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Comparative Evaluation of Cytotoxicity and Antioxidative Activity of 20 Flavonoids

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Flavonoids are common dietary components with many health benefits shown through epidemiological studies. However, the fact that flavonoids also act as pro-oxidants and mutagens makes the safety of flavonoids uncertain when used at higher doses. To give a preliminary evaluation on the correlation between beneficial and harmful effects of flavonoids, the antioxidative activity and cytotoxicity of 20 flavonoids from food and herbs were investigated in vitro. The results indicated that luteolin, hydroxygenkwanin, and kaempferol possessed significant dual properties, whereas flavokawain B, flavokawain C, cardamonin, and uvangoletin showed a marked cytotoxicity. The relationships between structure and antioxidant and cytotoxic activity are intensively discussed. In view of the toxicity, the intake of flavonoids in large amounts should not yet be encouraged.

KEYWORDS: Flavonoids; antioxidative activity; cytotoxicity; structures; chalcones

INTRODUCTION

Flavonoids are polyphenolic compounds that occur ubiquitously in food from plants (*1*). The daily human intake of flavonoids is quite different in amounts and classes due to various feeding habits. For example, the consumption of flavonols and flavones has been estimated to be approximately ~20−22 mg/day in the U.S. (*2*), whereas in Italy, the mean value was 35 mg/day (*3*). Epidemiological and pharmacological studies have shown that the intake of flavonoids is associated with many beneficial effects, such as antioxidative (*4*), antiviral (*5*), antitumor (*6*), anti-inflammatory (*7*), and hepatoprotective activities (*8*) and the prevention of cardiovascular diseases (*9*). In particular, the antioxidative activity has gained the most interest, and the biological effects of flavonoids are believed to come, in large part, from their antioxidative properties (*10*).

In contrast to beneficial effects, flavonoids also have been found to be pro-oxidant or mutagenic and to produce toxicity (*11, 12*). Interestingly, these contradictory activities of flavonoids may depend on the same determinants in chemical structures (*13, 14*), which may make the beneficial effects of flavonoids remain uncertain regarding the conditions and concentrations of the flavonoids used.

Considering the fact that flavonoids are present in considerable quantities in common food products, spices, beverages, as well as herbal medicine, and if flavonoids are used as therapeutic agents or functional food ingredients, even higher amounts may be ingested, it is extremely important to understand the relationships between beneficial and harmful effects of flavonoids, as well as the role of structures on these effects. This may be useful information for the safe use and structural modification of flavonoids.

In this paper, 20 specific and structure-related flavonoids including seven flavones, two flavanones, one isoflavone, two flavonols, four chalcones, one dihydrochalcone, and three glycosides (**Figure 1**) were selected as candidates to examine cytotoxicity (toxic effects) and antioxidative activity (protective effects) in vitro. These flavonoids are common in vegetables, fruits, spices, or medicinal herbs and are representative in structure. For example, quercetin and kaempferol are the main representatives of flavonols, which are the most ubiquitous flavonoids in foods and richest in onion (*15*). Luteolin, a flavone, is found in high concentrations in onion, celery, green pepper, and broccoli (*16*). The chalcones flavokawain A, B, and C are mainly in kava, which has been used for thousands of years in the Pacific islands, served as a beverage and as medication (*17*). Besides being common in food, many of these flavonoids possibly may be used as therapeutic agents or as functional food supplements, in the future based on related beneficial reports, and will be ingested at higher doses, such as quercetin, genistein, apigenin, and cardamonin (*18–21*). In view of safety, experiments were conducted to preliminarily evaluate the correlation between beneficial activities and harmful effects and structures of the 20 flavonoids in vitro. Cytotoxicity was investigated upon the inhibition of liver cell proliferation on L-02 (human normal hepatocytes) and HepG2 (human hepatoma cells)

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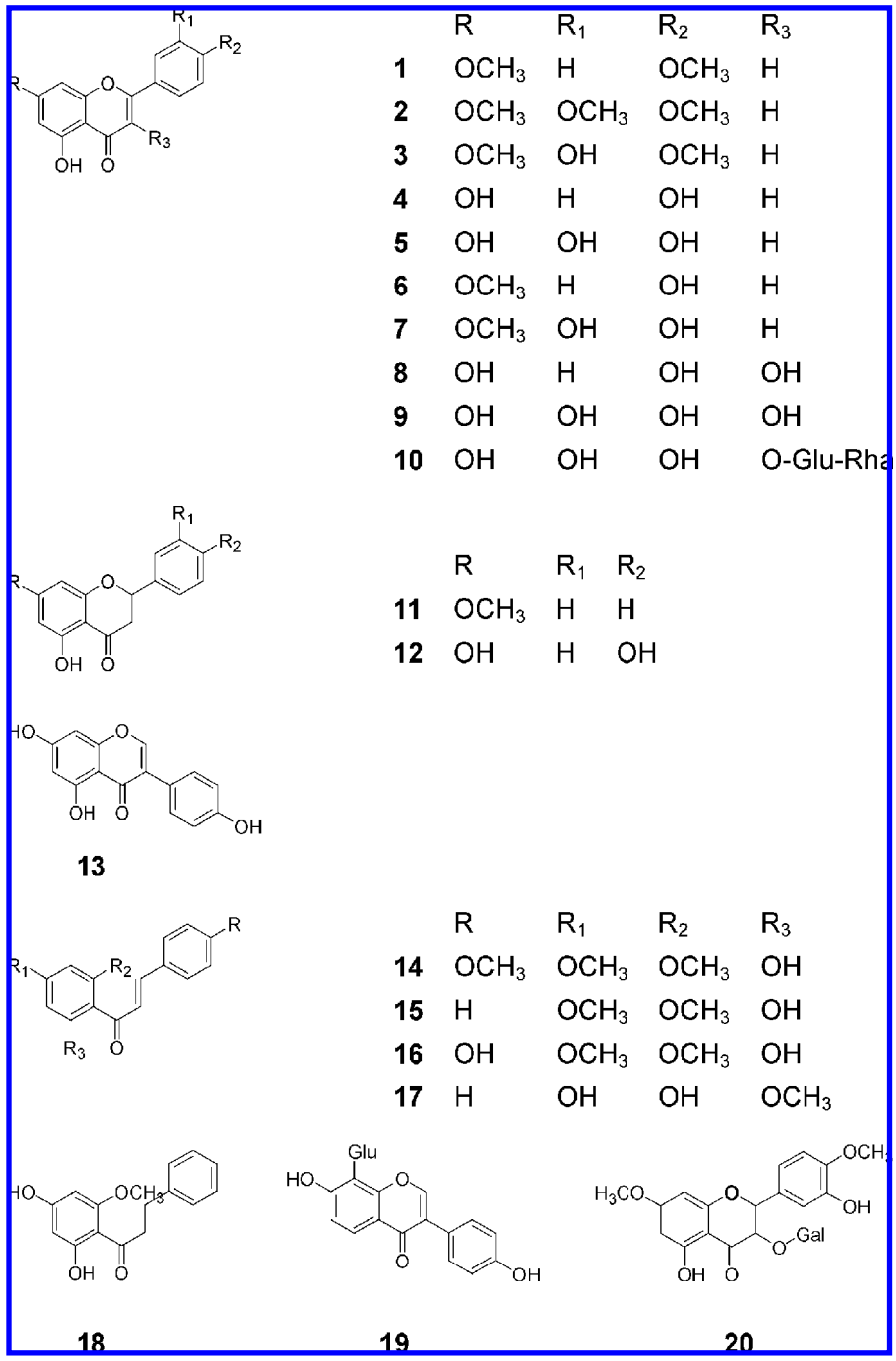

Figure 1. Chemical structures of flavonoids **1**−**20**.

since the liver is the central organ in the metabolism and detoxification of drugs, which affect the liver more frequently than any other organ and place the liver at increased risk for toxic damage (*22*), while the antioxidative activity was quantified from the scavenging ability of the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^{\bullet}$) and the superoxide anion ($O_2^{\bullet -}$). The different cytotoxic effects of flavonoids on L-02 and HepG2 also are discussed.

MATERIALS AND METHODS

Materials and Chemicals. RPMI 1640 medium, MEM, and FBS were purchased from Invitrogen (Grand Island, NY). 1,1-DPPH$^{\bullet}$, MTT, and 5-FU were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade. Apigenin (**4**), naringenin (**12**), genistein (**13**), kaempferol (**8**), quercetin (**9**), cardamonin (**17**), rutin (**10**), and puerarin (**19**) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and the other chemicals including apigenin-4′,7-dimethyl ether (**1**), luteolin-3′,4′,7-trimethyl ether (**2**), persicogein (**3**), luteolin (**5**), genkwanin (**6**), hydroxygenkwanin (**7**), 5-hydroxyl-7-methoxyl flavanone (**11**), flavokawain A (**14**), flavokawain B (**15**), flavokawain C (**16**), uvangoletin (**18**), and 3,5,3′-trihydroxy-7,4′-dimethoxyflavone-3-*O*-β-D-galactopyranoside (**20**) were obtained from our department. The purity of the chemicals was analyzed by HPLC and found to be at least 98% pure.

Cell Lines and Culture. Human normal liver cells L-02 and human hepatoma cells HepG2 were obtained from the Cell Bank of Type Culture Collection of Chinese Sciences (Shanghai, China). L-02 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.0 g/L sodium bicarbonate. HepG2 cells were cultured in MEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 1.5

Table 1. Cytotoxicity and Radical-Scavenging Activity of Various Flavonoids

class	compound	IC_{50}[a] (μM)		EC_{50}[a] (μM)	
		L-02	HepG2	DPPH·	$O_2^{\cdot-}$
flavones	apigenin-4′,7-dimethyl ether (**1**)	n.c.[b]	n.c.	n.c.[b]	n.c.
	luteolin-3′,4′,7-trimethyl ether (**2**)	n.c.	n.c.	n.c.	n.c.
	persicogein (**3**)	n.c.	n.c.	n.c.	48.55 ± 7.19
	apigenin (**4**)	30.29 ± 2.95	79.77 ± 3.13	n.c.	46.12 ± 3.29
	luteolin (**5**)	31.18 ± 3.34	78.95 ± 4.46	8.57 ± 0.11	5.04 ± 0.21
	genkwanin (**6**)	70.43 ± 4.70	n.c.	n.c.	43.47 ± 1.85
	hydroxygenkwanin (**7**)	39.67 ± 0.07	41.35 ± 1.98	14.85 ± 0.36	2.50 ± 0.12
flavonols	kaempferol (**8**)	57.05 ± 5.89	84.72 ± 8.53	24.27 ± 1.11	0.76 ± 0.08
	quercetin (**9**)	113.03 ± 6.02	188.84 ± 12.40	6.15 ± 0.03	0.87 ± 0.01
flavanones	5-hydroxyl-7-methoxyl flavanone (**11**)	143.88 ± 9.39	n.c.	n.c.	n.c.
	naringenin (**12**)	93.26 ± 3.58	n.c.	n.c.	n.c.
isoflavone	genistein (**13**)	50.08 ± 3.01	135.95 ± 8.19	n.c.	84.36 ± 2.86
chalcones	flavokawain A (**14**)	n.c.	n.c.	n.c.	n.c.
	flavokawain B (**15**)	35.15 ± 2.56	62.38 ± 5.04	n.c.	n.c.
	flavokawain C (**16**)	57.04 ± 2.32	59.48 ± 2.72	n.c.	52.94 ± 4.84
	cardamonin (**17**)	30.90 ± 4.03	22.63 ± 3.00	n.c.	n.c.
dihydrochalcone	uvangoletin (**18**)	9.69 ± 3.96	55.61 ± 5.35	n.c.	n.c.
glycosides	rutin (**10**)	n.c.	n.c.	4.49 ± 0.16	5.13 ± 0.08
	puerarin (**19**)	n.c.	n.c.	n.c.	n.c.
	3,5,3′-trihydroxy-7,4′-dimethoxyflavone-3-*O*-β-D-galactopyranoside (**20**)	190.52 ± 1.17	n.c.	n.c.	n.c.

[a] Results are expressed as mean ± SD of three experiments. [b] Effects can be neglected at tested concentrations.

g/L sodium bicarbonate. The cells were maintained in logarithmic phase under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Cytotoxicity Assay. The cytotoxicity was determined according to the method of Hsu et al. with slight changes (*23*). Cells were seeded in 96-well plates at a density of 10^4 per well for 24 h and then treated with various concentrations of flavonoids or 5-FU (as positive control), which were dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI 1640 or MEM medium without serum. The DMSO in culture medium never exceeded 0.1% (v/v), a concentration known not to affect cell proliferation. The medium solution was removed after 48 h of cultivation at 37 °C with 5% CO_2 and 95% air. An aliquot of 100 μL of RPMI 1640 or MEM medium containing 0.5 mg/mL MTT was reloaded onto the plate, and the cells were cultured for another 3 h. Then, the medium solution was removed, and an aliquot of 150 μL of DMSO was added to the plate. After shaking for 10 min at room temperature, the cytotoxicity was determined by measuring the absorbance of the converted dye at 570/650 nm in a microplate reader (Tecan, Austria).

DPPH Assay. To measure antioxidant activity, the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging assay was carried out according to a procedure described in ref *24* with a little modification. Briefly, the DPPH radical-scavenging activity was measured in a reaction mixture containing 0.1 mL of 0.2 mM DPPH radical solution and 0.1 mL of each one of the tested compounds (all were dissolved in absolute ethanol). The control was measured by using ethanol to replace the flavonoids in the reaction solution. The solution was rapidly mixed and incubated for 30 min in a water bath at 25 °C. Then, the scavenging capacity was measured by monitoring the decrease in absorbance at 517 nm. The DPPH radical-scavenging activity was calculated according to the following formula:

$$\text{DPPH}^{\bullet}\ \text{scavenging activity}\ (\%) = [1 - A_{\text{sample}}/A_{\text{control}}] \times 100\% \quad \text{(a)}$$

The antioxidative activity was expressed as EC_{50}, which was defined as the concentration of the compounds required for inhibition of the formation of DPPH radicals by 50%.

Superoxide Anion Assay. The superoxide anion ($O_2^{\cdot-}$) scavenging ability of flavonoids was determined by the pyrogallol autoxidation system according to a method described previously (*25, 26*). The reaction mixture contained 20 μL of pyrogallol (1 mM), 970 μL of solution mixed with luminol (1 mM) and bicarbonate buffer (pH 10.2), and 10 μL of flavonoid samples (0.1, 0.5, 1, 2.5, 5, and 10 mM) dissolved in DMSO. The sample without flavonoids was recorded as the control, and the final volume was always the same (1 mL) for all assays. The intensity of chemiluminescence (CL) was simultaneously recorded and processed by a BPCL-1-G-C instrument and BPCL Appl.7.2 software. The scavenging activity of $O_2^{\cdot-}$ by flavonoids was calculated according to the following formula:

$$O_2^{\bullet-}\ \text{scavenging avtivity}\ (\%) = [1 - \text{CL}_{\text{sample}}/\text{CL}_{\text{control}}] \times 100\% \quad \text{(b)}$$

The EC_{50} value, that is, the concentration of sample required to cause 50% inhibition of $O_2^{\cdot-}$, was estimated.

Statistical Analysis. All experiments were conducted for three independent replicates at least. The data are expressed in terms of mean and standard deviation. The experimental data were analyzed using Microsoft Excel software (Microsoft Software Inc.).

RESULTS

Cytotoxicity on L-02 and HepG2. L-02 and HepG2 were adopted to evaluate the cytotoxic effects of the 20 flavonoids. The results are expressed as IC_{50} values, which were calculated from the dose-survival curves obtained from the MTT assay. According to **Table 1**, **1**−**3**, **10**, **14**, and **19** did not exhibit significant cytotoxic effects on both cell lines under the tested concentrations (20, 40, 60, 80, and 100 μM), while the other compounds showed different cytotoxic behaviors on L-02 or HepG2, and a higher susceptibility was found in L-02 proliferation. In the case of L-02, three flavones, **4**, **5**, and **7**, an isoflavone, **13**, two chalcones, **15** and **17**, and a dihydrochalcone, **18**, showed relatively strong cytotoxic effects ($IC_{50} \leq 50$ μM), and among them, **18** was the most potent cytotoxic agent with an IC_{50} value of 9.69 ± 3.96 μM. Comparatively, **6**, **8**, **12**, and **16** exhibited a moderate cytotoxicity (50 μM < IC_{50} < 100 μM) on L-02, and their cytotoxic sequence was **8**, **16** > **6** < **12**. The cytotoxicity of other compounds containing **9**, **11**, and **20** was relatively weak (IC_{50} >100 μM). In comparison with L-02, the cytotoxic effects of these compounds on HepG2 were different. The chalcone, **17**, and a flavone, **7**, showed a strong cytotoxicity on HepG2 with IC_{50} values of 22.63 ± 3.00 and 41.35 ± 1.98 μM, respectively, while the others including **4**, **5**, **8**, **15**, **16**, and **18** displayed moderate cytotoxic activities (50 μM < IC_{50} > 100 μM). Compounds **9** (188.84 ± 12.40 μM) and **13** (135.95 ± 8.19 μM) only showed tenuous cytotoxicity

at the tested concentrations (20, 40, 60, 80, and 100 μM). Except for **17**, all these compounds showed relatively weaker cytotoxic effects on HepG2 than on L-02.

DPPH$^{\cdot}$ Scavenging Activity. The DPPH radical is a stable organic free radical with an absorption band in the range of 515−528 nm. The radical loses this absorption feature when accepting an electron or a free radical species, resulting in a visually noticeable discoloration from purple to yellow. Because the DPPH radical can accommodate many samples in a short time period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used to screen the antiradical activities of compounds or plant extracts (*27*). We examined the scavenging activity of the 20 flavonoids on DPPH$^{\cdot}$, and quercetin was used for a positive control (*28*). The EC_{50} values of the DPPH$^{\cdot}$ scavenging activity were calculated (**Table 1**). The results indicated that, besides **9** (quercetin), two flavones, **5** and **7**, one flavonol, **8**, and a glycoside, **10**, showed a significant DPPH$^{\cdot}$ scavenging activity with EC_{50} values of less than 25 μM. Their scavenging ability was declined in the order **10** > **9** > **5** > **7** > **8**. However, the other selected flavonoids did not show an obvious DPPH$^{\cdot}$ scavenging activity even at 200 μM (data not shown).

$O_2^{\cdot-}$ Scavenging Activity. The superoxide anion is a major source of many free radicals. Hence, compounds that can scavenge the superoxide anion can reduce the production of many other free radicals, such as peroxyl, alkoxyl, hydroxyl, and nitric oxide (*29*). To further validate the antioxidative activity of these flavonoids, we performed this experiment.

The pyrogallol method, which is easy to perform and does not interfere with the enzymatic system (*30*), was used here to evaluate the $O_2^{\cdot-}$ scavenging activity of the 20 flavonoids. Pyrogallol is unstable in basylous conditions and produces $O_2^{\cdot-}$through autoxidation. Then, the produced $O_2^{\cdot-}$ reacts with luminal to illuminate. Antioxidants that can remove $O_2^{\cdot-}$ will decrease the luminous intensity consequently. This method has been proven to be simple, direct, and effective for ROS and antioxidant studies (*26*).

The EC_{50} values of the $O_2^{\cdot-}$ scavenging activity were calculated (**Table 1**), and quercetin was selected for comparison (*31*). The results indicated that, besides **9** (quercetin), **5**, **7**, **8**, and **10** showed a relatively strong $O_2^{\cdot-}$ scavenging activity (EC_{50} < 6 μM). According to EC_{50}, their scavenging ability was **8** > **9** > **7** > **5**, **10**. The other flavonoids that showed a moderate or weak $O_2^{\cdot-}$ scavenging activity (40 μM < EC_{50} < 100 μM) were as follows: **6** > **4** > **3** > **16** > **13**. The $O_2^{\cdot-}$ scavenging ability of **1**, **2**, **11**, **12**, **14**, **15**, and **17**−**20** could be neglected at the tested concentrations (1, 5, 10, 25, 50, and 100 μM).

DISCUSSION

We evaluated the toxicity−beneficial effects−structure relationships of 20 structure-related flavonoids in vitro. The toxicity was tested by determining the cytotoxic effects of flavonoids on two liver cell lines, L-02 and HepG2, while the beneficial effects were evaluated by the antioxidative activity of flavonoids since many biological effects of flavonoids are related to their antioxidative activities (*32–34*).

In the present study, the two cell lines showed different sensitivities to the 20 compounds. Generally, the L-02 cells were more sensitive to the tested flavonoids than the hepatoma cells HepG2 by their IC_{50} value evaluation. This may be due to the fact that hepatoma cells, as compared to normal cells, tend to exhibit a higher level of intracellular antioxidant enzymes and GSH (*35, 36*), which are beneficial to maintain the redox balance in cells since cytotoxic effects of flavonoids are possibly associated with their pro-oxidant activity, according to many other reports (*11, 14, 37*). In addition, GSH itself can detoxify flavonoids by forming GSSG or GSH conjugates. However, we could not exclude the possibility that the flavonoids resulted in cytotoxicity through apoptosis-inducing properties in cells by actions at the protein kinase, lipid kinase, or other apoptosis-related signaling pathways (*38–40*), which could produce different responses to even the same compounds in different cell lines. Moreover, the difference in metabolic rate or enzyme activity in these two liver cell lines also may be involved (*41*). Thus, more detailed experiments should be performed to confirm these speculations. In contrast to the flavonoids, the anticancer drug 5-FU (positive control) showed stronger cytotoxic effects on HepG2 than on L-02 (IC_{50} = 70.82 ± 3.93 and 363.50 ± 35.05 μM for HepG2 and L-02, respectively). This phenomenon may suggest that most of the tested flavonoids, including the reported compounds **4** (apigenin), **5** (luteolin), and **13** (genistein), which could induce apoptosis of HepG2 (*42–44*), are not appropriate for clinical hepatoma treatment now because the cytotoxic effects of them on normal cells were even stronger than on cancer cells, at least in our study. Additionally, absorbed flavonoids always are modified in the liver to form methyl, glucuronate, or sulfate conjugates, which may change the toxic effects of flavonoids in vivo (*45*). Thus, when searching natural antitumor drugs, both the high selectivity toward cancer cells and the in vivo activity should be considered.

For the 20 compounds with different basic structures or substituent groups (**Figure 1**), the results showed that, except for **1**−**3**, **10**, **14**, and **19**, all other compounds exhibited different cytotoxic effects on L-02 or HepG2 at the tested concentrations (20, 40, 60, 80, and 100 μM). Structural analysis on these results demonstrated that, for the flavones, 4′-monohydroxyl (phenol B ring) or 3′,4′-dihydroxyl (catechol B ring) might be important for the cytotoxic effects on both cell lines (**4**, **5**, and **7**). When 3′- and/or 4′-hydroxyl were methylated (**1**−**3**), the cytotoxicity of flavones decreased or even disappeared, while the role of 7-OH in the cytotoxic effects was uncertain. For example, the cytotoxicity of **6** on L-02 and HepG2 was considerably declined when compared to **4**, but the cytotoxic effect of **7** (IC_{50} = 41.35 ± 1.98 μM) was even stronger than that of **5** (IC_{50} = 78.95 ± 4.46 μM) on HepG2. For flavonols **8** and **9**, which are flat structures with a characteristic 3-hydroxyl substituent, we found that **8** (IC_{50} = 57.05 ± 5.89 and 84.72 ± 8.53 μM for L-02 and HepG2, respectively) showed obviously stronger cytotoxic effects than **9** (IC_{50} = 113.03 ± 6.02 and 188.84 ± 12.40 μM for L-02 and HepG2, respectively). It was an unexpected result that **9** (quercetin) showed such lower cytotoxic effects on L-02 and HepG2. The existence of 3-OH seemed to attenuate the cytotoxicity of **8** (as compared to **4**) and **9** (as compared to **5**) on the both cell lines, while glycosylation of the 3-OH markedly decreased the cytotoxicity (comparing **9** with **10**). On both L-02 and HepG2, **1** (apigenin-4′,7-dimethyl ether), **2** (luteolin-3′,4′,7-trimethyl ether), **3** (persicogein), **4** (apigenin), **5** (luteolin), **8** (kaempferol), and **9** (quercetin) showed the same cytotoxic sequence: **4** ≈ **5** > **8** > **9** ≫ **1**, **2**, **3** by evaluating their IC_{50} values. According to this result, both 3′,4′-OH and 4′-OH played certain roles in cytotoxic effects, but we are unable to briefly conclude that 3′,4′-OH contributes more to the cytotoxicity than 4′-OH and vice versa. Besides 3′,4′-OH or 4′-OH, the other hydroxyl group or the total number of hydroxyl groups in the skeleton also may influence the cytotoxic effects of flavonoids (*46, 47*).

However, we may be able to explain the previous result as follows: the higher IC_{50} values of **8** and **9** as compared to those of **4** and **5** might be due to the existence of 3-OH (*48*), while the extremely high IC_{50} of **9** (quercetin) may also be related to its low incorporation efficiency and fast metabolism in liver cells (*49, 50*), which could be influenced by the total number of hydroxyls. Furthermore, flavonoids may not act as conventional hydrogen donators but exert toxic effects in cells through acting at survival or apoptosis-related signaling molecules (*51, 52*), which requires specific structures. Further experiments were performed to elucidate the role of structures in the cytotoxic mechanism of flavonoids. The flavanones, **11** and **12**, affected cell viability of L-02 at high concentrations (IC_{50} = 143.88 ± 9.39 and 93.26 ± 3.58 μM, respectively) and had no effect on HepG2 cells. Compound **13** (genistein), a kind of isoflavone with 4′-OH, showed a cytotoxic activity on L-02 cells (IC_{50} = 47.95 ± 3.01 μM), which is obviously stronger than that of HepG2 (IC_{50} = 135.95 ± 8.19 μM). Although the flavanone and isoflavone exerted certain cytotoxic effects, it could be seen that the lack of a double bond between C2 and C3 as well as ring B at the C3 position could reduce the cytotoxicity of flavones on both L-02 and HepG2, by comparison of **12**, **13**, and **4**. The greater activity of the flavones as compared to the flavanones also was observed in studies by Agullo et al. (*53*) and Fotsis et al. (*54*) in other cell lines.

Both L-02 and HepG2 cells were relatively sensitive to **15**−**18**. The finding of **15** (flavokawain B) was consistent with the result reported by Jhoo et al. (*55*). The cytotoxic effects of **16** (flavokawain C), **17** (cardamonin), and **18** (uvangoletin) on liver cells were first reported, to our knowledge. Although **14** (flavokawain A) could induce apoptosis in bladder cancer cells (*56*), there were no effects on any of these two selected liver cell lines. The only difference between chalcones **14** and **15** in terms of structure is whether 4-OCH_3 is located on the B ring, but their cytotoxic activity is quite different. We speculated that the different cytotoxicities of **14** and **15** may be related to the particular cell type. However, other influential factors, such as the different intracellular metabolism in liver cells and the stereochemical structures of compounds, also may be involved. The cytotoxicity of **14**−**18** on L-02 was found to decline in the order **18** > **17** > **15** > **16** > **14**, whereas on HepG2, the order was changed to **17** > **18** > **16** > **15** > **14**. The results indicated that chalcones and dihydrochalcones may have different structure requirements in cytotoxic activity as compared to flavones, flavonols, and dihydroflavones, at least on these two cell lines. The lack of 4′-OH (flavokawain B **15**) and the C2═C3 double bond (uvangoletin **18**) did not significantly decrease the cytotoxicity of the compounds. In contrast, **18** showed an even stronger toxicity than the other chalcones on L-02. The hydroxyl groups on the A ring seemed to contribute more to the cytotoxic effects when **17** (IC_{50} = 30.90 ± 4.03 and 22.63 ± 3.00 μM for L-02 and HepG2, respectively) is compared to **16** (IC_{50} = 57.04 ± 2.32 and 59.48 ± 2.72 μM for L-02 and HepG2, respectively). Glycosides **19**, **20**, and **10** showed a weak or no effect on the two cell lines, suggesting that sugar moieties could reduce the cytotoxic activity and that glycosides had a weaker cytotoxicity than their corresponding aglycones, such as **9** and **10**.

Among the 20 flavonoids, only five showed a strong DPPH$^{\cdot}$ scavenging activity (EC_{50} < 25 μM) under the tested concentrations, including **5** and **7**−**10**, and four of them possessed a 3′,4′-dihydroxyl group in the basic skeleton of the flavonoids. These results indicated that the 3′, 4′-dihydroxyl on the B ring was a key functional group for scavenging DPPH$^{\cdot}$ (*57*). However, **8** (kaempferol), without the 3′,4′-dihydroxyl group, also showed a DPPH$^{\cdot}$ scavenging activity, suggesting that 3-OH in conjunction with the 4-carbonyl group on the C ring also could contribute to the anti-DPPH activity. The possible role of 3-OH in the anti-DPPH activity may be related to the transformation of the catechol group (just like 3′,4′ -OH) with 4-keto by keto−enolic tautomerism, which needs further validation. Compound **9** (quercetin) exhibited a higher anti-DPPH activity than **5** (luteolin) and **8** (kaempferol) because both 3-OH and 3′,4′-OH are in its structure. However, compound **10** (rutin) with 3-OH blocked by rutinose showed an activity similar to or greater than that of **9** (quercetin), indicating that glycosylation of 3-OH also could influence anti-DPPH activity. This result was confirmed by Cho et al., who reported that the anti-DPPH activity of flavonoids may be influenced greatly by glycosylation, related to the type and configuration of glycons (*57*).

Compounds **5** and **7**−**10** also showed strong $O_2^{\cdot -}$ removal activities. However, 3-OH in conjunction with the 4-carbonyl groups seemed to contribute more than 3′,4′-dihydroxyl when **8** (EC_{50} = 0.76 ± 0.08 μM) was compared to **5** (EC_{50} = 5.04 ± 0.21 μM). When 3-OH is glycosylated, the $O_2^{\cdot -}$ scavenging effect decreased, such as **10** (EC_{50} = 5.13 ± 0.08 μM) and **9** (EC_{50} = 0.87 ± 0.01 μM). The methylation of 7-OH did not reduce the antioxidative activity of **7** (EC_{50} = 2.50 ± 0.12 μM) and **6** (EC_{50} = 43.47 ± 1.85 μM) as compared to **5** (EC_{50} = 5.04 ± 0.21 μM) and **4** (EC_{50} = 46.12 ± 3.29 μM). Compound **13** (EC_{50} = 84.36 ± 2.86 μM) showed a lower activity than **4** (EC_{50} = 46.12 ± 3.29 μM), indicating that ring B at the C3 position decreased the $O_2^{\cdot -}$ scavenging effect as compared to ring B at the C2 position.

In our study, **5** (luteolin), **7** (hydroxygenkwanin), and **8** (kaempferol) showed significant dual properties, whereas **15** (flavokawain B), **16** (flavokawain C), **17** (cardamonin), and **18** (uvangoletin) exhibited a marked cytotoxicity on both L-02 and HepG2. Structural analysis concerning the flavones (**1**−**7**), flavonols (**8** and **9**), flavanones (**11** and **12**), and isoflavone (**13**) on their antioxidant−cytotoxicity relationship showed that (a) the 3′,4′-dihydroxyl group on the B ring was important to both antioxidative and cytotoxic activities (**5** and **7**), while methylation of the catechol group reduced the radical-scavenging activity and cytotoxic effects (by comparing **5** and **7** with **2**); (b) hydrogenation of the C2═C3 double bond significantly decreased the antioxidative effects as well as the cytotoxicity (by comparing **12** with **4**); (c) the existence of 3-OH markedly increased the antioxidative activity of flavonoids but attenuated the cytotoxicity relatively (by comparing **8** and **9** with **4** and **5**), which enables us to find good natural antioxidants from flavonols; and (d) as compared to ring B at the C2 position, ring B at the C3 position could decrease the radical-scavenging activity and cytotoxicity (by comparing **13** with **4**). However, the chalcones and dihydrochalcone **15**−**18** demonstrated different structure requirements from the previous flavonoids. They showed relatively strong cytotoxic effects against L-02 and HepG2, while a weak or no antioxidative activity was observed. Hydrogenation of the C2═C3 double bond did not significantly decrease the cytotoxic actions of **18** as compared to **15** or **16**, and the hydroxyl group on the A ring contributed more to the cytotoxicity when **17** is compared to **15** or **16**.

The previous analysis suggests that although to some flavonoids their cytotoxic and antioxidative activities may have similar structural requirements, such as the catechol group in the B ring and the C2═C3 double bond (*47, 58*), the structure-dependent correlation between cytotoxicity and antioxidative

activity is not applicable to all flavonoids. This may be because the antioxidative activity of flavonoids is, in large apart, dependent on their ability to donate hydrogen or to form complexation with a metal ion (*59, 60*), which is mainly influenced by the number or position of hydroxyl groups in the skeleton of flavonoids, while the cytotoxicity of flavonoids can be caused by multiple mechanisms, such as inhibiting specific kinases of the cell cycle, depleting GSH, acting on mitochondria, regulating apoptosis-related proteins, and pro-oxidant effects (*61, 62*), which may require particular structures in a particular cell line. However, it is possible that flavonoids produce both antioxidant activity and cytotoxic effects as conventional hydrogen donators, such as **5** (luteolin), **7** (hydroxygenkwanin), **8** (kaempferol), and other flavonoids with multihydroxyls, which may on one hand be hydrogen-donating antioxidants and on the other hand form phenoxyl radicals to induce cytotoxicity (*11, 13*). Flavonoids with a strong antioxidative activity also may result in cytotoxicity by destroying the intracellular antioxidant system or influencing the antioxidant-sensitive signaling pathway (*63–65*). In addition, **15**−**18**, which are in the chalcone skeleton and have no antioxidant activity, showed relatively strong cytotoxic effects on both cell lines. We proposed that their cytotoxicity may be related to the disruption of the mitochondrial respiratory chain or depletion of hepatic GSH by forming GSSG or GSH conjugates (*66*).

Besides the flavonoids in our study, there are also numerous compounds that possess similar structures in them. According to our results, attention should be paid when large amounts of flavonoids are ingested for health maintenance, especially those with a strong antioxidative activity or in chalcone structures. The antioxidative activity and cytotoxic effects of flavonoids may depend on the same active groups in the chemical structures, and chalcones as the intermediate precursors for all flavonoids in the phenylpropanoid pathway in plants may have a more reactive activity than other flavonoids due to their flexible structures.

The antioxidative or cytotoxic activity of flavonoids is a comprehensive effect of multiple factors, and observing the cytotoxic and antioxidant activity of flavonoids in vitro from the aspect of structures is only a preliminary study. Although the structure-dependent correlation between cytotoxicity and antioxidative activity is not applicable to all flavonoids, it exists in certain flavonoids. Further experiments are in progress to study particular flavonoids with both strong antioxidative and cytotoxic effects, such as **5**, **7**, and **8**, which attempts to elucidate the role of structures in the contradictory effects. In addition, in view of potential toxicity, the consumption of large quantities of flavonoids as supplements to functional food should not yet be encouraged.

ABBREVIATIONS USED

CL, chemiluminescence; DMSO, dimethyl sulfoxide; DPPH$^{\bullet}$, 1,1-diphenyl-2-picrylhydrazyl radical; FBS, fetal bovine serum; MEM, minimal essential medium; MTT, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide; 5-FU, 5-fluoro-2,4(1*H*,3*H*)-pyrimidinedione.

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