

The potency of bay leaves extract (*Syzygiumpolyanthum*) as anti-inflammation in *Rattus norvegicus* induced with Complete Freund's Adjuvant

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HIGHLIGHTS

Bay leaves extract (*Syzygiumpolyanthum*) can prevent inflammation in rat (*Rattus norvegicus*) induced with Complete Freund's Adjuvant (CFA)

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ABSTRACT

The using of herbal plants in Indonesia as medicine based on traditional knowledge. One of the herbal plants that believed has medication effect is bay leaves (*Syzygiumpolyanthum*). Bay leaves contain a flavonoid that can prevent inflammation. The study aims to prove the potency of bay leaves extract to prevent the inflammation, which is indicated by decreasing the paw edema thickness in rat induced with Complete Freund's Adjuvant (CFA). This study used 25 animal models of male rat (*Rattus norvegicus*) strain Wistar that divided into five groups, one group for a normal group (no treatment), one group for positive control which was injected with CFA sub plantar and given treatment with Na CMC and three test groups which is got three different doses of bay leaves extract peroral and the doses were 100 mg/kg BW, 300 mg/kg BW, 400 mg/kg BW, and injected with CFA into the sub plantar. Bay leaves extract was given one hour per oral before injected with CFA. The measurement of paw edema was done at 24 hours after CFA injection. The result of this study showed that bay leaves extract gave the anti-inflammation effect to the rats which were injected with CFA. It is concluded that bay leaves extract containing flavonoid plays a role in inhibit cytokines proinflammatory synthesis.

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1. INTRODUCTION

Inflammation is a response of the host to protect itself from infection and tissue damage which involve of immune cells and gathering of plasma protein to the area of infection and tissue damages by removing the injurious stimuli as well as initiate the healing process for the tissue and also vasodilatation of blood vessels.^{1,2} Inflammatory process involves a series of phenomena that can be triggered by various stimuli such as infectious agents, ischemia, antigen-antibody interactions and thermal or injury caused by other physical agents.³ This series of events leads to the release of damage-associated molecular patterns (DAMPs) then stimulate TLRs on macrophage inducing upregulation of cytokines TNF- α and IL-1 β by way of NF κ B activation.⁴ The inflammation is characterized with five cardinal signs namely redness, swelling, heat, pain, and loss of function.⁵

Bay leaves is mostly distributed in the tropic and subtropic area especially in Southeast Asia including Indonesia and Malaysia.⁶ Bay leaves usually use as a traditional medication for treating diabetes mellitus, hyperuricemia, hyper cholesterol, gastritis, hypertension, diarrhea and rheumatism.⁷ Bay leaves contain of tannin, flavonoid, alkaloid and essential oil whereas flavonoid has an active role as anti-inflammation by inhibiting of transcription factors such as NF- κ B.^{8,9} Previous study showed that bay leaves extract with concentration 200% could reduce the inflammation to the rats that were dipped with phenol 50%.¹⁰ Flavonoid based the chemical features are divided into subclasses: flavonols, flavones, flavanones, flavanols, anthocyanins, isoflavones, chalcones and dihydrochalcones.¹¹

Complete Freund's Adjuvant (CFA) is a mixture of paraffin oil and surfactant with heat-killed *Mycobacterium tuberculosis* or *M. butryicum* in which aqueous antigen solutions are emulsified.¹² CFA has been used to induce inflammation and study inflammatory pain in several animal models, and it attempts to mimic human conditions of persistent or chronic inflammatory pain. Role of CFA is concluded that adjuvant activity from sustain release of antigens from the oily deposit and stimulation of local innate immune response causing delayed hypersensitivity reaction that can react with intense inflammatory reaction and hyperalgesia at the site of injection.¹³ Therefore, this study aimed to prove the potency of bay leaves extract to prevent the inflammation, which is indicated by decreasing the paw edema thickness in rats induced with CFA.

2. MATERIALS AND METHOD

This study used adult male Wistar rats aged 12 weeks, weighing 200-250 gram, were acclimated one week before the experiment in a room with constant temperature (25-26 °C). Food and water were available *ad libitum*, and all the procedure were approved by Biochemical Laboratory of Medicine Faculty of Universitas Airlangga. This study was approved by the Ethical Committee of Dental Medicine Faculty, Universitas Airlangga 066/HRECC.FODM/III/2019. The data were analyzed by calculating mean and standard deviation.

Bay leaves derived and determined from Material Medika, Batu, Indonesia. Phytochemical screening test for bay leaves included was standardized of flavonoid. Stages to be performed were following: 500 g powder of bay leaves is macerated with 3L of ethanol 70% for 24hours, after maceration by mixing the processed bay leaves into rotary evaporator for 90 minutes (3 hours) then we get 370 mL of solution then added 8% of amyllum (29.6 gram) then we get 50 grams dry extract of bay leaves. From the extract has done Phytochemical screening to know the presence of flavonoid in this extract given.

The Phytochemical screening was performed through Shinoda's test. The one mg bay leaf extract is added with 0.5 mL of hydrochloric acid and magnesium metal, and then reddish colouration appeared to confirm the presence of flavonoid. Inflammation model in this study used CFA (Invivogen) 0.1 mL mixed with phosphate buffer saline (PBS) 0.1 mL. This solution was injected into right hind paw sub plantar. Animals were then divided into five groups (n = 5/group) named normal group/ no treatment (Kn), CFA-control group (Kk), CFA with peroral prevention of bay leaves extract group (Kp1, Kp2, Kp3) with dose 100 mg/kg BW, 300 mg/kg BW and 400 mg/kg BW. The bay leaves extract was given per oral one hour before CFA induction. The measurement of hind paw edema used digital calliper at 24 hours after CFA injection.

The mean of group kn recorded lowest amongst all because of no experimental method was applied. Group Kk with CFA injected and Kp1 with 100 mg/kg BW mean is higher as compared to others with CFA and bay leaf higher dose i.e. \geq 100 mg/kg BW subsequently we have to increase to dose to achieve the results. Consequently, dose increased to 300 and 400 mg/kg BW hence the

mean of group kp2 and kp3 are decreased. We can still evaluate the higher dose, i.e. ≥ 400 mg/kg BW, to achieve the maximum results. The dose 400 mg/kg BW is effective to inhibit the inflammation as compare to other compared groups in the study hence a future study is recommended to prolong the treatment and duration to observe the effects. We also applied Levene's test for homogeneity, i.e. one-way ANOVA in [table 2](#). There is a difference between the control group and treatment groups, because of non-homogeneity of data, we used Tamhane posthoc multiple comparisons. Tahmane test results show that CFA applied to group and treatment with dose 400 mg/kg BW has a significant effect determined by p-value, which is $p \leq 0.05$. Hence it can be concluded that there is a difference between the control group with treatment group having applied dose of 400 mg/kg BW.

3. RESULTS AND DISCUSSION

There are many studies discussing the inflammation and flavonoids¹⁴ and bay leaf and flavonoids¹⁵ and its role in diseases.¹⁶ The role of flavonoids and how to decrease inflammation by using it is discussed in details by Levya-Lopez.¹⁷ According to Levya-Lopez¹⁷ flavonoid can function in propelling the activation of NFkB so the synthesis of pro-inflammation cytokine, i.e. TNF- α can be inhibited. The flavonoids are also correlated with the structure-activity and have the ability to inhibit neutrophil degranulation.² Hence, we can assume that through neutrophil degranulation, we can identify inflammation easily. In view of discussion and explanation by Rathee² we can thus, assume that the current study hypothesises the functions of bay leaves as the anti-inflammation agent.

The measurement of hind paw edema thickness used a digital calliper and was done at 24 hours after CFA injection because subcutaneous injection in right hind paw of CFA induces a more prolonged swelling that becomes maximal at 24 hours and persists for at least seven days.^{18,19}

Table 1. Hind Paw Edema Thickness (in mm)

	Minimum	Maximum		Mean	
	Statistic	Statistic	Statistic	Statistic	Std. Error
kn	2.25	2.28	2.28	2.2620	0.00583
kk	5.49	5.70	5.70	5.5880	0.03904
kp1	4.69	6.01	6.01	5.3680	0.20958
kp2	3.71	5.42	5.42	4.9300	0.31056
kp3	4.48	4.94	4.94	4.7460	0.10127

Noted: Kn = (Normal);kk = (CFA Injected);kp1= (treatment with 100 mg/kgBW);kp2 (treatment with 300 mg/kgBW);kp3 (treatment with 400 mg/kgBW)

From the [Table 1](#) showed that the mean of each treatment group showed that all variances dose of bay leaves extract has the capability to inhibit the inflammation than a group of CFA with no treatment (Kk) and a group of kp3 showed the lowest mean than the other treatment groups.

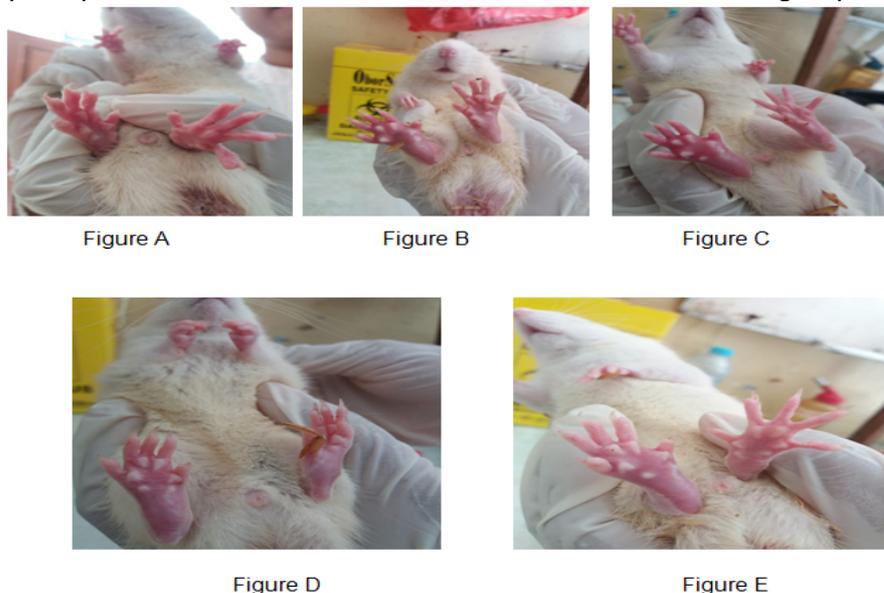


Figure 1. Hind paw edema of Wistar rats after 24 hours CFA induction

Noted:

Figure A: Hind paw of a normal rat with no treatment (Kn).

Figure B: Hind paw edema of rat with CMC Na and CFA induction (Kk).

Figure C: Hind paw edema of rat with bay leave extract with dose 100 mg/kg BW and CFA induction (Kp1).

Figure D: Hind paw edema of rat with bay leave extract with dose 300 mg/kg BW (Kp2).

Figure E: Hind paw edema of rat with bay leave extract with dose 400 mg/kg BW (Kp3).

From [figure 1](#) showed the inflammation of right hind paw on each group, from figure E showed less inflammation after 24 hours of CFA induction, it means that dose 400 mg/kg BW has a possibility to inhibit the inflammation than no treatment group (kp3)

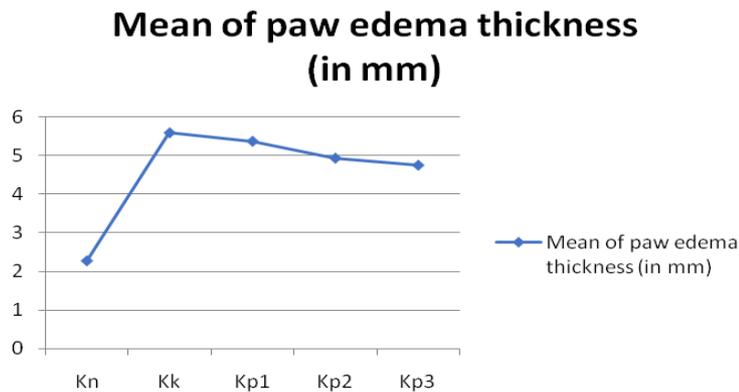


Figure 2. Graphic decreasing of paw edema thickness after 24 hours CFA injection

From [figure 2](#) showed that there is increasing of mean value from Kn group to Kk group, but it also shows that there is decreasing of mean value from Kk group and all variances dose of treatments and Kp3 group shows the lowest mean value than the other treatment groups.

Table 2. Test of Homogeneity of Variances (for treatment)

Levene Statistic	df1	df2	Sig.
2.954	4	20	0.045

The [table 2](#) is explaining homogeneity of variance for treatment through Levene's, and the result shows that there is no homogeneity noted in these groups. Thus, data was not homogeneous; we keep using posthoc multiple comparisons using Tamhane.

Table 3. ANOVA (Treatment)

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	35.801	4	8.950	58,810	0.000
Within Groups	3.044	20	0.152		
Total	38.845	24			

To see the difference between the group, we applied the One-Way ANOVA test, it shows the value of $p < 0.05$ indicates the significance of the current study, from [table 3](#) shows that between the CFA applied to group and treatment 400 mg /kg BW is significant with a value of $p = 0.005$ ($p < 0.05$), so that means it has a difference between the control group and the treatment group 400 mg /kg BW, from the result based on table 4 using Tamhane Post Hoc Multiple Comparison shows that there was difference result on each group of treatment with bay leaves extract (Kp1, Kp2, Kp3) compared with the CFA group (Kk) and the group of Kp3 showed the stronger inhibitory activity against inflammatory paw edema with value of $p = 0,005$ ($p < 0,05$). It means that dose 400 mg/kg BW is an effective dose to inhibit the inflammation as compare to other compered groups in the study.

Table 4. Post Hoc Multiple Comparison

Tamhane		Sig
Kk	Kn	0.000
	Kp1	0.988
	Kp2	0.656
	Kp3	0.005

CFA consist of mineral oil, containing a suspension of whole or pulverized heat-killed mycobacteria and its adjuvant activity results from the sustained release of antigens from the oily deposit and stimulation of a local innate immune response causing a delayed hyper-sensitivity reaction with an intense inflammatory reaction at the side of injection.¹³ A previous study showed that sub-plantar injection of CFA resulted in a significant elevation in IL-1B as a proinflammatory cytokine.²⁰ The paw edema thickness of treatment group (Kp1, Kp2, Kp3) when compared to normal group (Kn) still have big difference in value (figure 1) because subcutaneous injection of CFA induced inflammation is still in the acute phase (0-10 days) is caused by various mediators such as histamine, serotonin, kinins and prostaglandins, released by leukocytes that migrate to the affected region and provoked a vasculo-exsudatif phenomena responsible edema.²¹ When CFA was injected subplantar into paw, then it will make DAMPs forming to the protein of cell and cytoplasm. One of the receptor that binding to DAMPs is a *toll-like receptor* (TLR) then the inflammation cells which is inside the cytosol will activate MyD-88 then MyD-88 will activate TRAF-6 then TRAF-6 will activate IRAK-1 and IRAK-4, these 2 part will activate NFκB then NFκB will translocate to the cell nucleus to induce DNA to synthesize proinflammation cytokines like TNF-α and IL-1β.²²

The anti-inflammation action of bay leaves extract might be related to flavonoid as antiinflammation. The activity of flavonoid in the inflammatory response include the inhibition of transcription factor-like NFκB and activating protein-1 (AP-1).¹⁷ Flavonoid also inhibits of cyclooxygenase (COX) which has an important role as inflammatory mediator and is involved in the release of arachidonic acid that could be affected of starting point for general inflammatory response because it is a precursor for the biosynthesis of eicosanoids like prostaglandins and prostacyclin.² When the transcription factor is inhibited then it would be affected to the synthesis of pro-inflammatory cytokines like TNF-α, IL-1β and IL-6 and IL-8 and indicated with inhibiting leukocyte migration and edema formation.^{3,9,23}

4. CONCLUSION

Bay leaves (*Syzygiumpolyanthum*) extract with dose 100mg/kg, 300 mg/kg BW and 400 mg/kg BW were applied and found 400 mg/kg BW effective in preventing inflammation in rats with induced CFA. Bay leaves extract contains flavonoid that may inhibit NFκB, which has important role in cytokines proinflammatory synthesis like TNF-α dan IL-1β.

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CONFLICT OF INTEREST

We declared in this work, not any conflict of interest.

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