

Saponins from *Plumeria acuminata* Ait Induce Cell Growth Inhibition and Apoptosis of Oral Squamous Carcinoma Cells

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Objective: To investigate the cytotoxic and apoptotic effects of saponins from *Plumeria acuminata* Ait on oral squamous carcinoma cells (OSCC).

Methods: OSCC cells seeded at 2×10^4 cells/well in a 96-well plate were treated with saponins from *P. acuminata* Ait and cisplatin in various concentrations for 24 h. Trypan blue dye exclusion assay and ethidium bromide/acridine orange were used to evaluate their cytotoxic and apoptotic effects on the cells, respectively.

Results: The results showed that both saponins and cisplatin had cytotoxic and apoptotic effects on OSCC cells.

Conclusion: Saponins from *P. acuminata* Ait may be potential anti-cancer agents for OSCC.

Key words: apoptosis, OSCC, *Plumeria acuminata* Ait, saponins

Ninety per cent of oral cancer is oral squamous carcinoma (OSC), and the prevalence is increasing in the world population¹. Forty per cent of OSC is more prevalent in the tongue². According to one study, 86% of OSC patients are detected in the late stage in the dental clinic³. Surgery is the most effective treatment of oral cancer. However, surgery also produces several adverse effects, such as facial asymmetry, and speech and mastication problems⁴. Therefore, combinations of surgery with chemotherapy or radiotherapy are often the choice for the treatment of oral cancer. Owing to the surgery's adverse effects and the expensive cost of chemotherapy and radiotherapy, some oral cancer patients fail to receive any of the above treatments^{5,6}, and some seek alternative treatment. Herbal treatment is popular in Indonesia, and many types of herbs are used⁷.

Secretions from Kamboja plants (*Plumeria acuminata* Ait) have been placed topically into carious teeth by some Indonesians (mostly people from Madura island, East Java) to treat toothache^{8,9}. Previous studies have found that saponins from *P. acuminata* Ait have cytotoxicity on dental pulp fibroblast cells¹⁰. The saponins induce cellular apoptosis susceptibility (CAS), a protein transport factor, which is needed in the induction of apoptosis of the human dental fibroblast cell¹⁰. However, whether saponins can induce apoptosis in oral squamous carcinoma cells (OSCC) remains unexplored. The aim of the present study was to explore the cytotoxic effects of saponins on OSCC cells.

Materials and methods

Extraction of saponins from P. acuminata Ait

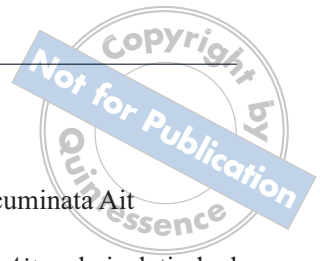
The saponins were purified by a serial extraction processing as described in a previous study¹⁰. The stem of *P. acuminata* Ait (350 g) was dried and macerated in hexane solution for 24 h. The extracts were further extracted in ethanol solution for 24 h three times. The ethanol extracts were applied to a column, in which silica gel was the stationary phase and the mobile phase

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was 100% ethyl, a mixture of ethyl acetate and methanol (9:1), ethyl acetate and methanol (8:1), ethyl acetate and methanol (1:1), and methanol 100%. The extraction process continued until a mixture of saponin compounds was obtained. Column chromatography was used to identify the extract.

Cell culture

B88 cells, an OSCC line¹¹, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), 100 µg/ml penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. The media and sera were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Trypan blue exclusion assay for growth inhibition

B88 cells were seeded at 2×10^4 cells/well in 96-well plate and treated with saponins at concentrations of 0, 1, 10, 40, 80, 150, 600, 1250 and 5000 µg/ml and cisplatin (as a positive control) at concentrations of 0, 6.25, 12.5, 25, 50 and 100 µg/ml for 24 h. The viability of cells was determined using the trypan blue exclusion test. Data for the viability of cells were obtained from three separate experiments.

Ethidium bromide/acridine orange (EB/AO) assay for apoptosis examination

B88 cells were seeded at 2×10^4 cells/well in a 96-well plate and treated with saponins at concentrations of 37.5, 75 and 150 µg/ml and cisplatin at concentrations of 10, 15 and 20 µg/ml for 24 h. The cells were washed with phosphate buffered saline (PBS) and further incubated in the medium without any treatment and mounted on slides. The cells were fixed in 4% methanol for 25 min at 4°C. The cells were stained with EB/AO (ratio 1:1) for 1 min at room temperature (24°C) and examined using a fluorescence microscope. Apoptotic cells presented orange colour. Cells without any treatment were used for negative control.

Statistical analysis

Probit analysis was used to determine the half maximal inhibitory concentration (IC₅₀) of saponins and cisplatin. Pearson correlation was used to examine the relationship between saponin concentration and apoptosis percentage.

Results

Cytotoxicity of saponins from P. acuminata Ait

Both saponins from *P. acuminata Ait* and cisplatin had cytotoxic activity on B88 cells. The IC₅₀ of saponins and cisplatin in B88 cells were 309.62 µg/ml and 33.68 µg/ml, respectively (Table 1).

Apoptotic effects of Saponins from P. acuminata Ait

Saponins from *P. acuminata Ait* induced apoptosis of OSCC cells (Fig 1). According to Pearson correlation analysis, there was a negative correlation between the concentrations of saponins and the apoptotic B88 cells, but this was not statistically significant ($P > 0.05$) (Table 3). The correlation between concentrations of cisplatin and the apoptotic B88 cells was statistically significant ($P < 0.05$).

Discussion

The present study is the first report that saponins from *P. acuminata Ait* have cytotoxic and apoptotic effects on OSCC cells. The mechanism underlying the saponin-induced OSCC apoptosis might be the same as that in previous studies^{10,12-14}. The differences in saponin structure were not important for their apoptotic effects on cells¹³. A previous study showed that saponins from *P. acuminata Ait* induce CAS in the human pulp fibroblast cells¹⁰. Saponins from *Acacia victoriae* (avicins) induce apoptosis in cultured human fibroblast cells through mitochondrial membrane potential, independent of the membrane-bound death receptor^{14,15}. This transition induces the release of mitochondrial factors (apoptosis inducing factors and cytochrome *c*), which activate caspase cascades¹⁶.

The present study reported a negative correlation between the concentration of saponins from *P. acuminata Ait* and the percentage of OSCC apoptosis; the relationship, however, was not statistically significant ($P > 0.05$). It was also found that there was a significant negative relationship between the percentage of OSCC apoptosis and the concentration of cisplatin ($P < 0.05$). Cisplatin is one of the antitumour drugs used in many types of malignancies such as oral cancer^{6,17}. Cisplatin, as a positive control in the present study, also induced apoptosis of OSCC cells. The mechanism of cisplatin-induced apoptosis may not only induce DNA damage, but also induce proteins of cell damage¹⁸. The difference in the correlation between saponin concentration and OSCC apoptosis, and the correlation between cis-

Table 1 Cell growth inhibition and IC₅₀

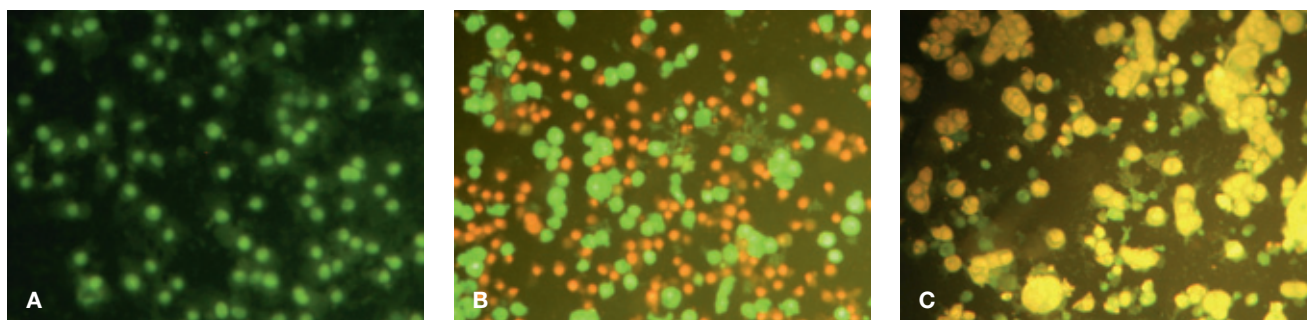
Treatment	No.	Concentration ($\mu\text{g/ml}$)	% Cell growth inhibition (mean)	IC ₅₀ ($\mu\text{g/ml}$)
Saponin	1	1	16.42	309.62
	2	40	29.76	
	3	80	43.60	
	4	150	56.42	
	5	600	71.80	
	6	1250	76.93	
	7	5000	100.00	
Cisplatin	1	6.25	8.27	33.68
	2	12.5	36.57	
	3	25	43.64	
	4	50	58.50	
	5	100	72.89	
Negative control	1	0	0	-
	2	0	0	
	3	0	0	

Table 2 Percentage of apoptotic cells

Saponin ($\mu\text{g/ml}$)	% Cell apoptosis (mean)	Cisplatin ($\mu\text{g/ml}$)	% Cell apoptosis
37.5	75.17	10	56.00
75	60.37	15	76.00
150	54.87	20	87.00

Table 3 The Pearson correlation analysis between saponins of *P. acuminata* Ait and cisplatin concentration and the percentage of OSCC apoptosis

Pearson correlation	R	P
apoptosis (%): saponins concentration	-0.901	0.143
apoptosis (%): cisplatin concentration	0.969	0.000*

* significant correlation ($P < 0.05$); -, negative correlation**Fig 1** Ethidium bromide/acridine orange staining of apoptotic cells. The OSCC cells were not treated (A) or were treated with saponins from *P. acuminata* Ait (75 $\mu\text{g/ml}$) (B) or cisplatin (15 $\mu\text{g/ml}$) (C) for 24 h (100 \times magnification).

platin concentration and OSCC apoptosis may be due to the different threshold of OSCC cells responding to the saponins and cisplatin¹⁶.

In conclusion, saponins from *P. acuminata Ait* had cytotoxic and apoptotic effects on OSCC cells. Saponins from *P. acuminata Ait* may be potential anti-cancer agents for OSCC.

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