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 Susilowati5
1Department of Microbiology, Faculty of Medicine, Universitas Kristen Duta Wacana
2Doctoral Program, Faculty of Medicine, Universitas Gadjah Mada
3International PhD Program in Medicine, Taipei Medical University, Taipei, Taiwan
4Department of Physiology, Faculty of Medicine, Universitas Kristen Duta Wacana
5Department 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 Phyllanthus 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 100 μg/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

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.
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.\textsuperscript{10} In addition, extract of \textit{P. niruri} has been observed to induce macrophage activity by increasing phagocytosis and NO in mice macrophage infected with \textit{Salmonella typhi} and in the macrophages of tuberculosis patients.\textsuperscript{13} Despite numerous studies in elucidating the medicinal use of \textit{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 \textit{P. niruri} aqueous extract on macrophage proliferation and NO production on \textit{S. sanguinis} infection. From this study, it was expected to gain new insights into the potential use of \textit{P. niruri} as an immunomodulatory agent in the management of infections caused by \textit{S. sanguinis}.

MATERIALS AND METHODS

\textit{Streptococcus sanguinis} was prepared as in our previous study.\textsuperscript{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\textsuperscript{8} colony forming units (CFU). Dried \textit{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-density separation, as described above.\textsuperscript{15} After centrifugation, the PBMC layer containing lymphocytes and monocytes were 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\textsuperscript{5} cells/well and incubated at 37°C in 5% CO\textsubscript{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 \textit{P. niruri} aqueous extract for four hours and stimulated with 1.5x10\textsuperscript{5} CFU (0.5 mc Farland) \textit{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 homogeneity 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.\textsuperscript{15} Griess reagent was made by adding 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (NED, Sigma) dissolved in sterile water and 1% Sulfanilamide (Sigma) dissolved in 5% phosphoric acid mixed in equal volume. For standard nitric, NaNO\textsubscript{2} was dissolved in sterile water (2mL 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 \textit{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, Illions, USA) after 15 minutes of incubation at room temperature. For further analysis, a standard curve was made based on simple linear regression from standard NaNO\textsubscript{2} 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 \textit{P. niruri} supplementation in both groups, characterized by an increased optical density (OD) value in both groups at
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 (R²: 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 400 μg/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

<table>
<thead>
<tr>
<th>Time exposure</th>
<th>Concentration (μg/ml)</th>
<th>0</th>
<th>25</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>0.076±0.0001</td>
<td>0.075±0.003</td>
<td>0.078±0.0001</td>
<td>0.085±0.0001</td>
<td>0.097±0.004</td>
<td>0.223±0.002</td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>0.081±0.003</td>
<td>0.08±0.0001</td>
<td>0.086±0.002</td>
<td>0.096±0.0001</td>
<td>0.215±0.003</td>
<td>0.311±0.003</td>
<td></td>
</tr>
</tbody>
</table>

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

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

**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 (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).
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. RNIs 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-γ. TNF-α and IFN-γ 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, Exe 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 inductive 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.9 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.17 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


