Selective apoptotic effect of *Zelkova serrata* twig extract on mouth epidermoid carcinoma through p53 activation

Hoe-Jin Kang and Young-Joo Jang

Apoptosis or programmed cell death plays an essential role in chemotherapy-induced tumor cell killing, and inducers of apoptosis are commonly used in cancer therapy. Treatment with *Zelkova serrata* extracts was performed in human gingival fibroblast (HGF), mouth epidermoid carcinoma cell (KB), lower gingival squamous cancer cell (YD38) and tongue mucoepidermoid carcinoma cells (YD15). We observed that extract prepared from *Zelkova serrata* twig selectively inhibited proliferation of various oral cancer cells, but not normal gingival fibroblasts, in a dose-dependent manner. Caspase-8-mediated apoptosis was induced by treatment with the extract only in mouth epidermoid carcinoma and not in other types of cancer cells, including lower gingival squamous cell carcinoma. The selective apoptotic effect of *Zelkova serrata* twig extract in mouth epidermoid carcinoma was dependent on normal p53 status. Apoptosis was not remarkably induced by treatment with the extract in either lower gingival squamous or tongue mucoepidermoid carcinoma cells, both of which contain abnormalities of p53. Upon treatment with *Zelkova serrata* twig extract, mouth epidermoid carcinoma cells accumulated in S phase by activation of p21. These data indicate that *Zelkova serrata* twig extract exerted a cancer type-specific, p53-dependent apoptotic effect and disturbed the cell cycle, which suggests that herbal medicine could be a treatment for specific types of cancers.

**Keywords:** anticancer; apoptosis; oral carcinomas; p53; *Zelkova serrata*

**INTRODUCTION**

Oral cancer is the sixth most common cancer in the world and the third most common cancer in Asia with an average incidence rate of 1–10 per 100,000 people. The incidence of oral cancer is increasing in developed countries. Oral cancer constitutes a serious health problem, as evidenced by its poor survival rates and severe functional and cosmetic defects resulting from this cancer and its treatment. Despite significant medical advances in the treatment of oral cancer over the past 30 years, the 5-year survival rate of this cancer remains low at approximately 50%. Especially, squamous cell carcinoma of the oral cavity is a severe cancer type as patients fail to respond to treatment frequently. In this situation, development of an oral cancer-specific, anti-cancer drug is needed. Apoptosis is an essential process in the physiology of normal development and in the maintenance of constant cell numbers. As numerous cancers are caused by improper cell death, cancer cell-specific inducers of apoptosis are being developed as anti-cancer agents. These studies suggested that induction of apoptosis has become a principal mechanism through which anticancer therapy exerts its effects. Caspase-8 is an upstream caspase that is activated by a receptor-mediated pathway through the external apoptotic pathway. Caspase-9 is the primary caspase in the mitochondria-initiated apoptosis pathway and requires release of cytochrome C from mitochondria as well as interaction with Apaf-1. This internal apoptotic pathway, which is associated with changes in the permeability of the outer mitochondrial membrane and the collapse of membrane potential, results in release of cytotoxic proteins and caspase activation. Caspases-8 and -9 activate downstream effector caspases such as caspase-3 and therefore play a pivotal role in the induction of apoptosis. As a key tumor suppressor, p53 plays a critical role in tumor prevention. In response to various stress signals, p53 selectively regulates a set of target genes and initiates various stress responses, including cell cycle arrest, apoptosis and/or senescence, to exert its tumor suppression function. Generally, p53 is the most frequently mutated gene in human tumors, and p53 mutations occur in more than 50% of all tumors. Disruption of normal p53 function is often a prerequisite for the development and/or progression of tumors. Specifically, p53 triggers cell cycle arrest through specific transcriptional activation of the cyclin-dependent kinase inhibitor p21 (also known as p21WAF1/Cip1), which promotes cell cycle arrest in response to many stimuli. Additionally, biochemical and genetic studies have indicated that p21 acts as a master effector for promoting anti-proliferative activities that are independent of the classical p53 tumor suppressor pathway. These reports suggested that, paradoxically, p21 might also promote apoptosis through both p53-dependent and p53-independent mechanisms under certain cellular stresses. Use of natural products has increased based on their potential
chemotherapeutic activity. Especially, medical plants are widely used in drug development as anti-cancer and anti-inflammatory agents, health food materials and resources of new functional materials. *Zelkova serrata* is a deciduous tree with very slender, zigzag-shaped, and red-brown twigs that contain widely divergent, pointed and cone-shaped buds, and it is found growing naturally in Japan, Korea and Taiwan. Young leaves of *Z. serrata* are cooked to make vegetables, whereas its bark and leaves are used medicinally. Whole tree extract of *Z. serrata* is also widely used for the treatment of cancer. For example, a cadal compound extracted from this tree was shown to possess antioxidant effects based on inhibition of lung tumorigenesis in mice. Even though the whole tree of *Z. serrata*, including the bark and leaves, is often used to treat various diseases, there have been no reports on the specific uses of twigs of *Z. serrata*. Therefore, the goals of this study were to examine the effects of methanol extract and fractions of *Z. serrata* twig on various oral cancer cell growths and to investigate the signal pathway mediating *Z. serrata*-induced growth inhibition in oral cancer cells. For this, we used human oral cancer cell lines, such as mouth epidermoid carcinoma cells, lower gingival squamous cancer cells and tongue mucoepidermoid carcinoma cells, to investigate the anti-cancer activities of extract and fractions of *Z. serrata* and elucidate their selective apoptotic effects on specific types of oral cancers.

**MATERIALS AND METHODS**

**Preparation of extracts**

Twigs of *Z. serrata* were collected, dried at room temperature naturally and powdered. The powders were extracted with 100% methanol twice at room temperature in a magnetic forced stirrer. By using an accelerated solvent extractor, the extracts were filtered and concentrated to remove methanol at 74 °C and freeze dried. Of the dry extract, 50% was water-soluble, 40% was soluble in ethyl acetate and ~10% was soluble in hexane. The ethyl acetate-soluble fraction containing a cancer-specific cytotoxicity was used for further study.

**Cell lines and culture**

Human oral carcinoma cell lines, such as mouth epidermoid carcinoma cell (KB), lower gingival squamous cancer cell (YD38) and tongue mucoepidermoid carcinoma cells (YD15) were obtained from Korea Cell Line Bank (KCLB, http://cellbank.snu.ac.kr). Human gingival fibroblast (HGF) was cultured primarily from tissue around an extracted tooth with the consent of patient. Cancer cells and primary cells were maintained in Dulbecco’s modified Eagle medium and 10% minimum essential medium, respectively. Both cell cultures were supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and maintained in a humidified atmosphere of 5% CO2 at 37 °C.

**Cell cytotoxicity test**

The cytotoxicity of twig extracts of *Z. serrata* on both oral carcinoma and primary cells was determined by using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, cells were seeded in 100 μL of medium in 96-well plate (5×103 cells per well). After incubation overnight, extracts were added in various concentrations (0.01, 0.1, 1, 20, 100, 500 and 2 000 ng·mL−1) for indicated times, and then the cytotoxicity test was performed. 10 μL CCK-8 solution was added to each well, and cultured for another 2–4 h. By using a microplate reader (Molecular Device, Sunnyvale, CA, USA), soluble tetrazolium salt (WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfoophenyl)-2H-tetrazolium, monosodium salt) was measured as absorbance at 450 nm.

**Sequence analysis of p53 gene**

Total RNA was isolated from three kinds of oral carcinoma cell lines by using total RNA extraction kit (Intron Biotechnology, Seongnam-si, Korea) according to the manufacturer’s instructions. Then cDNAs were synthesized from total RNAs with the cDNA synthesis kit (Intron biotechnology). The exponential phase in reverse transcriptional PCR was determined on 15–30 cycles to allow semiquantitative comparisons among cDNAs developed from identical reactions. PCR regime involved a 2-min initial denaturation step at 94 °C for 30 s, 55–59 °C for 30 s and 72 °C for 1 min. Primer sequences were for p53: axon1 forward, 5′-ATGGAGGAGCGAGCTA-3′, axon1 reverse, 5′-CATCGCTATCTGACACG-3′; axon2 forward, 5′-GTCTTGCC CCTCTCACG-3′, axon2 reverse, 5′-CTGAGGGTGAAATATTG-3′, axon3 forward, 5′-ATCGCTGGCCTGAGGAGC-3′, axon3 reverse, 5′-TCATCTGAGTCGAGGC-3′. PCR products were separated by electrophoresis on 1% agarose gels, and used for DNA sequencing reaction.

**Western blot and antibodies**

Whole cells were lysed in a lysis buffer (0.5% Nonidet P-40; 120 mMol·L−1 NaCl; 20 mMol·L−1 Tris-HCl, pH 8.0; 2 mMol·L−1 EDTA, pH 8.0; 2 mMol·L−1 EGTA, pH 8.0; protease inhibitors), and protein concentration of lysates was determined by using protein assay kit (BioRad, Hercules, CA, USA). Cell lysates were subjected to SDS-PAGE on 10% gel. The proteins were then electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA), and blocked in TBST buffer with 5% skim milk. The blots were then probed with primary antibodies, and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Bioscience, Uppsala, Sweden). Signals were visualized using enhanced chemiluminescence reagent (ECL; Amersham Biosciences). Antibodies for caspase-3, caspase-8, caspase-9, PARP-1 and p21 were obtained from Santa Cruz Biotechnology. Anti-p53 and various anti-phospho-p53 antibodies were purchased from Cell Signaling.

**Fluorescence-activated cell sorting analysis**

Trypsinized cells were collected by centrifugation and fixed in ice-cold 80% ethanol. Cells were washed with PBS, stained with propidium iodide (40 μg·mL−1) and 100 μg·mL−1 RNaseA, and were analyzed in 10 000 events by flow cytometry (FACSCaliber; Becton Dickinson, Franklin Lakes, NJ, USA). Histograms by flow cytometry were generated in Cell Quest program (Becton Dickinson), and analyzed by ModFit software (Becton Dickinson).

**RESULTS**

*Z. serrata* twig extract has significant cytotoxicity on human oral carcinoma cell lines

To investigate whether or not primary methanol extract of *Z. serrata* twigs has cytotoxic effects on normal primary and oral cancer cells, various concentrations of the methanol extract were treated to cells and the number of viable cells was measured by cytotoxicity test (Figure 1). Methanol extract showed a dose-dependent cytotoxic effect on cells from mouth epidermoid carcinoma, lower gingival squamous cancer and tongue mucoepidermoid carcinoma (Figure 1, KB, YD38 and YD15). The IC50 in mouth epidermoid carcinoma cells (KB) was 200–250 ng·mL−1 upon treatment with the methanol extract for 18 h. Reversely, methanol extract did not show a cytotoxic effect on normal cells cultured from gingival tissue (Figure 1, HGF).
The primary methanol extract was further partitioned into three
to the solvent (hexane, ethyl acetate and water). The ethyl acetate
effect of the methanol extract (EtOAc fraction) showed a dose-dependent
to mesenchymal carcinoma, and the IC$_{50}$ in KB cells was 20 ng·mL$^{-1}$ upon treatment with the EtOAc
fraction for 18 h (Figure 2a, KB), indicating an approximately 10-fold
increase in IC$_{50}$ by the partition process. The cytotoxic effect of the
EtOAc fraction on mouth epidermoid carcinoma cells was further
analyzed according to morphological changes in response to treatment.
The EtOAc fraction induced severe cell death at 20 ng·mL$^{-1}$,
and most of the cells were detached from the culture dish at 18 h after
treatment (Figure 2b-II).

**Z. serrata** twig extract has selective apoptotic activity in oral
carcinoma cells

To examine whether or not these cytotoxic effects were the result of
apoptosis, the methanol extract (200 ng·mL$^{-1}$) and its three fractions
(each of 20 ng·mL$^{-1}$) prepared from the second partitioning were
treated to primary and cancer cells for 24 h. When mouth epidermoid
 carcinomas (KB) cells were treated with both the methanol extract
and EtOAc fraction, the levels of procaspases-3 and -8 decreased (Figure 3a,
lanes 1 and 3), suggesting that cleavage of procaspases increased and
the apoptosis pathway was activated. As a positive control for the
detection of apoptosis, 10 μmol·L$^{-1}$ camptothecin was applied. Indeed,
the levels of the procaspases intensively decreased according to treatment with camptothecin (Figure 3a, lane 5). Conversely, other
fractions partitioned from the methanol extract, such as the hexane
and water fractions, had no effects on caspase activation (Figure 3a,
lanes 2 and 4). In the case of tongue mucoepidermoid carcinoma cells
(YD15), there was only a slight decrease in the levels of these proteins,
suggesting that induction of apoptosis occurred very weakly upon
treatment with both the methanol extract and EtOAc fraction
(Figure 3b, lanes 1 and 3). On the contrary, the levels of procaspases
did not remarkably change upon treatment with any of the twig fractions
or camptothecin in lower gingival carcinoma cells (YD38)
(Figure 3c, lanes 1–5), suggesting that this cell line might have resistance
to apoptosis inducers.

Additionally, to confirm the selective apoptotic effect of the EtOAc
fraction of **Z. serrata** twig extract on mouth epidermoid carcinoma
cells, we analyzed the cleavage products of active caspase-3 and poly-
(ADP-ribose)-polymerase (PARP-1). When lower gingival squamous
(YD38) and epidermoid (KB) cells were treated with 20 ng·mL$^{-1}$ of the
EtOAc fraction for 24 h, active fragments of the apoptotic proteins
were detected in KB cell lysate (Figure 4a, lane 2 in upper and middle
panels). There are two major apoptotic pathways, the caspase-8-
coupled death receptor pathway and the caspase-9-coupled intrinsic
mitochondrial pathway with crosstalk between them. To determine
which apoptotic pathway is induced by treatment with **Z. serrata**
extract in KB cells, we examined the active cleavage of caspase-8 and
caspase-9. When EtOAc fractions of twig extract and camptothecin
were applied to KB epidermoid carcinoma cells for 24 h, active fragments
of caspase-8 were observed in the cell lysates (Figure 4b, lanes 2
and 3 in upper panel). However, under the same conditions, caspase-9
activation was not detected at all in either lysate (Figure 4b, lanes 2
and 3 in middle panel). These data suggest that **Z. serrata** twig extract
induced the caspase-8-dependent external apoptotic pathway in an
epidermoid carcinoma cell-specific manner.

**p53 activation is involved in **Z. serrata** twig extract-induced
apoptosis**

Modulation of apoptosis by treatment with twig extract was observed in
mouth epidermoid carcinoma cells, but not in the other two types of
oral cancer cells. Based on this selective apoptotic effect, we analyzed
the differences in the molecular aspect of p53 protein among the three
types of oral carcinomas. By performing p53 cDNA sequence analysis, we found that both mouth epidermoid carcinoma (KB) and tongue mucoepidermoid carcinoma (YD15) cells contained wild-type p53 gene, whereas lower gingival squamous carcinoma (YD38) cells did not contain the p53 gene due to deletion (Figure 5a). In addition to p53 gene status, p53 protein expression was also detected by Western blot. Under the conditions of treatment with the EtOAc fraction, p53 protein expression was strongly induced in mouth epidermoid carcinoma cells (Figure 5b, lane 2 in upper panel). However, although the p53 gene was determined to be wild-type without any mutation, p53 protein expression in tongue mucoepidermoid carcinoma cells was not induced by treatment with the EtOAc fraction. Interestingly, the basal level of endogenous p53 protein in tongue mucoepidermoid carcinoma cells was much higher than that in mouth epidermoid carcinoma cells, indicating that p53 protein expression in tongue mucoepidermoid carcinoma cells was not inducible and that these cells contained a much amount of p53 protein even under normal conditions (Figure 5b, lanes 3 and 4 in upper panel). As expected, p53 protein was not detected in lower gingival squamous carcinoma (YD38) cells upon either EtOAc fraction treatment or non-treatment (Figure 5b, lanes 5 and 6 in upper panel), as the p53 gene was deleted.

Generally, phosphorylation is important to the activation of p53, and specific residues in p53 are phosphorylated by specific upstream protein kinases and activation signals.24–26 To investigate the functional mechanism of p53 in modulating apoptosis upon treatment with the EtOAc fraction of Z. serrata twigs, we observed phosphorylation of p53 upon treatment with the EtOAc fraction using

Figure 3 Activation of caspases in oral carcinoma cells by treatment with fractions prepared from Zelkova serrata twigs. Cells originated from mouth epidermoid carcinoma (a), lower gingival squamous cancer (b) and tongue mucoepidermoid carcinoma (c) were treated with each fractions of twig extract, and the total cell lysates were subjected to western blot analysis with the indicated antibodies, which were specific to procaspases. Lanes 1, primary methanol extract of twigs; lanes 2, hexane fraction from secondary partitioning of methanol extract; lanes 3, ethyl acetate (EtOAc) fraction from secondary partitioning of methanol extract; lanes 4, water fraction from secondary partitioning of methanol extract; lanes 5, treatment with 10 μmol·L⁻¹ of camptothecin as a apoptosis positive control; lanes 6, untreated control.

Figure 4 EtOAc fraction from twig extract induced an internal apoptotic pathway in mouth epidermoid carcinoma cells. (a) Treatment of EtOAc fraction induced the active cleavages of caspase-3 and PARP-1 in epidermoid carcinoma cells (lane 2, KB), not in lower gingival squamous cancer cells (lane 1, YD38). (b) Treatment of EtOAc fraction induced the active cleavages of caspase-8 (α-casp8), but not that of caspase-9 (α-casp9) in mouth epidermoid carcinoma cells. Lane 1, untreated control; lane 2, treatment with 10 μmol·L⁻¹ of camptothecin; lane 3, treatment with 20 ng·μL⁻¹ of EtOAc fraction.

Figure 5 Features of p53 in three different oral cancer cell lines. (a) By DNA sequence analysis of p53 cDNAs prepared from oral cancer cells, endogenous p53 gene in both mouth epidermoid carcinoma (KB) and tongue mucoepidermoid carcinoma (YD15) was wild type. In contrast, p53 gene was deleted in lower gingival squamous cancer (YD38) by deletion mutation. (b) The expression level of p53 protein in three different oral cancer cell lines as described in (a). Total cell extracts were prepared from cells untreated with EtOAc fraction (lanes 1, 3 and 5) and from those treated with EtOAc fraction (lanes 2, 4 and 6), and were subjected to Western blot analysis with anti-p53 specific antibody (α-p53). (c) The phosphorylations on Serine-15 and -46 of p53 were increased by treatment with EtOAc fraction in mouth epidermoid carcinoma (KB) cells. Total cell extracts were prepared from cells untreated with EtOAc fraction (lane 1) and from those treated with EtOAc fraction (lane 2), and were subjected to Western blot analysis with phosphor-specific antibodies of p53.
phospho-specific p53 antibodies during Western blot analysis. The phosphorylation of Ser-15 and Ser-46 increased upon treatment with the EtOAc fraction (Figure 5c, lanes 2 in upper and middle panels). These data indicate that treatment with twig extract in KB cells induced phosphorylation and activation of p53, resulting in apoptosis.

**Z. serrata** twig extract disturbed cell division cycle

To validate the role of the EtOAc fraction in disturbing cell division, fluorescence-activated cell sorter analysis was performed. For this, we examined the DNA content of cells after reaction with 7 ng·µL⁻¹ of the EtOAc fraction for 18 h, which were mild treatment conditions. In unsynchronous culture of KB, YD15 and YD38, cells were mainly in G1/S phase (70.4%, 70.4% and 71.5%, respectively), whereas a small population of cells was in G2/M phase (29.6%, 29.6% and 28.5%, respectively) (Figure 6a, upper panels). As shown in Figure 6, treatment with the EtOAc fraction induced accumulation of KB and YD15 cells in S phase from 10.9% to 41.8% and 10.9% to 56.9%, respectively (Figure 6a, KB and YD15). The percentages of G2/M phase cells in both cultures dramatically decreased from 29.6% to 6.7% and 29.6% to 5.9%, respectively (Figure 6a, KB and YD15). However, lower gingival squamous carcinoma (YD38) cells did not display disturbance of the cell cycle, and there were no remarkable changes in the percentages of cells in G1, S or G2/M phase with and without treatment with EtOAc fraction (Figure 6a, YD38). To investigate the mechanism through which the twig EtOAc fraction disturbs the cell division cycle, the expression level of p21Waf1/Cip1, a cdk inhibitor, was measured. Expression of p21 protein increased upon treatment with the EtOAc fraction in both mouth epidermoid and tongue mucoepidermoid carcinoma cells (Figure 6b, lanes 2 and 4). Induction of p21 protein in gingival squamous carcinoma cells did not significantly increase upon EtOAc fraction treatment (Figure 6b, lane 6).

**Figure 6** Treatment of EtOAc fraction of *Zelkova serrata* twig disturbed cell division cycle in cells originated from mouth epidermoid and tongue mucoepidermoid carcinomas. (a) FACS analysis of cell cycle distribution. DNA content of oral cancer cells in untreated (−) or treated (+) with 7 ng·µL⁻¹ of EtOAc fraction for 18 h was determined by flow cytometry. After fixation of cells with 70% ethanol for 24 h, DNA was stained by adding propidium iodide. The population of cells in G1, S and G2/M phase is indicated. (b) Effect of EtOAc fraction of *Zelkova serrata* twig on p21 expression. Total cell extracts were prepared from cells untreated with EtOAc fraction (lanes 1, 3 and 5) and from those treated with EtOAc fraction (lanes 2, 4 and 6), and were subjected to Western blot analysis with anti-p21Waf1/Cip1 antibody (α-p21). In (a) and (b), KB, YD15 and YD38 indicated mouth epidermoid carcinoma cells, tongue mucoepidermoid carcinoma cells and lower gingival squamous cancer cells, respectively. FACS, fluorescence-activated cell sorter analysis.
**DISCUSSION**

Oral cancer, a subtype of head and neck cancer, is any cancerous tissue growth located in the oral cavity.²⁷ Oral cancer was reportedly diagnosed in almost 35,310 US adults in 2008. Oral or mouth cancer most commonly occurs on the tongue, but may also occur on the cheek lining, gingival (gums) or lips. Based on this information, we chose three different types of oral cancer cell lines, mouth epidermoid carcinoma, lower gingival squamous carcinoma and tongue mucoepidermoid carcinoma, and investigated the anti-cancer effect of a natural product originating from *Z. serrata*. Interestingly, the molecular aspects of p53 tumor suppressor protein were remarkably different among these three cell lines. Whereas lower gingival squamous carcinoma was a p53-deletion mutant, p53 gene in both mouth epidermoid and tongue mucoepidermoid carcinoma cells did not contain any mutations, as determined by cDNA sequencing. Although the gene status of p53 was normal, its protein expression level was extremely high and was not controlled at all in tongue mucoepidermoid carcinoma cells (Figure 5). Due to the differences in p53 expression, we expect that the three carcinoma cell types could differ in their response to an anti-cancer agent. So far, apoptotic inducers have been used in cancer therapy, as the apoptotic pathway is frequently impaired in many cancers. Several studies have attempted to induce apoptosis in cancer cells by triggering cell death proteins directly and indirectly,²⁸–³⁰ and some of these approaches have shown promising results. Previously, we analyzed the cytotoxic effects of crude extracts from 35 natural local plants on various cancer cell lines.²² *Z. serrata* is called Keyaki or Japanese Zelkova and is native to Japan, Korea, Taiwan and eastern China. Traditionally, in Korea, crude extract of *Z. serrata* is known to exhibit anti-inflammatory and anti-cancer properties. Recently, a secondary metabolite classified as a flavonoid, cadalene, was isolated from whole tree and bark ethanol extract of *Z. serrata* Makino, and this compound was shown to exert anti-oxidative effects in mice.³⁰ In this report, we used only twigs from whole tree to prepare the extract. Methanol extract of the twig powder showed cancer cell-specific cytotoxicity (Figure 1). The average IC₅₀ value of methanol extract in oral cancer cells was about 200–250 ng·µL⁻¹. After partitioning this methanol extract with hexane, ethyl acetate and water, the EtOAc fraction showed cancer cell-specific cytotoxicity (Figure 2). The IC₅₀ Value of the EtOAc fraction (~20 ng·µL⁻¹) was about 10 times higher than that of primary methanol extract, indicating that the ingredient possessing cytotoxicity could be enriched by purification. Interestingly, this fraction modulated caspase activation in only KB epidermoid carcinoma cells (Figure 3a) and had no remarkable apoptotic effects on either gingival squamous or tongue mucoepidermoid carcinoma cells (Figure 3b and 3c). Additionally, the EtOAc fraction induced the external apoptotic pathway involving caspases-8 and -3 (Figure 4b), indicating that this fraction possibly modulates cell surface death receptors by ligand stimulation. Generally, induction of p53 tumor suppressor initiates blockage of the cell division cycle and apoptosis. Indeed, only in mouth epidermoid carcinoma cells, both expression and modification of p53 protein markedly increased upon treatment with 20 ng·µL⁻¹ of EtOAc fraction of *Z. serrata* twigs (Figure 5b and 5c), indicating that apoptosis induced by EtOAc fraction treatment is dependent on the induction and activation of p53. In addition to apoptosis induction, the percentage of cells in S phase increased, and G₂/M populations of KB cells dramatically decreased upon treatment with the EtOAc fraction. These data indicate that induction and activation of p53 modulate cell cycle disturbance. To further investigate how twig extract induced cells to arrest in G₁–S phase, we examined changes in the level of p21^{Waf1/Cip1}, which is a target gene of p53 and a major factor in G₁–S arrest in response to stress.¹⁶–¹⁸ The results show that the expression of p21^{Waf1/Cip1} was upregulated by treatment with the EtOAc fraction in KB cells (Figure 6b, lanes 1 and 2). Although expression of p53 was not controlled in YD15 tongue mucoepidermoid carcinoma cells, p21 protein increased upon treatment with the EtOAc fraction, and cell proliferation was disturbed (Figure 6a and lanes 3 and 4 in Figure 6b), which indicates that tongue mucoepidermoid carcinoma cells might express p21 in a p53-independent manner.³¹–³² Due to p53-deletion and lower expression of p21, treatment with the EtOAc fraction of *Z. serrata* did not stimulate cell cycle arrest or apoptosis in YD38 gingival carcinoma cells (Figure 6a and lanes 5 and 6 in Figure 6b). In conclusion, partially purified fraction of *Z. serrata* twigs exerted a selective apoptotic effect on mouth epidermoid carcinoma cells, which express functional p53, by inducing p21^{Waf1/Cip1} expression. In addition, induction of cancer type-specific apoptosis by *Z. serrata* twig extract suggests that this material could be developed as a promising p53-dependent cancer therapeutic agent in the future.

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