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Original Article

Effect of *Clinacanthus nutans* Leaf Chloroform Extract on Reducing Nitric Oxide in Fibroblasts

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ABSTRACT

Background: *Clinacanthus nutans* or dandang gendis is a plant that can be an effective herbal treatment for inflammation, in addition the Indonesian people are still looking for alternative treatments. Research shows that the antiinflammatory properties of *C. nutans* leaves are effective in reducing inflammation against fibroblasts. Nitric oxide (NO) is one of the inflammatory mediators, that are released by fibroblast when inflamed. **Objective:** To observe the effect of *C. nutans* leaf chloroform extract on reducing NO in inflamed fibroblasts. **Methods:** Fibroblasts stimulated with *Escherichia coli* lipopolysaccharide were treated with chloroform extracts of *C. nutans* leaves at concentrations of 25, 50, 100, and 200 µg/mL for 24 h, then a NO inhibition test was performed. **Result:** The *C. nutans* leaf extract at a concentration of 200 µg/mL resulted in significantly ($P < 0.05$) lower NO production than the negative control. **Conclusion:** *C. nutans* leaf chloroform extract has the effect of reducing NO in fibroblasts. This extract has the potential as an antiinflammatory agent.

Keywords: *Dandang gendis (Clinacanthus nutans), fibroblasts, nitric oxide*

Background

Inflammation, a biological reaction of the immune system, may be caused by pathogens, injured cells, and poisonous substances.[1] Inflammation is the result of prolonged exposure to stimulation or an inappropriate reaction to the cell's molecules and may result in an acute or chronic phase, where tissue damage and fibrosis may occur.[2] The acute phase is when inflammation occurs for a short time and functions as the body's defense against infection—where immune cells migrate to the injured area, facilitated by soluble mediators, such as cytokines, chemokines, and proteins. If the inflammation occurs repeatedly, it progresses to the second stage—chronic inflammation, which lasts for a long period and can trigger various chronic diseases, such as arthritis, cancer, cardiovascular disease, diabetes, neurological disease, and respiratory disease.[3] Inflammatory conditions depend on the severity, chronicity, and mechanisms involved in the process, as well as the species and capacity of the individual's immune system to respond and adapt. Inflammation has a role in the development of several diseases.[4] Most human diseases have an inflammatory component and inflammatory mediators, such as inducible nitric oxide synthase (iNOS), which produces nitric oxide (NO) from l-arginine.[5] NO is a reactive gas molecule that has several functions in biological systems, depending on its concentration. At low concentrations, NO functions as a signaling molecule, whereas at high concentrations, it becomes highly toxic due to its ability to react with several cellular targets.[6] NO is a potent vasodilatory and antiinflammatory signaling molecule.[7] NO is also

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an inflammatory mediator[8] formed by iNOS, which has many homeostatic functions and plays an important role in inflammation. Astrocytes and microglia in an inflamed brain produce high NO levels during inflammation. When inflammation occurs, NO can cause vasodilation and increased leukocyte adhesion.[9] There are several nitrovasodilator compounds capable of releasing NO, such as glyceryl trinitrate, isosorbide dinitrate, sodium nitroprusside, and s-nitroso glutathione.[10]

Fibroblasts are cells in connective tissue that play a major role in wound healing. When fibroblasts are injured, NO is secreted and provides an antiinflammatory effect.[11] When a tissue experiences an inflammatory state, fibroblasts move toward the wound to proliferate and synthesize collagen and an extracellular matrix, and then a new epithelium forms on the mucosa to cover the wound. Fibroblast proliferation indicates rapid healing.[12] Fibroblasts also assume the role of inflammatory cells and recruit leukocytes, promote angiogenesis, and enable chronic inflammation in tissues.[13]

Clinacanthus nutans is a plant that belongs to the family Acanthaceae. This plant has antiinflammatory properties,[14] and the leaf extract can be an effective alternative herbal treatment.[15] *C. nutans* has attracted worldwide attention for its hidden therapeutic potential.[16] The biological activities of *C. nutans* include antioxidant, antiinflammatory, anticancer, antidiabetic, antiviral, antimicrobial, and antiproliferative effects. As such, it is used to treat several antiinflammatory conditions.[17]

Research on the effect of *C. nutans extracts* on NO in fibroblasts has not been conducted. Therefore, this study aimed to investigate the effect of the chloroform extract of *C. nutans* leaves on NO production in fibroblasts.

Materials and Methods

This study was an *in vitro* laboratory experimental research to investigate the effects of *C. nutans* chloroform extract on NO in fibroblasts. The study location was the BioCore Laboratory, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia.

The leaves of *C. nutans*, obtained from the Indonesian Spice and Medicinal Crops Research Institute, Bogor, Indonesia, were washed and dried. Then, the extraction stage was performed by the maceration method using 85 g of *C. nutans* leaf powder and 425 mL of chloroform solvent with a ratio of 1:5 at room temperature. Periodic agitation was performed during maceration for 3 days (every 15 min for 8 h/day). Then the suspension was filtered using Whatman filter paper no. 1, and the filtrate was evaporated using a rotary evaporator to separate the chloroform from the extract at a set temperature of 61°C. This procedure was repeated three times.

Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (Gibco, New York, NY, USA) supplemented with 20% fetal bovine serum (Gibco), and 1% amphotericin B/1% penicillin–streptomycin (Invitrogen, Waltham, MA, USA) at 37°C in a 5% CO_2 incubator. The fibroblasts were subcultured when the cells reached 80%–90% confluency in the flask.

Fibroblasts were incubated for 24 h at 37°C and 5% CO_2. The medium was then aspirated, and cells were stimulated with 2 µg/mL of *Escherichia coli* lipopolysaccharide (LPS, 0111:B4, Sigma–Aldrich, Burlington, MA, United States) for 24 h. Then the medium was replaced with various concentrations of the extract (25, 50, 100, and 200 µg/mL) and genistein 1000 µM as a positive control. The concentration of nitrite, a stable metabolite of NO, in the supernatant, was measured as an indicator of NO production using an NO detection kit (Griess Reagent System, Promega, Madison, WI, USA), according to the manufacturer's instructions. The 96-well plate containing 50 µL of supernatant and Griess reagent was incubated at room temperature for 10 min. The absorbance was then measured in a microplate reader at a wavelength of 490 nm. The nitric concentration is determined from the standard sodium nitrite curve.

The data obtained will be presented as mean ± standard deviation. The results of the detection of NO secretion in fibroblasts, which were analyzed using the NO detection kit, were then tested for normality using the Shapiro–Wilk method. If the results of the normality test showed that the distribution of the data was not normal, a nonparametric test was conducted. If the results of the test showed normal data ($P > 0.05$), then the analysis proceeded to a one-way Analysis of variance test. If the results showed a significant difference ($P < 0.05$), then Tukey's *post hoc* test was indicated.

Results

In this study, the variables used were *C. nutans* leaf chloroform extracts with respective concentrations of 25, 50, 100, and 200 µg/mL. The negative control was fibroblasts with 2 µg/mL LPS *E. coli* added without treatment, whereas the positive control used Genistein 1000 µM. Each variable was tested for its ability to determine the effect of *C. nutans* leaf chloroform extract on inflamed fibroblasts.

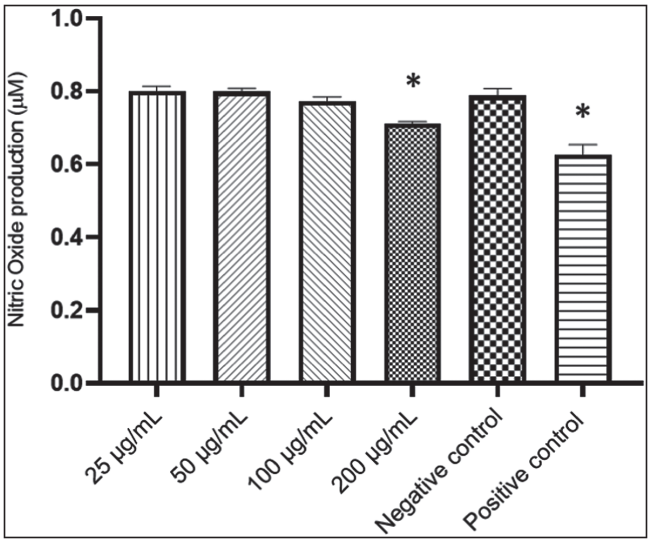

Figure 1: Bar graph of nitric oxide inhibition test results with the chloroform extract of *C. nutans* leaves against inflamed fibroblasts. *Indicates a significant difference from the negative control ($P < 0.05$)

The results revealed that *C. nutans* at a concentration of 200 µg/mL, and the positive control group had a significant difference in NO production compared with the negative control group [Figure 1]. Other concentrations (25, 50, and 100 µg/mL) have no significant difference compared with the negative control group.

Discussion

The biological activities of *C. nutans* leaves are antioxidant, antiinflammatory, anticancer, antidiabetic and antiviral, antimicrobial, and antiproliferative effects.[18] In previous studies, *C. nutans* leaves indicated the presence of flavonoids.[19] In addition, the results of phytochemical tests performed in previous studies indicated that the chloroform extract of *C. nutans* leaves has the highest total flavonoid and phenolic content compared to extracts with other solvents.[20]

In this study, *C. nutans* leaf chloroform extract at a concentration of 200 µg/mL produced lower levels of NO compared with the negative control. The positive control group (Genistein 1000 µM) also produced lower NO compared with the negative control group. At concentrations of 25, 50, and 100 µg/mL, the extracts did not produce lower NO levels compared with the negative control. This implies that *C. nutans* leaf chloroform extract requires a minimum concentration of 200 µg/mL to reduce NO production in fibroblasts stimulated by *E. coli*.

A previous study indicated that *C. nutans* leaf chloroform extract with a concentration of 100 µg/mL can reduce NO production in macrophages.[19] However, in this study, NO production decreased at a concentration of 200 µg/mL. This difference may be due to variations in the cell types used; this study utilized fibroblasts, whereas the previous research used macrophages. Differences in extraction methods could also account for variations in results. The previous study employed the Soxhlet method for extraction, whereas this study used the maceration method. Variances in extraction methods can influence the compounds present in the extract.[19] In this study, fibroblasts were stimulated with 2 µg/mL LPS, whereas in the previous study, macrophages were stimulated with 100 ng/mL of LPS.[19] The difference in concentration is due to differences in the cells used. This difference in LPS concentration caused a difference in the amount of NO produced by cells.

The anti-inflammatory activity of *C. nutans* can be attributed to its flavonoid content. Flavonoids can inhibit the tyrosine kinase enzyme by binding to adenosine triphosphate in the active enzyme.[20] Inhibition of this enzyme can result in a downregulation of various proteins, such as NO synthase, cyclooxygenase, and lipoxygenase, which can stop the proliferation of inflammatory cells and inhibit inflammation.[21] The phenolic compounds contained in the chloroform extract of *C. nutans* leaves also play an active role as an antiinflammatory. The antiinflammatory activity of its natural compounds exerts its biological properties by blocking two main signaling pathways: the nuclear factor kappa B (NF-kB) and mitogen-activated protein kinase pathways, which play a major role in the production of various proinflammatory mediators.[22]

In addition, the phenolic compounds in the chloroform extract of *C. nutans* leaves also have the highest levels of antioxidant activity.[23] In a normal and healthy body, there is a balance between the formation of reactive oxygen/free radicals and endogenous antioxidant defense mechanisms. However, if this balance is disturbed, it can cause oxidative stress, which in turn can result in injury to all cellular components (such as DNA, proteins, and membrane lipids) and cell death. Antioxidants based on natural compounds play a preventive role in protecting against the formation of free radicals. Antioxidant compounds can inhibit and prevent oxidation processes that can produce free radicals and break chains that can damage cells and tissues; hence, these compounds can be used as a therapeutic option for inflammation.[24]

In general, antiinflammatory properties can suppress the NF-kB pathway and the production of iNOS and proinflammatory cytokines, such as interleukin-1β

and tumor necrosis factor alpha.[25] Inflammation is prevented by the binding of LPS to toll-like receptor 4 (TLR4). Simultaneously, LPS and TLR4 are catalyzed by lipopolysaccharide-binding protein (LBP) and cluster of differentiation 14 (CD14). After LPS is released, it LPS binds to LBP to form LPS-LBP and targets CD14 on macrophages. LBP catalyzes LPS to CD14 so that LPS combines with CD14, which transfers LPS to TLR4 to then activate the TLR pathway and be directed at inflammatory factors. LPS that binds to TLRs can be neutralized by adenosine monophosphate and can inhibit the release of inflammatory factors.[24] Another antiinflammatory mechanism is the inhibition and regulation of inflammatory-related signaling pathways. Signal transduction is dependent on a toll-interleukin-1 receptor (TIR) domain-containing adapter protein (TRIF/TIR domain-containing adaptor-inducing interferon-β) associated with endocytosis of activated TLR4. LPS binding to TLRs activated by TLR4 can also activate the NF-kB and mitogen-activated protein kinase pathways, where inhibition of TLR4 endocytosis can be performed to suppress inflammatory pathways.[26]

In a previous study, *C. nutans* extracts showed positive results on a fibroblast migration assay compared to *Strobilanthes crispus* extracts. Chloroform extract of *C. nutans* has also been demonstrated to accelerate healing, including in inflammatory processes, compared with acetone and ethanol extracts. However, *C. nutans* extracts showed less antimicrobial activity against *Staphylococcus aureus*.[27] In addition, previous studies have shown that chloroform extract of *C. nutans* is the most active at inhibiting NO production compared with hexane and ethanol extracts.[19] In addition, chloroform extracts of *C. nutans* and aloe vera can upregulate the synthesis of basic fibroblast growth factor (bFGF) in wound healing.[28] Another previous study showed that *C. nutans* leaf could increase fibroblast count in mucosal burn and tongue wounds *in vivo*.[29] This proves that the chloroform extract of *C. nutans* leaves is more effective in reducing or inhibiting inflammation.

During inflammation, macrophages and fibroblasts are the main source of NO synthesis.[30] NO has a small size and lacks charge, so the penetration of NO molecules through cell membranes and subcellular organelles is high. Moreover, NO can be transferred by S-nitrosothiols through cells with protein binding. Therefore, when inflammation occurs, NO is not only in the cells where it is produced but also in the surrounding cells. Among the immune cells that are involved in inflammation, macrophages synthesize the most NO.[31] Several other factors influence the effect of the chloroform extract of *C. nutans* leaves on inflamed fibroblasts, such as differences in the metabolite compounds contained in the extract, the amount of extract concentration used, and the extraction method.[32,33]

The limitation of this study was the use of a limited concentration range. If concentrations above 200 µg/mL and up to 1000 µg/mL were used, we could have determined definitively whether 200 µg/mL is indeed the minimum concentration needed to reduce NO production in fibroblasts.

Conclusion

Based on the study results, the chloroform extract of *C. nutans* leaves at a concentration of 200 µg/mL has the effect of reducing NO synthesis in inflamed fibroblasts. This extract has the potential to be an antiinflammatory agent.

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Conflicts of interest

There are no conflicts of interest.

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