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Bioactive compounds assessment and antioxidant capacity of bitter orange

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Abstract

Citrus is the more important fruit crop produced and used up from all over world. *Citrus aurantium L* is the member of citrus genus Rutaceae. Citrus bioactive compounds prevent from oxidative damage and these compounds also possess their activity by rendering the chain reaction and constraining the lipid oxidation for preventing the oxidation damage. Flavones chalcones, flavan-3,4-diol and flavan-3-ols are biosynthetic origin classes of flavonoids and also end product of biosynthesis. On the base of molecular structure they are categories into eight classes that contains catechin, chalcones, is flavones, flavanones, flavanols and dihydroflavonols. Means values titratable acidity for bitter orange varieties presented in table obtained for V3 depicted the highest value of titratable acidity 4.01% and followed by V2 % was 4.93% while lowest was found in V1 4.82. Total soluble solid probably represent the sugars and mainly used to check the maturity of fruit. Total soluble solids of bitter orange varieties was shown in Figure 4.7. Results show that V3 has higher Total soluble solids contents 9.1 as compared to V1 8.1 and V2 8.5.

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Introduction

Citrus is the more important fruit crop produced and used up from all over world. *Citrus aurantium L* is the member of citrus genus Rutaceae. It has been distribute in tropic and subtropics of Asia especially in Pakistan. Pakistan is located in citrus belt hence citrus growing potential of this country highly prospective. Citrus fruit resided at the area of 199,000 hectares with total production of 200 thousand tones in Pakistan (FAO, 2014) and regarding to this data Pakistan ranked 21st in citrus production. Edible portion of Citrus aurantium contain 8-12% total soluble solid, out of which 76% are carbohydrates mainly sucrose, glucose, fructose and trace amount of other sugars. Organic acid mainly citric acid and malic acid less than 10% of TSS (Spiegel-Roy and Goldschmidt, 1996). Different studies investigate the role of this compound against various health disorders.

These bioactive compounds prove health benefits against different diseases such as antioxidant, anti-inflammatory, antimicrobial and heart diseases (Marin *et al.,* 2007). Citrus bioactive compounds also prevent from oxidative damage(Liu *et al.*,2000) and health-promoting effects of citrus. Antioxidants that are naturally present in citrus quench with free radicals and stop the chain reaction (Dorman *et al.,* 2003) and these compounds also possess their activity by rendering the chain reaction and constraining the lipid oxidation for preventing the oxidation damage (Huang *et al.,* 2005).Several studies reveal the source of antioxidants that may prove to be a potential barrier against degenerative disease such as cardiovascular and carcinogenic diseases. The antioxidant capacity of citrus increase with presence of higher concentration of phenol contents (Sawalha *et al.,* 2005).Flavonoids compound shown a diverse classification and they are divided into four major categories. Flavones chalcones, flavan-3,4-diol and flavan-3-ols are biosynthetic origin classes of flavonoids and also end product of biosynthesis (Cushnie and Lamb, 2005)while flavanols, flavones, anthocyanidins and proanthocyanidins are end products that are accumulates at the end of biosynthesis (Sannomiya *et al.,* 2005).On the base of molecular structure they are categories into eight classes that contains catechin, chalcones, isoflavones, flavanones, flavanols and dihydroflavonols (Tsuchiya, 2010).Isoflavones, anthocyanidins, catechin, flavanones and flavanols are the classes based on differentiation of heterocyclic ring C of flavonoids (Hollman and Katan, 1999) andflavanones and flavan-3-ol, anthocyanidins, proanthocyanidins are classes based on central pyran ring. Recent research mainly focus on the natural bioactive compounds and there antioxidant capacity of bitter orange.

Drugs that may use as a source of antioxidants promote limited effect and also cause a serious health consequences most of them are carcinogenic. In order to minimize the side effect of these synthetic drugsits need of time to replace the synthetic substance with natural substance.

Materials and methods

Collection of samples

The fruit was ripened, fresh and no sign of injury were purchased from National Agriculture Research Center (NARC) Islamabad. The sample were brought to the laboratory of Department of Food Technology Pir Mehr Ali Shah Arid Agriculture University Rawalpindi. Fruit was washed with tap water to remove dust and any other particles.

Physicochemical analysis

Physicochemical analysis including moisture, pH, Titrable Acidity, Total soluble solids were determined by AOAC (1990).

Preparation of extract

Five grams of dried powder was mixed with 50ml of methanol-water (80:20 v/v) at room temperature for 22 hrs using magnetic blender. Then, the extract was filtered through filter paper and residue was again taken up with 50 ml of acetone-water (70:30 v/v).

The volume of two obtained filtrates was mix and centrifuged for 5min and filter using Whatman No.1

paper. The obtained aqueous organic extract was concentrated and reduce pressure in rotary evaporation at 40°C until organic solvent completely evaporate.

Determination of Total Phenolic Content

Total phenolic compounds was determined by the Folin-Ciocalteau method (Ebrahimzadeh and N. Nabavi, 2009).

Antioxidant activity

DPPH radical scavenging assay

Free radical scavenging activity of sample was determined by using the stable radical 1, 1-diphenyl 1-2-picrylhydrazyl (DPPH) method (Dehpour *et al.,* 2009).

ABTS Assay

The ABTS (2, 20- azinobis (3-ethylbenzthiazoline -6-sulphonic acid) radial Cation decolorization assay was based on method describe by (Silva *et al*., 2013).

Determination of ascorbic acid

Ascorbic acid was determined by using AOAC (2000) official method No.967.21.

Flavonoids contents determination

Total flavonoids determined by using method described by (Park *et al.,* 2008).

Chromatographic evaluation for flavonoiods

HPLC chromatogram was used for the identification of flavonoids in peel and pulp of bitter orange varieties. Results were analyzed by matching the retention time of standard compounds.

Sample preparation

Take 10g of sample and extract was obtain by mixing the sample with 20ml methanol and 6ml of HCL 25%. Filter the sample in volumetric flask and dilute the sample with methanol raise volume up to 20ml. Mix the extract with methanol up to 100ml. Extract was filter through micro filter and transfer into flask. Filter the extract with micro filters and transfer into volumetric flask. Sample was injected into HPLC for further analysis (He *et al.,* 1996).

HPLC Determination of flavonoids

High performance liquid chromatography (HPLC) involving the specification of reverse phase column C18, Ultraviolet visible (SPD-10A) detector column oven (CTO-10AS), system controller (SCL-10A) and auto injection pump having model (SIL-10AS) was used to analyzed the sample. Wave length was detected at 270-280nm. Two solvent A (water to acetonitrile, (97:3 v/v) to solvent B (methanol) was used as mobile phase by dual pumping system gradient was formed. Compounds were determined by their corresponding retention time and curve making against standard (Rijke *et al.,* 2006).

Minerals contents

Sample was analyzed for following minerals Ca, Na and Mg by using atomic absorption and spectrophotometer (1990).

Statistical analysis

The results will be analyzed using standard statistical tools and procedures (Steel *et al.,* 1997*).*

Results and discussion

Physicochemical chemical analysis

Analysis of variance for moisture contents show significant difference among the varieties and parts. However, interactive effect of variety and parts depict the non-significantly difference (Table 4.1).

Means value for moisture in V3 (Martyfola) shown highest among all other (51.60%) and is followed by V1 (Amara) having means value of moisture content of 48.94% while lowest was (47.85%) observed in V2 (Bergamo rate). Means value of moisture contents in parts of varieties was significantly difference from each other. Highest value was found in pulp of which was (88.49%) due to present of more water content while peel show lowest value (10.43%). Higher value of moisture content in pulp due to presence of more water content and proximate components such as

crude protein crude fat, ash, crude fiber and carbohydrates was found in less amount.

Analysis of variance for pH shown significantly difference from each. Cooperative effect of pH on the varieties and parts also significantly difference from each other as described in (Table 4.3).

Means values is given in (Table 4.4) that variety V3 (Martyfola) possess the significantly high value of pH (2.84) as followed by V1 (3.78) while least value of pH was found in V2 (4.29). Means value of pH in parts (pulp and peel) demonstrated the significantly difference from one another. Means value of Pulp (P1) have significantly high (2.95) as compared to Peel (P2) which was (4.33). High value of pH in V3 is may be due to the presence of high acid content of citric acid as described by (Sarala *et al.,* 2011). Interaction between the varieties and parts shows significance difference. Means value was represent in Table 4.4. Analysis of variance for titratable acidity was given in Table 4.5. Result shown the significance difference between varities. Means values titratable acidity for bitter orange varieties presented in table obtained for V3 depicted the highest value of titratable acidity 4.01% and followed by V2 % was 4.93% while lowest was found in V1 4.82. Total soluble solid probably represent the sugars and mainly used to check the maturity of fruit. Total soluble solids of bitter orange varieties was shown in Figure 4.7. Results shows that V3 has higher Total soluble solids contents 9.1 as compared to V1 8.1 and V2 8.5.

Total phenolic contents of pulp and peel

Total phenolic compounds of three varieties V1, V2, V3 were determined by folin-ciolcalteu reagent . Phenolic contents of pulp extract of three varieties was described by gallic acid equalent and comparing with standerd curve of gallic acid.

Analysis of varience of Total phenolic contents was depicted in Table 4.9.

The results shows high significant effect (P>0.05) among the varieties and parts.

Means value of variety V3 (Martyfola) show higher amount of phenolic contents 225.06 mg/g as followed by V2 143.25mg/g and lowest phenolic content was found in V1 134.92 mg/g dry wt. Table 4.9. Mean values of parts of varities also shown significance difference of their means, pulp (P1) contain 207mg/g dry wt and peel (P2) contain 128.44mg/g dry wt.Interaction between the parts (pulp, peel) and varities in phenolics are not significanlty different from each other. Results shown in the Table 4.9 describe that peel (P2) of V3 show the highest content of total phenolic content 290.39 mg/g followed by peel of Higher amount of phenolic content present in citrus peel was also studied by Kamran *et al.,* 2009). Variation in phenolic contents may effected by different factor such sample prepartion, time, temperature, variety differenceand genetic factor(Rapisarda *et al.,* 1999). Among these three varieties pulp of V3 present highest amount of phenolic contents. Phenolic contents of citrus varieties possessing potential health benefits because they act as antioxidant and prevention many diseases.

Determination of antioxidant activity

DPPH R Adical scavenging activity of pulp and peel

DPPH is usually most steady radical that may use to pattern the ability of hydrogen ions of compounds and quenching the free radical ions. Citrus flavonoids has demonstrate the strong antioxidant activity and having potency to sharing the hydrogen atom to form stable radical intermediate.

Analysis of variance bitter orange varieties and parts are significantly subjective to DPPH radical scavenging Table 4.11. Means Value shows that highest DPPH radial scavenging was observed inV3 68.93%. It has been followed by V1 68.024V3 68.93% and V2 66.79 respectively. Interactive between parts and varities also shows the significantly difference (P>0.5). Means values of parts of bitter orange varieties depicted that highest DPPH radical scavenging activity was found in Peel (P2) 72.12% and as follow by pulp (P2) 67.93%. Variation in DPPH activity of three varieties were due to the antioxidant compound present in it and that difference occur due

to the genetically variation and the growth region (Bourgou *et al.,* 2008). Highest amount DPPH radical scavenging activity in V1 is due to presence of highest amount of antioxidant. The higher antioxidant activity of make it more potential source for health benefits. Means show that bitter orange variety V1 possess high antioxidant activity as compared to V3 and V2. Bitter orange peel present sufficient amount of antioxidants and possess potential antioxidant activity. Difference in DPPH Radical scavenging Activity of peel due to the different in concentration of antioxidant compounds present in it. Different factor such as extract preparation, time, re and variety difference. The mechanism of DPPH comprises with reaction between DPPH radicals and antioxidants hence it may cause the radical molecule decrease in number consistent to the hydroxyl group. These free radical donate electron that may be associated with disappearance of DPPH absorbance.

ABTS activity of peel and pulp

The analysis of variance of ABTS radical scavenging activity of pulp of citrus varieties was presented in Table 4.12. Means of ABTS radical scavenging activity of pulp of citrus varieties represented that ABTS radical scavenging activity of V2 shown a significance difference and was highest (23.66 percent) which was followed by V1 having means of ABTS radical scavenging of (29.957 percent). This was followed by means of ABTS radical scavenging activity of V3 (32.54 %). Means of ABTS radical scavenging activity of peel of three citrus varieties shown that ABTS radical scavenging activity of peel of V3 shown significantly difference and depicted the high ABTS radical scavenging activity (41.45 percent) and which was followed by V1 having means of ABTS radical scavenging of (39.50 percent).

This was followed by ABTS radical scavenging activity of V2 (37.21 percent) which shown non-significant difference. There was a difference observed in ABTS radical scavenging activity of peel and pulp of three citrus varieties this may be due to genotypic variation which are responsible for difference in antioxidant activity. Peel shown a highest amount ABTS radical activity due to presence of antioxidant. Peel shown a highest amount ABTS radical activity due to presence of antioxidant.

Minerals contents of peel and pulp

Calcium

The analysis of variance of calcium contents in pulp of citrus varieties are represented in Table 4.13. Results shown that all three means are significantly difference from each other. Calcium contents in pulp of citrus varieties was found in highest in V3 21.0 mg /100g and as followed by V1 19.0 mg /100g and V2 17.0 mg/100 g of sample weight on dry basis. Results shown that all means of three varieties are significantly difference from each other. Peel shown highest value calcium contents as compared to pulp (Ladaniya, 2008). Highest calcium was found in V2 160.3 mg/ 100g and as followed by V3 155.0 mg/ 100g and lowest were founds in V1 141.0mg/100g respectively.

In addition to the formation of insoluble salts citric acid present in citrus act as chelating agent increase the absorption of calcium in order to prevent the formation of insoluble salt in human body. Amount of calcium contents was shown in Fig. No. 4.15.

Sodium

Analysis of variance for the sodium content of part and varieties shows significantly difference from each other Table. 4.16.

Results shown that all means of three varieties are significantly different from each other. Pulp contain lowest amount sodium contents as compared to peel. Sodium contents was found in V3 3.2 mg/100g and as followed by V1 2.76 mg/100g and lowest was found in V2 1.96mg/100g.

Results of means value of parts of bitter orange varieties also shows the significantly difference from each other. Highest value of sodium content was observed in peel of V3 84.0 mg/ 100g.This was followed by V2 62.0 mg/100g and lowest was found in V1 50.33 mg/ 100g. Sodium play important role in

electrolyte balance of cell hence prevent human body from cell damage and cardiovascular disease (Ladaniya, 2008).

Magnesium

Analysis of variance for magnesium contents of pulp of citrus varieties was shown in Table 4.18 which shows the significance difference among varieties. There is also to be significance difference of between the interactive effect on parts and varities. Results shown that means of two varieties V3 and V1 are not significantly different from each other. Magnesium contents of pulp of citrus varieties was shown in Fig no. 11.

V3 possess high amount of magnesium contents 11.0mg/100g and as followed by V1 9.9 mg/100g while lowest magnesium contents was observed in V2 7.16mg/100g. Fig. 4.13 represent the graphical representation of magnesium content for pulp. Results depicted that means values of peel of citrus varieties was significantly different from one another. It was shown that highest value of magnesium content was found in peel of V3 33.33 mg/100g. That was followed by V1 25.0 mg/100g and lowest contents was found in V2 19.0 mg/100g. Magnesium is present in mitochondria which is power house of cell and provide energy to cell. Peel contain high amount of magnesium as compared to pulp hence it has been classified as good source of magnesium.

Ascorbic acid contents

Analysis of variance for ascorbic contents of parts and varities of bitter orange shown a significantly difference Table. 4.20. Means values shown that the ascorbic acid contents in pulp of citrus varieties was significantly different from each other. Highest value was found in V3 60.48 mg/100g and as followed by V2 40.56 mg/100g but V1 34.44 mg/ 100g depicted the lowest content of ascorbic acid contents as compared to other. Variation in ascorbic acid contents of varieties is due to the genotypic difference. The higher ascorbic acid content in V3 can be utilized for nutraceutical purpose. This property may allow it to use as replacement of artificial vitamin C supplements. Means value shown that the ascorbic acid content in peel of two varieties V1 and V2 are not significantly difference from each other. High value of ascorbic acid was found in peel of V3 21.64 mg/100g and as followed by V2 18.56mg/100g while V1 depicted the lowest value of ascorbic acid contents 15.77 mg/100g. Means values are given in Table. 4.21.

Total flavonoids content

Analysis of variance for total flavonoids contents for part and varieties of bitter orange Table. 4.22. Which shows that there is no significantly difference between parts and varieties. Means value shows that total flavonoids content was found high in V3 8.58 mg/100g and as followed by V1 7.43mg/ 100g while V2 shown the lowest flavonoids contents 6.66mg/100g. Interactive effect between parts and varieties also non-significantly difference from each other. The highest flavonoids in V3 recognize it's for promoting beneficial health effects and lower flavonoids health benefits are attained by V1 and V2 respectively. Regarding to flavonoids health benefits, citrus flavonoids promote nutraceutical potential and aid to prevent from different degenerative disease. Means values represented that total flavonoids contents for peel of citrus varieties shown that V1 and V2 depicted non-significantly difference while means value of V3 shown a significantly difference. Highest values was found in V3 20.77mg/100g and as followed by V1 19.29 mg/100g and lowest was found in V2 14.61 mg/100g. Flavonoids contents was found higher in peel studies revealed by (Gorinstein *et al.,* 2001). Graphical representation of flavonoids contents of peel was shown in Fig. no.4.20.

Hplc analysis of flavonoids

HPLC chromatogram has been used to determine the two flavonoid compound rutin and quercetin in peel and pulp of bitter orange variety. Quercetin and rutin standard solution was at different concentration from6-125ppm. Peaks of peel and pulp were computed at 270-280nm wavelength. By using area under curve graph was conducted. Concentration of compounds was calculated by linear equation. Chromatogram of standard compound quercetin

shows in Fig. 4.24. Retention time of quercetin was 1.23 and detected at wavelength of 271nm. By using standard curve relating with linear equation of y= 0.984x -229.45 and R^2 = 0.9988 concentration of quercetin in peel and pulp of citrus varieties has been calculated. In pulp of citrus varieties highest quercetin contents was present in V3 (10.40mg/gm.) and as followed by V1 which present (8.62mg/ gm) and lowest was found in V2 (6.66.48mg/ gm). Means value shown a non-significant difference from one another, while in peel all three varieties are shown in significantly difference from each other. Highest amount of quercetin was found in V3 (15.54 mg/gm) and as followed by V1 (13.33mg/gm) and V3 depicted the lowest quercetin contents (6.517 mg/gm) as compared to other. Citrus peel contain higher amount of flavonoids contents (Gorinstein *et al.,* 2001) which make it potential source for making citrus by products. Highest value of quercetin in V3 make him potential source for health benefits which may include anti-inflammatory, anti-cancer, antiviral and antioxidant (Cao *et al.*, 1996). HPLC chromatogram of standard compound rutin was represented in Fig.4.25. Rutin was detected best at wavelength of 274nm with retention time RT of 0.952min. Amount of rutin in peel and pulp of bitter orange varieties was calculated by comparing the linear equation y= 0.9836x - 30.756 and R^2 = 0.9883. In pulp means value shown non-significant difference from each other. Higher concentration of rutin was found in V1 (9.22mg/gm) and as followed by V2 (8.55mg/gm) while V3 depicted the lowest value of rutin contents (5.48 mg/gm).In peel means values also shown the non-significant difference and highest value of rutin was found in V1 (12.30 mg/gm) as followed by V3 (10.44 mg/gm) and lowest Was found in V2 (8.60 mg/gm).Peel is rich source of flavonoids compounds (Alexandra *et al.,* 1998). All the results of rutin and quercetin of peel and pulp of bitter orange varieties may vary and depend upon the environmental factor, genetically difference and source of plant.

HPLC chromatogram for pulp of citrus varieties V1, V2, V3 was shown in Fig. 4.26, 4.27 and 4.28 at 270nm wavelength for quercetin and 274 nm wavelength for rutin respectively while HPLC chromatogram for peel was shown in Fig. 4.29, 4.30 and 4.31. All the results were summarized in Table 4.1.

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