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Citrus residues: A potential source of phenolics with high antioxidant values

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Abstract

This work explores underutilized Lemon *(Citrus limon)* and Galgal *(Citrus pseudolimon)* residues for their phenolic contents and *in-vitro* antioxidant activities. Methanolic extracts from different parts of these two *Citrus* species were tested for total phenolic contents (TPC), total flavonoid contents (TFC), DPPH radical scavenging activity, % inhibition of linoleic acid peroxidation and reducing power by using respective *in-vitro* antioxidant model assays. The percentage yield of extracts, total phenolic and flavonoid contents among parts tested varied from 6.13 – 24.20 g/100g, 98.20 – 199.18 (mg gallic acid equivalent/g of extract) and 19.95 – 39.60 (mg catechin equivalent/g of extract), respectively. Percent inhibition of linoleic acid peroxidation and DPPH radical scavenging capacity for the tested extracts ranged between 31-60 and 40-62%, respectively. The overall order of antioxidant potential among parts of *Citrus* species was established to be: *C. pseudolimon* leave > *C. limon* leave > *C. pseudolimon* peel > *C. limon* peel > *C. pseudolimon* seed ≈ *C. limon* seed. It could be concluded that extracts of *Citrus* fruit residues, especially, leaves can be explored as an economically viable source of natural antioxidants and nutraceuticals.

Introduction

Plants naturally produce a wide array of secondary metabolites which not only protect them from environmental and oxidative stresses but also contribute towards their flavor and sensory properties. Free radicals and other reactive oxygen species produced as result of oxidation have been implicated in different health disorders such as atherosclerosis, cancer, diabetic mellitus, neurodegenerative disorders, hypertension, and aging. The plant metabolites (antioxidants) have capacity to retard the progress of chronic diseases as well as inhibit the development of rancidity and off flavors in foods (Biglari *et al*., 2008).

Citrus belongs to the family *Rutaceae* which comprises about 40 species; mainly distributed in Brazil, China, , India, Mexico, United States, Spain and Pakistan (sixth largest producer of citrus) *(www.fao.com)*. Citrus are one of the most important fruit crops having medicinal benefits against coronary diseases, chronic asthma, inflammatory, tumor and blood clotting (Dugo and Giacomo, 2002; Abeysinghe *et al*., 2007). Such multiple medicinal properties of citrus might be ascribed to the presence of bioactive compounds, such as hydrocinnamic acid, cyaniding glucoside, hisperidine, vitamin C, carotenoids and flavonoids. The main flavonoids found in citrus are hesperidine, naringin, narirutin and eriocitrin (Schieber *et al*., 2001; Calabro *et al*., 2004; Xu *et al*., 2008).

Citrus species of various origins have been assessed for their antioxidant activity and phenolic constituents using different *in-vitro* assays (Guimaraes *et al*., 2009; Fattahi *et al*., 2011). In addition to edible portion of citrus other parts of these species such as peel and leaf are also rich in phenolic antioxidants (Anagnostopoulou *et al*., 2006) but rarely investigated. Recently, plant antioxidants have gained much attention due to their growing applications as chemotherapic, anticancer (Taxol), antiviral and anti-inflammatory drugs as well as functional food additives (Amin *et al*., 2004; Wansi *et al*., 2006).

In view of the medicinal and functional food attributes, it is fascinating to explore more and more plant-based antioxidant resources. The peels, leaves and seeds of citrus fruits are usually discarded as waste during the processing of fruits. This study was therefore designed to appraise the

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phenolic compounds and antioxidant potential of different parts/residues (leaves, seed and peel) of locally available two citrus species (*C. limon* and *C. pseudolimon*).

Materials and Methods

Samples collection

Fresh sample of leaves and fruits of *C. limon* and *C. pseudolimon* were collected from the orchards in the vicinity of District Faisalabad, Pakistan. A taxonomist at the Department of Botany, University of Agriculture Faisalabad (UAF), Pakistan, further authenticated the identity of the specimens.

Sample preparation and extraction

Peels and seeds separated from the fruits and leaves were washed with distilled water, dried at ambient temperature and finally ground into a fine powder. For extraction, 10 g of ground sample of the tested parts of *C. limon* and *C. pseudolimon* were mixed with aqueous methanol (methanol/water with 80/20 v/v ratio). The extraction was performed by shaking overnight using an orbital shaker (Gallenkamp, UK). All extracts were filtered through Whatman filter paper No. 1 and the residues were re-extracted as described above. The filtrates obtained were concentrated to dryness under vacuum at 45°C, in a rotary evaporator apparatus. The extracts yields were calculated gravimetrically and the extract materials preserved at freezing temperature.

Phenolic contents

Folin–Ciocalteu reagent (FCR) based colorimetric assay was used to assess the amount of total phenolics (Chaovanalikit and Wrolstad 2004; Ebrahimzaded *et al*., 2008; Nabavi *et al.*, 2008). Briefly, 50 mg of dry matter for each extract was taken in a test tube and mixed with 0.5 mL of FRC and deionized water 7.5 mL. The mixture was mixed well, kept at room temperature for ten min, after that about 1.5 mL of aqueous 20% sodium carbonate (w/v) solution was added. Absorbance of the finally obtained solution mixture, after incubation at 40°C for 20 min, followed by cooling, was recorded at 755 nm spectrophotomterically (Hitachi U-2001 spectrophotometer, model 121-0032). The Total Phenolics (mg/g of dry matter) were calculated as gallic acid equivalents (GAE) for triplicate measurements.

Flavonoid contents (FC)

Total flavonoids were determined following the method as earlier reported by Dewanto *et al.* (2002). As per said method, 1 mL aqueous extract (containing 0.1 g/mL of dry matter) was diluted further with 5 mL of distilled water; 0.3 mL of 5% $NaNO_2$ was added and after 5 min, 0.6 mL of 10% $AlCl_3$ was added. Finally after 5 min 2 mL of 1 M NaOH was added and absorbance measured at 510 nm. Total flavonoids were calculated as catechin equivalents (mg/g of dry weight/matter).

Antioxidant activity in linoleic acid system

The antioxidant activity (AA) of the extracts produced was also appraised in relation to inhibition of linoleic acid (C18:2) peroxidation as per method described previously by Iqbal and Bhanger (2005). In this experiment, test extract (5 mg) was mixed with linoleic acid solution (0.13 mL), 10 mL of ethanol (99.8%) and 0.2 M sodium phosphate buffer (10 mL) of pH 7. The mixture was diluted to 25 ml with distilled water and incubated at 40°C. The extent of linoleic acid oxidation was followed by thiocyanate method (Yen *et al*., 2000; Gulçin *et al.*, 2004) with some modification. Briefly, analytical/sample solution (0.2 mL), 75% ethanol (10 mL), an aq. solution of ammonium thiocyanate (30%), and 0.2 mL $FeCl_2$ solution and (20 mM in 3.5% HCl) were mixed and incubated at 40°C with constant stirring. After 3 min absorbance was measured at 500 nm and peroxide contents were calculated in comparison with butylated hydroxytoluene (BHT) and ascorbic acid (200 ppm) as positive controls.

Ferric reducing power (FRAP) and DPPH· scavenging assay

FRAP and DPPH· scavenging potential of the tested sample extracts was determined by using procedure described by Iqbal and Bhanger (2005), respectively. For FRAP Equivalent volume of different extracts containing 2.5–10.0 mg of dry mass was mixed with 5.0 mL buffer (sodium phosphate, 0.2 M, pH 6.6) and 5.0 mL potassium ferricyanide (1.0%). After 20 min incubation at 50°C, 5 mL of 10% trichloroacetic acid was added and the reaction mixture centrifuged at 980 rpm for 10 min at 5oC in a refrigerated centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water mixed with 1.0 mL ferric chloride (0.1%) and absorbance measured at 700 nm (Hitachi U-2001). DPPH· (2, 2-dipheny-l-picrylhydrazyl) scavenging activities of the extracts of different parts of citrus plant were measured by mixing 1.0 mL methanolic extract (25 µg/mL dry matter) with 5.0 mL of freshly prepared solution of DPPH (0.025 g/L) and absorbance at 515 nm was measured at different intervals of time using spectrophotometer (Hitachi

Table 1. Percentage Yield, TPC and TFC of two citrus species residues (leaves, peels, and seeds)

Citrus species	Part[L]	Yield of extract[M]	Total Phenolic Contents[N]	Total Flavonoid Contents[O]
C. limon	Leaves	$20.81^{cd} \pm 0.93$	$192.46^{d} \pm 0.12$	$36.34^{d} \pm 0.15$
	Peels	$9.44^{b} \pm 0.81$	$158.79^{c} \pm 0.72$	$29.33^{bc} \pm 0.38$
	Seeds	$6.13^{a} \pm 1.05$	$98.23^{a} \pm 0.84$	$19.95^{a} \pm 0.45$
C. pseudolimon	Laves	$24.20^{d} \pm 1.17$	$199.18^{d} \pm 1.20$	$39.63^{d} \pm 0.38$
	Peels	$16.31^{c} \pm 1.25$	$162.30^{c} \pm 0.54$	$32.21^{c} \pm 0.21$
	Seeds	$7.14^{ab} \pm 1.25$	$106.06^{a} \pm 1.34$	$21.18^{a} \pm 0.12$

[M] Values are (g /100g) mean±SD, [N] Values are given as mg/g of dry matter, calculated as gallic acid equivalent, [O] The result were calculated as catechin equivalents (CE) mg/g of dry matter, Different letters in superscript within the same column indicate significant differences (p<0.05) among parts of *Citrus* species

U- 2001, model 121-0032) to calculate DPPH radical scavenging capacity.

Statistical analysis

Triplicate samples of peel, leave and seed of citrus species were analyzed individually to report the data as mean coupled with SD for three replicates. Statistical variations for the analyzed attributes among the three parts analyzed were evaluated by analysis of variance (ANOVA) with probability value (p) 0.05 considered to be significant at 5% significance level.

Results and Discussion

Extract yield, total phenolic and total flavonoid contents

The yield of crude extracts, total phenolic contents (TPC), total flavonoid contents (TFC) for different parts tested are given in Table 1. The analysis of variance (ANOVA) showed that variation in measured parameter among the parts tested was generally significant (p<0.05). The percentage yield of extractable antioxidant components from leave, seed and peel of the investigated *Citrus* species varied widely (6.13 - 24.20 %).

The maximum amount of bioactive compounds was obtained for *C. pseudolimon* leaves and minimum for *C. limon* seed. The difference in the yield might be attributed to the availability extractable components in different parts analyzed depending upon their chemical composition and the agro-climatic conditions (Hsu *et al*., 2006). The crude extract yield of *C. pseudolimon* and *C. limon* leaves was comparable with those (19.87% and 21.5%) investigated for *Citrus* peel by Zia-Ur-Rehman (2006) and Sultana *et al.* (2008), respectively. The overall order of crude extracts yield among parts and species was as: *C. pseudolimon* leave ≈ *C. limon* leave > *C. pseudolimon* peel > *C. limon* peel > *C. pseudolimon* seed ≈ *C. limon* seed.

The key role of phenolic compounds to scavenge free radicals produced during the lipid oxidation has been emphasized in several reports. Previous literature showed that the phenolic contents are strongly associated with the antioxidant activity of plants, fruit, grains and vegetables (Huang *et al.*, 2005). In the present experiment, the total phenolic contents of extracts from leaves, peels and seeds of *C. limon* and *C. pseudolimon* varied over 98.20-199.18 mg/g of dry matter determined as Gallic acid Equivalent (GAE) revealing the citrus fruit residues a potential source of phenolic bioactives (Table 1). Of the parts tested, *C. pseudolimon* leaves had the highest amount of total phenolics (199.18 mg/g of dry matter) which was contradictory to the data of previous reports indicating higher TPC in citrus peel as compared to other parts (Li *et al*., 2005; Kamran *et al*., 2009). Furthermore, TPC determined in different parts of selected *Citrus* species during this study were higher as compared to certain fruits and followed the order of *C. pseudolimon* leave > *C. limon* leave > *C. pseudolimon* peel > *C. limon* peel > *C. pseudolimon* seed > *C. limon* seed. When compared with the literature, the present TPC were found to be higher than those investigated by some other researchers in different *Citrus* species (Someya *et al*., 2002; Anagnostopoulou *et al.*, 2006; Kamran *et al*., 2009), some citrus fruits (Hashempour *et al*., 2013) whereas, relatively lower as compared to that reported by Sultana *et al.* (2008).

Considerable interest has been displayed recently in flavonoids due to their beneficial effects related to human health. Flavonoids have been reported to have anti-viral, anti-allergic, anti-platelet, anti-

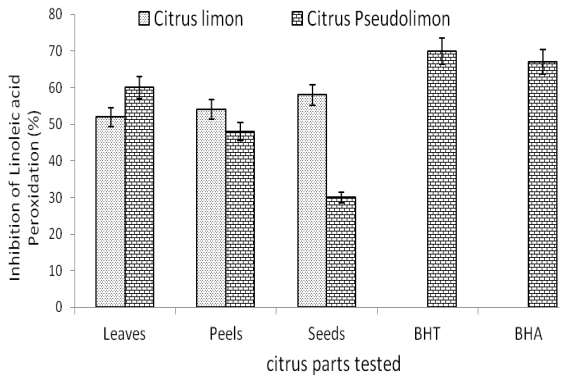

Figure 1. Percent inhibition of linoleic acid oxidation of extracts from different parts of *Citrus* species assessed versus synthetic antioxidants (BHT and BHA)

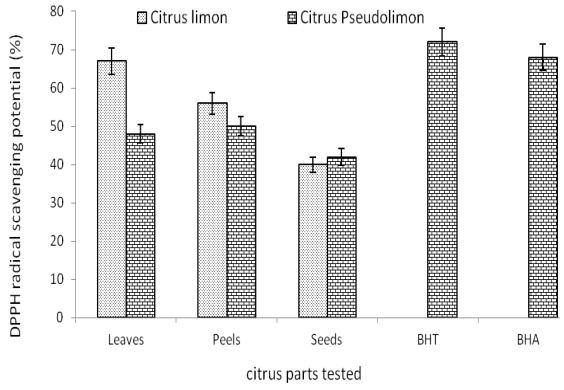

Figure 2. DPPH radical scavenging activity of extracts from different parts of *Citrus* species as compared to BHT and BHA

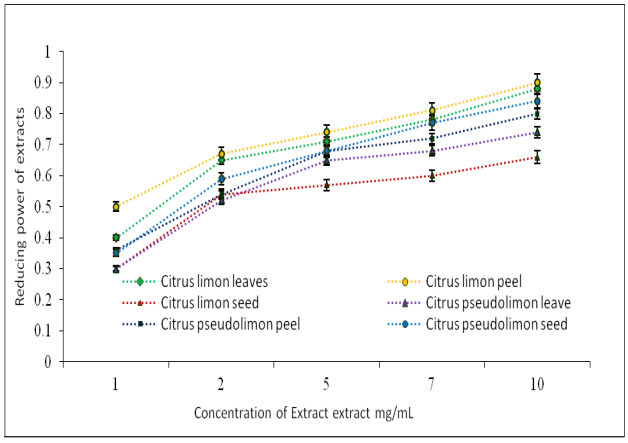

Figure 3. Comparison of reducing power of extracts from different fruit residue of *Citrus* species

inflammatory, anticancer and antioxidant properties. Citrus fruits are good source of antioxidants/ phenolic compounds that can play a beneficial and preventive role towards reducing different health disorders including aging, cardiovascular diseases, neurodegenerative diseases and cancer. Total flavonoid contents (TFC) were expressed as mg/g dry matter, measured as catechin equivalent. TFC determined in extracts of leaves, peels and seeds of *C. limon* and *C. pseudolimon* ranged from 19.95-39.60 mg CE /g dry matter. The maximum value of flavonoids (39.60 mg) was obtained for *C. pseudolimon* leaves while the minimum (19.95 mg) in *C. limon* seeds extracts. The present flavonoids contents (39.6 mg/ g) in *Citrus* leaves were higher than that reported by Kamran *et al*. (2009), who revealed that in general the total phenolic and flavonoid contents in citrus peel are higher as compared to other parts.

Furthermore, the presently determined total flavonoid contents in different parts of *Citrus* species were also found to be higher than those reported earlier by Someya *et al* (2002), Anagnostopoulou *et al*. (2006) and Sultana *et al.* (2008).

Linoleic acid peroxidation inhibition potential

It is well established that the antioxidant activity strongly correlates to the ability of extracts to prevent the linoleic acid peroxidation. Therefore, inhibition of linoleic acid oxidation was used to express the antioxidant activity of leave, peel and seeds extracts of *C. limon* and *C. pseudolimon.* The oxidation of linoleic acid was observed to be concentration dependent ($p<0.05$) as depicted in the Figure 1. All the extracts showed considerable percent inhibition of linoleic acid ranging from 31-60%. The methanolic extract of *C. pseudolimon* leaves showed the highest inhibition of peroxidation (60%) thus reflecting greater antioxidant potential while the lowest (31%) was observed in the case of *C. pseudolimon* seed extract. The values of inhibition of linoleic acid oxidation determined in the present investigation were correlated with their total phenolic contents and higher than those in some lichen species (5.27-26.43%) reported by Odabasoglu *et al.* (2005) and citrus species by Kamran *et al.* (2009).

DPPH radical scavenging activity

DPPH radical, a stable organic free radical, with deep violet color, which showed absorption maxima within the range of 515-518 nm, was used to assess the antioxidant activity of different parts of *Citrus* species (*C. limon* and *C. pseudolimon*). When DPPH radical absorbs proton denoted by extract it becomes yellow and this color change is proportional to proton denoting or antioxidant potential of the extract. Higher the concentration of phenolics compounds, or the degree of hydroxylation of the phenolics compounds larger will be the DPPH radical scavenging activity and vice versa (Roginsky and Lissi, 2005). The free radical scavenging activity of the extracts from leaves, peels and seeds of two *Citrus* species (*C. limon* and *C. pseudolimon*) ranged from 39.98-64.38%. The highest DPPH radical scavenging activity (64.38%)

was achieved for *C. limon* leaves extract while the lowest (39.98%) in the case of *C. limon* seeds extract as shown in Figure 2. The values of DPPH scavenging activities found in the present study were higher than those in wild edible mushrooms ranging 8.52-40.2% from northeast Portugal (Ferreira *et al*., 2007) whereas, lower than those of four *Citrus* species ranging 38.65-87.63% (Ghafar *et al.* 2010).

Reducing power

The reducing power (RP) of a typical compound can be linked to its ability to transfer electrons and is therefore taken as a good indicator of the antioxidant activity/potential. The presence of reductones is detected by the breakdown of free radical chain caused by hydrogen atoms donated. In this assay the presence of reductones in the antioxidant sample reduces Fe^{3+}/ferricyanide complex to Fe^{2+}/ferrous ions with a change in color from yellow to bluish green.

The intensity of color depends on the reducing potential of the compound present in the medium. A greater intensity of the color is associated with greater absorption value and hence higher the antioxidant activity (AA). The RP of the extracts generally increases with the increases in the concentration/ amount (Yen *et al*., 2000; Zhou and Yu, 2006).

The RP of extracts derived from the leaves, peels and seeds of *C. limon* and *C. pseudolimon* at 10 mg/ mL concentration ranged from 0.63-0.88 (absorbance data). It was observed that the extract of *C. limon* leaves had highest 0.88 reducing power while that of *C. limon* seeds expressed the lowest 0.63 (Figure 3). At the tested concentration (10 mg/mL), the reducing effect of different parts were significantly ($p<0.05$) varied between *Citrus* species tested. The values of the reducing power obtained from the present investigation were found to be higher than those documented by Hashempour *et al.* (2013) in six *citrus* species juices and some lichen species (Odabasoglu *et al.*, 2005) however, lower than those found in pomegranate marc by Qu *et al* (2010). The antioxidant activity exhibited by different parts of *Citrus* species analyzed can be linked to the presence of considerable amounts of phenolics detected such as flavone glycosides, flavonones, hydroxy cinnamate, polymethoxylated flavones, and some diverse phenolic glycosides as well as amines (Someya *et al.*, 2002; Ziaur-Rehman, 2006).

Conclusions

The findings of this study explored that leave extracts from the tested *Citrus* species contained higher amounts of TP and TF as well as superior antioxidant potential. Of the species selected, *C. pseudolimon* exhibited better antioxidant activity which might be in due part to the occurrence (distribution) of greater levels of phenolic and flavonoids in this species. Besides, it was further indicated that phenolics and antioxidant activity was varied notably among parts of the species tested.

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