The study of anti-inflammatory and anti-oxidant effects of the five edible plants

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The anti-inflammatory and anti-oxidant effects of the five edible plants (floral herbal tea, FHT) commonly utilized as a drink in Guangdong of China were discussed. Firstly, DPPH and ABTS methods were used to determine antioxidant activity of methanolic extract from floral herbal tea (MEFH). Subsequently, the relationship between total content of phenol and flavonoids with respect to antioxidant activity were studied. Finally, anti-inflammatory activity of MEFH in RAW 264.7 cell line was tested. The scavenging activity of DPPH radicals in the order from highest to lowest was: Flos sophorae, Lonicera japonica Thunb, Flos chrysanthemi Indici, Flos puerariae, Bombax ceiba. F. Sophorae not only possessed the highest anti-oxidant activity, but also the high contents of total phenol and flavonoids. NO induced by lipopolysaccharide (LPS) in mouse macrophages can be effectively inhibited by MEFH, in which the anti-inflammatory effect of F. Sophorae was highest. Amongst five edible plants, F. sophorae demonstrated extraordinary anti-inflammatory as well as anti-oxidant effects. These effects were mainly due to high content of total phenol and flavonoids, which could serve as a natural resource of food and medicine.

Key words: Anti-inflammatory, anti-oxidant, floral herbal tea, Flos sophorae.

INTRODUCTION

There is ample evidence that reactive oxygen/nitrogen species generated in the human body can cause oxidative damages associated with many chronic and degenerative diseases, such as cardiovascular diseases, cancer, diabetes mellitus, ageing and neurodegenerative disease (Finkel and Holbrook, 2000; Young and Woodside, 2001). Chronic inflammation is implicated in the pathogenesis of a variety of diseases, such as atherosclerosis, obesity, metabolic syndrome, diabetes, neurodegenerative diseases, and several types of cancers (Casserly and Topol, 2004; Lee et al., 2010; Pradhan, 2007). Macrophages play a crucial role in the inflammatory response through the release of a variety of factors, such as nitric oxide (NO), proinflammatory cytokines (TNF-α, IL-1β, IL-6), in response to an activating stimulus, e.g. lipopolysaccharide (LPS) (Surh et al., 2001).

It is well known that long-term use of synthetic drugs often causes numerous side effects and sometimes resistance (Karaman et al., 2003). Unlike synthetic drugs, natural products including medicinal plants has become more and more important in primary health care especially in developing countries. It has been estimated by WHO that in 2002, about 90% of the world’s population from developing countries relied mainly on traditional medicines for their primary health care (Astin et al., 1998; Yawar, 2001).

In many oriental countries, for a long time, medicinal plants have been utilized for treatment and prevention of diseases. It was believed in Chinese traditional medicine that many foods had effects similar to medicine, and that there was no major difference between food and medicine. Hence, medicine plant played double roles in nutrition and medicine. Floral herbal tea consumed as a traditional drink in China, is one of the most popular herbal teas in Guangdong. The FHT with thick perfume was that the five medicine plants including Flos sophorae, Lonicera japonica Thunb (honesuckle bud and flower), Flos chrysanthemi Indici (wild chrysanthemum flower), Flos Puerariae (puerari lobata), and Bombax ceiba (kapok) were boiled in water. In

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Chinese traditional medicine, the main function of FHT included heat-clearing and detoxifying effects, which lead to prevention of anemopyretic cold and influenza, and the tea was especially for drinking in summer. Though FHT was used as a drink for long time, the functions of anti-oxidation and anti-inflammatory were not fully explored.

In order to develop and make full use of the resources in basic research, the paper employed FHT as materials to study the anti-oxidation and anti-inflammatory function of MEFH. Therefore, the total phenol and flavonoids content were determined. Subsequently, DPPH and ABTS methods were employed to determine the antioxidant activity of MEFH. The anti-inflammatory activity was measured by the effect of MEFH on the nitric oxide (NO) that serves as an inflammatory mediator secreted by the macrophages.

MATERIALS AND METHODS

Plant materials

The FHT were obtained from herb shop, Er-Tian-Tang drug retail outlet in Guangzhou. Identification of the investigated plants was done by Dr. Yang Yan-jun at the Institute of Chinese Medicine of Guangdong Province.

Sample preparation

The plant samples were ground to fine powder using a high-speed pulverizer (Kunjie Ltd., China). A precisely weighted amount (~5 g of each) of the powder was extracted with 100 ml 80% methanol at 40°C for 24 h. The samples were cooled down to room temperature and centrifuged at 5000 r/min for 15 min. The supernatant was obtained and evaporated using a rotary evaporator (R-215, Buchi, Switzerland) and freeze dryer (Alphal-2, Christ, Germany). The dried extracts were stored at -20°C until used.

Chemicals

Standards of 2, 2-diphenyl-1-picyrylhydrazylradical (DPPH), Trolox, 2’-azinobis (3-ethylbenethiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulfate, folin-ciocalteu phenol reagent, butylated hydroxytoluene (BHT), gallic acid, rutin were purchased from Sigma.

Dulbecco’s modified eagle’s minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin, Griess reagent, dimethyl sulfoxide (DMSO), MTT reagent (3-(4,5-dimethylthiazol-2,5-diphenyltetrazolium bromide) and lipopolysaccharide (LPS) were obtained from Sigma–Aldrich Co. All other chemicals used were of analytical grade.

Cell culture

Mouse macrophage-like cell line (RAW 264.7), a commonly used model for inflammatory processes was obtained from the European Collection of Cell Cultures (ECACC) and cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin and maintained at 37°C in a 5% CO₂ humidified atmosphere (CO₂ incubator, Thermo). The cells were treated with the MEFH at different concentrations and stimulated with LPS (1 μg/ml) for the indicated period.

Cell viability

Cell viability was evaluated by the MTT assay (Denizot and Lang, 1986). Cell was seeded in 96-well plants in the absence and/or presence of MEFH for 24 h as described in the preceding section. At the end of the incubation period, 100 μl of a MTT solution (100 μg/ml in PBS buffer) was added to each well and incubated for 4 h further. Further 200 μl of DMSO was added to each well to dissolve and extract tetrazolium dye. After 15 min of gentle agitation at 37°C, absorbance was determined at 550 nm.

Nitric oxide (NO) assay

Cells were seeded in 96-well plates with 5 × 10⁵ cells/well and allowed to adhere for 24 h. Then, medium was removed and replaced with 0.2 ml of fresh medium containing 1 μg/ml LPS alone or LPS along-with containing various concentrations MEFH, and DMSO was used as control group. After 24 h of incubation, NO was measured as nitrite in the culture supernatant using the Griess reagent with absorbance at 542 nm (Park et al., 2006). Results were expressed as %inhibition calculated according to the following equation:

\[ \text{%Inhibition of NO} = \frac{\text{Nitrite in LPS-treated cells} - \text{Nitrite in control cells}}{\text{Nitrite in LPS-treated cells} - \text{Nitrite in isolate cells}} \times 100 \]

The level of isolated required to inhibit NO evolution by 50% was defined as an IC₅₀ value and was interpolated from the dose-response results.

Determination of antioxidant activity

Scavenging activity of DPPH radicals

The scavenging activity of MEFH on DPPH radicals was measured according to the method of Liu and Chu but was modified in details (Liu and Ng, 2000). The dried MEFH were re-dissolved in methanol to various concentrations. An aliquot of 0.5 ml of 0.1 mM DPPH radical in methanol was added with 1 ml of MEFH of different concentrations. Similarly, concentrations of butylated hydroxytoluene (BHT) were used as positive control. Methanol was used instead of plants sample as a control. The reaction mixture was shaken thoroughly and incubated at 25°C for 30 min in darkness. The absorbance was measured later at 515 nm, against a blank of methanol without DPPH. Results were expressed as percentage of inhibition of the DPPH radical. Percentage of inhibition of the DPPH radical was calculated according to the following equation:

\[ \text{%Inhibition of DPPH} = \left( \frac{\text{Abs control} - \text{Abs sample}}{\text{Abs control}} \right) \times 100 \]

Plants extract concentration providing 50% inhibition (IC₅₀, expressed in μg/ml) was calculated from the graph plotted inhibition percentage against extract concentration.

Trolox equivalent antioxidant capacity (TEAC) assay

The spectrophotometric analysis of ABTS⁺ method was carried out as described by Re with slight modification (Re et al., 1999). The ABTS⁺ radical cation was generated by a reaction of 7 mmol/L ABTS and 2.45 mmol/L potassium persulfate. The reaction mixture
was allowed to stand in the dark for 12 to 16 h at room temperature and used within 2 days. Before usage, the ABTS solution was diluted with phosphate buffer (0.1 M, pH 7.4) to absorbance of 0.700 ± 0.050 at 734 nm. All samples were diluted appropriately to provide 20 to 80% inhibition of the blank absorbance. 100 μl of the diluted sample were mixed with 3.8 ml ABTS working solution. The absorbance of the mixture was measured at 734 nm of 6 min in dark at room temperature, and the percent of inhibition of absorbance at 734 nm was calculated. The assay with the mixture was carried out in triplicate. Trolox solution (final concentration, 0 to 15 μmol/L) was used as a reference standard, and the results were expressed as μmol Trolox/g dry weight of plant extract.

Total phenolic content determination

Total soluble phenolic compounds in MEFH were determined with Folin-Ciocalteu reagent according to the method using gallic acid as a standard phenolic compound (Li et al., 2008). In brief, 200 μl diluted sample were added to 1 ml of 1:10 diluted Folin-Ciocalteu reagent. After 4 min, 800 μl of saturated sodium carbonate solution (75 g/L) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured in triplicate. Gallic acid (0 to 500 mg/L) was used for calibration of standard curve. The results were expressed as milligram gallic acid equivalent (mg GAE)/g dry weight of plant material.

Assay for total flavonoids

The total flavonoids content was determined using the Brainslav method (Rankovic et al., 2011). Two milliliters of 2% aluminium trichloride (AlCl₃) in methanol was mixed with the same volume of the extract solution (1 mg/ml). The mixture was incubated at room temperature for 10 min, and the absorbance was measured at 415 nm in spectrophotometer against blank samples. The total flavonoids content was determined as microgram of rutin equivalent by using an equation that was obtained from standard rutin graph. The results were expressed as milligram rutin equivalent mg/g dry weight of plant material.

Statistical analysis

Data are expressed as mean values ± standard errors of the mean (SEM). Comparisons between extracts-treated groups and non-treated group were performed with the Student’s t-test. P-value less than 0.05 was considered to be statistically significant. The experiments were performed in triplicate and at least three times each.

RESULTS AND DISCUSSION

Antioxidant activity

The classic method of DPPH free radical scavenging was used for evaluating the anti-oxidant activity of medicine in vitro (Yoshiki et al., 2001). DPPH is a purple stable free radical and has absorption at wavelength of 517 nm. In the presence of a free radical scavenging agent, the lone electron is paired and hence, a reduction in absorption maxima at wavelength 517 nm is observed. The absorption peak and the matched electron presented the relation of stoichiometric coefficient. Hence, it is a convenient and reliable method that could be used to evaluate the anti-oxidant activity, providing a reference for free radical scavenging (Parejo et al., 2000).

Figure 1 indicates a dose dependent increase in the scavenging activity of DPPH radicals on addition of MEFH. In comparison with the control and the other four plants, the scavenging capacity of DPPH radicals of Flos sophorae was highest and IC₅₀ was 37.24 μg/ml, indicating that F. sophorae was excellent anti-oxidation edible plant resource. Compared with control, the scavenging activity of DPPH radicals of the four plants were significantly increased and from high to low, the order was as follows: L. japonica Thunb (IC₅₀ 86.9 μg/ml), F. chrysanthemi Indici (IC₅₀ 171.0 μg/ml), F. puerariae (IC₅₀ 193.9 μg/ml), B. ceiba (IC₅₀ 266.6 μg/ml).

The study on phenol of botany was the one of the hot points in plant chemical research. Phenolic hydroxyl group with strong ability in scavenging active oxygen is well known for its anti-mutation, anti-cancer, anti-virus, anti-microbiology and anti-aging effects. Flavonoids as the most widespread phenol components had broader biological effects, especially on reducing or removing scavenging free radicals.

The total phenolic and flavonoids contents were shown in Table 1. The total phenol content in MEFH ranged from 96 ± 3.4 to 20 ± 2.9 mg GAE/g from highest to lowest, the order of the plants was as follows: F. sophorae, L. japonica Thunb, F. Puerariae, F. chrysanthemi Indici, B. ceiba; Flavonoids content in MEFH ranged from 65 ± 3.6 to 28 ± 2.3 mg/g; from high to low, the order was: L. japonica Thunb, F. sophorae, F. puerariae, B. ceiba, F. chrysanthemi Indici.

ABTS method was widely used in determination of scavenging free radicals and evaluating anti-oxidant activity of both lipophilic and hydrophilic antioxidants. A stable blue/green ABTS⁺ was generated by oxidation of ABTS, and had maximum absorption at wavelength 734 nm. The ABTS⁺ was transformed into ABTS in the presence of the antioxidant (Re et al., 1999).

Determination of anti-oxidant activity of MEFH by ABTS was shown in Table 1, according to the ATBS values arranged from the highest to lowest, the order was F. Sophorae, L. japonica Thunb, F. chrysanthemi Indici, F. Puerariae, B. ceiba. F. Sophorae had the highest anti-oxidant activity (60 ± 0.77 μmol Trolox/g) due to the highest total phenol and flavonoids content. These results were in accordance with the values measured via the DPPH method.

In this study, the variety and contents of flavonoids in the five plants utilized were different from each other. F. sophorae of FHT is drying flower, and alabastrum of Sophora japonica L., which is grown in northern, southern and southwestern part of China, is utilized not only as food but also as a medicine. The main components of F. Sophorae include rutin, small amount of quercetin and naphthol. L. japonica Thunb grows in ShanDong and HeNan of China, and also is named honeysuckle bud and

Chen et al
flower, belonging to Lonicera of Caprifoliaceae. The antioxidant activity and scavenging activity free radicals effects of L. japonica Thunb may be due to the flavonoids such as caffeotannic acid, isochlorogenic acid and luteolin (Xiang and Ning, 2008). F. chrysanthemi Indici was used as regular Chinese medicine material. Its chief components contained flavonoids, chlorogenic acid, polysaccharide and many kinds of essential oil including monoterpene, sesquiterpene and its derivatives, triterpenes and aliphatic compound. F. puerariae is the flower of Pueraria lobata (Wild) Ohwi or P. thomsoii Beeth, and was used in relieving vomiting of a drunken person, providing the effects of anti-inebriation and liver protection. The chief components include isoflavone and saponin.

The values of ABTS from Figures 2 and 3, demonstrate a direct correlation with the total phenolic content for $R^2 = 0.728$, indicating that the anti-oxidant activity of MEFH was due to the total phenolic content. Meanwhile, ABTS values was related to the content for $R^2 = 0.486$, indicating that the total phenolic content could be utilized as judgmental basis for anti-oxidant activity of the screening plants. The indistinct linear relations between the total phenolic content and anti-oxidant activity were due to the variety of phenol in different plants, representing the different dose-effect relationship.

As previously reported, nine kinds of natural flavonoids include almost 3000 monomer, the anti-oxidant activity being different between the different kinds of monomers, mainly due to the chemical structure of the compounds (Okawa et al., 2001, Zhang and Chen, 2000). Free radicals scavenging ability of flavonoids was closely related to the positions of their hydroxyl groups and 3', 4'-hydroxyl groups on B ring of flavonoids was the key structure. 3', 4'-hydroxyl groups on B ring of flavonoids formed the key structure. When the hydroxyl group at 3rd position on the C ring was glycosylated, its DPPH scavenging activity was decreased (Zhang et al., 2003).
Figure 2. Correlation between total phenolic content and antioxidant capacities measured by the ABTS assay.

Figure 3. Correlation between flavonoids content and antioxidant capacities measured by the ABTS assay.
In addition, the components of anti-oxidant activity was not particularly limited to phenol or flavonoids.

The results of anti-inflammatory activity

Macrophages play a crucial role in the inflammatory response through the release of a variety of factors, such as nitric oxide (NO), prostaglandin mediators and proinflammatory cytokines (TNF-α, IL-1β, IL-6), in response to an activating stimulus, e.g. lipopolysaccharide (LPS). NO is an important proinflammatory mediator and inhibition of production of NO via the inhibition of its synthases such as inducible nitric oxide synthase (iNOS), has been demonstrated beneficial in treating inflammatory diseases (Bogdan, 2001; Cheng et al., 2005; Panaro et al., 2003; Ryu et al., 2010).

To assess the potential anti-inflammatory activity of MEFH, macrophage RAW 264.7 cell line was used. First, the cytotoxicity of the extracts administered to the cells was examined. Results were shown in Figure 4 and expressed as percentage of viability with respect to the LPS-treated cells which were taken as 100% viability. The MEFH were tested to guarantee that the remaining vehicle in the extracts had no effect on cell viability. Among the tested concentrations, all samples did not affect the cell viability.

The effects of MEFH on NO induced by LPS in mouse macrophages RAW 264.7 was shown in Figure 5. MEFH could inhibit NO generation in a dose dependent manner. The inhibitory effects of MEFH, from highest to lowest, were in the following order: F. sophorae (IC₅₀ 27.29 μg/ml), L. japonica Thunb (IC₅₀ 125.45 μg/ml), F. chrysanthemi Indici (IC₅₀ 160.56 μg/ml), F. puerariae (IC₅₀ 480.85μg/ml), B. ceiba (IC₅₀ 512.53 μg/ml). Amongst five plants, the NO inhibition effects of F. sophorae and L. japonica Thunb were significant, demonstrating the effective anti-inflammatory effects of MEFH.

A tannin and saponin-free Lonicera japonica extract was studied and its anti-inflammatory and analgesic activities in vivo experimental models of inflammation and pain was reported, in which flavanoids were found to be the effective component (Ryu et al., 2010). Additionally, the anti-inflammatory, humoral and cellular immunomodulatory and mononuclear phagocytic activities of ethanol extraction from Chrysanthemum indicum Linne on animal inflammation model was reported (Cheng et al., 2005). The reports on anti-inflammatory effects of other four plants were not found.

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**Figure 4.** Effects of MEFH on cell viability in RAW 264.7 cells. Viability of cells treated with LPS alone has been taken as reference (100%). Bars represent the mean standard deviations from four different experiments performed in triplicate.
Concentration of extracts (µg/ml) 
Flos chrysanthemi Indici 
Lonicera japonica Thunb 
Flos sophorae 
Flos puerariae 
Bombax ceiba 

Figure 5. Effects of MEFH in inhibition NO production in LPS-activated RAW 264.7 cells. Values are means, n = 3, * P < 0.05 vs. LPS.

Conclusion

The FHT consisted of F. Sophorae, L. japonica Thunb, F. chrysanthemi Indici, F. Puerariae, and B. ceiba was the popular drink in southern areas of China, which are also consumed as conventional food amongst the common people. Based on the wide consumption of the herbal tea, anti-inflammatory and anti-oxidant activities of MEFH were studied in the paper. The results obtained demonstrated that Flos Sophorae presented good anti-oxidant as well as DPPH free radicals scavenging activity, which was better than BHT. These activities could be due to the high total phenolic (> 85 mg/g) and flavonoids content (> 30 mg/g). The scavenging ABTS·⁺ activity of per gram of F. sophorae was the equal of 60 µmol Trolox. In mouse macrophage model stimulated by LPS, F. sophorae exhibited a significant anti-inflammatory effect. Therefore, in conclusion it can be said that F. sophorae is an extraordinary natural resource that exhibits both anti-inflammatory as well as anti-oxidant activity.

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