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Biotransformation of Ginsenoside Rb1, Crocin, Amygdalin, Geniposide, Puerarin, Ginsenoside Re, Hesperidin, Poncirin, Glycyrrhizin, and Baicalin by Human Fecal Microflora and Its Relation to Cytotoxicity Against Tumor Cells

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To understand the role of intestinal microflora in the biological effect of functional herbs, which have been used in Korea, Japan, and China as traditional medicines, and suggest new bioactive compounds transformed from herbal constituents, the metabolic activities of the functional herb components (ginsenoside Rb1, crocin, amygdalin, geniposide, puerarin, ginsenoside Re, poncirin, hesperidin, glycyrrhizin, and baicalin) toward their bioactive compounds (compound K, crocetin, benzaldehyde, genipin, daidzein, ginsenoside Rh1, ponciretin, hesperetin, 18b-glycyrrhetic acid, and baicalein) were measured in fecal specimens. The metabolic activities of these components were 882.7±814.5, 3,938.1±2,700.8, 2,375.5±913.7, 1,179.4±795.7, 24.6±10.5, 11.4±10.8, 578.8±206.1, 1,150.0±266.1, 47.3±58.6, and 12,253.0±6,527.6 μmol/h/g, respectively. No differences were found in the metabolic activities of the tested components between males and females, although these metabolic activities between individuals are extensively different. The metabolites of functional herb components showed more potent cytotoxicity against tumor cells than nonmetabolites. These findings suggest that intestinal microflora may activate the pharmacological effect of herbal food and medicines and must be the biocatalytic converter for the transformation of herbal components to bioactive compounds.

Keywords: Intestinal microflora, metabolism, functional herb, cytotoxicity, biotransformation

All individuals possess their own characteristic indigenous strain of intestinal microflora, which are thought to be rather stable over time within individuals in the absence of disease and antimicrobial therapy [15, 20, 25, 28]. Kobashi *et al.* [15] reported that intestinal bacteria between individual Japanese were not significantly different, but some of their enzyme activities were significantly different. The fecal metabolic activities of herbal components are variable among individuals [32]. Nevertheless, the intestinal bacterial enzyme activities are not associated with specific populations [12]. These fecal bacterial enzyme activities are affected by diet [11, 24, 28], but rebound if diet or supplements were stopped for a short term [19, 21].

Functional herbs have been used in China, Korea, and Japan as traditional medicines. If these herbs are orally administered to humans, their components are inevitably brought into contact with intestinal microflora in the alimentary tract and transformed before their absorption from the gastrointestinal tract [14, 16, 26, 27]. For example, when ginsenoside Rb1, which is a major component of ginseng, is administered to humans or rats, it is transformed by intestinal microflora 20-*O*-β-D-glucopyranosyl-20(*S*)-protopanaxadiol (compound K), which shows more potent antiallergic and antiinflammatory effects than ginsenoside Rb1 (Fig. 1), and the compound K is subsequently absorbed [1, 2, 30]. Ginsenoside Re of ginseng, crocin and geniposide of *Gardeniae fructus*, amygdalin of *Armeniacae semen*, puerarin of *Puerariae Radix*, poncirin of *Ponciri fructus*, hesperidin of *Aurantii pericarpium*, glycyrrhizin of liquorice, and baicalin of *Scutellariae Radix* are also transformed by intestinal microflora prior to their absorption from the gastrointestinal tract, and it is the transformed metabolites that exhibit pharmacological actions [3, 5, 8, 17, 24, 29, 31]. The metabolites also exhibit biological activities, such as antiviral, antibacterial, antiallergic, and antioxidant activities [5, 13, 17, 23]. Therefore, intestinal bacteria related to the metabolism of the herbal components should be an

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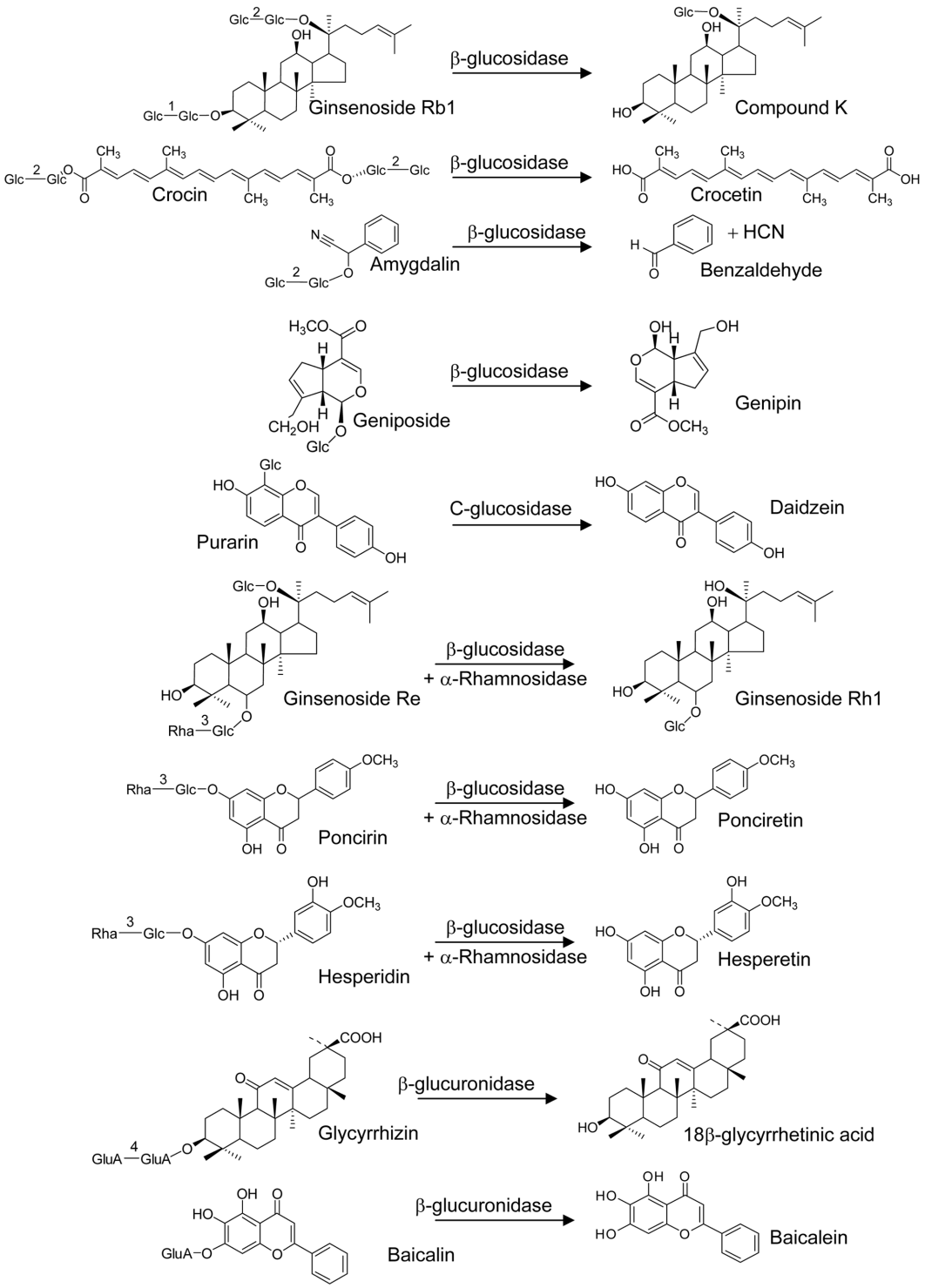

Fig. 1. Metabolic pathways of natural product components by intestinal microflora and their catalyzing enzymes.
Glc, D-Glucose; GluA, D-glucuronic acid; Rha, L-Rhamnose. Arabic numbers indicate the linkages bound between glycosides as follows: 1, β-D-Glc-(1→2)-β-D-Glc; 2, β-D-Glc-(1→6)-β-D-Glc; 3, α-L-Rha-(1→2)-β-D-Glc; 4, β-D-GluA(1→2)-β-D-GluA.

important factor in understanding the biological activities of functional herbs.

Related to these pharmacological effects of some functional herbs, fecal bacterial enzymatic activities have previously been reported [18, 19]. However, the metabolism of many herbal constituents has not been thoroughly studied.

Therefore, the metabolic activities of several herbal components by human fecal microflora were determined,

and the relationship between the metabolic activities and the cytotoxicity of their metabolites against tumor cells investigated.

MATERIALS AND METHODS

Subjects

The subjects were 100 healthy Korean males and females (average, 26.8±6.5 years; 63 males; 37 females). Exclusion criteria included smoking and current medication, especially regular or current use of antibiotics.

Materials

Glycyrrhizin, 18β-glycyrrhetinic acid (GA), hesperidin, hesperetin, baicalin, baicalein, amygdalin, and benzaldehyde were purchased from Sigma Chem. Co. (U.S.A.). Genipin and geniposide were purchased from Wako Pure Chem. Ind. (Japan). Crocin, crocetin, poncirin, ponciretin, ginsenosides Rb1, Re, and Rh1, and compound K were isolated according to a previous method [5–8, 17].

Specimen Preparation

The human fecal specimens (about 3 g), prepared according to a previous method [18], were collected in plastic cups 9 h after fasting, and then carefully mixed with a spatula and suspended with 27 ml of cold saline. The fecal suspension was centrifuged at 100 ×*g* for 5 min. The supernatant was then centrifuged at 10,000 ×*g* for 20 min. The resulting precipitates (about 0.3 g) were used as a metabolic enzyme source for the assay of enzyme activity. The preparation and assay of the enzyme source were performed within 24 h.

Assay of Herbal Medicine Component Metabolic Activities by Human Fecal Microflora

To measure the metabolic activity of herbal medicine components, the above fecal precipitate (0.2 g) was suspended with 1.8 ml of 50 mM phosphate buffer (pH 7.0) and then used in the present experiment. The reaction mixture (1 ml), containing 0.2 ml of the fecal suspension, 0.2 ml of 0.5 mM natural glycosides, and 0.6 ml of 25 mM phosphate buffer (pH 7.0), was incubated at 37°C for 1 h (or 10 h in the case of glycyrrhizin, puerarin, and ginsenoside Re), and the reaction mixture was extracted twice with 5 ml of ethyl acetate, and then evaporated *in vacuo*. The ethyl acetate fraction was dissolved in methanol and then analyzed by TLC.

Thin-Layer Chromatography

TLC for amygdalin, benzaldehyde, crocin, crocetin, genipin, and geniposide was performed on silica gel plates (silica gel 60F-254; Merck, Germany) with a developing solvent system of $CHCl_3$:MeOH=6:1 (v/v). TLC for puerarin, daidzein, poncirin, ponciretin, hesperidin, and hesperetin was performed on silica gel plates (silica gel 60F-254; Merck, Germany) with a developing solvent system of $CHCl_3$:MeOH=5:1 (v/v). TLC for glycyrrhizin and 18β-glycyrrhetinic acid was performed on silica gel plate with $CHCl_3$:petroleum ether: acetic acid=6:6:1 (v/v). TLC for baicalin, baicalein, ginsenosides Rb1, Re, Rh1, and compound K was performed on silica gel plate with $CHCl_3$:MeOH:H_2O=65:35:10 (lower layer, v/v). The chromatograms of these compounds were quantitatively assayed with a TLC scanner (CS-9301PC, Shimadzu Co.) according to a previously described method [6, 18, 19].

The metabolic activities (μmol/h) were defined as the amount required to catalyze the formation of products per hour under the standard assay conditions. Specific activity was defined in terms of the metabolic activities per wet weight (g) of fecal microflora.

In Vitro Cytotoxicity Assay

The *in vitro* cytotoxicity was tested against P388 (mouse lymphoid neoplasma cell line), A549 (human lung carcinoma), and HeLa (human cervix uterine adenocarcinoma) cells by MTT [3-(3,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to a previously described method [9, 13]. Each cultured cell line was harvested, counted, and inoculated at the appropriate concentrations (180 μl volume: 1.5×10^4 cells/well) into a 96-well microtiter plate. P388, A549, and HeLa cells were cultured for 24 h in media with or without fetal bovine serum (FBS) and treated with the samples. These cells were exposed to the test compounds for 5 d at 37°C. Fifty μl of MTT solution (2 mg/ml in PBS) was added to each well and the plates were incubated for 30 min. After aspiration of the medium, DMSO (100 μl) was added to solubilize the MTT-formazan product. The plates were read on a microplate reader (540 nm). The 50% cytotoxic concentration (IC_{50}) was defined by comparing with the control cell culture.

Statistics

The SPSSWin 8.0 program was used for statistical analysis of the data. The differences in fecal enzyme activities between males and females and between ages were assessed by ANOVA.

RESULTS AND DISCUSSION

The subjects studied in the present experiment were 100 healthy persons. To evaluate the role of intestinal microflora in the pharmacological action of herbal medicines, the metabolic activities of their main components, ginsenosides Rb1 and Re of ginseng, crocin and geniposide of *Gardeniae fructus*, amygdalin of *Armeniacae semen*, puerarin of *Puerariae Radix*, poncirin of *Ponciri fructus*, hesperidin of *Aurantii pericarpium*, glycyrrhizin of liquorice, and baicalin of *Scutellariae Radix* in relation to their bioactive compounds compound K, ginsenoside Rh1, crocetin, genipin, benzaldehyde, ponciretin, hesperetin, 18β-glycyrrhetinic acid, and baicalein, by fecal specimens, were investigated (Table 1). The metabolic activities of these compounds in relation to the bioactive compounds were 0–5,084.6, 0.3–54.5, 2.5–8,992.8, 0–3,442.8, 19.3–3,321.7, 9.0–86.0, 95.6–1,522.0, 350.5–2,262.3, 0.6–385.0, and 3,032.7–38,843.4 μmol/h/g, respectively. These activities were extensively different in individuals, but were not different between males and females. These average metabolic activities (mean±SD) were 882.7±814.5, 3,938.1±2,700.8, 2,375.5±913.7, 1,179.4±795.7, 24.6±10.5, 11.4±10.8, 578.8±206.1, 1,150.0±266.1, 47.3±58.6, and 12,253.0±6,527.6 μmol/h/g, respectively. Of these components,

Table 1. Metabolic activity of herbal constituents to their bioactive compounds in 100 humen subjects.

Component	Activity (μmol/h/g)						
	Highest		Lowest		Mean±SD		
	Male	Female	Male	Female	Male	Female	All
RB	5,084.6	2,448.1	0	165.0	947.6±633.5	844.5±906.7	882.7±814.5
CR	8,457.5	8,992.8	2.5	3.4	3,482.5±2,765.8	4,205.6±2,647.4	3,938.1±2,700.8
AM	3,321.7	3,320.7	60.3	19.3	2,460.8±1,029.9	2,325.5±842.8	2,375.5±913.7
GE	3,143.6	3,442.8	0	0	1,331.8±861.6	1,089.9±746.9	1,179.4±795.7
PU	86.0	37.9	9.5	9.0	25.5±11.5	23.2±8.7	24.6±10.5
RE	54.5	43.7	0.3	1.0	10.3±10.2	16.7±12.4	11.4±10.8
PO	1,522.0	989.3	95.6	321.9	586.3±168.6	574.5±226.4	578.8±206.1
HE	2,262.3	2,078.2	350.5	614.2	1,189.5±365.9	1,126.8±367.1	1,150.0±266.1
GL	385.0	141.3	0.6	1.0	36.7±40.3	78.2±65.4	47.3±58.6
BA	25,178.6	38,843.4	3,032.7	5,418.0	15,090.8±8,231.7	10,586.3±4589.5	12,253.0±6527.6

RB, ginsenoside Rb1; Cr, crocin; AM, amygdalin; GE, geniposide; PU, puerarin; RE, ginsenoside Re; PO, poncirin; HE, hesperidin; GL, glycyrrhizin; BA, baicalin.

baicalin was more potently metabolized to its bioactive component. These variable metabolic activities among individuals may be due to the metabolic enzyme productivity rather than the species difference of intestinal microflora. The suggestion is supported by the report of Kobashi *et al.* [15] that intestinal bacteria between Jitsu-syo and Kyo-syo Japanese are not different, but their enzyme activities, such as β-glucuronidase and urease, are significantly different.

To understand the relationship between the metabolic activities of natural components in individuals, the correlation index and its significance between their metabolic activities were investigated (Table 2). The metabolic potencies of ginsenoside Rb1 in individuals are significantly in proportion to those of geniposide and poncirin. Those of crocin are in proportion to those of amygdalin, geniposide, puerarin, poncirin, hesperidin, and glycyrrhizin. Those of amygdalin are in proportion to those of geniposide, puerarin, poncirin, and baicalin. There of geniposide are in proportion to those of poncirin and hesperidin. Those of puerarin, except crocin and amygdalin, are out of proportion to those of other components tested. These results suggest that the hydrolyzing enzyme(s) of C-glycoside, which is bound of C-glycoside to an aglycone, such as puerarin, seem to be different to those of O-glycosides such as geniposide, crocin, and amygdalin. These results are supported by the report that geniposide-hydrolyzing bacteria and β-D-glucosidase did

Table 2. Correlation indexes and their significance among fecal metabolic activities of herbal constituents.

	RB	CR	AM	GE	PU	RE	PO	HE	GL	BA
RB	–									
CR	0.067	–								
AM	0.115	0.441** (0.001)	–							
GE	0.330** (0.001)	0.295** (0.003)	0.288** (0.004)	–						
PU	0.041	0.275** (0.006)	0.219* (0.028)	0.111	–					
RE	−0.068	0.052	0.009	0.149	0.097	–				
PO	0.232* (0.020)	0.323** (0.001)	0.257** (0.010)	0.262** (0.008)	0.168	0.198* (0.048)	–			
HE	0.115	0.337** (0.001)	0.185	0.285** (0.004)	0.096	0.202* (0.044)	0.232* (0.020)	–		
GL	−0.082	0.266** (0.008)	0.061	−0.087	0.075	0.128	0.231* (0.021)	0.074	–	
BA	−0.015	−0.008	0.213* (0.033)	0.156	−0.170	0.169	0.207* (0.039)	0.403** (0.000)	−0.204* (0.042)	

Arabic numbers out and in parentheses indicate correlation index and *p*-values, respectively.
RB, ginsenoside Rb1; Cr, crocin; AM, amygdalin; GE, geniposide; PU, puerarin; RE, ginsenoside Re; PO, poncirin; HE, hesperidin; GL, glycyrrhizin; BA, baicalin.

not hydrolyze puerarin [13, 23, 31]. Those of ginsenoside Re are significantly in proportion to those of poncirin and hesperidin. Those of poncirin are in proportion to those of hesperidin, glycyrrhizin, and baicalin. Herbal rhamnoglycoside components are metabolized to their bioactive compounds by intestinal bacterial β-D-glucosidase and α-L-rhamnosidase in the intestine to express their pharmacological effects [8, 13]. The metabolic activity potencies of rhamnoglycosides, poncirin, hesperidin, and ginsenoside Re in individuals were significantly proportional, even if poncirin is a α1,2-bound rhamnoglycoside, hesperidin is a α1,6-bound one, and ginsenoside Re is a α1,2-bound one. These results suggest that the metabolic activity of these rhamnoglycosides may be mainly dependent on their aglycones. Glycyrrhizin and baicalin, which are glucuronate conjugated in algycones, are metabolized to their bioactive compounds by β-D-glucuronidases in intestinal bacteria [13, 32]. Nevertheless, the glycyrrhizin-metabolic activity potency of individuals was out of proportion to baicalin-metabolic activity. On the contrary, these activity potencies were inversely proportional. These results are supported by the previous reports that glycyrrhizin-metabolic β-D-glucuronidase may be different to the baicalin-metabolic enzyme, although both these compounds are hydrolyzed by β-D-glucuronidases [18, 32]. The former may recognize and hydrolyze β-D-diglucuronide, whereas the latter may recognize and hydrolyze β-D-monoglucuronide. However, those of hesperidin are in proportion to those of baicalin and those of glycyrrhizin are proportional to those of crocin and poncirin. These results suggest that intestinal bacteria metabolizing hesperidin and glycyrrhizin may produce the metabolic enzymes of baicalin and crocin and poncirin, respectively. In addition, Ameer *et al*. [4] and Erlund *et al*. [10] reported that hesperidin and naringin were easily metabolized to hesperetin and naringenin upon the oral administration of *Citrus* juice (hesperidin and naringin) to humans and their metabolites were detected in their plasma and urine. Park *et al*. [22] reported that, when hesperidin or ponicirn was orally administered to rats, the metabolites hesperetin and ponciretin were detected in the plasma and urine. Akao *et al*. [1, 2] reported that when ginsenoside Rb1 was orally administered to conventional, germ-free, and gnotobiotic rats, compound K, not ginsenoside Rb1, was detected in the plasma of conventional and gnotobiotic rats, but ginsenoside Rb1 and compound K were not in the germ-free animals. Based on these reports, if some constituents of natural products were orally administered, these may be metabolized by intestinal microflora and be absorbed into the blood. Therefore, the role of intestinal microflora may be important in the biological activities of the constituents of natural products.

The intestinal bacteria transform glycosides of natural products, such as ginsenoside Rb1, glycyrrhzin, and baicalin, to their bioactive compounds, such as compound K, glycyrrhizin, and baicalein, respectively [5, 13, 17, 23]. These metabolites showed potent pharmacological effects, such as antiinflammatory and antiallergic effects. Therefore, in the present study, the cytotoxic effects of herbal constituents and their metabolites transformed by intestinal bacteria against some tumor cells were investigated (Table 3). Of the tested compounds, compound K, ginsenoside Rh1, 18β-glycyrrhetinic acid, and baicalien showed potent cytotoxic effects. Generally, the metabolites showed more potent cytotoxic activity against tumor cells than nonmetabolized herbal constituents. These results suggest that intestinal bacteria may be the convenient bioreactor, transforming herbal constituents to their bioactive compounds.

These findings suggest that intestinal microflora may activate the pharmacological effect of herbal foods and medicines for intestinal as well as systemic diseases and must be the catalytic converter for the transformation of herbal components to bioactive compounds.

Table 3. Cytotoxicity of herbal constituents and their metabolites against some tumor cells.

Test compound	IC_{50} (mM)		
	P388	A 549	HeLa
Ginsenoside Rb1	>0.1 (32)	>0.1 (21)	>0.1 (31)
Compound K	0.027	0.045	0.040
Glycyrrhizin	>0.1 (21)	>0.1 (15)	>0.1 (19)
18β-Glycyrrhetic acid	0.047	0.075	0.063
Ginsenoside Re	0.093	>0.1 (5)	>0.1 (5)
Ginsenoside Rh1	0.037	0.1 (15)	>0.1 (30)
Geniposide	>0.1 (7)	>0.1 (6)	>0.1 (7)
Genipin	>0.1 (23)	>0.1 (18)	>0.1 (17)
Baicalin	0.038	>0.1	>0.1
Baicalein	0.038	>0.1	0.051
Crocin	>0.1 (18)	>0.1 (11)	>0.1 (17)
Crocetin	>0.1 (36)	>0.1 (45)	>0.1 (46)
Amygdalin	>0.1 (9)	>0.1 (3)	>0.1 (8)
Benzaldehyde	>0.1 (11)	>0.1 (5)	>0.1 (10)
Poncirin	>0.1 (38)	>0.1 (11)	>0.1 (15)
Pociretin	0.1	>0.1 (12)	>0.1 (44)
Hesperidin	>0.1 (23)	>0.1 (9))	>0.1 (14)
Hesperetin	>0.1 (39)	>0.1 (11)	>0.1 (32)
Puerarin	>0.1 (20)	>0.1 (5)	>0.1 (9)
Daidzein	0.018	>0.1 (36)	>0.1 (43)
Adriamycin	0.002	0.011	0.004

IC_{50} indicates 50% cytotoxic concentration, compared with the viability of the non-treated group.
The values in parenthesis indicate the cytotoxic percents at a concentration of 0.1 mM, compared with the viability of the non-treated group.

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