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2'-Hydroxylation of Genistein Enhanced Antioxidant and Antiproliferative Activities in MCF-7 Human Breast Cancer Cells

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Bioconversion of the isoflavonoid genistein to 2'-hydroxygenistein (2'-HG) was performed using isoflavone 2'-hydroxylase (CYP81E1) heterologously expressed in yeast. A monohydroxylated product was analyzed by liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) and NMR spectrometry and was identified as 2'-HG. An initial bioconversion rate of 6% was increased up to 14% under optimized conditions. After recovery, the biological activity of 2'-HG was evaluated. Bioconverted 2'-HG showed higher antioxidant activity against 1,1-diphenyl-2-picryl hydrazine (DPPH) and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radicals than did genistein. Furthermore, 2'-HG exhibited greater antiproliferative effects in MCF-7 human breast cancer cells than did genistein. These results suggest that 2'-hydroxylation of genistein enhanced its antioxidant activity and cell cytotoxicity in MCF-7 human breast cancer cells.

Keywords: Genistein, 2'-hydroxygenistein, bioconversion, antioxidant activity, antiproliferation activity, MCF-7 cells

Isoflavonoids have been found to possess a broad range of biological activities that include antioxidative [26, 34], antiestrogenic [5, 28], anticancer [22, 28], anti-inflammatory [36], and enzyme inhibitory effects [37]. Investigation of genistein (4',5,7-trihydroxyisoflavone), one of the predominant soy isoflavones, has been stimulated over the past 10 years owing to the significant antioxidant activity characteristic of phytoestrogenic isoflavones [26, 28, 32], and it is playing an important role in reducing breast and prostate cancers [14–16, 27].

Antioxidant activity, being one of the most important features of food ingredients, dietary supplements, and natural anticancer products [6], has been established in phytoestrogen-rich plants such as legumes. Genistein and daidzein isolated from soybean seeds showed stronger antioxidant capacity than the *O*-glycosides, because their antioxidant activity is associated with the number and positions of hydroxyl groups attached to ring structures [3, 4, 16, 26]. Previous research has demonstrated the contribution of the 5,7-dihydroxy groups in the A ring to total antioxidant activity in isoflavones and the crucial importance of the hydroxyl group at the 4'-position of the B ring to antioxidant activity in genistein [4, 26, 34]. Thus, genistein has greater antioxidant capacity than the other soy isoflavones, which include daidzein, glycitein, and their glycosides. In the present study, increasing the number of hydroxyl groups in genistein will thus be essential to the elevation of antioxidant activity.

Genistein has been a controversial candidate for breast cancer chemoprevention, with several studies appearing to support this idea [8, 21, 29, 30]. Because the structure of genistein is closely similar to that of estrogens, it can bind to estrogen receptors (ERs) and may either induce cell proliferation or prevent hormone-dependent growth of cancer cells, depending on its concentration [28]. In addition, since genistein is a known inhibitor of protein tyrosine kinase, it may attenuate the growth of cancer cells by inhibiting protein tyrosine kinase-mediated signaling pathways [2]. Inhibition of cell proliferation by genistein could also be due to increased apoptosis [28]. Recently, it has been shown that isoflavones may be activated *via* hydroxylation *in vitro* and *in vivo* and that the activated isoflavone may have biological activities that differ from the parent isoflavone [17, 18].

Recent progress in the investigation of 2'-hydroxyisoflavones has made possible the cloning of isoflavone 2'-hydroxylase by the research group of Shin-ichi Ayabe [1]. In this report, we demonstrate that heterologously expressed isoflavone 2'-hydroxylase catalyzes the hydroxylation of genistein at

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C-2'. In addition, we developed a method for obtaining a larger quantity of 2'-HG using genistein as a substrate and evaluated its biological activities, which include antioxidant and antiproliferative properties in MCF-7 human breast cancer cells.

MATERIALS AND METHODS

Bioconversion of Genistein

Previously, cDNA for isoflavone 2'-hydroxylase (CYP81E1) from *Glycyrrhiza echinata* was inserted into the pYES2 yeast expression vector under the control of the Gal10 promoter (pYES2 I2H) [1]. Isoflavone 2'-hydroxylase was expressed singly in the WAT11 strain of the yeast *Saccharomyces cerevisiae* [13, 24, 33]. pYES2 I2'H (CYP81E1) was transformed into WAT11 competent cells prepared using a Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA, U.S.A.) [24]. Non-transformed cells were used as a control. Since the pYES2 vector has a Ura selection marker, a selection medium (SC DO-Ura) was used for the transformed WAT11 (designated JNC-1), which consisted of 0.5 g of Synthetic Complete Drop-Out Supplement Minus Uracil (Clonetech, Otsu, Shiga, Japan), 4.0 g of yeast nitrogen base without amino acid (Difco, Franklin Lakes, NJ, U.S.A.), and 12 g of glucose in 600 ml of distilled water adjusted to pH 5.6 with 10 N NaOH. JNC-1 cells were cultured for 1–2 days (pale red color) at 30°C in 50 ml of SC DO-Ura liquid medium, and control cells were cultured under the same conditions, except that Synthetic Complete Supplement was substituted for Synthetic Complete Drop-Out Supplement [7, 23, 24]. The cultures were collected by centrifugation at 6,000 ×*g* for 30 min and washed in distilled water. For induction of isoflavone 2'-hydroxylase, cell pellets were resuspended in liquid medium containing galactose instead of glucose [24].

Genistein (Sigma, St. Louis, MO, U.S.A.) was added to a final concentration of 60 μM. Approximately 12–15 h after induction, the JNC-1 culture was centrifuged at 6,000 ×*g* for 30 min and the supernatant was extracted with an equal volume of ethyl acetate to concentrate phenolic compounds [24]. The extracts were concentrated to dryness in a rotary evaporator at 30°C.

To increase the conversion rate and obtain a larger quantity of product, we used a 0.1 M potassium phosphate buffer (pH 7.5) instead of the selection medium and performed the extraction after 9 h. The product was then analyzed by HPLC, ^{1}H-NMR, and LC–ESI–MS.

Separation and Identification of 2'-Hydroxygenistein

HPLC samples were analyzed on a Hitachi LaChrom Elite HPLC (Hitachi High-Technologies Co., Tokyo, Japan) equipped with a photodiode array detector using a YMC Pack-Pro C18 column (Waters, Milford, MA, U.S.A.) and high-purity grade reagents (Burdick and Jackson, Muskegon, MI, U.S.A.). Samples were diluted in methanol and then separated using a 40 min linear gradient from 20% acetonitrile/80% distilled water to 50% acetonitrile, followed by 40–50 min using 100% acetonitrile at a flow rate of 1 ml/min. Elution of metabolites was monitored with a photodiode array detector and compared with a control consisting of transformed cell metabolites. Retention time and UV spectra were compared with samples and authentic standards (genistein) when available.

LC–ESI–MS analysis was used to confirm the identity of the various flavonoid and isoflavonoid metabolites [19]. Mass spectra were obtained *via* either direct infusion or liquid chromatographic introduction into a Finnigan LCQ Advantage MAX ion trap mass spectrometer equipped with a Finnigan Surveyor Modular HPLC system (Thermo Electron Co., MA, U.S.A.). Chromatographic separation of compounds was achieved using a YMC Hydrosphere C18 column (50×2.0 mm, 5 μm; Waters, Milford, MA, U.S.A.) at a flow rate of 0.2 ml/min. Mobile phases A and B were high-purity water and acetonitrile, respectively, both containing 0.1% formic acid. Elution was conducted as follows: 0–30 min with a linear gradient of 5–60% B, followed by 30–45 min with 100% B [19, 35].

Each HPLC-purified sample was dissolved in CD_3OD and analyzed by ^{1}H-NMR on a Varian Unity 500 spectrometer (Varian, Inc., Palo Alto, CA, U.S.A.)

DPPH Radical Scavenging Activity

Free radical scavenging activity was measured with DPPH dissolved in 80% aqueous methanol [20]. For each compound, 100 μl of a 2–1,000 μg/ml solution in methanol was added to 100 μl of 200 mM DPPH solution and reacted at 37°C for 30 min. Decoloration of the solution indicated the scavenging efficiency of the added substance. A reference sample was prepared with 100 μl of methanol. The absorbance was measured at 517 nm on a microplate reader. Radical scavenging activity was calculated as a percentage using the following equation:

$$\text{Radical scavenging activity (\%)} = 100 \times (1 - \text{absorbance of sample} / \text{absorbance of reference})$$

ABTS Radical Scavenging Activity

A method developed by Strube *et al.* [31] was slightly altered to measure the ABTS radical scavenging activity. One mM 2,2'-azo-bis-(2-amidinopropane) hydrochloride (AAPH) was mixed with 2.5 mM ABTS as a diammonium salt in a phosphate-buffered saline solution (0.1 M potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl) and heated at 70°C for 45 min. The concentration of the resulting blue-green ABTS radical solution was calibrated to an absorbance of 0.650±0.020 (mean±SD) at 734 nm. Samples were diluted to various concentrations and added to 980 μl of the resulting blue-green ABTS radical solution. Mixtures were incubated in darkness in a 37°C water bath for 10 min, and the decrease in absorbance was measured at 734 nm. The control solution consisted of 20 μl of 50% methanol and 980 μl of ABTS radical solution.

Cell Cultures and Conditions

MCF-7 human breast cancer cells from the American Type Culture Collection (ATCC) were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2 and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Cellgro, VA, U.S.A.) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, NY, U.S.A.) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) (Sigma, St. Louis, MO, U.S.A.).

Cell Viability Assay

The effect of 2'-HG and genistein on the viability of MCF-7 cells was determined using the MTT [3-(4,5-dimethyl thiazolyl)-2,5-diphenyl tetrazolium bromide] assay, which is based on the reduction of a

tetrazolium salt by mitochondrial dehydrogenases in viable cells. MCF-7 cells were seeded in a 96-well plate at a concentration of 1.5×10^5 cells per well. One day after plating, cells were treated with three concentrations of 2'-HG and genistein and incubated for 24 h at 37°C. During the last 4 h, cells were incubated with 20 μl of MTT stock solution in 100 μl of medium. Cells were then extracted with acidic isopropanol and the absorbance was measured with an ELISA reader at 570 nm. MCF-7 cells treated with DMSO only for 24 h were used as the control. Relative cell viability was determined by the amount of MTT converted to the insoluble formazan salt. A reduction in the absorbance of a sample compared with the control was used as a measure of the reduction in cell viability. All data are presented as mean±standard deviation (SD) for at least six replications for each prepared sample.

Results

Identification of 2'-HG

When an ethyl acetate-extractable metabolite produced by JNC-1 bioconversion was analyzed by HPLC, a new peak was detected at 15.09 min prior to the substrate genistein (17.81 min) (Fig. 1.). This result suggests that the new metabolite is a more hydroxylated and hydrophilic derivative of genistein. In contrast with JNC-1, the control cells did not produce the 15.09 min metabolite. To confirm this assumption, the ethyl acetate extract containing the genistein derivative was analyzed by LC–ESI–MS. As shown in the total ion chromatograms (negative ion mode) and mass spectra (Fig. 1), two significant peaks having molecular weights of *m/z* 285 ($[M-H]^-$) and 269 ($[M-H]^-$) appeared at 15.09 and 17.81 min, respectively. The molecular weight and retention time of the 17.81 min compound were identical to those of the genistein substrate. The parent ion *m/z* value of 285 obtained for the 15.09 min metabolite indicates that the genistein derivative has one additional oxygen atom (an *m/z* value of 16) compared with genistein. The present mass spectra data support our previous assumption that genistein could be hydroxylated at the C-2' position by isoflavone 2'-hydroxylase (CYP81E1), resulting in 2'-HG. The putative 2'-HG detected at 15.09 min was purified through HPLC and confirmed to be 2'-HG by comparison with the ^{1}H-NMR spectrum of genistein (Table 1). Three protons on the A and C rings were detected at δ_H (CD_3OD): 6.36 (1H, d, $J_{6,8}$=2.0 Hz), 6.24 (1H, d, $J_{6,8}$=2.0 Hz), and 8.07 (1H, s). However, the B ring protons that are reciprocally coupled in genistein were absorbed at δ_H (CD_3OD): 7.39 (2H, dd, $J_{2',3'}$=8.5 Hz, $J_{2',6'}$=2.0 Hz) and 6.86 (2H, dd, $J_{2',3'}$=8.5 Hz, $J_{3',5'}$=2.0 Hz), whereas the 2'-HG protons were detected at δ_H (CD_3OD): 7.05 (1H, d, $J_{5',6'}$=8.5 Hz), 6.41 (1H, dd, $J_{5',6'}$=8.5 Hz, $J_{3',5'}$=2.5 Hz), and 7.03 (1H, d, $J_{3',5'}$

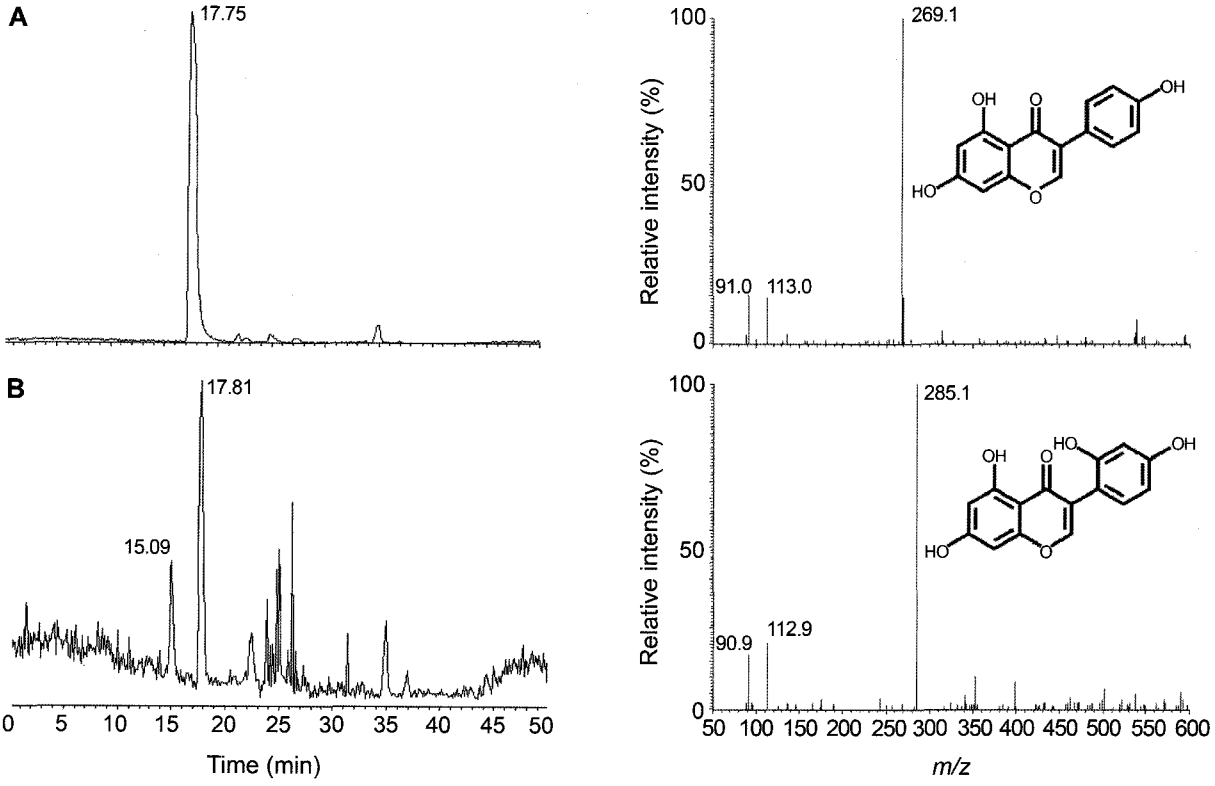
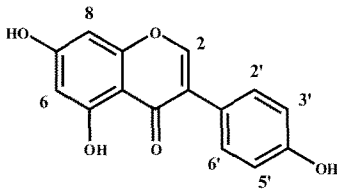
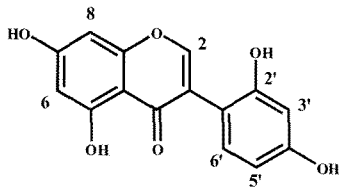


Fig. 1. HPLC and LC–ESI–MS analyses of genistein (**A**) and its bioconversion product (**B**).
Extracts of non-transformed WAT11 cells (**A**) and JNC-1 cells (**B**) were reacted with genistein.

Table 1. ^{1}H-NMR (CD_3OD, 500 MHz) chemical shifts of genistein and 2'-hydroxygenistein.

Compound	Position	^{1}H(Hz)	Structure
Genistein	C-2	8.07 (s)	
	C-6	6.24 (d, $J_{6,8}$=2.0)	
	C-8	6.36 (d, $J_{6,8}$=2.0)	
	C-2', 6'	7.39 (dd, $J_{2',3'}$=8.5, $J_{2',6'}$=2.0)	
	C-3', 5'	6.86 (dd, $J_{2'',3''}$=8.5, $J_{3'',5''}$=2.0)	
2-Hydroxygenistein	C-2	8.07 (s)	
	C-6	6.24 (d, $J_{6,8}$=2.0)	
	C-8	6.36 (d, $J_{6,8}$=2.0)	
	C-2'	-	
	C-3'	7.03 (d, $J_{3',5'}$=2.0)	
	C-5'	6.41 (dd, $J_{5',6'}$=8.5, $J_{3',5'}$=2.0)	
	C-6'	7.05 (d, $J_{5',6'}$=8.5)	

=2.5 Hz). Based on this result, we concluded that the hydroxyl group was bound at the C-2' position, demonstrating that genistein was converted to 2'-HG by JNC-1.

Method for Obtaining a Larger Quantity of 2'-HG

The rate of bioconversion of genistein to 2'-HG was very low (2–6%). Thus, we attempted to increase the yield by optimizing the conversion time and environment. As shown in Fig. 2, the optimum reaction time was 9 h after treatment with galactose. A greater quantity of 2'-HG was obtained when a potassium phosphate buffer (pH 7.5) was used instead of liquid medium containing galactose. By combining these two modifications, a bioconversion rate of 14% was obtained after induction with galactose in a phosphate buffer (Fig. 2). Peak area values from the LC–ESI–MS analysis were used to calculate the conversion rate with the following equation:

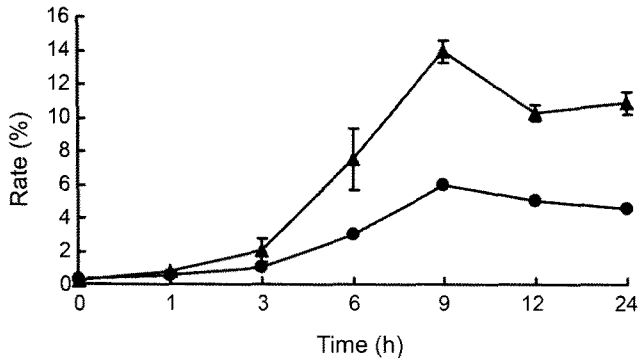


Fig. 2. Rate of bioconversion of genistein to 2'-HG performed in potassium phosphate buffer (▲) or SC DO-Ura induction medium (●).

Conversion rate (%)=(product peak area/internal standard peak area)/(substrate peak area/internal standard peak area)×100.

Isoliquiritigenin was used as the internal standard, and unreacted genistein was used to obtain the value for the substrate peak area (0 h value in Fig. 2).

DPPH Radical Scavenging Activity

The antioxidant capacities of genistein and 2'-HG were determined by monitoring the scavenging of DPPH radicals, and the results are shown in Fig. 3A. 2'-HG exhibited significant DPPH radical scavenging activity, ranging from 13.9 to 50.9% inhibition at 5 μg/ml to 50 μg/ml in a concentration-dependent manner, whereas genistein showed very weak inhibition, ranging from 0.9 to 1.1% at the same concentrations.

ABTS Radical Scavenging Activity

The antioxidant capacities of genistein and 2'-HG were also evaluated by measuring ABTS radical scavenging activity and comparing it with that of vitamin C. The results are presented in Fig. 3B. 2'-HG exhibited greater ABTS radical scavenging activity than either genistein or vitamin C, all of which reacted in a concentration-dependent fashion.

Effect of 2'-HG on Cell Viability of MCF-7 Human Breast Cancer Cells

Since genistein has shown some promise as a chemopreventive agent for breast cancer [8, 21, 29, 30], its 2'-HG derivative was expected to have an inhibitory effect on MCF-7 human breast cancer cells. As shown in Fig. 4, MCF-7 cell viability was significantly decreased following treatment with various concentrations of 2'-HG. Furthermore, 2'-HG

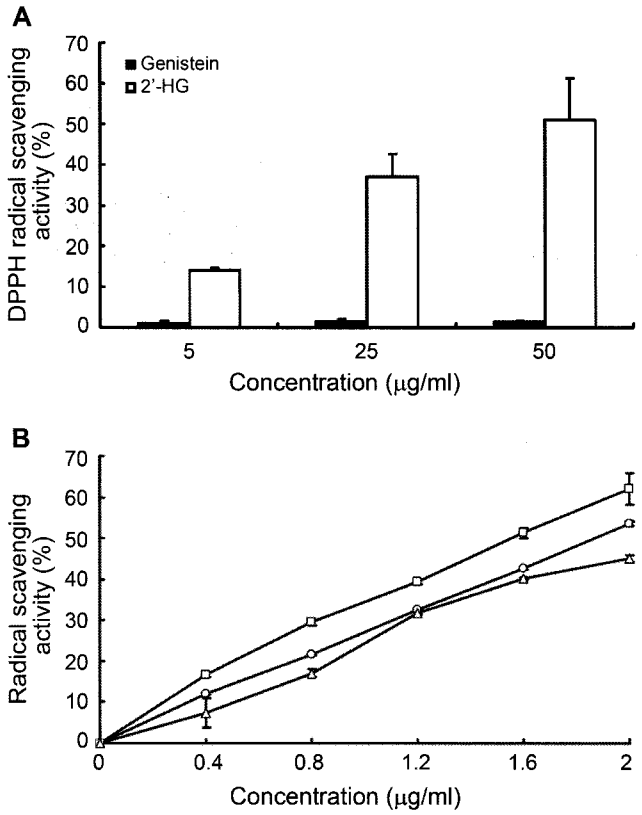


Fig. 3. DPPH (**A**) and ABTS (**B**) radical scavenging activities of 2'-HG and genistein.
In (**B**), the data for 2'-HG (□) and genistein (△) are compared with that for vitamin C (○). The results are presented as the percentage of radical scavenging activity relative to the reference.

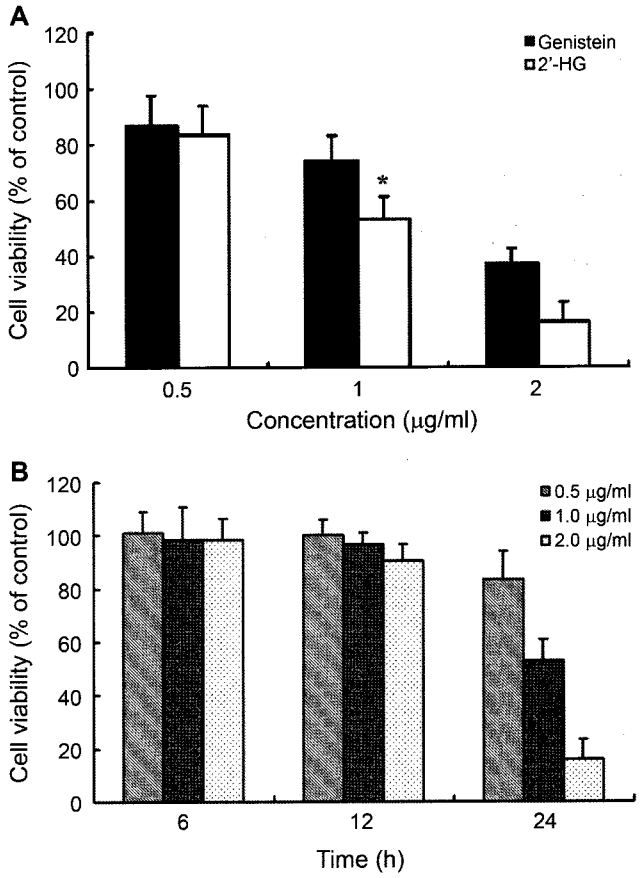


Fig. 4. Viability of MCF-7 cells incubated with 2'-HG and genistein for 24 h (**A**), and MCF-7 cell viability time course in the presence of 2'-HG (**B**).
The data are expressed as a percentage of the value obtained for untreated control cells after incubation for 24 h. The results were obtained from six individual replications. Error bars denote standard deviation. The asterisk *denotes a significant difference from the control.

was found to be more effective than genistein in this regard. Statistical analysis indicated that the influence of the compounds on cell viability was significant ($p<0.05$). Cell viability was 86.9%, 73.9%, and 36.9% in the presence of genistein at 0.5, 1, and 2 μg/ml, respectively, whereas it was 83.5%, 53%, and 16% in the presence of 2'-HG at the same concentrations (Fig. 4A). At 2 μg/ml, 2'-HG decreased cell viability twice as much as did genistein. Moreover, cell viability in the presence of 2'-HG was significantly decreased with increasing exposure time (Fig. 4B). These results suggest that 2'-HG may have an antiproliferative effect on MCF-7 breast cancer cells.

Discussion

The hydroxylation of an isoflavone is an essential step in the biosynthesis of a biologically active isoflavonoid. In particular, 2'-hydroxylation has been shown to increase the antimicrobial activity of phytoestrogenic isoflavonoids [9, 10, 11].

In this study, we successfully bioconverted genistein to 2'-HG in yeast using the pYES2 I2'H expression vector and increased the yield to 14% by utilizing a potassium phosphate buffer and optimizing the conversion time. The 2'-HG conversion rate decreased as the reaction proceeded beyond 9 h. Using this method, we obtained a greater yield of 2'-HG than has been previously reported, making possible the evaluation of its biological activity.

Bioconverted 2'-HG showed a greater antioxidant capacity than genistein. Genistein is known for its antioxidant activity in biological systems, where it scavenges peroxyl radicals and protects against iron-induced free radical reactions [25, 26]. In previous reports, genistein showed relatively low DPPH radical scavenging activity while showing high ABTS radical scavenging activity [12, 26]. In the present study, 2'-HG exhibited significantly higher DPPH and ABTS free radical scavenging activity than genistein. This result suggests that the presence of a hydroxyl group at the C-2' position of the B ring effectively increased the radical scavenging activity of genistein. In addition, 2'-HG was more cytotoxic to MCF-7 human

breast cancer cells than was genistein. The use of genistein as a chemopreventive agent for breast cancer has been controversial because its estrogen-like structure enables it to potentially interact with ERs, thereby causing either positive or negative effects on the growth of cancer cells [8, 21, 28–30]. Accordingly, we regard 2'-HG as possibly of therapeutic value in preventing human breast cancer because it inhibited cell viability more strongly than genistein. We are in the process of verifying the efficacy of 2'-HG as a chemopreventive agent for breast cancer. Additional studies on the chemopreventive mechanisms of genistein and 2'-HG in breast cancer cells are also in progress.

This is the first report of the development of a method for obtaining a larger quantity of 2'-HG and of the evaluation of its biological activity.

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