Dental Journal
Majalah Kedokteran Gigi

Dental Journal
(Majalah Kedokteran Gigi)
2018 September; 51(3): 124–128

Research Report

Potential immunomodulatory activity of *Phyllanthus niruri* aqueous extract on macrophage infected with *Streptococcus sanguinis*

Suryani Hutomo,[1] Denise Utami Putri,[2,3] Yanti Ivana Suryanto,[4] and Heni Susilowati[5]
[1]Department of Microbiology, Faculty of Medicine, Universitas Kristen Duta Wacana
[2]Doctoral Program, Faculty of Medicine, Universitas Gadjah Mada
[3]International PhD Program in Medicine, Taipei Medical University, Taipei, Taiwan
[4]Department of Physiology, Faculty of Medicine, Universitas Kristen Duta Wacana
[5]Department of Oral Biology, Faculty of Dentistry, Universitas Gadjah Mada
Yogyakarta - Indonesia

ABSTRACT

Background: *Streptococcus sanguinis is an oral commensal bacterium commonly found in periodontal lesions and deep abscesses that are usually dominated by anaerobic bacteria. As an important causative agent of systemic diseases, and with the increasingly numerous cases of antimicrobial resistance, some means of modulating the immune response to bacterial infection is thus necessary. Phyllanthus niruri Linn is widely used as a medicinal herb to both prevent and treat disease and demonstrates immunomodulatory properties.* ***Purpose:*** *This study aimed to observe the potential for aqueous extract of Phylanthus niruri to induce macrophage proliferation and NO production following S. sanguinis infection.* ***Methods:*** *Macrophages were isolated from the peripheral blood of healthy subjects, stimulated with P. niruri aqueous extract in graded doses and infected with S. sanguinis ATCC 10556 bacterial suspension. Cell proliferation and nitric oxide release was observed at 24 and 48 hours to determine macrophage activities.* ***Results:*** *NO production and cell proliferation started to increase upon 50 and 100µg/ml P niruri respective stimulation.* Statistical analysis using One-way Anova demonstrated a significant difference of cell proliferation after stimulation *with P. niruri aqueous extract at various doses* ($p<0.05$). ***Conclusion:*** *P. niruri aqueous extract induced macrophage proliferation and NO secretion upon S sanguinis infection, showing potential antibacterial and immunomodulatory activities. At the same concentrations, NO production and macrophage were higher at 48 hours than at 24 hours.*

Keywords: *Streptococcus sanguinis; macrophage; Phyllanthus niruri; medicinal plant; oral bacteria*

Correspondence: Suryani Hutomo, Department of Microbiology, Faculty of Medicine, Universitas Kristen Duta Wacana, Yogyakarta 55224, Indonesia, Email: suryanihutomo_drg@yahoo.com;

INTRODUCTION

Streptococcus sanguinis is an oral commensal bacterium belonging to the viridans streptococci group and one of the initial species to colonize tooth surfaces. Its role in oral disease is uncertain, but this species is often implicated in infective endocarditis and is, in fact, the most frequently involved.[1] Regular dental procedures can sometimes carry the risk of exposure to oral microorganisms in the circulatory system. These bacteria, either naturally commensal or pathogenic, may induce unexpected infection and inflammation, including infective endocarditis. Poor oral hygiene is another predisposing factor in bacterial endocarditis, although the bacteria can also enter the body through the daily diet. Infection is more likely in the presence of an abnormal heart valve such as that caused by congenital heart disease.[2,3]

Upon exposure to a microbial antigen, macrophage of the immune system serves as one of the initial defenders of the host. Macrophages play an important role in the immune system, not only as phagocytic cells, but also as antigen-presenting cells. As phagocytic cells, they function by direct engulfment and secretion of chemokines including reactive oxygen species (ROS) and nitric oxide

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v51.i3.p124–128

(NO) that destroy bacteria, viruses and parasites. They also process the engulfed antigen and introduce it into T cells, while simultaneously secreting pro-inflammatory cytokines which, together, will induce activation of the cellular immune response cascade, recruitment of other macrophages and further clearance of pathogen.[4–6] In an immunocompetent subject, the macrophage functions, further activating immunity, resulting in eradication of pathogens. However, in some patients with impairment of the immune system, prophylactic measures are often necessary to minimize the risk of systemic infection. In fact, prophylactic interventions are not always effective since previous studies have reported multiple cases of resistance, including that of *Streptococcus viridans* to both penicillin and fluoroquinolone.[7,8] Therefore, the application of antibiotic agents should be minimized and every attempt to modulate the immune response to harmful pathogens be made.

Phyllanthus niruri Linn, is a tropical plant commonly found in South East Asia, India, China and the USA. In Indonesia, known as *Meniran,* it is widely used as a medicinal herb to both prevent and treat diseases. Plants from the genus Phyllanthus have been shown to have anti-bacterial and anti-viral effects, including against *Staphylococcus aureus* and *Streptococcus agalactiae*.[9–11] In addition, extract of *P. niruri* has been observed to induce macrophage activity by increasing phagocytosis and NO in mice macrophage infected with *Salmonella typhii*[12] and in the macrophages of tuberculosis patients.[13] Despite numerous studies in elucidating the medicinal use of *P. niruri*, little is known about its antibacterial potential in relation to human commensal bacteria, as well as its effect on human macrophage. Therefore, in this study, human macrophages from the peripheral blood of healthy subjects were extracted in order to observe the effect of *P. niruri* aqueous extract on macrophage proliferation and NO production on *S. sanguinis* infection. From this study, it was expected to gain new insights into the potential use of *P. niruri* as an immunomodulatory agent in the management of infections caused by *S. sanguinis.*

MATERIALS AND METHODS

Streptococcus sanguinis was prepared as in our previous study.[14] The bacteria was grown in Brain-Heart Infusion Broth (BHI; Difco Inc., Detroit, MI) under microaerobic conditions at 37°C. A bacterial stock suspension was prepared at a concentration of $1.5x10^8$ colony forming units (CFU). Dried *Phyllanthus niruri* leaves were obtained and confirmed by a botanical expert at the herbal manufacturing company, CV Merapi Farma, Yogyakarta. The dried leaves were made into an aqueous extract by means of a maceration technique at the Integrated Research and Testing Laboratory, Universitas Gadjah Mada, Yogyakarta. Extract in paste form was dissolved in dimethyl sulfoxide (DMSO) solution to form a stock which was then diluted with culture medium to yield concentrations of 25, 50, 100, 200 and 400 µg/mL Peripheral blood mononuclear cells (PBMC) were isolated from the peripheral venous blood of three healthy subjects. Mononuclear cells were isolated by Ficoll-gradient density separation, as described above.[13] After centrifugation, the PBMC layer containing lymphocytes and monocytes weas transferred to a RPMI 1640 (Sigma) culture medium with 10% FBS, 2% PenStrep, and 0.5% Fungizone. PBMC numbers were calculated using a 1:20 dilution of tryphane blue dye. The cells were seeded into a 24-well plate with coverslips at a density of $5x10^4$ cells/well and incubated at 37°C in 5% CO_2 for maturation. The culture media were changed every three days. After six days, mature macrophages were present as adherent cells on the cover slips ready for further studies.

Macrophage cells were cultured with 0, 25, 50, 100, and 200 µg/ml of *P. niruri* aqueous extract for four hours and stimulated with $1.5x10^8$ CFU (0.5 mc Farland) *S. sanguinis.* This culture was then incubated for either 24 hours (Group 1) or 48 hours (Group 2) for time dependent study. Macrophage proliferation was observed under a phase contrast microscope after hematoxylin-eosin (HE) staining and calculated based on the number of cells visible in ten observation fields. A Kolmogorov-Smirnov Normality statistic test was performed, followed by a Levene homogenity statistic test. The difference between the groups of macrophage proliferation was established by the conducting of a one-way Anova test at a significance level of 0.05.

A NO release assay was performed using a previously described modified Griess method by Green.[15] Griess reagent was made by adding 0.1% N-(1-napthyl) ethylenediamine dihydrochloride (NED, Sigma) dissolved in sterile water and 1% Sulfanilamide (Sigma) dissolved in 5% phosphoric acid mixed in equal volume. For standard nitric, NaNO2 was dissolved in sterile water (2mm stock), while graded dilution of 0-200 µm was undertaken by dilution of the standard nitric with culture medium. 100 µl of Griess reagent was distributed into a 96-well plate and 100 µl of supernatant from the macrophage culture or graded doses of *nitric standard* was added to the wells. The control medium was used as a blank. The absorbance of the pink colour was measured at 550 nm using a microplate reader (Thermo Scientific, Rockford, Illionis, USA) after 15 minutes of incubation at room temperature. For further analysis, a standard curve was made based on simple linear regression from standard NaNO2 reading. NO concentration was quantified based on the standard curve in µm units.

RESULTS

The increase in NO production occurred at concentrations of 50 µg/ml, 100 µg/ml, 200 µg/ml, and 400 µg/ml of *P. niruri* supplementation in both groups, characterized by an increased optical density (OD) value in both groups at

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v51.i3.p124–128

24 hours and 48 hours (Table 1). A Modified Griess assay was used to calculate NO concentration in the culture supernatant. A regression line equation from standard nitric absorbance was obtained (R^2: 0.9975) and the means of OD values were plotted on the curve (Figure 1).

Upon *S. sanguinis* stimulation, control samples produced 19.70 µm of NO at 24 hours (group 1) and 20.74 µm at 48 hours (group 2). With 25 µg/ml *P. niruri* supplementation, NO concentrations were lower in both groups (19.35 and 20.51 µm for group 1 and group 2, respectively) compared to the controls. However, with the addition of 50, 100, 200 and 400 µg/ml of *P. niruri* there was a corresponding increase in NO concentration observed in both groups. The highest NO concentration was recorded with the addition of 400µg/ml *P. niruri,* 53.77 for group 1 and 74.23 µm for group 2. At the same concentrations, NO production was higher in group 2 than in group 1.

This study also demonstrated that *P. niruri* extract has a proliferative effect on macrophages reflected in an increase in the number of macrophage cells per field of view. Proliferation occurs from a concentration of 100 µg ml as shown in Table 2. Cell proliferation increased with greater extract concentration and exposure time. Similar to NO production, cell proliferation was greater with 48 hours of treatment than with 24 hours (Figure 2). The data was analyzed using a One-way ANOVA test at a significance level of 0.05 and there was significant difference between groups (p=0.000).

Table 1. Mean and standard deviation of optical density (OD) of nitric oxide production of *P. niruri* supplementation at various concentrations

Time exposure	Concentration (µg/ml)					
	0	25	50	100	200	400
24 h	0.076+0.0001	0.075+0.003	0.078+0.0001	0.085+0.0001	0.097+0.004	0.223+0.002
48 h	0.081+0.003	0.08+0.0001	0.086+0.002	0.096+0.0001	0.215+0.003	0.311+0.003

Table 2. Mean and Standard Deviation of the number of macrophage cells upon *P. niruri* supplementation at various concentrations and time exposures

Time exposure	Concentration (µg/ml)			
	0	100	200	400
24 h	2±1.83	4±0.67	5±0.82	6.4±0.97
48 h	2.3±2.0	7.8±1.23	12±1.89	17±2.05

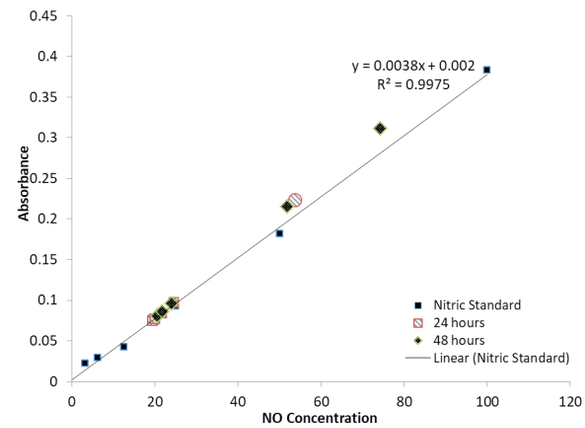

Figure 1. Standard curve and plotting of nitric oxide concentration

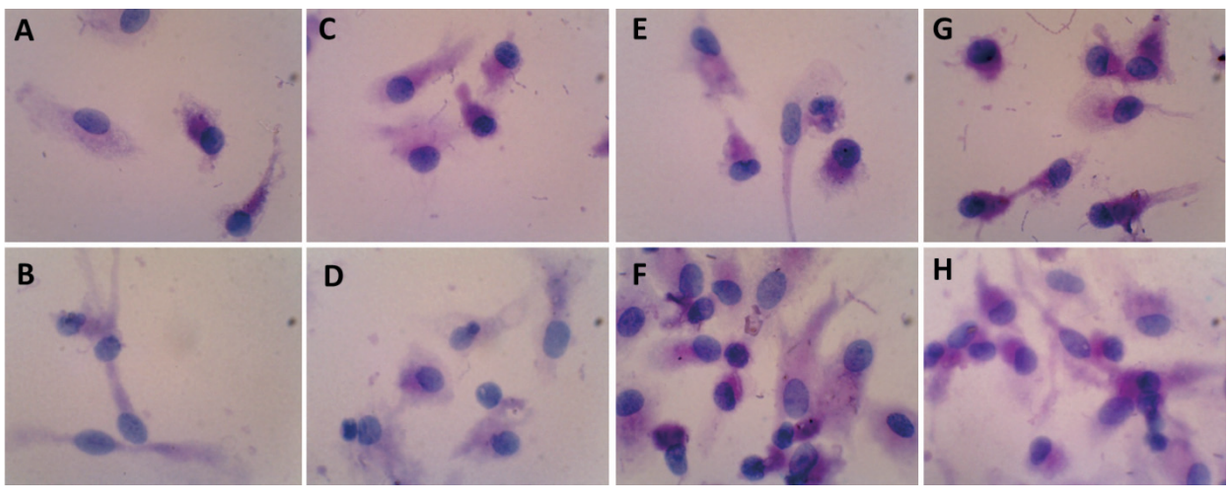

Figure 2. Macrophage proliferation (40x magnification). Untreated cells at 24 h (A) and 48h (B), no visible proliferation. Macrophages with 100 µg/ml *P. niruri* extract and *S.sanguinis* at 48h (D) showed greater macrophage proliferation compared with 24 h exposure (C). Similarly, 200 µg/ml *P. niruri* at 48h (F) there was greater proliferation compared with 200 µg/ml 24h (E). Likewise, 400 µg/ml *P. niruri* at 48h (H) resulted in greater macrophage proliferation than at 24h exposure (G).

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v51.i3.p124–128

DISCUSSION

Before the development of specific immune response, bacterial infection is predominantly countered by circulating macrophages and dendritic cells (DCs). Bacteria ingested by macrophages are internalized in a phagosome where they can be killed by a number of mechanisms, including that of reactive nitrogen intermediates (RNIs), particularly NO. ROIs alone have been shown to be insufficient to kill mycobacteria inside macrophage, but they enhance killing by RNIs.[16,17] Macrophages also produce cytokines such as TNF-α and IL-12, which subsequently induce NK-cells to secrete IFN-ɣ. TNF-α and IFN-ɣ stimulated more IL-12 production from the infected macrophages, creating a positive feedback loop. Moreover, these cytokines also play important functions in the activation of cellular immune response mediated by T cells, as well as further recruitment of macrophages to the infection site.[16,17]

Our study observed induced macrophage proliferation upon stimulation with *P. niruri* aqueous extract. It indicates that this substance has mitogenic ability in macrophages. In line with our findings, Eze *et al* used methanol extract of *P niruri* and observed an increase in mobilization and proliferation of polymorphonuclear (PMN) neutrophils in rodent peritoneal fluid.[18] Another study conducted by Amin *et al* also reported induced proliferation of PBMC after *P. niruri* aqueous extract stimulation.[9]

NO, a substance that results from catalyzing inducible nitric oxide synthase, is an enzyme in the phagolysosomes that are activated during phagocytosis. Other enzymes that are also activated during phagocytosis: inducible nitric oxide synthase, phagocyte oxidase and lysosomal proteases, referred to as ROS, destroy ingested pathogen. The increases in NO and ROS production reflects an intensification of phagocytic processes by macrophages.[6] In our study, the NO release assay demonstrated that NO production upon *S. sanguinis* infection gradually increased beginning at 50 µg/ml of *P. niruri* supplementation in both groups, and the increase observed was both dose- and time-dependent. This suggests that *P. niruri* aqueous extract may modulate an increase in phagocytic activity upon *S. sanguinis* infection in macrophages. A similar dose response phenomenon was observed in a study conducted by Putri *et al*.[13] using macrophage cells from tuberculosis patients. The results of this study are also consistent with one conducted by Nworu *et al*.[19] which reported that treatment with *P. niruri* extract in animal model macrophages significantly enhance the activation and function of these cells, such as phagocytosis, lysosomal enzyme activity and TNF-α release, which also modulate nitric oxide release from macrophages. While the increase in NO secretion may reflect both macrophage phagocytic activity and the destruction of intracellular bacterial, these observed phenomena can also be the result of higher macrophage numbers due to increased proliferation. Nevertheless, it was demonstrated that the aqueous extract of *P niruri* did subsequently exhibit toxicity upon macrophages.

In conclusion, this study concludes that *P. niruri* aqueous extracts showed an ability to potentiate macrophage responses to *S. sanguinis* infection by inducing proliferation and NO production in dose- and time-dependent ways, with the highest effect observed at a concentration of 400 µg/ml at 48 hours. This study adds to the existing knowledge of the antibacterial properties of *P niruri,* that may not only act directly against bacteria, but also induce strong immune protection against them. Further studies are required to establish the effects of *P niruri* stimulation to other types of immune cells including the subsequently activated cellular immune cells: T and B cells, upon bacterial infection.

ACKNOWLEDGEMENT

The authors would like to thank the Faculty of Medicine, Universitas Kristen Duta Wacana, Yogyakarta, Indonesia for the grant which funded the research reported here.

REFERENCES

1. Ge X, Kitten T, Chen Z, Lee SP, Munro CL, Xu P. Identification of Streptococcus sanguinis genes required for biofilm formation and examination of their role in endocarditis virulence. Infect Immun. 2008; 76(6): 2551–9.
2. Lockhart PB, Brennan MT, Sasser HC, Fox PC, Paster BJ, Bahrani-Mougeot FK. Bacteremia associated with toothbrushing and dental extraction. Circulation. 2008; 117(24): 3118–25.
3. Westling K. Viridans group Streptococci septicaemia and endocarditis. Thesis. Stockholm: Karolinska Institutet; 2005. p. 9-31.
4. Bah A, Vergne I. Macrophage autophagy and bacterial infections. Front Immunol. 2017; 8: 1–9.
5. Vazquez-Torres A, Stevanin T, Jones-Carson J, Castor M, Read RC, Fang FC. Analysis of nitric oxide-dependent antimicrobial actions in macrophages and mice. Methods Enzymol. 2008; 437: 521–38.
6. Abbas AK, Lichtman AH. Basic immunology : functions and disorders of the immune system. 3rd ed. Philadelphia: Saunders; 2011. p. 312.
7. Masuda K, Nemoto H, Nakano K, Naka S, Nomura R, Ooshima T. Amoxicillin-resistant oral streptococci identified in dental plaque specimens from healthy Japanese adults. J Cardiol. 2012; 59(3): 285–90.
8. Sahasrabhojane P, Galloway-Peña J, Velazquez L, Saldaña M, Horstmann N, Tarrand J, Shelburne SA. Species-level assessment of the molecular basis of fluoroquinolone resistance among viridans group streptococci causing bacteraemia in cancer patients. Int J Antimicrob Agents. 2014; 43(6): 558–62.
9. Amin ZA, Abdulla MA, Ali HM, Alshawsh MA, Qadir SW. Assessment of In vitro antioxidant, antibacterial and immune activation potentials of aqueous and ethanol extracts of Phyllanthus niruri. J Sci Food Agric. 2012; 92(9): 1874–7.
10. Ibrahim D, Hong LS, Kuppan N. Antimicrobial activity of crude methanolic extract from Phyllanthus niruri. Nat Prod Commun. 2013; 8(4): 493–6.
11. Liu S, Wei W, Shi K, Cao X, Zhou M, Liu Z. In vitro and in vivo anti-hepatitis B virus activities of the lignan niranthin isolated from Phyllanthus niruri L. J Ethnopharmacol. 2014; 155(2): 1061–7.
12. Ibnul A. Uji komparasi aktivitas fagositosis makrofag dan produksi nitrit oksida pada mencit Balb/c akibat perlakuan ekstrak meniran hijau (Phyllanthus niruri) dan meniran merah (Phyllanthus urinaria) yang diinfeksi bakteri Salmonella typhi. Thesis. Surakarta: Universitas Sebelas Maret; 2012.
13. Putri DU, Rintiswati N, Soesatyo MH, Haryana SM. Immune modulation properties of herbal plant leaves: Phyllanthus niruri aqueous

DOI: 10.20473/j.djmkg.v51.i3.p124–128

extract on immune cells of tuberculosis patient - in vitro study. Nat Prod Res. 2018; 32(4): 463–7.
14. Hutomo S, Susilowati H, Agustina D, Asmara W. Analysis of anti-Streptococcus sanguinis IgY ability to inhibit Streptococcus sanguinis adherence. Dent J (Maj Ked Gigi). 2018; 51: 33–6.
15. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem. 1982; 126: 131–8.
16. Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev. 2015; 264(1): 182–203.
17. Myers JT, Tsang AW, Swanson JA. Localized reactive oxygen and nitrogen intermediates inhibit escape of Listeria monocytogenes from vacuoles in activated macrophages. J Immunol. 2003; 171(10): 5447–53.
18. Eze CO, Nworu CS, Esimone CO, Okore VC. Immunomodulatory activities of methanol extract of the whole aerial part of Phyllantus niruri L. J Pharmacogn Phyther. 2014; 6(4): 41–6.
19. Nworu CS, Akah PA, Okoye FBC, Esimone CO. Aqueous extract of Phyllanthus niruri (Euphorbiaceae) enhances the phenotypic and functional maturation of bone marrow-derived dendritic cells and their antigen-presentation function. Immunopharmacol Immunotoxicol. 2010; 32(3): 393–401.

Dental Journal (Majalah Kedokteran Gigi) p-ISSN: 1978-3728; e-ISSN: 2442-9740. Accredited No. 32a/E/KPT/2017.

DOI: 10.20473/j.djmkg.v51.i3.p124–128