Spectrophotometric Quantification of Antioxidant Phytochemicals in Juices from Four Different Varieties of *Citrus limon*, Indigenous to Pakistan

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Summary: This research work was carried out to quantify the total phenolics, flavonoids and carotenoides, vitamin C contents, reported as antioxidants, in juices of four varieties of Pakistani *Citrus limon*. Juice was extracted from fresh lemon samples using classical method and subjected to study the various quality parameters. The total phenolic contents from juices of *Citrus limon*, determined following the Folin-Ciocalteu assay were found in the range of 690.62–998.29 mg/L, showing the significant inter-varietals variations. The total flavonoids and vitamin C contents from juices of *Citrus limon* were found in the range of 211.36–220.34 and 18.87–25.1 mg/L, respectively. Whereas, the total carotenoides contents of *Citrus limon* juices were found in a low concentrations *i.e.* 0.05–0.08 mg/L. The statistical analysis showed significant ($p < 0.05$) variations in the total phenolic, carotenoides and vitamin C contents while non-significant ($p > 0.05$) variation in total flavonoids contents among different varieties of *Citrus limon*.

Introduction

Citrus fruits are universally well-liked raw material for the production of juices, customary well admired drinks worldwide because of their appetizing flavor and health benefits [1-3]. The consumption of fruit juices whose health affects has been ascribed to inhibit the development of major clinical circumstances including heart diseases and certain cancers has acquired the dietary recommendations due to their functional constituents like vitamin C and natural antioxidant compounds [4, 5]. Most of the photochemicals are an integral part of the diet and also being used in medicinal formulations. A number of health protective effects of phenolic compounds have been reported due to their antioxidant, antimutagenic, anticarcinogenic, antiinflammatory, antimicrobial, and other biological possessions [6-8]. Currently citrus fruits are popular in daily utilization because of their first-class flavor and health-promoting compounds, such as flavonoids, phenolics and carotenoides conents [2, 9-12]. Phenolic compounds constitute one of the most plentiful and extensively distributed groups of substances in the plant kingdom and foods and fruit juices may have a very complex phenolic composition These are biogenetically arise secondary metabolic products of plants and their activity is reported due to the polyphenols which demonstrate antibacterial, anti-inflammatory and vasodilatory actions that might be attributed to polyphenolic compounds with antioxidant activity [2, 12, 13].

Dietetic carotenoid antioxidants from fruits and vegetables have been reported to play a vital role in human health and, particularly, their inference in cancer prevention has grown a very immense interest in the antioxidant composition of fruits and vegetables. The carotenoides content of different fruit products like juices, provide a very useful clue about the quality of product [8, 14, 15].

Citrus fruits juices contain a considerable amount of phenolics, flavonoids and carotenoids and their quantitative fingerprint shows differences in the proportions because of various locations of origins, agro-climatic conditions and dreadful diversity in species. A number of reports revealed that in recent years a number of articles have focused on the investigation of these fruits [2-5, 14, 15].

Although a huge amount of literature is available with major focus on the polyphenolic and carotenoid contents of citrus juices but such type of investigations are lacking regarding the intervarietal variations of these phytochemicals in *Citrus limon* indigenous to Pakistan. The main objective of this work was to determine the total phenolics, flavonoids and carotenoid contents and amount of vitamin C in various varieties of *Citrus limon* using spectrophotometric methods.

Results and Discussion

Physico-Chemical Analysis

The physico-chemical parameters of the various varieties of *Citrus limon* (*C. limon*) are

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presented in Table-1. The pH values ranged from 2.50-2.69. The *C. limon* juice from Lisbon variety was found to be more acidic (pH 2.50) followed by Eureka (pH 2.56), Mayre (pH 2.62) and Bush (pH 2.69). Density and viscosity of all the citrus juices were in the range of 1.026-1.029 g/cm^3 and 1.044-1.127 cP, respectively. Among all the citrus varieties, Bush variety offered highest values of total soluble solid contents (112.38 g/L) while, Mayre contained the lowest (103.15 g/L). Titrate-able acidity, measured at 25 $^{\circ}$C, of all the citrus juice samples was found to be analogous concentrations. Analysis of variance (ANOVA) revealed non-significant ($p > 0.05$) difference in the physico-chemical parameters of juices from various varieties of *C. limon*.

Table-1: Physio-chemical quality parameters of juices from various varieties of *Citrus limon.*

Varieties	pH	Density (g/cm^3)	Viscosity (cP)	TSS (g/L)	TA at 25 ^{0}C (g/L)
Lisbon	2.50± 0.07	1.028± 0.02	1.127± 0.01	110.02± 4.40	3.720± 0.11
Eureka	2.56± 0.12	1.026± 0.03	1.044± 0.02	107.34± 5.12	3.660± 0.13
Mayre	2.62± 0.08	1.027± 0.02	1.047± 0.03	103.15± 6.01	3.540± 0.21
Bush	2.69± 0.11	1.029± 0.05	1.103± 0.02	112.38± 5.47	3.401± 0.15

Antioxidant Potentials

Total Phenolics Contents (TPC)

The amount of total phenolics contents found in the *C. limon* juice samples varied widely among varieties. The values of TPC ranged from 690.62-998.29 mg/ L as gallic acid equivalent (GAE) (Fig. 1). The maximum TPC was found from the Bush variety while the minimum from the Eureka variety. Lison and Mayer varieties of citrus showed 730.46 and 825.37 mg/L TPC as GAE, respectively. Statistical analysis showed the significant ($p < 0.05$) variation in the TPC with respect to citrus varieties.

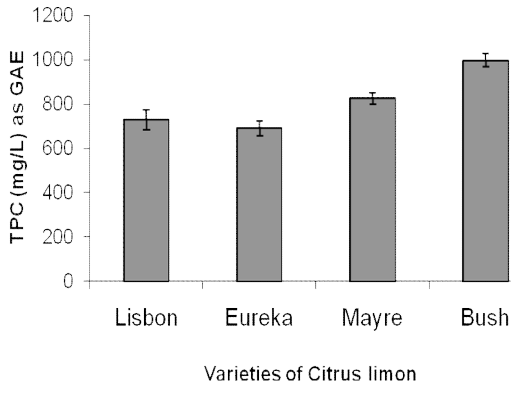

Fig. 1: Total phenolic contents in the juices of *Citrus limon.*

Total Flavonoid Contents (TFC)

The data regarding the TFC is presented in Fig. 2. It was observed that the amount of TFC found in the *C. limon* juice samples varied widely among varieties. The overall TFC found in juice samples of various varieties of *C. limon* were in the range of 211.36-220.34 mg/ L as catichine equivalents (CE). The maximum TFC was found from the Bush variety while the minimum from the Lisbon variety. Eureka and Mayer varieties of citrus showed 219.27 and 216.61 mg/L TFC as CE, respectively. Statistical analysis showed that variations in TFC with respect to citrus varieties were non-significant ($p > 0.05$).

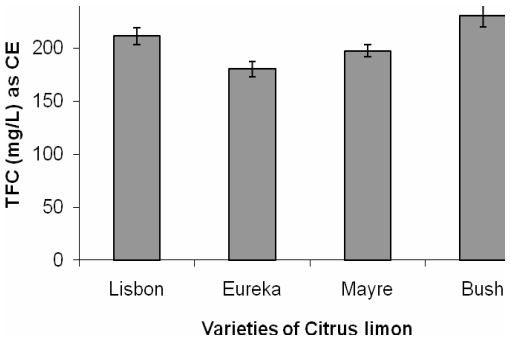

Fig. 2: Total flavonoid contents in the juices of *Citrus limon.*

Total Carotenoides Contents (TCC)

The carotenoides contents were found in juice samples of various varieties of *C. limon* in very low concentration (Fig. 3). The overall range of carotenoides contents were 0.05-0.08 mg/L measured as β-carotene equivalent. The statistical analysis showed the significant ($p < 0.05$) variation in TCC with respect to citrus varieties.

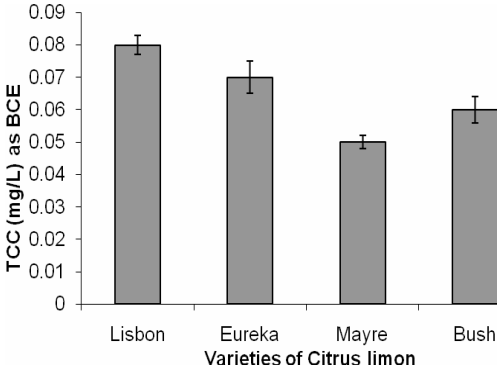

Fig. 3: Total carotenoid contents in the juices of *Citrus limon*.

Vitamin C Contents

The amount of vitamin C found in the *C. limon* juice samples varied widely among varieties (Fig. 4). The concentration of vitamin C was found maximum in Lisbon variety (25.13 mg/L) and that of least in bush (18.87 mg/L). Based on these findings, the order of vitamin C contents in various varieties of *C. limon* was as follows; Lisbon > Eureka > Mayre > Bush. Statistical analysis showed the significant ($p < 0.05$) variation in vitamin C with respect to citrus varieties.

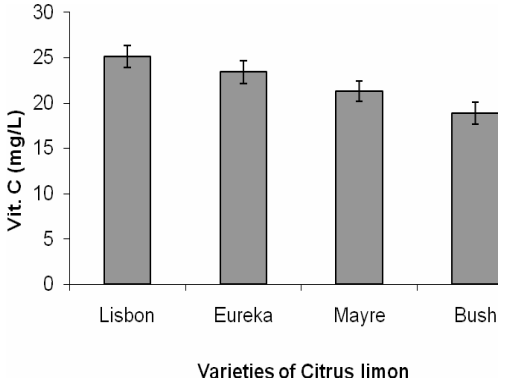

Fig. 4: Estimation of vitamin C contents in the juices of *Citrus limon.*

In nature, there are a number of various types of antioxidant compounds that play an important **role** as radical scavengers, inhibit lipid peroxidation and other free radical-mediated processes and able to protect the human body as well as processed foods from oxidative damage ascribed to the reaction of free radicals [16, 17]. The uses of plant-based antioxidants like polyphenols, flavonoids, vitamin C are related to reduce the risk of incidence of coronary heart diseases, neurological disorders, and certain type of cancers [16, 17] The ability of fruit juices to exhibit antioxidant potential was closely related to their total phenolic, flavonoids and carotenoids contents [17, 18]. There are few reports in literature on the antioxidant potentials of citrus fruit juices [19, 20].

All the juices samples analyzed in the present study contained considerable amount of total phenolics, total flavonoids as well as good concentration of vitamin C. As reported earlier vitamin C is the major antioxidant present in citrus juices [19, 21, 22]. The contents of antioxidant phytochemicals varied among species, cultivars, varieties and geographical origins [23].

Experimental

Collection of Samples

Fresh samples (12 each) of four indigenous varieties of *Citrus limon L.*, e.g. Lisbon, Eureka, Mayre and Bush lemon were procured from the Supermarket of District Jhang, Pakistan and transferred to research laboratory of Department of Chemistry, Government College University, Faisalabad. After washing with tap water to remove the dust particles, these samples were identified and authenticated by Dr. Muhammad Naeem, Assistant Professor of Botany, Government College University Faisalabad, Pakistan.

Chemicals and Reagents

Folin-Ciocalteu's phenol reagent, gallic acid, catichine, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), linoleic acid, butylated hydroxytoluene (BHT), β-carotene and Tween 40 were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All the other chemicals/reagents and solvents used in this study were purchased from Merck (Darmstadt, Germany), unless stated otherwise.

Extraction of Juice

Juice of each variety of *Citrus limon* was extracted in separate 250 mL beaker by using the classical approach *i.e.* by squeezing the lemon. This extracted juice was filtered through 2mm steel sieve and stored in labeled glass viols in refrigerator at 4 ^{o}C till further analysis.

Evaluation of Juice Quality

The quality of extracted juice of each variety was assessed by studying the various quality parameters like pH, total dissolved solids, viscosity, density and acidity. For pH determination, 10 g of lemon juice of each sample, blended with 20 mL deionized water was heated to 100 $^{\circ}$C and then cooled to 25 $^{\circ}$C followed by the addition of 20 mL deionized water. The pH was measured at 25 $^{\circ}$C with a pH meter (Inolab pH 720, wtw 82362, Weiheim, Germany) [24]. The acidity of juice samples was determined according to the method reported by Sanchez-Moreno [5]. Each sample was titrated with 0.1 M NaOH to pH 8.1 monitoring with an electrode pH meter (Inolab pH 720, wtw 82362, Weiheim, Germany) at both 20 $^{\circ}$C and 32 $^{\circ}$C temperatures. Results were expressed as grams of citric acid per liter juice. Total solids were measured following the

official method of AOAC [24]. Briefly; after heating 5 mL of sample juice in china dish in electric oven at 105 °C to a constant weight. Results were expressed as grams of total solids per liter juice. Viscosity of each juice sample was measured by viscometer and results were expressed as centipoises (cP). Density (g/cm^3) of each juice sample was measured by specific gravity bottle at 25 °C.

Antioxidant Activity

Determination of Total Phenolic Contents

Total phenolic contents (TPC) were determined by following the Folin-Ciocalteu method [25] with modifications. Briefly, 1 mL of citrus juice was extracted with 9 mL of 80% methanol for 30 min at room temperature. After centrifugation **at** 4000 rpm for 10 min, an aliquot (1 mL) of appropriately diluted 80% methanol extracts were added to a 25 mL volumetric flask filled with 9 mL distilled water. Folin–Ciocalteu phenol reagent (0.5 mL) was added to the mixture and shaken vigorously. After 5 min, 5 mL of Na_2CO_3 solution was added with mixing. The solution was immediately diluted to 25 mL with distilled water and mixed thoroughly and then allowed to stand for 60 min before measurement, and the absorbance was measured at 750 nm versus the prepared blank. Quantification was done on the basis of a standard curve with gallic acid. Results were expressed as gram of gallic acid equivalents (GAE) per 100 g dry weight.

Determination of Total Flavonoid Contents

Total flavonoid contents (TFC) were determined by following the method of B. Sultana *et al.* [26] with modifications. In brief, 2.5 g of sample placed in a Soxlet extractor and refluxed with methanol for more then 2 h at 80 °C. The extract was evaporated to dryness in a rotary vacuum evaporated at less than 40 °C and dissolved with methanol. Exactly 0.3 mL of 5% $NaNO_2$ was added to 1 mL of extract in a 10 mL volumetric flask and the mixture was kept for 10 min at room temperature. Addition of 0.3 mL of $Al(NO_3)_3$ to the mixture and incubated for 10 min again followed by the addition of 4 mL of 1 NaOH and of methanol up to volume. After incubating for 30 min at room temperature for colour development, absorbance at 500 nm was measured and TFC was expressed as CE.

Determination of Total Carotenoid Contents

The method of Lee [27] was used for total carotenoid quantization. In brief, 5 g of each sample and 50 mL of n-hexane-acetone-ethanol (v/v; 2:1:1) were placed in a flask, extracted on a shaker at 200 rpm for 20 min at room temperature, centrifuged at 4000 rpm for 10 min at 4 °C, and the supernatants were collected and made up to 50 mL with extraction solvent. Two layers were observed after mixing the solvent, an organic (upper layer) and aqueous (lower layer). After carefully separating the layers, the absorbance was measured at 450 nm. Total carotenoid was expressed as β-carotene equivalents.

Determination of Vitamin C Contents

Procedures used to determine the vitamin C content were as described by Wright and Kader [28] for the determination of ascorbic acid by HPLC.

Statistical Analysis

All the experiments were conducted in three replicates and data obtained was analyzed statistically by one way ANOVA technique using Minitab Software.

Conclusion

Our study, on four limon varieties, of bioactive compounds showed variation among different varieties with respect to the content of antioxidant phytochemicals and quality attributes of selected limon juices. Bush variety contained significantly higher amounts of total phenolics and total flavonoids contents. While vitamin C contributed considerably to the Lisbon variety. It is thus recommended to consume citrus fruit juice for uptake of antioxidant phytochemicals and vitamin C.

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