Antioxidant Activity of Flavonoids Isolated from *Scutellaria rehderiana*

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ABSTRACT: The present study examined the antioxidant activity in heated canola oil of hexane, acetone, and methanol extracts of dry roots of gansu huangqin *(Scutellaria rehderiana)* as well as six flavonoids isolated from the acetone and methanol extracts. The oxidation was conducted at 95°C by monitoring oxygen consumption and decreases in both linoleic and α-linolenic acids. The acetone extract was most effective in inhibiting oxidation of canola oil, followed by the methanol extract. The antioxidant activity of gansu huangqin acetone extract was dose-dependent. Among the six flavonoids, baicalein and ganhuangenin were more effective than butylated hydroxytoluene (BHT) in protecting canola oil from oxidation. The present results suggest that the acetone extract of this root may be a potential source of natural antioxidants for use in processed foods.

Paper no. J9399 in *JAOCS 77,* 807–812 (August 2000).

KEY WORDS: Antioxidant, baicalein, baicalin, canola oil, ganhuangenin, huangqin, *Scutellaria rehderiana*.

The dry roots of *Scutellaria baicalensis* Georgi, common name huangqin, are used widely in traditional Chinese herbal medicine. The roots reportedly have antiinflammatory (1), antiviral (2), sedative (3), hypocholesterolemic (3), antithrombotic (4), and antioxidant (5,6) activity. Baicalein, a major flavonoid present in the roots of *S. baicalensis* Georgi, was reported to have hypotensive and antiproliferative effects on vascular smooth muscle cells (7). We found that baicalein at low concentration contracted while at high concentration relaxed arterial smooth muscle (8). Baicalein may also have an inhibitory effect on the production of plasminogen activator inhibitor-1 induced by thrombin (9) and may act as a lipoxygenase inhibitor (10).

Natural antioxidants are highly desirable because of the general public's reluctance to use synthetic antioxidants in foods (11,12). We previously studied the antioxidant activity of acetone, hexane, and methanol extracts of *S. baicalensis* and found that only the acetone extract had an antioxidant activity greater than that of butylated hydroxytoluene (BHT) when added to canola oil (13). Baicalein was demonstrated to be responsible for the antioxidant activity of the acetone extract (13). In the present study, we purified six flavonoids from the dry roots of *S. rehderiana* Diel, which belongs to the same genus and is used in traditional Chinese medicine in a manner similar to *S. baicalensis.* This species is cultivated in Gansu Province, China, and is commonly called gansu huangqin. The present study examined the antioxidant activity of these individual flavonoids isolated from gansu huangqin on canola oil oxidation.

MATERIALS AND METHODS

Plant material. The dry roots of gansu huangqin were purchased from a local store of traditional Chinese medicine in Gansu Province, China, and were then cut into small pieces and ground into powder in a coffee grinder. The botanical origin and purity of gansu huangqin was not confirmed. However, the chemical composition described elsewhere in this paper was similar to that reported by Liu *et al*. (14), confirming the species studied was *S. rehderiana*. Canola oil without addition of any synthetic antioxidants was obtained from Lam Soon Marketing Service Ltd. (Kowloon, Hong Kong).

Extraction of gansu huangqin. The ground roots of gansu huangqin were first extracted with hexane. In brief, 5 g of sample and 100 mL of hexane were placed into a 500-mL flask attached to a water-cooled condenser. The flask was then placed on a heater, and after refluxing for 2 h, the flask was cooled to room temperature. After filtration (using filter paper), the residue was refluxed once more with an additional 100 mL of hexane for 2 h. The hexane extracts were then pooled and saved. Similarly, the acetone extract was obtained by refluxing the residue remaining after the two hexane extractions twice with 100 mL of acetone each. Finally, the methanol extract was obtained by twice extracting the residue remaining after two acetone extractions with 100 mL of methanol in a similar way. The solvents in the three extracts were then evaporated using a vacuum evaporator. The resulting three extracts were then weighed and stored at −20°C prior to the antioxidant activity test. The weights (± standard deviation) of methanol, acetone, and hexane extracts as a percentage of dry roots were 18.96 (±3.68), 6.58 (±0.84), and

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1.04 (±0.06), respectively.

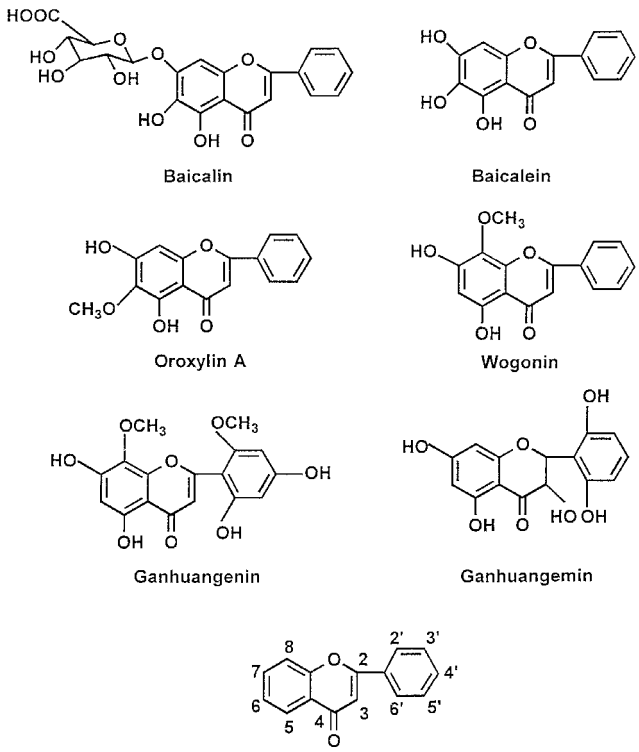
Isolation and purification of gansu huangqin flavonoids. The ground root powder of gansu huangqin was first extracted twice using hexane, acetone, and methanol as just described. The acetone extract was first applied onto silica gel column A (50 × 3.5 cm, i.d.; silica gel 60M, 230–240 mesh, Macherey-Nagel, Duren, Germany). The column was then eluted with a mixture of chloroform, methanol, and acetic acid (200:20:1, vol/vol/vol) to obtain 24 fractions (AF = fractions obtained from column A) using a fraction collector (15 mL/AF). Each fraction was subjected to thin-layer chromatography (TLC; aluminum sheet, 6.5 × 4.0 cm, silica gel 60 F_{254}; Merck KGaA, Darmstadt, Germany). After development in a solvent mixture of chloroform/methanol/acetic acid (10:1:0.1, vol/vol/vol), the flavonoids were visualized under ultraviolet (UV) light. The fractions with a profile similar to known flavonoids were combined. The pooled AF2–6 were concentrated under reduced pressure and applied to silica gel column B (30 × 1.5 cm, i.d.). The same eluting solvent system was used to yield 80 fractions (BF = fractions obtained from column B, 15 mL/BF); three pure flavonoids, namely, oroxylin (BF14), wogonin (BF15–16), and ganhuangenin (BF69–79), were obtained by using aluminum TLC as already described here. Similarly, the pooled AF7–13 were applied onto silica gel column C (30 × 1.5 cm, i.d.) and eluted using the same solvents to produce 67 fractions (CF = fractions obtained from column C, 15 mL/CF). Baicalein (CF13–24) and ganhuangenin (CF37–44) were isolated. The pooled AF14–24 were applied onto silica gel column D (30 × 1.5 cm, i.d.) and eluted using the same solvent system to produce 32 fractions (DF = fractions obtained from column D, 17 mL/DF). Ganhuangemin (DF21–30) was obtained.

The methanol extract was placed in a freezer at –4°C. A yellow precipitate formed, which was separated using filter paper, redissolved in methanol, and recrystallized. The crystallization process was performed four more times in methanol at –4°C to obtain baicalin as previously described (13).

The six purified flavonoids were subjected to verification of their chemical structures using the melting point test, TLC, UV spectrometry, liquid chromatography–mass spectrometry (MS), and ^{1}H nuclear magnetic resonance spectrometry. The results were in agreement with those previously reported (14,15). Five flavonoids purified from gansu huangqin were confirmed as baicalein, baicalin, ganhuangenin, oroxylin A, and wogonin (Scheme 1).

The presence of the sixth compound, i.e., 2′,3,5,6′,7-pentahydroxy-flavanone, trivial name ganhuangemin, has been reported in other species (15,16), but this was the first time it was identified in gansu huangqin. The structure of ganhuangemin was further verified using its electron impact MS spectrum (*m/z*: 304, 286, 269, 195, 153, and 123) and infrared spectrum, which had characteristic absorptions at 3496, 3330 (OH), 1640 (C=O), 1620, and 1610 (aromatic ring) cm^{-1}.

High-performance liquid chromatography (HPLC) analysis of individual flavonoids. To quantify and check their purity, the flavonoids were analyzed using a Shimadzu LC-10AD



SCHEME 1

HPLC (Tokyo, Japan). In brief, 10 µL of the acetone extract (10 mg/mL) was injected onto a column (Hypersil ODS, 250 × 4.6 mm, 5 µm; Alltech, Deerfield, IL) *via* a Rheodyne valve (20 µL capacity; Cotati, CA). A water solution containing 14.5% tetrahydrofuran, 12.5% dioxane, 5.0% methanol, 2.0% acetic acid, and 0.01% phosphoric acid was used at a flow rate of 1.0 mL/min (Fig. 1). The individual flavonoids were separated and quantified by using a UV detector at 275 nm (UVIS-205, Alltech) and quercetin as an internal standard.

Oxygen consumption test. Oxygen uptake by canola oil was monitored as previously described (17,18). In brief, 200 mg of canola oil was placed in a glass tube (150 × 16 mm, o.d.) followed by adding 1 mL of acetone containing varying amounts of the extracts or purified flavonoids. The solvent was removed under a gentle stream of nitrogen at 45°C. The reaction tube was then flushed with air using an air pump and sealed tightly with a rubber stopper obtained from an evacuated blood collection tube (100 × 16 mm, o.d.; Becton-Dickinson, Rutherford, NJ), which usually maintains a vacuum for 2–3 yr. The reaction tubes were then placed in a heating block at 95°C with variation of ±3°C. Temperature fluctuation would lead to an inconsistent oxygen consumption rate over time. Therefore, the comparison of antioxidant activity was made only among the samples that were placed in the same heating block and heated at the same time. The headspace air (50 µL) was sampled every 4–6 h for a total of 40 h with a gas-tight syringe and injected into a Hewlett-Packard 5890 series II gas–solid chromatograph (Palo Alto, CA) fitted with a 1/8″ × 6′ stainless-steel column packed with Molecular Sieve 5A (60–80 mesh) and a thermal conductivity detector.

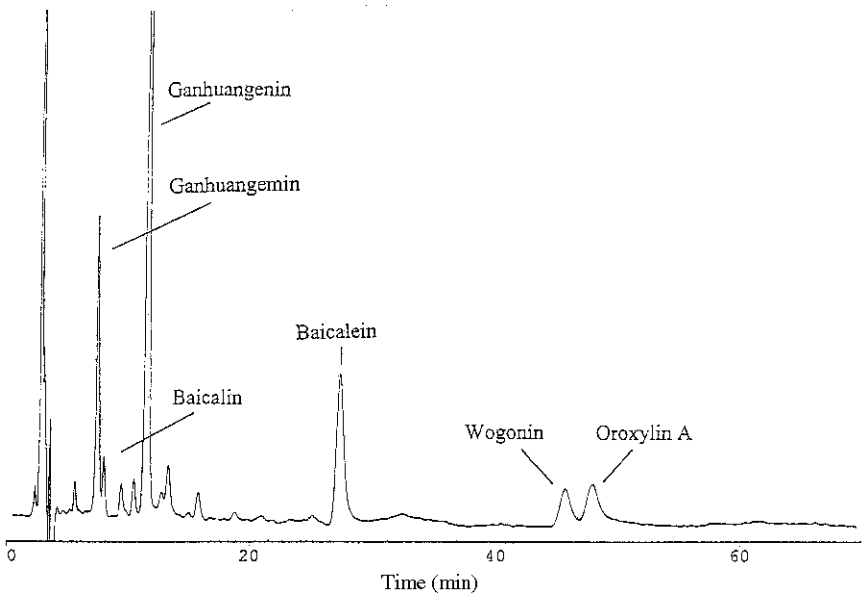


FIG. 1. High-performance liquid chromatography profile of flavonoids in the acetone extract without addition of quercetin as an internal standard. See text for conditions and the Results section for percentage composition.

The percentage oxygen in the headspace was calculated from the ratio of oxygen to nitrogen. The canola oil was sampled for fatty acid analysis at 24 and 40 h.

Fatty acid analysis. Fatty acids of the heated canola oil sample with or without addition of individual flavonoids purified from gansu huangqin were converted to the corresponding methyl esters with a mixture of 14% BF_3 in methanol (Sigma Chemical Co., St. Louis, MO) and toluene (1:1, vol/vol) under nitrogen at 90°C for 45 min (3). Fatty acid methyl esters were analyzed on a flexible silica capillary column (SP 2560, 100 m × 0.25 mm, i.d.; Supelco, Inc., Bellefonte, PA) in a Hewlett-Packard 5980 Series II gas–liquid chromatograph equipped with a flame–ionization detector. Column temperature was programmed from 180 to 220°C at a rate of 1°C/min and then held for 10 min. Injector and detector temperatures were set at 250 and 300°C, respectively. Hydrogen was used as the carrier gas at a head pressure of 15 psi.

Statistics. All the experiments were repeated two or three times. Each experiment consisted of four or five sample replicates. Data for the headspace oxygen consumption and fatty acid analysis were subjected to analysis of variance, and the means were compared between treatments by using Duncan's multiple range test (19). This was done by running data on PC ANOVA software (PC ANOVA for the IBM Personal Computer, Version 1.1, 1985, IBM, Armonk, New York). Significance was set at 5%.

RESULTS AND DISCUSSION

Antioxidant activity of the three extracts was first compared at a concentration of 200 ppm. As shown in Figure 2, the headspace oxygen concentration of samples to which acetone extract had been added remained above 20%, whereas that of the samples to which methanol extract had been added dropped to 8.5% after 40 h of heating at 95°C. The headspace oxygen for the control and the samples to which hexane extract was added dropped to below 6.5% after 40 h of heating at 95°C. The protective effect of the acetone extract on oxidation of canola oil was dose-dependent (Fig. 3). Compared with BHT at 200 ppm, the acetone extract at 100 ppm or above was more effective against lipid oxidation of canola oil heated at 95°C. The present results suggest that the acetone

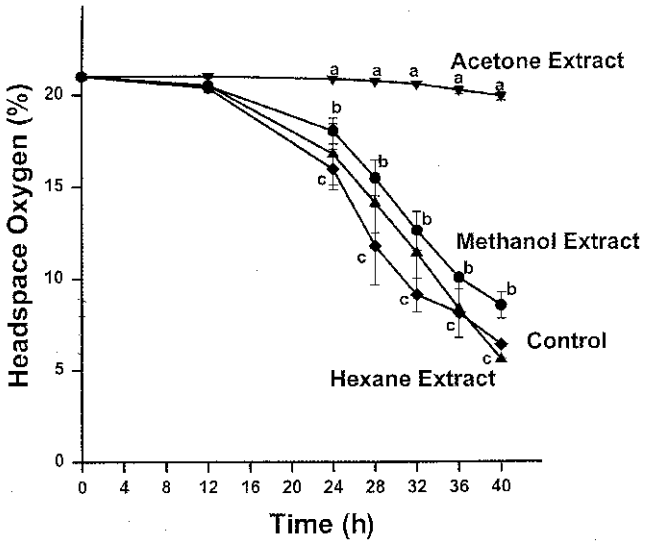


FIG. 2. Effect of 200 ppm gansu huangqin *(Scutellaria rehderiana)* hexane extract, acetone extract, and methanol extract on oxidation of canola oil heated at 95 ± 3°C. Data are expressed as mean ± SD (n = 5 samples). Means at the same time point with different superscript letters (a–c) differ significantly ($P < 0.05$).

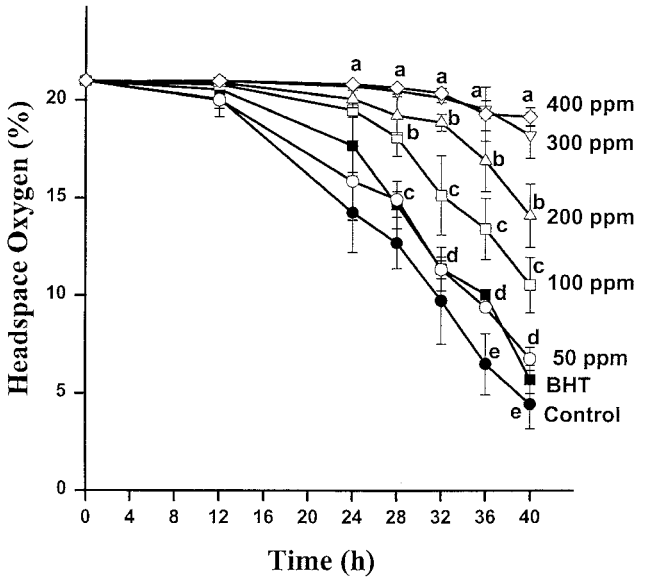


FIG. 3. Dose-dependent effect of gansu huangqin *(Scutellaria rehderiana)* acetone extract (50–400 ppm) on oxidation of canola oil heated at 95 ± 3°C as compared with that of 200 ppm butylated hydroxytoluene (BHT). Data are expressed as mean ± SD of $n = 4$ or 5 samples. Means at the same time point with different superscript letters (a–e) differ significantly ($P < 0.05$).

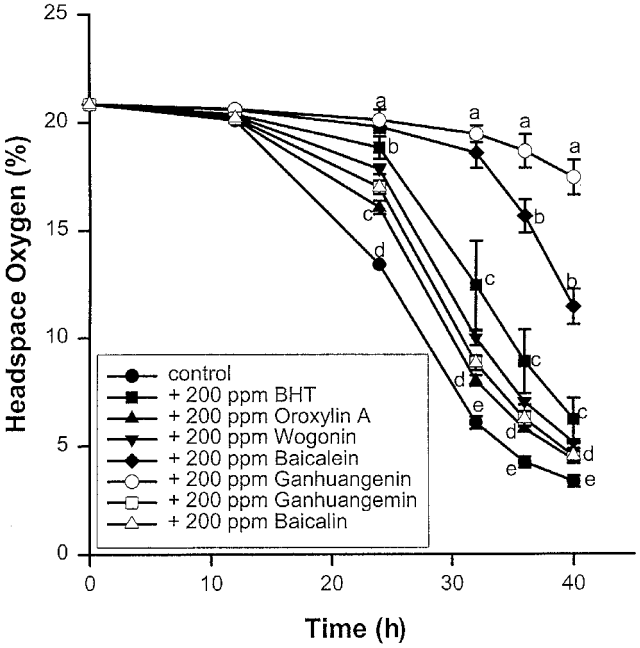


FIG. 4. Effect of six flavonoids (200 ppm) isolated from gansu huangqin *(S. rehderiana)* on the oxidation of canola oil heated at 95 ± 3°C as compared with BHT. Data are expressed as mean ± SD of $n = 4$ samples. Means at the same time point with different superscript letters (a–d) differ significantly ($P < 0.05$). For abbreviations see Figure 3.

extract contains antioxidants that may be more effective than BHT. Therefore, we concentrated on purification and isolation of antioxidants present in the acetone extract.

To identify the possible antioxidants, six flavonoids, namely, oroxylin A, wogonin, ganhuangenin, baicalein, baicalin and ganhuangemin, were purified from the acetone extract. The antioxidant activity of these flavonoids was tested by measuring oxygen consumption at a concentration of 200 ppm. As shown in Figure 4, the antioxidant activity of ganhuangenin was greatest, followed by baicalein, wogonin, baicalin, ganhuangemin, and oroxylin A. Under the same conditions, ganhuangenin and baicalein were more effective than BHT in inhibiting oxidation of canola oil (Fig. 4). To verify the antioxidant activity of both baicalein and ganhuangenin, 50–400 ppm was added to canola oil and oxidation was conducted at 95°C. As shown in Figure 5, baicalein had dose-dependent antioxidant activity. As the concentration of baicalein increased from 50 to 400 ppm, the oxygen consumption of canola oil decreased. Similarly, ganhuangenin also showed dose-dependent antioxidant activity (Fig. 6). At concentrations above 100 ppm both baicalein and ganhuangenin were more effective than BHT at 200 ppm against canola oil oxidation.

The present results demonstrated clearly that the crude gansu huangqin acetone extract contained a mixture of antioxidants that was more effective than BHT if the oxidation was measured under the conditions of this study. The six flavonoids in the acetone extract were quantified using HPLC. Ganhuangenin accounted for 21.8 ± 3 wt% (mean ± SD, $n = 3$) followed by ganhuangemin (6.7 ± 0.4 wt%), baicalein (5.6 ± 0.5 wt%), baicalin (1.6 ± 0.3 wt%), oroxylin A (2.6 ± 0.5 wt%), and wogonin (2.3 ± 0.4 wt%). Ganhuangenin and baicalein were probably the two major antioxidants responsible for the antioxidant activity of the acetone extract.

To further verify the antioxidant activity of baicalein and

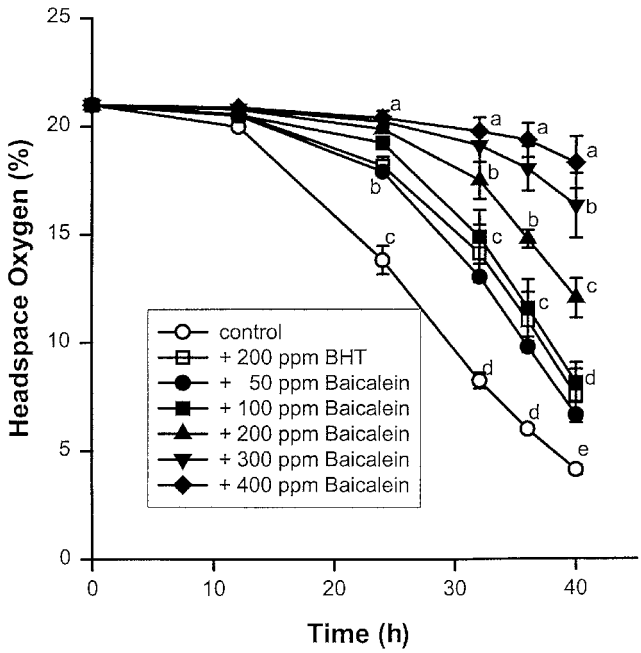


FIG. 5. Dose-dependent effect of baicalein (50–400 ppm) on oxidation of canola oil heated at 95 ± 3°C as compared with that of 200 ppm BHT. Data are expressed as mean ± SD of $n = 4$ or 5 samples. Means at the same time point with different superscript letters (a–d) differ significantly ($P < 0.05$). For abbreviation see Figure 3.

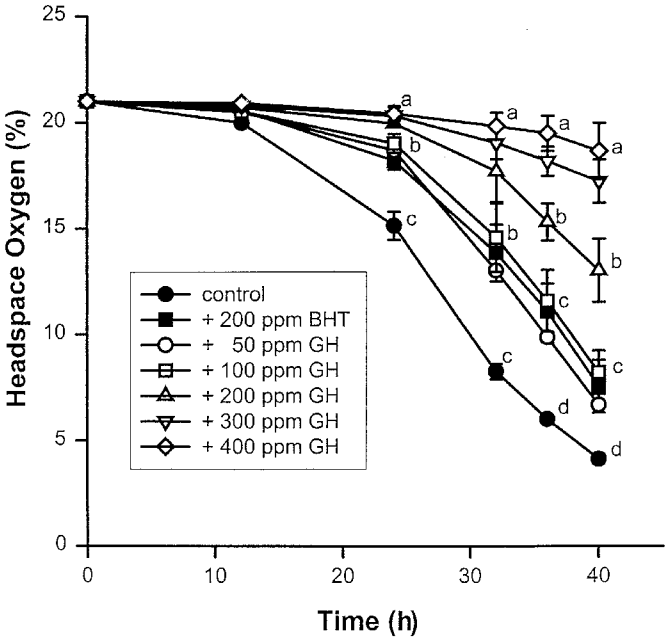


FIG. 6. Dose-dependent effect of ganhuangenin ± (GH) on oxidation of canola oil heated at 95 ± 3°C as compared with that of 200 ppm BHT. Data are expressed as mean ± SD of $n = 5$ samples. Means at the same time point with different superscript letters (a–d) differ significantly ($P < 0.05$). For abbreviation see Figure 3.

ganhuangenin observed in the oxygen consumption test, the effect of baicalein and ganhuangenin on changes in content of linoleic and α-linolenic acids was monitored in canola oil heated at 95°C for 24 and 40 h. As shown in Table 1, both baicalein and ganhuangenin significantly prevented the oxidative losses of these two unsaturated fatty acids in canola oil. For example, the control canola oil had 3.49% α-linolenic acid after 40 h heating at 95°C whereas the samples with added BHT, baicalein, and ganhuangenin had 4.49, 6.05, and 5.37% α-linolenic acid, respectively. The data were consistent with those obtained in the oxygen consumption test.

We previously examined the antioxidant activity of baicalein and baicalin isolated from *S. baicalensis* (13). The present data confirmed that baicalein possessed stronger antioxidant activity than BHT. Baicalein was also able to inhibit the lipid oxidation in phosphatidylcholine liposome membrane (5) and rat liver microsome (6). The mechanism by which baicalein was effective against lipid oxidation remains unexplained. Perhaps the three adjacent hydroxyl groups at positions 5, 6, and 7 are more able to donate a proton because of resonance delocalization (Scheme 1). It was interesting to note that baicalein's derivatives, baicalin and oroxylin A, exhibited no or weaker antioxidant activity. This indicates that blockage of C7-OH by the glucuronate group or the C6-OH of baicalein by methyl group almost completely abolishes its antioxidant activity.

The antioxidant activity of a flavonoid is governed by many factors including its structure and the number and location of phenolic hydroxyl groups on the A, B, and C rings (20,21). The present study was the first to examine the antioxidant activity of ganhuangenin, ganhuangemin, and wogonin. Only ganhuangenin was more effective than BHT as an antioxidant, indicating that the number and position of hydroxyl groups on rings A and C play an important role in contributing to antioxidant activity. When the antioxidant activity of ganhuangenin was compared with that of wogonin, the role of the methoxyl group at position 2′ and the two hydroxyl groups at positions 4′ and 6′ was clearly illustrated.

Any vegetable oil including canola oil contains α-tocopherol (22). The present study did not use canola oil free of α-tocopherol. It was possible that baicalein, ganhuangenin and the α-tocopherol naturally present in canola oil have a synergistic effect on lipid oxidation. It will be interesting to examine the antioxidant activity of the acetone extract, baicalein, and ganhuangenin in the absence of other antioxidants.

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TABLE 1
Effect of Ganhuangenin and Baicalein on Change in Fatty Acids of Canola Oil (wt% of total fatty acids) Heated at 95¡C[a]

	Linoleic acid	α-Linolenic acid	Oleic acid	Palmitic acid	Stearic acid	Other acid
Unheated canola oil	20.27 ± 0.04	6.68 ± 0.03	61.69 ± 0.23	4.29 ± 0.05	2.19 ± 0.02	4.48 ± 0.12
Canola oil heated 24 h	19.07 ± 0.16^{c}	5.73 ± 0.10^{c}	63.75 ± 0.28^{b}	4.44 ± 0.07	2.28 ± 0.02	4.73 ± 0.21
+ BHT	19.08 ± 0.71^{c}	5.73 ± 0.54^{c}	63.41 ± 1.21^{b}	4.50 ± 0.08	2.29 ± 0.03	4.99 ± 0.15
+ Baicalein	20.21 ± 0.21^{b}	6.60 ± 0.14^{b}	61.81 ± 0.25^{c}	4.34 ± 0.04	2.18 ± 0.05	4.86 ± 0.11
+ Ganhuangenin	20.15 ± 0.03^{b}	6.58 ± 0.03^{b}	61.91 ± 0.07^{c}	4.30 ± 0.02	2.18 ± 0.02	4.88 ± 0.09
Canola oil heated 40 h	15.52 ± 0.10^{d}	3.49 ± 0.04^{e}	68.99 ± 0.99^{b}	5.14 ± 0.05^{b}	2.60 ± 0.01	4.26 ± 0.11^{b}
+ BHT	17.52 ± 0.72^{c}	4.49 ± 0.22^{d}	67.67 ± 1.25^{b}	4.89 ± 0.10^{b}	2.46 ± 0.04	2.97 ± 0.08^{c}
+ Baicalein	19.45 ± 0.03^{b}	6.05 ± 0.03^{b}	63.03 ± 0.02^{c}	4.41 ± 0.02^{c}	2.25 ± 0.03	4.81 ± 0.21^{b}
+ Ganhuangenin	18.56 ± 0.82^{b}	5.37 ± 0.58^{c}	64.50 ± 1.33^{c}	4.56 ± 0.13^{c}	2.32 ± 0.07	4.69 ± 0.13^{b}

[a]Data are expressed as mean ± SD of $n = 6$ samples. Means in the same column and the same time point with different roman superscripts differ significantly ($P < 0.05$). BHT, butylated hydroxytoluene.

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[Received September 20, 1999; accepted June 13, 2000]