Inhibition of Nitric Oxide Production in Lipopolysaccharide-induced Macrophages Cell by *Plectranthus scutellarioides* (l.) R.br Leaves

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Abstract
Inflammation is a certain tissue response against physical stimulation or harmful chemicals. This stimulus releases inflammatory mediator such as histamine and prostaglandin that causes reaction in the form of redness, pain, swollen and functional disorders. Inflammation is a protective response from the body, but if it goes on (chronic), it can cause damage to the tissue. Nowadays, the commonly used drug is the non-steroid anti-inflammatory group which has side effects of gastrointestinal and cardiovascular disorders. Hence, there is a need to develop anti-inflammatory drugs derived from plants due to their less side effects.

One of the known medicinal plants which has been widely used by people is Plectranthus scutellarioides (L.) R.Br. This research was aimed to determine the anti-inflammatory activity of ethanol extract, ethyl acetate fraction and water fraction of Plectranthus scutellarioides (L.) R.Br leaves. The assay was conducted in vitro using nitric oxide (NO) inhibitory assay on lipopolysaccharide-induced macrophage cells. Nitric oxide concentration was determined using Griess methods.

The results showed that IC_{50} value of the ethanol extract, ethyl acetate fraction and water fraction of Plectranthus scutellarioides (L.) R.Br leaves were 79.46 µg/mL, 327.58 µg/mL and 286.58 µg/mL respectively. Thus, ethanol extract was the most potent in inhibiting nitric oxide in the macrophages cell.

Keywords: *Plectranthus scutellarioides* (L.) R.Br., anti-inflammatory, nitric oxide, macrophage cells, Griess method.

Introduction

An important molecule for host defense response against various pathogens such as bacteria, viruses, fungi and parasites is called Nitric oxide (NO).[1] NO plays an important role in the regulation of various pathophysiological processes such as vasodilatation, neurotoxicity and neuronal communication.[11,12] Overproduction of NO induces tissue damage associated with acute and chronic inflammations.[17] There are 3 isoforms of Nitric Oxide Synthase (NOS), namely endothelium NO synthase (eNOS), neural NO synthase (nNOS) which are constitutively expressed and inducible NO synthase (iNOS) specifically expressed in response to various pro-inflammatory cytokines such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS).[9,11]

Inflammation is a normal protective response to tissue injury with the aim of drawing plasma proteins and phagocytes to the site of injury or invasion to enable them to isolate, destroy or inactivate incoming agents. Clean debris and prepare the network for the healing process.[3] The iNOS enzyme is the most important pro-inflammatory enzyme because under inflammatory conditions, NO will be produced in large quantities that were a thousand times more by the iNOS found in macrophages.[14]

Macrophages is a mediator that can subsequently initiate and regulate inflammatory responses and detect pathogenic substances through pattern-recognition receptors.[10] A component from the cell walls of gram-negative bacteria and one of the most powerful activators to produce inflammatory mediators such as nitric oxide (NO) and other free radicals is lipopolysaccharide (LPS).[13] Therefore, inhibition of NO production in LPS-stimulated macrophage cells is one of the possible ways to screen various anti-inflammatory drugs.

Nonsteroidal anti-inflammatory drugs (NSAID) are the most widely used over-the-counter drugs as well as the most prescribed class of drugs for inflammation.[4] However, NSAIDs also have undesirable side effects including ulcers, bleeding, kidney failure and increased risk of heart attack and stroke.[8]

Plectranthus scutellarioides (L.) R.Br is a popular ornamental plant which belongs to Lamiaceae or Labiatae family. *Plectranthus scutellarioides* (L.) R.Br are widely grown in Indonesia, purple in color, a shrub ornamental plant and grows in the lowlands to an altitude of 1,300 m above sea level.[7]

People boiled the leaves and consumed the tea daily to cure various diseases.[15] This research was aimed to study the anti-inflammatory activity of *Plectranthus scutellarioides* (L.) R. Br leaves in lipopolysaccharide-induced macrophages cell.

Material and Methods

Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, USA), Fetal Bovine Serum (FBS) (Gibco, USA), penicillin and streptomycin (Sigma-Aldrich, USA), sulfanilamide (Merck), phosphoric acid (Merck), naphthyl-ethylenediamine (Sigma-Aldrich, USA) and lipopolysaccharide (LPS) were purchased from Sigma–Aldrich, USA.

Instrument: Rotary evaporator (Buchi, Switzerland), UV lamps λ_{366} dan λ_{254} (Camag UV-Betrachte, USA), microplate reader (Epoch Biotech, USA), micropipet (Socorex, Switzerland), digital scales (Mettler Toledo PL 601, Colombus, USA), oven (Memmert, Germany), waterbath (Memmert, Germany), laminar airflow (Robust, UK).

Leaves of *Plectranthus scutellarioides* (L.) R.Br: The fresh leaves of *Plectranthus scutellarioides* (L.) R.Br were identified and authenticated at Department of Biology, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Bandung, West Java, Indonesia with the document number 557/HB/11/2016.

Extraction and Fraction method: Extraction method was carried out in two steps. One kilogram of fresh leaves of *Plectranthus scutellarioides* (L.) R. Br was put into a macerator, ethanol 96% acidified by concentrated HCl 0.5% was added into the macerator until all of the leaves soaked, repeat the maceration up to 3 times and change the mixture every 24 hours. Then, evaporate and concentrate the resultant extracts in a rotary evaporator at 35-40°C until a concentrated extract was obtained.

The concentrated extract of *Plectranthus scutellarioides* (L.) R.Br (PSE) was fractionated using ethyl acetate, n-hexane and water. Fifty grams of PSE were dissolved into 250 mL of water and add n-hexane as much as water in separating tunnel, repeat until the fraction of n-hexane and water is completely separated. Then, the water fraction was fractionated using ethyl acetate, repeat until the fraction of ethyl acetate and water is completely separated. The ethyl acetate fraction (EAF) was concentrated in a rotary evaporator at 35-40°C and water fraction (WF) was concentrated by freeze dry -80°C until a viscous fraction was obtained.

Cell culture: The isolation of macrophages-cell from peritoneum of mice was diluted in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin, streptomycin sulfate and Fetal Bovine Serum (FBS). The medium added was used as a test cell for *in-vitro* assay. 100 µL cells (9x106 / mL) were distributed into each well of a 96-well plate ($9x10^6$/mL), incubated for 12 hours in humidified atmosphere of 5% CO_2. After that, the medium was removed, then add PSE, EAF and WF in concentration of 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL dan 12,5 µg/mL respectively as sample, incubate at room temperature for 1 hour, add 1 µg / mL LPS and incubate for 24 hours at atmosphere of CO_2 moisture. After that, 100 µL of supernatant was collected for nitrite assay.

Nitrite determination: This method was performed as described previously by Yun et al1[8] with slight modification. Indicator of Nitric Oxide (NO) production was measured by quantity of nitrite in the culture medium. Amounts of nitrite were measured. First, mix 100 µL of cell culture medium with 100 µL of Griess reagent, then incubate for 10 minutes at room temperature. The absorbance was measured by microplate reader at 540 nm. Fresh culture medium was used as a blank and the sodium nitrite standard curve was used to determine the quantity of nitrite.

Results and Discussion

Extraction and fractionation: About 1 kg of fresh *Plectranthus scutellarioides* (L.) R.Br leaves were soaked in a mixture of 96% ethanol solvent and 0.1% HCl of total ethanol for three times in 24 hours. Time of 24 hours and room temperature were the effective time and temperature for the solvent to be trapped into the cell.[5] This solvent was used based on the general principle of maceration, which is "like dissolves like" which means the solubility of a substance is based on its polarity. Meanwhile, HCl in ethanol was used for plant cell membrane denaturation, then dissolve the anthocyanin pigment out of the cell. The anthocyanin pigments were soluble in ethanol because they were equally polar.[2] The concentrated extract has a dark red color and the yield of extract is 8.625%. The yields of n-hexane, ethyl acetate and water fraction were 0.04%, 2.16% and 5.22% respectively.

Phytochemical screening of *Plectranthus scutellarioides* (L.) R.Br extract: The *Plectranthus scutellarioides* (L.) R.Br extract was qualitatively tested using phytochemical screening for the presence of secondary metabolite (table 1).

Anti-inflammatory Activity Assay:

Nitrite Standard Curve: Nitrite standard curve was made with an absorbance determination of known concentration of NO solution. Absorbance determined was plotted into the curve. NO concentrations used were: 0.125 µg/mL; 0.25 µg/mL; 0.5 µg/mL; 1 µg/mL; 2 µg/m; 4 µg/mL; 6 µg/mL; 8 µg/mL and 12 µg/mL. The reaction between nitrite and Griess reactor would produce colored diazonium so that the absorbance can be measured at 540 nm. The sodium nitrite standard curve was used to determine the quantity of nitrite (figure 1).

NO Concentration Determination in Macrophage cell: This research used concentration of ethanol extract, ethyl acetate fraction and water fractions as 200 µg / mL, 100 µg / mL, 50 µg / mL, 25 µg / mL and 12.5 µg / mL respectively. After treatment, the absorbance value was plotted into the equation obtained from NO standard curve

to get the result of NO values. The results of anti-inflammatory activity assay showed that extract, ethyl acetate fraction and water fraction were alleged to have the activity of inhibiting NO formation in LPS-induced macrophage cells characterized by significant decrease of NO concentration when compared with LPS control (table 2 and figure 2).

From the data in figure 1, the percent inhibition of NO (%) production was calculated by activity of PSE, EAF and WF (table 3).

The data from table 2 indicate that the ethanol extract of *Plectranthus scutellarioides* (L.) R.Br leaves was allegedly anti-inflammatory based on the decreasing amount of NO concentration in response to inflammation occurring after induction with lipopolysaccharide (LPS). Table 2 also showed the ability of PSE, EAF and WF at the concentration of 200 µg / mL having a highest inhibition compared with other concentrations. In addition, the data also showed that ethanol extract of 200 µg/mL was the best concentration sample in inhibiting the occurrence of NO secretion; after induction, the percentage of NO inhibition was 62,24%. From the data of NO inhibition on some concentrations, the determination of the best concentration or value of IC_{50} (inhibition concentration) could be calculated (table 4).

IC_{50} is a concentration that can inhibit or reduce anti-inflammatory in 50% by entering the number 50 as y in the existing equation. The result showed that IC_{50} of ethanol extract value is the best sample. Potential as an anti-inflammatory drug candidate is better if IC50 values are smaller because only low doses were required to inhibit nitric oxide in macrophage cells by 50%.

Nitric Oxide (NO) is a mediator that plays an important role in the inflammatory process. One of the isoforms produced through nitric oxide synthase (NOS) is inducible nitric oxide synthase (iNOS) which produces large amounts of NO during the body's defense processes and inflammation. High concentration of NO could have a detrimental effect on cell metabolism and induce DNA fragmentation. In addition, increased permeability of blood vessels caused by NO could enhance pro-inflammation[16] so that NO inhibition can be used as one way to overcome inflammation.

One of the most important chemical constituents in *Plectranthus scutellarioides* (L.) R.Br leaves was flavonoids. Based on the screening content results in leaves and *Plectranthus scutellarioides* (L.) R.Br, extracts were positive to have flavonoid content. There was a study that suggests that the flavonoid content in plants has a cellular mechanism as anti-inflammatory. Recent studies also showed that certain flavonoids, especially flavones, have anti-inflammatory activity in modulating pro-inflammatory gene expression such as cyclooxygenase-2 (COX-2), nitric oxide (NOS) and some important cytokines.[6]

Table 1
Phytochemical screening of *Plecranthus scutellaroides* (L.) R. Br

Secondary metabolite	Leaves	Concentrated Extract
Alkaloid	-	-
Polyphenols	+	+
Tannin	-	-
Flavonoid	+	+
Kuinon	+	+
Saponin	-	-
Monoterpenoid/ Sequiterpenoid	-	-
Steroid dan Terpenoid	-	-

+ : detected - : undetected

Table 2
NO Production Level of Ethanol Extract, Ethyl Acetate Fraction, Water Fraction

S. No.	Concentration (µg/mL)	NO Production (µM) ± SD		
		PSE	EAF	WF
1	12.5	5,49 ± 0,20	5,62 ± 1,02	7,00 ± 0,25
2	25,0	4,85 ± 0,24	5,01 ± 0,06	6,23 ± 0,25
3	50,0	4,53 ± 0,47	4,79 ± 0,17	5,33 ± 0,11
4	100,0	3,57 ± 1,06	4,47 ± 0,22	4,69 ± 0,69
5	200,0	2,90 ± 0,15	4,12 ± 0,06	4,40 ± 0,42
LPS	1 µg/mL	7,673 ± 0,19		

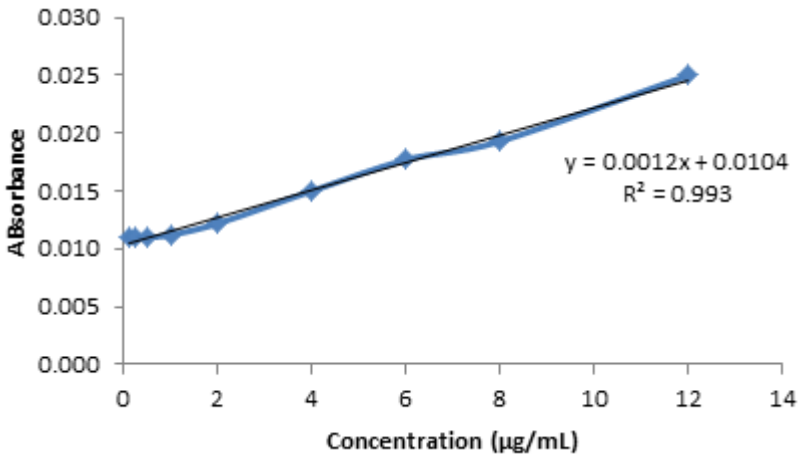

Figure 1: Sodium nitrite standard curve with Griess method

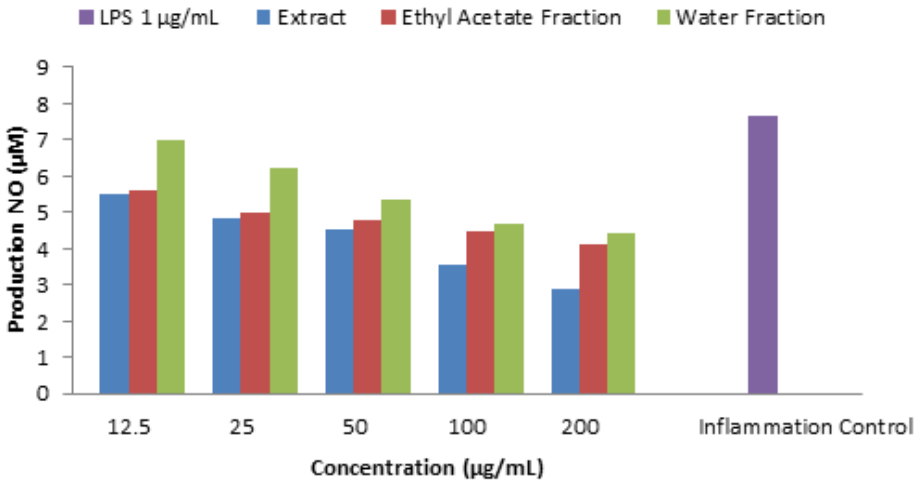

Figure 2: The graph of Inhibition of NO secretion of Ethanol Extract, Ethyl Acetate Fraction, Water Fraction

Table 3
Percentage of NO Production Inhibition by Ethanol Extract, Ethyl Acetate Fraction, Water Fraction of *Plectranthus scutellarioides* (L.) R. Br on LPS Induced Macrophage Cells.

S. No.	Concentration (µg/mL)	NO Inhibition Concentration Mean (%) ± SD		
		PSE	EAF	WF
1	12.5	28.40 ± 1.91	26.73 ± 0.72	8.77 ± 5.46
2	25	36.76 ± 13.86	34.67 ± 2.89	18.80 ± 9.04
3	50	40.94 ± 6.18	37.59 ± 2.17	30.49 ± 1.45
4	100	53.47 ± 3.15	41.77 ± 0.72	38.85 ± 3.32
5	200	62.24 ± 2.61	46.37 ± 13.26	42.61 ± 3.32

Table 4
IC_{50} values of extract ethanol, ethyl acetate fraction and water fraction on NO inhibition of induced LPS macrophage cells.

S. No.	Sample	IC_{50} (µg/mL)
1	PSE	79.46
2	EAF	327.58
3	WF	286.58

Conclusion

The *Plectranthus scutellarioides* (L.) R. Br leaves potentially inhibited the formation of nitric oxide (NO). Concentration with IC_{50} value for ethanol extract of *Plectranthus scutellarioides* (L.) R.Br (PSE) was 79.46 µg/mL, ethyl acetate fraction of *Plectranthus scutellarioides* (L.) R.Br (EAF) was 327.58 µg/mL and water fraction of *Plectranthus scutellarioides* (L.) R.Br (WF) was 286.58 µg/mL. Thus, ethanol extract is thought to be the best potential in inhibiting the occurrence of NO secretion.

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