Original Article

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# Cytotoxic effect of *Kaempferia parviflora* extract on normal oral keratinocytes and a human squamous carcinoma cell line

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**Objective:** The purpose of this study was to investigate the cytotoxic effect of *K. parviflora* extract on normal oral keratinocytes (NOK) and a human squamous carcinoma cell line (HSC-4).

**Materials and Methods:** The cytotoxic effect of ethanolic extract of *K. parviflora* root (0.46, 0.93, 1.87, 3.75, 7.5, 15, and 30 mg/ml) on normal oral keratinocytes and a human squamous carcinoma cell line was evaluated using an MTT assay per ISO 10993-5/2009.

**Results:** The results revealed that after exposure to 0.46, 0.93, 1.87, 3.75, and 7.5 mg/ml *K. parviflora* extract NOK cell viability was 113%, 99%, 96%, 91%, and 75%, respectively, whereas 15 mg/ml and 30 mg/ml *K. parviflora* root treatment decreased cell viability to ~15–31%. In HSC-4 cells, no cytotoxicity was demonstrated at concentrations ≤ 30 mg/ml.

**Conclusion:** *K. parviflora* extract at concentrations ≤ 7.5 mg/ml was not cytotoxic to NOKs, and concentrations from 0.46–30 mg/ml were not cytotoxic to HSC-4 cells. This study provides additional basic knowledge concerning the biological response of NOK and HSC-4 cells to *K. parviflora* extract.

Keywords: *Kaempferia parviflora*, Normal oral keratinocytes, Human squamous carcinoma cell, Cytotoxicity

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## Introduction

*K. parviflora* or, a native plant of the South Asia, is used extensively in Thailand and Myanmar (Figure 1). Its botanical name is Kaempferia parviflora Wall. Ex Baker, and its scientific name is Boesenbergia Pendurata (Roxb.) Holtt [1]. In Thailand, it is commonly called Krachaidam, however, it is also known as Kalaholood, Kala Halud (Chakma), Kalahalood (Tonchonga), and Cheilanki (Khumi) [2]. *K. parviflora* has demonstrated many pharmacological effects, such as Anti-peptic ulcer, anti-inflammatory, anti-allergy, anti-mutagenicity, anti-depression, antimicrobial, anti-cholinesterase, anti-cancer, cardioprotection, and anti-obesity

Figure 1 Transverse section of *K. parviflora* rhizome (9).

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effects [3]. Many extracts from *K. parviflora* have been investigated for their biological effects. The known chemical constituents of *K. parviflora* include polyphenolic glycosides [4], methoxyflavones [5], three kinds of acetophenones, flavones [6], chalcone, volatile oil, and essential oil components (borneol and sylvestrene) [7] and kaempferiaosides [8]. The major compounds of *K. parviflora* are flavonoids, such as 5 dimethoxyflavone (DMF), 5, 7, 4-trimethoxyflavone (TMF), and 3, 5, 7, 3', 4'-pentamethoxyflavone (PMF) [9]. Potikanond *et al*. revealed that the 95% ethanolic extract of *K. parviflora* was cytotoxic to human HeLa cell line with an $IC_{50}$ value of 0.22 mg/ml [10].

There is a lack of pharmacological information on the cytotoxicity of *K. parviflora* on oral mucosal barrier cells. Thus, aim of the present study was to evaluate the cytotoxic effects of *K. parviflora* on different oral mucosa epithelial sub-types: normal oral keratinocytes (NOKs) and a human squamous carcinoma cell line (HSC-4). NOKs were used to represent oral mucosal barrier cells comparable to human native oral mucosa *in vivo*. HSC-4 cells were used as representative oral mucosa cancer cells.

## Materials and methods

### *Kaempferia Parviflora* Preparation

Dried *K. parviflora* rhizomes were purchased from Phitsanulok province, Thailand. The *K. parviflora* roots were dried and immersed in 95% ethanol for 3 d at room temperature. The extracted solution was filtered through No. 1 Whatman filter paper, concentrated under a rotary evaporator, and lyophilized to obtain the dried crude extract. The dried crude extract was dissolved in distilled water for 24 h before using it in the experiments.

### Cell Culture

The human squamous carcinoma cell line (HSC-4) (a gift from Dr.Yohko Kohno School of Dentistry, Showa University, Japan) was seeded and cultured in Dulbecco's Modified Eagle Medium-high glucose (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Thermo Fisher Scientific, USA) in a humidified 5% $CO_2$ atmosphere at 37ºC.

The normal oral keratinocytes were established by Piboonniyom *et al*. [11]. The oral keratinocytes were immortalized using human telomerase reverse transcriptase (*h-TERT*, a kind gift from R.A. Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA, USA) and cdk4. The NOKs were grown in keratinocyte serum-free medium (Keratinocyte-SFM, GIBCO™, Invitrogen Corporation) supplemented with EGF and bovine pituitary extract.

### Cytotoxicity Assay

The cytotoxic effect of *K. parviflora* on the HSC-4 and NOK cells was evaluated per ISO 10993-5:2009 using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay [12]. The NOK and HSC-4 cells were seeded in 96-well plates (Costar®, Corning, USA) at a density of $2\times10^4$ cells/well in a humidified 5% $CO_2$ atmosphere at 37 °C for 24 h. The cells were treated with 0.46, 0.93, 1.87, 3.75, 7.5, 15, or 30 mg/ml *K. parviflora* extract, and DMEM without extract was used as the control. After 24 h incubation, the cells were washed with phosphate buffer saline solution (PBS) and incubated with 50 µl 1 mg/ml MTT (Sigma-Aldrich, Inc., USA) for 2 h. The formazan product was dissolved and shaken in 100 µl dimethyl sulfoxide (DMSO, Riedel-de Haën, Sigma-Aldrich Laborchemikalien GmbH) for 30 min. The amount of viable cells was determined by measuring the absorbance of the solution at 570 nm using Epoch microplate spectrophotometer

(Biotek®, USA). The results with a coefficient of variation of less than 15% were analyzed and reported. All experiments were performed in triplicate and repeated three times.

### Statistical Analysis

The cytotoxicity data were calculated and presented as mean ± standard deviation of % cell viability. The comparison of the cytotoxicity between the various concentrations of *K. parviflora* was performed using the Kruskal-Wallis test. *P* value of ≤ 0.05 was considered to be statistically significant.

## Results

The results demonstrated that the NOK cell viability after exposure to 15 mg/ml and 30 mg/ml *K. parviflora* was 31.41±1.82 and 14.61±2.27%, respectively. In contrast, cell viability in the ≤ 15 mg/ml *K. parviflora* groups ranged from ~75%–113% (Table 1). Furthermore, the cell viability in the 15 mg/ml and 30 mg/m groups were significantly lower compared with the other groups. The cell viability in the 0.46–3.75 mg/ml groups were not significantly different (Figure 2).

When evaluating the cytotoxic effect on HSC-4 cells, the percentage of cell viability after exposure to 0.46, 0.93, 1.87, 3.75, 7.5, 15, and 30 mg/ml *K. parviflora* extract was 101%, 99%, 96%, 95%, 89%, 84%, and 74%, respectively (Figure 3). The percentage of cell viability at in the 30 mg/ml group was significantly different from the other groups. The *K. parviflora* extract $IC_{50}$ value on NOK and HSC-4 cells was 11.69 and 55.50 mg/ml, respectively (Figure 4).

## Discussion

Cell viability is one of the major criteria for evaluating the cytotoxic effect of crude extracts. Decreased cell viability with increasing concentrations of crude extracts indicates the potency of the specific extracts. Here, we investigated the cytotoxic effect of *K. parviflora* extract on NOK cells. We found that the *K. parviflora* was not cytotoxic at a concentration of ≤ 7.5 mg/ml, however, cell viabilty decreased to 31% and 15% after exposure to 15.0 mg/ml and 30 mg/ml, respectively. When treating the HSC-4 cells, none of the *K. parviflora* concentrations had a cytotoxic effect on the HSC-4 cells.

Table 1 The percentages of cell viability of the NOK and HSC-4 cells.

| Concentration of *K. parviflora* (mg/ml) | mean±S.D. | |
|---|---|---|
| | NOK | HSC-4 |
| 0.46 | 112.80±3.64 | 100.78±2.29 |
| 0.93 | 99.23±1.32 | 99.14±3.12 |
| 1.87 | 96.17±1.81 | 96.39±5.33 |
| 3.75 | 90.69±6.60 | 94.51±1.43 |
| 7.5 | 75.02±5.79 | 89.41±1.77 |
| 15 | 31.41±1.82 | 83.54±3.01 |
| 30 | 14.61±2.27 | 73.92±2.23 |

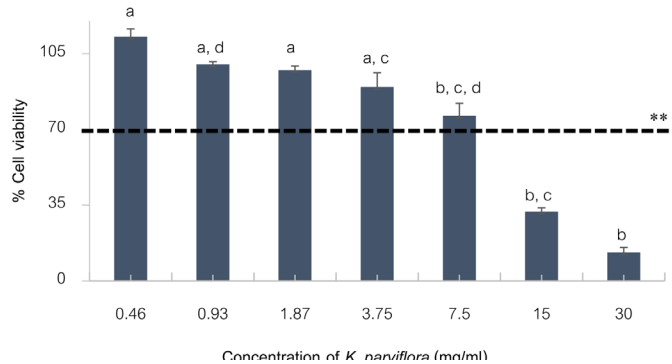


Figure 2 The percentages of NOK cell viability after exposure to *K. parviflora* extract.
**cytotoxicity potential margin was set at 70% cell viability.
a,b,c,d different superscripts are significantly different at $p < 0.05$.

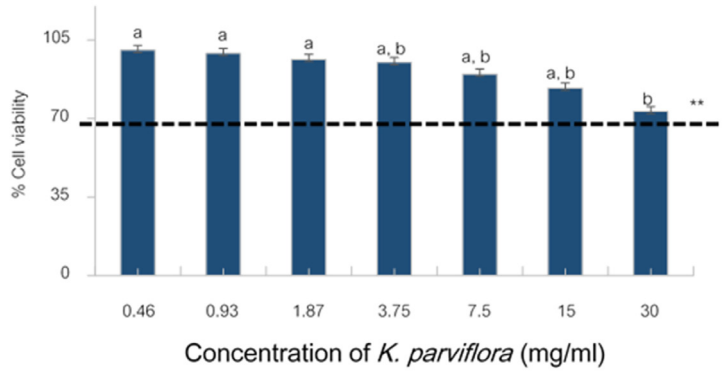


Figure 3 The percentages of HSC-4 cells viability after exposure to *K. parviflora* extract.
**cytotoxicity potential margin was set at 70% cell viability.
a,b,c,d different superscripts are significantly different at $p < 0.05$.

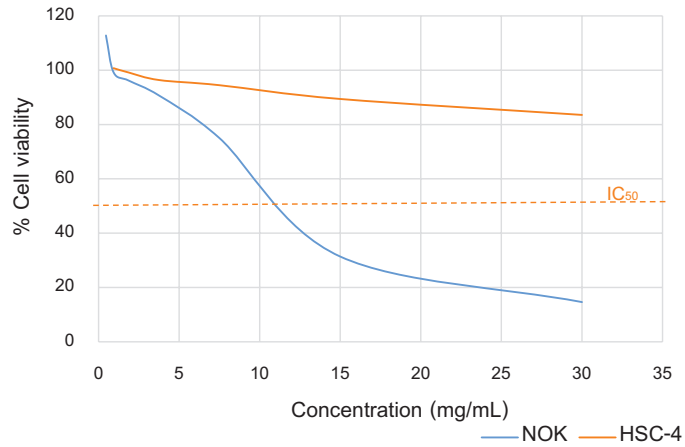


Figure 4 The percentages of HSC-4 and NOK cells viability after exposure to *K. parviflora* extract. The $IC_{50}$ margin was set at 50% cell viability.

A previous study in dermal fibroblasts (Hs68) found that 1-10 µg/ml *K. parviflora* ethanol extract was non-toxic to $H_2O_2$-stimulated cells [13]. Similarly, Srivastava *et al*. revealed that *Kaempferia galanga* rhizome extracted with ethyl p-methoxy cinnamate, methanol, and hydro-distillate at concentrations of 10–15 mg/ml was non-toxic to normal peritoneal macrophage cells [14].

*K. parviflora* has been demonstrated to contain chalcones [15], phenolic glycosides [4], volatile oils [7], and flavonoids as the major constituents, including polymethoxyflavonoids, and acetophenones [8]. Although the results from the present study did not indicate any cytotoxic effect of *K. parviflora* on HSC-4 cells, the mechanisms of the anticancer action of flavonoids in plant extracts have been described as the downregulation of mutant p53 protein, cell cycle arrest, tyrosine kinase inhibition, inhibition of heat shock proteins, estrogen receptor binding capacity, and inhibition of Ras protein expression [16].

Many studies have demonstrated that 95% ethanolic extract of *K. parviflora* suppressed human promyelocytic leukaemia cell (HL-60) proliferation and decreased cell viability. The $IC_{50}$ at 24, 48, and 72 h was 25.5, 18.5, and 14.5 mg/ml, respectively [17]. Similarly, Potikanond *et al.* found that the 95% ethanolic extract of *K. parviflora* was cytotoxic to a human cervical cancer cell line with an $IC_{50}$ value of 0.22 mg/ml [10]. The disparate cytotoxic efficacies of *K. parviflora* leaves ethanol extract observed among studies may also be due to different times of herb harvesting [18], [19], extraction methods [20], and types of cell tested [21].

An MTT cytotoxicity test was performed per ISO 10993-5 and ISO 10993-12 [22] to evaluate the cytotoxicity of *K. parviflora* leaves ethanol extract using their dissolved solution products. As defined in ISO 10993 (Biological evaluation of medical devices), a reduction in cell viability by more than 30% compared with the non-toxic control sampled is considered a cytotoxic effect [23]. In conclusion, *K. parviflora* extract at concentrations of ≤ 7.5 mg/ml was not cytotoxic to NOK cells, and none of the evaluated concentrations was cytotoxic to HSC-4 cells. This study provides additional basic knowledge concerning the biological responses of NOK and HSC-4 cells to *K. parviflora* extract.

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