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

Hendri Susanto[1], Nanang Fakhrudin[2], Yosi Bayu Murti[2], Supriatno[1], Widowati Siswomiharjo[3]

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 population[1]. Forty per cent of OSC is more prevalent in the tongue[2]. According to one study, 86% of OSC patients are detected in the late stage in the dental clinic[3]. Surgery is the most effective treatment of oral cancer. However, surgery also produces several adverse effects, such as facial asymmetry, and speech and mastication problems[4]. 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 used[7].

1 Oral Medicine Department, Faculty of Dentistry, University of Gadjah Mada, Yogyakarta, Indonesia.
2 Biology Pharmacy Department, Faculty of Pharmacy, University of Gadjah Mada, Yogyakarta, Indonesia.
3 Biomaterial Department, Faculty of Dentistry, Univeristy of Gadjah Mada, Yogyakarta, Indonesia.

Corresponding author: Hendri Susanto, DMD, Oral Medicine Department, Faculty of Dentistry, University of Gadjah Mada, Yogyakarta 55281, Indonesia. Tel: 62-274-515307; E-mail: drghendri@ugm.ac.id

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 cells[10]. The saponins induce cellular apoptosis susceptibility (CAS), a protein transport factor, which is needed in the induction of apoptosis of the human dental fibroblast cell[10]. 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 study[10]. 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

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 line[11], 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% CO_2 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 (IC_{50}) 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 IC_{50} 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 cells[13]. A previous study showed that saponins from *P. acuminata Ait* induce CAS in the human pulp fibroblast cells[10]. 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 cascades[16].

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 damage[18]. The difference in the correlation between saponin concentration and OSCC apoptosis, and the correlation between cis-

Table 1 Cell growth inhibition and IC_{50}

Treatment	No.	Concentration	% Cell growth	IC_{50}
		(μg/ml)	inhibition (mean)	(μg/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		Cisplatin	
(μg/ml)	% Cell apoptosis	(μg/ml)	% Cell apoptosis
	(mean)		
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

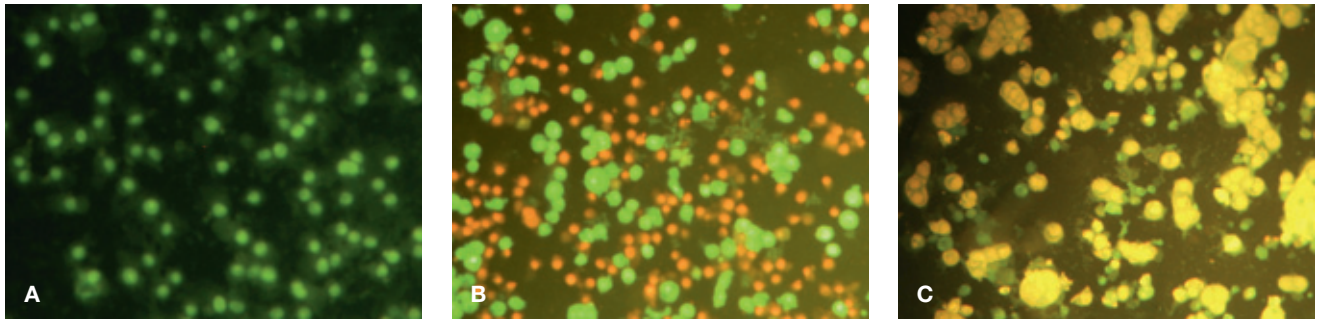

Fig 1 Ethidium bromide/acridine orange staining of apoptotic cells. The OSCC cells were not treated (A) or were treated with saponins from *P. acuminata Ait* (75 μg/ml) (B) or cisplatin (15 μg/ml) (C) for 24 h (100× magnification).

platin concentration and OSCC apoptosis may be due to the different threshold of OSCC cells responding to the saponins and cisplatin[16].

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.

References

1. Bsoul SA, Huber MA, Terenzhalmy GT. Squamous cell carcinoma of the oral tissues: a comperehensive review for oral healthcare providers. J Contempt Dent Pract 2005;6:1-16.
2. Neville BW, Day TA. Oral cancer and precancerous lesions. CA Cancer J Clin 2002;52:195-215.
3. Moerniati S. Frequency of the oral cancer at the Dental Clinic of the Dr. Sardjito Hospital during 3 years. Scientific Abstracts: Free Paper Session on First International Congress on Oral Cancer and Jaw Tumours. Singapore: University of Singapore, 1987.
4. Canto MT, Horowitz AM, Child WL. Views of oral cancer prevention and early detection: Maryland physicians. Oral Oncol 2002;38:373-377.
5. Amemiya K, Shibuya H, Yoshimura R, Okada N. The risk of radiation-induced cancer in patients with squamous cell carcinoma of the head and neck and its result of treatment. Br J Radiol 2005;78:1028-1033.
6. Shibuya Y, Tanimoto H, Umeda M, Yokoo S, Komori T. Induction chemotherapy with docetaxel, cisplatin, and 5-flourouracil for tongue cancer. Kobe J Med Sci 2004;50:1-7.
7. Wahyuningsih MSH, Mubarika S, Ganjar IG, Wahyono S. Searching of anticancer from nature. Trad Drug J 2003;8:1-7 (in Indonesian).
8. Umiati NS, Eko S, Odijati R, Suwanto AMF, Rudiyanto. Traditional medicine in Madura. In: Inventory and Cultural Value Supervision. Jakarta: Department of Education and Culture of Indonesia, 1990 (in Indonesian).
9. Widowati S. The use of saponins from *Plumeria acuminate Ait* as dental devitalization agent (an *in vitro* study). Timnas Fkg Unair Surabaya 2005;4:13-16 (in Indonesian).
10. Hendri S, Harijadi, Astuti I. Cellular apoptosis susceptibility expression in cultured pulp fibroblast cell induced by saponin from *Plumeria acuminate Ait*. Hong Kong Dent J 2006;3:94-97.
11. Supriatno, Harada K, Kawaguchi S, Onoue T, Yoshida H, Sato M. Characteristics of antitumor activity of mutant type *p27Kip1* gene in an oral cancer cell line. Oral Oncol 2004;40:679-687.
12. Brinkmann U. CAS, the human homologue of the yeast chromosome-segregation gene CSE1, in proliferation, apoptosis, and cancer. Am J Hum Genet 1998;62:509-513.
13. Candra E, Matsunaga K, Fujiwara H, Mimaki Y, Sashida Y, Yamakuni T, Ohizumi Y. Two steroidal saponins *Camassia cusickii* induce L1210 cell death through the apoptotic mechanism. Can J Physiol Pharmacol 2001;79:953-958.
14. Haridas V, Higuchi M, Jayatilaku GS, Bailey D, Mujoo K, Blake ME et al. Avicins: triterpenoid saponins from *Acacia victoria* (*Bentham*) induced apoptosis by mitochondrial perturbation. Proc Natl Acad Sci 2001;98:5821-5826.
15. Fisher DA. Apoptosis in cancer therapy: crossing the threshold. Cell 1994;78:539-542.
16. Melzig MF, Bader G, Loose R. Investigation of the mechanism of membrane activity of selected triterpenoid saponins. Planta Med 2001;6:43-48.
17. Lemasters JJ. V. Necrapoptosis and the mitochondrial permeability transition: shared pathways to necrosis and apoptosis. Am J Physiol 1999;276:G1-6.
18. Gonzalez VM, Fuertes MA, Alonso C, Perez JM. Is cisplatin cell death always produced by apoptosis? Mol Pharmacol 2001;59:657-663.