Progress in Nutrition 2016; Vol. 18, N. 1: 74-80

Original article

Antioxidant activity of hydro-alcoholic extracts of 4 citrus species flower

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Summary. *Objectives:* During past two decades, much attention has been paid to antioxidant activity of different edible parts of medicinal plants. Present study aimed to evaluate antioxidant activity of hydro-alcoholic extract of flowers of *Citrus aurantium*, *Citrus nobilis*, *Citrus limon* and *Citrus sinensis*. *Methods:* Biological activities of hydro-alcoholic extract of *Citrus aurantium*, *Citrus nobilis*, *Citrus limon* and *Citrus sinensis* flowers were investigated employing different *in vivo* and *in vitro* assay systems including DPPH, nitric oxide and H_2O_2 scavenging models, Fe^{2+} chelating, reducing power model, linoleic acid system test, as well as H_2O_2-induced hemolysis test. *Results: Citrus limon* extract showed best activity in DPPH and nitric oxide radicals scavenging activity and Fe^{2+} chelating activity than others. *Citrus nobilis* extract exhibited better H_2O_2 scavenging. Extracts exhibited good antioxidant activity in linoleic acid system test that were comparable with vitamin C ($p>0.05$). The extracts showed weak reducing power activity. *Citrus sinensis* showed better antihemolytic activity against H_2O_2 induced hemolysis. *Conclusion:* Combining data showed that hydro-alcoholic extract of flowers of 4 *Citrus* species possess antioxidant activity in different assay systems.

Keywords: antioxidant, *citrus*, flower extract, free radical scavenging, lipid peroxidation

Introduction

Free radicals have been found to play an important role in the initiation and/ or progression of various diseases such as atherosclerosis, inflammatory injury, cancer, and cardiovascular disease (1-4). Recent studies have investigated the potential of natural originated antioxidants against various diseases induced by free radicals (5-7). However, synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxyltoluene (BHT), have restricted use in foods as they are suspected to be carcinogenic (8, 9). Therefore, natural originated compounds with medicinal properties are being sought (10-12). Several species of *citrus* genus have been shown to have radical scavenging activity (13, 14), immonutoxicity and TNF- α inhibitory (15), anti-platelet activity (16), cholesterol lowering (17) and antiproliferative (18). Also different class of phenols including hydroxycinnamates, flavonoid glycosides, and polymethoxylated flavones was reported from different part *citrus* (18). To best of our knowledge, nothing has been reported about the antioxidant activity of extract of *Citrus aurantium*, *Citrus nobilis*, *Citrus limon* and *Citrus sinensis* flowers.

Methods

Chemicals

Ferrozine, Linoleic acid, Trichloroacetic acid (TCA), 1, 1-Diphenyl-2-picryl hydrazyl (DPPH),

Potassium ferricyanide, Hydrogen peroxide were purchased from Sigma Chemicals Co. (USA). Gallic acid, Quercetin, Butylated hydroxyanisole (BHA), Vitamin C, Sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, EDTA and Ferric chloride were purchased from Merck (Germany). All other chemicals were of analytical grade or purer.

Sample preparation

Citrus flowers were collected from Panbeh chuleh, near the Caspian Sea, Sari, Mazandaran, Iran. A voucher specimen (No 700-703) was deposited in the school of pharmacy herbarium. Flowers were transported to the laboratory and kept at < 4°C within 24 h prior to sample preparation.

Preparation of extract

The materials were oven dried at 38°C, for 5 days. Dried materials were coarsely ground (2-3 mm) before extraction. Materials were extracted by percolation method using ethanol/distilled water (70/30) for 24 h at room temperature. Extracts were filtered and concentrated under reduced pressure at 40°C using a rotary evaporator.

Determination of total phenolic compounds and flavonoid content

Total phenolic compound contents were determined by the Folin-Ciocalteau method (19). The extracts samples (0.5 ml) were mixed with 2.5 ml of 0.2 N Folin-Ciocalteau reagent for 5 min and 2.0 ml of 75 g l^{-1} sodium carbonate were then added. The absorbance of reaction was measured at 760 nm after 2 h of incubation at room temperature. Result was expressed as gallic acid equivalents. Total flavonoids content were estimated as previously described (20). Briefly, 0.5 ml solution of extracts in methanol were separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water and left at room temperature for 30 minutes. The absorbance of the reaction mixture was measured at 415 nm with a double beam spectrophotometer (Perkin Elmer). Total flavonoid content was calculated as quercetin from a calibration curve.

Antioxidant activity

DPPH radical-scavenging activity

The stable 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) was used for determination of free radical-scavenging activity of the sample (21). Different concentrations of samples were added, at an equal volume, to ethanolic solution of DPPH (100 μM). After 15 min at room temperature, the absorbance was recorded at 517 nm. The experiment was repeated for three times. Vitamin C, BHA and quercetin were used as standard controls. IC_{50} values denote the concentration of sample, which is required to scavenge 50% of DPPH free radicals.

Metal chelating activity

The chelating of ferrous ions by the extract was estimated by the method of Dinis et al. (22). Briefly, the samples (0.2-3.2 mg ml^{-1}) were added to a solution of 2 mM $FeCl_2$ (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml), the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as $[(A_0 - A_s)/A_0] \times 100$, where A_0 was the absorbance of the control, and A_s was the absorbance of the extract/standard. Na_2EDTA was used as positive control.

Assay of nitric oxide-scavenging activity

For the experiment, sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of samples dissolved in water and incubated at room temperature for 150 min. The same reaction mixture, without extract, but with an equivalent amount of water, served as control. After the incubation period, 0.5 ml of griess reagent was added. The absorbance of the chromophore formed was read at 546 nm. Quercetin was used as positive control (19).

Scavenging of hydrogen peroxide

The ability of the extract to scavenge hydrogen peroxide was determined according to our recently publisher paper (23). A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Samples (0.1-1 mg ml^{-1}) in distilled water were added to a hydrogen peroxide solution (0.6 ml, 40 mM). The absorbance of sample at 230 nm was determined after ten minutes against a blank solution containing phosphate buffer without hydrogen peroxide. The percentage of hydrogen peroxide scavenging by the extract and standard was calculated as follows: % Scavenged $[H_2O_2] = [(A_o - A_1)/A_o] \times 100$ where A_o was the absorbance of the control and A_1 was the absorbance in the presence of the sample of extract and standard.

Reducing power determination

Fe (III) reduction is often used as an indicator of electron- donating activity, which is an important mechanism of phenolic antioxidant action (19). The reducing power of extracts was determined according to our recently publish paper (19). Different amounts of each extracts (25-800 μg ml^{-1}) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 ml, 1%). The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Antioxidant activity in a hemoglobin-induced linoleic acid system

The antioxidant activities of extracts were determined by a modified photometry assay (24). Reaction mixtures (200 μl) containing 50 μl of extracts (125–1000 mg), 1 mmol l^{-1} of linoleic acid emulsion, 40 mmol l^{-1} of phosphate buffer (pH 6.5), and 0.0016% hemoglobin, were incubated at 37 ° C for 45 min. After the incubation, 2.5 ml of 0.6% HCl in ethanol was added to stop the lipid peroxidation. The amount of peroxide value was measured in triplicate using the thiocyanate method by reading the absorbance at 480 nm after colouring with 100 ml of 0.02 mol l^{-1} of $FeCl_2$ and 50 ml of ammonium thiocyanate (0.3 g ml^{-1}). Vitamin C was used as positive control.

Antihemolytic activity of extract

Preparation of rat erythrocytes

All the animal experiments were carried out with the approval of institutional animal ethical committee. Male rats in the body weight range of 180–220 g were housed in individual polypropylene cages and had free access to food and water. The animals were fed with standard diet. The animals were sacrificed under anesthesia and blood was collected by heart puncture in heparinized tubes. Erythrocytes were isolated and stored according to the method described by Yuan et al. (24). Briefly blood samples collected were centrifuged (1500 rpm, 10 min) at 4°C, erythrocytes were separated from the plasma and Buffy coat and were washed three times by centrifugation (1500 rpm 5 min) in 10 volumes of 10 mM phosphate buffered saline (pH 7.4). The supernatant and Buffy coats of white cells were carefully removed with each wash. Washed erythrocytes stored at 4°C and used within 6 h for further studies.

Antihemolytic activity of extract against H_2O_2 induced hemolysis

Briefly, erythrocytes from male rat blood were separated by centrifugation and washed with phosphate buffer (pH 7.4). Erythrocytes were then diluted with phosphate buffered saline to give 4% suspension. 1g of samples/ml of saline buffer was added to 2 ml of erythrocyte suspension and the volume was made up to 5 ml with saline buffer. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H_2O_2 solution in saline buffer was added to induce the oxidative degradation of the membrane lipids. The concentration of H_2O_2 in the reaction mixture was adjusted to bring about 90% hemolysis of blood cells after 240 min. After incubation the reaction mixture

was centrifuged at 1500 rpm for 10 min and the extend of hemolysis was determined by measuring the absorbance at 540 nm corresponding to hemoglobin liberation (25).

Statistical analysis

The values are presented as means ± SEM. Differences between group means were estimated using a one-way ANOVA followed by Duncan's multiple range test. Results were considered statistically significant when $p<0.05$.

Results and Discussion

The yield percent and total phenolic content of extracts obtained from *citrus* species flowers are shown in Table 1. The maximum of extractable polyphenolic content was recorded in *Citrus aurantium* extract with 78.76 ± 2.21 mg gallic acid equivalent/g of extract, by reference to standard curve ($y = 0.0063x$, $r^2 = 0.987$). Also maximum flavonoid content recorded in *Citrus limon* extract with 18.55 ± 0.54 mg quercetin equivalent/g of extract, by reference to standard curve ($y = 0.0067x + 0.0132$, $r^2 = 0.999$). Polyphenolic compounds, such as flavonoids, were widely found in naturally originated food products, and they have been shown to possess significant Biological activities (26). DPPH is a stable nitrogen-centered free radical. Any Substances which can donate hydrogen or electron and can change it color of which changes from violet to yellow so can be considered as antioxidants and therefore radical scavengers (27). IC_{50} for DPPH radical-scavenging activity exist in Table 1. The IC_{50} values for Ascorbic acid, quercetin and BHA were 5.05 ± 0.1, 5.28 ± 0.2 and 53.96 ± 3.1μg ml^{-1}, respectively. Flavonoid contents of *Citrus limon* flower seem to have direct roles for its good DPPH-scavenging activity (23). Chelation therapy reduces iron-related complications in human and thereby improves quality of life and overall survival in some diseases such as thalassemia major, cancer, HIV or wilson's disease (28). For example In addition, brain iron dysregulation and its association with amyloid precursor protein plaque formation are implicated in alzheimer's disease (AD) pathology and so iron chelation could be considered a rational therapeutic strategy for AD (29). Recently, many of researches focused on some natural product especially flavonoids that possess direct influence on iron (II) ions level within tissues ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases.

Table 1. Phenol and flavonoid contents and antioxidant activities of *Citrus* genus

Sample name	Extraction yield	Total phenol contents (mg/g)[a]	Total flavonoid Contents (mg/g)[b]	DPPH free radical scavenging, IC_{50} (μg/ml)	Nitric oxide scavenging, IC^{50} (μg ml^{-1})	H_2O_2 scavenging activity, IC^{50} (μg/ml)	Antihemolytic activity (μg/ml)	Fe^{2+} chelating ability (%)[c]
Citrus aurantium var. amara	12%	78.76 ± 2.21	12.11 ± 0.41	723.1 ± 23.67	30 % at 800 μg/ml	902.3 ± 29.78	757.5 ± 24.75	6.3%
Citrus nobilis var. unshiu	11.6%	62.10 ± 1.86	11.55 ± 0.40	1223.1 ± 38.27	942.9 ± 31.95	191.1 ± 6.11	781.25 ± 22.12	15.8%
Citrus limon	9.8%	60.01 ± 1.92	18.55 ± 0.54	654.05 ± 23.51	335.4 ± 10.39	604.2 ± 22.73	925.9 ± 30.39	24%
Citrus sinensis var. thompson novel	11%	78.47 ± 2.05	9.87 ± 0.29	835.2 ± 25.49	43% at 800 μg/ml	1001.1 ± 34.59	746.2 ± 20.88	8.3%
BHA	-	-	-	53.96 ± 3.1	-	-	-	-
Vit C	-	-	-	5.05 ± 0.1	-	21.4 ± 1.1	235 ± 9	-
Quercetin	-	-	-	5.28 ± 0.2	20 ± 0.01	52 ± 2.6	-	-

[a] mg gallic acid equivalent/g of extract powder [b.] mg quercetin equivalent/g of extract powder [c.] Inhibition at 800 mg ml^{-1}. EDTA used as control (IC_{50} = 18 ± 1.5 μg ml^{-1}).

In this assay, extract and EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. Although, all of the extract showed weak metal chelating activity, EDTA showed better activity (IC_{50} = 18 μg ml^{-1}). The nitric oxide assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce nitrite ions that can be estimated using griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. The % inhibition increased by increasing concentration of the extracts. In addition to reactive oxygen species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions (30). A number of disease states including sepsis and hepatic failure are characterized by abnormally high NO production and removing the excess NO could have salutary effects (31). The plant/plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Further, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health. *Citrus limon* extract showed better activity than others (IC_{50} = 335.4 ± 10.39 μg ml^{-1} vs. quercetin 20 ± 0.01 μg ml^{-1}). Although quercetin showed very potent NO radical scavenging, but its carcinogenic activity has been reported (32). Scavenging of H_2O_2 by extracts may be attributed to their phenolics, and other active components which can donate electrons to H_2O_2, thus neutralizing it to water (33). The extracts were capable of scavenging hydrogen peroxide in a concentration dependent manner. Results exist in Table 1. *Citrus nobilis* showed better activity than others (IC_{50} = 191.1 ± 6.11 μg ml^{-1}). Although hydrogen peroxide itself is not very reactive, it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm (20). Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1 shows the dose response curves for the reducing powers of the extracts. It was found that the reducing powers of all the extracts also increased with the increase of their concentrations. There were no significant differences ($p > 0.05$) among the extracts in reducing power that were not comparable with Vitamin C ($p < 0.001$). Polyphenolic contents of all the sample extracts appear to function as good electron and hydrogen atom donors and therefore should be able to terminate radical chain reaction by converting free radicals and reactive oxygen species to more stable products. Similar observation between the polyphenolic constituents in terms of dose dependent and reducing power activity have been reported for several plant extracts (20). Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (34). Erythrocytes are considered as prime targets for free radical attack owing to the presence of both high membrane concentration of polyunsaturated fatty acids (PUFA) and the O_2 transport associated with redox active hemoglobin molecules, which are potent promoters of reactive O_2 species. Specially, linoleic acid and arachidonic acid are targets of lipid peroxidation (34). The inhibition of lipid peroxidation by antioxidants may be due to their

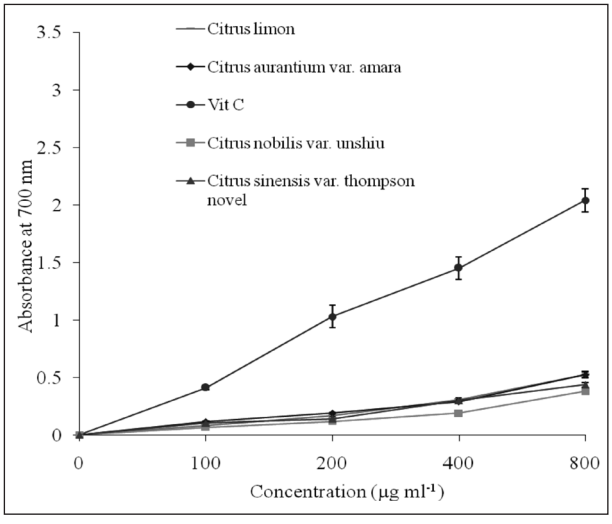

Figure 1. Reducing power activity of *citrus* species. Each value is expressed as mean 3 standard deviation positive control (vitamin C).* Significant difference from baseline within group ($p < 0.05$)

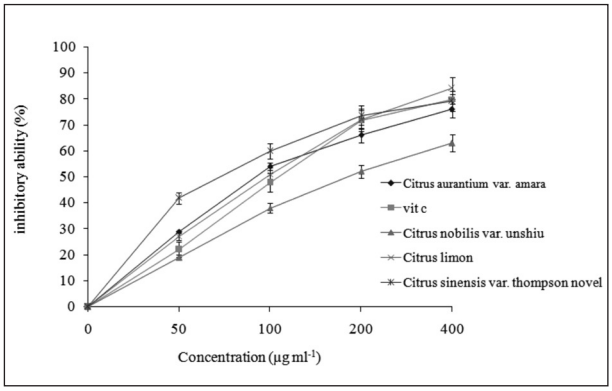

Figure 2. Antioxidant activities of *citrus* extracts against linoleic acid peroxidation induced by hemoglobin. Each value is expressed as mean 3 standard deviation positive control (vitamin C).

free radical-scavenging activities. Superoxide indirectly initiates lipid peroxidation because superoxide anion acts as a precursor of singlet oxygen and hydroxyl radical (9). Hydroxyl radicals eliminate hydrogen atoms from the membrane lipids, which results in lipid peroxidation. Tested extracts show good activity in hemoglobin-induced linoleic acid system that There were significant differences between extracts and vit C ($p < 0.001$) (Fig. 2). Effect of extract was tested and found that they did not show any harmful effects on erythrocytes. Results have been showed in table 1. *Citrus sinensis* extract showed potent antihemolytic activity (IC_{50} was 746.2 ± 20.88 vs. 235 ± 9.1 µg ml^{-1} for vitamin C). Antihemolytic activity of quercetin and other flavonoid have previously reported and good activity of extracts maybe result of high flavonoid content especially quercetin (35).

Conclusion

Present study indicate that the hydroalcoholic extract of *Citrus* species show different level of antioxidant and antihemolytic effect that maybe results of high phenol and flavonoid content. These results can be useful as a starting point of view for further applications of this plant or its constituents in pharmaceutical preparations after performing clinical researches.

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