Methanol Extract of *Katuk* (*Sauropus androgynus*) Leaves as an Anti-inflammatory Agent: Animal Study in Carrageenan-induced Rat Models of Inflammation

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**Background:** Inflammation is a response in the human body to survive during infection, injury and tissue damage. Acute inflammation causes edema and polymorphonuclear neutrophils (PMNs) release. *Sauropus androgynus* leaves which contain flavonoids, tannins, saponins, steroids and triterpenoids may have anti-inflammatory properties. These compounds can be extracted with methanol. This research aimed to determine the anti-inflammatory effect of *S. androgynus* leaves methanol extract (SALME) on carrageenan induced-rats.

**Materials and methods:** True experimental study was conducted using 12 Wistar rats. Rats were induced with carrageenan subcutaneously on the plantar pedis. Carrageenan-induced rats were treated with/without various doses of SALME. Edema volume was measured with a plethysmometer. The plantar pedis tissues were collected and stained with haematoxylin-eosin (HE) staining, then PMNs were observed and counted under a light microscope. All data were analyzed by one-way ANOVA, Bonferroni post hoc test, Pearson correlation and linear regression.

**Results:** SALME had significant effects on the volume of edema ($p=0.000$) and the number of PMNs ($p=0.000$). The most effective dose to reduce the edema volume and decrease the PMNs cell number was 37.80 mg/200 g body weight (BW). SALME doses may affect 91.0% of edema volume and 89.2% of PMNs cell number. Edema volume had a significant, robust correlation (92.3%) with PMNs cell number.

**Conclusion:** SALME is confirmed to have an anti-inflammatory activity by reducing the edema volume and decreasing the PMNs cell number.

**Keywords:** *Sauropus androgynus*, methanol extract, anti-inflammamatory, carrageenan, plantar pedis, rats

**Introduction**

Inflammation is a process that occurs in the body in response to harmful external stimuli, such as microbial infections and tissue damage, as well as other physiological conditions, including tissue healing. The acute inflammatory process is characterized by redness, edema, increase of temperature...
Acute inflammation causes changes in blood flow and vessel permeability, which are intended to increase the exudation of fluid and plasma proteins, and the emigration of leukocytes, predominantly polymorphonuclear neutrophils (PMNs) to injury site. Administration of anti-inflammatory drugs both steroid (corticosteroid) and non-steroidal (aspirin and acetaminophen) is one of the methods used to treat inflammation. Unfortunately, these drugs may cause several side effects. The most common side effects caused by short-term oral corticosteroid therapy in children are vomiting, behavioral changes, sleep disorders and increase the susceptibility to infections. In adults, the risk of developing various corticosteroid-related complications, such as infections, diabetes, osteoporosis, and psychiatric disorders increases with long-term exposure to oral corticosteroids. Aspirin is also known to be involved in acid-induced injury and may increase the risk of cardiovascular and cerebrovascular events.

Katuk (Sauropus androgynus) leaves are known to have antioxidant compounds. Phytochemical test using thin layer chromatography (TLC) shows that S. androgynus leaves contain flavonoids (flavones and flavonols), saponins, tannin gallate, steroids and triterpenoids. S. androgynus leaves extracted with maceration method have been reported to produce more extract yield compared to soxhletation. Methanol as a solvent has the advantage over the more commonly used 70% ethanol since methanol can extract the triterpenoid content. S. androgynus leaf extraction with methanol also results in higher antioxidant activity than other solvents, such as n-hexane and chloroform.

S. androgynus leaves methanol extract (SALME) may have anti-inflammatory properties since it contains flavonoid, tannin, saponin, steroid and triterpenoid. Higher intakes of flavonoids are associated with lower plasma concentrations of inflammatory biomarkers, such as soluble vascular cell adhesion molecule-1 (sVCAM-1) and interleukin (IL)-18. Steroids exert their anti-inflammatory effect by switching off several inflammatory genes. At low concentrations, triterpenes show an immunomodulatory activity by inhibiting the production of pro-inflammatory mediators, such as nitric oxide (NO), IL-6 and cyclooxygenase-2 (COX-2). The aim of this research was to determine the effect of SALME as an anti-inflammatory using carrageenan-induced rat models of inflammation. In this study, S. androgynus leaves were extracted using methanol as a solvent to yield more active compounds, particularly triterpenoids, which cannot be extracted with ethanol in previous studies. The parameters measured as indicators of inflammation in this study were edema volume and the number of PMNs. This study also determined the correlation between the volume of edema and the PMNs number.

Materials and methods

Preparation of SALME
S. androgynus leaves were obtained from Materia Medika Batu, Malang. Three hundred g of S. androgynus leaf powder was macerated three times using methanol at the ratio of 15% for 3 days at room temperature and concentrated using a rotary evaporator at 60°C. The resulting extract was stored at 4°C for further use.

Experimental Animals and Study Design
This research was a true experimental study using 12 male Wistar rats (180-200 g). Rats were acclimatized under controlled room temperature, fed with standard diet and water ad libitum for 7 days prior to the experiment. Rats were induced with 0.1 mL 1% carrageenan suspended in saline subcutaneously on the plantar pedis. Carrageenan-induced rats were then divided into 4 groups, which were control and SALME groups at dose 2.48, 6.15, and 37.80 mg/200 g body weight (BW). Each group contained three rat models of inflammation. SALME was administered orally to rat models 4 hours after fasting. This protocol was approved by Health Research Ethics Committee, University of Muhammadiyah Malang (number: E.5.a/147/KEPK-UMM/V/2018).

Edema Volume Measurement and PMNs Calculation
To measure the edema volume, plantar pedis of rats were inserted into the plethysmometer before and 4 hours after carrageenan injection. The rats were sacrificed under anesthesia and the plantar pedis tissues were collected and stained with haematoxylin-eosin (HE) staining. PMNs in plantar pedis were observed and counted by 2 observers (supervised by a pathologist) under a light microscope in 5 fields of view (averaged) with 400× magnification.

Statistical Analysis
All data were analyzed by one-way ANOVA, Bonferroni post hoc test, Pearson correlation and linear regression using SPSS version 24 (IBM, Armonk, NY, USA).
Results

Administration of SALME decreased the average of PMNs cell number in plantar pedis tissue of rats in a dose-dependent manner (Figure 1). One-way ANOVA test results showed that SALME had significant effects on the volume of edema ($p=0.000$) and the number of PMNs ($p=0.000$). PMNs were the most abundant in the control group (Figure 1A). The groups that received 2.48 and 6.15 mg/200 g BW of SALME had fewer PMNs (Figure 1B, Figure 1C). Meanwhile, the group that received 37.80 mg/200 g BW SALME had the least number of PMNs (Figure 1D). The increase in SALME doses also reduced the volume of edema. Bonferroni post hoc test results showed that 37.80 mg/200 g BW SALME had the most significant effect on the volume of edema and the number of PMNs (Figure 2, Figure 3). R² values suggested that 91.0% edema volume could be affected by SALME doses. Meanwhile, 89.2% PMNs cell number could be affected by SALME doses (Table 1). There was a strong (92.3%), significant ($p=0.000$) correlation between the PMNs cell number and the volume of edema.

Discussion

According to a previous study, SALME has a half-maximal inhibitory concentration (IC₅₀) of 80.81.¹⁴ This value is lower compared to the S. androgynus leaves extracted with 80% (IC₅₀ =813) and 96% ethanol (IC₅₀ =1,024).¹⁵ The IC₅₀ value of a compound is inversely proportional to its antioxidant capacity. Lower IC₅₀ values indicate stronger antioxidant activity.²⁰

Extracts obtained from several plant and fungi species have been reported to have anti-inflammatory properties.¹⁶⁻¹⁹ In this study, SALME had a significant effect on the volume of edema and the number of PMNs. SALME has been reported to contain flavonoids, saponins, tannin gallate, steroids and triterpenoids which have anti-inflammatory properties.⁵,²¹ Numerous studies show that the high-flavonoid diets can reduce inflammatory markers, such as C-reactive protein (CRP)⁹,²²,²³, IL-1β²⁴,²⁵, IL-8²⁶,²⁷, IL-18⁹, IL-6²⁸ and tumor necrosis factor-alpha (TNF-α).²⁹,³⁰ In addition, saponin extracts have been reported to reduce the volume of edema in the feet of rats that have been induced by carrageenan. The reduction in edema volume is thought

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*Figure 1. PMNs in plantar pedis tissue of carrageenan-induced rat models of inflammation.* A: Control group. B: SALME group at a dose of 2.48 mg/200 g BW. C: SALME group at a dose of 6.15 mg/200 g BW. D: SALME group at a dose of 37.80 mg/200 g BW. White bar: 100 µm.
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Steroids are known as anti-inflammatory agents that work through several pathways. The main function of steroids is to inhibit the activation of several inflammatory genes, including nuclear factor kappa B (NF-κB), a regulator of inflammatory genes. Triterpenoids have been known to have antioxidant and anti-inflammatory properties in both animals and humans. Previous study had shown that triterpenoids inhibit the production of pro-inflammatory mediators, such as TNF-α, NO, IL-6 and COX-2, as well as induce the production of anti-inflammatory cytokines, such as IL-10. In addition, triterpenoids show no potential cytotoxicity to macrophages.

Carrageenan has been known to be a potent inflammatory agent in rodents that induces the production of TNF-α. Inflammation caused by carrageenan upregulates the production of prostaglandins and COX-2, increases vascular permeability, and causes edema. Carrageenan has low toxicity and has not been shown to be teratogenic. Carrageenan-induced rat foot edema is widely used to determine anti-inflammatory activity, a simple and routine way to evaluate pain in the inflamed area without any injury or damage to the inflamed foot.

SALME at a dose of 37.80 mg/200 g BW had the most significant effect on the volume of edema and the number of PMNs. This result is slightly different from the result of a previous study which concludes that S. androgynus leaves extract at a dose of 48.6 mg/200 g BW is the best in decreasing TNF-α expression, improving histopathological features of duodenum (epithelium and villi), decreasing the number of inflammatory cells (neutrophils) and vasodilation of blood vessels.

Lower doses (37.80 mg/200 g BW) of SALME in this study had a significant result in decreasing the number of PMNs. This is consistent with the results of a previous study. SALME also decreased the edema volume. This is in accordance with the previous studies that flavonoids, steroids, saponins, tannins and terpenoids have anti-inflammatory properties, including reducing edema.

Higher intakes of selected subclasses of flavonoids (flavones and flavanones) is associated with a 20% modestly lower

Table 1. Linear regression results between SALME and edema, and SALME and number of PMNs.

<table>
<thead>
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<th>Model</th>
<th>R</th>
<th>R²</th>
<th>Adjusted R²</th>
<th>Standard Error of the Estimate</th>
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<tr>
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<td>0.910</td>
<td>0.900</td>
<td>0.05666</td>
</tr>
<tr>
<td>Number of PMNs</td>
<td>0.945a</td>
<td>0.892</td>
<td>0.882</td>
<td>2.54527</td>
</tr>
</tbody>
</table>

aPredictors: (Constant), dose.
plasma concentrations of inflammatory biomarkers, such as IL-1β as well as TNF-α and NO. Several mechanisms have been described regarding the anti-inflammatory activity of flavonoids. These compounds have free radical scavenging and antioxidative activities. They also regulate the activities of inflammation-related cells and enzymes which are involved in arachidonic acid metabolism (lipooxygenase, cyclooxygenase, phospholipase A2) and nitrogen monoxide synthase. Moreover, flavonoids regulate the expression of pro-inflammatory gene and production of other pro-inflammatory molecules. Triterpenoids as antioxidants are characterized by their abilities to remove free radicals and inhibit their enzymatic generation, as well as block the oxidation of cells and extracellular compounds.

The volume of edema had a strong, significant correlation with the number of PMNs. The cardinal characteristic of the inflammatory reaction is the emigration of leukocytes from small blood vessels into the injured tissues, causing leukocytes accumulation at the injury site. This emigration is due to vascular dilatation, which is followed by an increase in vascular permeability and exudation of PMNs-predominant fluid, leading to edema.

Conclusion

SALME is confirmed to have an anti-inflammatory activity by reducing the edema volume and decreasing the PMNs cell number in plantar pedis tissue. Edema volume has a strong correlation with PMNs cell number.

References

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