC value, highest anti-inflammatory power, and stronger suppression on leukocyte count. The extract was composed of some potent anti-inflammatory compounds including hexadecanoic acid, geranylgeraniol, geraniol, 3-aminobenzamide, otadecanoic acid, and 4-hydroxycinnamic acid. Therefore, the velvet bean leaf is a potential candidate to be developed as natural anti-inflammatory drugs.

5. MATERIALS AND METHODS

5.1. Plant material

Velvet bean leaves were collected in Tanjuang Bonai, Tanah Datar Regency, West Sumatra, Indonesia. Plant identification was carried out by a certifed plant taxonomist in the herbarium of Andalas University (ANDA) (Identification number: 111/K-ID/ANDA/III/2021).

5.2. Plant extraction

The fresh velvet bean leaves were air dried before being extracted by maceration using 70% ethanol solvent for 3-5 days. Thereafter, the macerate was filtered using filter paper and collected in a container. Subsequently, the sample was concentrated using a rotary evaporator and dried over a water bath to obtain the extract [25].

5.3. Preparation of test animals

The adult male BALB/c mice (40 individuals, weight 20-30 grams, 2-3 months old) were purchased from the the Veterinary Center, Baso, West Sumatra, Indonesia (ISO 17025:2017). The mice were firstly acclimatized individually in a single cage and fed with standard rodent chow diet (RATBIO, PT Citra Ina Feedmil, Jakarta, Indonesia) and tap water ad libitum for one week. The temperature, humidity and ligh-dark cycle of the rearing room were regularly controlled. Before treated, the mice were fasted for ± 18 hours by removing the food, but drink remained provided. The procedures for handling and treating test animals have been approved by the Research and Ethics Committee of Andalas University (Approval number: 528/UN.16.2/KEP-FK/2021).

5.4. Preparation of carrageenan suspension

Carrageenan cappa was purchased from Pharmaprenurstore (Depok, Indonesia). A 100 mg of carrageenan, was dissolved in 10 ml of 0.9% NaCl solution to achieve a suspension with a concentration of 1% [26].

5.5. Anti-inflammatory testing procedures

This study was performed using an experimental method with a completely randomized design (CRD) consisting of 4 treatment and 10 replications. The treatment was as follows:
G1 = carrageenan + 1% Na-CMC (Sodium-Carboxy Methyl Cellulose) suspension 10 ml/kgBW (negative control)
G2 = carrageenan + diclofenac sodium 4.5 mg/kg BW (positive control)
G3 = carrageenan + velvet bean leaf extract 200 mg/kgBW
G4 = carrageenan + velvet bean leaf extract 400 mg/kgBW

The dosage of the extract was chosen based on previous study [9] showing that the aerial part of velvet bean at the doses of 200 and 400 mg/kg BW effectively exerted an anti-inflammatory effect in rats. In our study, prior to test, the mice were weighted and their left hind legs were marked using a permanent marker. Then, the initial volume of marked paw was determined by putting it into a measuring glass fully filled with distillated water. The volume of spilled water out of the glass was subsequently measured and presents as initial paw volume (V0), namely the volume of the mouse paw before the carrageenan injection and treatments. Thereafter, each mouse was injected intraplantar with 1% carrageenan as much 0.05 ml. After 60 minutes of carrageenan injection, the volume of edema of the paw was measured using a measuring glass as previously described. Furthermore, each mouse was given a suspension of the velvet bean leaf extract or diclofenac sodium orally according to the respective treatment groups. Eventually, the volumes of paw edema of the mice were determined at 1, 2, 3, 4, and 5 hours after being treated with the extract or diclofenac [27, 28].

5.5.1. Edema volume calculation

Edema volume was calculated using the formula [27]:

$$V_u = V_t - V_u$$

Information:
Vu : Volume of edema of the feet of mice at time t
Vt : Volume of mice feet after being induced by 1% carrageenan at time t
V0 : Initial volume of mice feet before being induced by carrageenan

5.5.2. Calculation of AUC nilai value

The AUC value is the average area under the curve which is the relationship between the average edema volume per unit of time used in the formula [27]:

$$AUC_{tn-1}^{tn} = [\frac{(Vu_{n-1} + Vu_n)}{2}(t_n - t_{n-1})]$$

Information:
Vun-1 : Average edema volume at time tn-1
Vun : Average edema volume at time tn

5.5.3. Calculation of the percentage of anti-inflammatory power

The percentage of anti-inflammatory power (inhibition of edema volume) was calculated based on the percent reduction edema used in the formula [27]:

$$Antiinflammatory\ Percentage\ (\%) = \frac{AUC_k - AUC_p}{AUC_k} \times 100\%$$

Information:
AUCk : The mean AUC of the negative control
AUCp : The mean AUC of the treatment group

5.6. Analysis of the quantity of mice leukocyte components

At the end of the treatment, the mice were dislocated vertebrae. Then, the mice were dissected and blood was isolated from the heart. Blood hematology examination was carried out using an automatic hematology analyzer machine to determine the total leukocyte quantity (White Blood Cell) which includes lymphocytes, monocytes, and granulocytes. A 500 l whole blood sample is applied to the analyzer column and then the quantity of blood values is presented automatically on the monitor screen [29].

5.7. Content analysis of velvet bean leaf extract using GC-MS

A total of 1 µl of the velvet bean leaf ethanolic extract was injected into the GC-MS glass column. The gas carrier was high purity helium with a pressure of 45.1 kPa and a total rate of 81.1 ml/min and a split ratio of 1:100. The eluted component was detected on the mass detector. The spectral profiles were used to match the identity of the compound in the NIST library database [30].

5.8. Statistical analysis

The data were analyzed with SPSS 23 software (International Business Machines Corporation). The one-way analysis of variance (ANOVA) was deployed to elucidate the difference among groups of treatment, followed by a least significant difference (LSD) post-test.

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Author contributions: Concept – F.F., P.S.; Design – F.F.; Supervision – P.S.; Resources – R.R.; Materials – F.F.; Data Collection and/or Processing – F.F., R.R.; Analysis and/or Interpretation – F.F., P.S.; Literature Search – P.S., R.R.; Writing – F.F., P.S., R.R.; Critical Reviews – F.F., P.S., R.R.

Conflict of interest statement: The authors declared no conflict of interest.

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