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Research Report

The potency of *Andrographis paniculata Nees* extract to increase the viability of monocytes following exposure to *Porphyromonas gingivalis*

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ABSTRACT

Background: *Periodontitis is a chronic infectious disease affecting the global population. In Indonesia, the prevalence of periodontal disease has reached 57.6% across all age groups. The bacterium considered as the orginator factor of periodontitis is Porphyromonas gingivalis (P. gingivalis). Herbal ingredients are currently being promoted as a form of treatment because of the minimal side effects they induce. Andrographis paniculata Nees (ApN) extract produces pharmacological effects, including ones immunomodulatory in character, rendering possible its application as a preparation for treating periodontitis.* ***Purpose:*** *The purpose of the study was to prove the potency of Andrographis paniculata Nees extract in increasing the viability of monocytes following exposure to P. gingivalis.* ***Methods:*** *The sample was divided into four groups, namely; Control negative (C-): monocytes in the medium, not exposed to P. gingivalis; Control positive (C+): monocytes in the medium, exposed to P. gingivalis; Treatment I (AP25): monocytes with 25% ApN extract, exposed to P. gingivalis; Treatment II (AP50): monocytes with 50% ApN extract, exposed with P. gingivalis. The monocytes were exposed to 100 uL P. gingivalis for 4.5 hours and stained with trypan blue. Observations were conducted using an inverted microscope at 200x magnification. The percentage of viable monocytes was calculated based on the ratio of the number of the cells which absorbed trypan blue staining to that which did not. Data was tested using a one-way ANOVA followed by an LSD test.* ***Results:*** *There were significant differences between the treatment groups in the number of viable monocytes (p=0.001) they contained. Monocyte viability was higher in the 25% ApN extract group than that exposed to 50% P. gingivalis.* ***Conclusion:*** *Andrographis paniculata Nees extract demonstrates the potency to increase monocyte viability following exposure to P. gingivalis.*

Keywords: *Andrographis paniculata Nees extract; monocytes viability; Porphyromonas gingivalis*

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INTRODUCTION

Periodontitis, a disease potentially causing damage to supporting tissues, is triggered by *Porphyromonas gingivalis* (*P. gingivalis*), a gram-negative anaerobic bacterium which can invade periodontal tissues, while evading the host's defences.[1,2] During chronic inflammation, *P. gingivalis*-mediated periodontal disease constitutes a risk factor in several systemic diseases among others; diabetes, pre-term birth, strokes, and atherosclerotic cardiovascular disease.[3] It has major virulence factors, namely; lipopolysaccharide, capsule, gingipains and fimbriae.[4]

During bacterial attack, lipopolysaccharide (LPS) of *P. gingivalis* stimulates an increase in monocyte levels in the periodontal tissues and activates monocytes in peripheral blood vessels. Monocytes which are not activated will produce free radicals through a metabolic process .[5] Free radical production in activated monocytes increases as the result of phagocytosis against infection. Free radical production from various biological and environmental

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sources is caused by an imbalance of natural antioxidants which leads to various disease-associated inflammation.[6] The effect of oxidative stress and its associated factors are an important problem within human health. Endogenous enzymatic and non-enzymatic antioxidant substances are incapable of coping with the overload of reactive oxygen species (ROS) which leads to imbalances within the process, cell damage, and health problems.[7] Therefore, in this case, the body requires external antioxidants, referred to as exogenous antioxidants, to increase monocytes viability.[8]

Andrographis paniculata Nees (ApN), commonly known as the "king of bitters," is an herbaceous plant which includes the Acanthaceae family found in all regions of tropical and subtropical Asia and Southeast Asia. In Indonesia, most people refer to it as sambiloto (Java) to. The Andrographolide contained in ApN exhibits immunostimulatory, antiviral, and antibacterial pharmacological properties.[9,10] The purpose of this research was to prove the potency of ApN extract to increase monocytes viability after exposure to *P. gingivalis*.

MATERIALS AND METHODS

This study was approved by the Ethical Committee, University of Jember (Permit Number: 000651/KKEP/FKG-UGM/EC/2016). *P. gingivalis* strain ATCC 33277 was supplied by the Department of Microbiology, Faculty of Dental Medicine, Universitas Jember (Identification number: 0970Mikro/S. Ket/2016).

ApN powder was obtained from Materia Medica, Batu, East Java, Indonesia which had utilized the following extraction process. First, n 400 g of ApN leaf powder was moistened with 96% ethanol solvent until 2,000 ml of solution was obtained. The jar containing it was closed for 48 hours and agitated in a digital shaker at 50 rpm. The liquid extract was subsequently filtered and placed in an erlenmeyer flask. Remaseration was performed twice with 1,000 ml of 96% ethanol before being soaked overnight in a shaker. Finally, the liquid extract was evaporated in a rotary evaporator for 2.5 hours.

Monocytes were isolated by collecting 9cc of blood using 1 mM ethylene diamine tetraacetic acid (EDTA) as an anticoagulant. The blood, all solutions, and equipment were conditioned at 4°C prior to use and agitated gently before extraction of an aliquot. Iodixanol working solution (WS) at 40% (w/v) concentration was prepared with OptiPrep 4 vol of diluted with 2 vol of diluent (Axis-Shield Density Gradient Media, USA). This solution had a density of approximately 1.217 g/ml. 1.072 g/ml-1.074 g/ml density barrier solution was prepared by WS diluted with 2.14 ml + 5 ml and 2.27 + 5 ml diluent, respectively. 4.24 ml of WS was mixed with 10 ml of whole blood (WB) in a 15 ml centrifuge tube; 5 ml of one of the density barrier solutions over 5 ml of the blood, and then layered with 0.5 ml of diluents (WS mix WB) on top. The solution was centrifuged at 700g in a swinging-bucket rotator for 30 minutes at 4°C. 100 µl of mononuclear cells was pipetted onto the microplate, suspended again with 1000 µl RPMI media, and then added to 20 µl fungizon.[11] The *P. gingivalis* used was *P. gingivalis* ATCC 33277 at a concentration of 10^6 which was measured by densicheck until a density of 0.5 McFarland was achieved.

In the subsequent stage, monocytes were divided into four groups. Control negative (C-): monocytes not exposed to *P. gingivalis*; Control positive (C+): monocytes exposed to *P. gingivalis*; Treatment I (AP25): monocytes with 25% ApN extract and exposed to *P. gingivalis*; and, Treatment II (AP50): monocytes with 50% ApN extract and exposed to *P. gingivalis*.

A 0.4% trypan blue exclusion test was conducted to determine monocyte viability. Monocytes that did not absorb trypan blue staining were counted as viable. The percentage of monocytes viability was calculated by dividing the number of viable monocyte cells by the total number of monocyte cells and multiplying the result by 100%. The data produced was subsequently tested statistically using IBM with SPSS 20.0 software. A one-way analysis of variance (ANOVA) and a LSD test were administered for pairwise comparison with a significance of 0.05.

RESULTS

The results of data analysis can be observed in Table 1 indicated that significant differences existed in all experimental groups. The results of data analysis contained in Table 2 revealed that 25% of the ApN extract group (AP25) had a significant difference compared to the negative control group (C-), positive control group (C+),

Table 1. The different monocyte viability test results for all groups produced by a one-way ANOVA test

Groups	Mean ± SD	one-way ANOVA (p)
C-	73.5 ± 2.38	0.0001
C+	64.25 ± 2.36	
AP25	82.75 ± 2.62	
AP50	96 ± 2.16	

Table 2. The different monocyte viability results for groups produced by a LSD test

Treatment groups	C-	C+	AP25	AP50
C-		.004	.003	.000
C+			.000	.000
AP25				.000
AP50				

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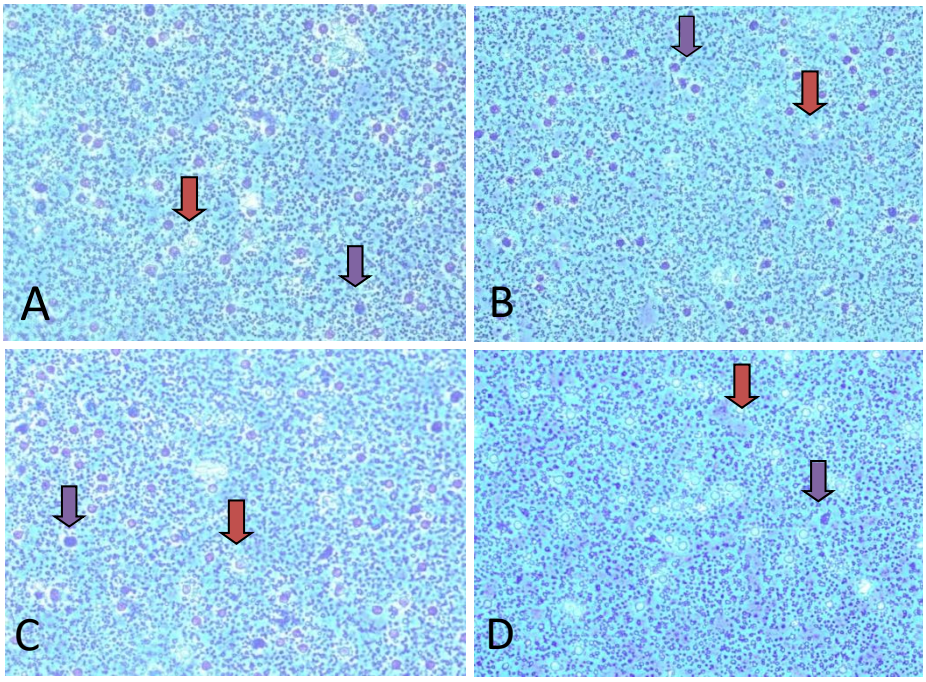

Figure 1. Monocytes viability counted using an inverted microscope (200x) and trypan blue staining. Viable monocytes are light-colored (red arrows), while unviable monocytes are dark-colored (purple arrows). Monocytes viability in C- group (A), C+ group (B), AP25 group (C), and AP50 group (D). In the AP50 group, monocyte viability was higher than in the other groups.

and 50% ApN extract group (AP50). The 50% ApN extract group (AP50) had a significant difference compared to the negative control group (C-), positive control group (C+), and 25% ApN extract (AP25) group.

Figure 1 contains images of monocyte viability counted by means of an inverted microscope (200x) and trypan blue staining. Viable monocytes were light-colored (red arrows), while unviable monocytes were dark-colored (purple arrows). Monocyte viability was highest in the AP50 group compared to the other groups (light-colored).

DISCUSSION

The overall results of this research indicated that the presence of ApN leaf extract induced a higher increase in monocyte viability in the treatment group compared to the control group. Based on the contents of Table 1, the results of this study showed that there were significant differences in all experimental groups. ApN is an effective antiinflammatory and a correlation existed between increasing the extract dose and a more potent anti-inflammatory effect. The positive control group had the lowest average monocyte viability of all the groups due to the virulence factors of *P. gingivalis*, i.e. LPS, fimbria, and gingipain.[4]

The LPS in *P. gingivalis* induced an increase in the superoxide and nitric oxides contained in monocytes. Nitric oxide in the form of gas molecules constitutes another reactive species of ROS which is toxic.[12] The imbalance between free radical production and antioxidant defenses leads to an oxidative stress causing lipid peroxidation of cell membranes. The presence of excessive lipid peroxidation causes damage to cell membranes precipitating lysis in the monocytes. LPS acts as protypical endotoxin which binds CD14/TLR-4, a complex receptor in different types of cells, especially monocytes. Binding of CD14 by LPS results in the release of proinflammatory cytokines, namely; IL-1α, IL-1β, IL-16, TNF-α and lipid inflammatory prostalglandins E_2 (PGE_2) mediators. [13]

During the inflamatory process, inflammatory cells produce inflammatory mediators such as arachidonic acid and chemokines that demonstrate higher solubility. Both of these inflammatory mediators will work by activating inflammatory cells in the infection site and releasing more reactive species. Certain markers, in addition to being capable of stimulating signal transduction cascade, can also cause changes in transcription factors, including; nuclear factor kappa B (NF-κB), signal transducers and activators in transcription 3, NF-E2 related factor-2, nuclear factor in activated T cells (NFAT), and hypoxia-inducible factor-1α (HIF1-α), which mediate cellular stress reactions. The subsequent stage is the initiation of cyclooxygenase-2 (COX-2), inducing of nitric oxide synthase (iNOS), and high expression of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and chemokines.[14–16] However, if the number of inflammatory mediators in monocytes is excessive, tissue damage may ensue commencing with cell lysis.[17]

Gingipain in *P. gingivalis* degrades CD14 receptors which causes the hyper-responsiveness of monocytes to bacterial infection.[18] Gingipain in *P. gingivalis* manipulates host molecules proactively by intervening in cross-talk

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between C5a receptors and TLR signaling to avoid bacterial clearance. In-vitro studies have indicated that gingipain plays a role in the regulation of inflammatory mediators in a number host cells, including IL-1α, IL-1β, and IL-18.[19] The excessive amount of inflammatory mediators in monocytes causes tissue damage which leads to lysis.[20]

Anti-inflammatory activity of andrographolide has been studied using a number of in vivo and in vitro experimental paradigms, including human whole genome DNA microarrays. The most common anti-inflammatory and immunomodulatoy activities of andrographolide are barriers to mitogen-activated protein kinase/extracellular signal (MAPK/ERK) or regulated kinase signalling (specifically p38 MAPK/ERK1/2) and final transcription factors such as nuclear factor kappa B (NF-κB) and nuclear factor of activated T cells (NFAT).[21] Inflammation is a complex interaction between organisms and pathogens which induces macrophage activation and secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, as the body's immune system response. Research previously conducted confirmed that LPS is a powerful inflammatory trigger capable of stimulating macrophages to synthesize TNF-α, IL-1β, and IL-6.[22]

As indicated by the contents of Table 2, this study showed that the AP25 and AP50 groups had a significant difference to C- and C+ group, while AP25 also demonstrated a significant difference to AP50. It was suspected that the active ingredients in ApN leaves are andrographolide and flavonoids capable of suppressing the inflammatory process. Flavonoids contained in the ApN leaves extract act as antioxidants which inhibit the formation of free radical reactions (peroxide) during lipid oxidation by donating one or more electrons to free radicals causing them to become muted. Antioxidants delay or prevent the formation of free radical reactions (peroxide) in the lipid oxidation.[23] In vitro studies have confirmed that flavonoids are strong inhibitors of lipid peroxidation, as traps of ROS or reactive nitrogen, and are also able to inhibit cyclooxygenase and lipooxygenase enzyme activity.[24] Flavonoids derived from polyphenols can inhibit the oxidation reaction through a radical arrest mechanism (radical scavenging) by donating an electron to the unpaired electrons in free radicals causing a reduction in their number.[23] Flavonoids significantly inhibit the protease activity of Porphyromonas gingivalis gingipain depending on the size of the dose administered.[24]

Monocyte viability of the AP25 group was higher compared to the C- and C+ groups. The AP50 group demonstrated the highest level of monocyte viability. The results of this study are compatible with those of the research conducted by Chandrasekaran (2011) which found that andrographolide is the main active component of Andrographis paniculate which provides anti-inflammatory effects by inhibiting the activation of the NF-κB/MAPK signaling pathway and inducing pro-inflammatory cytokines. Andrographolide can inhibit the expression of iNOS through LPS, which is activated by macrophages and prostaglandin E2 (PGE2) production.[25]

ApN can suppress isoform iNOS and COX-2 and decrease nitric oxide and the production of PGE2.[14] Andrographolide reduces the expression and production of pro-inflammatory cytokines (IL-1α, IL-6, TNF-α) and pro-inflammatory mediators PGE2-stimulated by LPS, while also reducing the production of superoxide anion radicals and hydrogen peroxide.[13] It was concluded that ApN extract is highly effective in increasing monocyte viability after exposure to *P. gingivalis*.

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