RESEARCH ARTICLE

Parkia speciosa Seeds Ethanol Extract as Co-chemotherapeutic Agent for Doxorubicin Toward Tongue Cancer

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Abstract

BACKGROUND: *Parkia speciosa* seeds have been reported to have an anticancer property due to the presence of various antioxidant compounds. Since the potential uses of *P. speciosa* for the tongue cancer has not been clearly disclosed, we conducted a study to investigate anticancer properties of *P. speciosa* seed ethanol extract (PSSEE) as well as its effect on cardiac cells.

METHODS: Tongue cancer rat model were treated with/without Doxorubicin and various concentrations of PSSEE. After treatment, tongue and heart samples were collected and processed further for histological examinations. Tongue epithelium thickness and damaged heart tissues was observed by HE staining, while tongue cancer cell proliferation was assessed by Ki-67 immunohistochemistry. Analyses were performed under an upright light microscope to measure tongue epithelium thickness, state of cancer cell proliferation, and degree of heart tissue damage.

RESULTS: Addition of 400 mg/kg body weight (BW) PSSEE to 4.6 mg/kg BW Doxorubicin reduced the average tongue epithelial thickness and Ki-67$^+$ cells number. Upon addition of PSSEE to Doxorubicin, the damage of heart tissue was reduced in a concentration dependent manner. Among all groups, the group of tongue cancer treated with 4.6 mg/kg BW Doxorubicin and 400 mg/kg BW PSSEE had the lowest percentage as well as the lowest degree of heart tissue damage.

CONCLUSION: Since addition of PSSEE to Doxorubicin reduced epithelial thickness, number of Ki-67$^+$ cells and heart tissue damage, PSSEE could be a potential co-chemotherapeutic agent for Doxorubicin toward tongue cancer.

KEYWORDS: *Parkia speciosa*, Doxorubicin, tongue cancer, epithelial thickness, Ki-67, cardiotoxicity, co-chemotherapy

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Introduction

Oral cancer ranks the eighth of all cancer cases that often occur.(1) More than 90% of the oral cancers are squamous cell carcinoma (SCC) with approximately 300,000 new cases/year.(2) Tongue squamous cell carcinoma is often found on the lateral side of the tongue.(3) Detection of cancer invasion can be determined histopathologically by looking at the thickness of the cancer epithelium.(4) Hematoxylin-eosin (HE) staining was usually performed to determine the state of tongue organ, while Ki-67 detection with immunohistochemistry (IHC) was performed to observe the state of cancer cell proliferation.(5,6)

Anti-cancer chemotherapeutic drugs, including Doxorubicin have a good efficacy. Anticancer action of Doxorubicin involves DNA damage through inhibition of topoisomerase II. However, Doxorubicin activities have cytotoxic effects on normal cells. For example, on cardiac cells.(7) Doxorubicin triggers the accumulation of iron

that mediates the systemic formation of free radicals in the mitochondria of cardiac cells.(8) Damage caused by Doxorubicin is frequently found in the left ventricle of the heart, leading to cardiomyopathy and congestive heart failure.(9,10)

Since anti-cancer chemotherapeutic drugs are not only attacking cancer cells but also normal cells, various studies have been conducted to overcome the issue. Exploration in potential natural resources, including herbal extracts, have been widely investigated in several countries, including Indonesia.(11) Various herbal plants are considered as anti-cancerous, such as *Parkia speciosa*. *P. speciosa* is known to have efficacy as an antioxidant, especially in the seeds. *P. speciosa* has been frequently consumed in Indonesia along with other vegetables. *P. speciosa* is believed to have medicinal properties to cure hepatalgia, kidney inflammation, diabetes, cholera, intestinal worms and cancer.(12) Anticancer property of *P. speciosa* allegedly appears due to its antioxidant compounds such as alkaloids, saponins, tannins, and flavonoids (13), those supposedly prevent the development of cancer cells through the inhibition of angiogenesis and proliferation as well as the induction of apoptosis.(14,15) However, potential of *P. speciosa* for the tongue cancer has not been clearly disclosed, therefore current research was conducted to investigate its anti-cancer property as well as its effect on cardiac cells.

Methods

Preparation of *P. speciosa* Seeds Ethanol Extract (PSSEE)

P. speciosa seeds were collected in Yogyakarta, washed, dried and grinded. One kg grinded sample was macerated using 70% ethanol for 48 hours, filtered and evaporated in a rotary evaporator (Buchi, Flawil, Switzerland) at 70°C. The extract was collected after all solvent was evaporated. The extract was weighed for 100, 200 or 400 mg/kg body weight (BW) and solubilized with 1 mL distilled water.

Tongue Cancer Animal Model

Tongue cancer was induced by applying 4-Nitroquinoline 1-oxide (4-NQO) (Sigma-Aldrich, St. Louis, MO, USA) on the dorsal tongue from posterior to anterior, 3 times/week for 12 weeks. The 4-NQO, a synthetic carcinogen derived from quinoline, has been used to stimulate carcinogenesis in animal model through the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS).(2,16)

Animal Treatment Protocol

Male Sprague Dawley rats with approximate weight of 100-150 g, aged 5-6 weeks, were divided in 6 groups: control, tongue cancer, tongue cancer with treatment of Doxorubicin (4.6 mg/kg BW) and PSSEE (0, 100, 200 or 400 mg/kg BW). Treatments of Doxorubicin and *P. speciosa* extract were performed every day for 2 weeks using oral gavage. Three rats were used for each group. The research protocol was approved by the Research Ethics Committee of Faculty of Medicine and Health Sciences, Universitas Muhammadiyah Yogyakarta (049/EP-FKIK-UMY/2015).

Hematoxylin-Eosin (HE) Stain and Ki-67 Immunohistochemistry

After 2-weeks treatment periods, rats were euthanized and operated to collect tongue and heart. Collected tongues and hearts were fixed in 10% buffered formalin and processed for making paraffin blocks. The blocks were sliced in 4 µm and de-paraffinized. HE stain was performed for tongue and heart sections, while Ki-67 immunohistochemistry was performed for tongue sections merely.

For the immunohistochemistry, the sections were antigen retrieved, treated with 3% hydrogen peroxide and 2% bovine serum albumin. For primary antibody, mouse monoclonal anti- Ki-67 [MIB-1] antibody (BioCare Medical, Pacheco, CA, USA) was used. After that, N-Histofine High Stain HRP (MULTI) (Nichirei Biosciences, Tokyo, Japan) kit was applied. The peroxidase activity was visualized by immersing tissue sections in N-Histofine DAB-2V (Nichirei Biosciences), resulting in brown nuclei. Tissue sections were finally counterstained and mounted.

Histopathological Analysis

All stainings were documented with an upright light microscope (Olympus, Tokyo, Japan). For epithelial thickness, tongue HE-stained slides were microscopically observed, then the maximum and minimum limits of the stratum corneum layer-basement membrane were measured.

For Ki-67 analysis, tongue immunohistochemically stained slides were microscopically observed. Stained cells were counted in randomly-selected-5-visual fields at 400x magnification. Counting was performed with single-blind method by 2 independent operators. More than 100 stained and unstained cells were confirmed in each field.

For heart tissue analysis, heart HE-stained slides were microscopically observed. Damaged heart tissues could be identified through the formation of vacuoles as well as the loss of myofibrils. The damage was measured in five

degrees as follows: 1 degree: <5% damages; 1.5 degrees: 5-15% damages; 2 degrees: 16-25% damages; 2.5 degrees: 26-35%; and 3 degrees: > 35% damages. The measurement was performed with single-blind method by 2 independent operators.

Results

PSSEE Enhanced Potential of Doxorubicin in Reducing Epithelial Thickness

Based on the average of the maximum and minimum limits of the basement membrane and stratum corneum layer (Figure 1), tongue cancer group had the highest epithelial thickness. The average of epithelial thickness was reduced with the treatment of 4.6 mg/kg BW Doxorubicin. By addition of 400 mg/kg BW PSSEE, the average could be reduced further. Among all groups, the group of tongue cancer treated with 4.6 mg/kg BW Doxorubicin and 400 mg/kg BW PSSEE had the significant lowest epithelial thickness (Table 1). Statistical analyses showed that *p*-value<0.05 based on one way ANOVA test, followed by the Tukey HSD test.

PSSEE Enhanced Potential of Doxorubicin in Reducing Ki-67$^+$ Cells

Immunohistochemical results showed that tongue cancer group had the highest number of Ki-67$^+$ cells, marked by cells with brown nuclei (Figure 2). Number of Ki-67$^+$ cells were reduced by treatment of 4.6 mg/kg BW Doxorubicin.

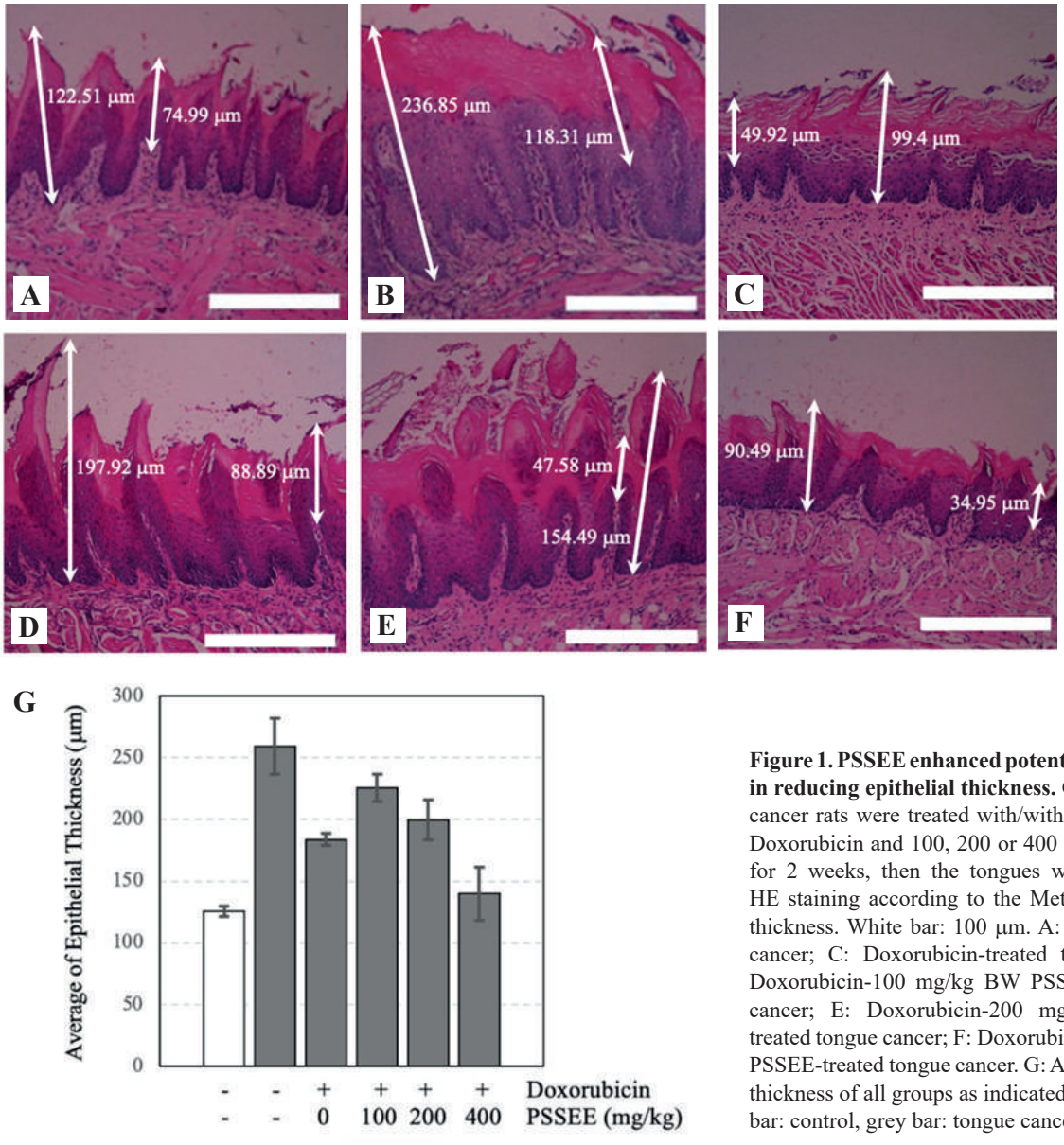

Figure 1. PSSEE enhanced potential of Doxorubicin in reducing epithelial thickness. Control and tongue cancer rats were treated with/without 4.6 mg/kg BW Doxorubicin and 100, 200 or 400 mg/kg BW PSSEE for 2 weeks, then the tongues were analyzed with HE staining according to the Methods for epithelial thickness. White bar: 100 µm. A: control; B: tongue cancer; C: Doxorubicin-treated tongue cancer; D: Doxorubicin-100 mg/kg BW PSSEE-treated tongue cancer; E: Doxorubicin-200 mg/kg BW PSSEE-treated tongue cancer; F: Doxorubicin-400 mg/kg BW PSSEE-treated tongue cancer. G: Average of epithelial thickness of all groups as indicated in the panel. Open bar: control, grey bar: tongue cancer.

Table 1. Tukey's HSD analysis of the HE results for epithelial thickness.

Compared Group			Tukey's HSD	
			Mean Difference	*p*-value
4.6 mg/kg Doxorubicin + 400 mg/kg PSSEE	*vs.*	4.6 mg/kg Doxorubicin	-44.005	0.027
		4.6 mg/kg Doxorubicin + 100 mg/kg PSSEE	-85.714	0.000
		4.6 mg/kg Doxorubicin + 200 mg/kg PSSEE	-60.108	0.005

The reduction of Ki-67$^+$ cells number could be additionally reduced by addition of 400 mg/kg BW PSSEE to 4.6 mg/kg BW Doxorubicin. Among all groups, the group of tongue cancer treated with 4.6 mg/kg BW Doxorubicin and 400 mg/kg BW PSSEE had the significant lowest Ki-67$^+$ cells (Table 2).

PSSEE Reduced Damage of Heart Tissue Caused by Doxorubicin

Based on analysis of heart tissue damage by observing the formation of vacuoles and myofibril loss (Figure 3), the group of tongue cancer treated with 4.6 mg/kg BW Doxorubicin has the highest percentage of heart tissue

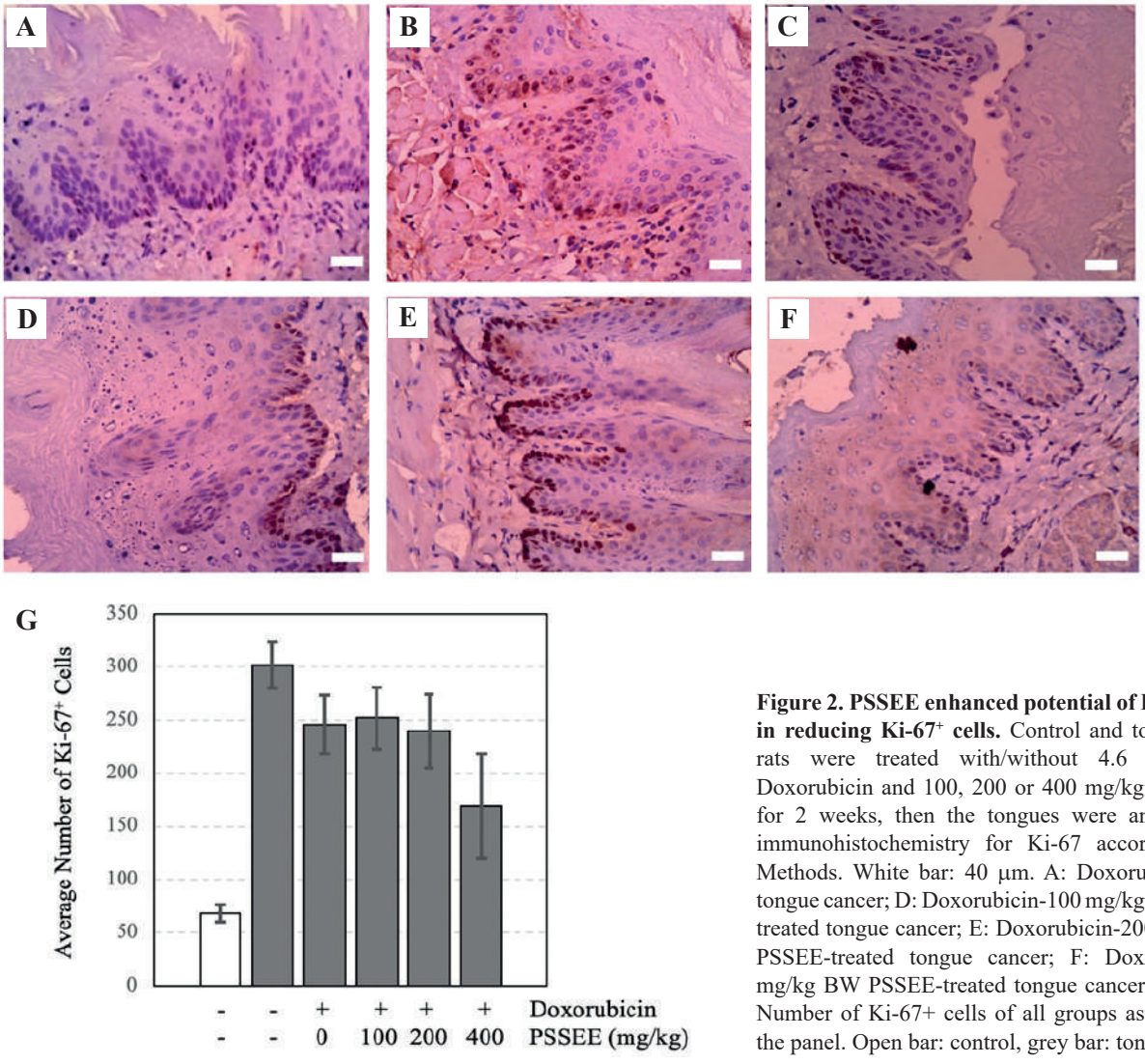

Figure 2. PSSEE enhanced potential of Doxorubicin in reducing Ki-67$^+$ cells. Control and tongue cancer rats were treated with/without 4.6 mg/kg BW Doxorubicin and 100, 200 or 400 mg/kg BW PSSEE for 2 weeks, then the tongues were analyzed with immunohistochemistry for Ki-67 according to the Methods. White bar: 40 µm. A: Doxorubicin-treated tongue cancer; D: Doxorubicin-100 mg/kg BW PSSEE-treated tongue cancer; E: Doxorubicin-200 mg/kg BW PSSEE-treated tongue cancer; F: Doxorubicin-400 mg/kg BW PSSEE-treated tongue cancer. G: Average Number of Ki-67+ cells of all groups as indicated in the panel. Open bar: control, grey bar: tongue cancer.

Table 2. Tukey's HSD Analysis of the Immunohistochemical results for Ki-67$^+$ cells.

Compared Group			Tukey's HSD	
			Mean Difference	*p*-value
4.6 mg/kg Doxorubicin + 400 mg/kg PSSEE	*vs.*	4.6 mg/kg Doxorubicin	-71.000	0.050
		4.6 mg/kg Doxorubicin + 100 mg/kg PSSEE	-76.667	0.035
		4.6 mg/kg Doxorubicin + 200 mg/kg PSSEE	-83.333	0.023

damage and classified as 2.5-degree damage (Table 3). Upon addition of PSSEE to the Doxorubicin the damage of heart tissue was reduced in a concentration dependent manner. Hence, the group of tongue cancer treated with 4.6 mg/kg BW Doxorubicin and 400 mg/kg BW PSSEE had the lowest percentage as well as the lowest degree of heart tissue damage (Table 3).

Discussion

Doxorubicin has been widely used in laboratory and clinical setting as anti-cancer drug. In the present study, Doxorubicin has proven its potential as well, marked by reduction of epithelial thickness and Ki-67$^+$ cells, suggesting that Doxorubicin could reduce the growth of tongue cancer by suppressing number of proliferating cancer cell. However, Doxorubicin was shown to have side effect on heart tissue, marked by vacuoles and myofibril loss in the heart tissue.

PSSEE in a concentration dependent manner could reduce the heart damage caused by Doxorubicin, especially in concentration of 400 mg/kg BW. The 400 mg/kg BW PSSEE was shown promising in providing additional reduction of epithelial thickness and Ki-67$^+$ cells. This suggested that PSSEE could be combined with Doxorubicin, to enhance Doxorubicin's potential as anti-cancer as well as to reduce Doxorubicin's side effect on heart tissue.

The ability of PSSEE as a co-chemotherapy agent in inhibiting the progression of tongue cancer cells might be related with its content of flavonoid. Flavonoid could inhibit cancer cell growth by increasing the production of gamma interferon that has a function as a stimulator activity of Natural Killer (NK) cells and Cytotoxic T lymphocytes (CTL) immune system against cancer cells.(17) Active NK cells and CTL in the body caused the shutdown or killing process/apoptosis against cancer cells. When the apoptotic process can run properly, cell proliferation can be controlled. Induction of apoptosis toward tumor cell has been suggested

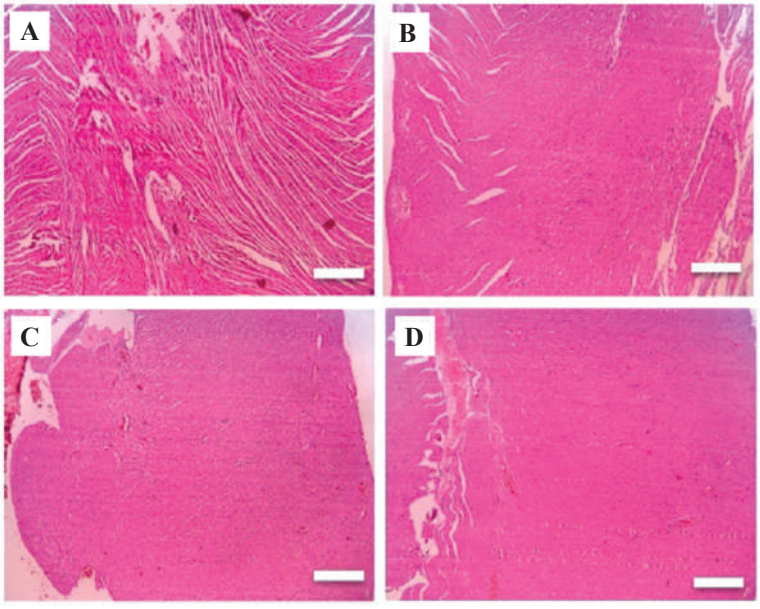

Figure 3. PSSEE reduced damage of heart tissue caused by Doxorubicin. Control and tongue cancer rats were treated with/without 4.6 mg/kg BW Doxorubicin and 100, 200 or 400 mg/kg BW PSSEE for 2 weeks, then the hearts were analyzed with HE staining according to the Methods. White bar: 100 μm. A: Doxorubicin-treated tongue cancer; B: Doxorubicin-100 mg/kg BW PSSEE-treated tongue cancer; C: Doxorubicin-200 mg/kg BW PSSEE-treated tongue cancer; D: Doxorubicin-400 mg/kg BW PSSEE-treated tongue cancer.

Table 3. HE results for the damage of heart tissue.

Group			Damage of Heart Tissue					
			1		2		3	
			%	Degree	%	Degree	%	Degree
4.6 mg/kg Doxorubicin	+	0 mg/kg PSSEE	27.67	2.5	28	2.5	27.33	2.5
		100 mg/kg PSSEE	25.67	2.5	26.33	2.5	24.67	2
		200 mg/kg PSSEE	26	2.5	25.67	2.5	24	2
		400 mg/kg PSSEE	10	1.5	11.67	1.5	9	1.5

as one of important approach and has been investigated widely.(18) To achieve optimal induction of apoptosis, specific markers and signaling pathway of the targeted cancer cell should be investigated.(19-23) Information of targeted cancer genetics and epigenetics will provide a better approach and risk assessment.(24) Due to angiogenesis, signaling pathways in angiogenesis as well as endothelial generation were also taken placed.(25,26)

Herbal extracts have been intensively investigated to find substitutes of current drug as well as novel agents with novel mechanism of action. Herbal extract has been explored for various purposes, including for inhibiting cancer cell and inducing stem cell.(27,28)

Cardiotoxicity of doxorubicin toxicity could be proceeded by an increasing amount of iron-mediated reactive oxygen species (ROS). Specifically, iron accumulates in the mitochondria of cardiac cells, causing tissue destruction and degradation.(29,30) Doxorubicin-caused cardiomyopathy can be identified by the area of the myocardium, interstitial fibrosis, and scattered vacuolated cardiomyocyte.(9) The manifestation of cardiotoxicity by Doxorubicin is a decreasing function in the left ventricle of the heart, which can lead to irreversible heart failure.(7) PSSEE which has been reported to contain flavonoids, can fight free radicals. As a chelating agent, flavonoid prevent the formation of ROS in the mitochondria of cardiac cells.(31)

Flavonoid was reported to have anti-inflammatory effects in ischemic reperfusion in rat hearts.(31) Flavonoids found in propolis was revealed to inhibit the growth of various cancer cells *in vitro* and *in vivo* without causing toxicity to normal cells. Flavonoids might regulate genes that organize cell proliferation, cell cycle, apoptosis, oncogenesis, angiogenesis and metastasis.(32)

Conclusion

PSSEE could enhance potential of Doxorubicin in reducing epithelial thickness and Ki-67$^+$ cells as well as damage of heart tissue caused by Doxorubicin. Taken together, PSSEE could be a potential co-chemotherapeutic agent for Doxorubicin toward tongue cancer.

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Authors Contribution

ESM, INA, ANI, and YL were involved in planning, ESM supervised the work. INA, ANI, YL, ESM and FS performed the measurements, processed the experimental data, performed the analysis, drafted the manuscript and designed the figures. ESM, INA and FS aided in interpreting the results and worked on the manuscript. All authors discussed the results and commented on the manuscript.

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