

# Cellular apoptosis susceptibility expression in cultured pulp fibroblast cell induced by saponins from *Plumeria acuminatae Ait.*

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**ABSTRACT Objective.** Saponins from *Plumeria acuminatae Ait.* have been proven to have cytotoxic activity in cultured human mucosal fibroblast cells. However, there is no evidence that these saponins can induce cellular apoptosis susceptibility expression. The aim of this study was to investigate the cellular apoptosis susceptibility expression of pulp fibroblast cells induced by saponins from *Plumeria acuminatae Ait.* **Methods.** Cultured human dental pulp fibroblast cells ( $2 \times 10^4$  cells/well in a 96-well plate) were treated with saponins from *Plumeria acuminatae Ait.* in different concentrations (0.01; 0.1; 1; 100; 1000  $\mu\text{g}/\text{mL}$ ) and incubated at  $37^\circ\text{C}$  for 24 and 48 hours. A dye exclusion test was used to study cytotoxicity and the immunohistochemistry assessed with a monoclonal antibody to cellular apoptosis susceptibility in cultured pulp fibroblast cell samples. **Results.** Saponins from *Plumeria acuminatae Ait.* induced cellular apoptosis susceptibility expression in cultured pulp fibroblast cells, and the percentage of the cells expressing cellular apoptosis susceptibility decreased as the saponin concentration and time exposure increased. **Conclusion.** Saponins from *Plumeria acuminatae Ait.* induced cellular apoptosis susceptibility expression in cultured human dental pulp fibroblast cells. Further study of the pulp fibroblast cell death via apoptosis is required.

## Introduction

Secretions from *Plumeria acuminatae Ait.* have been used by Indonesians (mostly people from Madura) to treat toothache. They place it topically in the decayed tooth<sup>1</sup>. A study has reported that the *Plumeria acuminatae Ait.* secretion devitalized guinea pig (*Cavia cobaya*) teeth (unpublished data, 1985). This study used a decayed molar tooth in which the secretion was placed. It caused devitalization of the tooth within several days. The devitalization was probably caused by the saponins in *Plumeria acuminatae Ait.* (unpublished data, 1985). Cell death of pulp tissue is the

main effect exerted by a devitalization agent on teeth<sup>2</sup>. A previous study has found that saponins from *Plumeria acuminatae Ait.* have cytotoxic activity in cultured mucosal fibroblast cells<sup>3</sup>.

A chemical compound's cytotoxic activity can be indicated by its ability to induce cell death. There are two mechanisms of cell death, namely, necrosis and apoptosis<sup>4</sup>. Both can be distinguished by morphological and biochemical changes<sup>5</sup> and can be detected by examination of those morphological or biochemical changes<sup>6</sup>. Today, apoptosis has been used as a basic therapy for several diseases, including malignancy and those caused by tissue ischemia<sup>7</sup>. In endodontics, achieving apoptosis is also a treatment target. There is evidence that calcium hydroxide can induce apoptosis in human periodontal ligament cells<sup>8</sup>.

It has been reported that normal lymphoid tissue and lymphoma cells express the cellular apoptosis susceptibility (CAS) protein. The expression of CAS correlates with proliferation of normal and malignant lymphoid cells. However, it is not possible to distinguish between the functions of CAS in apoptosis and proliferation in normal and malignant lymphoid tissue<sup>9</sup>. The CAS protein is a 100-kDa

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protein, which is homologous with the *CSE1* yeast chromosome segregation gene and can also be found on the 20q13 human chromosome. Within cells, CAS lies not only in the nucleus but also in the cytoplasm where it is associated with the cell microtubules but is not an integrated part of the microtubules<sup>10,11</sup>. Cellular apoptosis susceptibility has a role as a protein transport factor, which is needed in the apoptosis induction and execution phase<sup>12</sup>.

In this study, we present the first report showing that CAS proteins are expressed in saponin-mediated cell death. We used dental pulp fibroblast cells because saponins will produce this effect in dental pulp tissue.

## Materials and methods

### Extraction of saponins from *Plumeria acuminatae* Ait.

The saponins were purified using serial extraction processing. The Biology of Pharmacy Department, Faculty of Pharmacy, Gadjah Mada University identified the plant. Three hundred and fifty grams of *Plumeria acuminatae* Ait. stem were dried then macerated in hexane solution for 24 hours. The extract obtained was then macerated in an ethanol solution for 24 hours, and this procedure repeated 3 times. The ethanol extract was analyzed by column using silica gel for the stationary phase and in the mobile phase, ethyl 100%, ethyl acetate and methanol (9:1), ethyl acetate and methanol (8:1), ethyl acetate and methanol (1:1), and methanol 100% were used. The extraction process continued until a complex mixture of saponin compounds was obtained. Column chromatography was used to identify the extract.

### Cell culture

A human dental pulp fibroblast cell line was obtained by following a modified version of previously reported procedures<sup>13</sup>. Human dental pulp fibroblast cells from a molar tooth extracted to relieve impaction were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO<sub>2</sub>-95% air humidified incubator. The media and sera were purchased from Sigma-Aldrich (St. Louis [MO], US).

### Assay for growth inhibition

The fibroblast cells were seeded at 2 x 10<sup>4</sup> cells/well in a 96-well plate, and saponins in varying concentrations (0.01; 0.1; 1; 100; 1000 µg/mL) were added to each well.

The cells were incubated at 37°C for 24 hours and 48 hours. The general viability of the cultured cells was determined using the trypan blue dye exclusion test. This experiment was performed in triplicate.

### Immunohistochemistry for cellular apoptosis susceptibility expression

Cells (2 x 10<sup>4</sup>) were treated with saponins and incubated for 24 hours. Twenty-four and 48 hours later, the cells were washed with phosphate-buffered saline (PBS) and incubated in growth medium. Cells were mounted on polylysine slides. They were fixed in methanol and acetone for 15 minutes in order to block endogenous peroxidase activity then washed with PBS 3 times. Subsequently, an overnight incubation with anti-CAS monoclonal antibody was performed at room temperature. A biotinylated anti-mouse immunoglobulin secondary antibody was used for detection of anti-CAS binding, according to the manufacturer's instructions, and streptavidin-peroxidase was used for the CAS immunoreaction. After development of the peroxidase reaction, the cells were incubated for 60 minutes at room temperature. All cells were counterstained with hematoxylin. Untreated cells were used as negative controls in this study.

Apoptotic pulp fibroblast cells were revealed by CAS expression and they were examined using an inverted phase contrast microscope. This experiment was performed in triplicate.

### Statistical analysis

A Pearson correlation analysis was used to describe the relationship between the saponin concentration and the CAS expression percentage.

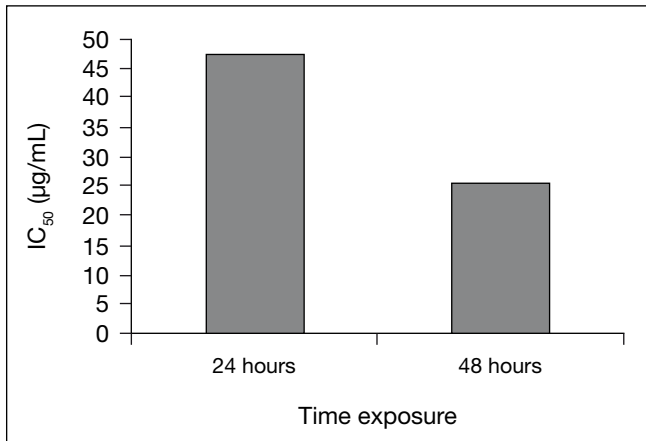
## Results

### Cytotoxicity of saponins from *Plumeria acuminatae* Ait.

The results of this study showed that *Plumeria acuminatae* Ait. saponins have cytotoxic activity in cultured pulp fibroblast cells. The concentrations for 50% inhibition (IC<sub>50</sub>) of the *Plumeria acuminatae* Ait. saponins in cultured pulp fibroblast cells were 47.4 µg/mL (24 hours) and 25.6 µg/mL (48 hours) [Figure 1].

### Cellular apoptosis susceptibility expression

This study showed that *Plumeria acuminatae* Ait. saponins



**Figure 1** IC<sub>50</sub> of saponins from *Plumeria acuminatae* Ait. in cultured pulp fibroblast cells

induced CAS expression in cultured pulp fibroblast cells (Figure 2). A Pearson correlation analysis (Table) indicated a negative correlation between each of the *Plumeria acuminatae* Ait. saponin concentration groups and the percentage of pulp fibroblast cells with CAS expressions, and a significant negative correlation between the time exposure and the CAS expression percentage in the pulp fibroblast cells (P<0.05) [Figure 3].

**Discussion**

This study is the first to show that pulp fibroblast cells can express the CAS protein. The CAS protein is needed for the apoptosis induction and execution phase<sup>12</sup>. The CAS expression in pulp fibroblast cells treated with saponins suggests fibroblast cell death occurs via apoptosis although not all cells express CAS. In this immunohistochemical study, CAS expression showed as a brown, intact nucleus (Figures 2a and 2b), and the percentage of

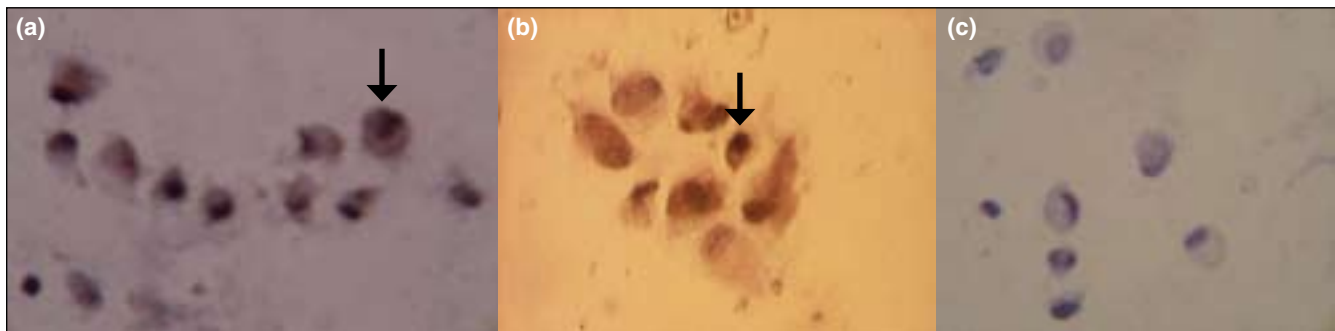
**Table** Pearson correlation analysis of *Plumeria acuminatae* Ait. saponin concentration levels and the percentage of pulp fibroblast cells with apoptosis after 24 and 48 hours' exposure

	r	P value
CAS * expression % vs saponin concentration	-0.524	0.09
CAS expression % vs time exposure	-0.799	0.00

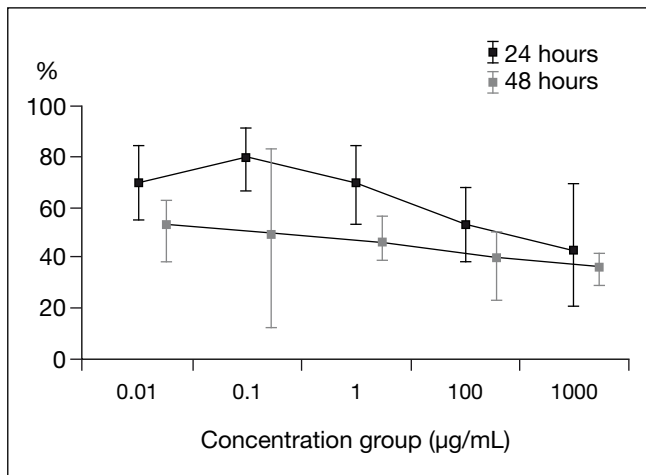
\* CAS denotes cellular apoptosis susceptibility

cells with CAS expression decreased with an increasing concentration of saponins. The CAS expression was not seen in the control cells (Figure 2c). This study suggests that the mechanism by which pulp fibroblast cell apoptosis is induced by *Plumeria acuminatae* Ait. saponins might be similar to that seen in previous studies<sup>14-16</sup>. Saponin structural differences are not an important factor in their apoptotic effect on cells<sup>14</sup>. Saponins from *Acacia victoriae* (Avicin) induce apoptosis in cultured human fibroblast cells. These saponins induce cell apoptosis by affecting the mitochondrial membrane potential independently of the membrane-bound death receptors<sup>15</sup>. This transition will induce the release of mitochondrial factors (apoptosis-inducing factor and cytochrome c), which will activate caspase. Cytochrome c will interact with Apaf-1 inside the cytosol. The interaction of Apaf-1 with adenosine triphosphate (ATP) will activate caspase-9, which will then activate caspase-3. Caspase-3 is the caspase responsible for execution of apoptosis<sup>5</sup>.

This study reports a correlation between CAS expression and length of exposure to and concentration of saponins from *Plumeria acuminatae* Ait. A Pearson analysis found a negative relationship between the percentage



**Figure 2** The CAS expression of cultured pulp fibroblast cells treated with *Plumeria acuminatae* Ait. saponins after exposure for (a) 24 and (b) 48 hours, and that of (c) control cells (magnification, x 400)



**Figure 3** Percentage of pulp fibroblast cells with apoptosis in different *Plumeria acuminatae Ait.* saponin concentration groups, after exposure of 24 and 48 hours. Mean values represented by the square with a 95% confidence interval included

of pulp fibroblast cells showing CAS expression and the concentration of *Plumeria acuminatae Ait.* saponins, however the relationship was not significant ( $P > 0.05$ ). The same analysis also showed a significant negative relationship between the percentage of pulp fibroblast cells expressing CAS and the length of time of exposure to saponins from *Plumeria acuminatae Ait.* ( $P < 0.05$ ). Apoptosis and necrosis occur as a continuum depending on ATP concentration. If there is minimal ATP depletion, only apoptosis occurs but further ATP depletion switches apoptosis to the necrosis cell death process<sup>17,18</sup>. We suggest that there was minimal cell ATP concentration depletion in the low saponin concentration groups, allowing apoptosis to be induced; further depletion of the ATP concentrations in the pulp fibroblast cells caused by high saponin concentrations will switch apoptotic cell death to necrotic cell death<sup>2,17,18</sup>.

## Conclusion

This study has found that *Plumeria acuminatae Ait.* saponins induce CAS expression in pulp fibroblast cells and that the percentage of pulp fibroblast cells expressing CAS correlates negatively with saponin concentration levels and time exposure. The pulp fibroblast cell CAS expression induced by saponins from *Plumeria acuminatae Ait.* indicates that further study of the pulp fibroblast cell death via apoptosis is needed.

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