Evaluation of Antioxidant Potential of Citrus Peel Extracts

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Summary: The antioxidant potential of different solvent extracts of three different locally grown citrus varieties; grape fruit, lemon and mussambi, was assessed using some antioxidant assays like estimation of total phenolic contents (TPC), total flavonoids contents (TFC), percentage inhibition of linoleic acid oxidation and DPPH free radical scavenging capacity. The yield of extracts was found in the range of 17.92–30.8%. TPC, TFC, percent inhibition of linoleic acid oxidation and DPPH radical scavenging capacity of different citrus peel extracts were found in range of 2.72 – 3.77 g/100g as Gallic Acid Equivalent (GAE), 2.20–2.98 g/100g as Catechine Equivalent (CE), 68.20 – 91.78% and 19.53 – 41.88 mg/mL, respectively. Statistical analysis showed significant ($p < 0.05$) variations in the yield and antioxidant potentials of the extracts with respect to different species and solvent systems. From the results it is reasonable to say that methanolic extracts of citrus peels have exhibited varying degree of antioxidant potentials.

Introduction

The main characteristic of an antioxidant is its ability to trap or inhibit free radicals. Antioxidant compounds like polyphenols, phenolic acids and flavonoids scavenge free radicals and thus inhibit the oxidative process that lead to degenerative diseases such as atherosclerosis, stroke, diabetes, Alzheimer's disease, and cancer [1, 2]. From the last two-three decades, antioxidant based drugs/formulations are being mostly consumed for the prevention and treatment of these diseases [3].

Synthetic antioxidants are prohibited to use in food because they are suggested to be carcinogenic and very dangerous for human health [4, 5]. Some advanced countries, such as Japan and Europe, have put a strict ban on the use of tertiary butyl hydroquinone (TBHQ), the most effective synthetic antioxidant, and some other countries may ban the use of synthetic antioxidant in the future due to safety concerns [6]. This has encouraged the research scientists to utilize their potential to explore the plant based natural antioxidants to cope the existing challenges. Plant based natural antioxidants like vitamin C, vitamin E, carotenes, polyphenols, glycosides, tocopherols, phenolic acids, phytate and phytoestrogens have been recognized [7-9] that they have the ability to reduce the risk of diseases because of their anticarcinogenic potential and other health promoting effects [1, 10]. Over the past decade, there has been an increased interest in phytochemicals and the importance of antioxidants for the purpose of human health [11].

Pakistan has a status of the leading citrus producer in the world. The citrus peel and other agro wastes have not still received much attention to recycle for the purpose of antioxidants instead of being discharged or disposed due to the lack of commercial application of these wastes [12]. Isolation of antioxidants from citrus peels are of great interest to the food industry as they can retard oxidative damages in food and thereby improve the quality and nutritional value of food. It is reported that intake of orange is very favourable to reduce the incidence of asthma. Citrus fruit extracts have shown a very good antioxidant, antitumor and blood clot inhibition activities [13].

Agro waste like citrus peels is a byproduct of beverages and food industries and considered as a waste product and usually discarded. Therefore, the present research work was carried out to evaluate the antioxidant potential in citrus fruit peel extracts as an effort to utilize them as a substitute of synthetic antioxidants.

Results and Discussion

Yield of Extracts

The data regarding the yield of extracts with different solvents is presented in Fig. 1. The yield of citrus peel extracts was found in the range of 17.92–30.80 % on the basis of dry weight of extracted material. The maximum yield of extracts (30.80%) was obtained from grape fruit peel with 70% aqueous

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methanol and the minimum (17.92 %) from lemon peel with 90% aqueous methanol. The overall yield of citrus peel extracts and the extraction efficiency of different solvents was observed in the following decreasing order; grape fruit > mussambi > lemon in 70, 80 and 90% methanol-water blends. The efficient extraction in 70% methanol might be attributed to the polarity of solvent as the most of the plant based antioxidants are polar in nature. The statistical analysis showed that percentage yield of extracts was found to vary significantly ($p < 0.05$) among the species and solvent systems. The findings of present work regarding the yield of extracts were found in close agreement with the findings of Zia-ur-Rehman [14] and Sultana *et al.* [15] who reported 19.87% and 21.5% extract yield from citrus peels, respectively. Methanol is generally employed for the extraction of antioxidants components from plant materials due to their polarity and good solubility with many antioxidant components [16, 17]. Another reason is that the effectiveness and ability of methanol is increased by using water as a co-solvent especially when extraction of the antioxidants of various kinds is essential.

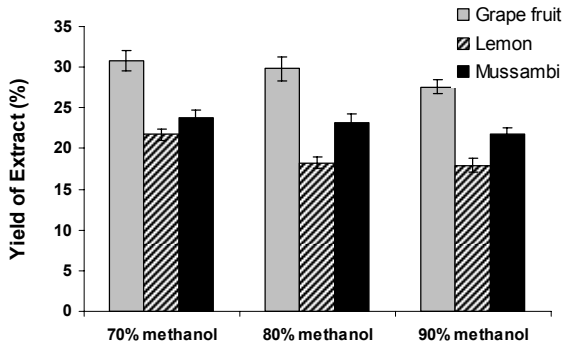

Fig. 1: Yield of extracts (g/100g of dry weight) of different cultivars

Total Phenolics Contents (TPC)

Total phenolic contents (TPC) of citrus peels extracts were determined by using Folin-Ciocalteu method due to its high sensitivity, lower interference and quickness to quantify the phenolics as compared to other competitive test [18]. Although exact reaction of the reagent with reducing species is not known but it is considered that a complex is formed between phospho-molybdic tungstate and reducing species, phenolic ion, changing color from yellow to blue where absorbance at 755nm is measured [19]. The data regarding the TPC (g /100 gm of dry weight as GAE) of extracts from different species is presented in Fig. 2. It was observed that TPC of extracts from different species of citrus peel and solvents were found in the range of 2.72–3.77 g/100g measured as gallic acid equivalent (GAE). The maximum amount of total phenolic contents (3.77 g/100g) were obtained from mussambi fruit peel extract with 70% methanol and minimum 2.72 g/100g from grape fruit peel extract by using 90% methanol. Overall, TPC of mussambi, lemon and grape fruits peels were found to be 3.26–3.77 g/100g GAE, 3.01–3.59 g/100g GAE and 2.72–3.13 g/100g GAE from 70, 80 and 90% methanol, respectively. In the present analysis 70% methanol was an efficient solvent. The amount of TPC among these different varieties of citrus peels and the efficiency of solvent systems was observed as follows; mussambi > lemon > grape fruit and 70% methanol > 80% methanol > 90% methanol. These variations might be attributed to different citrus varieties and also the polarity of solvents. The statistical analysis revealed that significant ($p < 0.05$) variations were found in phenolic contents of citrus peel extracts. The present findings regarding TPC were found in close agreement with those (1.7 g/100g GAE) reported for sweet orange peels [20]. Our results are also comparable with the finding of Sultana *et al.* [15] who reported the 2.4 g/100g GAE total phenolic contents in the methanol extracts of citrus peel.

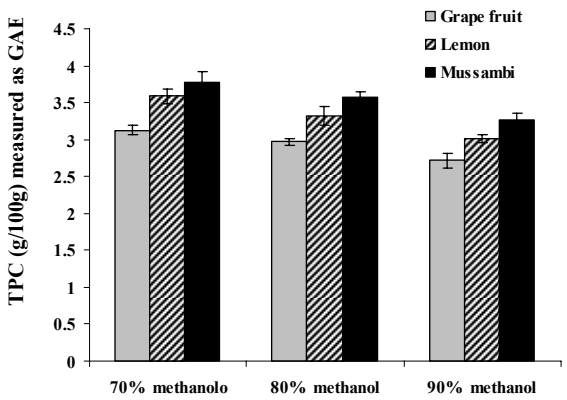

Fig. 2: Total phenolic contents (g/100g measured as GAE) of different extracts.

Total Flavonoid Contents (TFC)

The data regarding TFC of extracts from different peels is presented in Fig. 3. It was observed that TFC of extracts from different citrus peels varied significantly and found in the range of 2.20−2.98 g/ 100g CE. Overall the highest TF contents was found in mussambi fruit peel extract (2.98 g /100 g CE) followed by lemon peel extract (2.55 g /100 g CE) and grape fruit peel extract (2.20 g /100 g CE). The maximum TFC (2.98 g /100 g CE) was obtained from

mussambi fruit peels with 90% methanol and lowest (2.20 g /100 g CE) from grape fruit peel with 70% methanol. In the present analysis 90% methanol was efficient solvent. The statistical analysis showed that TFC of all the species were found to be varied significantly ($p < 0.05$) among the species and solvent systems. Our results were comparable with TFC 1.92 g/100g CE reported for citrus peels by Sultana *et al.* [15]. A significant change in the total flavonoid contents among different varieties of citrus fruit peels might be attributed to factors such as natural chemical composition, maturity at harvest, soil state and conditions of post harvest storage [19]. However, a good correlation of total flavonoid was observed in the present analysis.

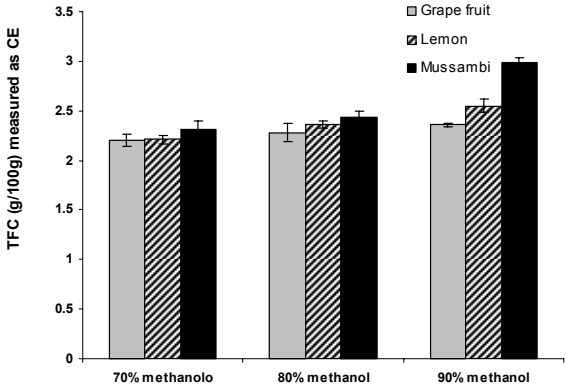

Fig. 3: Total flavonoid contents (g/100g measured as CE) of different extracts.

Percentage Inhibition of Linoleic Acid Oxidation

The antioxidant activity in terms of ability of extracts to prevent the linoleic acid oxidation is a very good parameter to assess the antioxidant capacity. Therefore, the antioxidant activity of citrus peel extracts was evaluated using this method as reported by Yen *et al.* [21]. The data regarding the percentage inhibition of linoleic oxidation with different citrus peels extracts is presented in Fig. 4. It was observed that % inhibition of extracts from different varieties of citrus peels varied widely and found in the range of 68.20−91.78%. The highest inhibition (91.78%) was obtained from the mussambi peel extract with 70% methanol and minimum inhibition (68.20%) from grape fruit peel extract with 90% methanol. The 70% methanol was found to be more efficient extracting solvent as compared to 80 and 90% methanol. The trends of inhibition with different citrus peels extracts was observed as mussambi > lemon > grape fruit. The statistical analysis showed that variation in the percentage inhibition of linoleic acid oxidation was found to be significant ($p < 0.05$) with respect to citrus peels samples and solvent systems. Our results were also comparable with BHT (synthetic antioxidant) i.e. 89.49 % and also were found to be comparable with reported by Sultana *et al.* [15] for citrus peels i.e. 86.9 %.

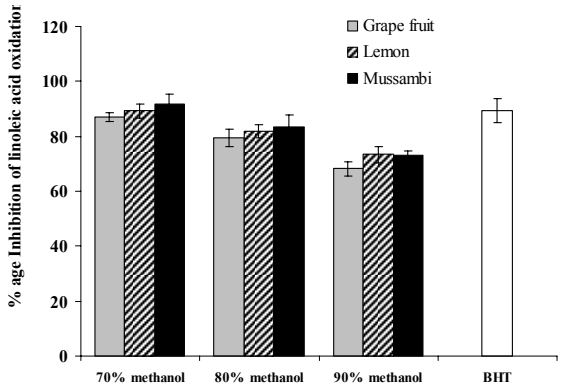

Fig. 4: Percent inhibition of linoleic acid oxidation by different extracts.

DPPH Free Radical Scavenging Activity

The DPPH radical is commonly used for the assessment of antioxidant activity. The DPPH scavenging activity of each sample was evaluated using the method of Roginsky and Lissi [22]. The data regarding the 50% inhibition (IC_{50} mg/mL) of extracts from different cultivars is presented in Fig. 5. Lower the IC_{50} value of extract, more effective it will be for the inhibition of DPPH. It was observed that IC_{50} value of extracts from different citrus peel extracts varied significantly and found in the range of 19.53–41.88 mg/mL. Overall, highest scavenging activity was achieved from the mussambi fruit peels extract because its IC_{50} value (19.53 mg/mL) was low and the less scavenging activity was obtained from the grape fruit peels extract because its IC_{50} value (41.88 mg/mL) was high as compared to other species. The 90% aqueous methanol was more efficient extracting solvent. The antioxidant activity of citrus peels extracts might be attributed to the presence of polyphenols, such as ellagic tannins, ellagic acid and gallic acid [23]. Citrus peels extracts exhibited good radical scavenging activity, which is comparable to the previous findings reported by Abrosca *et al.* [24] and Sultana *et al.* [15] for citrus peels i.e 21.8 IC_{50} ug/mL. The statistical analysis showed that scavenging activity of extracts of different verities of citrus peels was found to be varied significantly ($p < 0.05$) among the citrus fruit peels species and solvent systems.

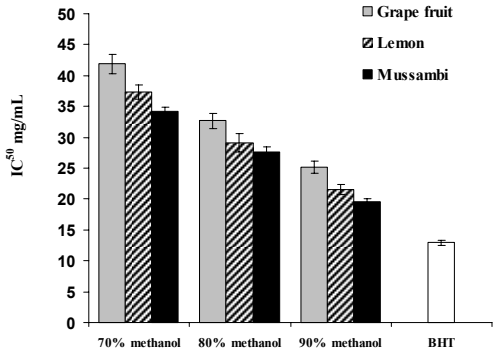

Fig. 5: DPPH free radical scavenging activity (IC_{50} mg/mL) of different extracts

Experimental

Chemicals and Reagents

All the reagents (Linoleic acid, Gallic acid, DPPH, Ethanol, Methanol, Folin-Ciocalteu reagent) used in research work were purchased from Sigma – Aldrich, UK. The analytical grade chemicals (Sodium carbonate Ammonium thiocyanate, Ferrous chloride, Butylated hydroxytoulene, Ascorbic acid, Catechin, Hydrochloric acid, Ferric chloride and Trichloroacetic acid were purchased from E. Merck, Germany.

Pretreatment and Storage of Samples

The fresh citrus peel samples of three locally grown varieties; grape fruit (*Citrus paradise*), lemon (*Citrus limon*) and mussambi (*Citrus sinensis*) were collected from a local juice industries, Faisalabad, Pakistan. The collected samples were authenticated in the research laboratory of Botany Department, G.C. University Faisalabad, Pakistan. Citrus peels samples were air dried till constant weight achieved. Dried samples of citrus peels were ground to semi powder using grinder (LG BL-999SP). The ground samples were stored in air tight polythene bags.

Preparation of Extracts

The antioxidant fractions of powdered citrus peels samples were extracted using three solvent systems; 70, 80 and 90% methanol blends with water. 20g of ground sample of citrus peels was taken in a 500 mL conical flask followed by the addition of 200 mL solvent and extraction was executed on orbital shaker for 12 hours at 120 rpm. The filtrate was separated from the solid residue by filtering through Whatman No. 1 filter paper and the solvent was removed under vacuum at 45 ^{o}C, using a vacuum rotary evaporator (Eyela, N-N Series, Rikakikai Co. Ltd). Viscous extracts were dried on water bath and were stored at –4 ^{0}C in refrigerator for further utilization in assays followed by the calculation of yield [25].

Determination of Total Phenolic Content (TPC)

The total phenolic contents were determined by using Folin-Ciocalteu reagent according to the method described by Chaovanalikit and Wrolstad [26]. Briefly, 50 mg of dry mass of extracts was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL deionized water. The mixture was kept at room temperature for 10 minutes, and then 1.5 mL of 20% sodium carbonate (w/v) was added. The mixture was heated in a water bath at 40 ^{o}C for 20 minutes and then cooled in an ice bath. Finally absorbance at 755 nm was measured with spectrophotometer (Hitachi U-2001 model 121-0032 Japan). Amounts of TPC were calculated using a calibration curve for Gallic acid (10-100 ppm). The results were expressed in g/100g as Gallic acid equivalents (GAE) on dry mass bases.

Determination of Total Flavonoid Content (TFC)

Total flavonoids were determined by the procedure reported by Dewanto *et al.* [27]. One milliliter of aqueous extract containing 0.01g/ mL of dry matter was placed in a 10 mL volumetric flask, then 5mL of distilled water added followed by 0.3 mL of 5% $NaNO_2$. After 5 min, 0.6 mL of 10% $AlCl_3$ was added. After another 5 min 2mL of 1M NaOH was added and volume made up with distilled water. After through mixing of contents, absorbance was measured at 510 nm using spectrophotometer. TFC amount was expressed in g/100g as catechin equivalents on dry mass bases.

Determination of Percentage Inhibition of Peroxidation in Linoleic Acid System

Antioxidant activity of extracts was determined by measuring inhibition of peroxidation of linoleic acid system following a method reported by Iqbal *et al.* [28]. Extracts of each treatment 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 having (p H = 7). Total mixture was diluted to 25 mL with distilled water. The degree of oxidation was measured following ammonium thiocyanate method [17]; with 10 mL of 75% ethanol, 0.2 mL of and aqueous solution of 30% ammonium thiocyanate, 0.2 mL of sample solution

and 0.2 mL of ferrous chloride ($FeCl_2$) solution (20 mm in 3.5% HCl) being added sequentially. After 3 minutes of stirring, the absorbance values of mixtures measured with spectrophotometer (Hitachi U-2001 model 121-0032 Japan) at 500 nm were taken as peroxide contents. A control was performed with linoleic acid but without extracts. Synthetic antioxidants, butylated hydroxytoluene (BHT) (200 ppm) were used as positive control. The system was incubated at 40 ^oC and maximum peroxidation level was observed after 360 hours (15 days) in the control (blank). Percent inhibition of linoleic acid was calculated by the following formula:

100 – [(Increase in A_{sample} at 360 h / Increase in A_{blank} at 360 h) × 100]

where A_{sample} is absorbance of sample and A_{blank} is absorbance of blank.

DPPH Radical Scavenging Assay

The antioxidant activity of the citrus peels extracts was assessed by measuring their scavenging abilities to 2, 2-diphenyl-1- picrylhydrazyl (DPPH) stable radical. The DPPH assay was performed as described by Bozin *et al.* [29]. The samples (from 0.2 to 500 mg/mL) were mixed with 1 mL of 90 mM DPPH solution and filled up with 95% Methanol, to a final volume of 4 mL. The absorbance of the resulting solutions and the blank were recorded after 1 hour at room temperature. Butylated hydroxytoulene (BHT) was used as a positive control. The disappearance of DPPH was studied at 515 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., and Tokyo, Japan). Scavenging of free radical DPPH in percent (%) was calculated in the following way.

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

where, A_{blank} is the absorbance of the control reaction mixture excluding the test compounds, and A_{sample} is the absorbance of the test compounds. IC_{50} (mg/ml) values, which represented the concentration of extracts that caused 50% neutralization of DPPH radicals, were calculated from the plot of inhibition percentage against concentration.

Statistical Analysis

All the experiments were conducted in three replicate and analyzed in triplicate. The results are presented as mean ± SD (standard deviation) of triplicate determinations. The data obtained was analyzed by a statistical technique ANOVA using Minitab Software.

Conclusion

Based on these results it could be concluded that methanol was considered as an efficient solvent for the extraction of antioxidant components from dry citrus peels. Statistical analysis revealed a significant difference among different solvent extracts of citrus peels analyzed. All the citrus peels extracts exhibited good antioxidant and radical scavenging potentials. However further research is recommended for the extraction, isolation and purification of bioactive constituents that might be utilized for the fortification of functional foods.

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