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Kaempferol and Quercetin, Components of *Ginkgo biloba* Extract (EGb 761), Induce Caspase-3-Dependent Apoptosis in Oral Cavity Cancer Cells

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EGb 761, extracted from *Ginkgo biloba* leaves, has been proven to induce caspase-3-dependent apoptosis in oral cavity cancer cells. Since EGb 761 is a composition of various components, it is important to identify which components are responsible for its anticancer effects to reduce the total dosage and to avoid toxicity. Therefore, the study aimed to determine the effective compounds of EGb 761 that induce apoptosis in oral cavity cancer cells and to identify whether caspase-3 was involved in apoptosis of oral cancer cells by EGb 761 components. The results of cell proliferation assays on oral cavity cancer cells showed that kaempferol and quercetin significantly inhibited cellular proliferation at a concentration of 40 μM. Flow cytometry showed that the antiproliferative effects of each component were due to increased apoptosis. Kaempferol and quercetin induced apoptosis in various oral cancer cell lines (SCC-1483, SCC-25 and SCC-QLL1) and showed cleavage of poly (ADP-ribose) polymerase (PARP). Caspase-3 activity assay revealed that induction of apoptosis by kaempferol and quercetin was caspase-3-dependent. In conclusion, the results suggest that kaempferol and quercetin, two components of EGb 761, effectively induce caspase-3-dependent apoptosis of oral cavity cancer cells and can be considered as possible anti-oral cavity cancer agents. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords: Ginkgo biloba extract; oral cavity cancer; apoptosis; caspase-3.

INTRODUCTION

Ginkgo has long been used in Oriental medicine and applications for *Ginkgo biloba* extract are being found in modern medicine (Jacobs and Browner, 2000). EGb 761 was clinically used for cognitive function disorder, peripheral blood flow insufficiency, tinnitus and vertigo (Jacobs and Browner, 2000; DeFeudis, 1991). Recently, the antiproliferative effects and induction of apoptosis in cancer cells by EGb 761 have been reported (DeFeudis, 1991; Mutoh *et al*., 2000; Raso *et al*., 2001). In a study performed on oral cavity cancer, 250 μg/mL of EGb 761 induced inhibition of cellular proliferation in oral cavity cancer cells by caspase-3-dependent apoptosis (Kim *et al*., 2005). Diverse groups of molecules are involved in the apoptosis pathway. One set of mediators implicated in apoptosis belongs to the aspartate-specific cysteinyl proteases or caspases. A member of this family, caspase-3 (CPP32, apopain, YAMA), has been identified as being a key mediator of apoptosis of mammalian cells. During apoptosis, the inhibitor of caspase-activated DNase is cleaved by caspase-3 resulting in the release of caspase-activated DNase, which in turn triggers the rapid fragmentation of DNA (Kothakota *et al*., 1997; Sakahira *et al.,* 1998). In addition, initiator caspases such as caspase-2, -8, -9 and -10 instigate the apoptotic cascade and lead to the activation of effector caspases, which include caspase-3, -6 and -7. PARP is a 116 kDa nuclear poly (ADP-ribose) polymerase, and can be cleaved by many IL-1β-converting enzyme (ICE)-like caspases such as caspase-3, and this cleavage induces apoptosis of cells (Lazebnik *et al.,* 1994; Nicholson, 2000; Tewari *et al.*, 1995). Therefore, our previous study focused on caspase-3 and PARP (Kim *et al*., 2005).

EGb 761 is a mixed compound comprising flavonoid glycosides (kaempferol, quercetin and luteolin), terpene trilactones (ginkgolides and bilobalides) and ginkgolic acid (less than 5 ppm) (Jacobs and Browner, 2000). Therefore, if the effective components of EGb 761 are identified, they can reduce the efficient dose needed to induce apoptosis, which will minimize adverse effects. For example, 2–200 μg/mL of IPS 200 and 0.2–20 μg/mL of ginkgolide B, components of EGb761, suppressed cellular proliferation in MDA-MB-231 breast cancer cells (Papadopoulos *et al*., 2000). The purpose of the present study was to determine the effective components of EGb 761 that induce apoptosis in oral cavity

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cancer cells and to identify whether caspase-3 was involved in apoptosis of oral cavity cancer cells by components of EGb 761.

MATERIALS AND METHODS

Cell lines and culture. SCC-1483 (squamous carcinoma cell line originated from retromolar trigone) and SCC-QLL1 (squamous carcinoma cell line originated from metastatic lymph nodes of oral cavity cancer) cells were a generous gift from Dr J. Shah (Memorial Sloan-Kettering Cancer Center, New York, NY, USA). The SCC-25 cell line (squamous carcinoma cell line originated from tongue) was purchased from the American Type Culture Collection (Rockville, MA, USA). Cells were cultured in the minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, penicillin (50 μg/mL) and streptomycin (50 μg/mL).

Materials. EGb 761 was provided by Yuyu Pharmaceutical Co. (Seoul, Korea). The terpenoids bilobalide, ginkgolides A and B, kaempferol and quercetin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Anti-poly (ADP-ribose) polymerase (anti-PARP) antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA) and benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk) as a caspase cascade inhibitor was purchased from Sigma Chemical Co.

Cell proliferation assay. The cells were plated in 96-well plates at a density of 2000 cells/well. After incubation for 12–16 h, they were washed with PBS and incubated for 24 h in serum-free medium containing the experimental agents. Cell proliferation was measured using Cell Titer 96 Aqueous One Solution Proliferation Assay kits (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. Twenty μL of the reagent was added to each well of the 96-well plates, incubated for 1 h at 37 ºC, and the absorbance was monitored at 490 nm using a spectrophotometer.

Measurement of apoptosis by flow cytometry. The annexin V method was used to examine apoptosis. Briefly, the cells were plated at 4×10^5 cells/well in 6-well plates, incubated for 16 h, and treated with components of EGb 761 in the absence of serum. After treatment, the cells were harvested, washed with PBS, stained with 1 mL of 20 μg/mL of propidium iodide (PI) containing 1 mg/mL RNase in PBS for 20 min, and conjugated with Annexin V-FITC using TACS Annexin V-FITC kit (Trevigen, Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol. The cells were examined by Becton Dickinson FACS Vantage SE (San Diego, CA, USA). Annexin V-positive/PI-positive and Annexin V-positive/PI-negative cell populations were defined as the apoptotic population among the total gated cells.

PARP cleavage analysis. Cells were scraped in 1 mL of radioimmunoprecipitation assay buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS). The amount of protein was analysed with bicinchonic acid protein assay using bovine serum albumin. After that, the lysates were centrifuged at 10000 × **g** for 20 min at 4 ºC and the supernatants were blotted. Proteins (30 μg) were separated by 6% SDS-PAGE and transferred to nitrocellulose membranes. The blots were blocked with 10% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBST) at 4 ºC overnight and probed with anti-PARP antibodies (1:1000 in 2% skim milk in TBST) for 4 h at room temperature. After washing, the blots were treated with horseradish peroxidase-conjugated secondary antibody diluted 1:5000 in TBST for 1 h and washed several times. Blots were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and autoradiography.

Caspase-3 activity assay. The cells were plated at 10^6 cells/plate in 10 cm plates, incubated for 16 h, and treated with 40 μM of kaempferol and quercetin for 24 h in the absence of serum. To investigate apoptosis inhibition caused by blocking the caspase cascade, the caspase inhibitor z-VAD-fmk (10 μM) was cotreated with kaempferol and quercetin. Caspase-3 activity was determined using the CaspACE assay system (Promega Co.). Briefly, after each treatment, the cells were harvested and washed with ice-cold PBS and resuspended in 1 mL of cell lysis buffer. The cells were frozen and thawed several times and incubated on ice for 15 min. The cell lysates were centrifuged at 15000 × **g** for 20 min at 4 ºC and the supernatants were collected. DEVD-pNA (2 μL) substrate in caspase assay buffer was then added to the supernatants and incubated at 37 ºC for 4 h. The absorbance was monitored at 405 nm using a spectrophotometer.

Statistical analysis. Data are expressed as mean ± SD. A minimum of at least three separate experiments were performed for each measurement. Differences between treatment groups were assessed by analysis of variance with post hoc tests, and differences were considered statistically significant at $p < 0.05$.

RESULTS

Inhibition of cell proliferation by EGb 761 components

SCC-1483 cells were treated with 20, 40 and 80 μM of EGb 761 components (ginkgolides A and B, bilobalide, kaempferol and quercetin) for 24 h and the cell proliferation was determined. The mean proliferation percentage was calculated as (mean proliferation of experimental group/mean proliferation of control) × 100. The mean proliferation in 40 μM of kaempferol- and quercetin-treated cells was less than 50% compared with the control (Table 1, Fig. 1A). This inhibition was increased in a dose-dependent manner. Given this result, kaempferol and quercetin were selected for all subsequent experiments.

Induction of apoptosis by kaempferol and quercetin

SCC-1483 cells were treated with various concentrations of kaempferol and quercetin for 24 h. After treat-

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ment, the cells were harvested and apoptosis was examined by flow cytometry. Apoptotic induction was 1.8-fold higher after treatment with 20 μM of kaempferol compared with the control, and this effect was dose dependent. With 40 and 80 μM of kaempferol, each induced apoptosis 3.5- and 5.5-fold higher than the control. Apoptosis induced from 40 μM of kaempferol was statistically higher than the control (Fig. 1B, D).

Table 1. Cell proliferation in treatment with the components of EGb 761 (%)

	20 μM	40 μM	80 μM
Ginkgolide A	74 ± 5	72 ± 6	73 ± 5
Ginkgolide B	83 ± 6	81 ± 7	78 ± 6
Bilobalide	80 ± 4	78 ± 4	77 ± 5
Kaempferol	63 ± 5	49 ± 6	37 ± 5
Quercetin	60 ± 7	46 ± 4	33 ± 3

The mean proliferation percentage was calculated as (mean proliferation of experimental group/mean proliferation of control) × 100.
The values are the mean ± SD.

Treatment with 20, 40 and 80 μM of quercetin induced apoptosis 2.1-, 4.4- and 5.5-fold higher than the control and apoptosis induced by 40 μM of quercetin was significantly higher than the control (Fig. 1C, D). However, there was no significant difference between kaempferol and quercetin (Fig. 1D).

Confirmation of apoptosis by kaempferol and quercetin in various oral cavity cancer cell lines

To confirm the effectiveness of the component treatment compared with EGb 761 treatment, apoptosis was examined by flow cytometry after treatment with 40 μM of kaempferol, 40 μM of quercetin and 250 μg/mL of EGb 761 in SCC-1483, SCC-25 and SCC-QLL1 cells. Apoptosis was induced by all treatments in all cancer cell lines (Table 2, Fig. 2A). Even though the degree of apoptosis differed between the cancer cell lines, there was no statistical significance between them (Table 2, Fig. 2B). To confirm apoptosis, cleavage of PARP was examined by Western blot analysis in all cancer cell lines. As a result, an 89 kDa band was identified and cleavage of 116 kDa PARP was confirmed (Fig. 3).

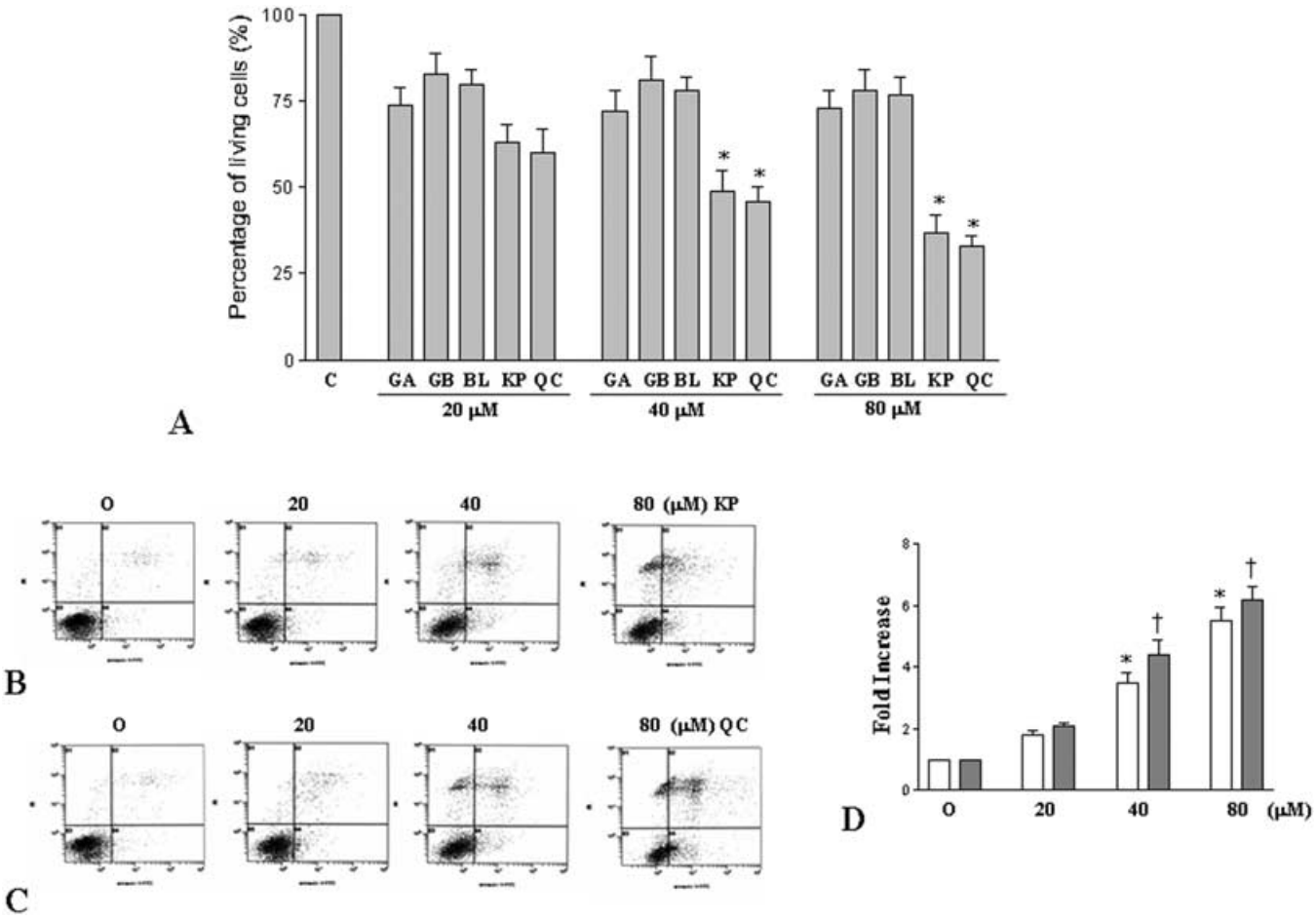

Figure 1. Inhibition of cell proliferation and induction of apoptosis by kaempferol and quercetin. (A) SCC-1483 oral cavity cancer cells were treated with various concentrations of ginkgolide A (GA), ginkgolide B (GB), bilobalide (BL), kaempferol (KP) and quercetin (QC) for 24 h, and the cell proliferation assay was done. At 40 μM concentration, kaempferol and quercetin showed a significant inhibition of cell proliferation compared with the negative control (C). *Cell proliferation less than 50% compared with the control. (B, C) Cells were treated, respectively, with various concentrations of kaempferol (B) and quercetin (C) for 24 h and apoptosis was examined with flow cytometry after Annexin V/PI double staining. Apoptosis induced by kaempferol and quercetin was observed at 40 μM concentration. (D) Bar graph of apoptosis induced by kaempferol (blank) and quercetin (filled). *† $p < 0.05$ compared with each control.

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Table 2. Ratio of apoptosis induced by EGb 761 and its components in various oral cancer cells

	SCC-1483	SCC-25	SCC-QLL1
Kaempferol (40 μM)	3.8 ± 0.5	4.0 ± 0.7	3.1 ± 0.3
Quercetin (40 μM)	4.6 ± 0.7	5.1 ± 0.6	4.5 ± 0.6
EGb 761 (250 μg/mL)	3.0 ± 0.4	3.2 ± 0.5	5.4 ± 0.7

The ratio was calculated as apoptosis of experimental group/apoptosis of the control.
The values are the mean ± SD.

Therefore, apoptosis was induced by all treatments in all cell lines.

Caspase-3-dependent apoptosis by kaempferol and quercetin

SCC-1483 cells were treated with 40 μM of kaempferol and 40 μM of quercetin with or without 10 μM z-VAD-fmk for 24 h, respectively. Apoptosis was examined by PARP cleavage analysis. PARP cleavage analysis showed an 89-kDa-sized PARP fragment in kaempferol- and quercetin-treated cells, which was not observed in the control or in the cells cotreated with z-VAD-fmk (Fig. 4A). SCC-1483 cells were treated with 40 μM of kaempferol and 40 μM of quercetin with or without 10 μM z-VAD-fmk for 24 h, respectively, and then caspase-3 activity assays were performed. The optical density of kaempferol-treated cells increased 6.5-fold vs the control (0.111 ± 0.018 vs 0.017 ± 0.006), and in quercetin, 7.1-fold higher than in the control (0.135 ± 0.013 vs 0.019 ± 0.005). However, the optical density of cells cotreated with kaempferol and z-VAD-fmk did not increase vs the control. In addition, in the case of quercetin, the same result was observed (Fig. 4B). The same experiments were performed on SCC-25 and SCC-QLL1 cells. The PARP cleavage was noted in kaempferol- and quercetin-treated SCC-25 and SCC-QLL1 cells to be identical to SCC-1483 cells (data not shown). The optical density of kaempferol-treated SCC-25 cells increased 7.0-fold vs the control (0.126 ± 0.020 vs 0.018 ± 0.005) and in quercetin, 7.5-fold higher than the control (0.150 ± 0.023 vs 0.020 ± 0.009). The optical density of kaempferol-treated SCC-QLL1 cells increased 7.4-fold vs the control (0.111 ± 0.012 vs 0.015 ± 0.006) and in quercetin, 7.8-fold higher than in the control (0.133 ± 0.019 vs 0.017 ± 0.004). However, the optical density of SCC-25 and SCC-QLL1 cells cotreated with kaempferol and z-VAD-fmk did not increase compared with the control. In addition, in the case of quercetin, the same result was observed in SCC-25 and SCC-QLL1 cells.

DISCUSSION

Although 250 μg/mL of EGb 761 is a safe dose in terms of acute and chronic toxicity, the use of components of EGb 761 could reduce the total amount and possible toxicity because EGb 761 is a mixed compound (Drieu, 1988). For example, two flavonoid constituents, kaempferol and quercetin, effectively inhibited the proliferation of colorectal cancer cells (Mutoh *et al*., 2000). Kaempferol reduced the incidence of prostate cancer development and rutin, a flavonoid glycoside, suppressed hepatoma induced by aflatoxin B1 and *N*-nitrosodimethylamine (Griffiths *et al*., 1999; Webster *et al.*, 1996). Therefore, the study investigated which components of EGb 761 have antiproliferative effects on oral cavity cancer cells. In this study, inhibition of proliferation by more than 50% in SCC-1483 was induced by 40 μM of kaempferol and 40 μM of quercetin, but ginkgolides A and B and bilobalide could not inhibit cell proliferation. Inhibition of proliferation of oral cavity cancer cells by kaempferol and quercetin was proven by flow cytometry and PARP cleavage analysis to be caused by apoptosis. Kaempferol and quercetin induced apoptosis in various cell lines, such as SCC-1483, SCC-25 and SCC-QLL1. Therefore, it was confirmed that kaempferol and quercetin induced apoptosis of oral cavity cancer cells. Furthermore, 40 μM of kaempferol is converted to 11.5 μg/mL and 40 μM of quercetin to 13.5 μg/mL. Compared with 250 μg/mL of EGb 761, this dose is very low and the use of kaempferol or quercetin is safer and more effective in inducing apoptosis. Even though the responses to kaempferol and quercetin showed no significant difference between cell types, the results were variable according to cell type, indicating that apoptosis is a cell-specific response. Further study on this subject will be needed.

Kaempferol is a flavonoid with antioxidative effects and is contained in strawberries (de Vries *et al*., 1997; DuPont *et al*., 2004). Compared with quercetin, kaempferol is not widely known. In this study, oral cavity cancer can be added to the list of kaempferol's anticancer effects. Quercetin is a flavonol that is mainly contained in fruit, tree bark, onions, red wine, green tea and wort (de Vries *et al*., 1997). It has antiulcer and protective functions in the gastrointestinal tract, as well as antiinflammatory and antiallergic effects through inhibition of degranulation of mast cells, basophils and monocytes (Martin *et al*., 1998). Recently, the anticancer effects of quercetin in colon cancer and leukemia have been reported but there are no reports of its effects in oral cavity cancer (Sato *et al*., 1997; Hollman *et al.*, 1997). Thus, oral cavity cancer can also be added to quercetin's list of anticancer effects.

In a previous study, caspase-3 was involved in apoptosis induced by EGb 761 in oral cavity cancer cells (Kim *et al*., 2005). This led us to investigate whether caspase-3 was involved in apoptosis induced by kaempferol and quercetin. The result of PRRP cleavage analysis (Fig. 3) revealed that the caspase cascade was involved in apoptosis of oral cavity cancer cells. Caspase-3 activity assay (Fig. 4) showed that caspase-3 was activated by kaempferol and quercetin and this activation was suppressed by a caspase cascade inhibitor, z-VAD-fmk. Therefore, these results show that caspase-3 is involved in apoptosis of oral cavity cancer cells by kaempferol and quercetin and are consistent with our previous results on the effect of EGb 761 (Kim *et al*., 2005). Brusselmans *et al*. reported that kaempferol and quercetin induced caspase-dependent apoptosis in prostate cancer cells (LNCaP) and breast cancer cells (MDA-MB-231) (Brusselmans *et al*., 2005), and these studies also corresponded with our results. Even though the results of PRRP cleavage analysis revealed that the

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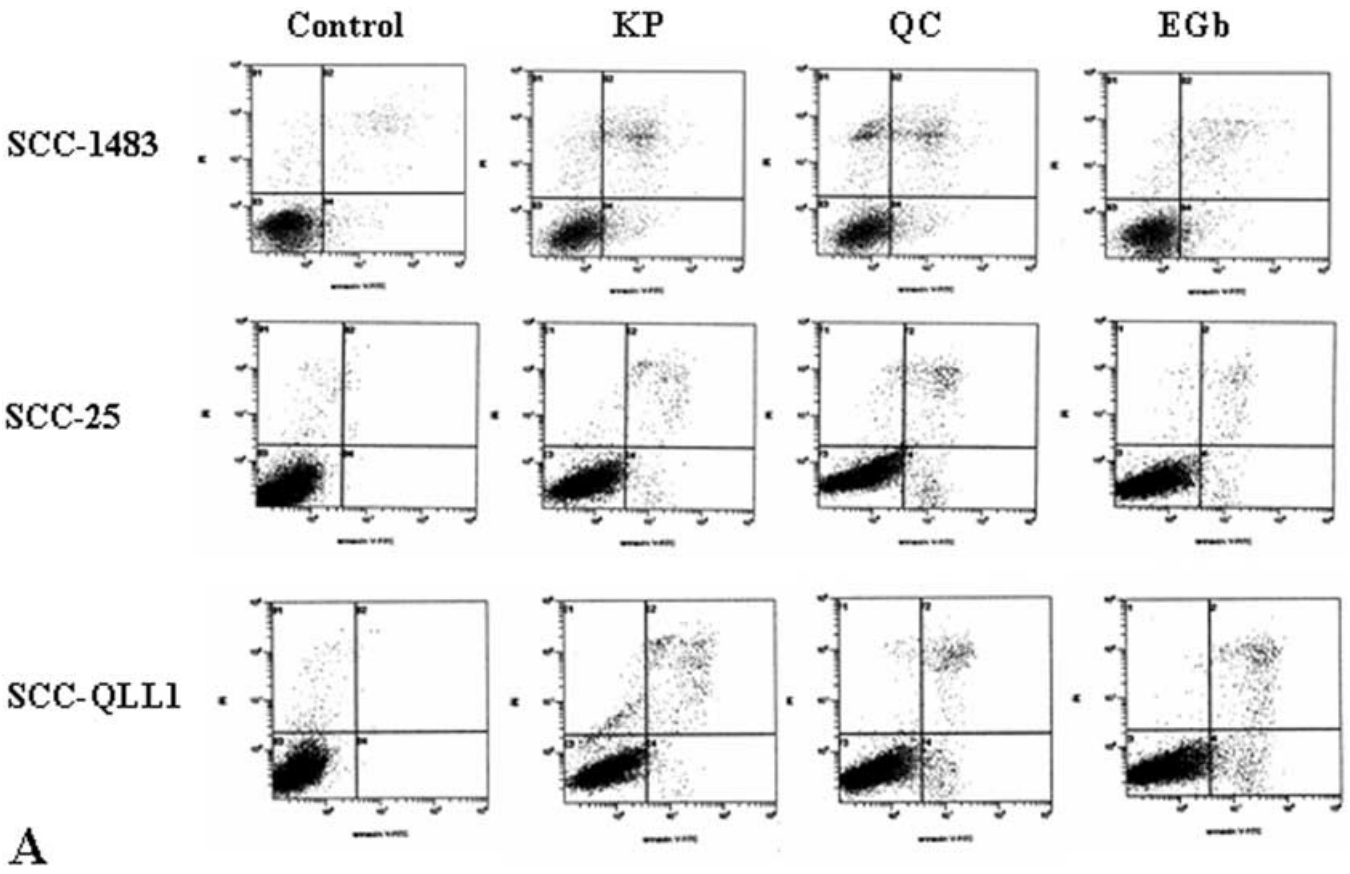

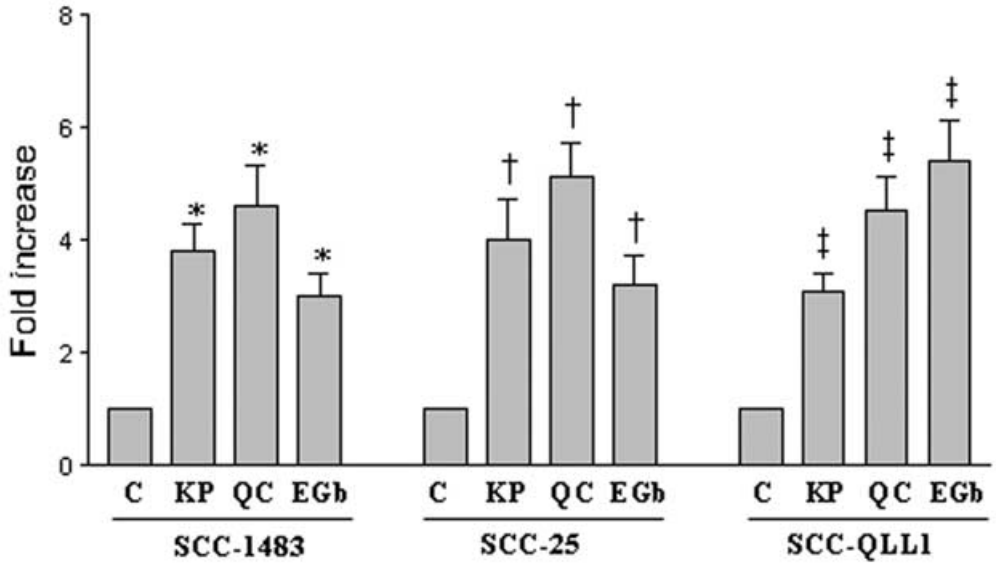

Figure 2. Apoptosis of various oral cavity cancer cells by kaempferol, quercetin and EGb 761. (A) SSC-1483, SCC-25 and SCC-QLL1 oral cavity cancer cells were treated, respectively, with 40 μM of kaempferol (KP), 40 μM of quercetin (QC) and 250 μg/mL EGb 761 (EGb) for 24 h and apoptosis was examined using flow cytometry after Annexin V/PI double staining. In all cells, apoptosis was significantly induced by EGb 761 and its components compared with the control ($p < 0.05$). (B) Bar graph of apoptosis by KP, QC and EGb. * $p < 0.05$ compared with the control of SCC-1483 cells, † $p < 0.05$ compared with the control of SCC-25 cells, ‡ $p < 0.05$ compared with the control of SCC-QLL1 cells.

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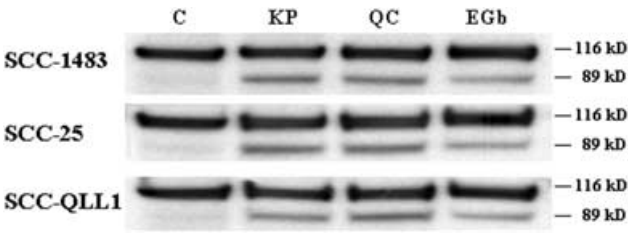

Figure 3. Confirmation of apoptosis of various oral cavity cancer cells by kaempferol, quercetin and EGb 761. SCC-1483, SCC-25 and SCC-QLL1 oral cavity cancer cells were treated, respectively, with 40 μM of kaempferol (KP), 40 μM of quercetin (QC) and 250 μg/mL EGb 761 (EGb) for 24 h and apoptosis was examined using PARP cleavage analysis. In all cell lines treated with KP, QC and EGb, the cleavage of PARP as 116 kDa- and 89 kDa-sized bands, which were absent in the control (C), are noted.

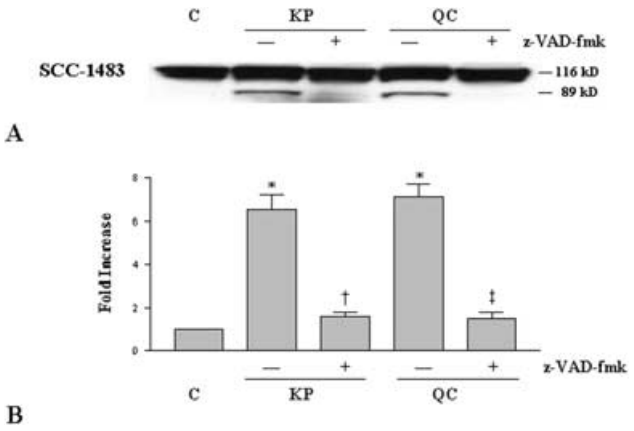

Figure 4. Caspase-3-dependent apoptosis by kaempferol and quercetin in SCC-1483 cells. (A) Inhibition of PARP cleavage by z-VAD-fmk. SCC-1483 cells were incubated, respectively, with 40 μM of kaempferol or quercetin with or without pretreatment by 10 μM z-VAD-fmk. PARP cleavage was examined by Western blot analysis. Compared with the control (C), cells (–) treated with kaempferol (KP) or quercetin (QC) showed cleavage of PARP as 116 kDa and 89 kDa. However, cells (+) pretreated with z-VAD-fmk showed uncleaved PARP as 116 kDa. (B) Inhibition of caspase-3 activity by z-VAD-fmk. Compared with the control (C), cells (–) treated with KP or QC, respectively, showed an increase of caspase-3 activity. However, cells (+) pretreated with z-VAD-fmk showed a significant decrease of caspase-3 activity. $*p < 0.05$ compared with the control, $\dagger p < 0.05$ compared with cells with KP treatment and without pretreatment by z-VAD-fmk, $\ddagger p < 0.05$ compared with cells with QC treatment and without pretreatment by z-VAD-fmk.

caspase cascade was involved in apoptosis of oral cavity cancer cells, caspase-9, activated in mitochondrial-mediated apoptosis, may be involved in this cascade but further investigation on mitochondrial-mediated apoptosis including Bcl-2, Bcl-xL and Bad is needed.

In conclusion, the results suggest that kaempferol and quercetin, two components of EGb 761, effectively induce caspase-3-dependent apoptosis of oral cavity cancer cells and can be considered as possible anti-oral cavity cancer agents.

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