*Full Length Research Paper*

# Radical-scavenging and rat liver mitochondria lipid peroxidative inhibitory effects of natural flavonoids from traditional medicinal herbs

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Baicalein (a), baicalin (b), daidzein (c), myricetin (d), oroxylin A (e), quercetin (f), quercetin pentaacetate (g), quercitrin (h), and wogonin (i) were isolated from regular anti-inflammatory Chinese herbs which contain many flavonoids compounds, also the radical-scavenging and liver mitochondrial lipid peroxidation (LPO) inhibitory capabilities were elucidated. The 50% inhibitory concentrations ($IC_{50}$) for the 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capability of (a), (b), (d), (f), and (g) were 3.61, 3.28, 2.41, 2.16, and 3.74 µg/ml, and for LPO inhibition were 9.32, 9.52, 19.63, 16.15, and 20.22 µg/ml, respectively. The antioxidant capacities of (d), (f), (g), (h), (b), and (a) were 20.46, 16.42, 14.46, 13.98, 12.74, and 8.13 µM Trolox equivalents, respectively. To compare with the structure-activity relationships of the flavonoids, two flavones of baicalein and baicalin which have 5,6-hydroxy group which contain ortho-hydroxyl group characteristic reveal better LPO inhibitory activity, and two flavonols of myricetin, quercetin which have 5,7-trihydroxy group have more DPPH activity and ferrous chelating ability. The hydroxyl groups might play a role in increasing the activity of the flavonoids and exhibit potent antioxidant activities. In view of these results, five of the nine natural flavonoids (baicalein, baicalin, myricetin, quercetin and quercetin pentaacetate) can be lead compounds for preventing liver damage and related iron-overload disease.

Key words: Anti-inflammatory Chinese herbs, ferrous ion, flavonoids, lipid peroxidation.

## INTRODUCTION

Iron is a general nutritional supplement for many people as a blood tonic and to prevent anemia, but iron overload is related to metal toxicity. In this situation, excessive iron deposition in the liver can lead to further injury such as hepatocellular necrosis, inflammation, fibrosis, and in some cases even to carcinoma (Zhao et al., 2005). This transition metal can catalyze the production of hydroxyl radicals from hydrogen peroxide by the Fenton reaction and react with lipid hydroperoxides to form peroxyl and alkoxyl radicals (Fischer et al., 2002). Otherwise, they are generated by normal metabolic processes and by

exogenous factors and agents. They are continuously produced during normal physiologic events and can easily initiate the peroxidation of membrane lipids, leading to the accumulation of lipid peroxidation (LPO) (Gulcin, 2006). When the liver is a primary target, iron overload causes tissue damage possibly leading to organ failure, hepatic fibrosis, and cirrhosis as often observed in patients (Pardo-Andreu et al., 2008). LPO is a general mechanism whereby oxygen free radicals induce tissue damage. Antioxidants, which protect against oxidative damage induced by free radicals, prevent the onset and progression of disease.

Flavonoids are a class of plant secondary metabolites derived from 2-phenyl-1,4-benzopyrone with a basic structural skeleton of diphenylpropane with different oxidation levels of the central pyran ring. They are the

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most common group of polyphenolic compounds in the human diet and are ubiquitously found in traditional herbal medicines. There are five subclasses of polyphenolic compounds: flavonols, flavones, isoflavones, flavanones, and flavanonols. Flavonols and flavanonols are thought to be the most important monomeric flavonoids in the human diet and have many biological activities (Delgado, 2009). It is now known that they provide health benefits against cancer and heart disease. They display many diverse types of biological properties such as antiproliferation activities against tumor cells, as well as antiviral, antioxidant, antifungal and anti-inflammatory properties (Prakash et al., 2008).

Flavonoids could be found in a diet with many different kinds of fruits, vegetables, tea and soy. In this study, natural flavonoids were isolated from traditional herbal medicines of *Scutellaria baicalensis, Pueraria lobata, Eugenia uniflora,* and *Sophora japonica.* Baicalein is a flavone originally isolated from the roots of *S. baicalensis,* and has antioxidant and free radical-scavenging effects that include baicalin which is a flavone that affects GABA receptors and was found in *Scutellaria lateriflora*; myricetin is a naturally occurring flavonol and is a particularly powerful antioxidant *in vitro.*

It is more readily reduced than vitamin E, a water-soluble vitamin E analogue (Bennett et al., 2004). Quercetin is a flavonoid, but more specifically, a flavonol. It is mainly found in onions, apples, and tea (Boadi et al., 2003). Quercetin pentaacetate is a flavone that effectively inhibits LPS-induced NO and PGE2 productions, while simultaneously inhibiting the enhanced expressions of iNOS and COX-2 genes (Chen et al., 2001). To know the free radical-scavenging and antioxidant capability is important to traditional medicinal herbs. We demonstrate many kinds of assays for DPPH radical-scavenging, superoxide anion radical-inhibitory, and ferrous ion-chelating activities, as well as the ferric ion-reducing antioxidant power (FRAP) and levels of the LPO product, $MDA(TBA)_2$, in Wistar rat liver mitochondrial solution were investigated to know the anti-oxidant and the structure-activity relationship of these flavonoids in traditional medicinal herbs.

## MATERIALS AND METHODS

### Chemicals and reagents

Dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), trolox, ferrous chloride ($FeCl_2$), 1,1,3,3-tetraethoxypropane (TEP), 2-thiobarbituric acid (TBA), allopurinol, the reduced form of β-nicotinamide adenine dinucleotide (β-NADH), catalase, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), nitro blue tetrazolium (NBT), peroxidase, phenazine methosulfate (PMS), phenol red, ferrozine, silymarin, superoxide dismutase (SOD), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), xanthine, and xanthine oxidase (XO) were purchased from Sigma Chemical (St. Louis, MO). All chemical solvents were analytical grade and purchased from Merck (Darmstadt, Germany).

### Animals

The Laboratory Animal Ethics Committee of Taipei Medical University (TMU) approved the study protocol. Sprague-Dawley (SD) rats were purchased from the Center of Experimental Animals, National Taiwan University, Taipei, Taiwan. Rats were housed in plastic cages in a temperature- and humidity-controlled environment and bred at the Experimental Animal Center of TMU. All experiments were performed in accordance with the guidelines for Experiments Animal Center of TMU and the guiding principles for the care and use of laboratory animals approved by the Chinese Society of Laboratory Animal Sciences, Taiwan. All efforts were made to minimize animal suffering and to reduce the number of animals used.

### Crude drugs and natural flavonoids

Anti-inflammatory traditional herbs of *S. baicalensis* roots*, P. lobata* roots*, E. uniflora* leaves, and *S. japonica* flowers were purchased from a crude drug market in Taipei, Taiwan. Nine natural flavonoids, baicalein (a), baicalin (b), daidzein (c), myricetin (d), oroxylin A (e), quercetin (f), quercetin pentaacetate (g), quercitrin (h), and wogonin (i) were isolated from these traditional herbs. Their chemical structures are shown in Figure 1.

### Baicalein, baicalin, oroxylin A and wogonin isolated from the root of S. baicalensis (Lee et al., 2003; Shih et al., 2009)

Dried *S. baicalensis* roots were cut into small pieces, immersed, and extracted with 10-fold v/w acetone twice at room temperature for 2 weeks. After concentrating the acetone filtrate, residues were reflux-extracted with 4-fold v/w of 50% aqueous ethanol twice for 6 h. Aqueous ethanol (50%) extracts were concentrated to obtain crude crystals. These were recrystallized with aqueous ethanol to obtain baicalin. The acetone extracts were subjected to column chromatography on silica gel eluted with $CHCl_3$ and $CHCl_3$-MeOH, and re-chromatographed on silica gel eluted with n-hexane-acetone to yield oroxylin A and wogonin. A portion of the $CHCl_3$-MeOH elute was subjected to a Sephadex LH-20 column eluted with MeOH to yield baicalein.

### Daidzein isolated from the root of P. lobata (Rong et al., 1998)

Dried *P. lobata* roots were chopped up and extracted with 80% ethanol. The subsequent extract was separated by silica gel column chromatography, and the elution solvents were $CHCl_3$-ethyl acetate-MeOH. The ethyl acetate fraction was subjected to column chromatography on silica gel eluted with $CHCl_3$ and $CHCl_3$-MeOH, and was re-chromatographed on silica gel eluted with hexane/ethyl acetate to yield daidzein.

### Myricetin extracted from the leaves of E. uniflora (Lee, 1997)

The 70% aqueous acetone extract of dried *E. uniflora* leaves was subjected to a combination of column chromatography on Dia-ion HP-20, Fractogel TSK HW-40, and MCI-gel CHP-20P with aqueous methanol to yield myricetin.

### Quercetin, quercitrin and quercetin pentaacetate isolated from S. japonica (YenandYang, 1990)

*S. japonica* was extracted with a warm borax solution. The filtrates were combined and acidified with 1/6 volume of 10% hydrochloric
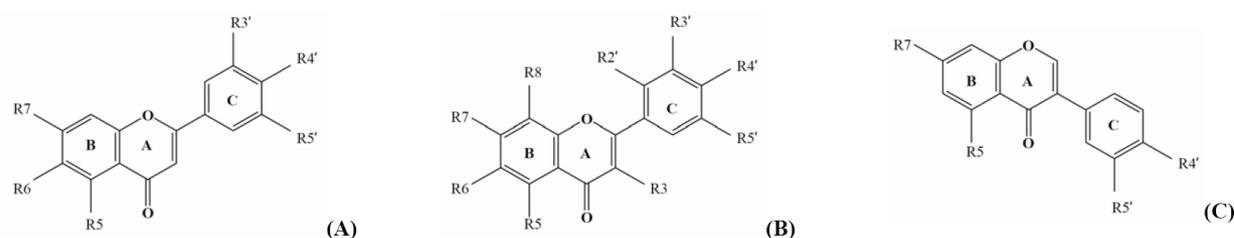
| Compounds | R3 | R5 | R6 | R7 | R8 | R2' | R3' | R4' | R5' |
|---|---|---|---|---|---|---|---|---|---|
| *(A) Flavones:* | | | | | | | | | |
| baicalein | | OH | OH | OH | | | H | H | H |
| baicalin | | OH | OH | H | | | H | H | H |
| oroxylin A | | OH | OCH$_3$ | OH | | | H | H | H |
| wogonin | | OH | H | OH | | | H | H | H |
| *(B) Flavonols:* | | | | | | | | | |
| myricetin | OH | OH | H | OH | H | H | OH | OH | OH |
| quercetin | OH | OH | H | OH | H | H | OH | OH | H |
| quercetin pentaacetate | O-AcOH | O-AcOH | H | O-AcOH | H | H | O-AcOH | O-AcOH | H |
| quercitrin | O-Rhamnoside | OH | H | OH | H | H | OH | OH | H |
| *(C) Isoflavonoids:* | | | | | | | | | |
| daidzein | | H | | OH | | | | OH | H |

**Figure 1.** Structures of (A) the flavones, baicalein, baicalin, oroxylin A, and wogonin, isolated from the root of *Scutellaria baicalensis*; (B) the flavonols, myricetin extracted from the leaves of *Eugenia uniflora*, and quercetin, quercetin pentaacetate, and quercitrin isolated from *Sophora japonica*; and (C) the isoflavonoid, daidzein isolated from the root of *Pueraria lobata*. The different flavonoid types are presented in italics.

acid to yield crude rutin. The crude rutin was dissolved in warm water and recrystallized with ethanol to obtain rutin. Rutin was partially and completely hydrolyzed with 3% sulfuric acid in a water bath for an hour to respectively yield quercitrin and quercetin. Quercetin was dissolved in ethyl alcohol, and an equal volume of acetic anhydride was added for acetylation to obtain crude crystals, which were recrystallized with 90% methanol to obtain the needle crystal form of quercetin pentaacetate.

**Test sample preparation**

Each isolated compound was subjected to high-performance liquid chromatography (HPLC). The HPLC system consisted of a Shimadzu model LC-10AT system (Kyoto, Japan) equipped with a Shimadzu model SIL-9A autoinjector and a Shimadzu model SPD-10 A detector. Peak areas were calculated with a Shimadzu model C-R8A recorder. A LiChrospher 100 RP-18e reversed-phase column (Merck, Darmstadt, Germany) and a LiChrospher 100 RP-18e guard column (Merck) were used. The purity of all compounds exceeded 98.5%. Each test compound was dissolved in DMSO for the following assay, and the concentration of DMSO was always < 0.05%.

***Preparation of liver mitochondrial solution (Lee et al., 2007)***

Male SD rats (4 to 6 weeks old) were killed by cervical dislocation and the livers were removed as soon as possible to perfuse with ice-cold PBS and homogenized in a Potter Elvehjem homogenizer (Lee et al., 2007). The homogenate was centrifuged at 2,000 rpm for 10 min at 4°C. The clear suspensions were re-centrifuged at 12,700 rpm for 10 min at 4°C to obtain mitochondrial s olution.

***Determination of protein content (Lee et al., 2007)***

Different concentrations of a protein solution were pipetted into 1.5 ml test tubes. The volume in the test tube was adjusted to 50 µl with PBS. Protein reagent was added to the test tube, and the contents were mixed with a vortex-mixer. The absorbance at 595 nm was measured. The weight of protein was plotted against the

corresponding absorbance resulting in a standard curve used to determine the protein in the mitochondrial solution.

### *FeCl$_2$ induced LPO of liver mitochondria (Lee et al., 2007)*

The reaction mixture solution had a total volume of 250 µl containing 50 µl of liver mitochondria (with a protein content of 0.5 mg/ml), 100 µl of PBS, 50 µl of a serial dilution of FeCl$_2$ solution (0.0625 to 8 mM), and 50 µl of distilled water; this was incubated at 37°C in an incubator for 60 min. The reaction was st opped by the addition of 375 µl of H$_3$PO$_4$, 200 µl of distilled water, and 125 µl of TBA.

The reaction mixture was incubated at 90°C in an in cubator for 66 min. At the end, the tubes were shifted to an ice bath and 350 µl of methanol-NaOH (9.1:0.9 v/v) was added, then the mixture was centrifuged at 13,000 rpm for 5 min at 4°C. The produ ct, MDA(TBA)$_2$, was quantitatively analyzed by HPLC at a wavelength of 532 nm.

### *LPO-inhibitory capability (Lee et al., 2007)*

The LPO-inhibitory activity of each compound was determined by a TBA-reactive substance (TBARS) assay, and the MDA(TBA)$_2$ product was quantitatively analyzed by HPLC. Briefly, the reaction mixture in a total volume of 250 µl contained 50 µl of liver mitochondria, 100 µl of PBS, 50 µl of an FeCl$_2$ solution (4 mM), and 50 µl of natural flavonoids (20 or 50 µg/ml) or standard compound was silymarin; this was incubated at 37°C in incuba tor for 60 min. The reaction was stopped by the addition of 375 µl of H$_3$PO$_4$, 200 µl of distilled water, and 125 µl TBA. The reaction mixture was incubated at 90°C in an incubator for 66 min. At the end, the tubes were shifted to an ice bath, and 350 µl of methanol-NaOH was added. This was centrifuged at 13,000 rpm for 5 min at 4°C. The inhibition of lipid peroxidation (%) was calculated by the following formula:

$$(A_{control\ (532\ nm)} - A_{sample\ (532\ nm)}) / (A_{control\ (532\ nm)} - A_{blank\ (532\ nm)}) \times 100\%.$$

### Quantitative analysis of MDA(TBA)$_2$ by HPLC

The MDA(TBA)$_2$ product was quantitatively analyzed by HPLC conducted on a Shimadzu system equipped with an ERC-3415α degasser, an LC-10AT pump, an SPD-M10A photodiode array detector, and an SIL-10AD auto-sampler. Peak areas were calculated with CLASS-VP software. A reversed-phase LiChroCART RP-18 column (4 × 250 mm, 5 µm, Merck) was used. The column oven was set to 25°C. The mobile phases consisted of 60% KH$_2$PO$_4$ (pH 8.2) and a 40% methanol solution. The detection wavelength was set to 532 nm. The flow rate was 1.0 ml/min. The volume for each sample injection was 20 µl.

### *DPPH free radical-scavenging activity* (Lee et al., 2007)

The free radical-scavenging activity assay was measured using DPPH. One hundred microliters of a serial dilution of each natural flavonoid (0.78 to100 µg/ml) solution or standard compound was gallic acid which was diluted with MeOH and mixed with 100 µl of 50 µM DPPH in methanol. After 30 min, the absorbance was measured on an ELISA reader at 517 nm. A lower absorbance of the reaction mixture solution indicated higher free radical-scavenging activity. The capability to scavenge DPPH radicals was calculated using the following equation:

$$\text{Scavenging effect (\%)} = (1 - A_{sample\ (517\ nm)} / A_{control\ (517\ nm)}) \times 100.$$

### Superoxide anion radical-scavenging activity

The measurement of the superoxide anion-scavenging activity of each natural flavonoid was based on the method described by Robak and Gryglewski, (1988). All solutions were prepared in 0.2 M sodium phosphate buffer (pH 7.4). The reaction mixture contained a serial dilution of each natural flavonoid (3.32 to 425 µg/ml) or standard compound was SOD, PMS (120 µM), β-NADH (936 µM), and NBT (300 µM). It was incubated at room temperature for 5 min, and the absorbance was read at 560 nm on an ELISA reader against a blank. Decreased absorbance of the reaction mixture indicated increased superoxide anion-scavenging activity. The capability to scavenge superoxide anions was calculated by the following equation:

$$\text{Scavenging effect (\%)} = (1 - A_{sample\ (560\ nm)} / A_{control\ (560\ nm)}) \times 100.$$

### Hydrogen peroxide-scavenging activity

The hydrogen peroxide-scavenging ability of each natural flavonoid was determined by the method of Rinkus and Taylor (1990). The principle of this method is that there is a decrease in absorbance of H$_2$O$_2$ upon its oxidation. The reaction mixture contained a serial dilution of each natural flavonoid (0.39 to 50 µg/ml) or standard compound was catalase; 0.4 ml of H$_2$O$_2$ solution (4 mM), and 0.6 ml of HRPase-phenol red incubated at 25°C, and the abs orbance of the reaction mixture was recorded at 610 nm on an ELISA reader. A lower absorbance of the reaction mixture indicated higher hydrogen peroxide-scavenging activity. The percentages of H$_2$O$_2$ scavenging by natural flavonoids and standard compounds were calculated using the following equation:

$$\text{scavenging effect (\%)} = (1 - A_{sample\ (610\ nm)} / A_{control\ (610\ nm)}) \times 100.$$

### Superoxide anion radical inhibition

Superoxide anion radical inhibition was assayed by the method of Kong et al. (2000). The reaction mixture contained a serial dilution of each natural flavonoid (0.78 to 100 µg/ml) or standard compound was allopurinol, PBS (75 mM), xanthine (480 µM), and XO (0.5 U/ml). Absorption increments at 295 nm on an ELISA reader indicating the formation of uric acid at 25°C were f ollowed, and the initial velocity was calculated using the following equation:

$$\text{Inhibitory effects (\%)} = (1 - A_{sample\ (295\ nm)} / A_{control\ (295\ nm)}) \times 100.$$

### Ferrous ion-chelating activity

The chelation of ferrous ions was determined by the method of Dinis et al. (1994) wherein the ferrous ion-chelating ability of each natural flavonoid was monitored by the absorbance of the ferrous ion-ferrozine complex at 562 nm. The reaction mixture, containing each natural flavonoid (50 µg/ml) or standard compound was EDTA, FeCl$_2$ (2 mM), and ferrozine (5 mM), was adjusted to a total volume of 1 ml with methanol, shaken well, and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm on an ELISA reader against a blank. A lower absorbance indicated higher metal-chelating activity. The ability of each natural flavonoid to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating- effect (\%)} = (1 - A_{sample\ (562\ nm)} / A_{control\ (562\ nm)}) \times 100.$$

### *Ferric-reducing ability of plasma (FRAP)* (Lee et al., 2007)

In the FRAP assay, excess Fe$^{3+}$ was used, and the reducing ability
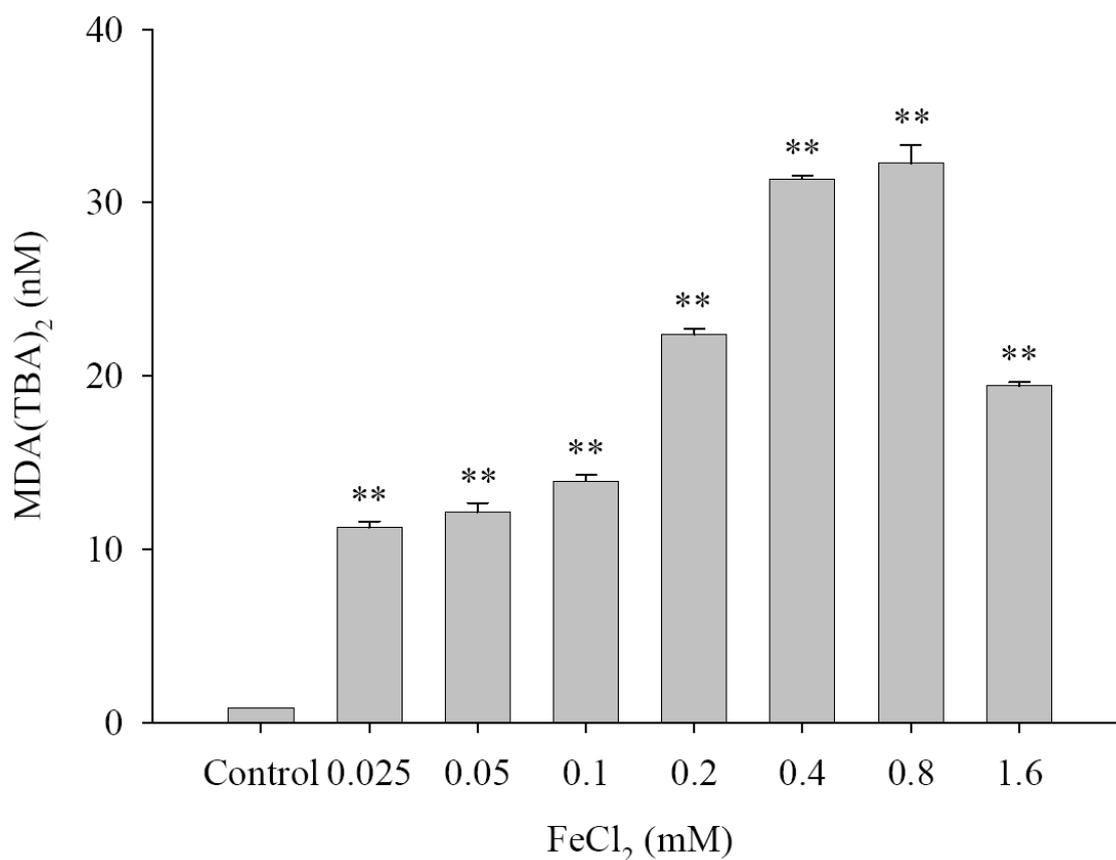
**Figure 2.** The capability of $FeCl_2$ (0.025 to 1.6 mM) -induced lipid peroxidation of liver mitochondria. The amount of $MDA(TBA)_2$ induced by $FeCl_2$ (0.8 mM) was significantly increased in liver mitochondria. Data derived from three independent experiments were statistically analyzed, and results are presented as the mean±SD. **$p < 0.01$ indicate significant differences from the control group as analyzed by Student's $t$-test.

of the sample is the rate-limiting factor of $Fe^{2+}$-TPTZ and hence color formation. The reaction mixture contained a serial dilution of each natural flavonoid (187.5 to 1500 μg/ml) or standard compound was trolox, acetate buffer solution (300 mM), TPTZ solution (5 mM), and $FeCl_3 \cdot 6H_2O$ (20 mM). The absorbance of the mixture was measured on an ELISA reader at 593 nm against a blank. An increased absorbance of the reaction mixture indicated an increase in the antioxidant capability.

**Statistical analysis**

Experiments were performed in triplicate. The data are presented as the mean ± SD and analyzed by Student's $t$-test. $p$ values of < 0.05 and < 0.01 were regarded as significantly and very significantly different from the control group.

**RESULTS**

**FeCl$_2$ induced LPO of liver mitochondria**

The effects of $FeCl_2$ at inducing LPO of liver mitochondria are shown in Figure 2. The amount of $MDA(TBA)_2$ induced by different concentrations (0.025 to 1.6 mM) of $FeCl_2$ were 11.23 ± 0.34, 12.13 ± 0.53, 13.92 ± 0.34, 22.38 ± 0.34, 31.36 ± 0.20, 32.26 ± 1.05, and 19.44 ± 0.20 nM, respectively. The highest amount of $MDA(TBA)_2$ induced by 0.8 mM $FeCl_2$ significantly increased in liver mitochondrial solution.

**LPO-inhibitory effects of each flavonoid on liver mitochondria**

LPO of polyunsaturated fatty acids leads to the formation of malondialdehyde (MDA) and causes cellular injury that result in several pathological conditions. The TBARS method measures the amount of $MDA(TBA)_2$, which is the product of LPO (Lee et al., 2007). In this study, LPO inhibitory activities of natural flavonoids and silymarin (as a positive control) were determined. Silymarin was derived from European botany-milk thistle as a liver supporter to prevent and treat cirrhosis, chronic hepatitis, and gall bladder problems. Table 1 shows the LPO-inhibitory effects of nine natural flavonoids, and five of

these compounds exerted significant effects as compared to the control group, including baicalein, baicalin, myricetin, quercetin, quercetin pentaacetate, and silymarin with respective values of 95.42 ± 5.14%, 98.76 ± 6.54%, 87.77 ± 8.06%, 80.25 ± 4.32%, 100.00 ± 8.72% and 95.56 ± 0.65%, at a concentration of 50 µg/ml. To evaluate the $IC_{50}$ values of baicalein, baicalin, myricetin, quercetin, quercetin pentaacetate, and silymarin were 9.32 ± 4.13, 9.52 ± 3.27, 19.63 ± 1.82, 16.15 ± 4.62, 20.22 ± 4.38, and 24.74 ± 0.47 µg/ml on SD rat liver mitochondrial solution, respectively. Meanwhile, the data exhibit the almost 2.65 fold stronger $IC_{50}$ value of baicalein and baicalin and both flavones reveal more powerful antioxidant activity of LPO (Table 1).

## DPPH free radical-scavenging activity

DPPH is widely used to evaluate the free radical-scavenging effects of various antioxidant substances. The DPPH radical absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. When a hydrogen atom or electron is transferred to the odd electron in DPPH•, the absorbance at 517 nm decreases proportionally to the increase in the non-radical form of DPPH (Ancerewicz et al., 1998). In this study, free radical-scavenging activities of each natural flavonoid and gallic acid as a positive control were determined using a DPPH method.

Gallic acid is an organic acid, also known as 3,4,5-trihydroxybenzoic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants. The potential DPPH radical-scavenging effects of nine natural flavonoids at a concentration of 50 µg/ml were in the decreasing order of baicalin (96.36%), baicalein (95.09%), quercetin pentaacetate (94.18%), quercetin (94.05%), quercitrin (91.78%), and myricetin (86.11%). The free radical-scavenging activities of these samples also revalued with an increasing concentration. The baicalein and baicalin exhibited the same inhibitory behavior, but not oroxylin A and wogonin, although they belonged to the flavones group. Quercetin and myricetin have extra 2 fold scavenging activity that is better than quercitrin and quercetin pentaacetate in flavonols group. In summary, we calculated the $IC_{50}$ concentrations of the DPPH-scavenging effect of baicalein, baicalin, myricetin, quercetin, quercetin pentaacetate and quercitrin as 3.61 ± 0.20, 3.28 ± 0.29, 2.41 ± 0.15, 2.16 ± 0.21, 3.74 ± 0.09, and 3.92 ± 0.20 µg/ml, respectively and we made a comparison of the relationship between their chemical structure and activity in the study's discussion (Table 2).

## Superoxide anion radical-scavenging activity

Superoxide anions are a precursor to active free radicals that have the potential to react with biological macro-

molecules, thereby inducing tissue damage. In addition, superoxide anions are a relatively weak oxidant; they decompose to form stronger reactive oxidative species, such as singlet oxygen and hydroxyl radicals, which initiate LPO. Superoxide anions derived from dissolved oxygen by the PMS-NADH coupling reaction reduced NBT in this system. In this method, superoxide anions reduce the yellow dye ($NBT^{2+}$) to produce blue formazan which was measured by an ELISA reader at 560 nm. Antioxidants are able to inhibit the blue NBT formation. In this study, free radical-scavenging activities of the nine natural flavonoids and SOD (as a positive control) were determined. The enzyme, SOD, catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. The superoxide anion radical-scavenging ability of wogonin at the concentration of 50 µg/ml was 18.66%. Results of the superoxide anion-scavenging effect of each natural flavonoid showed no significant decrease.

## Hydrogen peroxide ($H_2O_2$)-scavenging activity

$H_2O_2$, formed by the two-electron reduction of O, is an oxidizing agent. $H_2O_2$ can cross membranes and may slowly oxidize a number of biomolecules and compounds. In addition, $H_2O_2$ also forms OH• in the presence of metal ions, and oxygen facilitates this reaction. Hence, $H_2O_2$-scavenging processes are important for living organisms (Gulcin et al., 2003). The principle of this method is a decrease in absorbance of $H_2O_2$ upon its oxidation. In this study, free radical-scavenging activities of nine natural flavonoids and catalase (as a positive control) were determined. Catalase is a common enzyme found in nearly all living organisms, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. It contains four porphyrin heme (iron) groups that allow the enzyme to react with hydrogen peroxide. The reaction of catalase in the decomposition of hydrogen peroxide is: $2H_2O_2 \rightarrow 2H_2O + O_2$. The hydrogen peroxide-scavenging ability of wogonin at a concentration of 50 µg/ml was 6.04%. Results of the hydrogen peroxide-scavenging effect of each natural flavonoid showed no significant decrease.

## Inhibition of superoxide anion radicals

Superoxide anions are a precursor to active free radicals that have the potential to react with biological macromolecules, thereby inducing tissue damage. In this study, free radical-inhibitory activities of natural flavonoids and allopurinol (as a positive control) were determined. Allopurinol is an enzyme inhibitor that inhibits XO, which is responsible for the successive oxidation of hypoxanthine and xanthine resulting in the production of uric acid, a product of human purine metabolism (Acher et al., 2006). Allopurinol therefore decreases both uric

**Table 1.** Inhibitory effects of natural flavonoids on $FeCl_2$ induced rat liver mitochondria lipid peroxidation. LPO activities of natural flavonoids and silymarin as a positive control were determined. Five of the nine compounds exerted a significant effect from the control group. Data derived from three independent experiments were statistically analyzed, and results are presented as the mean±SD.

| S/N | Drug | Inhibition (%) | | | | | | | | $IC_{50}$ (µg/ml) |
|-----|------|------|------|------|------|------|------|------|------|------|
| | | 50 µg/ml | 25 µg/ml | 12.5 µg/ml | 6.25 µg/ml | 3.13 µg/ml | 1.56 µg/ml | 0.78 µg/ml | 0.39 µg/ml | |
| a | Baicalein | 95.42 ± 5.14 | 63.48 ± 0.93 | 58.21 ± 1.72 | 56.88 ± 2.17 | 19.63 ± 3.17 | 10.37 ± 2.07 | 2.74 ± 1.58 | 1.45 ± 1.54 | 9.32 ± 4.13 |
| b | Baicalin | 98.76 ± 6.54 | 51.05 ± 0.44 | 50.75 ± 2.59 | 46.25 ± 7.94 | 13.76 ± 6.90 | 1.35 ± 1.56 | 0.00 ± 3.90 | 0.00 ± 21.53 | 9.52 ± 3.27 |
| c | Daidzein | 9.03 ± 3.20 | — | — | — | — | — | — | — | — |
| d | Myricetin | 87.77 ± 8.06 | 63.24 ± 2.38 | 40.24 ± 0.84 | 26.18 ± 4.42 | 10.82 ± 1.62 | 2.29 ± 1.34 | — | — | 19.63 ± 1.82 |
| e | Oroxylin A | 11.68 ± 3.52 | — | — | — | — | — | — | — | — |
| f | Quercetin | 80.25 ± 4.32 | 54.67 ± 16.72 | 43.28 ± 1.72 | 34.38 ± 3.61 | 3.93 ± 1.54 | 2.86 ± 3.30 | 0.00 ± 0.18 | 0.00 ± 0.75 | 16.15 ± 4.62 |
| g | Quercetin pentaacetate | 100.00 ± 8.72 | 50.67 ± 0.00 | 35.82 ± 3.45 | 23.13 ± 5.05 | 13.08 ± 1.61 | 0.68 ± 0.78 | 0.00 ± 0.97 | — | 20.22 ± 4.38 |
| h | Quercitrin | 45.52 ± 2.74 | — | — | — | — | — | — | — | — |
| i | Wogonin | 0.00 ± 23.20 | — | — | — | — | — | — | — | — |
| Positive control | Silymarin | 95.56 ± 0.65 | 68.75 ± 3.42 | 50.36 ± 0.42 | 16.26 ± 2.07 | 26.49 ± 0.90 | 12.41 ± 5.96 | 4.09 ± 14.08 | 0.00 ± 0.00 | 24.74 ± 0.47 |

— Not detected

**Table 2.** DPPH free radical scavenging activity of the natural flavonoids. The high potential scavenging effects of nine natural flavonoids on the DPPH radical decreased in the order of baicalein, baicalin, quercetin pentaacetate, quercetin, quercitrin, and myricetin, at the concentration of 50 µg/ml. Scavenging activity of gallic acid (50 µM; 8.51 µg/ml) as a positive control was 94.01 ± 0.00%. $IC_{50}$ of gallic acid was 5.90 ± 0.32 µM (1.00 ± 0.05 µg/ml). Data derived from three independent experiments were statistically analyzed, and results are presented as the mean±SD.

| S/N | Drug | Scavenging (%) | | | | | | | | $IC_{50}$ (µg/ml) |
|-----|------|------|------|------|------|------|------|------|------|------|
| | | 50 µg/ml | 25 µg/ml | 12.5 µg/ml | 6.25 µg/ml | 3.13 µg/ml | 1.56 µg/ml | 0.78 µg/ml | 0.39 µg/ml | |
| a | Baicalein | 95.09 ± 0.00 | 95.33 ± 0.00 | 95.33 ± 0.00 | 77.69 ± 0.04 | 42.49 ± 0.01 | 25.33 ± 0.01 | 14.26 ± 0.02 | 10.89 ± 0.02 | 3.61 ± 0.20 |
| b | Baicalin | 96.36 ± 0.01 | 95.35 ± 0.00 | 95.35 ± 0.00 | 79.97 ± 0.06 | 47.13 ± 0.05 | 26.90 ± 0.05 | 15.85 ± 0.05 | 13.96 ± 0.04 | 3.28 ± 0.29 |
| c | Daidzein | 4.72 ± 5.28 | — | — | — | — | — | — | — | — |
| d | Myricetin | 86.11 ± 0.00 | 85.50 ± 0.01 | 87.12 ± 0.01 | 90.76 ± 0.08 | 60.49 ± 0.08 | 32.70 ± 0.05 | 16.45 ± 0.05 | — | 2.41 ± 0.15 |
| e | Oroxylin A | 0.00 ± 0.00 | — | — | — | — | — | — | — | — |
| f | Quercetin | 94.05 ± 0.00 | 93.25 ± 000 | 93.25 ± 0.00 | 92.51 ± 0.00 | 66.84 ± 0.00 | 34.09 ± 0.01 | 14.84 ± 0.01 | 8.42 ± 0.01 | 2.16 ± 0.21 |
| g | Quercetin pentaacetate | 94.18 ± 0.00 | 93.38 ± 0.00 | 93.05 ± 0.00 | 78.68 ± 0.00 | 43.58 ± 0.00 | 21.93 ± 0.00 | 8.09 ± 0.00 | 4.81 ± 0.00 | 3.74 ± 0.09 |
| h | Quercitrin | 91.78 ± 0.00 | 92.11 ± 0.00 | 90.84 ± 0.00 | 74.80 ± 0.03 | 40.37 ± 0.02 | 19.32 ± 0.01 | 8.29 ± 0.00 | 4.75 ± 0.00 | 3.92 ± 0.20 |
| i | Wogonin | 0.00 ± 0.00 | — | — | — | — | — | — | — | — |

— Not detected

**Table 3.** Chelated (%) of $Fe^{2+}$ by natural flavonoids. Chelating effect of EDTA (50 µg/ml) as a positive control was 99.67 ± 0.24%. Data derived from three independent experiments were statistically analyzed, and results are presented as the mean±SD.

| S/N | Drug (50 µg/ml) | Chelated (%) |
|---|---|---|
| a | Baicalein | 44.84 ± 2.26 |
| b | Baicalin | 38.48 ± 3.46 |
| c | Daidzein | 10.56 ± 2.34 |
| d | Myricetin | 14.62 ± 3.00 |
| e | Oroxylin A | 0.26 ± 0.30 |
| f | Quercetin | 3.39 ± 0.49 |
| g | Quercetin pentaacetate | 3.22 ± 2.65 |
| h | Quercitrin | 0.00 ± 0.00 |
| i | Wogonin | 0.14 ± 0.16 |

acid formation and purine synthesis. The inhibitory effects of natural flavonoids on superoxide anions were low at a concentration of 50 µg/ml. These results compared with the data of superoxide anion radical-scavenging ability and the fact demonstrates that each natural flavonoid had no significant effect on superoxide anion radical scavenging or inhibition.

**Ferrous ion-chelating activity**

Among the transition metals, iron is known to be the most important prooxidant of lipid oxidation due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton reaction[3]: $Fe^{2+}$ + $H_2O_2 \rightarrow Fe^{3+}$ + $OH^-$ + OH•. Bivalent ferrous ions ($Fe^{2+}$) are the most powerful prooxidant among the various species of metal ions. In this study, chelating activities of natural flavonoids and EDTA (as a positive control) were determined ferrous ion-chelating activity. EDTA is a very capable chelating agent with the formula $(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$. EDTA binds to metals via four carboxylate and two amine groups and it forms, in particular, strong complexes with Mn(II), Cu(II), Fe(III), Pb (II) and Co(III). These natural flavonoids had no significant chelating effect on ferrous ions, but baicalein and baicalin were shown to have almost 40% chelating power (Table 3).

**Antioxidant capability of each flavonoids by FRAP**

A simple, automated test to measure the ferric-reducing ability of plasma, the FRAP assay, can assess antioxidant power. Trolox concentrations are given as the calculated concentrations (Trolox equivalent antioxidant capacity, TEAC). In this study, reductive activities of each natural flavonoid were compared to trolox which was used as a standard, and were determined afterwards.

Trolox is Hoffman-LaRoche's trade name for 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, a water-soluble derivative of vitamin E and it is a vitamin E-like antioxidant which is used in biological and biochemical applications to reduce oxidative stress and damage. Table 4 shows the reductive abilities of the nine natural flavonoids. At a concentration 10 µg/ml, the flavonoids myricetin, quercetin, quercetin pentaacetate, quercitrin, baicalin and baicalein had trolox equivalent values of 20.46 ± 2.58, 16.42 ± 0.99, 14.46 ± 1.26, 13.98 ± 0.28, 12.73 ± 1.19, and 8.13 ± 1.62 µM, respectively. We found that four compounds, myricetin, quercetin, quercetin pentaacetate and quercitrin which belong to flavonols group are equivalent to a half trolox activity at least. The flavones group, baicalin and baicalein show less trolox activity to one third of its compounds, but its activity was not detected on daidzein, oroxylin A and wogonin.

**DISCUSSION**

In view of these results, we determine nine flavonoids of four traditional Chinese herbs which can protect the lipids in membrane are continuously subjected to oxidant challenges. Oxidant induced abstraction of a hydrogen atom from an unsaturated fatty acyl chain of membrane lipids initiates the process of LPO, which propagates as a chain reaction (Montiel et al., 2006). We examined the anti-inflammatory traditional herbs, *S. baicalensis* roots, *P. lobata* roots, *E. uniflora* leaves, and *S. japonica* flowers. *S. baicalensis* was confirmed as having strong anti-inflammatory properties (Kim et al., 2009), the ability to induce apoptosis in B-1F cells (Murashima et al., 2009), neuroprotective effects (Yune et al., 2009), and tocolytic effects (Shih et al., 2009). *P. lobata* inhibited the cytotoxicity of enterovirus 71 in a human foreskin fibroblast cell line (Su et al., 2008), inhibited the activity of advanced glycation end (AGE) product formation *in vitro* (Jang et al., 2006), and prevented secondary osteoporosis induced by dexamethasone (DXM) in rats

**Table 4.** Reductive ability from natural flavonoids as measured by FRAP method. Reductive activities of each natural flavonoid and trolox as a standard were determined. Trolox concentration given is the calculated concentrations (trolox equivalent antioxidant capacity, TEAC). Data derived from three independent experiments were statistically analyzed, and results are presented as the mean±SD.

| S/N | Drug (10 µg/ml) | TEAC (µM) | Relative activity (%) |
|-----|-----------------|-----------|------------------------|
| a | Baicalein | 8.13 ± 1.62 | 22 |
| b | Baicalin | 12.73 ± 1.19 | 57 |
| c | Daidzein | 0.22 ± 0.25 | 1 |
| d | Myricetin | 20.46 ± 2.58 | 65 |
| e | Oroxylin A | 0.20 ± 0.23 | 1 |
| f | Quercetin | 16.42 ± 0.99 | 50 |
| g | Quercetin pentaacetate | 14.46 ± 1.26 | 74 |
| h | Quercetrin | 13.98 ± 0.28 | 63 |
| i | Wogonin | 0.01 ± 0.01 | 0 |

(Zheng et al., 2002). *E. uniflora* was found to be effective against bacteria (Oliveira et al., 2008), to have antinociceptive and hypothermic effects (Amorim et al., 2009), and to have a dual effect on the heart related to its hypotensive action and is probably responsible for the therapeutic or adverse effects in patients under cardiac risk (Consolini and Sarubbio, 2002). *S. japonica* exhibits cellular tyrosinase inhibition in human epidermal melanocytes (Lo et al., 2009), and reduces the cerebral infarction area and neurological deficits induced by ischemia-reperfusion in rats (Lao et al., 2005). In brief, these four herbs could improve many biological events as reported symptoms which involve a widely free radical species generation. As a result, the flavonoids are to be regarded as the major antioxidant on their OH groups whose amount and location could be classified into flavanols, flavanones, flavones, flavonols, and isoflavonoids.

Reactive oxygen species (ROS) are responsible for numerous pathological conditions including cancers, cell injury, inflammation, Alzheimer's disease, intestinal diseases, and several other debilitating diseases. Hence, it was suggested that the consumption of antioxidant-rich diets would help alleviate diseases caused by ROS (Raghavan et al., 2008).

Mitochondrias are important intracellular sources and targets of ROS, while flavonoids, a large group of secondary plant metabolites, are important antioxidants. In view of the LPO-inhibitory activities and DPPH-scavenging effect, five of the nine natural flavonoids (baicalein, baicalin, myricetin, quercetin and quercetin pentaacetate) exerted significant effects compared to the control group.

The phenol groups are efficacious, as are the activities of flavones with 5,6 hydroxy groups in the B-ring (Table 1). The presence of an ortho-dihydroxy structure, baicalein and baicalin, in the B-ring increases the more antioxidant activity of flavones then meta-dihydroxyl structure, wogonin and oroxylin A. This ortho-dihydroxy structure in the B-ring is the radical target site (Cos et al., 2001).

Most studies concluded that both a catechol or pyrogallol moiety in the B-ring and a free hydroxy group at position C-3 are essential for high radical-scavenging effects.

At the position C-3, the different substituents reveal to decrease DPPH-scavenging activity because the ester group (quercetin pentaacetate and quercetrin) instead of OH group (myricetin and quercetin). In a word, the R3-OH group flavonoids (myricetin, quercetin, and quercetin pentaacetate) could enhance their ability as vitamin E and a powerful DPPH radical scavenger. Instead of R6-OH group flavonoids (baicalein and baicalin), these compound could protect liver damage by inhibiting LPO due to ferrous induced ROS and the parallel trend of $Fe^{2+}$ chelating ability, but isoflavonoids (diadzein) does not show the same protect potential as other flavones and flavonols groups.

The results showed that two flavones of baicalein and baicalin, and three flavonols of myricetin, quercetin, and quercetin pentaacetate have potential constituents for developing antioxidants and protectants against liver damage.

In conclusion, the hydroxy group might play a role in increasing the activity of the flavonoids and exhibited potent antioxidant activities. Here, we examined the inhibitory activity on different kinds of ROS generator and their scavenging ability of traditional medicinal herbs that we regularly use, and a combination of two or three complementary herbs that are popularly used in Asia. The high content of flavonoids of *S. baicalensis* and *S. japonica* might protect cell membrane and eliminate the later stage radical generator such as lipid peroxidation, but not the clearance superoxide anion or hydroxyl radical at the beginning of the ROS stage (Fischer et al., 2002).

## ACKNOWLEDGEMENT

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