Indonesian andaliman fruit (Zanthoxylum acanthopodium DC.) extract supresses the expression of inflammatory mediators on fibroblasts cells in vitro

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ABSTRACT

The recurrent aphthous stomatitis (RAS) is the oral mucosa inflammation that causes pain and interferes with daily activity. One of the RAS’ predisposing factors is Streptococcus sanguinis infection, which causes an antigenic reaction. Ulcer progression in the oral cavity is modulated by tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Zanthoxylum acanthopodium DC is known as andaliman is an Indonesian fruit that has been reported to have antimicrobial and anti-inflammatory activities. The objectives of this study were to analyze the cytotoxic concentration of andaliman fruit extract and determine the effect of andaliman fruit extract on TNF-α and IL-6 levels of fibroblasts infected with Streptococcus sanguinis. The cytotoxicity was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium (MTT) test. TNF-α and IL-6 levels were measured by enzyme-linked immunoassay (ELISA) tests. All the obtained data were statistically analyzed using the one-way analysis of variance (ANOVA) test (α=0.05). The MTT assays confirmed that 25 mg/mL concentration of Z. acanthopodium extract has no cytotoxic effects on fibroblast cells (viability=95.62%). TNF-α and IL-6 level on infected fibroblast cells were significantly decreased (P<0.05) after 25 mg/mL and 12.5 mg/mL concentration of Z. acanthopodium extract treatment, respectively. Our results concluded that Indonesian andaliman fruit significantly reduced the inflammatory biomarkers in the infected fibroblast cells. Furthermore, in vivo study using animal models is needed to confirm the anti-inflammatory potential of andaliman fruit extract.

Keywords:
Andaliman fruit, Anti-inflammation, Cytotoxicity, IL-6, TNF-α, Zanthoxylum acanthopodium

1. INTRODUCTION

The recurrent aphthous stomatitis (RAS) is a common disease of the oral mucosa, manifested by recurrent mucosal ulcers1,2. The etiology of RAS is still unclear, but the predisposing factors include trauma, microbial infection, indigestion, blood disorders, emotional disorders (e.g., stress and worry), immunological disorders, nutritional deficiency, genetic disorders, allergy, and hormones (e.g., menstruation cycle)3,4. The etiopathogenetic basis is an inflammation due to Streptococcus sanguinis; this organism is one of the normal flora in the oral cavity but can cause an antigenic reaction and recurrent ulcers on the oral mucosa. Immunological changes are an underlying etiology of the oral mucosa inflammation, and the tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) cytokines play a key role in ulcer progression of oral cavity4,5.

The primary strategy for RAS treatment is to control the inflammation, to suppress the inflammation-associated cells’ activity, reducing the pain of the lesion, and accelerating the healing process5. The most commonly used anti-inflammatory agents to treat oral cavity inflammations are topical corticosteroids6,7. However, the Indonesian government is currently promoting the use of herbal medicines as alternative treatments...
because Indonesia has many effective herbal plants, reasonably priced for its population. The Ministry of Health, Republic of Indonesia, now encourages the use and research of nutritious plants to reduce and treat illnesses, because these plants are easy to obtain and practical.

One promising alternative medicine is the andalimann (Zanthoxylum acanthopodium DC). The genus Zanthoxylum has known for its biological activities, including larvicidal, anti-inflammatory, analgesic, antioxidant, antibiotic, hepatoprotective, anti-plasmodial, cytotoxic, antiproliferative, anthelmintic, antiviral, anticonvulsant, and antifungal properties. This plant is originated from North Sumatra, where it is known as intir-intir, but it is also known abroad as Sichuan pepper or Indonesian lemon pepper. The Z. acanthopodium fruit is recognized for its chemical properties, such as 20.9% of oil, fat content, and the presence of terpenoids, flavonoids, limonene, and sabinene.

A previous study showed that Z. acanthopodium pure essential oil created a 16 mm inhibition zone against Staphylococcus aureus and 12 mm when diluted to a 50% concentration. The ethanol extract of Z. acanthopodium also had an antipyretic activity and suppressed the free radical activity by 61.81% at 200 ppm concentration. Therefore Z. acanthopodium extracts have the potential as food supplements and herbal medicines to treat inflammation, especially gastrointestinal inflammation. There are many potential health benefits from Z. acanthopodium, but this plant extracts’ effectiveness in reducing oral cavity inflations like RAS has not been studied.

Any investigation into new medicines, whether natural or synthetic, requires strict safety tests on host cells and the establishment of any cytotoxicity effects. The cell viability quantification and cell proliferation tests are the basis for many in vitro safety studies. However, no studies have been conducted to test the cytotoxicity of Z. acanthopodium extracts in fibroblasts, which are the key components of the oral mucosa and play an important protective role during the restoration of mucosa following a trauma event. Fibroblasts synthesize extracellular matrix and collagen that have important functions in epidermal proliferation and differentiation through the formation of cellular matrix and growth factors and cytokines secretion. The present study aims to examine the cytotoxic effects of Z. acanthopodium ethanol extract on fibroblasts using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium (MTT) assay method and determine the effects on TNF-α and IL-6 levels in fibroblasts infected with S. sanguinis.

2. MATERIALS AND METHODS

This study was a laboratory experiment with a post-test only control design aimed to determine the cytotoxicity of Z. acanthopodium ethanol extract on fibroblasts and its effects on chemical anti-inflammatory mediator’s, TNF-α, and IL-6 levels in fibroblasts infected with S. sanguinis.

2.1. Sample collection and extraction

Zanthoxylum acanthopodium DC fruit originates from plantations in Parsoburan Habinsaran, Tobasa, North Sumatera, Indonesia, and was picked in young conditions with a characteristic of dark reddish-green color. The Parsoburan Habinsaran area is about 700-1,650 meters above sea level. One kilogram of Zanthoxylum acanthopodium DC fruit was washed and dried at room temperature until completely dry. The dried Zanthoxylum acanthopodium DC fruit (267 grams) was ground and shifted until smooth. The simplicia was soaked in 70% ethanol inside a percolator overnight. Later, the ethanol was removed until the extract was obtained. Then 70% ethanol was added into the extract, filtered using a filter paper and the pulp was discarded. The ethanol was evaporated using an evaporator until the extract becomes thick. The extract was dried using the water bath method at 60-70°C and the obtained thick extract was ready to use. The thick extract of Zanthoxylum acanthopodium DC fruit was at 100% concentration. The dilution was performed using the 10% DMSO to obtain a series of concentration at 50%, 25%, 12.5%, and 6.25%.

2.2. Qualitative phytochemical screening analysis

Phytochemical analysis assay methods were done to determine the presence of bioactive compounds in the extracts. In this study, each phytochemical analysis assay was performed in The Indonesian Medicinal and Aromatic Crops Research Institute (local acronym BALITTO), Bogor, Indonesia to detect the presence of saponins, tannin, alkaloids, phenolic compounds, flavonoids, triterpenoids, glycosides, and plant sterols from Z. acanthopodium ethanol extract. The saponins were identified by heating. The tannin and phenolic were analyzed by soaking the sample in the FeCl3, I and 5% solution, respectively. The alkaloids were detected using Dragendorff’s and Mayer’s reagent. The flavonoids were detected using HCl + Mg and NaOH 10% solution. The triterpenoid and steroid were detected using H2SO4 + CH3COOH solution. The qualitative results were expressed as (+) for the presence and (-) for the absence of phytochemicals.

2.3. Streptococcus sanguinis culture

The bacterial culture used in this study was obtained from the MiCore laboratory collection of the
Faculty of Dentistry, Trisakti University, Jakarta. *S. sanguinis* was inoculated into the brain-heart infusion (BHI) broth (Thermo Scientific, Waltham, MA, USA) and incubated at 37°C in an anaerobic atmosphere. *S. sanguinis* was killed by heating at 80°C for 30 min before the exposure to the fibroblasts.

### 2.4. Fibroblast culture

The fibroblast culture from the mouse was incubated at 37°C in a 5% CO₂ atmosphere for 24-72 hours to allow the fibroblasts to mature into full fibroblast cells. The fibroblast growth was carried out at 3-4 days and the culture medium was changed regularly. A population of 10⁵ cells/mL fibroblast cell was treated by the ethanol extract of *Z. acanthopodium*.

### 2.5. Cytotoxicity evaluation using the 3-[4,5-dimethylthiazol-2yl]-2,5 diphenyltetrazolium (MTT) test

3-[4,5-dimethylthiazol-2yl]-2,5 diphenyltetrazolium assay was performed by measuring the amount of violet formazan product generated by the reduction of the MTT tetrazolium dye by fibroblasts’ mitochondrial dehydrogenase. Fibroblasts were added to the phosphate-buffered saline containing 1 mg/mL of MTT (Sigma-Aldrich, St. Louis, MO, USA) and incubated for three hours. The amount of formazan product was determined by measuring the absorbance at 490 nm with an AccuReader Microplate Reader (Metertech, Taipei, Taiwan). According to ISO 2010993-5:2009, regarding the biological evaluation of medical devices and in vitro cytotoxicity testing, it was determined if the cell viability showed less than 70% then the material has a toxicity potential.

### 2.6. Enzyme-linked Immunoassay (ELISA) measurements of fibroblast’s TNF-α and IL-6 levels

The confluent fibroblasts (10⁵ cell/mL) were exposed to *S. sanguinis* (10⁵ colony-forming units [CFU]/mL) for 24 h in a 5% CO₂ atmosphere at 37°C in 96 well-plate. The *Z. acanthopodium* ethanol extract was added and the fibroblasts were incubated for a further 24 hours. The TNF-α and IL-6 levels were determined using the ELISA method (BioLegend, San Diego, USA). The absorbance was measured at 490 nm with an AccuReader Microplate Reader (Metertech, Taipei, Taiwan). The ELISA assay was performed in triplicate. Wells without *Z. acanthopodium* ethanol extract was used as negative control and Triamcinolone (0.01%) was used as a positive control. In this study, all procedures were triplicate.

### 2.7. Statistical analysis

The Shapiro-Wilk test was used for the normality test. One-way analysis of variance (ANOVA) test was applied to reveal the significant differences between TNF-α and IL-6 levels in the fibroblast cells infected with *S. sanguinis* and *Zanthoxylum acanthopodium* fruits extract in different concentrations. The differences were considered statistically significant if *P*<0.05. Statistical calculations were performed with SPSS Statistics for Windows software version 20 (IBM, USA).

### 3. RESULTS

#### 3.1. Preliminary phytochemical qualitative analysis of *Zanthoxylum acanthopodium* extract

The use of spices in food has been practiced for centuries. The spices not only enhance the aroma and taste of the food but also provide health benefits. Apart from its unique flavor, several recent studies showed the various biological activities of these spices. *Zanthoxylum acanthopodium* DC is one of the spices that commonly used as a seasoning in North Sumatran foods, especially by the Tapanulis. The genus *Zanthoxylum* belongs to the Rutaceae family. The plants in this genus are mainly used as spices and medicines. The name *Zanthoxylum* was created by Linnaeus in 1757. This genus contains more than 546 species worldwide and 250 species were spread in tropical and subtropical regions of Asia and North America. *Z. acanthopodium* is one of the genera found in Indonesia. The findings of this present study demonstrated that the *Zanthoxylum acanthopodium* fruits extract contained phenolic, tannin, flavonoid, triterpenoid, steroid, and alkaloid compounds (Table 1).

#### 3.2. *Zanthoxylum acanthopodium* extract did not affect fibroblast cell viability in a concentration of 25%

As shown in figure 1, the MTT assay showed that the fibroblast viability was 0.73% following the exposure to 100 mg/mL and 63.5% following the exposure to 50 mg/mL *Z. acanthopodium* ethanol extract. The fibroblast cell viability was much higher at 95.62%, following the exposure to 25 mg/mL *Z. acanthopodium* extract.

#### 3.3. *Zanthoxylum acanthopodium* extract reduced TNF-α and IL-6 level on fibroblasts infected with *S. sanguinis*

The TNF-α level was significantly decreased (*P*<0.05) after *Z. acanthopodium* ethanol extract treatment in fibroblasts infected with *S. sanguinis*. The largest decrease in TNF-α level was obtained in the 50 mg/mL concentration, while 25 mg/mL concentration was almost
Table 1. Phytochemical analysis result of *Zanthoxylum aethiopodium* extract.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenolic</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Flavonoids</td>
<td></td>
</tr>
<tr>
<td>- HCl concentrated + Mg Reagent</td>
<td>+</td>
</tr>
<tr>
<td>- NaOH 10% Reagent</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>-</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>+</td>
</tr>
<tr>
<td>Steroids</td>
<td>+</td>
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<tr>
<td>Alkaloids</td>
<td></td>
</tr>
<tr>
<td>- Dragendorff’s Reagent</td>
<td>+</td>
</tr>
<tr>
<td>- Mayer’s Reagent</td>
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Figure 1. The viable fibroblast cells percentage after *andaliman* fruit (*Zanthoxylum aethiopodium* DC) extract treatment using MTT assay.

Figure 2. TNF-α level of *S. sanguinis*-infected fibroblast cells after treated with *andaliman* fruit (*Zanthoxylum aethiopodium* DC) extract.
almost equivalent to triamcinolone positive control (Figure 2). The one-way ANOVA results confirmed the statistically significant differences (P<0.05) in TNF-α level reduction of all concentrations compared to the negative control.

The IL-6 level was significantly decreased (P<0.05) after the Z. acanthopodium ethanol extract treatment. The 50 mg/mL concentration greatly reduced the cytokine level compared to the other concentrations (Figure 3). The ANOVA test confirmed a significant difference (P<0.05) in IL-6 reduction of all concentrations of Z. acanthopodium extract in fibroblasts infected with S. sanguinis compared to the negative control.

4. DISCUSSION

On cytotoxicity tests, the viable cells can reduce MTT to its colored formazan salt and the 490 nm measured absorbance is at parallels with the viable cell number, while dead cells are unable to change MTT into the colored formazan. The cytotoxic agent exposure to cells causes toxicity through various mechanisms, such as membrane destruction, protein synthesis prevention, irreversible ligands attachment to receptors, DNA synthesis inhibition, and enzymatic reactions disruption. Cell necrosis is initiated upon the disruption of the cell’s ability to maintain homeostasis, particularly water and ion homeostasis. Intracellular organelles, especially mitochondria and the cell will swell and rupture due to these disruptions, causing the release of cytoplasm, including the lysosome enzymes, into the extracellular fluid. The enzyme activity of the extracellular medium can be used to determine the necrosis level.

Toxicity effects can be harmful or advantageous, depending on the ecological context and the pharmacology. The ISO 2010993-5-2009 guidelines on the biological evaluation of medical equipment and in vitro cytotoxicity tests state that the potential toxicity is indicated by the cell viability measurements of less than 70%. In the present study, the fibroblast viability was 0.73% for the 100 mg/mL concentration of Z. acanthopodium ethanol extract and more than 70% for the 25 mg/mL. Therefore, Z. acanthopodium ethanol extract was toxic at 100 mg/mL concentration but non-toxic below 25 mg/mL. A concentration of 25 mg/mL is likely a safe concentration of Z. acanthopodium extract as an oral anti-inflammatory agent. The previous study reported that the IC50 of the Zanthoxylum acanthopodium extract was 48.94±0.32 µg/mL to T47D breast cancer cell lines. Different solvents might affect the cytotoxicity effect of the extract; Satria et al.’s study used ethyl acetate, while our study used ethanol as solvent.

TNF-α and IL-6 are cytokines associated with inflammation reactions. The secretion of TNF-α and IL-6 is stimulated by microbial products (e.g., bacterial endotoxins), immune complexes, and T-lymphocyte products that arise during the immune response. In this study, Z. acanthopodium ethanol extract treatment resulted in a significant reduction in the TNF-α level of fibroblasts infected by S. sanguinis. The median effect of the Z. acanthopodium ethanol extract on the TNF-α level was significantly decreased with the increase of concentration. The 25 mg/mL concentration of Z. acanthopodium ethanol extract showed the potency as a promising anti-inflammatory agent because it decreased the TNF-α level close to positive control and
was non-toxic to fibroblasts. The *Z. acanthopodium* extract significantly reduced the IL-6 levels in fibroblasts infected with *S. sanguinis*. The 25 mg/mL concentration of *Z. acanthopodium* extract was more effective and non-toxic at reducing the IL-6 levels than other concentrations. This suggests that 25 mg/mL concentration could be clinically useful as an anti-inflammatory treatment. IL-6 levels in each extract concentration did not have a specific trend or in a non-dose response fashion. This condition may be caused by the function of IL-6 which can be both pro and anti-inflammatory cytokine. However, this hypothesis still needs further studies.

Oral mucosa healing is preceded by cell migration and proliferation. The process of extra-cellular protein matrix secretion is highly controlled and the matrix is extensively remodeled to rebuild injured tissue. Epithelial cells are the first cell to migrate to the center of a wound to prevent pathogen contamination. The fibroblasts then migrate to sub-epithelial areas and produce collagen and healing factors. The inflammation process is an important part of the healing process because the presence of inflammation modulators can delay the healing of oral mucosa\(^{26,27}\). A high concentration of inflammatory cytokines inhibits the migration of epithelial cells and fibroblasts and increases the TNF-\(\alpha\) and IL-1\(\beta\) levels, thereby creating positive feedback for inflammation\(^{28}\).

In this study, the *Z. acanthopodium* extracts significantly reduce the inflammation mediator, TNF-\(\alpha\) and IL-6, level. Therefore, the extract has the potential to serve as an anti-inflammatory agent and accelerate the healing process of the oral mucosa. The observed reduction of TNF-\(\alpha\) and IL-6 levels in this study indicated that the *Z. acanthopodium* ethanol extract provided a greater reduction in TNF-\(\alpha\) than in IL-6 cytokine levels. Therefore, this extract may be useful for controlling inflammation in the oral cavity, because TNF-\(\alpha\) is a commonly found inflammation mediator in RAS cases.

Based on the phytochemical testing results, it can be seen that the ethanol extract of *Zanthoxylum acanthopodium* DC fruit in this study contains phenolic, tannin, flavonoid, triterpenoid, steroid, and alkaloid compounds. Flavonoid and tannin have potential benefits as an antioxidant and anti-inflammatory agents\(^{29,30}\). Flavonoids also have biochemical effects, which inhibit some enzymes, such as aldose reductase, xanthine oxidase, phosphodiesterase, Ca\(^{2+}\)-ATPase, lipooxygenase, and cyclooxygenase. They have been found to have anti-inflammatory activity in both proliferative and exudative phases of inflammation\(^{39}\). This study observed the significant decrease of TNF-\(\alpha\) and IL-6 levels as the effect of ethanol extract of *Zanthoxylum acanthopodium* DC on inflammation in fibroblasts infected with *Streptococcus sanguinis*.

5. CONCLUSIONS

Indonesian *andaliman* fruit (*Zanthoxylum acanthopodium* DC) ethanol extract was found to be non-toxic below the concentration of 25 mg/mL. *Z. acanthopodium* as herbal plant has the efficacy as an anti-inflammatory in the oral cavity since it could decrease the level of TNF-\(\alpha\) and IL-6 as chemical mediators. Indonesian *andaliman* fruit could be used as an herbal medicine to reduce inflammation, particularly within the oral mucosa. Further studies on molecular mechanisms on the *Zanthoxylum acanthopodium* substances to modulate the expression of inflammatory cytokines and proteins in fibroblast is still needed. Furthermore, *in vivo* study using an animal model is needed to confirm the anti-inflammatory potential of this herbal plant.

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Conflict of interest

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Author contribution statement

ASW, RA, SL conceived, and designed the study. ASW and SL conducted the laboratory experiments. ASW, RA, and SL analyzed the data. SL and ASW wrote the manuscript. All authors read and approved the manuscript.

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