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Characterization of Anti-Cancer Effects of the Ethyl Acetate Fraction from *Orostachys japonicus* on HT-29 Human Colon Cancer Cells

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Abstract The ethyl acetate fraction from *Orostachys japonicus* (OJE) is a mixture of flavonols (kaempferol and quercetin) and flavonol glycosides (afzelin, astragalin, quercitrin, and isoquercitrin), and there is little information about the interactive effects of these components on the anti-colon cancer activities. A comprehensive investigation of the anti-colon cancer activities obtained by combined or single treatment of OJE, kaempferol, or quercetin was performed to confirm the roles of key components contained in OJE. OJE alone or OJE supplemented with kaempferol and quercetin showed greater anti-colon cancer activities, namely, in inducing apoptosis, cell cycle arrest, anti-metastasis, and upstream signal transduction than the combination of kaempferol and quercetin without OJE, kaempferol alone or quercetin alone. The combination of kaempferol and quercetin without OJE was also superior to kaempferol or quercetin alone in exerting anti-colon cancer activities via various manners. The consistent cooperative effects were revealed among flavonols and flavonol glycosides.

Keywords: *Orostachys japonicus, apoptosis, cell cycle arrest, anti-metastasis, flavonol, flavonol glycoside*

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1. Introduction

Surgery, radiation therapy, immunotherapy, and chemotherapy are commonly used methods for cancer treatment [1,2]. Chemical anti-cancer drugs used to treat cancer attack not only cancer cells but also surrounding normal cells, resulting in serious side effects such as vomiting, hair loss, and white blood cell reduction [3,4]. Recently, interest has increased in identifying the anti-cancer activities of natural substances to alleviate side effects related to existing cancer treatments [5]. *Orostachys japonicus* is a perennial herbaceous plant belonging to the Crassulaceae family. *O. japonicus* is known to have anti-oxidant, anti-inflammatory, anti-atopic, anti-hypertensive, anti-bacterial, and anti-cancer activities [6,7,8,9,10]. The ethyl acetate (EtOAc) fraction of the ethanol (EtOH) extracts from *O. japonicus* (OJE) contains flavonols and flavonol glycosides such as kaempferol, quercetin, afzelin, astragalin, quercitrin, and isoquercitrin [11]. There are no systematic studies on the anti-cancer activities by interaction among these components. Among them, kaempferol and quercetin are particularly important components as flavonols showing anti-cancer activity [12,13]. On the other hand, it is very interesting to find out how much components in OJE will look cooperative. Apoptosis is essential for the balance of cells and maintenance of normal function, and is a programmed cell death process that occurs in response to biological, chemical, or physical stimuli, and plays an important role in various physiological and pathological situations of the cells [14]. When apoptosis occurs in a cell, it leads to exposure of phosphatidylserine (PS) on the outer plasma membrane, which impedes anti-apoptotic factors like B-cell lymphoma-2 (Bcl-2) and activates apoptosis-induced proteins such as Bax [15,16]. In cells where apoptosis occurs, cytochrome c is released from the mitochondria, and caspases, which are apoptosis-inducing proteins, are sequentially activated [17]. If apoptosis does not work properly, various diseases such as cancer, autoimmune diseases, and neurodegenerative disorders can occur [18]. The key properties seen in apoptosis include activation of caspases, cascade induction of caspases, chromatin condensation, DNA fragmentation, membrane blebbing, phosphatidylserine exposure, nuclear membrane fragmentation, and formation of apoptotic bodies [19,20,21].

The cell cycle, consisting of G1, S, G2, and M phases, undergoes stage-specific changes based on cellular conditions [22]. Critical checkpoints at G1/S, G2/M, and metaphase phases evaluate overall cellular status before

progressing to the next cycle [23]. Cyclins and cyclin-dependent kinases (CDK) play pivotal roles in regulating the cell cycle and affecting induction of apoptosis of cancer cells [24]. CDKs form complexes with cyclins at different stages, like CDK-4 and CDK-6 with cyclin D at G1, facilitating progression24. CDK-2 and cyclin E promote DNA synthesis at the S phase, CDK-1 is activated primarily at the G2 and M phases, and together with cyclin A, promotes the transition from G2 to M phases [25,26]. The arrest and progression of cell cycle are influenced by CDK inhibitors and extracellular signaling substances and depend on the expression of CDK and cyclin required at each stage.

Metastasis is a complex process where cancer cells spread from the primary tumor, forming secondary tumors in distant sites. This multistep process involves invasion, migration, and colonization, significantly contributing to cancer morbidity and mortality [27,28]. Cancer cells gain invasive ability by degrading the extracellular matrix, facilitated by enzymes like matrix metalloproteinases (MMPs). Adhesion, matrix degradation, and migration are key stages in invasion, orchestrated by cell adhesion molecules and MMPs [29]. The process involves breaking down structural barriers and invading surrounding tissues, crucial for cancer cell mobility. Cell adhesion, including cadherin-mediated adhesion, plays a pivotal role in cohesive cellular groups, influencing cancer progression and metastasis [30,31]. In addition to adhesion, collective cell migration relies on strong intercellular binding through cadherins, enabling coordinated movement and collective behaviors [32].

The MAP kinase (MAPK) cascade is a vital intracellular upstream signaling system comprised of three types of protein kinases, conveying external information into the cell [33]. It undergoes phosphorylation reactions, ultimately activating MAPK and influencing gene expression in the nucleus [34]. In response to cellular stress, pathways such as ERK, JNK, and p38 MAPK are activated, which affect processes such as cell survival, apoptosis, and phosphorylation of transcription factor. These pathways play critical roles in various cellular processes, including cell growth, differentiation, and response to environmental stimuli [35].

The objectives of this study were to compare and deeply explore the effects and cooperative effects of OJE, OJE supplemented with kaempferol and quercetin, the mixture of kaempferol and quercetin, and single substances of kaempferol or quercetin on induction of apoptosis, cell cycle arrest, anti-metastasis, activation of upstream signaling pathways, and cell viability in HT-29 human colon cancer cells.

2. Materials and Methods

2.1. Preparation of the EtOAc Fraction from *O. japonicus*

The OJE was prepared in a manner described as follows. The dried and sliced *O. japonicus* (200g) provided by Geobugwasong Inc. (Miryang-si, Korea) was powdered. The powdered *O. japonicus* was then boiled three times with 95 % EtOH for 3 h each. The EtOH extract was concentrated using at 40°C. The resulting concentrate was suspended in water and fractionated using a sequence of organic solvents. Each solvent fraction was concentrated using at 40°C and stored at -20 °C for future use.

2.2. Cell line and Reagents

The HT-29 human colon cancer cells No. 30038 and murine RAW 264.7 macrophage cells No. 40071 were bought from the Korean cell line bank (KCLB, Seoul, Korea). All other reagents in this study were of the highest grade or analytical grade.

2.3. Cell Culture

The HT-29 cells and RAW 264.7 were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin. Both cell lines were maintained at 37°C in a 5% CO_2 humidified atmosphere incubator until confluence was reached. Subcultures were performed every 4–6 days at a 1:3 split ratio, and the growth medium was changed every 2–3 days. Cells were used in experiments when they reached approximately 85% confluence.

2.4. Cell Growth Inhibition Assay

Cytotoxicity assessment

Cytotoxicity was assessed by cell viability using the CellTiter 96 aqueous cell proliferation assay kit (Promega, Pittsburgh, Wisconsin, USA). RAW 264.7 cells were seeded into well plates at a density of 5×10^5 cells/well and incubated at 37°C with 5% CO_2 for 24 h. Subsequently, cells were treated with various concentrations of kaempferol, quercetin, and OJE either alone or in combination for 12 and 24 h. After treatment, MTS solution was added to each well, and the plates were incubated for 2 h in the dark. Absorbance was then measured at 490 nm using a multi-detection reader (BioTek, Santa Clara, California, USA).

Cell viability assessment

In addition, cell viability was assessed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit. HT-29 Cells were seeded into well plates at a density of 4×10^5 cells/well, incubated for 24 h, and then treated with various concentrations of kaempferol, quercetin, and OJE either alone or in combination for 12 and 24 h. After treatment, MTS solution was added to each well, and the plates were incubated for 2 h in the dark before measuring absorbance at 490 nm using a multi-detection reader.

2.5. Nuclear Staining with 4′, 6-diamidino-2-phenylindole (DAPI)

Phosphatidylserine exposure on cell membranes was detected using annexin V-fluorescein isothiocyanate (Annexin V-FITC) and propidium iodide (PI) staining to assess apoptotic features. Cells cultured on glass coverslips were treated with varying concentrations of OJE and flavonols for 24 h. Subsequently, harvested cells were stained with 4', 6-diamidino-2-phenylindole (DAPI)

and morphology of cell nuclei stained were analyzed using a high-resolution confocal laser scanning microscope (CLSM).

2.6. Flow Cytometry Analysis of Apoptosis

To assess apoptosis in HT-29 cells (4×10^5 cells/mL in a 12-well plate), Annexin V-FITC and PI staining were conducted using the BD Pharmingen Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA), following the manufacturer's instructions. HT-29 cells were treated with various concentrations of OJE and flavonols for 12 h. Following treatment, cells were collected by centrifugation at 300 × g and washed twice with cold phosphate-buffered saline (PBS). The cell pellets were then resuspended in 100 µL of 1x binding buffer and stained with 5 µL of Annexin V-FITC and 5 µL of PI in the dark at room temperature for 15 m. After staining, 400 µL of 1x binding buffer was added to each tube. The stained cells were immediately analyzed using flow cytometry with the CellQuest Pro software (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

2.7. Wound Healing Assay

HT-29 cells were initially seeded at a concentration of 4 × 10^5 cells/mL in a 6-well plate for cell culture. After a 24 h stabilization period, cells were allowed to grow until reaching 80-90 % confluency. To induce a wound, a straight line was manually scraped in the center of each well (0 h), creating a linear wound. The healing process was then observed at 40× magnification using a phase-contrast microscope. Images were captured at 24 h after treatment with the various concentrations of OJE, kaempferol, and/or quercetin to assess the migration rate towards the center of the wound. The distances traveled by migrating cells in the image were measured and calculated.

2.8. Flow Cytometry Analysis of Cell Cycle Arrest

To assess the cell cycle phase in HT-29 cells (at a concentration of 4 × 10^5 cells/mL in a 12-well plate), DNA fragment staining with PI was performed using a Cell Cycle Phase Determination Kit from (Cayman Chemical, Ann Arbor, Michigan, USA) following the manufacturer's instructions. HT-29 cells were fixed and permeabilized by adding 1 mL of a cell cycle phase determination fixative solution to each tube and incubated at -20°C for more than 2 h. After centrifugation at 3,000 rpm for 5 m, the fixatives were removed, and cell pellets were suspended in 500 µL of staining solution containing 1 mL of cell-based assay buffer, 20 µL of RNase A solution, and 20 µL of PI reagent. The suspension was incubated for 30 m at room temperature in the dark. Finally, the cells were immediately analyzed by flow cytometry using CellQuest Pro software on a FACSCalibur instrument from BD Biosciences.

2.9. Analysis of Expression Levels of Proteins Using Western Blot

HT-29 cells were treated with varying concentrations of kaempferol, quercetin, and OJE for 12 h. After treatment, cells were harvested, washed with cold PBS, and lysed in 1x cell lysis buffer. The lysates were sonicated, kept on ice for 1 h, and then centrifuged. Protein concentrations were measured using the Pierce BCA protein assay kit. Equal amounts of protein (35 µg) were loaded onto 10 % SDS-polyacrylamide gels for electrophoresis and transferred to a PVDF membrane. The membrane was blocked, incubated with primary antibodies, washed, and then exposed to an HRP-conjugated secondary antibody. Protein signals were detected using Immunostar and X-ray hyperfilm and quantified with band intensities using ImageJ 1.8.0 software.

2.10. Statistical Analysis

The experimental data were presented as the mean ± standard deviation (SD). A Student's t-test was utilized to assess the statistical significance of differences between the control and experimental groups. Statistical significance was expressed as *$p<0.05$, **$p<0.01$, and ***$p<0.001$ to evaluate the significance level, and the lower the p-value, the greater the significance.

3. Results

3.1. Cytotoxic Effects of Kaempferol, Quercetin, and OJE on RAW 264.7 Macrophage Cells

To evaluate the potential cytotoxicity of kaempferol, quercetin, and OJE, cytotoxicity assay was performed using RAW 264.7 macrophages. Kaempferol or quercetin was administered at concentrations of 0, 0.1% DMSO, 25, 50, 75, and 100 µM, and OJE was administered at concentrations of 0, 0, 0.1% DMSO, 25, 50, 75, and 100 µg/mL to RAW 264.7 macrophages for 12 and 24 h. As shown in Figure 1 kaempferol, quercetin, and OJE did not exhibit cytotoxicity in RAW 264.7 cells. Based on the data from the cytotoxicity assays, the highest concentration of 100 µM for kaempferol or quercetin was used and the highest concentration of 100 µg/mL for OJE was used in the following experiments of this study.

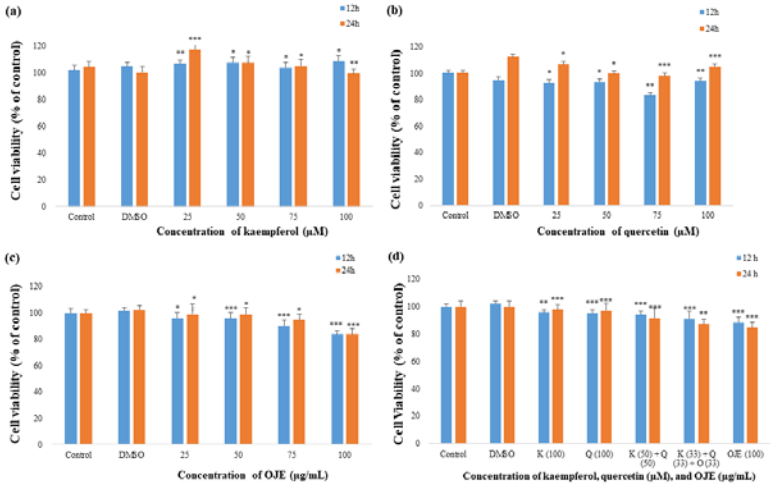

Figure 1. Effects of kaempferol alone (a), quercetin alone (b), OJE (c), and kaempferol alone, quercetin alone, OJE, and combinations of kaempferol, quercetin, and/or OJE (d) on viability of RAW 264.7 macrophage cells measured by cytotoxicity assay for 12 and 24 h. The results are presented as the mean ± standard deviation (SD) of three independent experiments. Statistical significance was determined as *p < 0.05, **p < 0.01, and ***p < 0.001

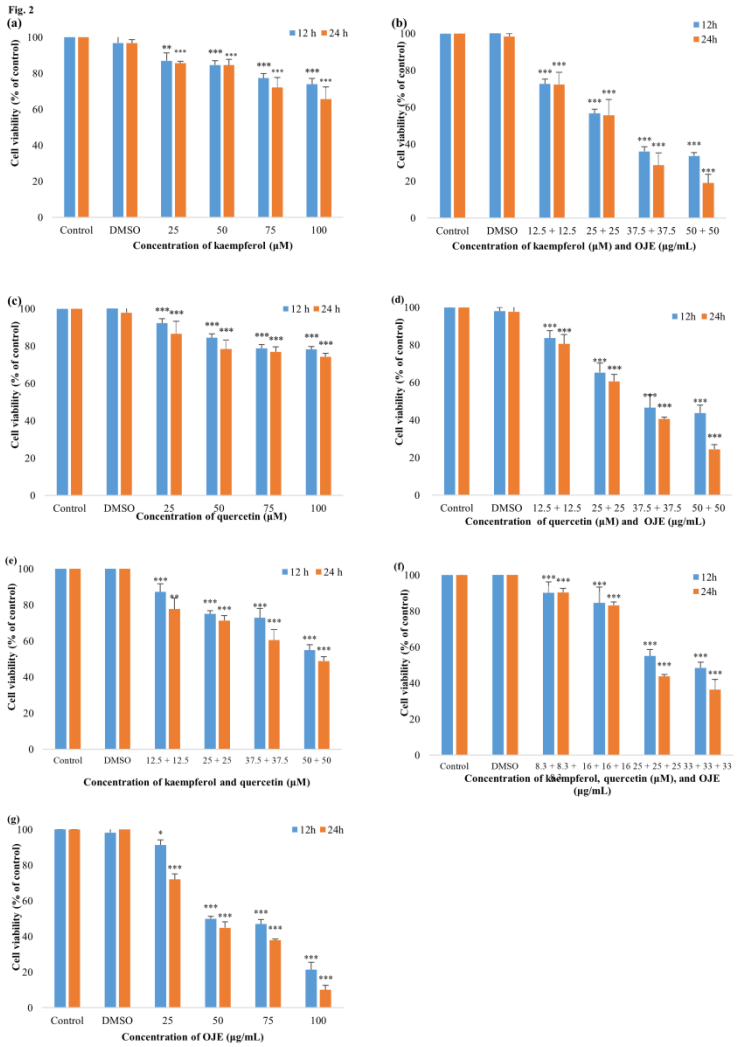

Figure 2. Inhibitory Effects of kaempferol (e), kaempferol + OJE (f), quercetin (g), quercetin + OJE (h), kaempferol + quercetin (i), kaempferol + quercetin + OJE (j), and OJE (k) on the proliferation of HT-29 cells. Cell viability was assessed using the MTS assay. The results are presented as the mean ± standard deviation (SD) of three independent experiments. Statistical significance was determined as *p < 0.05, **p < 0.01, and ***p < 0.001

3.2. Inhibitory Effects of Kaempferol, Quercetin, and/or OJE on the Proliferation of HT-29 Human Colon Cancer Cells

To investigate inhibitory effects of kaempferol, quercetin, and/or OJE on the proliferation of HT-29 human colon cancer cells, the MTS assay was employed. Kaempferol, quercetin, OJE with kaempferol, OJE with quercetin, a mixture of kaempferol and quercetin, OJE with kaempferol and quercetin, and OJE exhibited a dose- and time-dependent anti-proliferative effects on HT-29 cells. Figure 2 demonstrates that the inclusion of OJE exerted better inhibitory activities than the case without OJE. That is, kaempferol with OJE, quercetin with OJE, or a mixture of kaempferol and quercetin with OJE were found to exhibit more effective anti-proliferative activity than kaempferol alone, quercetin alone, or a mixture of kaempferol and quercetin. These findings suggest that the various flavonols and flavonol glycosides contained in OJE work together in cooperation, inducing inhibitory activities in proliferation of HT-29 cells more effectively than kaempferol alone or quercetin alone.

3.3. Apoptotic Effects of Kaempferol, Quercetin, and OJE on HT-29 Human Colon Cancer Cells Analyzed by DAPI Staining

DAPI staining and confocal microscopy analysis detected distinct apoptotic bodies, visualized by DAPI staining, in cells treated with different concentrations of kaempferol or quercetin (0, 0.1% DMSO, 25, 50, 75, and 100 μM), kaempferol alone or quercetin alone (100 μM), K + Q (kaempferol 50 μM + quercetin 50 μM), K + Q + OJE (kaempferol 33 μM + quercetin 33 μM + OJE 33 μg/mL), and OJE (OJE 100 μg/mL). Figure 3 shows that inclusion of OJE induces chromatin condensation and apoptotic body formation better than absence of OJE. These findings suggest that the various flavonols and flavonol glycosides contained in OJE work together in cooperation, inducing apoptosis more effectively than kaempferol alone or quercetin alone.

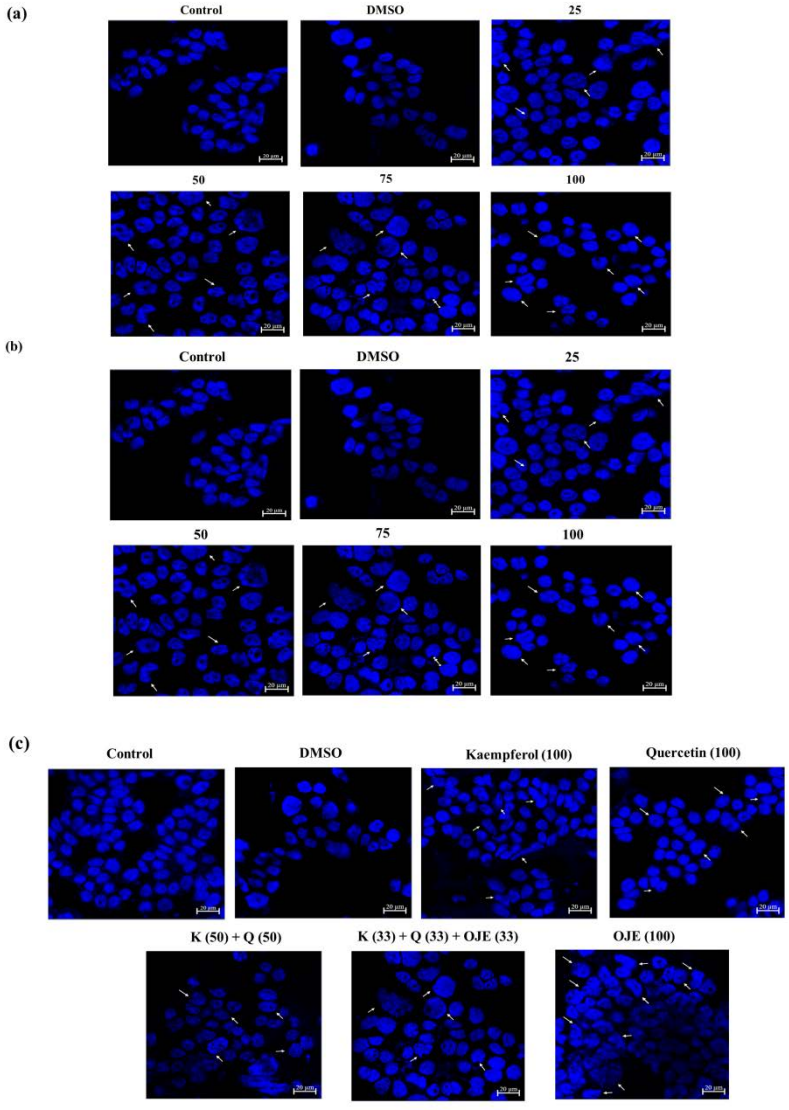

Figure 3. Nuclear morphological changes indicative of inducing apoptosis observed in HT-29 cells treated with kaempferol (a), quercetin (b), and kaempferol alone, quercetin alone, a mixture of kaempferol and quercetin, a mixture of kaempferol, quercetin, and OJE, and OJE (c). They were then stained with the DNA-specific fluorochrome DAPI. White arrows signify apoptotic nuclear morphological changes

3.4. Apoptotic Effects of Kaempferol, Quercetin, and OJE on HT-29 Human Colon Cancer Cells Analyzed by Flow Cytometry

To assess apoptosis induced by kaempferol, quercetin, and OJE, flow cytometry was employed to analyze Annexin V binding to phosphatidylserine (PS) during early apoptosis, alongside propidium iodide (PI) staining for evaluation of DNA damage. This dual staining approach facilitated thorough assessment of apoptosis progression using flow cytometry [36]. The staining results categorized cell populations as LL (lower-left, viable cells), LR (lower-right, early apoptotic cells), and UR (upper-right, late apoptotic or necrotic cells). Figure 4 shows that the inclusion of OJE induces early and late apoptosis better than absence of OJE. These findings suggest that the various flavonols and flavonol glycosides contained in OJE work together in cooperation, inducing apoptosis progression more effectively than kaempferol alone or quercetin alone.

3.5. Cell Cycle Arrest Effects of Kaempferol, Quercetin, and OJE on HT-29 Human Colon Cancer Cells Analyzed by Flow Cytometry

FACS analysis, employing PI staining, was conducted to assess cell cycle arrest in HT-29 cancer cells. The DNA contents of PI-stained HT-29 cells were determined through flow cytometry. Evidence of cell cycle arrest was observed on the left side of the G1 peak in the cell cycle. The increase in the sub-G1 peak indicates the cell cycle arrest at G1/S phase. As shown in Figure 5 (a, b), kaempferol and quercetin induced cell cycle arrest in HT-29 cells at G1/S phase in a concentration-dependent manner. As shown in Figure 5 (c), cell cycle arrest was induced better in the presence of OJE than in the absence of OJE it was found that OJE alone and the mixture of OJE, kaempferol, and quercetin more effectively induced cell cycle arrest at G1/S phase, than kaempferol alone, quercetin alone or the mixture of kaempferol and quercetin. These findings suggest that the various flavonols and flavonol glycosides contained in OJE work together in cooperation, inducing cell cycle arrest more effectively than kaempferol alone, quercetin alone or the mixture of kaempferol and quercetin.

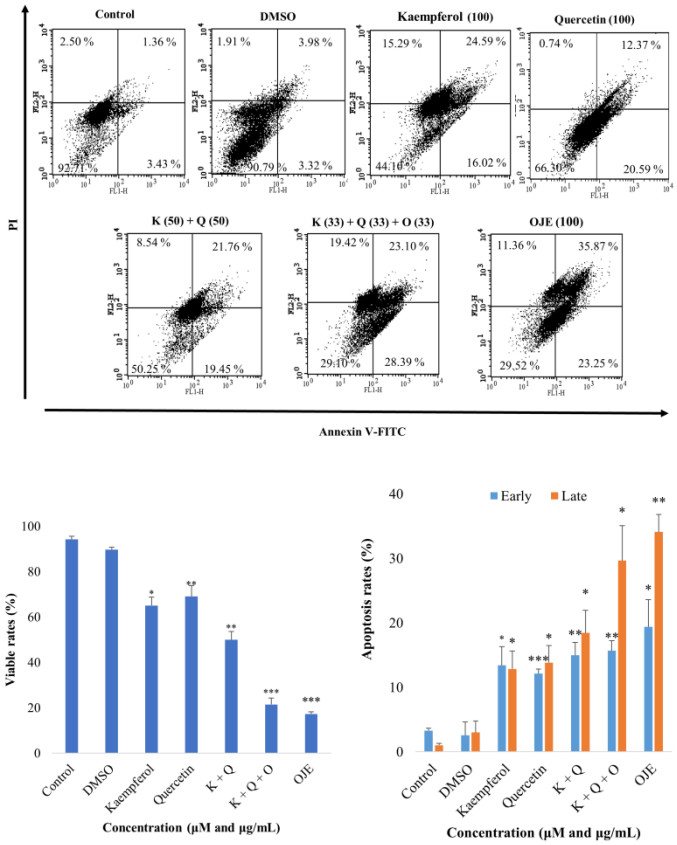

Figure 4. Flow cytometry analysis of apoptosis progression in HT-29 cells treated with kaempferol alone, quercetin alone, kaempferol + quercetin, kaempferol + quercetin + OJE, and OJE. The cells were exposed to various concentrations (kaempferol 100 μM, quercetin 100 μM, kaempferol 50 μM + quercetin 50 μM, kaempferol 33 μM + quercetin 33 μM + OJE 33μg/mL, and OJE 100μg/mL) fo 12 h. The cells were labeled with monoclonal antibodies specific to Annexin V-FITC and PI. Based on the staining pattern, cells were categorized as follows: Annexin−/PI− (LL) representing viable cells, Annexin+/PI− (LR) indicating cells undergoing apoptosis, and Annexin+/PI+ (UR) representing cells in end-stage of apoptosis or already deceased

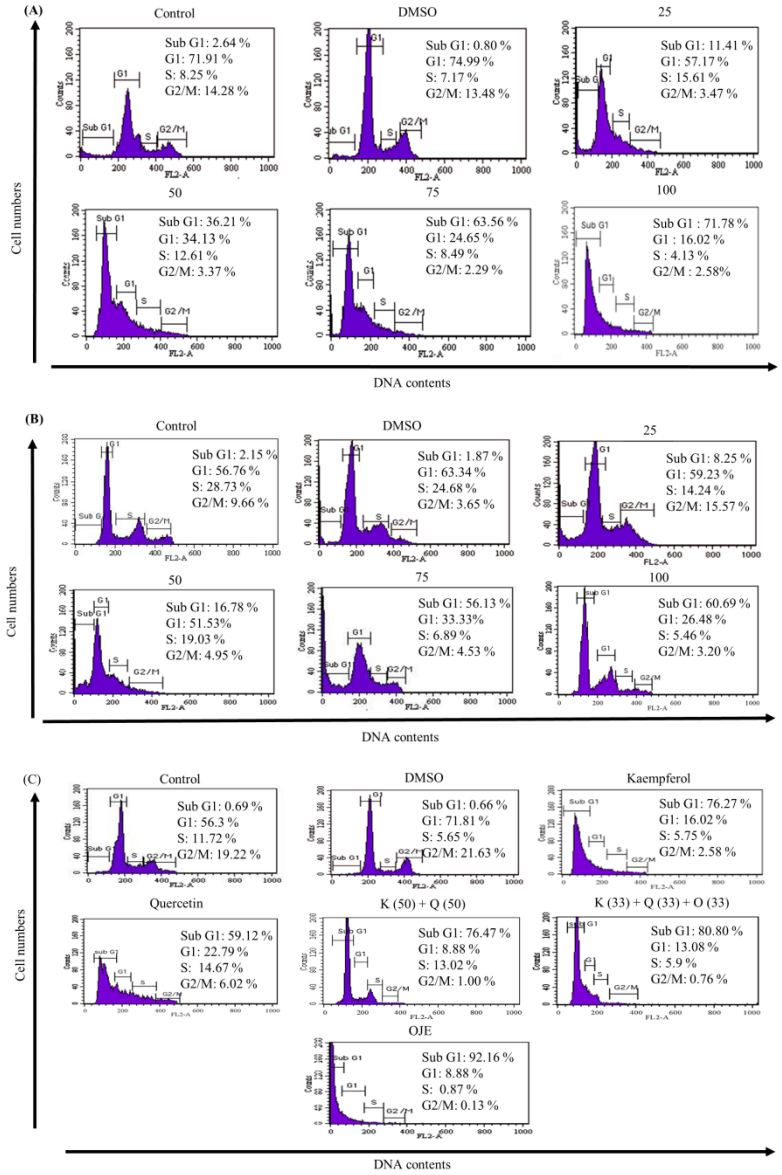

Figure 5. Flow cytometry analysis of cell cycle arrest in HT-29 cells treated with kaempferol (a), quercetin (b), kaempferol alone, quercetin alone, K + Q (kaempferol + quercetin), K + Q + O (kaempferol + quercetin + OJE), and OJE (c). Histograms represent sub-G1, G1, S, and G2/M phases of HT-29 cells. The results were expressed as percentage of total treated cells. Three experiments showed similar results

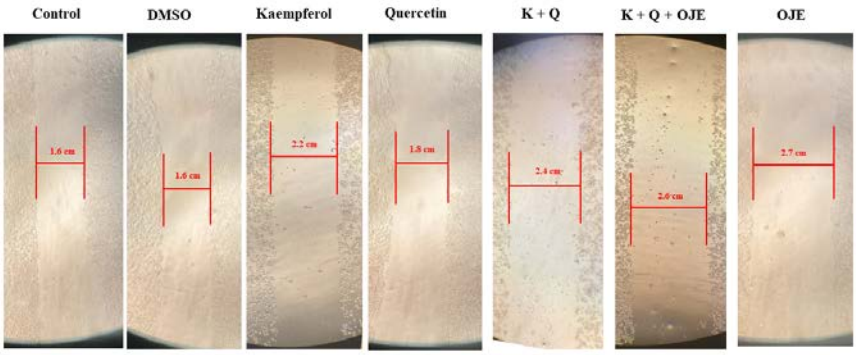

Figure 6. Wound healing assay for measurement of inhibitory effects of OJE, kaempferol, and quercetin alone or in combination on the migration of HT-29 cells. Three experiments showed similar results

3.6. Inhibitory Effects of Kaempferol, Quercetin, and OJE on the Metastasis of HT-29 Human Colon Cancer Cells Analyzed by Wound Healing Assay

To evaluate the effects of kaempferol, quercetin, and OJE alone or in combination on the metastasis of HT-29 cells, wound healing assay was employed. As shown in Figure 6, the inhibitory effects on wound healing in HT-29 cells were induced better in the presence of OJE than in the absence of OJE. These findings suggest that the various flavonols and flavonol glycosides contained in OJE work together in cooperation, inducing anti-migration more effectively than kaempferol alone or quercetin alone.

3.7. Effects of Kaempferol, Quercetin, and/or OJE on the Levels of Apoptosis Induction-related Proteins Analyzed by Western Blot

Western blot analysis was performed to evaluate the levels of key proteins related to mechanism and signaling pathways of inducing apoptosis. As shown in Figure 7 (a), it was confirmed that kaempferol especially activated biomarkers related to the intrinsic pathway of induction of apoptosis (reduction in procaspase-9, increase in cleaved caspase-9) and the ER stress-mediated pathway of induction of apoptosis (reduction in procaspase-12) in a concentration-dependent manner [37,38]. Meanwhile, kaempferol was also found to inactivate a protein (reduction in lamin A, increase in cleaved lamin A) involved in forming nuclear membrane in a concentration-dependent manner. As shown in Figure 7 (b), quercetin was found to specifically activate biomarkers related to the intrinsic pathway of induction of apoptosis (increase in cleaved caspase-9) and the extrinsic pathway of induction of apoptosis (increase in cleaved caspase-8) in a concentration-dependent manner. Meanwhile, quercetin was also revealed to inactivate proteins involved in forming nuclear membrane (reduction in lamin A and lamin C) in a concentration-dependent manner. As shown in Figure 7 (c), key biomarkers related to inducing apoptosis (cleaved caspase-9, procaspase-3, cleaved caspase-3, procaspase-8, and procaspase-12) were significantly more activated in the presence of OJE (OJE and K + Q + O) than in the absence of OJE (K, Q, and K + Q) while key biomarkers related to inhibiting apoptosis (PARP and Bcl-2) were significantly more inactivated or repressed in the presence of OJE (OJE and K + Q + O) than in the absence of OJE (K, Q, and K + Q). In particular, OJE and K + Q + O were superior to the single flavonol, kaempferol alone or quercetin alone, or the mixture of kaempferol and quercetin in activating signaling mediators involved in the intrinsic pathway (increase in cleaved caspase-9), extrinsic pathway (reduction in procaspase-8), and ER stress pathway (reduction in procaspase-12) of inducing apoptosis as well as in activating an executioner caspase (reduction in procaspase-3, increase in cleaved caspase-3). In addition, the mixture of kaempferol and quercetin (K + Q) exhibited superior effects in inducing apoptosis through various pathways compared to kaempferol alone or quercetin alone.

3.8. Effects of Kaempferol, Quercetin, and/or OJE on the Levels of Cell Cycle Arrest-related Proteins Analyzed by Western Blot

Western blot analysis was performed to evaluate the levels of key proteins related to mechanism and signaling pathways of inducing cell cycle arrest. As shown in Figure 8 (a), it was confirmed that quercetin especially reduced biomarker related to cell cycle progression at the S phase and G2/M phase in a concentration-dependent manner. As shown in Figure 8 (b), in all samples treated to HT-29 cells (kaempferol, quercetin, kaempferol + quercetin mixture, kaempferol + quercetin + OJE mixture, and OJE), CDK-2, CDK-4, and cyclin D1 inducing cell cycle progression at the G1/S phase were reduced, and cyclin A2 inducing cell cycle progression at the S phase and G2/M phase was also reduced. And the major biomarkers (CDK-2, CDK-4, cyclin D1, and cyclin A2) associated with induction of cell cycle progression were found to be significantly reduced in the presence of OJE (OJE and K + Q + O) than in the absence of OJE (K, Q, and K + Q). In particular, OJE was superior to the kaempferol + quercetin + OJE mixture in inducing cell cycle arrest at the G1/S, S, and G2/M phase. In addition, the kaempferol + quercetin mixture was superior to kaempferol alone or quercetin alone in inducing cell cycle arrest at the G1/S, S, and G2/M phase [39]. Therefore, it can be assumed that there is significant cooperative interaction among the two types of flavonols and the four types of flavonol glycosides in OJE in reducing the major signaling mediators that cause cell cycle progression. In addition, it can be speculated that there is cooperation between the two types of flavonols, kaempferol and quercetin, in reducing signaling mediators that induce cell cycle progression.

3.9. Effects of Kaempferol, Quercetin, and/or OJE on the Levels of Anti-metastasis-related Proteins Analyzed by Western Blot

Western blot analysis was performed to evaluate the levels of key proteins related to mechanism and signaling pathways of inhibition of metastasis. As shown in Figure 9 (a), it was confirmed that quercetin especially reduced a biomarker, MMP-9 related to inducing metastasis in a concentration-dependent manner. As shown in Fig. 9 (b), in all samples treated to HT-29 cells (kaempferol, quercetin, kaempferol + quercetin mixture, kaempferol + quercetin + OJE mixture, and OJE), E-cadherin, claudin-1, integrin β1, and MMP-9 which promote metastasis, including invasion and migration, were reduced. And the major biomarkers associated with induction of metastasis (E-cadherin, claudin-1, integrin β1, and MMP-9) were found to be significantly reduced in the presence of OJE (OJE and K + Q + O) than in the absence of OJE (K, Q, and K + Q). In particular, OJE was superior to the kaempferol + quercetin + OJE mixture in inhibiting

metastasis. Therefore, it can be assumed that there is signficant cooperative interaction within the two types of flavonols and the four types of flavonol glycosides contained in OJE in reducing the major signaling mediators that cause progression of metastasis.

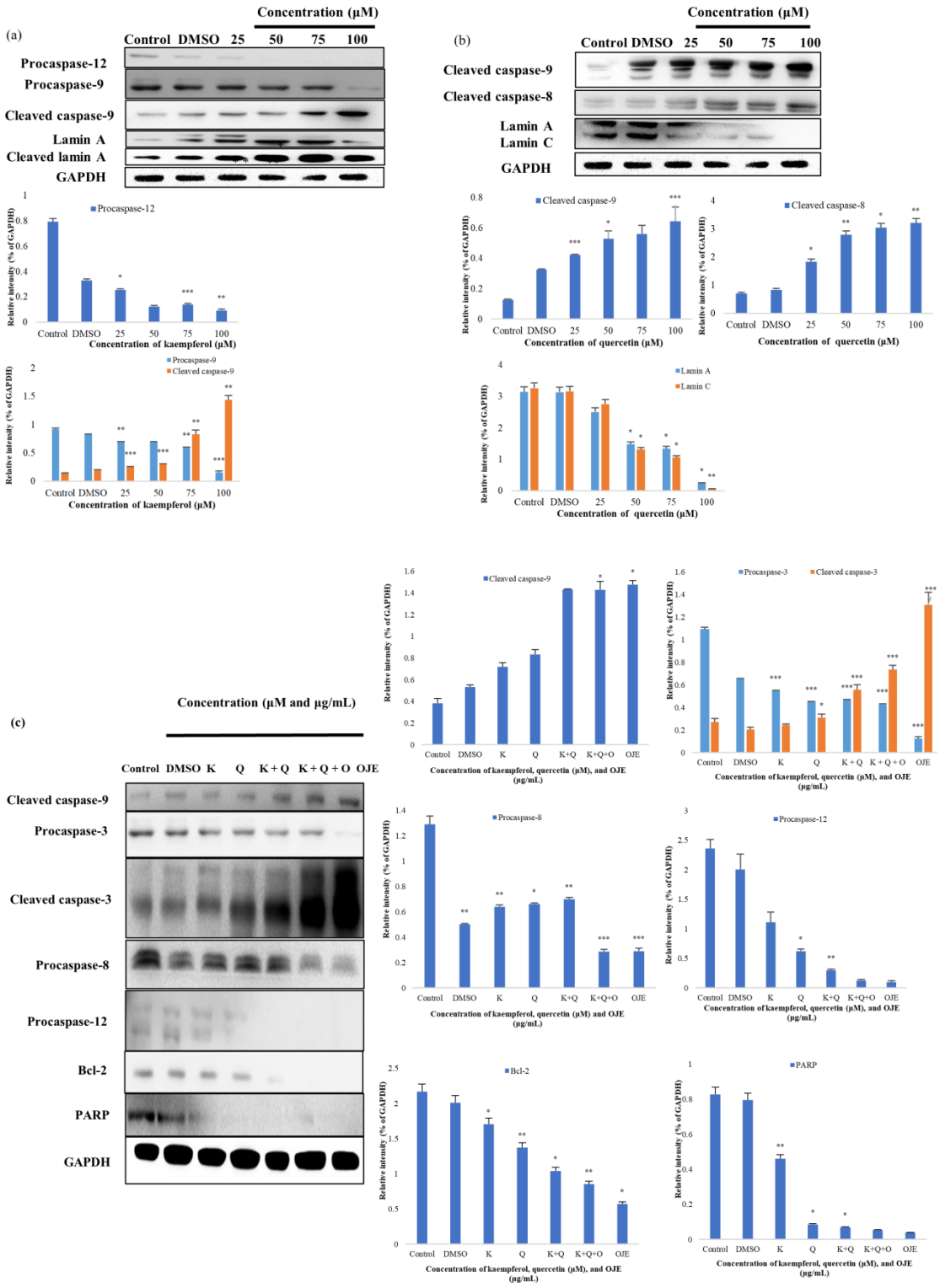

Figure 7. Western blot analysis of apoptosis-related key biomarkers produced or remained in HT-29 cells treated with kaempferol (a), quercetin (b), and single samples or combinations of kaempferol, quercetin, and/or OJE (c). The density of bands was quantitated. GAPDH was used as an internal control. Band intensities were measured by densitometry in three separate experiments with similar results

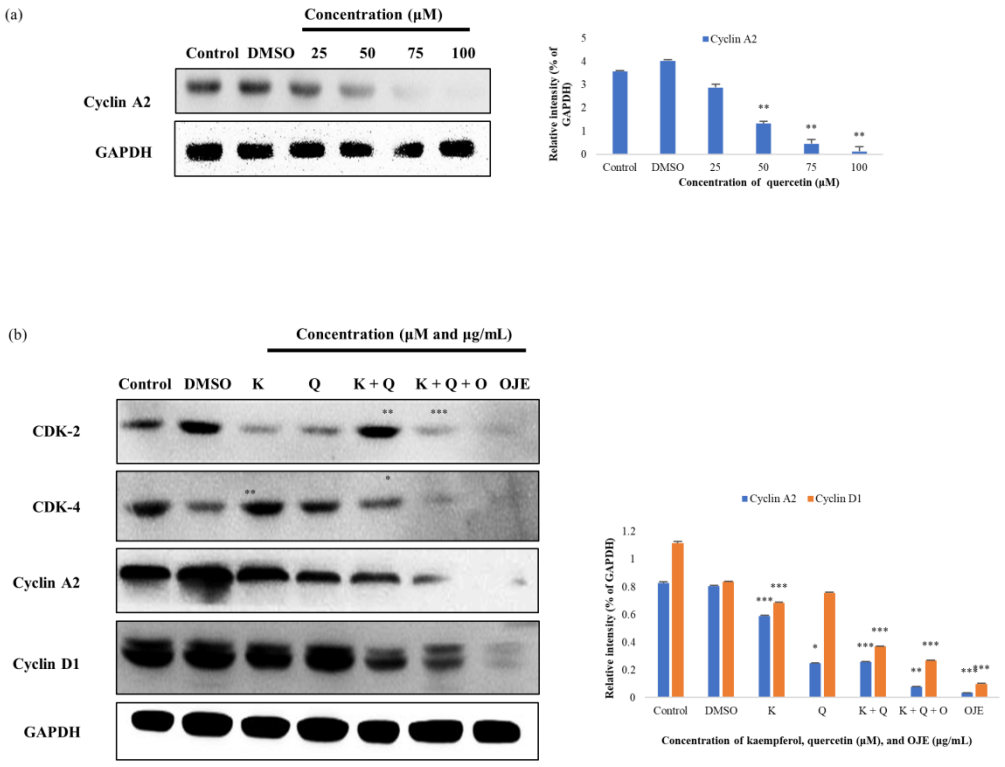

Figure 8. Western blot analysis of cell cycle arrest-related key biomarkers produced or remained in HT-29 cells treated with quercetin (a), and single samples or combinations of kaempferol, quercetin, and/or OJE (b). The density of bands was quantitated. GAPDH was used as an internal control. Band intensities were measured by densitometry in three separate experiments with similar results

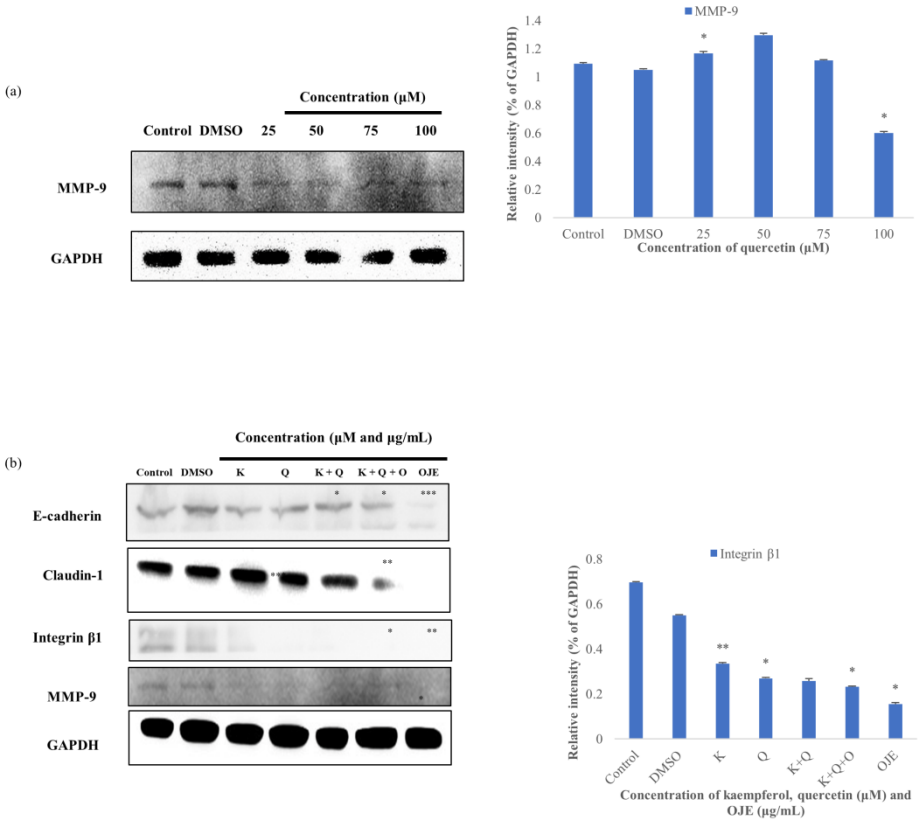

Figure 9. Western blot analysis of anti-metastasis-related key biomarkers produced or remained in HT-29 cells treated with quercetin (a), and single samples or combinations of kaempferol, quercetin, and/or OJE (b). The density of bands was quantitated. GAPDH was used as an internal control. Band intensities were measured by densitometry in three separate experiments with similar results

3.10. Effects of Kaempferol, Quercetin, and/or OJE on the Levels of Key Proteins Involved in the Activation of Upstream Signal Transduction Pathways Analyzed by Western Blot

As shown in Figure 10, key biomarkers related to the activation of upstream signal transduction pathways (p-ERK, p-JNK, and p-p38) significantly more increased in the presence of OJE (OJE and K + Q + O) than in the absence of OJE (K, Q, and K + Q). The kaempferol + quercetin mixture was superior to kaempferol alone or quercetin alone in phosphorylating ERK, JNK, and p38. Therefore, it can be assumed that there is significant cooperative interaction among the two types of flavonols and the four types of flavonol glycosides contained in OJE in increasing key signaling mediators, including p-ERK, p-JNK, and p-p38, inducing the activation of upstream signal transduction pathways. In addition, it can be speculated that there is cooperation between the two types of flavonols, kaempferol and quercetin, in enhancing signaling mediators leading to the activation of upstream signaling pathways [40].

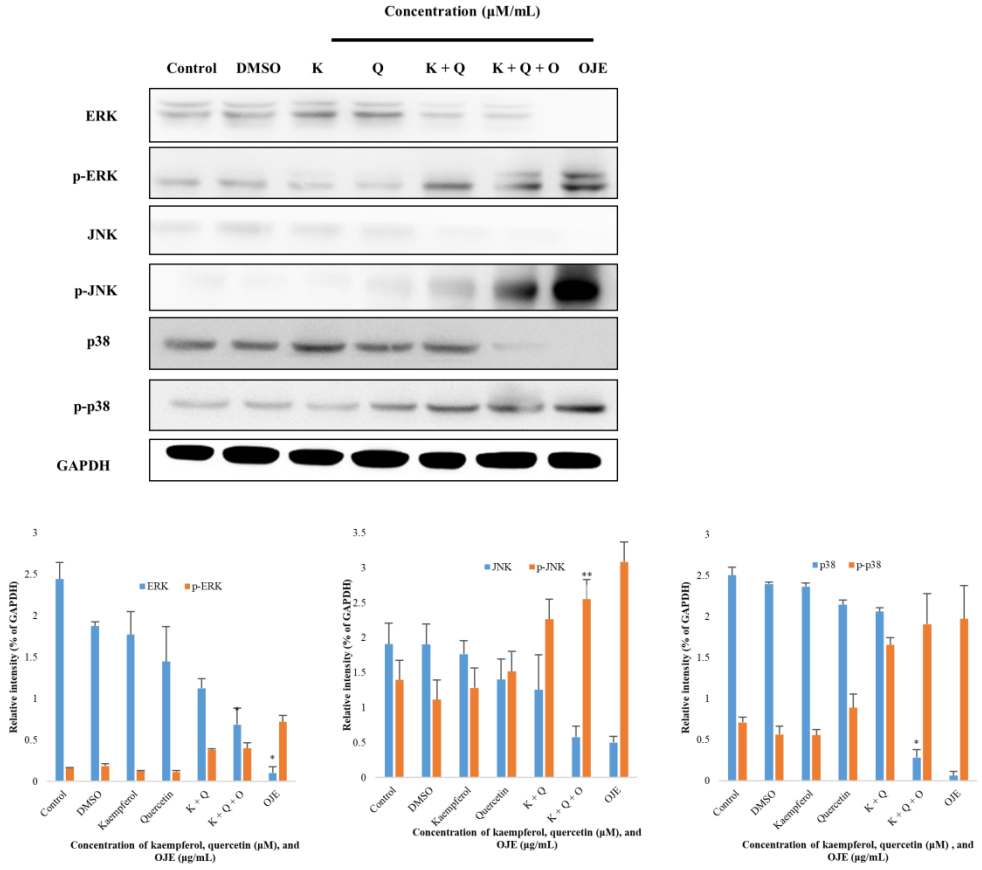

Figure 10. Western blot analysis of upstream signal transduction pathways-related key biomarkers produced or remained in HT-29 cells treated with single samples or combinations of kaempferol, quercetin, and/or OJE. The density of bands was quantitated. GAPDH was used as an internal control. Band intensities were measured by densitometry in three separate experiments with similar results

4. Discussion

Kaempferol and quercetin, the main components of *O. japonicus* are flavonols and are known to have anti-oxidant, anti-inflammatory, and anti-cancer effects [40]. In particular, though the researches on the anti-cancer activity of *O. japonicus* against some colons cancer continue to be conducted, but research on the roles and interactive effects of flavonols and flavonol glycosides, the major bioactive substances contained in *O. japonicus*, is lacking. In this study, OJE, a mixture of OJE enhanced with kaempferol and quercetin, a mixture of kaempferol and quercetin, and kaempferol or quercetin alone were treated to HT-29 human colon cancer cells, respectively, to investigate aspects of various anti-cancer activities, including degree of cytotoxicity to normal cells, degree of cytotoxicity to cancer cells, induction of apoptosis, induction of cell cycle arrest, induction of anti-metastasis, and activation of upstream signaling mediators. That is, the MTS assay was used to evaluate the cytotoxicity of RAW 264.7 macrophages and the survival rate of HT-29 human colon cancer cells. DAPI staining was used to examine apoptotic body formation and chromatin condensation by confocal microscope. After using Annexin V/PI staining, the apoptosis process was observed and analyzed using FACS, and after using PI staining, cell cycle arrest was confirmed using FACS. Key mediating factors involved in inducing apoptosis, cell cycle arrest, and anti-metastasis and activating upstream signaling pathways were systematically.

Among the five substances used in this study, those containing OJE (OJE, K + Q + O) exhibited better effects on the degree of cytotoxicity to normal cells, the degree of

cytotoxicity to cancer cells, the induction of apoptosis, cell cycle arrest, and anti-metastasis, and the activation of upstream MAPKs signal transduction pathways than those without OJE (K + Q, K, Q). In addition, the mixure of kaempferol and quercetin (K + Q) showed superior effects on the degree of cytotoxicity to normal cells, the degree of cytotoxicity to cancer cells, the induction of apoptosis, cell cycle arrest, and anti-metastasis, and the activation of upstream MAPKs signal transduction pathways compared to those with kaempferol alone or quercetin alone. These results suggest that there is a consistent and significant cooperative interaction between flavonols (kaempferol and quercetin) contained in OJE and furthermore, among flavonol glycosides (afzelin, astragalin, quercitrin, and isoquercitrin) and flavonols contained in OJE.

5. Conclusions

OJE, which contains flavonol glycosides (afzelin, astragalin, quercitrin, and isoquercitrin) as well as flavonols (kaempferol and quercetin), exhibits much higher anti-cancer activity in HT-29 colon cancer cells than either kaempferol alone or quercetin alone or in combination. The combination of kaempferol and quercetin shows superior anti-colon cancer activity compared to either kaempferol alone or quercetin alone. It is revealed that there is a consistent cooperative interaction between flavonols and more broadly among flavonol glycosides and flavonols contained in OJE in exerting effective and comprehensive anti-colon cancer activities including cytotoxicity against HT-29 cells, formation of apoptotic bodies, arresting cell cycles of HT-29 cells, anti-migration, and activation of various signal transduction mediators involved in inducing apoptosis, cell cycle arrest, anti-metastasis, and upstream signal transduction via various manners.

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