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# Antioxidant and anticancer activity of 3′-formyl-4′,6′-dihydroxy-2′-methoxy-5′-methylchalcone and (2S)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone

Chun-Lin Ye, Yu Liu and Dong-Zhi Wei

## Abstract

Two new flavonoids – 3′-formyl-4′,6′-dihydroxy-2′-methoxy-5′-methylchalcone (FMC) and (2S)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone (FMF) – isolated from the buds of *Cleistocalyx operculatus*, were investigated for their antioxidant and anticancer activity. Total antioxidant activity and reducing ability were measured. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and superoxide anion radical scavenging assays were carried out to evaluate the antioxidant potential of the two compounds. The antioxidant activity of the two compounds increased in a concentration-dependent manner. FMC and FMF at a concentration of 500 $\mu$M inhibited lipid peroxidation by 64.3 ± 2.5% and 60.3 ± 2.3%, respectively, an antioxidant activity approximately similar to that of 500 $\mu$M $\alpha$-tocopherol (66.3 ± 2.5%). Similarly, the effect of FMC and FMF on reducing power increased in a concentration-dependent manner. In DPPH radical scavenging assays, the IC50 values of FMC and FMF were 50.2 ± 2.8 $\mu$M and 75.8 ± 2.5 $\mu$M, respectively. Moreover, FMC and FMF scavenged the superoxide generated by the phenazine methosulfate (PMS)/reduced $\beta$-nicotinamide adenine dinucleotide (NADH) nitroblue tetrazolium (NBT) system, with IC50 values of 56.3 ± 2.3 $\mu$M and 317.5 ± 2.9 $\mu$M, respectively. The anticancer activity of the two compounds were determined in five human cancer cell lines, SMMC-7721 (liver cancer), 8898 (pancreatic cancer), K562 (chronic leukaemia), HeLa (tumour of cervix uteri) and 95-D (high metastic lung carcinoma). FMC and FMF showed broad-spectrum anticancer activity against all the human cancer cell lines tested. The results obtained in the current study indicate that the two flavonoids could be a potential source of natural antioxidant and anticancer agents. To our knowledge, this is the first report on bioactivity of FMC and FMF.

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## Introduction

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radicals, hydroxyl radicals and non-free-radical species such as hydrogen peroxide and singlet oxygen, are various forms of activated oxygen (Yildirim et al 2000; Gulcin et al 2002). The importance of free radicals and ROS has attracted increasing attention over the past decade (Gulcin et al 2002). Modern theories have revealed that ROS play a dual role in organisms. ROS are not only strongly associated with lipid peroxidation, which leads to the deterioration of food, but are also involved in the development of a variety of diseases, including cellular ageing, mutagenesis, carcinogenesis, coronary heart disease, diabetes and neurodegeneration (Harman 1980; Sasaki et al 1996; Moskovitz et al 2002).

Cancer is the largest single cause of death in both men and women. Recently, resistance to anticancer drugs has been observed. Therefore, the research and development of more effective and less toxic drugs by the pharmaceutical industry has become necessary (Russo et al 2005). Plants contain many phytochemicals with various bioactivities, including antioxidant, anti-inflammatory and anticancer activity. For example, some studies have reported that extracts from natural products, such as fruits, vegetables and medicinal herbs have positive effects against cancer, compared with chemotherapy or hormonal treatments (Pezzuto 1997; Wu et al 2002). In particular, interest has intensified in the flavonoid compounds present in normal human diet and in many folk medicines still in use. These compounds are known to possess the ability to scavenge free radicals (Torel et al 1986; Husain et al 1987). Furthermore, the redox properties of flavonoids allow them to act as reducing

agents and, in some cases, as chelating agents for transition metals (Rice-Evans & Miller 1996). The free-radical-scavenging properties of flavonoids can protect the human body from free-radical-mediated diseases, and epidemiological studies have indicated that consumption of flavonoids is associated with a reduced risk of cancer and inflammation (Wei et al 1990; Hertog et al 1993; Middleton 1996; Verma et al 1998). They have also been shown to protect cells from X-ray damage, block progression of the cell cycle, inhibit mutations, block prostaglandin synthesis and prevent multistage carcinogenesis in experimental animals (Abdulla & Gruber 2000). A number of flavonoids have been demonstrated to be cytotoxic (Mori et al 1988; Lin et al 1989; Cushman & Nagarathnam 1991; Yit & Das 1994) and to possess anticancer properties (Hirano et al 1989; Satomi 1993; Wattenberg et al 1994).

*Cleistocalyx operculatus* (Roxb.) Merr. et Perry (Myrtaceae) is a well-known medicinal plant, the buds of which are commonly used as an ingredient of tonic drinks in Southern China. A water extract of the buds of *C. operculatus* was reported to increase the contractility and decrease the frequency of contraction in an isolated rat heart perfusion system (Woo et al., 2002). Our previous phytochemical studies of the species has led to the characterisation of sterol, flavanone, chalcone and triterpene acid from its buds (Ye et al 2004). In this paper, the antioxidant activity of 3′-formyl-4′,6′-dihydroxy-2′-methoxy-5′-methylchalcone (FMC) and (2S)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone (FMF), two new flavonoids from the buds of *C. operculatus*, were evaluated by various tests and compared with commercial antioxidant agents. The anticancer activity of the two flavonoids was also examined.

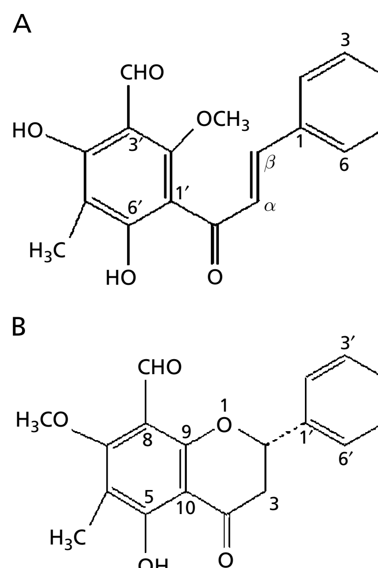


**Figure 1** Chemical structures of the flavonoids, 3′-formyl-4′,6′-dihydroxy-2′-methoxy-5′-methylchalcone (FMC) (A) and (2S)-8-formyl-5-hydroxy-7-methoxy-6-methylflavanone (FMF) (B).

## Materials and Methods

### Materials

FMF and FMC were isolated from *C. operculatus* in our laboratory, as described by Ye et al. (2004). Previous experiments have shown that the purity of the extract is above 96% (HPLC and spectral analysis). The structures of the compounds are shown in Figure 1. Linoleic acid (98%), 1,1-diphenyl-2-picrylhydrazyl (DPPH), $\alpha$-tocopherol, phenazine methosulfate (PMS), reduced $\beta$-nicotinamide adenine dinucleotide (NADH) and nitroblue tetrazolium (NBT) were purchased from Fluka Biochemika AG (Buchs, Switzerland); tertiary butyhydroquinone (TBHQ) was purchased from Acros Organics (Fairlawn, NJ, USA); butylated hydroxytoluene (BHT) and ascorbic acid (ASC) were purchased from Sigma (St Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Rosewell Park Memorial Institute (RPMI) 1640 medium were purchased from Life Technologies, Inc. (Gaithersburg, MD, USA). All other chemicals were of analytical grade.

### Measurement of antioxidant activity

*Determination of total antioxidant activity*

The antioxidant activity of compounds was determined using a linoleic acid system (Mistuda et al 1996). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween 20 as emulsifier and 50 mL phosphate buffer (0.2 M, pH 7.0); the mixture was then homogenised. Ethanol solution (0.5 mL) of different concentration (12.5–100 $\mu$M) of the compound to be tested was mixed with linoleic acid emulsion (2.5 mL, 0.2 M, pH 7.0) and phosphate buffer (2 mL, 0.2 M, pH 7.0) and the reaction mixture incubated at 37°C in the dark to accelerate the peroxidation process. The level of peroxidation was determined using the thiocyanate method by sequentially adding ethanol (5 mL, 75%), ammonium thiocyanate (0.1 mL, 30%), sample solution (0.1 mL) and ferrous chloride (0.1 mL, 20 mM in 3.5% HCl). The mixture was left for 3 min, then the peroxide value was determined by reading the absorbance at 500 nm on a spectrophotometer, using $\alpha$-tocopherol as the reference compound. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. All data reported are the average of triplicate analyses. Percentage inhibition of lipid peroxide generation was measured by comparing the absorbance values of control and test samples.

*Reducing power*

The reducing power of the prepared compounds was determined according to the method of Oyaizu (1986). Briefly, 1.0 mL of different concentrations of the samples (62.5–500 $\mu$M) was mixed with 2.5 mL phosphate buffer (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide solution (1% w/v). The mixture was incubated in a water bath at 50 °C for 20 min; 2.5 mL trichloroacetic acid solution (10% w/v) was added and the mixture was then centrifuged at 3000 rpm for 10 min. A 2.5 mL aliquot of the upper layer was combined with 2.5 mL distilled water and 0.5 mL ferric chloride solution (0.1% w/v), and absorbance was measured at 700 nm. ASC was used as a reference material. All tests were

performed in triplicate and the graph was plotted from the average of three determinations.

*Radical scavenging activity on DPPH*

The free-radical-scavenging activity of FMF and FMC was measured using DPPH by the method of Blois (1958). A 0.1 mM solution of DPPH in methanol was prepared and 1 mL of this solution was added to 3 mL of various concentrations (12.5–100 μM) of FMF and FMC and the reference compound, TBHQ. After 30 min, absorbance was measured at 517 nm. All tests were performed in triplicate. Percentage inhibition was calculated by comparing the absorbance values of control and samples.

*Superoxide anion radical scavenging activity*

Superoxide anion radicals are generated in a PMS–NADH system by oxidation of NADH and can be assayed by the reduction of NBT (Liu & Ng 2000). In this experiment, the superoxide anion radical were generated in 3 mL Tris-HCl buffer (16 mM, pH 8.0) containing 78 μM NADH, 50 μM NBT, 10 μM PMS, and samples at different concentrations (50–400 μM). PMS was added to initiate the reaction. The reaction mixture was incubated at room temperature for 5 min and absorbance at 560 nm was measured against control samples. BHT was used as reference compound. All tests were performed in triplicate. Percentage inhibition was calculated by comparing the results of control and test samples.

### Study on human tumour cell lines

*Cell lines and culture conditions*

The human cell lines used were: K562 human chronic leukaemia cells, liver cancer SMMC-7721 cells, pancreas cancer 8898 cells, tumour of cervix uteri HeLa cells and high metastatic lung carcinoma 95-D cells. All cells were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). K562, SMMC-7721 and 8898 cells were cultured in RPMI 1640 medium with 10% FBS, penicillin (100 units $mL^{1}$) and streptomycin (100 μg $mL^{-1}$). HeLa cells were cultured in DMEM medium with 10% FBS, penicillin (100 units $mL^{-1}$) and streptomycin (100 μg $mL^{-1}$); 95-D cells was cultured in RPMI 1640 medium with 15% FBS, penicillin (100 units $mL^{-1}$) and streptomycin (100 μg $mL^{-1}$). All cells were incubated at 37°C with 5% carbon dioxide in an air atmosphere.

*Cytotoxicity on cancer cells (MTT assay)*

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay was performed as described by Mosmann (1983). Compounds were dissolved in DMSO. The control cells were treated with the same amount of vehicle alone. The final DMSO concentration never exceeded 0.1% (v/v), in either control or treated samples. Previous experiments have shown that DMSO at this concentration does not modify the cellular activity that we were analysing. K562, SMMC-7721, 8898, HeLa and 95-D cells were placed in 96-well culture plates ($10^4$ cells/well) and allowed to attach for 24 h before treatment. Cells from each cell line were treated with compounds to be tested at concentrations ranging from 25 to 200 μM, or vehicle (0.1% DMSO) as a control. Cytotoxicity was measured after 2 days' culture, using the MTT assay. Absorbance in control and compound-treated wells was measured using an automated microplate reader (Bio-Rad 550) at 550 nm (Bio-Rad Laboratories, Hercules, CA, USA). The cytotoxicity of the compounds was expressed as the IC50 (concentration that caused 50% cytotoxicity), which was extrapolated from linear regression analysis of experimental data.

### Statistical analysis

Data are reported as the mean ± s.d. of three measurements. The scientific statistics software *GraphPad Prism* 3.03 (GraphPad Software Inc., San Diego, CA, USA) was used to evaluate the significance of differences between groups. Comparisons between groups were done using the Kruskal–Wallis test followed by Dunn's post hoc test. IC50 values for all the above experiments were determined by non-linear regression. $P < 0.05$ was regarded as significant.

## Results

### Antioxidant activity

The antioxidant activity of FMC and FMF was determined by peroxidation of linoleic acid using a thiocyanate method. During the linoleic acid peroxidation, peroxides are formed, which oxidise $Fe^{2+}$ to $Fe^{3+}$; the $Fe^{3+}$ ions form complexes with thiocyanate ions, which have a maximum absorbance at 500 nm. High absorbance is an indication of high concentration of peroxide formed during the emulsion incubation.

The antioxidant activity of both FMC and FMF was dose dependent (Figure 2). The IC50 values of FMC and FMF on lipid peroxidation were 169.3 ± 3.1 μM and 180.3 ± 2.9 μM, respectively. FMC and FMF at a concentration of 500 μM showed 64.3 ± 2.5% and 60.3 ± 2.3% inhibition, respectively, an antioxidant activity approximately equal to that of 500 μM α-tocopherol (66.3 ± 2.5%).

### Reducing ability

The reducing capacity of a compound, which may serve as a significant indicator of its potential antioxidant activity (Mier et al 1995), was determined using a modified $Fe^{3+}$-to-$Fe^{2+}$ reduction assay. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of extracts or compounds. The presence of reductants in the solution causes the reduction of the $Fe^{3+}$/ferricyanide complex to the ferrous form and the $Fe^{2+}$ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Figure 3 showed the reducing abilities of FMC and FMF compared with ASC. All of them had strong reducing powers; however, as anticipated, the reducing power of FMC and FMC was inferior to ASC, which is known to be a strong reducing agent. Like antioxidant activity, the reducing power of FMC and FMF increased with concentration. All FMC and FMF concentrations showed higher activity than control. The

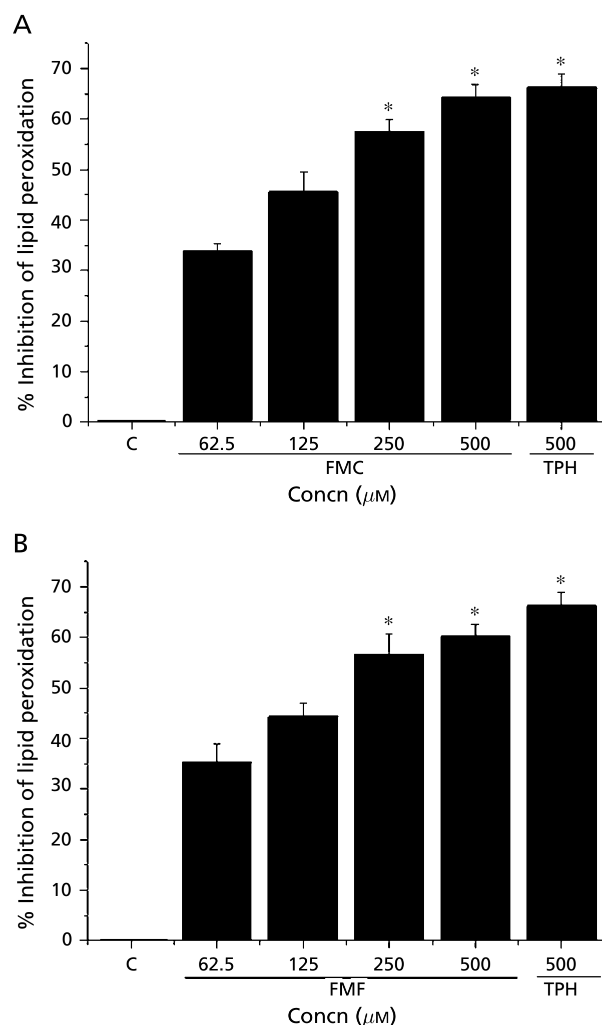


**Figure 2** Inhibition of lipid peroxidation in a linoleic acid emulsion by FMC and TPH (A) or FMF and TPH (B). Data are mean ± s.d. of three measurements. $*P < 0.05$ compared with control (C) (Kruskal–Wallis test followed by Dunn's post hoc test).

reducing power of these compounds followed the order: ASC > FMC > FMF.

## DPPH radical scavenging activity

The DPPH radical is considered to be a model lipophilic radical. A chain reaction in lipophilic radicals was initiated by lipid auto-oxidation. The radical scavenging activity of FMC and FMF was determined from the reduction in absorbance at 517 nm resulting from the scavenging of stable DPPH free radicals. The positive DPPH test suggested that the samples were free radical scavengers. The scavenging effects of FMC, FMF and TBHQ on the DPPH radical are illustrated and compared in Table 1. FMC and FMF had significant scavenging effects on the DPPH radical, which increased with increasing concentration in the 12.5–100 μM range. The IC50 values of TBHQ, FMC and FMF in the DPPH radical scavenging assay were 40.8 ± 2.1 μM, 50.2 ± 2.8 μM and 75.8 ± 2.5 μM, respectively.

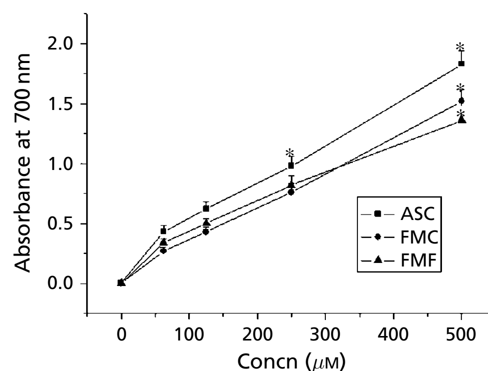


**Figure 3** Reducing power of FMC, FMF and ASC, determined using a modified $Fe^{3+}$-to-$Fe^{2+}$ reduction assay (see text for assay details). Data are mean ± s.d. for three measurements. $*P < 0.05$ compared with control (% inhibition by control = 0%; Kruskal–Wallis test followed by Dunn's post hoc test).

**Table 1** DPPH radical and superoxide anion scavenging activity of FMC, FMF TBHQ and BHT

| Treatment | Inhibition (%) | |
|---|---|---|
| | DPPH assay | Superoxide anion assay |
| TBHQ | | |
| 12.5 μM | 12.1 ± 1.9 | |
| 25 μM | 31.8 ± 1.8 | |
| 50 μM | 61.0 ± 2.5* | |
| 100 μM | 89.1 ± 2.6* | |
| BHT | | |
| 50 μM | | 62.1 ± 1.6 |
| 100 μM | | 70.3 ± 1.8 |
| 200 μM | | 75.2 ± 2.0* |
| 400 μM | | 81.3 ± 2.4* |
| FMC | | |
| 12.5 μM | 8.2 ± 1.0 | |
| 25 μM | 26.8 ± 1.9 | |
| 50 μM | 50.6 ± 3.3 | 48.6 ± 3.0 |
| 100 μM | 85.1 ± 2.5* | 58.7 ± 1.8 |
| 200 μM | | 65.6 ± 2.0* |
| 400 μM | | 70.8 ± 2.1* |
| FMF | | |
| 12.5 μM | 5.7 ± 1.1 | |
| 25 μM | 22.7 ± 2.3 | |
| 50 μM | 41.8 ± 3.2 | 12.6 ± 2.1 |
| 100 μM | 76.5 ± 3.1* | 22.3 ± 2.2 |
| 200 μM | | 35.4 ± 2.8 |
| 400 μM | | 60.7 ± 1.8* |

Data are mean ± s.d. of three measurements. $*P < 0.05$ compared with control (% inhibition by control = 0%; Kruskal–Wallis test followed by Dunn's post hoc test).

## Superoxide anion radical scavenging activity

In the PMS/NADH-NBT system, superoxide anions derived from dissolved oxygen by the PMS–NADH coupling reaction reduce NBT. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anions

**Table 2** Cytotoxicity of FMC and FMF

| Cell line | IC50 (μM) | |
|---|---|---|
| | FMC | FMF |
| SMMC-7721 | 79.8 ± 2.6 | 69.7 ± 2.9 |
| 8898 | 96.1 ± 4.7 | 89.2 ± 1.7 |
| K562 | 111.8 ± 3.4 | 55.3 ± 2.2 |
| Hela | 139.1 ± 5.9 | 102.3 ± 4.3 |
| 95-D | 165.7 ± 6.1 | 178.1 ± 6.2 |

Cells were treated for 48 h in the presence of the drug in medium. Cytotoxicity was determined by MTT assay and is expressed as mean ± s.d. of three separate experiments (n = 3 in each experiment).

in the reaction mixture. Table 1 shows the superoxide anion radical scavenging activity of different concentrations of BHT, FMC and FMF. All samples showed some degree of superoxide radical scavenging activity in a dose-dependent manner, but BHT, used as a positive control, exhibited the highest superoxide radical scavenging activity. The IC50 values of BHT, FMC and FMF were 38.2 ± 1.7 μM, 56.3 ± 2.3 μM and 317.5 ± 2.9 μM, respectively.

### Cytotoxicity activity on cancer cells

The cytotoxicity of FMC and FMF on various cells (measured using the MTT assay) are shown in Table 2. The different cells had different sensitivities to the inhibition effect of FMC and FMF. The IC50 values of FMC and FMF were between 50 μM and 180 μM. To investigate whether FMC and FMF have selective toxicities toward neoplastic rather than normal cells, the effects of FMC and FMF on primary cultures of normal human cells are now in progress in our laboratory.

## Discussion

ROS generated by mitochondria or from other intracellular or extracellular sites can cause cell damage and initiate various degradation processes (Davies & Hochstein 1982). Mild amounts of oxidative damage may actually stimulate physiological mitochondrial biogenesis, via the production of superoxide from ubisemiquinone. However, more severe or more prolonged oxidative damage clearly induces toxic reactions that can contribute significantly to the ageing process (Cadenas & Davies 2000). Flavonoids, abundant in fruits, teas, vegetables and medicinal plants, have received considerable attention and have been investigated extensively, since they are effective free radical scavengers and are assumed to be less toxic than synthetic antioxidants such as butylated hydroxyanisole and BHT, which are suspected to be carcinogenic and to cause liver damage (Hollman & Katan 1997; Pietta 2000). In this work, the antioxidant activity of FMC and FMF, two new flavonoids from the buds of *C. operculatus*, were determined by measuring their total antioxidant activity, reducing ability, DPPH scavenging activity and superoxide anion scavenging activity. Our results demonstrate that both FMC and FMF exhibited interesting total antioxidant activity in a linoleic acid system. Both FMC and FMF at 500 μM showed approximately the same antioxidant activity as 500 μM α-tocopherol (66.3 ± 2.5%). Antioxidant activity has been reported to be concomitant with the development of reducing power (Tanaka et al 1988). The reducing capacity of a compound may serve as a significant indicator of its total potential antioxidant activity (Mier et al 1995). Our data indicate that FMC and FMF had strong reducing power, although, as anticipated, their reducing power was less than that of ASC, which is known to be a strong reducing agent.

The DPPH radical is a stable organic free radical with an absorption band at 515–528 nm. It loses this absorption when accepting an electron or a free radical species, which results in a discoloration from purple to yellow. The DPPH radical scavenging assay enables many samples to be tested in a short period, and is sensitive enough to detect active ingredients at low concentrations; it has therefore been used extensively for screening antiradical activity of antioxidants (Sánchez-Moreno 2002). In this study, both FMC and FMF were found to have DPPH scavenging activity. The DPPH scavenging activity of FMC was similar to that of TBHQ; that of FMF was lower than that of TBHQ.

Superoxide radicals are one of the most representative free radicals. In cellular oxidation reactions, superoxide radicals are normally formed first, and their effects can be magnified because they produce other kinds of cell-damaging free radicals and oxidising agents (Liu & Ng 2000). Our results showed that FMC and FMF had some degree of superoxide radical scavenging activity. The superoxide radical scavenging activity of FMC was almost similar to that of BHT. The superoxide radical scavenging activity of FMF was markedly less effective than that of BHT at all concentrations tested.

Although surgery, chemotherapy and radiation therapy are the mainstream therapeutic methods for the treatment of cancer, more and more people are opting for alternative therapeutic methods, in an effort to minimise damage to normal cells and maintain better general health in the patient. In light of this, several potential antitumor flavonoids have been isolated from Chinese herbal medicines (Ikezoe et al 2001; Fukai et al 2002; Kajimoto et al 2002; Tyagi et al 2002; Kanazawa et al 2003). Our results demonstrated that the IC50 values of FMC and FMF on the six human cancer cell lines tested were between 50 μM and 180 μM. From a comparison of our results with values reported in the literature, it is interesting that FMC and FMF showed a growth inhibitory effect in the same concentration range as the most active flavonoids and related compounds (Parmar et al 1997).

### Conclusions

It is well-known that free radicals are one of the causes of several diseases, such as Parkinson's disease, coronary heart disease and cancer (Cerutti 1994; Adams & Odunze 1991; Hertog et al 1993). Our experimental evidence showed that FMC and FMF, especially FMC, had excellent antioxidant activity. The two compounds exhibited an inhibitory effect on all the human cancer cells examined. On the basis of the results obtained in this study, we conclude that FMC and FMF have significant antioxidant and anticancer activity. Further studies are needed to evaluate

their bioavailability and potential toxicity in-vivo. These points will be addressed in a forthcoming series of studies of the two compounds.

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