

ANTIOXIDANT POTENTIAL OF PEEL ESSENTIAL OILS OF THREE PAKISTANI CITRUS SPECIES: *CITRUS RETICULATA*, *CITRUS SINENSIS* AND *CITRUS PARADISII*

GHULAM MUSTAFA KAMAL¹, MUHAMMAD YASIN ASHRAF^{1*}, ABDULLAH IJAZ HUSSAIN²
AND LEEB SHAHZADI³ AND MUHAMMAD ISMAIL CHUGHTAI¹

²Institute of Chemistry, GC University, Faisalabad, Pakistan

¹Nuclear Institute for Agriculture and Biology, P.O. Box 128 Faisalabad, Pakistan

³Department of Medical Pharmacology, Cerrahpasa, Istanbul University, Turkey

*Corresponding author e-mail: niabmyashraf@gmail.com

Abstract

Citrus peel essential oils are a very important source of natural antioxidants. In the present study the essential oils isolated from peels of three Citrus species namely *Citrus reticulata*, *Citrus sinensis* and *Citrus paradisi* were studied for their antioxidant potential. The hydro-distilled essential oil content from peels of *C. reticulata*, *C. sinensis* and *C. paradisi* were 0.30, 0.24 and 0.20 g/100g, respectively. The maximum amount of essential oil was found in *C. reticulata* while the minimum in *C. paradisi* peel samples. The essential oils isolated by hydro distillation were characterized using GC and GC/MS. The antioxidant activity of the investigated essential oils was evaluated by testing their ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical, percent inhibition of linoleic acid peroxidation and bleaching of β -carotene in linoleic acid system. A significant difference was noticed in the antioxidant activities of the studied essential oils.

Introduction

Synthesized antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, and tertiary butylhydroquinone, are very commonly used in food items to cut short the lipid peroxidation. However, the foods supplemented with these synthetic antioxidants are not promoted due to their toxic effects (Buxiang & Fukuhara, 1997) and carcinogenicity (Hirose *et al.*, 1998). Therefore, some essential oils and their components have got much importance due to their antioxidant potentials as alternatives to the artificially synthesized antioxidants without showing any secondary effects (Carson & Rilly, 2003).

Family *Rutaceae* consists of about 160 genera and citrus is the most important genus of it. Genus citrus includes several fruits of high economic importance like mandarins, oranges, limes, lemons and grapefruits. Citrus are mostly grown in regions with temperate summers and mild winters, particularly in Mediterranean countries like Brazil, Japan, Argentina, USA and Australia (Singh *et al.*, 1983; Anwar *et al.*, 2008). Citrus fruit yield in Pakistan is 9.5 tons ha⁻¹ and 1.28 million tons per season (Balal *et al.*, 2011). The average yield in Pakistan is far below than other citrus producing countries like Brazil where it is 40 to 60 tons ha⁻¹ (Ibrahim *et al.*, 2011; Ashraf *et al.*, 2012). Pakistan with respect to annual production of citrus fruits stands among the ten top citrus producing countries of the world (Mahmood, 2005; Khan, 2005; Ashraf *et al.*, 2013).

Citrus peels which are considered as agro industrial waste are potential source of plant secondary metabolites in the form of essential oils (Andrea *et al.*, 2003). Citrus peel essential oils have a wide range of potential activities in food, perfumery, sanitary, cosmetics and pharmaceuticals (Mondello *et al.*, 2005). The most important applications of citrus peel essential oils is the presence of some bioactive compounds in them which serve as alternatives to the synthetic antioxidants (Tepe *et al.*, 2006; Viuda-Martos *et al.*, 2008; Choi *et al.*, 2000).

Therefore, the main objective of the present study was to evaluate the antioxidant property and free radical scavenging capacity of essential oils from the peel of *C. reticulata*, *C. sinensis* and *C. paradisi*. Furthermore; the selected essential oils were also characterized quantitatively by GC/MS.

Materials and Methods

Plant materials: Fully ripened fresh fruits of three citrus species (*C. reticulata*, *C. sinensis* and *C. paradisi*) were collected from citrus orchards of Nuclear Institute for Agriculture and Biology (NIAB), Faisalabad, Pakistan. The fruits were then peeled carefully with the help of a sharp knife to avoid damage of oil glands.

Isolation of the essential oil: The citrus peels were subjected to hydro-distillation for 3 h using a Clevenger-type apparatus. Distillates of essential oils were dried over anhydrous sodium sulfate, filtered and stored at -4°C until analyzed.

Analysis of the essential oil

Physical analysis: The refractive index and density of citrus peel essential oils were determined following standard methods (Guenther, 1964). A digital refractometer RX-7000 α (Atago Co. Ltd., Tokyo, Japan) was used for the determination of refractive index of oils.

Gas chromatography/mass spectrometry analysis: GC-MS analysis of the essential oils was performed using an Agilent-Technologies (Little Falls, California, USA) 6890N Network gas chromatographic (GC) system, equipped with an Agilent-Technologies 5975 inert XL Mass selective detector and Agilent-Technologies 7683B series auto injector. Compounds were separated on HP-5 MS capillary column (30 m x 0.25 mm, film thickness 0.25 μ m; Little Falls, CA, USA). A sample of 1.0 μ L was

injected in the split mode with split ratio 1:100. For GC/MS detection, an electron ionization system, with ionization energy of 70 eV, was used. Column oven temperature was programmed from 80°C to 220°C at the rate of 4°C min⁻¹; initial and final temperatures were held for 3 and 10 minutes, respectively. Helium was used as a carrier gas at a flow rate of 1.5 mL min⁻¹. Mass range was 50-550 *m/z* while injector and MS transfer line temperatures were set at 220 and 290°C, respectively. All quantification was done by a built-in data-handling program provided by the manufacturer of the gas chromatograph (Perkin-Elmer, Norwalk, CT, USA). The composition was reported as a relative percentage of the total peak area.

Compounds identification: The identification of the oil constituents was based on a comparison of their retention indices relative to (C₉-C₂₄) *n*-alkanes either with those of published data or with authentic compounds (Anwar *et al.*, 2008). Compounds were also identified using their MS data compared to those from the NIST mass spectral library and published mass spectra (Adam, 2001).

Antioxidant activity of essential oils

DPPH radical scavenging assay: The antioxidant activity of the citrus peel essential oils and the major components was assessed by measuring their scavenging ability to 2, 2'-diphenyl-1-picrylhydrazyl stable radicals (DPPH). The DPPH assay was performed as described by Mimica-Dukic *et al.*, (2004) and Bozin *et al.*, (2006). The samples (100.0 µg mL⁻¹) were mixed with 1 mL of 90 µM DPPH solution and made up with 95% MeOH, to a final volume of 4 mL. Synthetic antioxidant, BHT was used as control. After 1h incubation period at room temperature, the absorbance was recorded at 515 nm using spectrophotometer (U-2001, model 121-0032 Hitachi, Tokyo, Japan). Percent radical scavenging activity was calculated in the following way:

$$RS (\%) = 100 \times (A_{\text{blank}} - A_{\text{sample}} / A_{\text{blank}})$$

Where *A*_{blank} is the absorbance of the control reaction (containing all reagents except the test compounds), and *A*_{sample} is the absorbance of the test compounds.

Percent inhibition in linoleic acid system: The antioxidant activity of citrus peel essential oils was determined in terms of measurement of % inhibition of peroxidation in linoleic acid system following the method described by Mata *et al.*, (2007). Essential oils (5 mg) were added to a solution mixture of linoleic acid (0.13 ml), 99.8% ethanol (10 ml) and 10 ml of 0.2 M sodium phosphate buffer (pH 7). Total mixture was diluted to 25 ml with distilled water. The solution was incubated at 40°C for 175 h. The extent of oxidation was measured by peroxide value using the colorimetric method as described by Yen *et al.*, (2000). To 0.2 ml sample solution, 10 ml of ethanol (75%), 0.2 ml of an aqueous solution of

ammonium thiocyanate (30%) and 0.2 ml of ferrous chloride solution (20 mM in 3.5% HCl) were added sequentially. After 3 min of stirring, the absorbance was measured at 500 nm using spectrophotometer (U-2001, Hitachi instruments Inc., Tokyo, Japan). A control was performed with linoleic acid but without essential oils. Butylated hydroxytoluene (BHT) was used as positive control. Inhibition of linoleic acid oxidation expressed as percent was calculated as follows:

$$\% \text{ inhibition of linoleic acid oxidation} = 100 - [(Abs. \text{ increase of sample at 175h} / Abs. \text{ increase of control at 175h}) \times 100]$$

Bleachability of β-carotene in linoleic acid system:

Antioxidant activity of the *C. sinensis*, *C. reticulata* and *C. paradisi* essential oils was assessed by bleaching of β-carotene/linoleic acid emulsion system as described by Hussain *et al.*, (2008). A stock solution of β-carotene linoleic acid mixture was prepared by dissolving 0.1 mg β-carotene, 20 mg linoleic acid and 100 mg Tween 40 in 1.0 mL of chloroform (HPLC grade). The chloroform was removed under vacuum in rotary evaporator at 50°C. Then, 50 mL of distilled water saturated with oxygen (30 min, 100 mL/min) was added and mixture was shaken. A 5.0 mL of this reaction mixture was dispensed to test tubes containing 200 µL of the essential oil prepared at 4.0 g/L concentrations and the absorbance at *t* = 0 measured at 490 nm against a blank, consisting of an emulsion without β-carotene. Then emulsion was incubated for 50 h at room temperature and the absorbance was recorded at different time intervals. The same procedure was applied for BHT and blank. The values thus obtained were then plotted against BHT to estimate the extent of antioxidant activities of essential oils.

Results and Discussion

Yield, physico-chemical analysis of citrus essential oils: The yield (g/100g), physical properties and major chemical constituents of *C. reticulata*, *C. sinensis* and *C. paradisi* are summarized in Table 1. A significant variation among the yield of essential oil from citrus species was noted. The maximum oil yield was observed in *C. reticulata* (0.30%) while minimum in *C. paradisi* (0.20%) while 0.24% oil yield was found in *C. sinensis*. The results drawn in present study are in accordance with the findings of Tue *et al.*, (2002), who observed in his studies that the yield of citrus essential oils was differing with individual plant species ranging in most of the cases from 0.2-2.0%. The results of density and refractive indices of essential oils of *C. reticulata*, *C. sinensis* and *C. paradisi* are also given in table 1. There were no significant variations observed in density and refractive index with respect to citrus species.

Chemical analysis of the essential oils: The chemical analysis of peel essential oils of *C. reticulata*, *C. sinensis* and *C. paradisi* is presented in Table 1. The data from GC and GC-MS showed a significant variation among number and types of compounds. The major compounds (>1%) identified are listed in Table 1.

Table 1. Physico-chemical characterization (\pm SD) of selected Citrus essential oils.

Species/ common name	Essential oil yield (g/100g)	Refractive index (25°C)	Density (g cm ⁻³) (25°C)	Major components identified >1%
<i>Citrus reticulata</i> (Kinnow)	0.30 \pm 0.01 ^c	1.4596 \pm 0.03	0.834 \pm 0.02	β -myrcene, Limonene, α -terpinene, Linalool, α -terpineol, Decanal, Z-carveol, Isopropyl cresol, β -caryophyllene, Germacarene-D, γ -munrolene, Farnesol and Nootkatone
<i>Citrus sinensis</i> (Musammi)	0.24 \pm 0.01 ^b	1.4631 \pm 0.03	0.815 \pm 0.02	β -myrcene, Limonene, Linalool, Decanal and Valencene
<i>Citrus paradisi</i> (Grape fruit)	0.20 \pm 0.01 ^a	1.4634 \pm 0.03	0.831 \pm 0.02	β -myrcene, Limonene, Linalool oxide, Linalool, Decanal, Citronellol, Z-carveol, Citral, β -caryophyllene, Valencene Δ -cadinene, Farnesol and Nootkatone

Mean with different superscript alphabets differ significantly at $p < 0.05$

β -myrcene, limonene, α -terpinene, linalool, α -terpineol, decanal, Z-carveol, isopropyl cresol, β -caryophyllene, germacarene-D, γ -munrolene, farnesol and nootkatone were the major constituents (>1%) identified in *Citrus reticulata* (Kinnow). Similarly β -myrcene, limonene, linalool, decanal and valencene were the major compounds (>1%) identified in *Citrus sinensis* (Musammi) peel essential oil while β -myrcene, limonene, linalool oxide, linalool, decanal, citronellol, Z-carveol, citral, β -caryophyllene, valencene Δ -cadinene, farnesol and nootkatone were present as major constituents (>1%) in *Citrus paradisi* (Grape fruit).

In literature some reports were found on the composition of citrus peel essential oils all over the world. Lota *et al.*, (2001b) found two major monoterpenes: limonene and γ -terpinene in peel essential oil of *Citrus reticulata* (mandarin). Limonene was also found to be the major component in peel oils of commercial Brazilian Murcot Tangerines by Feger *et al.*, (2003). Limonene and citral are the major components contributing to tangerine peel oil composition (Anonymous, 2004). Choi and Sawanura (2000) reported limonene (80.35-82.39%), α -terpinene (7.71-9.03%), myrcene (2.11-2.28%), linalool (1.37-2.01%), and α -pinene (1.17-1.43%), the most abundant components in Hyuganatsu oils. The results in the present work are also in close agreement with the findings of Gancel *et al.*, (2003) who worked on the chemical composition of *Citrus paradisi* oils. Vekari *et al.*, (2002) reported that the main components of these oils were limonene, β -pinene, myrcene, neral, geranial, neryl acetate and β -caryophyllene. Lota *et al.*, (2001a) found that limonene and α -pinene were majorly distinguished for peel oils of sour orange. Ahmad *et al.*, (2006) isolated essential oils from the peels of Malta (*C. sinensis*), Mousami (*C. sinensis*), Grapefruit (*C. paradisi*) and Eureka lemon (*C. limon*) through cold pressing method. Maximum oil yield (1.21%) was obtained from Malta peel followed by Eureka lemon (1.12%), Mousami (0.98%) and Grape fruit (0.73%). According to them the main constituents separated in Malta peel oil were limonene (61.08%), citronellol (4.18%), citral (7.74%),

borneol (7.63%), α -terpinolene (2.06%) and linalool (1.28%). In Mousami, the principal compounds were limonene (76.28%), α -pinene (1.26%), β -pinene (5.45%), citral (1.74%), and linalool (2.32%). In Grapefruit peel oil, limonene (86.27%), myrcene (6.28%), γ -terpinene (2.11%) and α -pinene (1.26%), were among the principal components. Major constituents present in Eureka lemon oil were limonene (53.61%), α -terpinene (18.57%), α -pinene (11.80%), myrcene (11.16%) and β -pinene (2.63%). Chemical composition of essential oils of these species varied significantly, which may be due to the difference in their genetic makeup.

Antioxidant activities of peel essential oils: The *in vitro* antioxidant activity of the extracted Citrus peel essential oils was assessed by three different *In vitro* assays: the DPPH radical scavenging activity, bleachability of β -carotene and percent inhibition in oxidation of linoleic acid system. Free radical-scavenging capacities of the oils were measured by the DPPH assay and the results are given in Table 2. In the DPPH assay, the ability of the examined essential oils to act as donor of hydrogen atoms or electrons in transformation of DPPH into its reduced form DPPH-H was investigated. The examined Citrus essential oils were able to reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H. Essential oil obtained from *Citrus reticulata* showed highest radical-scavenging activity while *Citrus sinensis* showed the least radical scavenging activity.

Table 2. Percent radical scavenging activity (\pm SD) of citrus essential oils.

Essential oils	Percent radical scavenging activity
<i>Citrus reticulata</i>	24.08 \pm 0.48 ^c
<i>Citrus sinensis</i>	14.05 \pm 0.28 ^a
<i>Citrus paradisi</i>	18.47 \pm 0.55 ^b
BHT	87.30 \pm 1.20 ^a

Mean with different superscript alphabets differ significantly at $p < 0.05$

When DPPH scavenging activity of citrus peel essential oils was compared with synthetic antioxidant BHT (Table 2), all the oils showed significantly lower antioxidant activity. *C. reticulata*, *C. sinensis* and *C. paradisi* showed 24.08, 14.05 and 18.47% DPPH inhibition while BHT exhibited 87.30% activity. These results are in agreement with the poor performance given by other oils with similar patterns and by single monoterpene hydrocarbons (Ruberto & Baratta, 2000).

Level of % inhibition of linoleic acid oxidation as exhibited by the citrus peel essential oils is also shown in Fig. 1. The higher absorbance for iron based peroxides indicates the high concentration of peroxides formed

during reaction, and low antioxidant activity. The assessed citrus essential oils inhibited the oxidation of linoleic acid by 54.98%-67.80%. *Citrus sinensis* showed the highest antioxidant activity (67.80%) in linoleic acid system followed by *Citrus paradisi* (57.12%) and *Citrus reticulata* (54.98%), respectively. Synthetic antioxidant BHT exhibited 90.30% of percent inhibition significantly higher than the essential oils. Singh and Marimuthu (2006) found that essential oils effectively suppress the peroxide formation in linoleic acid system during incubation. The antioxidant activity of citrus essential oils might be attributed to the presence of phenolic compounds in them (Lu & Foo, 2001).

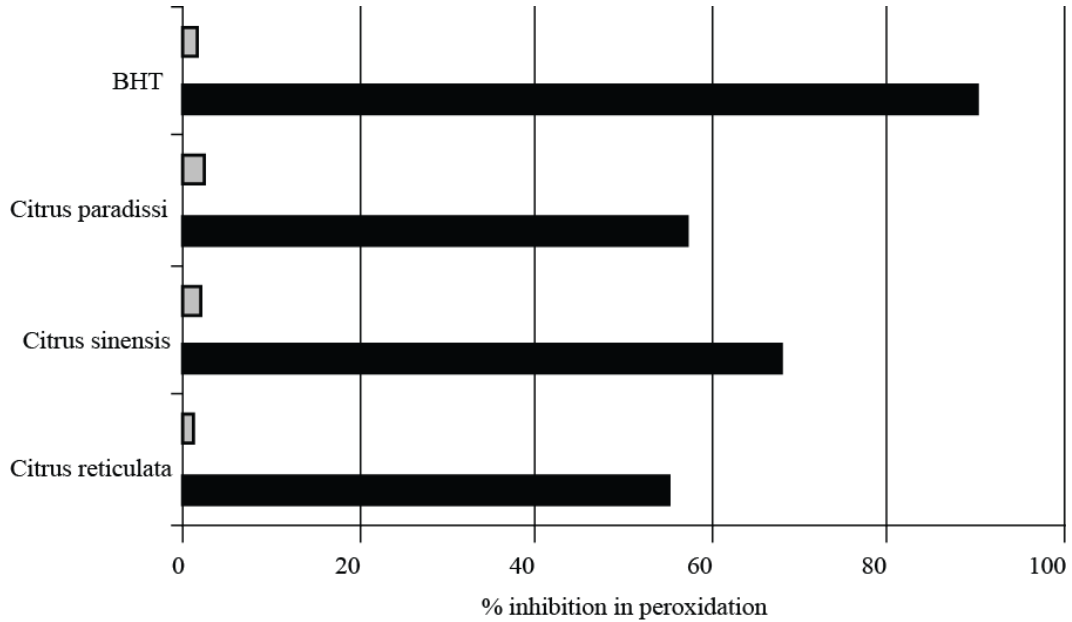


Fig. 1. Percent inhibition in linoleic acid peroxidation.

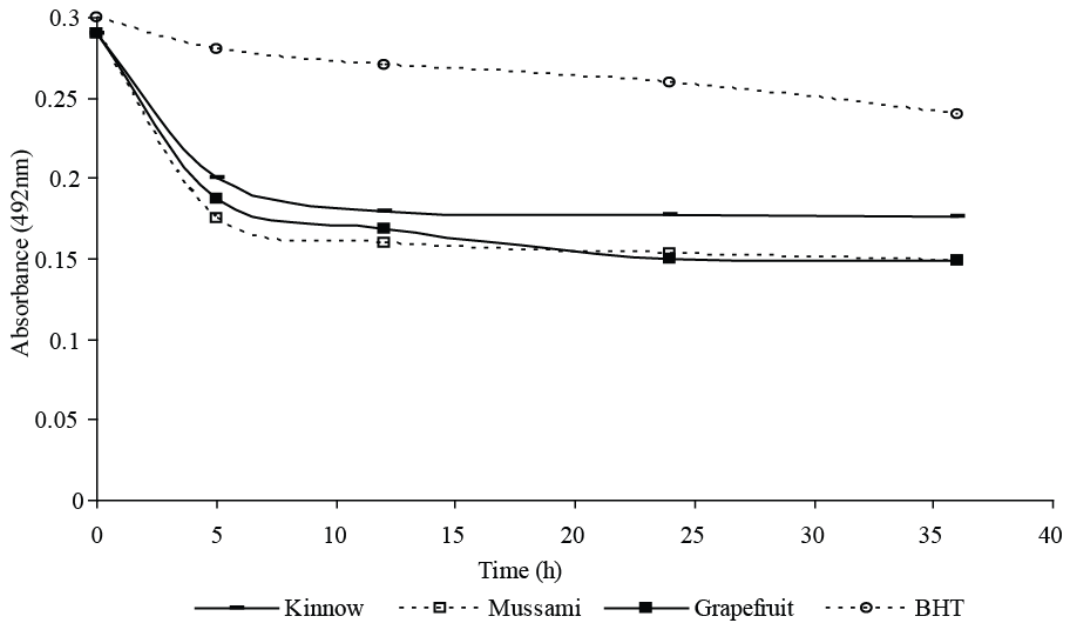


Fig. 2. Bleaching of beta-carotene in Linoleic acid system showing A.A. of essential oils.

Bleaching of β -carotene in linoleic acid system as antioxidant activity of Citrus essential oils is presented in Fig. 2. The greater the effectiveness of an antioxidant, the slower will be the color depletion. The smaller decrease in absorbance of β -carotene, indicates a lower rate of oxidation of linoleic acid and higher antioxidant activity in the presence of citrus essential oils.

Control showed the highest rate of color depletion and the least antioxidant activity. Essential oils of *C. reticulata* (Kinnow) exhibited better antioxidant activity than *C. sinensis* and *C. paradisii*. Based on these results, order of antioxidant activity of citrus peel essential oils was as follows: BHT > *Citrus reticulata* > *Citrus paradisii* \geq *Citrus sinensis*. Overall, results of β -carotene bleaching were better than those provided by the radical-scavenging activity. These results are comparable to the findings of Matta *et al.*, 2007, who worked on the antioxidant activity of essential oil and extract from different Portuguese food plants and reported the order of antioxidant activity as BHT > ethanol > extract > essential oil.

Conclusion

Based on results, it may be concluded that the differences in antioxidant activities of peel essential oils of different citrus species under investigation may be due to difference in their chemical compositions which in turn depends upon their unique genetic makeup.

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