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In vitro evaluation of the antioxidant activities in fruit extracts from citron and blood orange

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Abstract

Consumers are increasingly aware of diet related health problems and therefore demanding natural ingredients which are expected to be safe and health-promoting. Recently, number of studies on health benefits associated with citrus phytochemicals have been demonstrated. In the present study, an attempt has been made to isolate antioxidant fractions from two different citrus species such as Citron (*Citrus medica*) and blood orange (*C. sinensis*). Antioxidant fractions were extracted from mature, ripe fruits using five different solvents using a Soxhlet extractor. The total phenolic content of the extracts was determined by Folin–Ciocalteu method. MeOH:water (80:20) extract of citron and acetone extract of blood orange was found to contain maximum phenolics. The dried fractions were screened for their antioxidant activity potential using in vitro model systems such as 1, 1-diphenyl-2-picryl hydrazyl (DPPH), phosphomolybdenum method and as well as by the nitroblue tetrazolium (NBT) reduction test at different concentrations. The MeOH:water (80:20) fraction of citron showed highest radical scavenging activity 42.5%, 77.8% and 92.1% at 250, 500 and 1000 ppm, respectively, while MeOH:water (80:20) fraction of blood orange showed lowest DPPH radical scavenging activity at all the tested concentrations. Furthermore, all the fractions showed remarkable antioxidant capacity by the formation of phosphomolybdenum complex. In addition, superoxide radical scavenging activity was assayed using non-enzymatic (NADH/phenaxine methosulfate) superoxide generating system. All the extracts showed variable radical scavenging activity. The data obtained in the in vitro models clearly establish the antioxidant potency of citrus fruit extracts. However, comprehensive studies need to be conducted to ascertain the in vivo safety of such extracts in experimental animal models. To the best of our knowledge, this is the first report on antioxidant activity of citron and blood orange varieties of citrus fruits.

Keywords: Citron; Blood orange; Antioxidant activity; Radical scavenging activity; DPPH; Phosphomolybdenum; NADH/phenaxine methosulfate

1. Introduction

Consumers are becoming more conscious of the nutritional value and safety of their food and ingredients. Preference for natural foods and food ingredients that are believed to be safer, healthier and less subject to hazards is increasing compared to their synthetic counterparts (Farag, Badei, Heweij, & El-Baroty, 1986). In addition, it has been reported that dietary administration of synthetic antioxidants such as BHT (butylated hydroxytoluene) to rats can result in fatal hemorrhages. In recent years, evaluation of antioxidative activity of naturally occurring substances has been our focus of interest (Jayaprakasha, Jena, Negi, & Sakariah, 2002; Jayaprakasha, Singh, & Sakariah, 2001; Jayaprakasha, Tamil Selvi, & Sakariah, 2003; Jayaprakasha, Jaganmohan Rao, & Sakariah, 2004). However, the use of natural antioxidants is limited by lack of knowledge about their molecular composition, amount of active ingredients in the source material and the availability of relevant toxicity data (Shahidi, Wanasundara, & Amarowicj, 1994).

Several natural antioxidants have already been isolated from plant materials, such as oil seeds, cereal crops, vegetables, fruits, leaves, roots, spices, and herbs (Gil,

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Tom'as-Barber'an, Hess-Pierce, Holcroft, & Kader, 2000; Jayaprakasha et al., 2003; Ramarathnam, Osawa, Ochi, & Kawakishi, 1995; Shahidi et al., 1994). Antioxidant compounds have been identified in the seeds of citrus (Gorinstein et al., 2004), grape (Jayaprakasha et al., 2001), pomegranate (Singh, Murthy, & Jayaprakasha, 2002). However, little information is available on studies relating to the antioxidant activity of citron and blood orange.

Citron (*Citrus medica*) trees have an irregular shape and small size fruits, compared with lemons, oranges and grapefruit. Shape of the Citron fruit often has a persistent style. The rind is yellow, with a smooth but very uneven surface (Saunt, 1990). Blood oranges are characterized by their unique flesh and rind color due to phenolic pigments belonging to the anthocyanins. Blood orange fresh tissue and juice contain a predominance of anthocyanins (e.g. cyanidin-3-glucoside and cyanidin-3-(6″-malonyl)-glucoside) (Maccarone, Rapisarda, Fanella, Arena, & Mondello, 1998; Dugo, Mondello, Morabito, & Dugo, 2003). Recently, delphinidin, cyaniding, petunidin, pelargonidi, peonidin and malvidin were identified by ESI-MS in Sicilian blood orange juice. Rapisarda et al. (1999) reported in vitro studies on antioxidant capacity of blood orange juice was due to their anthocyanins rather than vitamin C content.

Citrus fruits and juices have long been recognized to contain secondary metabolites including antioxidants such as ascorbic acid, flavanones, phenolics and pectin that are important to human nutrition. Limonoids are secondary metabolites present in all citrus fruit tissues whose role in human nutrition has not been established. Our recent study showed the inhibition of human cancer cell proliferation, induction of apoptosis of human breast cancer cells by citrus limonoids (Tian, Miller, Ahmad, Tang, & Patil, 2001). In certain species of citrus, flavonoids are found as methoxylated flavones and glycoside flavones and they being used as indicators of authenticity of commercial juices (Bocco, Cuvelier, Richard, & Berset, 1998). Our recent study demonstrated the antiproliferative activity of citrus components such as pectin, pulp, naringin and limonin against colon cancer in vivo systems (Vanamala et al., 2004). Subsequently, citrus flavonoids such as apigenin, naringenin, hesperidin and nobiletin have been tested against the promotion stage of colon cancer. It was found that, apigenin and naringenin were low the proliferative index as compared to hesperidin and nobiletin (Leonardi et al., 2004). The present study was performed to evaluate the antioxidant activity of citron and blood orange, to investigate its potential as natural antioxidants in different in vitro models systems.

2. Materials and methods

2.1. Materials and equipments

1,1-diphenyl-2-picryl hydrazyl (DPPH), butylated hydroxyltoluene (BHT), folin–ciocaleteau reagent; ascorbic acid, phenazine methosulphate (PMS), nicotinamide dinucleotide phosphate (NADH), nitroblue tetrazolium (NBT) and catechin were obtained from Sigma Chemical Co. (St. Louis, MO). Visible spectra measurements were done using Genesys-20 visible spectrophotometer (Milton Roy, NY, USA).

2.2. Plant material

Citrus fruits of citron (*C. medica*) and blood red orange (*C. sinensis*) were harvested from Citrus Centre orchard, Citrus Centre, Texas A&M University-Kingsville, Weslaco. Peels were removed manually and slices (1270 g of citron and 1560 g of blood orange) were freeze dried. The yield of dried material obtained was 152 g and 227.7 g and stored at −20 °C until further use.

2.3. Extraction

Freeze dried citrus fruit powder of citron (140 g) and blood orange (150 g) was successively extracted in a Soxhlet extractor with 1200 ml of hexane, ethyl acetate (EtOAc), acetone, methanol (MeOH) and MeOH:water (80:20) for 8 h each. All the extracts were filtered, concentrated to 5–10 ml and freeze dried separately.

2.4. Determination of total phenolics

The concentration of total phenolic compounds in the extracts was determined as modified by Negi and Jayaprakasha (2003) and the results were expressed as catechin equivalents. The freeze dried extracts were dissolved in a mixture of methanol and water (6:4 v/v). Different concentrations (10, 20, 30, 40, 50, 75 and 100 μg) of standard (+)-catechin and extracts were taken in test tubes and the volume was adjusted to 0.2 ml by addition of distilled water. One milli liter of 10-fold diluted Folin–Ciocalteu reagent and 0.8 ml of 7.5% sodium carbonate solution were added to all the tubes. After 30 min incubation at room temperature the absorbance was measured at 765 nm using a spectrophotometer. The estimation of total phenolics in all the extracts was carried out in triplicate and the mean results were presented.

2.5. HPLC analysis

The high performance liquid chromatographic system consisted of a Thermo Separations Instrument (FL, USA), fitted with a Hamilton column C18 column (150 × 4.6 mm ID) and AS3000 autosampler was used. Detection was done by a photo diode array detector at wavelength of 210 and 280 nm for limonoids and flavonoids, respectively. The elution was carried out with 40% acetonitrile and flow rate was 1.0 ml min^{-1} under isocratic condition. All the extracts were filtered through 0.45 micron filters and injected to HPLC. The compounds were quantified using ChromQuest software.

2.6. Radical scavenging activity using DPPH method

Various concentrations (175, 350 and 700 μl equivalent to 250, 500 and 1000 ppm) of freeze dried extracts and ascorbic acid was prepared in different test tubes. The volume of the samples and ascorbic acid were adjusted to 700 μl by adding MeOH. Three milli liter of methanolic solution of DPPH (100 μM) was added shaken vigorously and the tubes were allowed to stand at 27 °C for 20 min (Singh et al., 2002). A control was prepared as described above without samples or standards. MeOH was used for the baseline correction. The changes in the absorbance of the all the samples and standards were measured at 517 nm. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula, % Radical scavenging activity = (Control optical density − sample optical density/control optical density) × 100.

2.7. Evaluation of antioxidant capacity by phosphomolybdenum method

The total antioxidant capacity of extracts of ethyl acetate, acetone, methanol and MeOH:water (80:20) and BHT were evaluated according to the method described by Prieto, Ineda, and Aguilar (1999). An aliquot of 0.1 ml of sample solution and BHT (equivalent to 200 and 400 ppm) was combined with 1 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank, 0.1 ml of methanol was used in place of sample. The tubes were incubated in a boiling water bath at 95 °C for 90 min. After the samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank in Genesys-20-Visible spectrophotometer (Milton Roy, New York, USA). Antioxidant capacity was expressed as equivalents of ascorbic acid (μmol g^{-1}).

2.8. Superoxide anion scavenging activity

The phenazine-methosulfate (PMS)-NADH method (Robak & Gryglewski, 1998) was used for the generation of O^{2-}. The test tubes contained 12 μM PMS, 100 μM NADH, and 100 μM NBT in 0.1 M phosphate buffer (K_2HPO_4–KH_2PO_4) at pH 7.8. After 2 min of incubation at room temperature, 100 μl of HCl (0.1 M) were added to stop the reaction. The spectrophotometric measurement was recorded at 560 nm against blank samples, in the absence of PMS. Different series of concentrations of all the fractions and ascorbic acid were added to the test tubes before adding PMS.

3. Results and discussion

Freeze dried fruit powder of citron and blood orange were successively extracted with hexane, EtOAc, acetone, MeOH and MeOH:water (80:20). All the extracts were separately freeze dried and stored at −20 °C until further use. Generally, hexane was used for the extraction of non-polar compounds like fatty material or some carotenoids, while EtOAc for carotenoids and some phenolics. The other solvents were used for the extraction of polar compounds like aglycones and glucosides of flavonoids and limonoids depending upon their polarity. Table 1 shows the yield and phenolic content of each fraction. MeOH extract of citron provided maximum yield (40.60%), whereas acetone provided minimum yield (1.30%). On the other hand, hexane extract of blood orange gave minimum yield (0.83%) and MeOH gave maximum yield (68%). All the extracts were subjected to HPLC analysis using 40% acetonitrile as a mobile phase and the compounds were detected at 210 nm and 280 nm. The major limonoids found in EtOAc and acetone extracts of citron are limonin, nomilin, obacunone and deacetyl nomiln, where as flavonoid glucosides and limonoid glucosides were found in MeOH and MeOH:water extracts. On the other hand, EtOAc extract of blood orange showed limonin and deacetyl nomiln. The other three extracts showed flavonoids and glucosides of flavonoids and limonoids. The identity of limonin, nomiln, obacunone and deacetyl nomiln were confirmed by relative retention times of authentic standards. Recently, Sun, Chen, Chen, and Chen (2005) reported antioxidant capacity of limonin and nomilin were found to be higher than vitamin C. In this context, the HPLC analyses of limonoids and flavonoids have been carried out.

The results of free radical scavenging potentials of extracts from citron and blood orange and ascorbic acid at different concentrations were tested by DPPH method and the results are depicted in Figs. 1 and 2. Antioxidants react with DPPH, which is a nitrogen-centered radical with a characteristic absorption at 517 nm and convert to 1,1,-diphenyl-2-picryl hydrazine, due to its hydrogen donating ability at a very rapid rate (Jayaprakasha et al., 2004). The degree of discoloration indicates the scavenging potentials of the antioxidant extracts. At 1000 ppm, citron MeOH:water (80:20) extract and ascorbic acid exhibited 92.06% and 97.0% free radical scavenging activity, respec-

Table 1
Percentage yield and phenolic content present in citrus fruits

Varieties	Solvents used for extraction	Yield (g/100 g of fruit)	Phenolics as catechin equivalents (mg/g of extract)
Citron	Hexane	1.50	0.00
	EtOAc	20.01	12.77
	Acetone	1.30	29.64
	MeOH	40.60	39.28
	MeOH:water (80:20)	7.31	71.81
Blood orange	Hexane	0.83	0.00
	EtOAc	7.19	29.6
	Acetone	2.76	48.8
	MeOH	68.01	8.0
	MeOH:water (80:20)	20.01	2.0

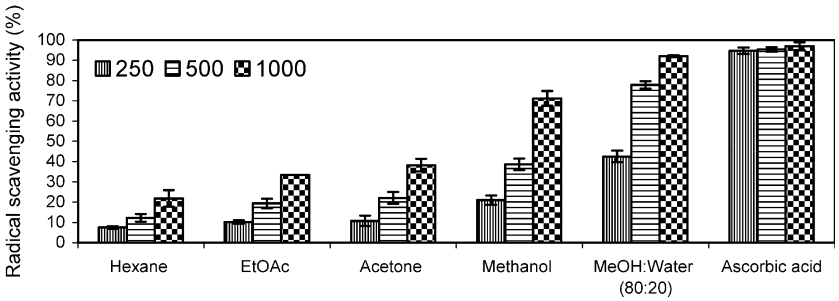

Fig. 1. Radical scavenging activity of citron extracts obtained from freeze dried fruits and ascorbic acid by DPPH method at different concentrations (ppm).

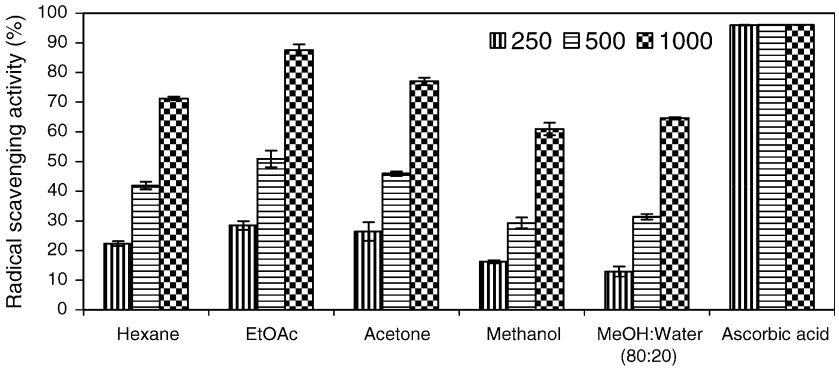

Fig. 2. Radical scavenging activity of blood orange extracts obtained from freeze dried fruit and ascorbic acid by DPPH method at different concentrations (ppm).

tively, and hexane extract showed minimum activity at all the tested concentrations (Fig. 1). Similarly, EtOAc and MeOH extract of blood orange showed maximum (87.6%) and minimum (60.9%) activity, respectively, at 1000 ppm. The antioxidant activity of the fractions was attributed to their hydrogen donating ability (Shimada, Fujikawa, & Nakamura, 1992). It is known that free radicals cause autooxidation of unsaturated lipids in food (Kaur & Perkins, 1991). On the other hand, antioxidants are believed to intercept the free radical chain of oxidation and to donate hydrogen from the phenolic hydroxyl groups, thereby forming stable end product, which does not initiate or propagate further oxidation of lipid (Sherwin, 1978). The data obtained revealed that the certain fractions isolated from citrus fruits are free radical scavengers and primary antioxidants that react with DPPH radical, which may be attributed to its proton donating ability.

The phosphomolybdenum method is based on the reduction of molybdenum (VI) to molybdenum (V) by the antioxidant compounds and the formation of a green molybdenum (V) complex, which has a maximal absorption at 695 nm. The different citrus fruit extracts exhibited

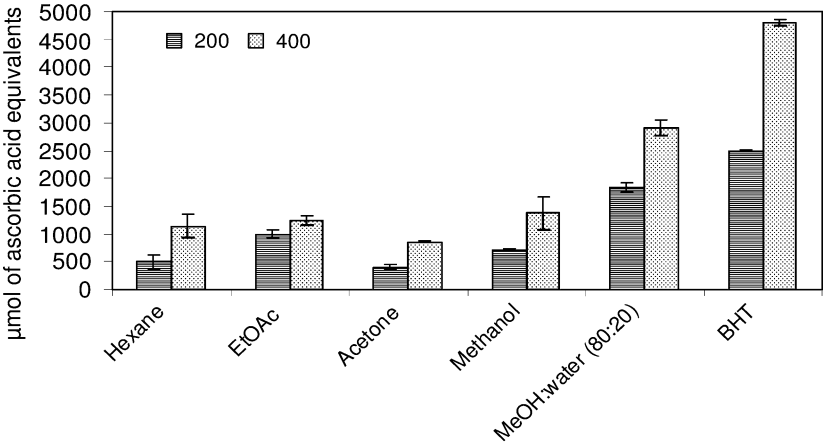

Fig. 3. Antioxidant capacity of citron extracts and BHT by phosphomolybdenum method at different concentrations (ppm).

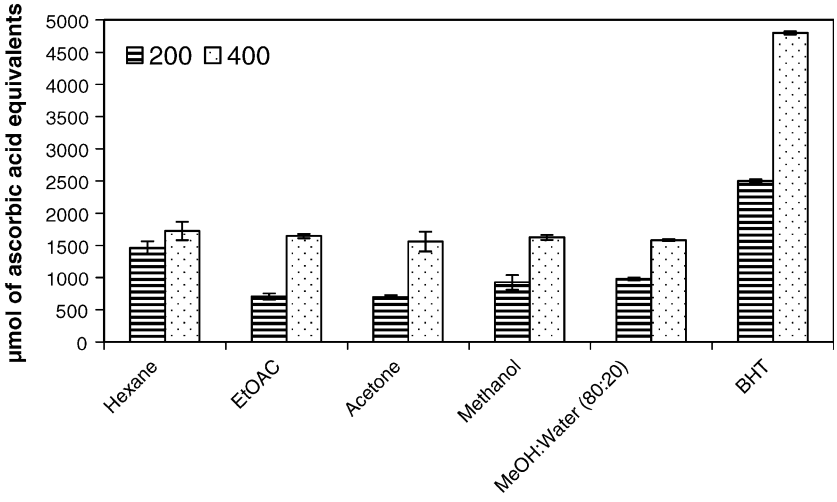

Fig. 4. Antioxidant capacity of blood orange extracts by phosphomolybdnum method at different concentractions (ppm).

various degrees of antioxidant capacity (Figs. 3 and 4). It is difficult to ascertain an order of antioxidant capacities of different extracts because of the differential responses at two concentrations. Maximum antioxidant capacities were observed in the MeOH:water (80:20) extract of citron and hexane extract of blood orange. The order of antioxidant capacity of citron is MeOH:water (80:20) > MeOH > EtOAc > hexane > acetone and of blood orange is hexane > EtOAc > MeOH > MeOH:water (80:20) > acetone. Variations in antioxidant capacity of different extracts may be attributed to differences in their chemical composition such as phenolics, ascorbic acid and carotenoids. It is clear that, all the extracts showed an increase in antioxidant capacity with increase in dose of sample. Our recent results indicated that certain citrus limonoids are found to posses good antioxidant activity (Patil et al., 2004; Poulose, Harris, & Patil, 2005; Yu et al., 2005). The antioxidant activity shown by the citrus fruit extracts may be due to the

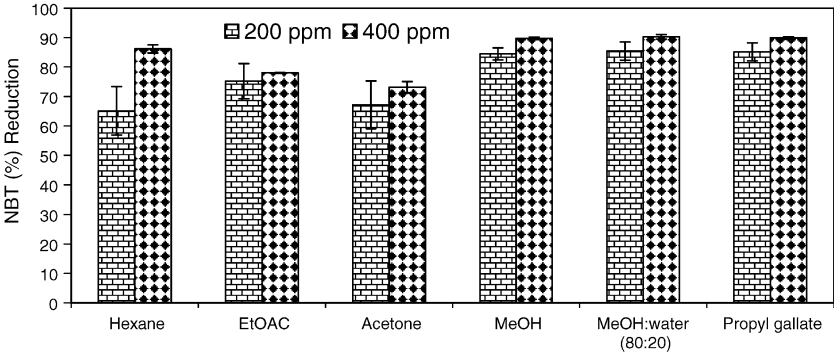

Fig. 5. Superoxide radical scavenging activity of different extracts from citron.

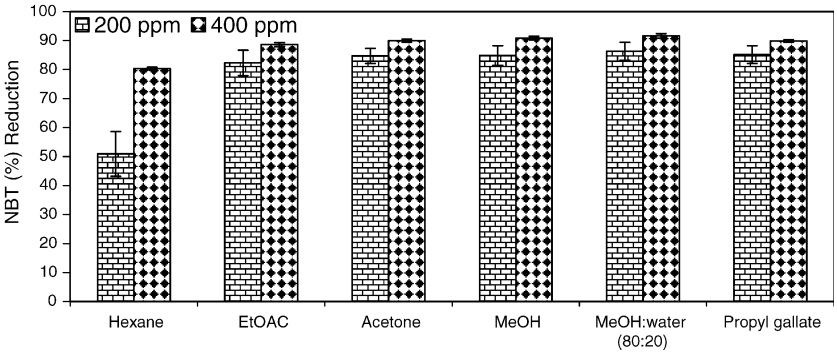

Fig. 6. Superoxide radical scavenging activity of different extracts from blood orange.

presence of flavonoids, carotenoids and ascorbic acid (Gorinstein et al., 2004).

In the PMS/NADH-NBT system, superoxide anion is generated using a non-enzymatic reaction of phenazine methosulphate in the presence of NADH and molecular oxygen (Robak & Gryglewski, 1998; Yen & Hsieh, 1998). In both the strategies, superoxide anion reduces NBT into formazan at pH 7.8 at room temperature and formazan generation is followed by spectrophotometry at 560 nm. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. In this assay, all the extracts exhibited very strong superoxide anion scavenging activity and the results are presented in Figs. 5 and 6. Among the fractions assayed, the MeOH:water (80:20) extract of citron and blood orange extracts showed the strongest activity. Most of the antioxidant activity results of citrus fractions showed a moderate correlation with other two methods. The superoxide anion radical-scavenging activity of the different extracts may be due to the presence of phenolic compounds except hexane extract. The increase in activity is due to increase in number of phenolic hydroxyl groups in the molecule. The antioxidant activity of hexane extract may be due to the presence of carotenoids in the citrus fruits (Gorinstein et al., 2004).

Correlation between the content of the total phenolics and radical scavenging activity of the citrus fruit extracts was studied and the results are presented in Fig. 7. Some of the studies reported no correlations between the total phenolic content and the radical scavenging activity (Yu et al., 2002), but in the present study showed very high

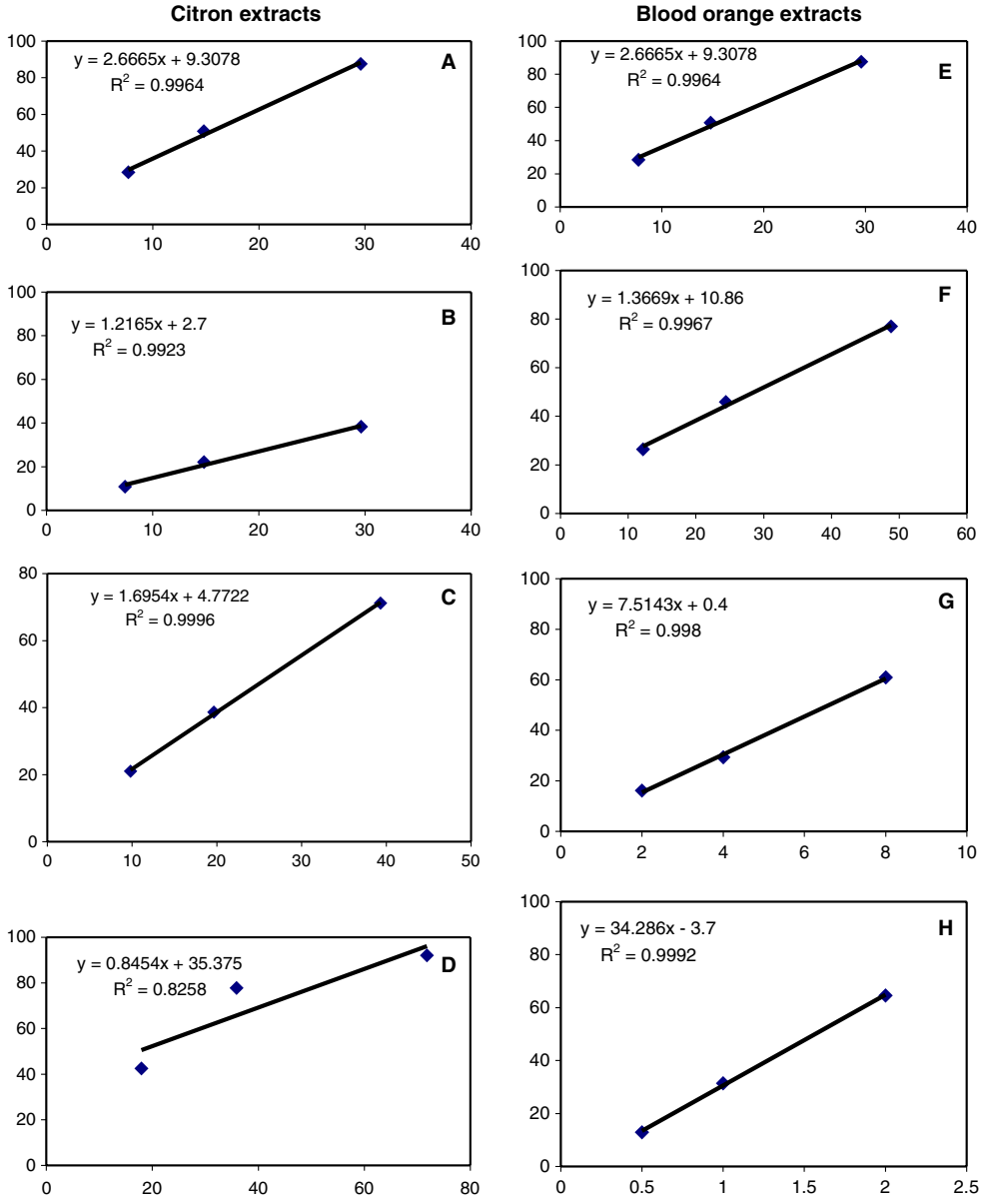

Fig. 7. Correlation between citrus phenolics and their radical scavenging activity by DPPH method: [A] EtOAc extract; [B] Acetone extract; [C] MeOH extract; [D] MeOH:water (8:2); [E] EtOAC; [F] Acetone; [G] MeOH and [H] MeOH:water (8:2).

correlation coefficient of the total phenolics and radical scavenging activity of all the samples ($r^2 = 0.99$) except MeOH:water (80:20) extract of citron. While there are many methods for the total antioxidant determination, most of the methods have their own limitations (Gorinstein et al., 2001; Yu et al., 2002). It was shown that some antioxidant assay methods give different antioxidant activity trends (Ou, Huang, Hampsch-Woodill, Flanagan, & Deemer, 2002). Therefore, the antioxidant properties of two citrus species different extracts were determined by three methods. Based on our results, the highest activity was found in MeOH:water extract of citron for DPPH and phosphomolybdenum and PMS-NADH methods (Figs. 1, 3 and 5). In blood orange, the highest activity was found in EtOAc extract by the DPPH method (Fig. 2), but in the hexane extract by the phosphomolybdenum method (Fig. 4) and in all the fractions, except in the hexane one, by the PMS-NADH method (Fig. 6). The DPPH approach seems to be rapid and accurate method for assessing the antioxidant activity of fruit and vegetable extracts. The results are highly reproducible and comparable to other free radical scavenging methods such as ABTS (Gil-Izquierdo, Gil, Ferreres, & Tom'as-Barber'an, 2001).

Phenolic compounds and ascorbic acid were identified as possible antioxidants in orange juice. Phenolic compounds were able to scavenge radicals and to chelate metals (Halliwell & Gutteridge, 2000), while ascorbic acid can play a pro-oxidant role in the presence of transition metals (Halliwell, 2001). These compounds can also act as antioxidants because of their ability to trap superoxide anions (Stadler, Turesky, Müller, Markovic, & Leong-Morgenthaler, 1994). Depending on the concentrations of phenolic compounds and of transition metals, a complex can be formed that facilitates the redox process (Khan, Ahmad, & Hadi, 2000). In general, the phenolic compounds at low concentrations show antioxidant behavior. At higher concentrations, they show pro-oxidant behavior; upon further increasing of their concentration they again show antioxidant behavior. This always depends on the type (position and number of hydroxyl in the molecule) and the concentration of the phenolic compound, as well on that of the transition metal (Khan et al., 2000). Thus, different citrus fruit extracts at different concentrations showed different degree of antioxidant activity due to the presence various compounds.

Very little information is available on antioxidant activity of all bioactive compounds in citrus (Gil-Izquierdo et al., 2001; Rapisarda et al., 1999). Number of studies has reported on the antioxidant capacity of foods and a significant in vitro antioxidant activity of fruit juices (Gil et al., 2000; Sluis Van der, Dekker, Verkerk, & Jongen, 2000). Recently, the study of antioxidant activity of orange juices through the scavenging of the DPPH radical was reported (Miller, Rigelhof, Marquart, Prakash, & Kanter, 2000; Sluis et al., 2000). The antioxidant capacities of limonin and nomilin in the four tissues of mature fruit were determined by β-carotene bleaching assay. The antioxidant capacities of limonin and nomilin varied in different tissues and cultivars. In the three tissues other than albedo, the antioxidant capacities of limonin and nomilin were higher 2.9–8.3 times than that of ascorbic acid (Sun et al., 2005). On the basis of these considerations, evaluation of antioxidant activity and their compounds of different solvent extracts from two different citrus fruits were undertaken.

The results obtained in the present study demonstrate that the citrus fruit extracts which can effectively scavenge various reactive oxygen species or free radicals under in vitro conditions. This may be due to the number of stable oxidized products that can form after oxidation or radical scavenging. The broad range of activity of the extracts suggests that multiple mechanisms are responsible for the antioxidant activity. The multiple antioxidant activity of extracts demonstrated in this study clearly indicates the potential application value of the citrus fruits. However, the in vivo safety of extracts needs to be thoroughly investigated in experimental rodent models prior to its possible application as an antioxidant ingredient, either in animal feeds or in human health foods. Currently, relevance of in vitro and in vivo tests of measuring antioxidant activity both in vitro and in vivo tests are also vital before launching human clinical trails. It is not desirable to assay any biological activities through in vivo methods directly without conducting in vitro tests using chemical methods or cell lines. Hence, we have conducted antioxidant activity of citrus fruit extracts in different in vitro models. By using these results, the most potent extract can be used for the assay of antioxidant activity using in vivo assays. Alternatively, the active components can be enriched in a particular extract and that can be used for the in vivo assay in our future studies. Similar type of work has been reported in our earlier studies (Chidamabara Murthy, Jayaprakasha, & Singh, 2002a; Chidamabara Murthy, Singh, & Jayaprakasha, 2002b; Singh et al., 2002; Vanamala et al., in press).

The above results demonstrated that, some of the extracts could exhibit antioxidant properties approximately comparable to commercial synthetic antioxidants. Further studies are needed on the isolation and characterization of individual compounds to elucidate their different antioxidant mechanisms and the existence of possible synergism, if any, among the compounds.

Acknowledgements

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