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Original Research Article

In vivo Anti-Inflammatory Activity of *Coleus atropurpureus* Leaves Extract and Fractions

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ABSTRACT

Inflammation is a normal body response to injury. However, inflammation also contributes to the progression of various diseases including cancer, atherosclerosis, asthma, obesity, and rheumatoid arthritis. Thus, the discovery of antiinflammatory agents remains chalenging. Medicinal plant is a potential source of drug discovery, including for antiinflammatory agents. One of the medicinal plants traditionally used for treating inflammatory diseases is *Coleus atropurpureus*. This study evaluated the *in vivo* anti-inflammatory effect of *Coleus atropurpureus* leaves extract and its fractions. Wistar rats were divided into 5 groups: negative control (solvent treatment), positive control (Diclofenac 9 mg/kg BW), ethanol extract (EE), *n*-hexane fraction (HF), and ethanol-water fraction (EWF). The extract and fractions were given at the same dose (45 mg/kg BW) 6 h prior to the induction of inflammation using carrageenan. Thin layer chromatography (TLC) analysis was done to identify the chemical components of the extract and fractions. The ethanol extract (EE), *n*-hexane fraction (HF), and ethanol-water fraction (EWF) reduced paw oedema thickness by 23.66, 19.01, and 20.80%, respectively, compared to the negative control. TLC analysis revealed the difference in the phytochemical content of the fractions. The HF mainly contained terpenoids, whereas the EFW contained flavonoids. This study demonstrated that EE, HF, and EWF of *C. atropurpureus* leaves have antiinflammatory activity in carrageenan-induced paw edema in rats.

Keywords: Coleus, Paw oedema, Inflammation, Thin layer chromatography.

Introduction

Inflammation is a body response to cell and tissue injuries affected by a various factors including microbial infection, chemical, thermal as well as mechanical inducers.[1,2] Inflammation-related disorders are characterized by pain, reddish colour and inflamed tissues. Drugs targeting inflammation are divided into steroidal anti-inflammatory drug (SAID) and non-steroidal anti-inflammatory drug (NSAID). Although many drugs from those groups have been approved, the need for discovery and development of novel anti-inflammatory agents is still promising to obtain more acceptable drugs.[3] Aspirin, the first generation of anti-inflammatory agent showed good efficacy but it retained the undesired side effects including gastrointestinal ulcerations, abdominal pain, and heartburn. Celecoxib was the first NSAID inhibitor of cyclooxgenase-2 (COX-2) inhibitor having less gastric disorders. However, it was gradually withdrawn from market due to the cardiovascular risks.[4] In another side, the use of corticosteroids was hampered by their undesirable side effects such as fluid retention, weight gain, fat deposits, and high blood pressure.[5] Therefore, the discovery of safe and effective anti-inflammatory agents is urgently needed. Natural product has been long time believed as one of the medications with lower toxic and adverse effects.[6]

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Many plants and their phytochemical constituents such as curcumin, colchicine, resveratrol, capsaicin, epigallocatechin-3-gallate, and quercetin demonstrated promising anti-inflammatory activity in various experimental models.[7] These included pre-clinical tests and clinical trials in humans, as well as mechanistic studies on the reduction in pro-inflammatory mediators such as leukotrienes and prostaglandins, inhibition of COX-2 and NFκB activities, and attenuation of pro-inflammatory measures such as CRP, IL-1β, IL-6, and TNF-α.[8,9] This indicated that medicinal plants provide various phytochemicals that are potential to be explored as anti-inflammatory agents.[10] *Coleus atropurpureus* is one of Indonesian herbal medicines locally known as "iler". This plant is traditionally used in Indonesia for the treatment of various diseases including inflammatory-related disorders. A scientific evidence is required to confirm the anti-inflammatory efficacy of the plant.

Previous study reported that *C. atropurpureus* contained flavonoid, saponin, polyphenol, and terpenoid in the leaves of *C. atropurpureus*.[11,12] The water extract of *C. atropurpureus* (at the doses of 100; 200; dan 400 mg/kg BW) demonstrated anti-inflammatory effect.[10] Another study showed the oedema reduction in rat paw oedema experimental model treated with 140 mg/kg BW of *C. atropurpureus* water extract.[12] In addition, a combination of the water extract of *C. atropurpureus* leaf with other herbal medicine, *Clitoria ternatea* flower at the dose of 328 mg/kg BW was able to reduce paw oedema with 9.1 mg/kg BW of Potassium diclofenac in mice.[11] In this study, the investigation of the anti-inflammatory activity of *C. atropurpureus* leaf was done by testing the ethanol extract (EE), *n*-hexane fraction (HF), and ethanol-water fraction (EWF) in carrageenan-induced paw oedema in rat.

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Materials and Methods

Plant material and Chemicals

The leaves of *C. atropurpureus* leaves were collected from the herbal garden of the Faculty of Pharmacy, Sanata Dharma University, Indonesia, in August 2017. The plant was authenticated by a botanist of the Faculty of Pharmacy, Universitas Sanata Dharma, Indonesia (Yohanes Dwiatmaka, M.Sc.) with the voucher specimen number 481/LKTO/far-USD/05/13. The organic solvents (*n*-hexane, ethanol, ethyl acetate) were purchased from Merck. The other chemicals used in this study were Cerium sulphate (Sigma), carrageenan (Sigma), Potassium diclofenac (Novartis), silica gel F254 (Merck).

Animal and ethics

The healthy male Wistar rats (150-200 g) were obtained from Imono Laboratory Animal House, Faculty of Pharmacy, Sanata Dharma University. All rats have the same treatment about their feeding and housing with a standard feed and water *ad libitum*. The animal house temperature was maintained at 22± 3°C with 30-70% in a relative humidity. The rats were maintained in a 12 h dark -light cycle. The use of animal was been approved by the Medical Health Research and Ethic Committee (MHREC) of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada–Dr. Sardjito Public Hospital, Indonesia (number: KE/FK/0209/EC/2020). All animals were acclimatized for two weeks prior to the evaluation of anti-inflammatory activity.

Extraction and fractionation

The extraction of the plant material was done using maceration method according to the previous study.[13] The fresh leaves (2.4 kg) were washed with flowing water and let in room temperature overnight. The leaves were then dried in the oven for 48 h at 50°C. The dried leaves (264 g) were powdered and then macerated in ethanol (1:10). After filtration, the macerate was evaporated in a rotary evaporator at 45°C under reduced pressure to obtain a concentrated crude extract (32.36 g). This crude extract (15 g) was fractionated by adding the mixture of *n*-hexane, ethanol, and water (1:1:2) in separating funnel and shaken intensively to obtain 2 separate layers: the upper layer or *n*-hexane fraction (HF; 8.84 g), and the lower fraction or ethanol-water fraction (EWF; 5.88 g).

Thin layer chromatography analysis

The extract and fractions were analysed using thin layer chromatography (TLC). For the detection of terpenoids, silica gel F_{254} and *n*-hexane-ethyl acetate (4:1) were utilized as the stationary and mobile phases, respectively. The spots were applied 1 cm from the bottom plate and eluted in TLC chamber with the 10 cm distance. The compound spots were visualized under UV254, UV366 nm, and derivatization using $CeSO_4$. For the detection of flavonoids, the TLC was done using the same stationary phase with *n*-butanol-acetic acid-water (4:1:5) as a mobile phase. The TLC spots were visualized under UV366 before and after derivatization using citroboric reagent.

In vivo study

The *in vivo* anti-inflammatory activity was done according to the previous study.[14] Before experiment, the rats were acclimatized and fasted for 24 h. The animals were randomly divided into 5 groups (5 rats per group): positive control (PC; diclofenac potassium), negative control (NC: solvent), HF, EWF and EE treatment groups. Carrageenan (0.5% w/v) was given through sub-plantar route to induce oedema. The doses of diclofenac potassium (9.1 mg/kg BW), FH, EWF, EE (each at 45 mg/kg BW), and distilled water were given orally. The oedema was measured every 30 min for six h post-carrageenan induction (0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 dan 360 min) using digital callipers. The oedema thickness was calculated using formula as follow:

$$Tu = Tt - To \quad (1)$$

Tu = the thickness of paw oedema at the certain time
Tt = the thickness of paw oedema post carrageenan induction
To = the thickness of paw oedema pre carrageenan induction

The area under curve (AUC) was measured every 30 min for 6 h after carrageenan induction and calculated using the trapezoid method as follow:

$$tn = \mathrm{Ttn} - 1 + \mathrm{Ttn} \quad (2)$$
$$UCtn - 1 = (tn - tn - 1) \quad (3)$$

Ttn-1 : the average of oedema volume at tn-1
Ttn : the average of oedema volume at tn

The percentage of inflammatory inhibition was calculated using this following formula:

$$Inhibition\ of\ Inflammation = \frac{(\mathrm{AUC_{0-x}})0 - (\mathrm{AUC_{n-x}})n}{(\mathrm{AUC_{0-x}})0} \quad (4)$$

(AUC0-x)0 = the average of AUC0-x for the negative control group, (AUCn-x)n = the AUCn-x for each animal given the tested samples at dose of n.

Statistical analysis

The data obtained from the anti-inflammatory activity was statistically analysed using Kolmogorov-Smirnov test, followed by Mann-Whitney ($p < 0.05$). The data were mean ± SD from 5 replicates ($n = 5$).

Results and Discussion

The herb of *C. atropurpureus* is an Indonesian traditional herbal medicine commonly used to treat inflammatory-related diseases. Scientific evidence is required to justify the traditional usage of the plant for inflammatory related diseases. The study investigated the anti-inflammatory activity of the extract and fractions of *C. atropurpureus* leaf in carrageenan-induced paw oedema in rat. The study also analysed the phytochemical profile of the extract and fractions using TLC. The phytochemical identification using TLC employing Cerium sulphate reagent resulted in the red to brown colour spots suggested that EE and HF contained terpenoids. Figure 1A showed that the spots at the Rf of 0.25-0.35 and at the Rf of 0.85-0.90 were typical of terpenoid compounds as they were not visible under UV254 and UV366 nm and they formed red to brown colour upon addition of Cerium sulphate reagent.[15] Apart from that, the EWF contained distinct phytochemical profile compared to the HF. Figure 1B clearly demonstrated that at least 3 flavonoids present in the HF indicated by the blue fluorescence spots at the Rf of 0.4, 0.48, and 0.6 after derivatization using citroboric acid reagent which indicated the presence of flavonoid compounds.[16] In the anti-inflammation activity assessment (Figure 2), HF, EWF, and EE were able to reduce paw oedema in 6 h of observation. The oedema of the negative control group was dramatically increased upon carrageenan injection (min 0 - 30). This increase was higher than the positive control, extract and fraction treatments. The oedema thickness of all treatment gradually declined as time of observation increase. However, the total oedema thickness of the positive control, extract and fractions were lower than the negative control. This indicated that HF, EWF, and EE demonstrated anti-inflammatory activity by reducing oedema thickness. As expected, Potassium diclofenac, the reference anti-inflammatory drug exhibited anti-inflammatory activity. Based on the AUC data, we calculated the percentage of anti-inflammatory activity of the extract and fractions compared to the negative control. Figure 3 showed that EE, HF, and EWF, significantly inhibited inflammation by reducing paw oedema by 23.66, 19.01, and 20.80, respectively, compared to the negative control (solvent-treated group). As expected, Potassium diclofenac, the positive control showed anti-inflammatory activity in this experimental model. Interestingly, the water-ethanol fraction and ethanol extract, but not *n*-hexane fraction showed a significant decrease ($p < 0.05$) in the paw oedema compared to the Potassium diclofenac. This indicated that EWF and EE have stronger anti-inflammatory activity than HF, and equal to Potassium diclofenac at the lower dosage.

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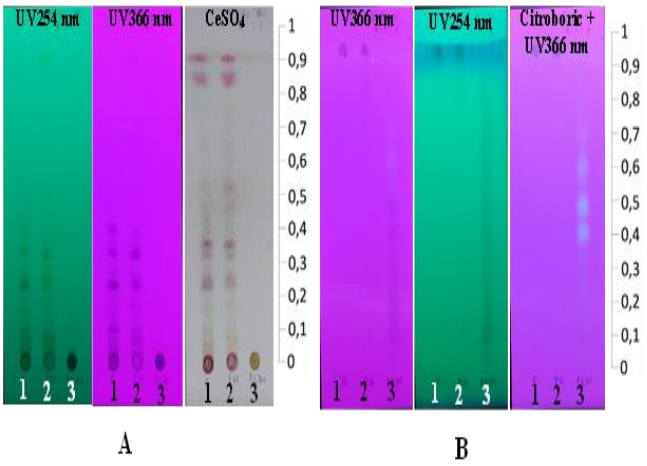

Figure 1: TLC profiles of the extract and fractions of *C. atropurpureus* leaves.
The ethanol extract of C. *atropurpureus* leaves (**1**) were fractionated using using a mixture of *n*-hexane, ethanol, and water with a ratio of1:1:2, yielding the *n*-hexane (upper) fraction (**2**), and ethanol-water (lower) fraction (**3**). **A**: detection of terpenoid compounds; **B**: Detection of flavonoid compounds. The TLC spots were visualized in UV254, UV366, and visible light (after derivatization using $CeSO_4$).

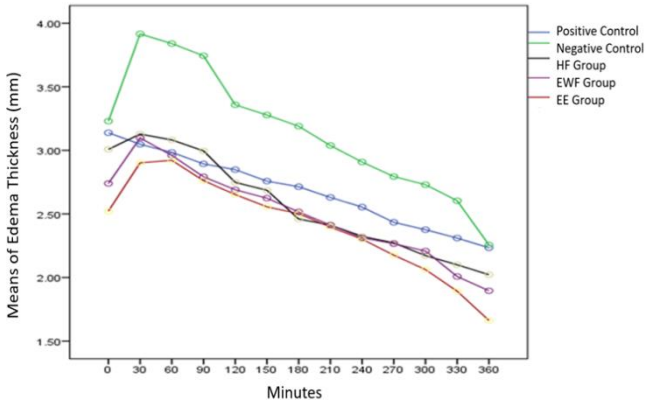

Figure 2: The *C. atropurpureus* extract and fractions reduced paw oedema in carrageenan-induced paw oedema in rat.
The paw oedema thickness was measured every 30 min for 6 h, and the AUC was calculated. HF: hexane fraction; EWF: ethanol-water fraction; EE: ethanol extract. The extract and fractions were tested at 45 mg/kg BW (n = 5).

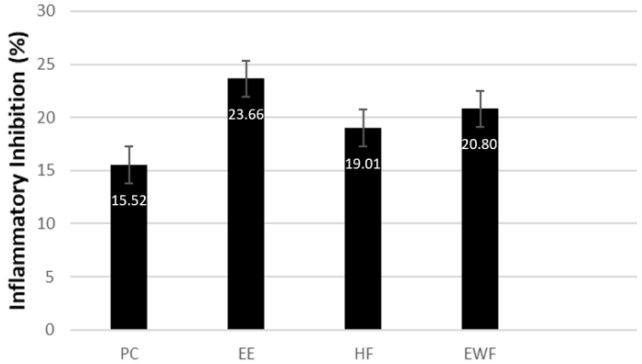

Figure 3: The percentage of inflammatory inhibition of the *C. atropurpureus* extract and fractions in carrageenan-induced paw oedema in rat.
EE: ethanol extract of *C. atropurpureus* leaves; HF: *n*-hexane fraction; EWF: ethanol-water fraction of the extract. Statistical analysis was done using Kolmogorov-Smirnov test, followed by Mann-Whitney test (p = 0.05).

The extract and fractions showed anti-inflammatory activities in carrageenan-induced paw oedema in rat. This method is simple and relatively safe without causing a serious injury or defect of the rat.[17,18] In addition, carrageenan-induced paw oedema is well-known for its sensitivity for the screening of anti-inflammatory agents.[19] Carrageenan is an irritant able of inducing cell injury and causing the release of inflammatory mediators. The oedema induced by carrageenan is a biphasic response. The first phase (after 1 h) is mainly caused by a neutrophils infiltration to the inflammation area along with the production of a pro-inflammation mediators such as prostaglandin (PGE), and a diverse cytokine. Theoretically, inflammatory peak is observed after 3-6 h and then gradually declines,[20] followed by the second late response after 72 h, and started to decline after 96 h.[21]
The study revealed that the extract and fractions of *C. atropurpureus* demonstsrated anti-inflammatory activity in carrageenan-induced paw oedema in rat. Previous study indicated that this plant contained flavonoids and phenolics. As flavonoids and phenolics have antioxidant activity, this could be crucial for the anti-inflammatory activity of the plant.[22,23] Phenolics, flavonoids, and terpenoids were reported to be able to scavenge free radicals produced after induction of carrageenan and to neutralize reactive oxygen species (ROS).[24] It is well known that carrageenan results in ROS generation that triggers an initial tissues injury leading to inflammation and tissues defects.[25] Antioxidants including plant extract might prevent this process by inhibiting the destructive effect of ROS. This could explain the anti-inflammatory activity of the ethanol extract, *n*-hexane fraction and water-ethanol fraction. Other mechanisms could be through modulation of pro-inflammatory mediators such as cAMP and histamine.[26] Further study focusing on influence of the extract and fractions on pro-inflammatory mediators might be required to confirm this mechanism of action.
The TLC analysis identified the major presence of terpenoids and flavonoids in the HF and EWF, respectively. EWF demonstrated stronger effect compared to HF. The TLC analysis (Figure 1A) showed that the extract and the HF but not EWF contained terpenoids (red to brown spots after spraying with Cerium sulphate reagent); whereas the EWF but not the HF contained flavonoids (Figure 1 B). The flavonoids were not initially detected in the extract due to their low level and they were detected after fractionation. Since both fractions showed anti-inflammatory activity, both terpenoids and flavonoids might contribute to the anti-inflammatory activity. Previous studies reported that *C. atropurpureus* contained terpenoid compounds (eugenol and thymol) that demonstrated analgesic, anti-irritant, anti-parasitic, and antiseptic effects.[27] Additionally, several flavonoids (isoflavones and flavones) with substituents at C5, C7, dan C4'[28,29] were also characterized from the leaves of this plant. Further investigation is needed to identify the most active compound responsible for the anti-inflammatory activity.

Conclusion

By using carrageean-induced paw oedema in rat, the results revealed that the ethanol extract of *C. atropurpureus* leaves and its n-hexane and ethanol-water fractions exhibited anti-inflammatory activity in a carrageenan-induced paw oedema in rat. The TLC analysis revealed that the extract and fractions mainly contained terpenoids and flavonoids.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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