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Antioxidant Property of Genistein: Inhibitory Effect on HOCl Induced Protein Degradation, DNA Cleavage, and Cell Death

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Abstract The aim of this study was to investigate the *in vitro* antioxidant profiles of genistein and other isoflavonoids. The reactivity of genistein towards stable radical and reactive oxygen species including $\cdot ABTS^+$, $\cdot O_2^-$, H_2O_2 and HOCl has been investigated, and the effects were compared with other isoflavonoids and antioxidants. All the tested isoflavonoids showed remarkable $\cdot ABTS^+$ scavenging activity and genistein was more potent than BHT and ascorbic acid. Genistein was more effective in scavenging hypochlorous acid than superoxide and hydrogen peroxide. At 10 μM concentrations of genistein and genistin showed about 90% inhibitory effect on HOCl, while BHT and ascorbic acid showed lower than 50% inhibitory effect. Moreover, genistein could inhibit plasmid DNA cleavage, protein degradation and cell death from HOCl attack, while daidzein, BHT and ascorbic acid could not protect them effectively. These results suggest that genistein is a more potent radical scavenger than other isoflavonoids, and it can remarkably reduce cellular damage induced by HOCl.

Keywords: genistein, isoflavonoids, antioxidant, ROS, HOCl

Introduction

Isoflavonoids are plant polyphenolic antioxidants that are found in legumes, especially in soybean and various soybean-based food products (1, 2). Genistein and daidzein are primary isoflavones and the correlations between isoflavones and diseases have been well documented during the last decade.

Genistein (5,7,4'-trihydroxyisoflavone) is one of the naturally occurring isoflavones present at relatively high (3 mg/g) levels in soybeans. Several studies have demonstrated that genistein exerts a protective effect against lipid peroxidation of low density lipoproteins (LDL) (3), and inhibits the expression of tyrosine kinases and proliferation of human cancer cell lines (4, 5). The cardioprotective and antiosteoporosis activities of genistein have also been reported together in conjunction with its antioxidant effect (6-10). It has been proposed that pharmacological activity of genistein may also arise from its antioxidant properties.

Oxidative stress, induced by oxygen radicals, is believed to be a primary factor in inducing various degenerative diseases as well as for the normal process of aging. Reactive oxygen species (ROS) are involved in the course of aging, progression of cancer, cardiovascular disease, diabetes, neurodegenerative disease, osteoporosis, etc. (11, 12). These diseases are closely related to daily food intake, and supplementation of plant-derived antioxidants would help to prevent these diseases.

The objectives of the present study were to determine the radical scavenging properties of genistein and isoflavonoids by *in vitro* assays, to evaluate the specific ROS scavenging activity of genistein on HOCl in order to understand its role in the prevention of ROS-induced damage in human diseases and to elucidate the relationships between the structure of isoflavonoids and its antioxidant activity.

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Materials and Methods

Materials All reagents were of analytic grade. Genistin, genistein (4',5,7-trihydroxyisoflavone), daidzein (4'7-dihydroxyisoflavone), biochanin A, butylated hydroxy toluene (BHT), L-ascorbic acid, nitroblue tetrazolium (NBT), β-nicotinamide adenine dinucleotide reduced form (NADH), phenazine methosulfate (PMS), guaiacol, horseradish peroxidase (HRP), 2,2-azino-bis (3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS), and trolox were purchased from Sigma Chemical Company (St. Louis, MO, USA). Sodium hypochloride was from Aldrich Chemical Company (Milwaukee, WI, USA). Water-soluble tetrazolium, which is the sodium salt of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfate (WST-1), was purchased from Roche (Germany). DMEM, L-glutamine, penicillin, and streptomycin were obtained from Bio-Whittaker (USA). Penicillin-streptomycin, fetal bovine serum (FBS), and trypsin-EDTA were from GIBCOBRL (Grand Island, USA).

Trolox equivalent antioxidant capacity (TEAC) assay This assay is based on the scavenging of the relatively stable ABTS radical ($\cdot ABTS^+$) (13). ABTS radical was generated by the incubation of 7 mM ABTS with 2.5 mM

potassium persulfate in dark at room temperature for 12-16 h. The ABTS solution was diluted to 60 μM using a molar extinction coefficient of •$ABTS^+$ at 734 nm (ε = 15 mM^{-1}/cm^{-1}). The •$ABTS^+$ solution in water (2.5 ml) was mixed with the tested compounds, and after 15 min the absorbance of •$ABTS^+$ was measured at 734 nm. TEAC (Trolox Equivalent Antioxidant Capacity) values were calculated from the slope of a plot (A_0/A_i)-1 versus the compound concentration at (A_0/A_i)-1 = 1, where A_0 is the absorbance in the absence of tested compound and A_i is the absorbance in the presence of tested compounds.

PMS-NADH assay (superoxide radical scavenging effect) Superoxide radicals (•O_2^-) were generated non-enzymatically in a phenazine methosulfate-NADH system (14). The reaction mixture of 20 μM phenazine methsulfate, 78 μM NADH and 50 μM NBT in 0.1 M phosphate buffer (pH 7.4) was incubated with each of the samples. After 3 min of incubation at room temperature, the generation of superoxide radicals was detected at 540 nm by using a microplate reader (VERSAmax, Molecular Device, Seoul, Korea).

Guaiacol assay (hydrogen peroxide scavenging effect) Generation of hydrogen peroxide was measured by the formation of brown color in reaction mixture containing 150 mM potassium phosphate buffer, 0.2% (v/v) guaiacol solution, 10 μl of Sigma horseradish peroxidase (1,500 U/ml), isoflavonoids and other compounds. The absorbance change was detected at 436 nm after guaiacol solution was incubated with 10 mM H_2O_2 for 30 min at room temperature (15).

Chlorination of taurine (HOCl scavenging effect) Sodium hypochlorite (NaOCl) was used to determine the HOCl scavenging activity (16). The mixture containing 600 mM NaOCl, 2 M potassium iodide and 150 mM taurine were incubated for 20 min at 37°C and the absorbance was measured at 350 nm to monitor the HOCl scavenging activity.

Inhibition of protein degradation Human serum albumin (HSA) was used as a model protein to assay the antioxidant activity of selected substances against HOCl. Ten il of HOCl was added to 100 mM sodium phosphate buffer (pH 7.0) with 0.05% (w/v) HSA. After incubation for 15 min at room temperature, the protein in the reaction mixture was analyzed by 7.5% polyacrylamide gel electrophoresis (17).

Inhibition of plasmid DNA strand breakage We modified previously reported method to induce DNA cleavage by HOCl (18). pRSET-B DNA was incubated at 37°C for 15 min with the reaction mixture containing 1 μl of samples (negative control was DMF), and 50 μM of HOCl in PBS (pH 7.4). The mixture was then analyzed by electrophoresis on a 1% agarose gel. After staining in ethidium bromide (EtBr) for 10 min, DNA band was detected under the UV lamp.

Cell culture and cell survival assay Human HeLa (ATCC CCL-2) cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO/BRL), supplemented with 10% fetal bovine calf serum (FBS; GIBCO/BRL). About 10^5 cells in each well were incubated with various concentrations of genistein and other compounds. After treating HeLa cells with 500 μM HOCl, WST-1 dye was added to each well, and the absorbance change was detected at 405 nm with microplate reader (19)

Preparation of hypochlorous acid (HOCl) HOCl was prepared immediately prior to use by adjusting NaOCl to pH 6.2 with diluted H_2SO_4. The concentration of HOCl was calculated by using molar extinction coefficient of 142 $M^{-1}cm^{-1}$ at 291 nm (17).

Statistical analysis Data are presented as the means ± S.D. All statistical tests were performed using SPSS for windows (version 13). Statistical analysis of group differences was examined by using the non parametric Mann-Whitney-*U* test. *P* values of <0.05 were considered to be significant.

Results and Discussion

Determination of antioxidant activity using ABTS radical To determine the antioxidant activity of isoflavonoids, ABTS stable radical was used (Table 1). The reactivity of isoflavonoids with •$ABTS^+$ radical indicates hydrogen donation capacity of isoflavonoids and this method is used for the evaluation of antioxidant activity (20).

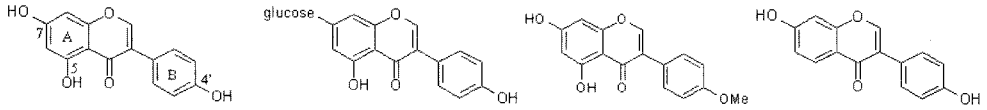
Genistein showed significant stable radical scavenging activity among other tested isoflavonoids and anti-oxidants. TEAC value is expressed as a millimolar concentration of a trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of tested compounds, and this value is used as an index of antioxidant capacity (13). The calculated TEAC value (0.79) of genistein signifies that though genistein is not as effective as trolox, but is the most effective scavenger of stable radical amongst the other tested isoflavonoids (Table 1).

The structure of genistein is similar to the other isoflavonoids such as daidzein and biochanin A (Fig. 1). The results showed that aglycon conferred more hydrogen

Table 1. Antioxidant capacity of genistein and other tested compounds by using trolox equivalent antioxidant capacity (TEAC) value

Compounds	TEAC (mM)[1)]
Genistin	0.65 ± 0.01**
Genistein	0.79 ± 0.02**
Biochanin A	0.49 ± 0.03**
Daidzein	0.72 ± 0.02**
Butylated hydroxy toluene (BHT)	0.95 ± 0.02**
Ascorbic acid	0.81 ± 0.01**

[1)]TEAC, millimolar concentration of a trolox solution having the anti-oxidant capacity equivalent to a 1.0 mM solution of genistein and other compounds.
Each data represents the mean ± standard deviation, n=3. **, $p < 0.01$ compared with DMF treated group



Genistein Genistin Biochanin A Daidzein

Fig. 1. Chemical structures of genistein and other isoflavonoids tested in the present study.

donation capacity than its glucoside. Genistein, aglycon of genistin, showed higher antioxidant activity than genistin. Also, as genistein has three -OH groups, it exhibited more potent antioxidant activity when compared with the other isoflavonoids. Next to genistein was daidzein which has two -OH groups in showing antioxidant activity. While genistin, daidzein and biochanin A have same number of -OH group, only biochanin A has 4'-methoxyl group instead of 4'-hydroxyl group in B ring and genistin is a glucoside not an aglycon (Fig. 1). Thus, it is conceivable that the number of -OH groups and the position of -OH groups in these isoflavonoids is an essential factor for their antioxidant activity (21).

Superoxide radical ($\cdot O_2^-$) scavenging activity Superoxide radical is naturally generated from electron transport chain of mitochondria, and superoxide as itself is directly not so toxic to cellular component, but it can react with H_2O_2 and HOCl and generate highly toxic $\cdot OH^-$ (11). Genistein showed superoxide radical scavenging activity depending on its concentration (data not shown). Genistein, genistin and daidzein showed about 40% scavenging activity at 100 μM of concentration, but biochanin A had no effect on $\cdot O_2^-$ (Table 2). This is because biochanin A has a methoxyl group in B ring instead of hydroxyl group (21). The number of -OH groups in B ring is an important factor for determining the superoxide radical scavenging activity, as has been reported previously (9, 11). It was found that genistein provided a stronger superoxide scavenging activity than other antioxidants, such as BHT and ascorbic acid.

Hydrogen peroxide (H_2O_2) scavenging activity Independently, hydrogen peroxide does not bring about any damage to cellular components, but it can react with superoxide radical, Fe^{2+} and Cu^+ to produce $\cdot OH^-$ (11). Hydroxyl radical is highly unstable and it can cause damage to DNA, proteins, lipids, etc. Also, high concentration of H_2O_2 has been found to induce apoptosis in many cells. ERK and JNK get activated by H_2O_2 and are involved in modulating nuclear accumulation and transcriptional activity of NF-kappa B (22, 23), and it is also related in TNF alpha pathway (24).

Hydrogen peroxide scavenging activities of isoflavonoids were found to be similar to superoxide radical, except daidzein (Table 2). Among the isoflavonoids, daidzein exclusively showed lower effect than BHT. However genistin and genistein showed similar activity with ascorbic acid. It might be considered that not only -OH group in B ring plays an important role to scavenge hydrogen peroxide but also hydroxyl group in A ring does play a crucial role (21). Genistein and genistin have significant H_2O_2 scavenging capacity and they might play a role in signal transduction pathway in apoptotic cells and protect cellular components from H_2O_2 induced toxicity.

Hypochlorous acid (HOCl) scavenging activity Hypochlorous acid (HOCl) is the most abundant ROS generated by phagocytic cells, and it may be the major mediator of inflammatory tissue damage (25). HOCl is a highly reactive compound that can oxidize and chlorinate biomolecules, and consequently damage the surrounding tissue by altering normal cellular function (25).

Genistein was the most potent scavenger of HOCl among the other isoflavonoids and antioxidants used for this assay (Table 2). Genistein had more than 80% of relative inhibition at 10 μM concentration (Table 2). All the tested isoflavonoids in this assay showed remarkable HOCl scavenging activities, while BHT and ascorbic acid showed lower than 40% of relative inhibition. In terms of HOCl scavenging, the number of oxidizing groups like -OH and methoxyl group are more important than the position of hydroxyl group in B ring. Some reports have stated that methoxyl and OH groups can be chlorinated by HOCl (10, 11). Daidzein has only two oxidizing groups to perform about 45% inhibitory function, while the other isoflavonoids have more than two oxidizing groups with higher scavenging activity.

Table 2. ROS scavenging activity of genistein and other tested compounds

Compounds	Scavenging effect(%)[1)]		
	$\cdot O_2^-$	H_2O_2	HOCl
Genistin	37.5 ± 6.2*	33.2 ± 5.3*	76 ± 1.4**
Genistein	38.3 ± 11.2*	34 ± 3.5*	86.5 ± 2.9**
Biochanin A	0.0 ± 2.6	11.72 ± 7.1	83 ± 6.1**
Daidzein	41.8 ± 1.7*	3.02 ± 1.5	45 ± 2.4*
Butylated hydroxy toluene (BHT)	7.5 ± 0.9	7.49 ± 1.4	18 ± 9.4
Ascorbic acid	12.1 ± 2.4	36 ± 3.2*	40 ± 4.6*

[1)]Scavenging effect(%) is the scavenging percentage of $\cdot O_2^-$, H_2O_2 at the test concentration of 100 μM and the scavenging percentage of HOCl at the test concentration of 10 μM.
Each data represents the mean ± standard deviation, n=3. *, $p < 0.05$ and **, $p < 0.01$ compared with DMF treated group

Inhibition of protein degradation To investigate the protein protection capacity of genistein against HOCl attack, human serum albumin (HSA) was used as a model protein. Genistein, at concentration levels higher than 100 μM showed protective effect on HSA from HOCl (Fig. 2A). Even at 1 mM levels, genistein had no toxic side effect on HSA even at. At the concentration level of 500 μM, genistin, genistein and biochanin A scavenged HOCl effectively, however BHT could not protect HSA from HOCl attack (Fig. 2B). Biochanin A also was evaluated as a HOCl scavenger and it could protect albumin protein, but daidzein and ascorbic acid partially inhibited protein degradation against HOCl damage. Thus, the number of OH and other oxidizing groups like methoxyl groups present in the scavenger could be an essential factor for scavenging HOCl (21).

Inhibition of plasmid DNA strand breakage To determine the inhibitory effect of genistein on DNA strand cleavage caused by HOCl, pRSET-B plasmid DNA was incubated with 50 μM HOCl and various concentrations of genistein for 15 min. Genistein protected the cleavage of plasmid DNA in a concentration dependent manner (Fig. 3A). When 1 μM of genistein was treated with DNA, the band was at the same position as that of the linear size DNA (2.9 Kb), while the bands represented open circular form at 10-100 μM of genistein. However, there was only supercoiled form present when over than 500 μM of genistein was treated with DNA and hence genistein protected DNA perfectly against HOCl attack. When genistin, genistein and biochanin A were treated with reaction mixture, there was no DNA strand breakage, whereas BHT and ascorbic acid could not protect DNA from damage (Fig. 3B). Daidzein partially inhibited plasmid DNA breakage because of its number of oxidizing group. The treatment of over than 100 μM of genistein alone with plasmid DNA without HOCl brought about no DNA strand breakage (data not shown). Similarly, inhibitory effect of genistein on H_2O_2/Cu(II) induced plasmid DNA strand breakage was also determined (18). In the previous study, genistein alone did not induce any DNA strand breakage and it inhibited DNA damage induced by hydroxyl radical in a concentration dependent manner.

Inhibition of cellular damage After genistein was treated with HeLa cells and incubated with 500 μM of HOCl for 12 h, cell viability was determined by WST-1 assay. Genistein protected cells from HOCl attack in a concentration dependent manner (Fig. 4A). At 500 μM of genistein, cell viability was over 60% and genistein showed the most powerful cellular protective effect against HOCl amongst the other tested isoflavonoids and antioxidants (Fig. 4B). Biochanin A had higher activity than genistin and lower than genistein. Interestingly, BHT and ascorbic acid slightly inhibited protein degradation, DNA strand breakage and scavenged HOCl in the chlorination of taurine assay, but they had no effect on HOCl-induced cell death. When over than 100 μM of genistein was incubated with HeLa cells for 24 h, it showed cytotoxic activity (data not shown). However, when it was incubated with HOCl together in cultured media, genistein did not induced cell death but lead to cell survival (Fig. 4A). In some of the previous reports, it has been reported that genistein can cause cytotoxic effect at high doses. Intracellular genistein may inhibit NF-κB activation and cell growth (26-28). At 1 mM concentration, of genistein, percentage of cell death was increased due its cytotoxicity. These results suggest that extracellular direct interaction of genistein with HOCl is the possible mechanism in bringing about the protection of HeLa cells from cell death.

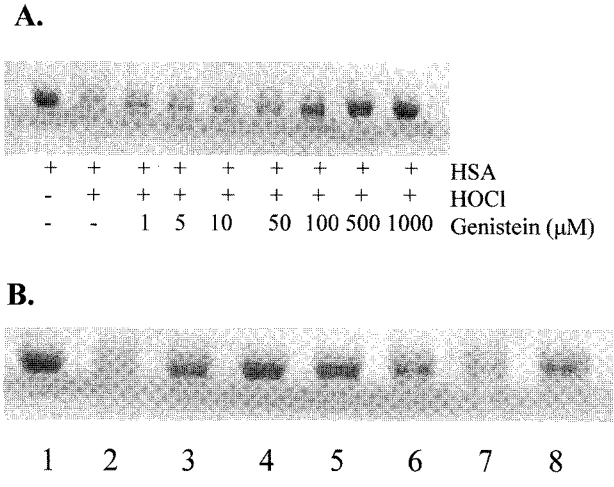


Fig. 2. Protective effect of genistein on protein degradation induced by hypochlorous acid. From 1 μM - 1 mM of genistein was incubated with 100 mM sodium phosphate buffer (pH 7.0), 0.05% (w/v) HSA, 100 μM HOCl at room temperature for 15 min, and 7.5% SDS-polyacrylamide-gel electrophoresis was performed (A). Inhibitory effect of genistein on HOCl induced HSA damage was compared with the other compounds at concentration level of 500 μM (B). Lanes are represented as follows: 1: HSA, 2: HSA + HOCl, 3: genistin, 4: genistein, 5: biochanin A, 6: daidzein, 7: BHT, 8: ascorbic acid.

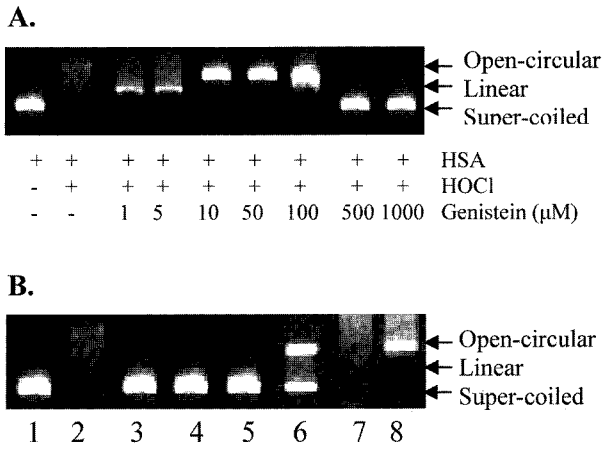


Fig. 3. Protective effect of genistein on plasmid DNA cleavage induced by hypochlorous acid. Plasmid DNA was incubated with 50 μM HOCl and 1 μM - 1 mM genistein for 5 min, and analyzed by 1% agarose gel electrophoresis (A). Inhibitory effect of genistein on HOCl induced plasmid DNA damage was compared with the other compounds at concentration level of 50 μM (B). Lanes are represented as follows: 1: DNA, 2: DNA + HOCl, 3: genistin, 4: genistein, 5: biochanin A, 6: daidzein, 7: BHT, 8: ascorbic acid.

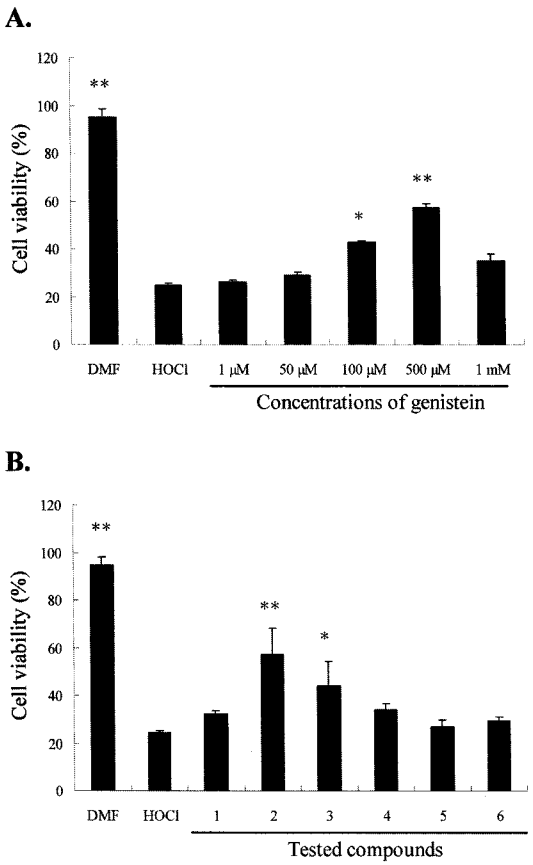


Fig. 4. Protective effect of genistein on cellular damage induced by hypochlorous acid. HeLa cells were incubated with various concentrations of genistein (1 μM - 1 mM) (A) and the protective activity was compared with the other compounds at concentration level of 500 μM (B). After treatment of HeLa cells with tested compounds and HOCl for 12 h, WST-1 dye was added to each well to determine the cell viability. Bars are represented as: 1: genistin, 2: genistein, 3: biochanin A, 4: daidzein, 5: BHT, 6: ascorbic acid. Each bar represents the mean ± standard deviation, n=3. *, $p < 0.05$ and **, $p < 0.01$ compared with HOCl treated group.

In this study, genistein was developed as a novel HOCl scavenger. While IC_{50} value of genistein against $\cdot O_2^-$ and H_2O_2 could not be reached at the highest concentration, IC_{50} against HOCl was 5.05 ± 0.88 (μM) (Table 3). Hypochlorous acid (HOCl), the most abundant end product of respiratory burst derived oxygen metabolites is generated by phagocytic cells and may be the major mediator of inflammatory tissue damage (25, 29). Also, it is a highly reactive compound that can oxidize, chlorinate biomolecules, and consequently damage surrounding tissues by altering normal cellular function (29). It has been demonstrated that the genistein, daidzein, and biochanin A can be chlorinated by hypochlorous acid (30). In previous studies, genistein has been reported to inhibit HOCl induced lysis of human erythrocytes (31), and another group reported that increasing consumption of genistein is associated with better lung function in patients with asthma (32). Because HOCl is much correlated with inflammatory diseases (21), genistein can contribute to the inhibitory action of inflammatory diseases.

Table 3. IC_{50} value of genistein against several ROS

ROS	IC_{50} (μM)[1)]
•ABTS+	1.9 ± 0.04
$\cdot O_2^-$	>100 (203.2 ± 9.3)
H_2O_2	>100 (218.9 ± 11.2)
HOCl	5.05 ± 0.88

[1)]When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. Data are presented as means ± S.D., n=3.

As described above, the antioxidant behavior of isoflavonoids is related to the structure of the compound. The number and the position of hydroxyl groups and oxidizing groups for electron donation are the keys for the activity of antioxidants. These results showed that the number of oxidizing groups like -OH and methoxyl groups in their structure is the critical factor for HOCl scavenging activity, because -OH and methoxyl groups could be chlorinated by HOCl (30). The chlorination of three -OH groups in genistein is a possible mechanism of antioxidant activity in HOCl-induced protein degradation, DNA strand cleavage and cell death. Likewise, the chlorination of two hydroxyl groups and one methoxyl group in Biochanin A might provide same mechanism as genistein.

In conclusion, our results demonstrated that genistein showed inhibitory effect on several major ROS as previously reported, but genistein was also evaluated as a potent HOCl scavenger and it could protect proteins, DNA and live cells from HOCl attack. This study implies the possibility of therapeutic effect of genistein on inflammatory diseases. Also, the intake of soy containing food might contribute positive effect in allergic patients. Further studies are necessary to elucidate anti-inflammatory activity of genistein at the cellular and molecular levels.
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