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Synthesis and Biological Evaluation of Novel C(6) Modified Baicalein Derivatives as Antioxidative Agents

JIN-YI WU,[†] KING-THOM CHUNG,[§] YI-WEN LIU,[†] FUNG-JOU LU,[#] RUEI-SHIUN TSAI,[†] CHI-HUNG CHEN,[‡] AND CHING-HSEIN CHEN*,[†]

Graduate Institute of Biomedical and Biopharmaceutical Sciences, College of Life Sciences, and Graduate Institute of Food Science and Biopharmaceutics, National Chiayi University, 300 University Road, Chiayi 60004, Taiwan; Department of Biology, University of Memphis, Memphis, Tennessee 38152; and Department of Applied Chemistry, Chung Shan Medical University, Taichung 402, Taiwan

Baicalein, one of the major flavones, was found to be responsible for the antioxidative activity of the traditional Chinese medicinal herb Huang-Qin (*Scutellaria baicalensis* Georgi), which is widely used as an antioxidative, anti-inflammatory, and antitumor agent. The hydroxyl group of the A ring of the baicalein was alkylated at position 6 with terpenoids such as prenyl, geranyl, and farnesyl groups, and their free radical scavenging activities and glutathione (GSH) depletion capacities were examined. Their free radical scavenging activity was measured according to the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) scavenging method. Baicalein and newly synthesized baicalein derivatives were found to be good free radical scavengers. Flow cytometrical method was employed to measure the intracellular antioxidative activity and GSH depletion capacity of these derivatives in human acute monocytic leukemia cell line (THP-1). It was also found that baicalein and its derivatives could decrease the levels of exogenous cumene hydroperoxide and H_2O_2 in THP-1 cells. These compounds also could significantly inhibit the intracellular GSH depletion induced by cumene hydroperoxide in THP-1 cells. The production of cumene hydroperoxide-induced *Bax*, a pro-apoptotic related protein, could also be inhibited by baicalein and its derivatives. These results suggested that baicalein and its derivatives could be beneficial to human health.

KEYWORDS: Baicalein; baicalein derivatives; antioxidative; reactive oxygen species; Bax

INTRODUCTION

Polyphenolic compounds, including a large group of flavonoids, are abundant in vegetables, fruits, tea, and Chinese traditional herbs. Baicalein (5,6,7-trihydroxyflavone), a flavonoid originating from the roots of *Scutellaria baicalensis* Georgi (Huang-Qin), is one of the most important medicinal herbs in traditional Chinese medicine (*1*). *S. baicalensis* Georgi contains three major polyphenolic components, that is, baicalein, oroxylin A, and wogonin. Baicalein is known as a selective inhibitor of 12-lipooxygenase, which is responsible for the production of reactive oxygen species (ROS) during arachidonic acid metabolism (*2*). Baicalein has been demonstrated to have a strong activity to scavenge superoxide radicals in cell-free systems (*3*). In the primary cultures of rat hepatocytes, baicalein can quench the cytotoxicity and genotoxicity of hepatocytes induced by *tert*-butyl hydroperoxide (*4*). Baicalein had also been reported to repress hydrogen peroxide-induced oxidative stress in human neuroblastoma HS-SY5Y cells (*5*). These polyphenols, including baicalein, may be important in preventing human oxidative stresses as they have been demonstrated to be capable of scavenging hydroxyl, 1,1-diphenyl-2-picrylhydrazyl (DPPH), and alkyl free radicals (*6*). These flavonoids, including baicalein, function as scavengers of free radicals by rapid donation of hydrogen atoms to radicals. Various methods including the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$) radical cation scavenging method have been used to estimate the antioxidative activities of baicalein derivatives in vitro (*7, 8*).

Terpenoid is an antioxidant with a molecular structure different from that of polyphenols. Several studies demonstrated the free radical (such as DPPH, superoxide, and lipid peroxide radicals) scavenging activity of terpenoid (*9*). Terpenoid could slow the oxidation of low-density lipid (LDL) (*10*). Atherogenesis may be caused by the combining actions of monocyte-derived macrophages and the oxidized LDL products (*11*). Terpenoids were also reported to be responsible for decreasing

* Author to whom correspondence should be addressed (telephone +886-5-2717837; fax +886-5-2717778; e-mail chench@mail.ncyu.edu.tw).

[†] Graduate Institute of Biomedical and Biopharmaceutical Sciences, National Chiayi University.

[§] University of Memphis.

[#] Chung Shan Medical University.

[‡] Graduate Institute of Food Science and Biopharmaceutics, National Chiayi University.

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β-amyloid-induced apoptosis in neuronal cells through their antioxidative properties (*12*). Some chemicals containing a terpenoid moiety (group) were reported to have antioxidative activities (*12*).

Many human diseases are related to the overproduction of ROS in living cells. ROS overproduction has been reported to induce lipid peroxidation, protein and enzyme oxidation, and DNA damage, which can lead to serious cellular damage such as mutations, death, and carcinogenesis (*13*). Moreover, aging, neurodegeneration, diabetes mellitus, hypertension, and inflammatory diseases were also reported to be directly related to ROS formation inside individual cells (*14*). Superoxide can be produced in mitochondrial electron transfer chain or during phagocytosis and can lead to other cellular reactions to produce H_2O_2, hydroxyl radical, and/or lipid peroxide. These ROS can cause intense cellular damage. Chemical compounds with the capacity of inhibiting the intracellular oxidative stresses caused by ROS were often considered to have potential in disease prevention.

Several studies have demonstrated that ROS generation by activated monocytes is especially important to exhibit leukocyte−endothelium interactions in the vascular dysfunction mechanism (*15*). Monocytes' differentiation into macrophages was possibly triggered by unquenched ROS, which might contribute to increased inflammatory response such as increased cycloxygenase-2 activity within atheromata (*16*). It had been reported that LDL oxidation might play a crucial role in early atherogenesis. The process of LDL oxidation appears to occur in all major cells within the arterial wall. Therefore, decreasing the intracellular oxidative stress by baicalein and its derivatives in monocytes may prevent the formation of monocyte-derived macrophages; atherogenesis can thus be prevented or minimized.

There are few studies illustrating that baicalein with a terpenoid moiety is able to increase ROS scavenging activity and thus decrease intracellular oxidative stresses. In this study, we synthesized and evaluated a few new baicalein derivatives that have a substitution at position 6 of the A ring with three terpenoid groups: prenyl, geranyl, and farnesyl. Our results indicated that baicalein derivatives with terpenoid groups on the A ring of the flavones would greatly increase the ROS scavenging activity and decrease the relative intracellular oxidative stresses in various ROS-treated human monocyte THP-1 cells.

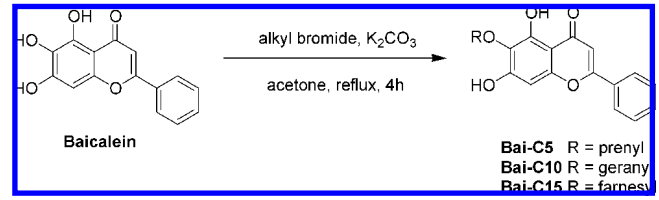
MATERIALS AND METHODS

Extraction and Isolation of Baicalein from *S. baicalensis* Georgi. Dried *S. baicalensis* roots were cut into small pieces, immersed, and extracted with a 10 times volume of acetone twice at room temperature for 2 weeks. Acetone extracts were concentrated and subjected to column chromatography on silica gel (7.5 cm i.d. × 30 cm) eluted with $CHCl_3$ and $CHCl_3$/MeOH (10:1) to yield two fractions. The $CHCl_3$/MeOH (10:1) eluent was subjected to chromatography on silica gel (3 cm i.d. × 20 cm) and eluted with CH_2Cl_2/acetone (9:1) to yield baicalein (2.30 g).

Baicalein (5,6,7-trihydroxyflavone). Baicalein was obtained as a yellow powder: mp 272−273 °C. [lit. (*17*) 268−271 °C]; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.62 (s, 1H, C5-OH), 10.63 (s, 1H, C6-OH), 8.83 (s, 1H, C7-OH), 8.03 (d, J = 7.2 Hz, 2H, H2′, H6′), 7.61−7.54 (m, 3H, H3′, H4′, H5′), 6.91 (s, 1H, H3), 6.62 (s, 1H, H8).

Chemistry. A series of 6-substituted baicalein derivatives were synthesized and evaluated by alkylation using alkyl bromide (including prenyl, geranyl, and farnesyl bromides) with anhydrous potassium carbonate in anhydrous acetone reflux for 4 h. The synthetic procedure and reaction conditions for all investigated compounds are shown in **Scheme 1**. The synthesized compounds were purified by flash silica gel column chromatography and the structures based on ^{1}H and ^{13}C NMR. Purity tests of baicalein derivatives were performed by HPLC equipped with a 280 nm detector and a LiChroCART RP-18e column (4.6 mm i.d. × 250 mm). The mobile phase was composed of MeOH/H_2O (0.05% TFA) (90:10), and the flow rate was 1.0 mL/min. The purity of all compounds was >98%.

Scheme 1. Synthesis of Alkoxy Derivatives of Baicalein



Bai-C5 (5,6-Dihydroxy-7-prenoxyflavone). Bai-C5 was obtained as a yellow needle crystal: mp 164−165 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.47 (s, 1H, C5-OH), 8.69 (s, 1H, C7-OH), 8.06 (dd, J = 8.0, 1.2 Hz, 2H, H2′, H6′), 7.59−7.54 (m, 3H, H3′, H4′, H5′), 6.96 (s, 1H, H3), 6.95 (s, 1H, H8), 5.50 (dd, J = 8.0, 6.4 Hz, 1H), 4.67 (d, J = 6.4 Hz, 2H), 1.77 (s, 3H, CH_3), 1.74 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.97, 162.84, 153.55, 149.47, 145.89, 137.57, 131.76, 130.66, 130.05, 128.94, 126.13, 119.11, 105.06, 104.54, 92.07, 65.72, 25.54, 18.18.

Bai-C10 (5,6-Dihydroxy-7-geranoxyflavone). Bai-C10 was obtained as a yellow powder: mp 121−124 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.47 (s, 1H, C5-OH), 8.70 (s, 1H, C7-OH), 8.06 (dd, J = 8.0, 1.2 Hz, 2H, H2′, H6′), 7.59−7.54 (m, 3H, H3′, H4′, H5′), 6.97 (s, 1H, H3), 6.94 (s, 1H, H8), 5.49 (t, J = 5.6 Hz, 1H), 5.05 (m, 1H), 4.70 (d, J = 6.4 Hz, 2H), 2.07 (m, 4H), 1.77 (s, 3H, CH_3), 1.64 (s, 3H, CH_3), 1.56 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 181.97, 162.84, 153.48, 149.44, 145.88, 140.49, 131.77, 130.86, 130.66, 130.10, 128.94, 126.12, 123.54, 118.95, 105.07, 104.54, 93.74, 92.17, 65.79, 25.78, 25.46, 17.63, 16.51.

Bai-C15 (5,6-Dihydroxy-7-farnesoxyflavone). Bai-C15 was obtained as a yellow powder: mp 103−104 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.47 (s, 1H, C5-OH), 8.71 (s, 1H, C7-OH), 8.05 (dd, J = 8.0, 1.6 Hz, 2H, H2′, H6′), 7.60−7.54 (m, 3H, H3′, H4′, H5′), 6.96 (s, 1H, H3), 6.92 (s, 1H, H8), 5.48 (t, J = 6.2 Hz, 1H), 5.05 (t, J = 6.2 Hz, 1H), 4.96 (t, J = 6.8 Hz, 1H), 4.71 (d, J = 6.4 Hz, 2H), 2.06 (m, 4H), 1.93 (m, 2H), 1.85 (m, 2H), 1.75 (s, 3H, CH_3), 1.61 (s, 3H, CH_3), 1.56 (s, 3H, CH_3), 1.49 (s, 3H, CH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ 182.00, 162.86, 153.46, 149.45, 145.90, 140.52, 134.53, 131.79, 130.68, 130.47, 130.14, 128.97, 126.12, 123.87, 123.32, 119.04, 105.09, 104.54, 92.15, 65.76, 26.20, 25.66, 25.54, 17.59, 16.56, 15.89.

ABTS$^{\bullet+}$ Decoloration Assay. The antioxidative activity was measured by the ability of hydrogen-donating antioxidants to scavenge the ABTS$^{\bullet+}$ radical cation using a previously reported method (*18*). The ABTS$^{\bullet+}$ radical cation was prepared by mixing a 7 mM ABTS stock solution with 2.45 mM potassium persulfate (1:1, v/v) and leaving the mixture for 12–16 h until the reaction was complete and absorbance was stable. The ABTS$^{\bullet+}$ solution was diluted in phosphate buffer (PBS) to an absorbance of 0.70 ± 0.02 at 734 nm for measurement. The photometric assay was conducted in 1.9 mL of the ABTS$^{\bullet+}$ solution and 0.1 mL of baicalein and its derivatives dissolved in an ethanol solution and mixed for 6 min. Measurements were taken immediately at 734 nm. The percentage inhibition (expressed as percent decrease of absorbance at 734 nm) is calculated as a function of concentration of baicalein derivatives and of Trolox for the standard reference data. All experiments were carried out in triplicates. The IC_{50} value was defined as the concentration that causes a 50% decrease of ABTS$^{\bullet+}$ radical cation.

Cell Line and Reagents. The human acute monocytic leukemia cell line THP-1 was obtained from the American type Culture Collection (Manassa, VA). Hydroethidine (HE) and chloromethylflourescein diacetate (CMF-DA) were acquired from Molecular Probes (Eugene, OR). Propidium iodide (PI), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), RPMI-1640 medium, menadione, H_2O_2, cumene hydroperoxide, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Treatment. The basal medium for the THP-1 cell line was cultured in RPMI-1640 medium supplemented with 10%

fetal bovine serum (FBS), 100 units/mL penicillin G, and 100 μg/mL streptomycin. The stock solution of baicalein and its derivatives was dissolved in DMSO, and different concentrations (micromolar) were prepared in the RPMI-1640 basal medium. The final DMSO concentration was 0.1%.

Analysis of Added Cumene Hydroperoxide Scavenging Activity of Baicalein and Its Derivatives in THP-1 Cells. THP-1 (10^6 cells/mL) cells were incubated with 20 μM DCFH-DA in the 5% CO_2 incubator for 10 min, then treated with 5, 10, and 20 μM baicalein and its derivatives for 10 min at 37 °C in a CO_2 incubator, and then treated with 20 μM cumene hydroperoxide for another 20 min. After treatment of cumene hydroperoxide, the THP-1 cells were washed with PBS buffer, collected by centrifugation, and suspended in the same buffer, and the level of DCF fluorescence was analyzed by flow cytometry.

Analysis of the Intracellular H_2O_2 Scavenging Activity of Baicalein and Its Derivatives in THP-1 Cells. THP-1 (10^6 cells/mL) cells were incubated with 20 μM DCFH-DA in the 5% CO_2 incubator for 10 min, then treated with 5, 10, and 20 μM baicalein and baicalein's derivatives for 10 min at 37 °C in a CO_2 incubator, and then treated with 1600 μM H_2O_2 for another 30 min. After the H_2O_2 treatment, the THP-1 cells were washed with PBS buffer, collected by centrifugation, and suspended in the same buffer, and the DCF fluorescence was analyzed by flow cytometry.

Analysis of Intracellular Superoxide Scavenging Activity of Baicalein and Its Derivatives in THP-1 Cells. THP-1 (10^6 cells/mL) cells were incubated with 15 μM HE in the 5% CO_2 incubator for 15 min, then treated with 5, 10, and 20 μM baicalein and baicalein's derivatives for 15 min at 37 °C in a CO_2 incubator, and then treated with 30 μM menadione for another 30 min. The intracellular NAD(P)H oxidase could generate superoxide from menadione. After the menadione treatment, the THP-1 cells were washed with PBS buffer, collected by centrifugation, and suspended in the same buffer, and the HE fluorescence was analyzed by flow cytometry.

Flow Cytometry Analysis of GSH Content in THP-1 Cells. THP-1 (10^6 cells/mL) cells were incubated with 10 μM baicalein and baicalein's derivatives for 1 h at 37 °C in a CO_2 incubator and then treated with 500 μM cumene hydroperoxide for another 30 min. After cumene hydroperoxide treatment, the THP-1 cells were incubated with 25 μM CMF-DA for 20 min at 37 °C in a CO_2 incubator. After CMF-DA staining, THP-1 cells were washed with PBS buffer, collected by centrifugation, and suspended in the PBS buffer, and the CMF fluorescence was analyzed by flow cytometry.

Measurement of Bcl-2, Bax, and Heme Oxygenase-1 (HO-1) by Western Blotting. THP-1 (10^6 cells/mL) cells were cultured in 100 mm tissue culture dishes for 24 h. The culture medium was replaced with new medium and then exposed to 10 μM baicalein and its derivatives separately for 1 h at 37 °C in the 5% CO_2 incubator and then treated with 500 μM cumene hydroperoxide for another 3 h. After treatment, cells were washed with PBS, resuspended in protein-extracted buffer for 10 min, and then centrifuged at 12000*g* for 10 min at 4 °C to obtain total extracted proteins (supernatant). The protein concentrations were measured with a commercial kit (Bio-Rad, Richmond, CA). The *Bax*, *Bcl-2*, HO-1, and actin expression was evaluated by Western blotting analysis. Briefly, the total extracted proteins were boiled in loading buffer, and an aliquot corresponding to 50 μg of protein was separated by SDS-PAGE. After blotting, the membranes were incubated with anti-Bax, anti-Bcl-2, anti-HO-1, and anti-actin antibodies (Laboratory Vision, Santa Cruz, CA) overnight and then washed with PBST solution (0.05% Tween 20 in PBS). Following washing, the second antibody labeled with horseradish peroxidase was adjacently incubated for 1 h and then washed with PBST solution (0.05% Tween 20 in PBS). The antigen–antibody complexes were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) with a chemiluminescence analyzer.

Statistical Analysis. Data are presented as means ± standard deviation from at least three independent experiments and analyzed using Student's *t* test. A *P* value of <0.05 was considered to be statistically significant (*19*).

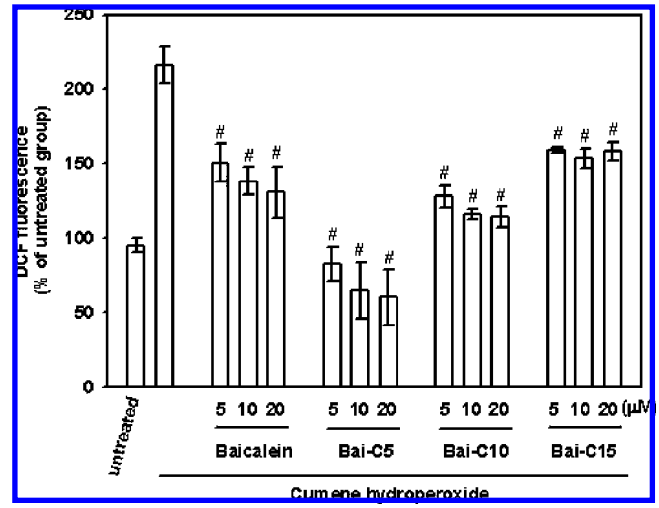


Figure 1. Scavenging activities of baicalein and its derivatives on added cumene hydroperoxide in THP-1 cells. THP-1 cells (10^6 cells/mL) were incubated with 20 μM DCFH-DA in the 5% CO_2 incubator for 10 min, then treated with 5, 10, and 20 μM baicalein and baicalein derivatives for 10 min at 37 °C in the 5% CO_2 incubator, and then treated with 20 μM cumene hydroperoxide for another 20 min. Data represent the fluorescence intensity within the THP-1 cells. Values shown are mean ± standard deviation (n = 3–8 of individual experiments). #, statistically significant, $p < 0.05$ to untreated group.

RESULTS AND DISCUSSION

Intracellular ROS Scavenging Activity of Baicalein and Its Derivatives. To evaluate the intracellular ROS scavenging activity of baicalein and its derivatives, three exogenous ROS sources were applied in the experiments. There were cumene hydroperoxide, H_2O_2, and menadione (an intracellular superoxide inducer). Two ROS detecting probes, DCFH-DA for cumene peroxide and H_2O_2 and HE for superoxide, were used in this study. As shown in **Figure 1**, 20 μM cumene hydroperoxide induced about a 2.2-fold increase of the intracellular DCF fluorescence in THP-1 cells as compared with the untreated group. Baicalein at 5, 10, and 20 μM concentrations could decrease the extent of the fluorescence of DCF to about 1.35−1.5-fold of the untreated group. It is of interest to note that Bai-C5 expressed an appreciable cumene hydroperoxide scavenging activity; it decreased the DCF fluorescence to less than that of the untreated group. The cumene hydroperoxide scavenging activity of Bai-C10 was slightly stronger than that of baicalein; it exhibited about 1.25−1.30-fold of the untreated DCF fluorescence. Bai-C15 displayed the weakest cumene hydroperoxide scavenging activity; the means of DCF fluorescence in three concentrations of Bai-C15 were larger than those of baicalein-treated groups. The order of intracellular cumene hydroperoxide scavenging activity is Bai-C5 > Bai-C10 > baicalein > Bai-C15.

Exogenous H_2O_2 could penetrate into the cellular membrane to reach the cytosolic or nuclear region and damage directly the intracellular biomolecules. The intracellular H_2O_2 scavenging activities of baicalein derivatives in THP-1 cells were then evaluated with a DCFH-DA probe. As shown in **Figure 2**, baicalein at the three concentrations tested could also depress the intracellular DCF fluorescence from 2.1- to 1.25−1.35-fold of the untreated group. The H_2O_2 scavenging activity of 5 μM Bai-C5 is similar to that of 5 μM baicalein. Bai-C5 at 10 and 20 μM appeared to have a stronger intracellular H_2O_2 scavenging activity than the untreated group, whereas the intracellular DCF fluorescence were less than that of the untreated group. The

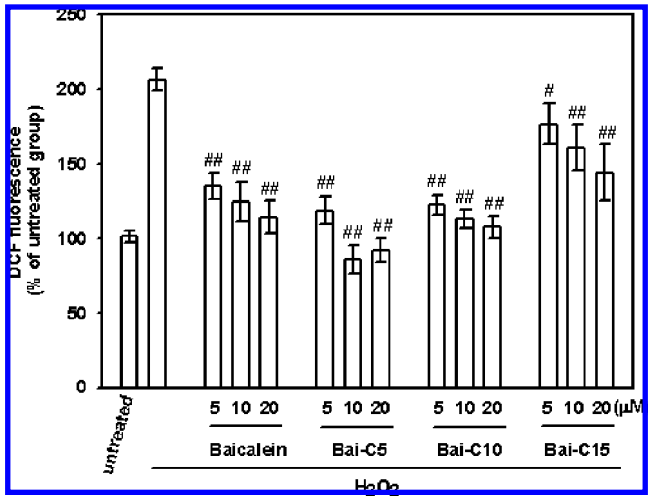


Figure 2. Scavenging activities of baicalein and its derivatives on added H_2O_2 in THP-1 cells. THP-1 cells (10^6 cells/mL) were incubated with 20 μM DCFH-DA in the 5% CO_2 incubator for 10 min, then treated with 5, 10, and 20 μM baicalein and its derivatives for 10 min at 37 °C in the 5% CO_2 incubator, and then treated with 1600 μM H_2O_2 for another 30 min. Data represent the fluorescence intensity within the THP-1 cells. Values shown are mean ± standard deviation (n = 3–8 of individual experiments). # and ##, statistically significant, $p < 0.05$ and $p < 0.01$ to untreated group, respectively.

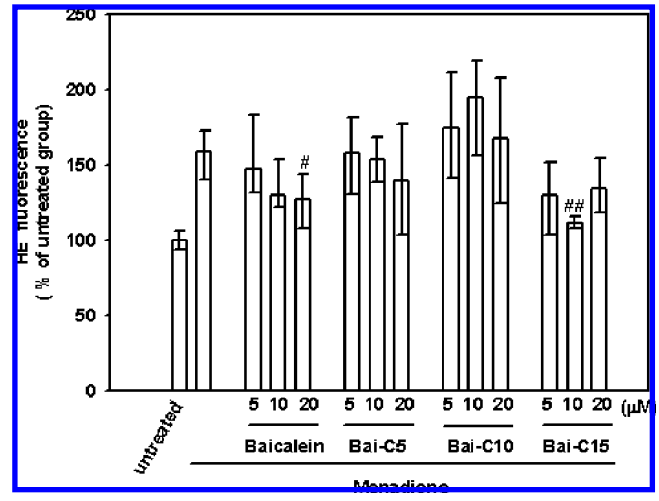


Figure 3. Scavenging activities of baicalein and its derivatives on intracellular superoxide in THP-1 cells. THP-1 (10^6 cells/mL) cells were incubated with 15 μM hydroethidine in the 5% CO_2 incubator for 15 min, then treated with 5, 10, and 20 μM baicalein and its derivatives for 15 min at 37 °C in the 5% CO_2 incubator, and then treated with 30 μM menadione for another 30 min. Data represent the fluorescence intensity within the THP-1 cells. Values shown are mean ± standard deviation (n = 3–8 of individual experiments). # and ##, dtatistically significant, $p < 0.05$ and $p < 0.01$ to untreated group, respectively.

H_2O_2 scavenging activity of Bai-C10 is similar to that of baicalein; it expressed 1.15−1.20-fold of the untreated DCF fluorescence. Although Bai-C15 could decrease the H_2O_2-induced intracellular DCF fluorescence in THP-1 cells, the H_2O_2 scavenging activity of Bai-C15 was still weaker than that of the baicalein. The order of intracellular H_2O_2 scavenging activity is Bai-C5 > Bai-C10 > baicalein > Bai-C15.

Superoxide is the first ROS production from mitochondrial electronic transfer chain. Superoxide overproduction can damage mitochondrial DNA and other intracellular biomolecules. To evaluate the intracellular superoxide scavenging activity of baicalein derivatives, menadione was used as an intracellular superoxide inducer, and hydroethidine was selected to analyze the intracellular superoxide in THP-1 cells. As shown in **Figure 3**, the intracellular superoxide scavenging activity of baicalein derivatives appeared irregularly. Only 20 μM baicalein and 10 μM Bai-C15 exhibited slight superoxide scavenging activity. All other concentrations of baicalein and its derivatives did not inhibit the HE fluorescence to less than that of the untreated group. These results indicated that the intracellular superoxide scavenging activity of these derivatives was negligible.

Effect of Baicalein Derivatives in Cumene Hydroperoxide-Induced Glutathione Depletion. Figures 1−3 indicate that baicalein derivatives exhibited good cumene hydroperoxide scavenging activity in THP-1 cells. We further evaluated the effect of baicalein derivatives on cumene peroxide-induced glutathione depletion. Glutathione is an important antioxidative tripeptide; it can cause the intracellular ROS levels to decrease. Glutathione also plays a detoxifying role to some toxic substances in cells. Once intracellular glutathione was depleted, the cells would proceed to die. CMF-DA was used as an intracellular glutathione probe, and a flow cytometrical method was employed; the intracellular glutathione depletion can be expressed in percentage of GSH-negative cells. In **Figure 1**, we first screened the intracellular cumene hydroperoxide scavenging activities among baicalein and its derivatives. The intracellular DCF fluorescence induced by cumene hydroperoxide was set for 2-fold of untreated cells at 1 h of treatment. Under the experimental condition, the intracellular DCF fluorescence was about 210 in the 20 μM cumene hydroperoxide treatment as compared with the 100 intracellular DCF fluorescence in untreated cells. Originally, we expected that GSH depletion could be induced by 20 μM cumene hydroperoxide at 1 h of treatment. Unfortunately, the intracellular GSH depletion did not markedly increase by 20 μM cumene hydroperoxide at 1 h of treatment. These results led us to reexamine the appropriate concentration and time of cumene hydroperoxide treatment in intracellular GSH depletion. As shown in **Figure 4**, 20 μM cumene hydroperoxide did not induce obvious GSH depletion at 3 h of treatment. The percentage of GSH-negative cells could be obviously increased to 40.8% in 500 μM cumene hydroperoxide treatment at 3 h. The 500 μM cumene hydroperoxide and 3 h treatment were used to induce intracellular GSH depletion. In **Figure 5A**, 500 μM cumene hydroperoxide induced 41.1% of GSH-negative cells. In cells treated with baicalein and its derivatives, the percentages of GSH-negative cells were <30%. Baicalein and its derivatives could significantly decrease the cumene hydroperoxide-induced glutathione depletion as shown in **Figure 5B**. There were no significant differences in the percentages of GSH-negative cells among all cells treated with either baicalein derivatives or baicalein.

Effect of Baicalein Derivatives on Cumene Hydroperoxide-Induced Oxidative Protein Expression. Overproduction of intracellular ROS could cause various cellular damages including apoptosis. *Bcl-2* and *Bax* are two important proteins that regulate the apoptotic process. *Bcl-2* is an anti-apoptotic protein, and its overexpression would prevent apoptosis. *Bcl-2* can also neutralize the harmful effects of cellular damage induced by free radicals, thereby gaining its well-known property of being an antioxidant (*20*). *Bax* is a pro-apoptotic protein (*21*). ROS could regulate the *Bax* expression to trigger apoptosis (*22*). We further evaluated *Bcl-2* and *Bax* expressions in cells pretreated with baicalein and its derivatives after cumene hydroperoxide treatment. In protein expressions, the 20 μM cumene hydroperoxide at 1 h of treatment also could not induce a marked difference as compared with the untreated cells. To

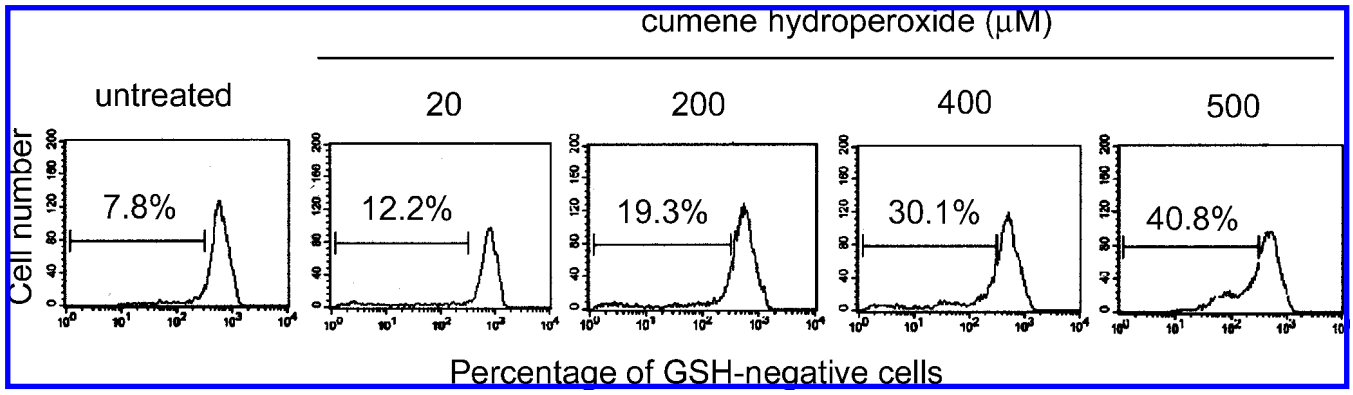


Figure 4. Cumene hydroperoxide-induced GSH depletion in THP-1 cells. THP-1 (10^6 cells/mL) cells were incubated with 0 (untreated), 20, 200, 400, and 500 μM cumene hydroperoxide for 3 h at 37 °C in the 5% CO_2 incubator. After treatment, the THP-1 cells were incubated with 25 μM CMF-DA for 20 min at 37 °C in a CO_2 incubator. The CMF fluorescence was analyzed by flow cytometry. Data in each panel represent the percentages of GSH-negative cells.

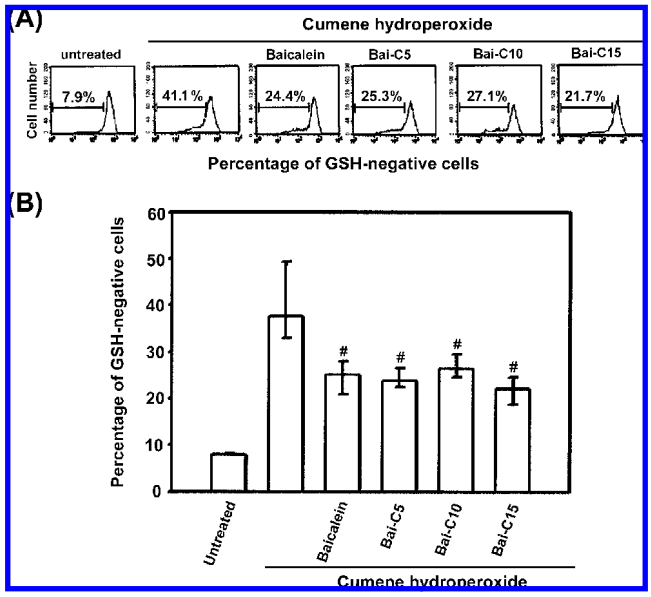


Figure 5. Effect of baicalein and its derivatives on cumene hydroperoxide-induced GSH depletion in THP-1 cells. THP-1 (10^6 cells/mL) cells were incubated with 10 μM baicalein and baicalein derivatives for 1 h at 37 °C in the 5% CO_2 incubator and then treated with 500 μM cumene hydroperoxide for another 3 h. After drug treatment, the THP-1 cells were incubated with 25 μM CMF-DA for 20 min at 37 °C in a CO_2 incubator. (**A**) Data in each panel represent the percentages of GSH-negative cells. (**B**) Data represent the percentages of GSH-negative cells. Values shown are mean ± standard deviation (n = 3–8 of individual experiments). #, statistically significant, $p < 0.05$ to untreated group.

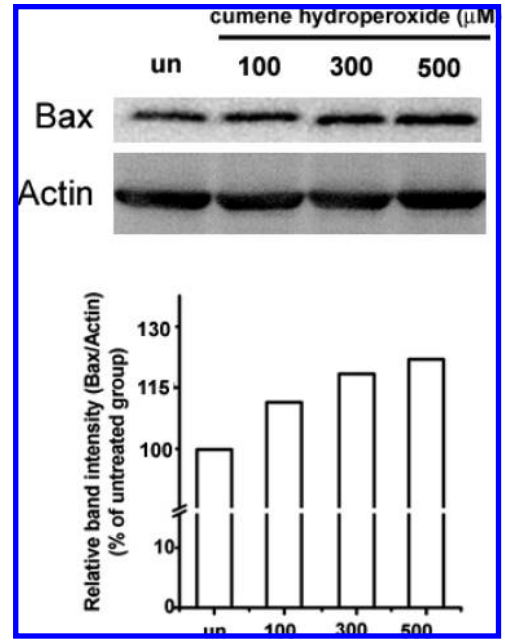


Figure 6. Cumene hydroperoxide-induced *Bax* expression in THP-1 cells. THP-1 (5×10^6 cells/mL) cells were incubated with 0 (untreated), 100, 300, and 500 μM cumene hydroperoxide for 3 h at 37 °C in the 5% CO_2 incubator. After treatment, the THP-1 cells were resuspended in protein-extracted buffer to obtain total extracted proteins. Fifty micrograms of protein was separated by SDS-PAGE. The expression of *Bax* and actin was examined by Western blotting. A densitometer was used to quantify *Bax* expression. Data are normalized for actin expression.

induce the obvious protein expression, the concentrations of cumene hydroperoxide were increased to 100, 300, and 500 μM and the time of treatment were prolonged to 3 h. As shown in **Figure 6**, *Bax* expression exhibited a concentration-dependent increase at 3 h of treatment. These results led us to select the 500 μM cumene hydroperoxide and 3 h treatment to evaluate the related protein expressions. As shown in **Figure 7A**, both *Bcl-2* and *Bax* expressions were increased at 3 h after 500 μM cumene hydroperoxide treatment. In baicalein-treated cells, *Bcl-2* expression was equal to that of cumene hydroperoxide-treated cells. However, *Bcl-2* expression was only slightly decreased in Bai-C5-treated cells but moderately decreased in Bai-C10- and Bai-C15-treated cells (**Figure 7B**). In **Figure 7C**, 500 μM cumene hydroperoxide also increased *Bax* expression after 3 h of treatment. *Bax* expression was slightly inhibited in baicalein-pretreated cells. Three baicalein derivatives caused more severe inhibitory effects in cumene hydroperoxide-induced *Bax* expression as compared with that of baicalein treatment alone. Heme oxygenase-1 (HO-1) is an oxidative stress defense enzyme, which could be induced by reactive oxygen species. In **Figure 8**, 500 μM cumene hydroperoxide could increase HO-1 expression. HO-1 expression could be slightly decreased by baicalein and Bai-C5 treatment. In Bai-C10- and Bai-C15-pretreated cells, HO-1 expression was dramatically decreased after 3 h of cumene hydroperoxide treatment.

Scavenging Activity of $ABTS^{\bullet+}$ by Baicalein and Its Derivatives and Trolox. The ROS scavenging activities of baicalein and its derivatives were evaluated using a cell-free system. Generation of the $ABTS^{\bullet+}$ radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the antioxidant activity of solution of

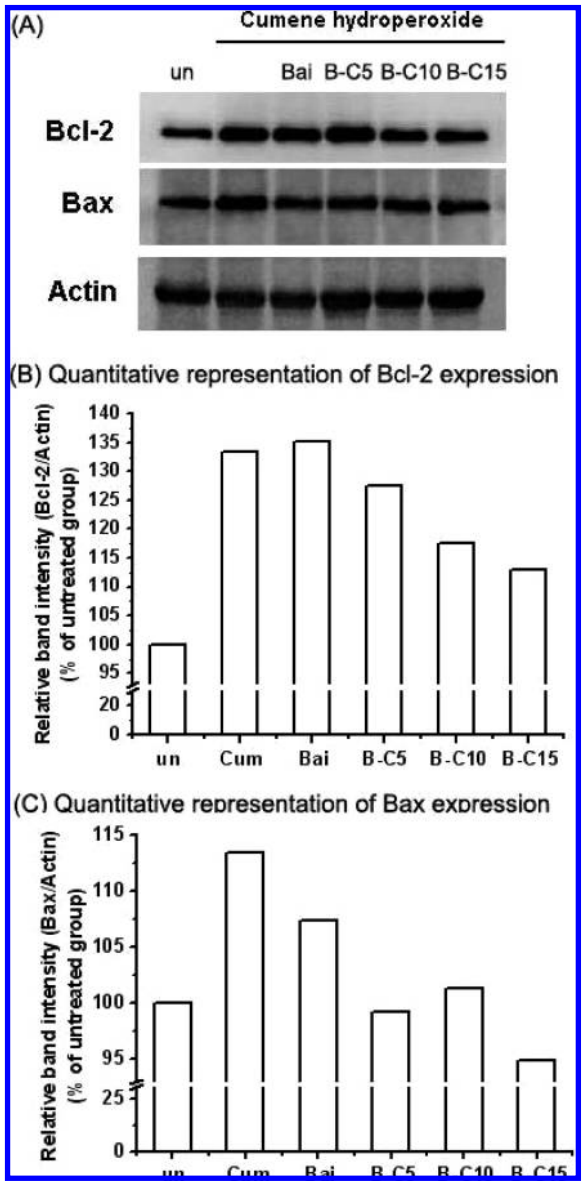


Figure 7. Effect of baicalein and its derivatives on *Bcl-2* and *Bax* expression in cumene hydroperoxide-treated THP-1 cells. THP-1 (5×10^6 cells/mL) cells were incubated with 10 μM baicalein and baicalein derivatives for 1 h at 37 °C in the 5% CO_2 incubator and then treated with 500 μM cumene hydroperoxide for another 3 h. After drug treatment, the THP-1 cells were resuspended in protein-extracted buffer to obtain total extracted proteins. Fifty micrograms of protein was separated by SDS-PAGE. (**A**) The expression of *Bcl-2*, *Bax*, and actin was examined by Western blotting. A densitometer was used to quantify (**B**) *Bcl-2* and (**C**) *Bax* expression. Data are normalized for actin expression.

pure substances and aqueous mixtures in vitro (*23*). The original $ABTS^{\bullet+}$ assay was based on the activation of ABTS with potassium persulfate to form the $ABTS^{\bullet+}$ radical cation in aqueous condition. The antioxidant activity reflects the ability of hydrogen-donating antioxidant to reduce the $ABTS^{\bullet+}$ radical cation, compared with that of Trolox, a water-soluble vitamin E analogue. Among the synthesized 6-position-substituted baicalein derivatives, the $ABTS^{\bullet+}$ scavenging activity of baicalein showed a slightly higher ROS scavenging activity than that of the three baicalein derivative compounds as shown in **Table 1**. The IC_{50} values of $ABTS^{\bullet+}$ scavenging activities of baicalein derivatives and baicalein were smaller than that of Trolox.

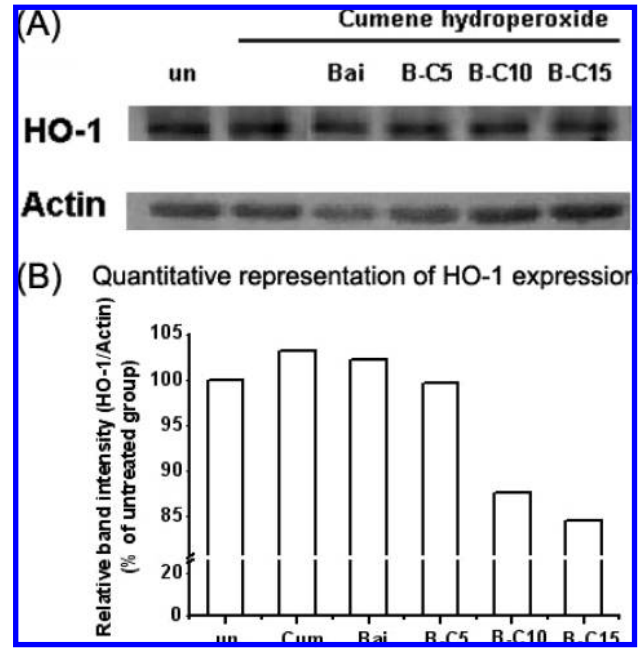


Figure 8. Effect of baicalein and its derivatives on HO-1 expression in cumene hydroperoxide-treated THP-1 cells. THP-1 (5×10^6 cells/mL) cells were incubated with 10 μM baicalein and baicalein derivatives for 1 h at 37 °C in the 5% CO_2 incubator and then treated with 500 μM cumene hydroperoxide for another 3 h. After the treatment, the THP-1 cells were resuspended in protein-extracted buffer to obtain total extracted proteins. Fifty micrograms of protein was separated by SDS-PAGE. (**A**) The expression of HO-1 and actin was examined by Western blotting. (**B**) A densitometer was used to quantitatively represent the HO-1 expression. Data are normalized for actin expression.

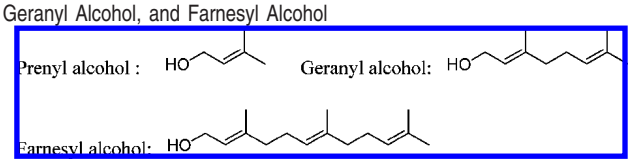
Table 1. Free Radical Scavenging Activity of $ABTS^{\bullet+}$ by Baicalein, Its Derivatives, and Trolox

flavone	IC_{50}[a] (μM)/$ABTS^{\bullet+}$
baicalein	5.5 ± 0.40
Bai-C5	8.8 ± 0.11
Bai-C10	8.7 ± 0.28
Bai-C15	10.6 ± 0.50
Trolox	12.6 ± 0.21

[a] IC_{50} values were calculated from the concentration at which 50% $ABTS^{\bullet+}$ free radicals were scavenged by the compounds ($n = 3$ or 4) tested. Trolox was used as a reference antioxidant.

In this cell-free system, the $ABTS^{\bullet+}$ scavenging activity might be determined by the number of hydroxyl groups in phenolic compounds. To view the chemical structure of baicalein, there are three hydroxyl groups in the phenolic structure. It might let baicalein express the best $ABTS^{\bullet+}$ scavenging activity as compared with its derivatives. In three baicalein derivatives, there are only two hydroxyl groups present in the phenolic structure. When one hydroxyl group on C(6) of baicalein was lost and substituted by a terpenoid group on C(6) of baicalein, the $ABTS^{\bullet+}$ scavenging activity was weakened. The results demonstrated that the order of the $ABTS^{\bullet+}$ scavenging activity of baicalein derivatives is Bai-C5 $\approx$ Bai-C10 $>$ Bai-C15. Of these baicalein derivatives, Bai-C15 expressed the weakest $ABTS^{\bullet+}$ scavenging activity. Bai-C15 contains a large farnesyl group on C(6) with a lower water solubility as compared with Bai-C5 and Bai-C10. Because the $ABTS^{\bullet+}$ is a water-soluble radical cation, the low water solubility of Bai-C15 might decrease the $ABTS^{\bullet+}$ scavenging activity. Only one hydroxyl group is present in the phenolic structure of Trolox. The $ABTS^{\bullet+}$ scavenging activity of Trolox was the weakest.

Table 2. Free Radical Scavenging Activity of $ABTS^{\bullet+}$ by Prenyl Alcohol, Geranyl Alcohol, and Farnesyl Alcohol



compound	IC_{50}[a] (μM)/$ABTS^{\bullet+}$
prenyl alcohol ($C_5H_{10}O$)	>200
geranyl alcohol ($C_{10}H_{18}O$)	>200
farnesyl alcohol ($C_{15}H_{26}O$)	>200

[a] IC_{50} values were calculated from the concentration at which 50% $ABTS^{\bullet+}$ free radicals were scavenged by the compounds ($n = 3$ or 4) tested.

To clarify the difference of scavenging activities among prenyl, geranyl, and farnesyl bromides, their radical scavenging activities were evaluated by $ABTS^{\bullet+}$ decoloration assay. Prenyl, geranyl, and farnesyl bromide can form prenyl, geranyl, and farnesyl alcohol in aqueous condition, because bromide is a good leaving group in organic synthesis. Therefore, we measured the radical scavenging activity of prenyl, geranyl, and farnesyl alcohol to prove that there were no effects of scavenging activity due to the length of carbohydrate of the terpenoid moiety. As shown in **Table 2**, the IC_{50} of prenyl, geranyl, and farnesyl alcohol on $ABTS^{\bullet+}$ scavenging activity was larger than 200 μM. There were no differences of scavenging activities between prenyl, geranyl, and farnesyl alcohol. These results explain the difference of intracellular scavenging activities against cumene hydroperoxide or H_2O_2 among baicalein, Bai-C5, Bai-C10, and Bai-C15 as perhaps being due to the lengths of carbohydrate of the terpenoid. The length of carbohydrate of the terpenoid in Bai-C10 and Bai-C15 is longer than that of Bai-C5 and may delay the penetration rates of Bai-C10 and Bai-C15 into the intracellular space.

The A ring on baicalein is often chemically modified to produce new drugs. For example, alkylation of baicalein on the A ring could increase the activity of anti-P-glycoprotein, a multiple drug resistant gene product (*24*). The ROS scavenging activity and effects of oxidative stress-related protein expression of baicalein and its derivatives in cellular systems had not been well addressed despite baicalein's being a well-known antioxidant. We showed for the first time the antioxidative effects of baicalein and its terpenoid derivatives on the A ring in human THP-1 cells.

In the cellular system, Bai-C5 was most effective in scavenging intracellular cumene hydroperoxide and H_2O_2 among the compounds tested. The cell membrane is a hydrophobic environment because it contains a large number of phospholipid and protein molecules. The terpenoid groups are hydrophobic. The addition of terpenoid groups on the A ring of baicalein derivatives might increase the hydrophobicity of the molecule and prompt these baicalein derivatives to penetrate readily through the cell membrane into the intracellular space. Our results demonstrated that Bai-C5 exhibited the best scavenging activity in THP-1 cells after cumene hydroperoxide or H_2O_2 treatment. However, the cumene hydroperoxide or H_2O_2 scavenging activity of Bai-C10 or Bai-C15 treatment was weaker than that of Bai-C5 treatment. This might be due to the fact that the length of carbohydrate of the terpenoid in Bai-C10 and Bai-C15 is longer than that of Bai-C5; it may hinder their rates of penetration into the intracellular space. This would explain why Bai-C15 expressed the weakest scavenging activity for cumene hydroperoxide or H_2O_2 among the three baicalein derivatives within a short period of 30 min of treatment.

Baicalein has been demonstrated to decrease the superoxide production induced by lipopolysaccharide in a rat model (*25, 26*). Baicalein has also been demonstrated to have superoxide scavenging activity in other cell-free experimental systems (*27*). When superoxide formation was induced by menadione, only 20 μM baicalein and 10 μM Bai-C15 exhibited some superoxide scavenging activity. The addition of terpenoid groups on the A ring of baicalein seemed to be ineffective in increasing the intracellular scavenging activity of superoxide. Nevertheless, whether this is true would require further study.

Glutathione is an antioxidative and detoxifying peptide. A large number of ROS or toxic substances present in the intracellular space could induce GSH depletion (*28, 29*). So far, there have been few reports on the preventive effects of baicalein on exogenous ROS-induced intracellular GSH depletion. In our present study, we demonstrated that baicalein and its derivatives could significantly decrease the percentage of GSH-negative cells induced by exogenous cumene hydroperoxide directly. However, there were no significant differences between baicalein and its derivatives in preventing GSH depletion after cumene hydroperoxide treatment (**Figure 5B**), despite Bai-C5's exhibiting the best scavenging activity on cumene hydroperoxide treatment (**Figure 1**). The differences between GSH depletion and cumene hydroperoxide scavenging activities of baicalein and its derivatives might be due to the difference of the concentrations of cumene hydroperoxide used and the length of time the experiment was conducted (3 h vs 30 min).

Our present studies demonstrate that 500 μM cumene hydroperoxide induced *Bax* and *Bcl-2* expression after 3 h of treatment (**Figure 7**). Both protein expressions would relate to apoptosis. ROS are capable of inducing apoptosis through *Bax* overexpression. To prevent ROS damage, the cells usually would increase *Bcl-2* or HO-1 expressions as a defense system. It is worth noting that three baicalein derivatives were better than baicalein in inhibiting *Bax* expression. The *Bcl-2* and HO-1 expression levels of the cells treated with three baicalein derivatives were also lower than that of baicalein-treated cells. In Bai-C15-pretreated cells, *Bax* expression was even lower than that in the untreated cells. This result contradicted the result in cumene hydroperoxide scavenging activity (**Figure 1**). One possible explanation is that the farnesyl group in Bai-C15 is more hydrophobic than the prenyl group in Bai-C5 and the geranyl group in Bai-C10. Due to the large size of the Bai-C15 molecule, cell membrane penetration might require a longer time, such as 3 or 4 h of treatment, to manifest its effect.

Conclusion. In conclusion, the presence of prenyl, geranyl, or farnesyl groups at position 6 of the A ring of baicalein derivatives would affect their antioxidative capabilities, which might be beneficial to human health. The in-depth mechanisms of these effects will require further study.

ABBREVIATIONS USED

ROS, reactive oxygen species; GSH, glutathione; DCF, 2′,7′-dichlorofluorescein; HE, hydroethidine; CMF, chloromethylfluorescein; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HO-1, heme oxygenase-1.

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