

## Research Report

## Cytotoxicity of Betel leaf (*Piper betel* L.) against primary culture of chicken embryo fibroblast and its effects on the production of proinflammatory cytokines by human peripheral blood mononuclear cells

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### ABSTRACT

**Background:** Betel leaf (*Piper betel* L.) has been used in modern and traditional medicine as antiseptic, antibacterial, and also prevention of plaque accumulation, but it still can stimulate cancer in lime-piper betel quid. Betel leaf also has anti-inflammatory properties. **Purpose:** The purpose of this study was examine the cytotoxicity of Betel leaf extract (BLE) against primary culture of chicken embryo fibroblast and its effects on the production of proinflammatory cytokines by peripheral blood mononuclear cells (PBMC) stimulated with LPS. **Methods:** MTT assay was used to investigate the survival rate of the culture with the survival rate result of the given culture extract 4%, 2% and 1% about 82%, 83.4% and 85%. There was no significant difference between treatment with various concentrations of the extract and the control ( $p > 0.05$ ). To evaluate the effect of Betel leaf extracts on the production of cytokines, proinflammatory was conducted by incubating the extracts of betel leaf with peripheral blood mononuclear cells stimulated with lipopolysaccharide. Peripheral blood mononuclear cells were obtained from healthy volunteers isolated by density centrifugation method using Ficoll-Hypaque. Once coupled with various concentrations of betel leaf extract and lipopolysaccharide, and then incubated for 24 hours, the culture supernatant was used to determine the level of IFN- $\gamma$  and TNF- $\alpha$  by ELISA method. **Results:** It is known that the survival rates of BLE 4%, 2% and 1% were 82%, 83.4% and 85%. There was no significant of difference between several concentrations of BLE and those in the control group ( $p > 0.05$ ). The production of IFN- $\gamma$  and TNF- $\alpha$  stimulated with LPS was no significant difference between BLE 4%, 2% and 1% and that in the control group ( $p > 0.05$ ). **Conclusion:** It can be concluded that BLE is not toxic against primary culture of chicken embryo fibroblast, and the production of IFN- $\gamma$  and TNF- $\alpha$  by PBMC was not affected by BLE.

**Key words:** Betel leaf extract, MTT assay, proinflammatory cytokines

### ABSTRAK

**Latar belakang:** Daun sirih (*Piper betel* L.) telah banyak digunakan dalam berbagai pengobatan tradisional maupun moderen sebagai antiseptik, antibakteri dan untuk pencegahan pembentukan plak, tetapi dapat juga menimbulkan kanker pada orang pengunyah sirih. Daun sirih juga memiliki aktivitas sebagai anti-inflamasi. **Tujuan:** Penelitian ini dimaksudkan untuk mengevaluasi sitotoksitas ekstrak daun sirih terhadap kultur primer sel fibroblas embrio ayam dan pengaruhnya terhadap produksi sitokin proinflamasi oleh sel mononuklear darah perifer yang distimulasi dengan LPS. **Metode:** Uji MTT digunakan untuk menginvestigasi survival rate kultur, dengan hasil: survival rate dari kultur yang diberi ekstrak 4%, 2% dan 1% adalah 82%, 83,4% dan 85%. Tidak terdapat perbedaan signifikan antara perlakuan dengan berbagai konsentrasi ekstrak terhadap kontrol ( $p > 0,05$ ). Untuk mengevaluasi pengaruh ekstrak daun sirih terhadap produksi sitokin proinflamasi dikerjakan dengan menginkubasi ekstrak daun sirih bersama sel mononuklear darah perifer yang distimulasi dengan lipopolisakarida. Sel mononuklear darah perifer diperoleh dari relawan sehat yang diisolasi dengan metode sentrifugasi densitas menggunakan ficoll-hypaque. Setelah ditambah dengan berbagai konsentrasi ekstrak daun sirih dan lipopolisakarida, diinkubasi selama 24 jam, supernatan kultur digunakan untuk menentukan level IFN- $\gamma$  dan TNF- $\alpha$  dengan metode ELISA. **Hasil:** Tidak terdapat perbedaan signifikan produksi IFN- $\gamma$  dan TNF- $\alpha$  antara kultur sel mononuklear darah perifer yang diinkubasi bersama ekstrak daun sirih dibandingkan dengan kontrol ( $p > 0,05$ ). **Kesimpulan:** Disimpulkan bahwa ekstrak daun sirih

tidak toksik terhadap kultur sel primer fibroblas embrio ayam dan tidak berpengaruh terhadap produksi sitokin proinflamasi IFN- $\gamma$  dan TNF- $\alpha$  oleh sel mononuklear darah perifer manusia yang distimulasi dengan LPS.

**Kata kunci:** Ekstrak daun sirih, uji MTT, sitokin proinflamasi

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## INTRODUCTION

Betel leaf from *Piper betel* L. is a traditional plant that is closely related to oral health. Betel leaf contains a variety of compounds, such as chavicol compound with powerful antiseptic activity which is stated, five times stronger than hydroxychavicol, phenol, allylrocatechol, cinneol, caryophyllene, menthone, eugenol and methyl ether compounds. 35% Betel leaf extracts have greater antibacterial activity against *Streptococcus viridans* than 10% povidone iodine.<sup>1</sup>

Betel leaf is widely used for various traditional and modern treatments in dental health such as oral antiseptic mouthwash (gargle). The content of essential oil in a betel leaf is antibacterial, which is oftenly used as additional agent in toothpaste to replace the use of fluoride. The use of toothpaste with high fluoride concentration can cause side effects, such as enamel fluorosis. Its antibacterial effect of fluoride only inhibit the differentiation rather than eliminate the bacteria.<sup>2</sup> Many experiments have been done to search better option. Such as the use of betel leaf essential oil in toothpaste as antibacterial agents. Toothpaste containing betel leaf essential oil is commercially available at the market recently. Betel leaf extract is also used to prevent plaque accumulation.<sup>3</sup> In some people chewing betel (*lime-piper betel quid*), the incidence of cancer was found, but it is still unknown which one from the chewing material, such as betel leaf, lime, betel nut (areca nut), or tobacco that has carcinogenic effect.<sup>4-6</sup> Compared with extracts of betel nut (areca nut), betel leaf extract has a lower toxicity towards human gingival keratinocyte cells.<sup>7</sup> It is also known that areca nut extract is considered as toxic towards *cell line* fibroblast culture (human buccal fibroblast culture),<sup>8</sup> but few study has been conducted to examine the toxicity of betel leaf towards primary cell culture. Therefore, clinical application of betel leaves in dentistry and others in the medical field is quite extensive and mostly applied topically (mouthwash, toothpaste, vagina antiseptics), which means more contacts with mucosa and epithelial cells, it is necessary to examine the toxicity of betel leaf against cells (cytotoxicity), and in this research cytotoxicity assay uses primary cell culture.

Betel leaves have anti-inflammatory activity that works by inhibiting lipoxygenase enzyme activity.<sup>9</sup> The content of betel leaf is hydroxychavicol, an anti-inflammatory that works by inhibiting cyclooxygenase activity and platelet aggregation, and it is also expected to prevent atherosclerosis.<sup>10</sup> One of various anti-inflammatory response

is to inhibit proinflammatory cytokine.<sup>11</sup> Inflammation is one of the immune mechanisms to eliminate microbial pathogens, and classified in natural immunity. In the inflammatory process, proinflammatory cytokines, such as TNF, IL-1 and IL-12, would be secreted. Furthermore, IL-12 induces NK cells and lymphocytes T to secrete IFN- $\gamma$  that can activate macrophage.<sup>12</sup> For those reasons, this research is aimed to determine the effect of betel leaf on the production of proinflammatory cytokines after incubation with human peripheral blood mononuclear cells (PBMC) stimulated with LPS. If proven that betel leaf can inhibit secretion of proinflammatory cytokines, particularly TNF, then it will likely be used in sepsis treatment.<sup>12</sup>

## MATERIALS AND METHODS

Betel leaf samples used in this research were obtained from traditional medicine industry, Tradimun Gresik, in the form of fine powder crude of dried leaves. The powder was extracted by maceration method using 70% alcohol. Two hundred grams of powder was extracted with 70% alcohol for 24 hours at room temperature, and then filtered through Whatmann filter paper. Afterwards, the filtrate was dried in a vacuum evaporator at temperature of 40° C-45° C. The final result obtained was in the form of 2.6% dried powder.

Cytotoxicity test was conducted on primary cell cultures of chicken embryo by preparing primary cell cultures made by standard methods of primary cell culture manufacturing.<sup>13</sup> This test was performed in a laminar flow cabinet. The chicken embryos obtained from embryonated eggs or hatched eggs at the age of 8-9 days old. Eggshells were disinfected with 70% alcohol. Next, they were opened and the embryos were removed and placed on a sterile petri dish. Afterwards, they were washed with sterile phosphate buffer, and its head, legs and wings were removed. The bodies were then cut with scissors into some pieces with the size of 2-3 mm. Those pieces of embryos were placed in erlenmeyer 100 ml equipped with a magnetic bar, added with trypsin-versene solution, phosphate buffer solution containing 0.25% trypsin and 1 mM ethylenediamine tetra acetic acid (EDTA), and then stirred on the magnetic stirrer for 10 minutes at 200 rpm.

Afterwards, those were left for 3 minutes until those pieces of tissue settled, and then supernatants, cell suspension and tissue debris, were separated into another erlenmeyer. Next, those were added with another trypsin-

versene solution were added and then stirred until the pieces of embryos were unraveled. Those cell suspensions were filtered by using three layers of sterile gauze to separate the remained tissue and debris. The filtrate containing fibroblasts was washed 3 times with phosphate buffer centrifugation, and then suspended in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin. Cell density was calculated by using hemocytometer, made with the size of  $4 \times 10^5$  cells/ml, and then ready for testing.

Moreover, preparation of the test was conducted by using modification of elution method (ISO 10993-5).<sup>14</sup> Two grams of extract was then added to 25 ml RPMI 1640 culture medium + 10% FBS (concentration of extract 8%), and sterilized by filtration using millipore filter with the size of 0.22  $\mu$ m. Next, 10 ml of it was taken for dilution conducted 2 times (double dilution) with RPMI 1640 culture medium to obtain the concentration of the extract about 4% and 2%. Afterwards, it was left at room temperature for 1 hour. The test was then conducted on cell culture plates with 96 wells. One hundred  $\mu$ l extract dilution was added into each well, 5 wells for each dilution, and then 100  $\mu$ l fibroblast suspension was added, so the series of extract concentration was 4%, 2%, and 1%. Each well then contained  $2 \times 10^5$  fibroblast cells, and a row of wells containing 200  $\mu$ l cell suspensions was used as control. The plates were incubated at 37° C in a 5% CO<sub>2</sub> incubator for 24 hours.

Cytotoxicity observation, was conducted on betel leaves by using MTT test method.<sup>15</sup> One hour before the incubation period ended, 5 mg/ml of 20  $\mu$ L MTT solution, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, M-5655), was added into all the wells. The incubation was continued for the next 1 hour. Culture medium was replaced with isopropanol containing 0.01 M HCl to dissolve formazan crystals. Optical density (OD) of purple color of the formazan was measured by using multiwell spectrophotometer (ELISA reader) at a wavelength of 570 nm. Survival rate was then calculated as survival rate, % = OD sample/OD control 100%.

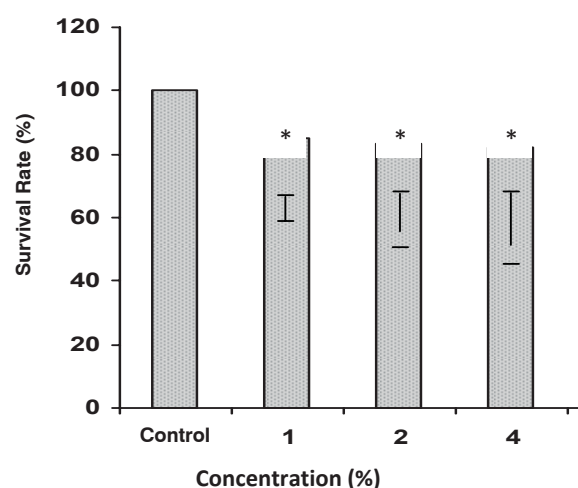
The effects of betel leaves on the production of proinflammatory cytokines incubated in peripheral blood mononuclear cells were evaluated by preparing peripheral blood mononuclear cells. Peripheral blood was obtained from healthy donor in Department of Clinical Pathology in Faculty of Medicine Airlangga University, and separated by gradient method using ficoll-hypaque 1.077 (ficoll hypaque density centrifugation). Buffy coat containing mononuclear cells was then separated and washed 3 times with phosphate buffer. Afterwards, the density of cells was measured by using hemocytometer, and adjusted to  $5 \times 10^5$  cells/ml in RPMI-1640 medium + FBS 10%, penicillin, and streptomycin. The test was then prepared by using cell culture plates with 96 wells, and conducted as the same as cytotoxicity test. Each well was filled with 80 mL suspension of mononuclear cells, and also added with 100  $\mu$ l betel leaf extract dilution, each of which used 5 wells.

Afterwards, mononuclear cell density changed to  $4 \times 10^4$  cells/ wells, and a series of wells containing 180  $\mu$ l cell suspension,  $4 \times 10^4$  cells/wells, was prepared as control. Each well was added with 10  $\mu$ g/ml of 20  $\mu$ l lipopolysaccharide (LPS) as a mitogen. The plates were then incubated at 37° C for 48 hours in CO<sub>2</sub> incubator. After incubated, the supernatant was used to analyze the content of IFN- $\gamma$  and TNF- $\alpha$  by ELISA method using Quantikine Colorimetric Sandwich ELISA kits. Finally, the results obtained were analyzed by using ANOVA, and then differences among treatment groups were analyzed with Student-Newman-Keul's test with  $p < 0.05$ .<sup>13</sup>

## RESULTS

In the cytotoxicity assay of betel leaf extracts on primary cell cultures of chicken embryos, the average survival rates of betel leaf extracts 4%, 2%, and 1% in primary cell cultures of chicken embryo fibroblasts were 82%, 83.4%, and 85% respectively (the percentage of living cells), with control of 100% (Figure 1). There was no significant difference between either the extract concentration of 4% ( $p = 0.5$ ), 2% ( $p = 0.5$ ), or 1% ( $p = 0.5$ ) and the control group. It means that betel leaf extract had no effect on the growth of culture. In other words, it was not toxic on primary cell cultures of chicken embryo fibroblasts.

Production of IFN- $\gamma$  and TNF- $\alpha$  from peripheral blood mononuclear cells after incubated with betel leaf extracts and stimulated with LPS can be seen in Table 1 and Table 2. There was no significant difference in the secretion of IFN- $\gamma$  in treatment group and controls group  $p = 0.285$ , 0.747 and 0.747 (Table 1). There was also no significant difference in TNF- $\alpha$  secretion in treatment group and controls group had  $p = 0.873$ , 0.873 and 0.747 (Table 2).



**Figure 1.** Survival rate (%) of primary cultures of chicken embryo fibroblasts with betel leaf extract treatment. K=control 1, 2, 4=treatment by 4%, 2%, and 1% extract \* =  $p > 0.05$

**Table 1.** The effect of betel leaf extract on ifn- $\gamma$  secretion of peripheral blood mononuclear cells. IFN- $\gamma$  concentrations in the picogram/ml

Control group pg/ml	1% Extract pg/ml	2% Extract pg/ml	4% Extract pg/ml
112	113.5	112.5	109.5
11.5	112	111	109
112.5	114	113	111
113	113	111.5	110
111	112.5	112	110.5
	p = 0.285	p = 0.747	p = 0.747

pg/ml = picogram/ml

**Table 2.** The effect of betel leaf extract on TNF- $\alpha$  secretion of peripheral blood mononuclear cells. TNF- $\alpha$  concentrations in the nanogram/ml

Control group ng/ml	1% Extract ng/ml	2% Extract ng/ml	4% Extract ng/ml
4.8	4.5	4.7	4.8
4.7	4.6	4.65	4.9
4.9	4.55	4.75	5.0
4.75	4.65	4.65	4.95
4.85	4.7	4.75	4.85
	p = 0.873	p = 0.873	p = 0.747

ng/ml = nanogram/ml

## DISCUSSION

Primary cultures are cultures of cells isolated directly from tissues. Their growth is limited compared to cell line culture and very easily inhibited by toxic materials. Cytotoxicity test on betel leaves used primary cell cultures in order to determine the toxicity effects more sensitively. Primary cell cultures of chicken embryo were considered as primary cell cultures with readily available raw materials and simple culture implementation. Therefore, the implementation was conducted by using a modified elution method according to ISO 10993-5 because betel extracts were soluble in water. According to ISO 10993-5, moreover, materials that would be tested was added to the cell culture after the culture reached a 80% density of growth (confluent) cell line culture was used.

In this research, if the primary cell culture was sub cultured, it might ruing its growth, so the tested betel leaf extracts were mixed with the cell suspension before incubation. There was no significant difference between the survival rates of the treatment group and the control groups. It was not considered as toxic to primary cell cultures of chicken embryos. Although other researchers found that betel leaf extract was considered as toxic to human gingival keratinocyte cell culture<sup>6</sup> and human buccal fibroblast culture,<sup>8</sup> chicken fibroblasts were more resistant to the toxicity of betel leaf extract possibly because human fibroblasts is different from avian fibroblasts.

Lipopolysaccharide (LPS) is the largest component of the cell wall of gram-negative bacteria, and also known as endotoxin. LPS activates a variety of mammalian cell types including monocytes/macrophages by activating NF- $\kappa$ B through intracellular signaling pathways and resulting in the synthesis and secretion of proinflammatory cytokines.<sup>16,17</sup> In the Buffy coat obtained by the gradient method using ficoll-hypaque 1.077 contained a population of lymphocytes T and lymphocytes B, as well as monocytes and NK cells. The giving of lipopolysaccharide mitogen, means that cells activated were B lymphocytes and monocyte cells. The activated monocyte cell would release IL-1 $\beta$  proinflammatory cytokines, IL-6, IFN- $\gamma$  and TNF- $\alpha$ . Monocytes have a central role in chronic inflammatory process,<sup>18</sup> and its inflammatory reactions can be classified as the natural immune response againts pathogen invasion.<sup>12</sup> Materials or drugs that can affect the production of proinflammatory cytokines are called immunomodulator,<sup>19</sup> including drug ingredients that can modulate proinflammatory cytokines work.

Several previous studies proved that betel leaf has anti-inflammatory effect,<sup>20</sup> and materials that can suppress the secretion of proinflammatory cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , are categorized as anti- inflammation.<sup>21</sup> Ingredients of medicine that are categorized anti-inflammation has several mechanism of action which are: 1) modulating inflammatory response with transcription factors as target, eg, cyclosporin A;<sup>22</sup> 2) inhibiting cyclo-oxygenase activity, eg diclofenac, piroxicam, and ibuprofen;<sup>23</sup> 3) inhibiting lipoxygenase, eg MK-886 and A-63162;<sup>22</sup> 4) inhibiting complement system activity;<sup>24</sup> and 5) inhibiting cytokine.<sup>25</sup> Anti-inflammation classified as cytokine inhibitors can be divided into two types. First, endogenous type, for example: 1) soluble cytokine receptors, such as TNF receptor (TNF-R) and IL-1 receptor type II (IL-1R); 2) cytokine receptor antagonists, such as receptor antagonist IL-1 (IL-1Ra); and 3) inhibitory cytokines, such as IL-4, IL-10, IL-11, IL-13, and TGF- $\beta$ . Second, exogenous type, such as antibodies against TNF- $\alpha$  (anti-TNF- $\alpha$ ), includes ingredients that inhibit the synthesis and secretion of proinflammatory cytokines.

In this research, there were no significant differences between the secretion of IFN- $\gamma$  and TNF- $\alpha$  by either betel extract 1%, 2%, or 4% ( $p > 0.05$ ) compared with the control group. It means that the anti-inflammatory properties of betel leaves was not working through suppression of proinflammatory cytokines, IFN- $\gamma$  and TNF- $\alpha$ , but through other channels in accordance with the research of Pin *et al.*,<sup>9</sup> which states that the anti-inflammatory properties of betel leaf works by suppressing the activity of lipoxygenase enzyme.<sup>8</sup> A research conducted by Ganguly *et al.*,<sup>26</sup> revealed that the anti-inflammatory activity of betel leaf works by suppressing the expression of IL-12 p40. Meanwhile, by Shalini T *et al.*,<sup>12</sup> reported showed that the anti-inflammatory activity of betel leaf works by suppressing cyclooxygenase and thrombosis aggregation. However, this research has several weaknesses: first, embryonated chicken eggs was not taken from SPF (specific

pathogenic free) chickens, and second, mononuclear cells were isolated from only one donor. It would be better when it is done with cells from several donors. Finally, it can be concluded that betel leaf extract (*Piper betel* L) is not toxic to primary fibroblast culture of chicken embryo. Betel leaf extract has no effect on the production of proinflammatory cytokines by human peripheral blood mononuclear cells stimulated with lipopolysaccharide.

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