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Dandelion (*Taraxacum officinale*) flower extract suppresses both reactive oxygen species and nitric oxide and prevents lipid oxidation *in vitro*

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Abstract

Flavonoids and coumaric acid derivatives were identified from dandelion flower (*Taraxacum officinale*). Characteristics of chain-breaking antioxidants, such as extended lag phase and reduced propagation rate, were observed in oxidation of linoleic acid emulsion with the addition of dandelion flower extract (DFE). DFE suppressed both superoxide and hydroxyl radical, while the latter was further distinguished by both site-specific and non-specific hydroxyl radical inhibition. DPPH-radical-scavenging activity and a synergistic effect with α-tocopherol were attributed to the reducing activity derived from phenolic content of DFE. A significant ($p<0.05$) and concentration-dependent, reduced nitric oxide production from bacterial-lipopolysaccharide-stimulated mouse macrophage RAW264.7 cells was observed with the addition of DFE. Moreover, peroxyl-radical-induced intracellular oxidation of RAW264.7 cells was inhibited significantly ($p<0.05$) by the addition of DFE over a range of concentrations. These results showed that the DFE possessed marked antioxidant activity in both biological and chemical models. Furthermore, the efficacy of DFE in inhibiting both reactive oxygen species and nitric oxide were attributed to its phenolic content.

Keywords: Dandelion; (*Taraxacum officinale*); Oxidation; Antioxidants; Reactive oxygen species; Nitric oxide

Introduction

Natural antioxidants not only protect food lipids from oxidation, but may also provide health benefits associated with preventing damage due to biological degeneration (Davies, 1995). Moreover, aerobic mammals use oxygen to maintain normal physiological functions, and up to 2% of oxygen consumption may end in the form of reactive oxygen species (ROS) (Davies, 1995; Yuan and Kitts, 1996). ROS are oxygen derivatives with unpaired orbital electrons and as a result are unstable and highly reactive. ROS include hydroxyl radical, superoxide radical, peroxyl radical and singlet oxygen (Halliwell, 1995). Though some ROS are part of normal metabolism (Davies, 1995; Halliwell, 1995), cigarette smoking and exposure to environmental oxidative stress (Halliwell and Auroma, 1997) can result in the production of exogenous sources of ROS, which may contribute to several forms of human cancer (Morse and Stoner, 1993). Exogenous antioxidants act to supplement endogenous primary antioxidants, such as α-tocopherol, in combating against

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cell injury induced by oxygen radicals. In addition to ROS, reactive nitrogen species (RNS), such as nitric oxide and peroxynitrite, also have high reactivity with potentially important biological significance (Halliwell, 1995).

Natural plant extracts, particularly those with abundant polyphenolic content, have been examined for their antioxidant potential in both food and biological models (Cuvelier et al., 1994; Teissedre et al., 1996; Marcocci et al., 1994; Hu and Kitts, 2000; Liao and Yin, 2000). The structure-activity relationships of polyphenolic antioxidants have been addressed in various model systems, and factors determining antioxidant activity include the amount and position of hydroxyl substitution (Rice-Evans, 1995).

Dandelions (*Taraxacum officinale*) have been used as a phytomedicine for their choleretic, diuretic, antirheumatic and anti-inflammatory properties (Bisset, 1994). The distribution of flavonoids and phenolic fractions in dandelion (*Taraxacum officinale*) has been documented (Williams et al., 1996), but antioxidant and free-radical-scavenging activities of dandelion flower have not been examined until recently. We demonstrated that a fractionated dandelion flower product inhibited both DNA scission and LDL oxidation induced by peroxyl radicals; however, the pro-oxidant character was shown, due to the reduction of transition metal ion, to initiate the Fenton reaction (Hu and Kitts, 2003). The purpose of this study was to examine antioxidant activity of a crude dandelion flower standardized extract against other common oxygen radicals, i.e., superoxide radical and hydroxyl radical. In addition, the effect of the dandelion flower extract (DFE) in scavenging nitric oxide and suppressing peroxyl radical-induced intracellular oxidation was also evaluated, using a mouse macrophage cell line.

Materials and methods

Nitro blue tetrazolium (NBT), xanthine, xanthine oxidase (EC1.1.3.22), deoxyribose, linoleic acid, 2-thiobarbituric acid, deoxyribose, bacterial lipopolysaccharide (LPS, *Escherichia coli,* serotype 0111:B4), superoxide dismutase (SOD, EC1.15.1.1), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2′, 7′-dichlorofluorescin diacetate (DCFH-DA), Dulbecco's modified Eagle medium (DMEM), Folin-Cioalteu reagent (2N) and gallic acid were purchased from Sigma Chemical Co. (St. Louis, MO). 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals USA Inc. (Richmond, VA). HPLC grade luteolin and luteolin-7-O-glucoside were obtained from Indofine Chemical Co. (Somerville, NJ). Other reagents were analytical or higher grade.

Extraction of dandelion flower

Dandelions flowers were collected from a certified dandelion farm (Naturally Nova Scotia, Dartmouth, NS) during early summer. Flowers were separated manually from aerial parts and washed with tap water prior to freeze-drying. Freeze dried dandelion flower was then extracted by reflux 40 times with 70% (v/v) ethanol solution for 8 h. The obtained 70% (v/v) ethanol extract was rotary evaporated ($<40\,^{\circ}C$) to one-third of original volume and stored at 4 °C overnight, followed by filtration (Whatman No. 4 filter paper). The filtrate was then dried in vacuo. The dry material (DFE) obtained here was used for the entire study.

Total phenolic content

Total phenolic content was measured by Folin–Ciocalteu assay (Shahidi and Naczk, 1995) with modification. Briefly, a 100 µl sample was mixed with 500 µl of 10-times diluted Folin–Ciocalteu regent and 400 µl 7.5% (w/v) Na_2CO_3. The absorbance at 765 nm was measured 10 min later at room temperature (Multiskan Spectrum, ThermoLabsystem). A standard curve was obtained from gallic acid and total phenolic content was expressed as a gallic acid equivalent (µg gallic acid/mg DFE).

Reducing power

Reducing power of DFE was measured (Hu and Kitts, 2000) using the standard reducing agent ascorbic acid as the reference. The reducing power of dandelion was expressed as ascorbic acid equivalent (µg ascorbic acid/mg DFE).

HPLC analysis

A phenolic profile assay was performed using a Waters Alliance 2690 separation module, equipped with a 996 photodiode array detector (Waters Corp., Franklin, MA). A Xterra MS C18 column (2.5 µm, 2.1 × 50 mm) (Waters Corp.) was used at 40 °C with a linear gradient mobile phase containing solvent A (water), solvent B (acetonitrile) and solvent C (5% formic acid in water, v/v) with flow rate set at 0.2 ml/min. The linear gradient program started with 88% A: 10% B: 2% C (v/v) and finished at 73% A: 25% B: 2% C (v/v) within 25 min. The absorbance was recorded at 350 nm. The identifications of luteolin, luteolin-7-glucoside, caffeic acid and chlorogenic acid were determined according to retention times obtained from authentic standards run at identical conditions.

Effect of DFE on inhibition of superoxide radical

Xanthine–xanthine oxidase mixture was used to generate superoxide radical (Aruoma et al., 1988). The reaction mixture contained 1 mM EDTA, 0.1 mM xanthine and 0.94 mM NBT in final volume adjusted to 3 ml by 50 mM phosphate buffer (pH 7.4). The reaction was started by adding 0.1 ml of 0.5 unit/ml xanthine oxidase. Absorbance at 560 nm was taken after 10 min at room temperature against the blank containing no xanthine oxidase. Percentage of inhibition was calculated according to the following equation:

$$\%\text{Inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100.$$

The effect of dandelion extract on xanthine oxidase was determined by measuring the formation of uric acid at 295 nm (Hochestein et al., 1984).

Effect of DFE on scavenging hydroxyl radical and site-specific hydroxyl radical

A test-tube-based deoxyribose assay for the evaluation of the suppression of hydroxyl radical was used (Halliwell et al., 1987). Briefly, for the non-site specific hydroxyl radical system, the reaction mixture contained 3.6 mM deoxyribose, 0.1 mM $FeCl_3$, 0.1 mM ascorbic acid, 0.1 mM EDTA and 1 mM H_2O_2 in 10 mM phosphate buffer solution (pH 7.4). For the site-specific hydroxyl radicals system, EDTA was replaced by phosphate buffer. The reaction mixture (1 ml) was incorporated with test materials and incubated at 37 °C for 1 h, followed by adding 1 ml of 10% (w/v) tricholoacetic acid and 1 ml of 0.5% (w/v) 2-thiobarbituric acid. Color was developed at 100 °C for 15 min in a boiling water bath. Absorbance at 532 nm was measured at room temperature. The effect on hydroxyl radical scavenging was expressed as inhibition percentage calculated from an equation similar to the one above.

Antioxidant activity of DFE in linoleic acid emulsion

The antioxidant activity of DFE in linoleic acid emulsion was determined using an ammonium thiocyanate method (Asamarai et al., 1996) with modification. Briefly, a pre-emulsion was prepared by mixing 3 g linoleic acid and 3 g Tween-20 thoroughly with 200 ml of 30% (v/v) ethanol (Kitts et al., 2000). Aliquots of 10 ml of the above emulsion were transferred to a 125 ml flask and mixed with 10 ml distilled deionized water, as well as different concentrations of DFE. The final volume was adjusted to 25 ml using distilled deionized water. The reaction mixtures were incubated at 50 °C in darkness and flasks were sealed with parafilm. Aliquots (0.1 ml) of incubated reaction mixture were mixed with 5 ml of 75% ethanol, 0.1 ml of 30% (w/v) NH_4SCN and 0.1 ml of 20 mM $FeCl_2$ (in 0.1 M HCl). The absorbance was taken at 500 nm (Shimadzu UV-160 spectrophotometer) and time-course of absorbance at 500 nm was then plotted.

Effect of DFE on scavenging DPPH radical and its synergistic effect with α-tocopherol

A DPPH assay (Hu and Kitts, 2001) was used with modifications made for measurement by a microplate reader. Briefly, 200 μl methanol, containing 0.1 mM DPPH were mixed with various amounts of test samples in a 96-well plate. The plate was covered to minimize the evaporation of methanol. The absorbance at 519 nm was taken 30 min later at room temperature by an UV-visible plate reader (Multiskan Spectrum, ThermoLabsystem). Both DFE and α-tocopherol, and combinations of each were measured by this method in an effort to determine if a synergistic effect between dandelion extract and α-tocopherol existed. A synergistic effect was defined as the inhibition of the combined samples that was markedly higher ($p < 0.05$) than the mathematic sum of the inhibition from individually tested samples.

Effect of DFE on preventing nitric oxide production in RAW264.7 cells

A murine macrophage cell line RAW264.7 (TIB-71, American Type Culture Collection, Manassas, VA) was cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37 °C under humidified air with 5% CO_2. Cells were plated at a density of 2×10^5 cell/ml in a 96-well plate. After overnight incubation, samples and 1 μg/ml LPS were added and the culture was incubated for another 24 h (Hu et al., 2003). Aliquots of media (100 μl) were transferred to another 96-well plate where 100 μl of Greiss reagent (50 μl of 1% sulfanilamide in 5% phosphoric acid and 50 μl of 0.1% naphthylethylenediamine dihydrocholide in water) was added. Absorbance at 540 nm was determined using a microplate reader (Multiskan Spectrum, ThermoLabsystem). The concentration of nitrite was calculated from a standard curve obtained from the same procedure with sodium nitrite. The inhibition of nitric oxide was calculated according to the following equation:

$$\%\text{Inhibition} = \frac{\text{Absorbance}_{\text{positive}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{positive}} - \text{Absorbance}_{\text{negative}}} \times 100.$$

Of which, $\text{Absorbance}_{\text{positive}}$, $\text{Absorbance}_{\text{negative}}$ and $\text{Absorbance}_{\text{sample}}$ represented absorbance of cultural media containing LPS, without LPS and sample with LPS.

Cell viability test

Cell viability was conducted according to the protocol of cell proliferation kit I (Roche Diagnostics Canada, Laval, Que.). Briefly, 10 µl of 5 mg/ml methylthiazolete-trazolium (MTT) was added to each well. After 4 h incubation at 37 °C under 5% CO_2, 100 µl of 10% SDS (w/v) was added and incubated overnight. Absorbance at 570 nm was then taken with microplate reader with reference wavelength at 690 nm (Multiskan Spectrum, ThermoLabsystem). Cell viability was calculated according to the following equation:

$$\text{Cell viability}\% = \frac{\text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100.$$

Effect of DFE on peroxyl radical-induced intracellular oxidation in RAW264.7 cells

RAW264.7 cells were plated in a 96-well plate at a density of 2×10^5 cell/ml overnight, followed by the addition of DFE, 1 µM DCFH-DA and 1 mM AAPH. Fluorescence readings were taken with an excitation wavelength at 485 nm and emission wavelength at 527 nm using a fluorescent plate reader (Fluoroskan Ascent FL, ThermoLabsystem).

Statistics

Data were expressed as mean ± s.e.m. of triplicate experiments (SPSS Inc, Chicago, IL). Student's *t*-test was applied to evaluate significant difference, set as $p < 0.05$ level.

Results

Total phenolic content, phenolic content profile and reducing power

Total phenolic content and reducing power of DFE are shown in Table 1. The phenolic content of DFE was calculated to be equivalent to 20% of gallic acid, with a reducing activity equivalent to 40% of ascorbic acid. In addition to total phenolic content, HPLC confirmed the presence of luteolin, luteolin-7-glucoside, caffeic acid and cholorgenic acid (Fig. 1).

Table 1. Total phenolic content[a] and reducing power[b] of standardized dandelion flower extract (DFE)

Total phenolic content (µg/mg)	195.4 ± 3.6
Reducing power (µg/mg)	417.0 ± 8.3

[a]Total phenolic content was expressed as µg gallic acid/mg dandelion flower extract.

[b]reducing power was expressed as µg ascorbic acid/mg dandelion flower extract.

Inhibitory effect on superoxide radical

The xanthine–xanthine oxidase system was used in this study to generate superoxide radical. The decline of absorbance at 560 nm reflected the elimination of superoxide radical. The generation of superoxide radical was confirmed with the addition of superoxide dismutase (SOD), which specifically scavenges superoxide radical. Under an identical experimental condition, flavonoids such as rutin, and its aglycone quercetin, exhibited a similar inhibition of superoxide radical (Table 2).

DFE was found to exhibit a concentration-dependent ($p < 0.05$) superoxide radical inhibiting activity (Table 2). The formation of uric acid, which is the product of enzymatic reaction, was reduced by less than 10% when the DFE concentration was added up to 166.7 µg/ml. This result suggested that the observed superoxide radical inhibiting activity was not due to the interference of enzymatic activity. Thus, the superoxide radical inhibition of DFE was associated with a direct affinity to scavenge superoxide radical.

Inhibitory effect on hydroxyl radical

DFE neutralized non-site-specific, hydroxyl-radical-induced deoxyribose cleavage in a concentration-dependent manner (Table 3). Hydroxyl radical is defined as site-specific hydroxyl radical when generated by replacing EDTA with buffer, and in this case, deoxyribose acts as a chelator of ferrous ion (Halliwell et al., 1987). DFE exhibited a site-specific, hydroxyl-radical-scavenging activity (Table 3), though such activity was weaker than that of non-site-specific, hydroxyl radical at the same concentration ($p < 0.05$).

The effects of luteolin and luteolin-7-glucoside on both types of hydroxyl radical are shown in Fig. 2. Increased, concentration-dependent inhibition of the non-site specific hydroxyl radical was seen with both luteolin and luteolin-7-glucoside. No difference was found between these two flavones in regard to non-site-specific hydroxyl radical suppression, suggesting that glysidation at the C-7 position did not affect the hydroxyl- radical-scavenging capacity of luteolin. In the case of site-specific hydroxyl radical assay where EDTA was replaced by buffer, however, a significant ($p < 0.05$) suppression of non-site-specific hydroxyl radical was found to be independent of the concentrations of both luteolin and luteolin-7-glucoside (Fig. 2).

Inhibitory effect on linoleic acid emulsion oxidation

A typical oxidation profile of a polyunsaturated fatty acid (e.g., linoleic acid), including reaction stages of

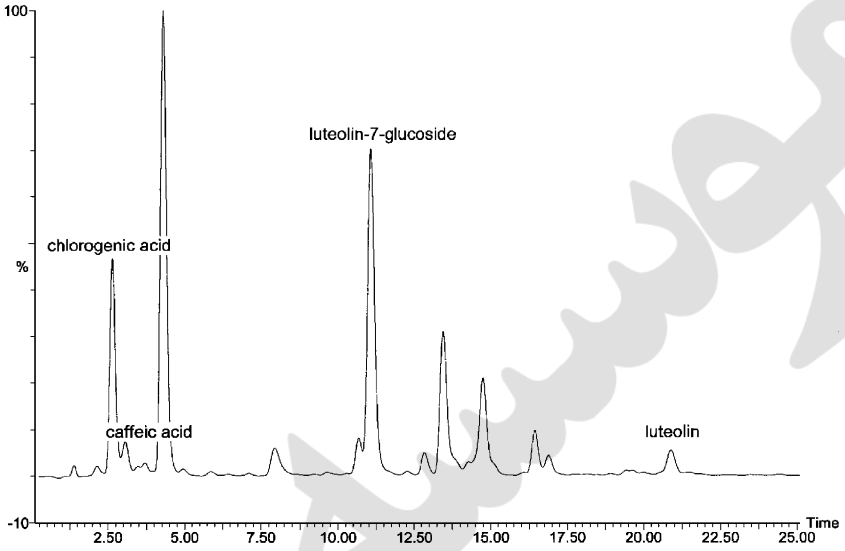

Fig. 1. Phenolic components profile of standardized dandelion flower extract (DFE) by HPLC.

Table 2. Scavenging effect of antioxidant standards and DFE[a] on superoxide radical

Sample[b]	Inhibition%
SOD	
0.67 unit/ml	63.9±3.9**
0.067 unit/ml	25.0±3.2*
Quercetin	
3.4 μg/ml	42.0±4.8**
0.34 μg/ml	21.6±0.6*
Rutin	
6.2 μg/ml	34.1±1.4**
0.62 μg/ml	17.7±2.6*
Dandelion flower extract	
50 μg/ml	22.4±0.6**
100 μg/ml	44.2±0.7**
150 μg/ml	63.6±1.2***

[a]DFE = dandelion flower extract.
[b]Concentration represent as final concentration in the test model; *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus control.

initiation, propagation and termination is shown in Fig. 3. The addition of 400 ppm DFE reduced linoleic acid autoxidation by extending the duration of the initiation phase, while reducing the propagation rate. The addition of 100 ppm of DFE did not extend the lag phase, compared to the control sample; however, the propagation rate was significantly lower as compared to that of control. In comparison, BHT was a relatively more powerful inhibitor of linoleic acid oxidation in this emulsion system.

Table 3. Scavenging effect (%) of standardized dandelion flower extract (DFE) on non-site and site-specific hydroxyl radical in deoxyribose assay

Concentration (μg/ml)	Non-site specific hydroxyl radical (%)	Site-specific hydroxyl radical (%)
25	36.4±0.9**[a]	23.4±1.8**[a]
50	49.9±1.3**[b]	28.6±2.5*[b]
100	62.5±0.3**[c]	35.4±1.7**[c]
200	71.0±0.6***[d]	54.6±1.7**[d]

*$p<0.05$, **$p<0.01$, ***$p<0.001$ versus control; data within same row with same superscript are significantly different ($p<0.05$).

DPPH radical-scavenging effect and synergistic effect with α-tocopherol

In addition to scavenging hydroxyl radicals and superoxide radical and preventing linoleic acid from oxidation, DFE also suppressed the stable DPPH radical in a concentration-dependent manner ($p<0.05$) (Table 4). Moreover, DFE also exhibited a significant ($p<0.05$) synergistic effect with α-tocopherol in scavenging DPPH radical in this study (Table 4).

Effect of DFE on peroxyl-radical-induced oxidation of macrophage RAW264.7 cells

Intracellular oxidation was confirmed by the significantly higher ($p<0.01$) fluorescence when AAPH was introduced to the cell culture media (Fig. 4). The intracellular oxidation also increased with increased

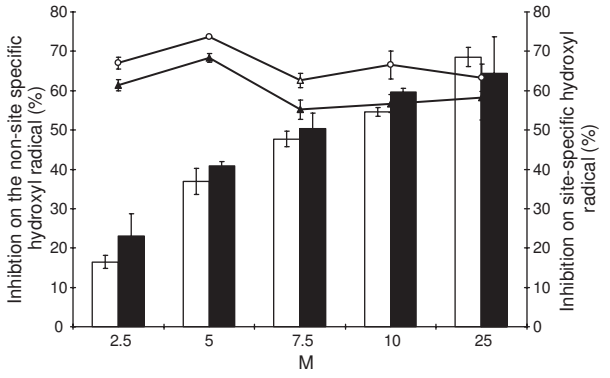

Fig. 2. Effects of luteolin and luteolin-7-glucoside on the inhibition of hydroxyl radical: open bar = luteolin on non-site specific hydroxyl radical; dotted bar = luteolin-7-glucoside on non-site specific hydroxyl radical. △ = luteolin on site-specific hydroxyl radical; ▲ = luteolin-7-glucoside on site-specific hydroxyl radical.

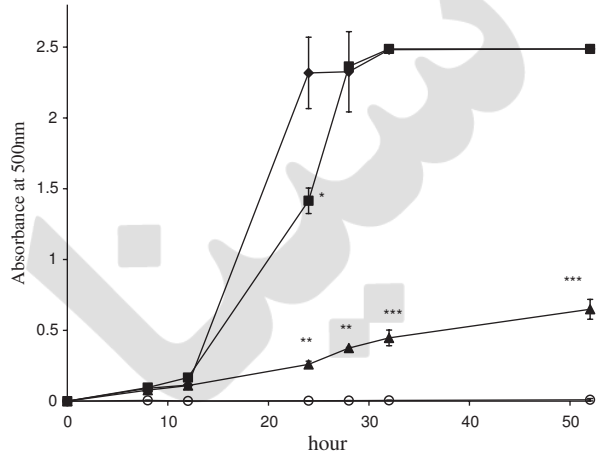

Fig. 3. Inhibitory effect of standardized dandelion flower extract (DFE) on oxidation of linoleic acid emulsion incubated at 50 °C. ♦ = control; ■ = 100 ppm DFE; ▲ = 400 ppm DFE; ○ = 100 ppm BHT. *$p<0.05$, **$p<0.01$ and $p<0.001$ versus control.

incubation time. Significant inhibition ($p<0.001$) was observed with the addition of DFE in RAW264.7 cells, stimulated by peroxyl radical during the course of incubation (Fig. 4). A concentration-dependent inhibition was also found for the standardized DFE in the peroxyl radical-induced cell oxidation model.

Suppressive effect of DFE on nitric oxide production in LPS-stimulated macrophage cells

DFE exhibited a suppressive effect on nitric oxide production in mouse macrophage cells stimulated with bacterial LPS (Table 5). The half-inhibition concentration was determined to be 130 μg/ml for DFE. The

Table 4. Scavenging capacities of standardized dandelion flower extract (DFE) and α-tocopherol on DPPH radical

DFE (μg/ml)	α-tocopherol (μg/ml)	Inhibition%
20	/	14.5±2.0*
40	/	35.6±3.0*
60	/	56.0±1.8**
80	/	80.1±2.7***
100	/	90.2±0.5***
/	2.2	18.2±2.0*
/	4.3	50.8±4.9**
/	6.5	74.9±4.5***
/	8.6	96.1±0.4***
20	2.2	45.9±2.8**[a]
40	2.2	71.5±2.1***[a]
60	2.2	90.6±0.5***[a]

[a]synergistic effect, *$p<0.05$, **$p<0.01$, ***$p<0.001$ versus control.

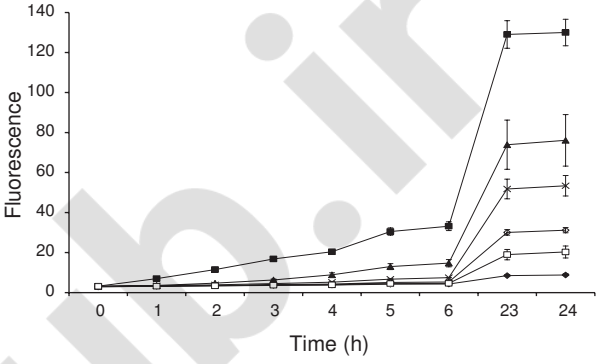

Fig. 4. Effect of standardized dandelion flower extract (DFE) in preventing RAW264.7 cell intracellular oxidation induced by 1 mM AAPH at 37 °C. ♦ = AAPH negative; ■ = AAPH positive; ▲ = AAPH + 7.8 μg/ml DFE; × = AAPH + 15.6 μg/ml DFE; ○ = AAPH + 31.3 μg/ml DFE; □ = AAPH + 62.5 μg/ml DFE.

Table 5. Effect of standardized dandelion flower extract (DFE) in suppressing nitric oxide production in RAW264.7 cells stimulated with LPS

Concentration (μg/ml)	Inhibition%	Cell viability (%)
31.3	7.5±2.8	95.7±3.1
62.5	15.4±4.2*	105.1±9.4
125	44.0±4.9***	108.3±2.4
250	74.3±8.4***	109.2±1.3
500	86.5±4.2***	84.1±3.9*

*$p<0.05$, **$p<0.01$, ***$p<0.001$ versus control.

cell viability test showed that concentrations effective at inhibiting nitric oxide production in culture did not induce a cytotoxic effect on cultured cells up to

500 μg/ml (Table 5). A weak cytotoxicity at 500 μg/ml did not compromise the efficacy of nitric oxide inhibition.

Discussion

Superoxide, hydroxyl and peroxyl radicals represent different forms of ROS (Morse and Stoner, 1993), although superoxide radical is more likely to be a mild reductant (Davies, 1995). Superoxide radical undergoes either enzymatic or non-enzymatic dismutation to generate hydrogen peroxide and a more active hydroxyl radical when transition metal (i.e., ferric or cupric ion) is present. For instance, superoxide radical generates hydroxyl radical through the Haber–Weiss reaction ($H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + HO^{\bullet} + HO^-$), although iron ion catalyzes this reaction through a Fenton reaction mechanism (Halliwell, 1995). Moreover, reactions between superoxide anion and nitric oxide can also result in the production of a highly reactive peroxynitrite radical (Halliwell, 1995). Therefore, it is important to remove excess superoxide radical to prevent potential damage to biological molecules from toxic ROS. The suppressive effect of DFE on the superoxide radical was confirmed in this study. This observed inhibition could be attributed in part to the phenolic content. As demonstrated here, and by others (Cos et al., 1998), phenolic components including quercetin and rutin both scavenge superoxide radical. In the present study, we showed that the inhibition of superoxide radical was not necessarily due to the suppression of xanthine oxidase activity, because the formation of uric acid (i.e., the product of xanthine oxidase with xanthine) remained unchanged. Therefore, we conclude that DFE was effective at directly scavenging superoxide radical.

Hydroxyl radical is a very reactive ROS with a short half-life (Halliwell, 1995). Both site-specific and non-site specific hydroxyl radical (Halliwell et al., 1987) were generated based on the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH$) in this study. The extent of prevention will depend on both the antioxidant concentration ratio to deoxyribose and the second-order rate constant for reaction with hydroxyl radical (Halliwell et al., 1987). In this study, we not only report the inhibitory effect of DFE on hydroxyl radical, but also show that a half-inhibition concentration (IC_{50} value) of DFE for the non-site specific hydroxyl radical was lower than that for the site-specific hydroxyl radical. This result indicates that the DFE exhibited a higher capacity to suppress the non-site specific hydroxyl radical, albeit both sources of hydroxyl radical generated were significantly inhibited (Table 2). This finding demonstrated that the DFE also acted as a potential transitional metal ion chelator. The chelating activity of flavonoids has been illustrated elsewhere (Rice-Evans, 1995) with the 3-hydroxyl-4-keto structure and *ortho*-di-hydroxyl groups being particularly critical for this effect. The presence of phenolic components in DFE, such as caffeic acid, chlorogenic acid, luteolin and luteolin-7-glucoside were confirmed by HPLC (Fig. 1). It is of interest that all of these compounds contain the ortho-di-hydroxyl group, which explains the observed concentration-independent inhibition on site-specific hydroxyl radical activity by luteolin and its glycoside. With this in mind, the hydroxyl-radical-suppressing effect of DFE in this study can be attributed at least in part to the presence of phenolic compounds, including flavonoids and coumaric acid.

Incubating linoleic acid in an emulsion at elevated temperature resulted in a shorter initiation period for autoxidation and a fast propagation rate of oxidized fatty acid (Fig. 3). Using a low concentration of 100 ppm, DFE did not extend the initiation phase, but reduced the propagation rate for oxidation. Furthermore, the presence of DFE at a higher concentration suppressed lipid oxidation by extending the lag phase of lipid oxidation, thus reflecting typical characteristics of a chain-breaking antioxidant, similar to that of known chain-breaking antioxidants such as BHT. Prevention against the thermal-induced linoleic acid emulsion oxidation has also been reported with other phenolic-rich plant extracts (Asamarai et al., 1996). Burda and Oleszek (2001) reported a prooxidant activity of luteolin-7-glucoside in a thermal induced co-oxidation of β-carotene and linoleic acid heterogeneous model, suggesting that a balance between hydrophilicity and hydrophobicity is also important for the activity a potential antioxidant (Hu and Kitts, 2001). In addition to the hydrophobic property, antioxidant or prooxidant behavior of phytochemicals relies on the environmental conditions. For example, we have observed prooxidant activity of luteolin-7-glucoside in a cupric-ion-induced liposome peroxidation due to the formation of oxidative reaction-favorable cuprous ion (Hu and Kitts, 2003). In our current study, however, the presence of transitional metal ion was minimized by applying metal chelating resin (Chelax 100) treatment to the buffers used and thereby reducing the peroxidation potential.

The synergy between antioxidants, such as vitamin E and ascorbic acid, is well known (Scarpa et al., 1984). The synergistic effect between ascorbic acid and α-tocopherol has been attributed to a reducing power that facilitates the recovery of tocopherol from tocopheryl radicals (Niki et al., 1982). The reducing power of the standardized DFE was shown along with a strong DPPH-radical-scavenging activity of luteolin-7-glucoside (Burda and Oleszek, 2000). Current results not only indicated a scavenging effect of DFE on DPPH radical, but also showed a synergistic effect with α-tocopherol in scavenging DPPH radical. We conclude that flavonoids and other phenolic components present in the DFE possibly regenerate α-tocopherol in addition to sequestering free

radicals, as observed with the DPPH radical. The 40% of ascorbic acid equivalent of the DFE is likely responsible for the synergistic effect with α-tocopherol. In our former study, we reported a synergistic effect of tea catechin with α-tocopherol (Hu and Kitts, 2001). Similarly, Liao and Yin (2000) showed that flavonoids and phenolic acids synergized with α-tocopherol in protecting both human erythrocyte membrane ghost and liposome systems from ferrous-ion-induced peroxidation. Taken together, it is clear that, in addition to contributing directly to radical-scavenging activity, DFE also has the potential to synergize with α-tocopherol to quench DPPH radical.

The non-polar, non-ionic fluorescent precursor DCFH-DA can cross the cell membrane and be hydrolyzed by intracellular esterase to non-fluorescent 2′,7′-dichlorofluorescin (DCFH). This intermediate is further oxidized to a highly fluorescent 2′,7′-dichlorofluorescein (DCF) derivative (LeBel et al., 1992). Therefore, using this fluorescent probe is ideal for studying peroxidation reaction in living cells (Ma et al., 1998; Wang and Joseph, 1999). We applied AAPH as an initiator of peroxyl radical in our previous *in vitro* studies (Hu and Kitts, 2000, 2001, 2003), where flavonoids exhibited significant effects to reduce peroxyl radical-induced biomolecular damage *in vitro*. In our current study, we employed AAPH to generate peroxyl radical for the living mouse macrophage RAW264.7 cells. The baseline cell fluorescence without AAPH treatment remained low during the 24 h incubation, indicating that baseline of intracellular oxidation is low (e.g. the DCFH requires a strong oxidative agent, such as ROS, for the detectable fluorescence burst). On the other hand, the finding that AAPH-treated cells exhibited significantly higher fluorescence is strong evidence that the peroxyl radical in fact crossed the cell membrane and oxidized DCFH resulting in a highly fluorescent DCF product. The addition of DFE significantly reduced this effect in a concentration-dependent manner, without inducing significant cytotoxicity. As mentioned earlier, flavonoids and other phenolic-rich extracts can suppress the peroxyl radical-induced liposome oxidation and DNA breakage *in vitro* (Hu and Kitts, 2001, 2003). Our unpublished data also indicated that luteolin and luteolin-7-glucoside at concentrations as low as 20 μM significantly reduce the peroxyl-radical-induced intracellular oxidation in RAW264.7 cells. Therefore, in this study, we confirm that a phenolic-rich extract derived from DFE was effective at protecting living cells from peroxyl-radical-induced intracellular oxidation. The mechanism of this prevention could be associated with scavenging of both intercellular and intracellular peroxyl radicals by antioxidant components of DFE that reach intracellular pools.

Bacterial LPS stimulates macrophage, such as RAW264.7 cells, to express nitric oxide by up-regulating the inducible nitric oxide (iNOS). We show that the standardized DFE suppressed nitric oxide in cultured mouse macrophage cells stimulated by bacterial LPS (Table 5), without inducing cytotoxicity. Similar inhibitory activities have been reported with other standardized phenolic-rich food (Hu et al., 2003) and herbal extracts, such as *Ginkgo biloba* extract (Wadsworth and Koop, 2001). In the latter example, the flavonoid-rich extract suppressed nitric oxide in the activated macrophage by reducing the iNOS mRNA level and inhibiting p38 mitogen activated protein kinase activity, important requirements for iNOS expression in LPS-stimulated RAW264.7 cells (Wadsworth and Koop, 2001). Although we have not confirmed at this time which signal transduction pathway step is affected by DFE or its constituents, we found that luteolin and luteolin-7-glucoside, instead of caffeic acid and cholorgenic acid, actually suppressed the nitric oxide production in LPS-activated macrophages cells (Hu and Kitts, unpublished data). Others have shown null activities of caffeic acid and chlorogenic acid on nitric oxide production (Wang and Mazza, 2002).

Conclusion

The cardiovascular protective benefit of fruits and vegetables has been attributed to the antioxidant activities of various phytochemicals, and particularly to natural antioxidants such as flavonoids, carotenoid, vitamin E and vitamin C. An average of 20–23 mg/day of dietary flavonol and flavone is consumed typically in a western diet, from sources such as tea, onion and apple (Sampson et al., 2002). An increased dietary intake of flavonoids has been found to be associated with the decreased incidence of ischemic heart disease (Hollman and Katan, 1999; Geleijnse et al., 2002), potentially due to antioxidant protection. Therefore, the recognition of a need for increased daily flavonoid intake from vegetable and fruits, or alternatively from dietary supplements, has occurred. From the data reported here, we conclude that a standardized DFE suppresses both ROS and RNS in chemical and biological models. Free-radical-scavenging capacity and a synergistic effect with α-tocopherol for DFE were attributed to the phenolic content. The prevention of living cells from peroxyl radical-induced oxidation in the presence of DFE suggests that the standardized extract has biological antioxidant activity. Further studies are required to focus on the metabolism and bioavailability of antioxidant components derived from DFE, and the mechanisms that suppress nitric oxide production.

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