Extraction of diverse polyphenols in relation with storage periods of *Citrus paradisi* CV. Shamber through HPLC–DAD technique using different solvent

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Revised: 10 October 2018 / Accepted: 6 November 2018 / Published online: 10 December 2018
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Abstract In this article, *Citrus paradisi*, (Shamber) an exceptional source of Vitamins A and C and full of nutrients, selected for extraction of diverse polyphenols including dietary flavonoids and essential flavonoids by HPLC–DAD technique using various solvents. These essential targeted compounds also analyze after keeping different storage periods and compare with fresh fruits for better efficacy of these compounds. The highest number of phenolic compounds including gallic acid, chlorogenic acid sinapic acid, ferulic acid, myricetin, quercetin, and kaempferol extracted in methanol solvent leading to the new compounds of tetra-O-methylscutellar and heptamethoxy flavone. The essential flavonoids determined by polyethersulfone filter and insoluble precipitation separated by the dimethyl sulfoxide. The results showed that the methanolic extraction exhibited higher essential flavonoids including nobiletin, sinensetin, tangeritin, and tetra-O-methylscutellarein and heptamethoxy flavone. The RP-HPLC analysis exposed the maximum number of nutritional flavonoids like naringin, hesperidin, total flavones, glycosyl. Moreover, it observed that dietary flavonoids and

phenolic compounds of stored fruits were unaffected in 30 days of storage periods while minor variations were pragmatic during 60–90 days storage. The investigation revealed that *C. paradisi* proves to be the valuable resource of different phenolic compounds and flavonoids which are effective against various oxidative stresses in the human body.

Keywords *Citrus paradisi* · Dietary flavonoids · Phenols · HPLC · Extraction

Introduction

Citrus paradisi Macf. is one of the essential citrus crops in the world due to its large nutritive values, ensuring the highest amount of phytochemicals (polyphenols) in juice contents (Buachan et al. 2014). These secondary metabolites possess a particular function in the human body to reduce many chronic diseases (Staudacher et al. 2013). Flavonoids

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are one of the stimulating primary classes of compounds due to their excellent properties, derived from their anti-oxidative characteristics as free-radical neutralizers. It acts as a disease preventer in dietary supplement involved in health promotion. Flavonoids including quercetin and rutin, narirutin, naringin gallic acid, hesperidin, sinensetin tangeritin, nobiletin, tetra-O-methylscutellarein, heptamethoxy flavone mostly found in citrus, fruits, vegetables and plant-derived beverages (Staudacher et al. 2013). The best-known flavonols are quercetin and kaempferol for its unique values in human health (Boligon and Athayde 2014). Hesperidin and narirutin, a vibrant source of flavanones found mainly in the juice of *C. paradisi* (Shamber).

Extraction is a technique to move a targeted compound from one phase to another. Several experimentations conducted for extraction of flavonoids in a purified state to save the nutritional status associated with citrus fruits (Rawson et al. 2014). Experimental observation of both in vitro and in vivo studies described the anti-carcinogenic, antitumor, and antimutagenic activities of these compounds which were found to be preventive against the controls of tumor formations. It controls the deformed cells of human and repaired DNA structure and severe myeloid leukemia (Ko et al. 2010; Rawson et al. 2014).

Citrus flavanones, mainly hesperidin, are quite insoluble in acidic aqueous solutions and form white crystals that formed a precipitate (Rawson et al. 2014). It is well known that flavonoid crystals integrate into the juice cloud. Proper solvent extraction requires to separate these precipitations in the juice of citrus (Sankhalkar and Vernekar 2016). The safe extraction process can lead to an appropriate quantity without changes in some flavonoids in the juice of citrus (Sankhalkar and Vernekar 2016).

This article deliberated for the first time the extraction of valuable bioactive compounds from fresh and stored *C. paradisi* (Shamber) fruits using solvent extraction methods, and HPLC–DAD analysis as perviously no study is reported on the extraction of these compounds via different solvents extraction methods up to my best knowledge yet. The dimethyl sulfoxide-methanol used to solubilize the impure precipitated flavones. The exploration of stored *C. paradisi* (Shamber) fruits juices accompanied and compared with the fresh *C. paradisi* (Shamber) juice. Furthermore; the comparison of both dietary and essential flavonoids extracted in different solvents discussed in this paper.

Materials and methods

Field of experiment and plant materials

Orange Research Station Sargodha, Pakistan was selected as a new resource of *C. paradisi* (Shamber) for trialing with

three replicates, during the mid-seasons. Fifteen uniforms 12-years-old *C. paradisi* (Shamber) trees grafted onto sour orange rootstock 6 m × 6 m spacing. Fruits of selected trees shifted into the lab and stored in cold Storage. Harvested fruits stored at 8 °C and RH at 85–90%. RH in cold chambers equipped with automatic relative humidity and temperature control system. Fresh *C. paradisi* (Shamber) samples analyzed for extraction of bioactive compounds with different solvents. The samples of *C. paradisi* (Shamber) taken randomly from each treatment for analysis after intervals of every 30, 60, 90 days during the whole periods of storage.

Preparation of different solvents extraction methods

The juice of *C. paradisi* (shamber) prepared with the help of a mixer, from which 2 ml taken for analysis. The filtration conducted followed by the addition of 1 ml of water. The clear solution then mixed with methanol for 3 days, and the soluble part filtered followed by evaporation at 40 °C on a rotary evaporator for a crude extract. The citrus juice was separately extracted twice at room temperature with 95% alcohol. The 50 g of a crude extract of juice sample was then dissolved in 150 ml of distilled water and sequentially portioned with 100 ml of methanol, 100 ml of ether; 150 ml of acetone and the crude solution was 150 ml, respectively. The concentrated extracts of methanol, ether, acetone, and crude extraction were transferred to sample vials and stored at − 4 °C for further analysis.

Sample preparation

The centrifuge machine (B. Braun Biotech International) used to separate juice cloud (insoluble fraction) of *C. paradisi* (Shamber) by taking 1 ml of juice at 12,000 rpm. The supernatants filtered through a 0.45 m polyethersulfone filter and analyzed by HPLC–DAD. The sediment obtained after centrifugation was treated with 0.1 ml of dimethyl sulfoxide (DMSO) and sonicated for 2 min to extract the phenolic compounds.

HPLC–DAD analysis

The individual phenolic compounds of hydroxy-cinnamic acid derivatives, flavones, and flavanones (hesperidin and narirutin) determined and quantified by HPLC–DAD. These analyses performed with a Merck-Hitachi gradient liquid chromatograph with fixed a pump model L-6200 and a diode array detector Merck-Hitachi model L-3000 is attached with HPLC–DAD.

Dietary and essential flavonoids compounds

Estimation of flavonoids on chromatograms of *C. paradisi* (Shamber) were recorded at 280 nm for flavanone and quantitation at band 10 nm for hydroxycinnamic derivatives. Flavonoids compounds were quantified as chlorogenic acid (5-caffeoylquinic acid) (Sigma, St. Louis, MO), flavanones as hesperidin (E. Merck AG Darmstadt, Germany), and chalcones as a hesperidin-methyl chalcone (E. Merck AG Darmstadt). The flavanones narirutin and hesperidin identified by their DAD (Diode array detector), and chromatographic comparisons with an authentic marker, placed in the beaker. The beakers were sealed with paraffin film-coated material (Parafilm, American National Can) and incubated at a 37 °C then shaking with a water bath until the pH reached ~ 5 (~ 30 min). The pH of the solution adjusted at 5, by adding 2 ml of HCl in a solvent such as methanol, ether, acetone and crude extraction and 1 ml of water.

Standard curves of dietary flavonoids

The standards curves of naringin, hesperidin, total flavones, glycosylflavone, gallic-acid, chlorogenic-acid, sinapic-acid, ferulic acid, myricetin, quercetin, kaempferol, narirutin, nobiletin, Sinensetin, tangeritin, tetra-O-methylscutellarein, heptamethoxyflavone were prepared from a stock of concentration of 25 mg/l. The Standard calibrations of samples prepared by suitable dilutions with a mixture of DMSO/ ethanol from the stock solution and these solutions were filtrated on Millipore paper with the size of (0.21 µm) before use.

Statistical analysis

The obtained data submitted to a factorial analysis of variance and the mean values compared with using the least significant difference test (LSD) at the 5% alpha level. While the data of HPLC measured by the help of software Chrom gate v 3.31 Knauer software. The number of replicates for each analyzed sample was three (Salkind 2010).

Results and discussion

The phytochemicals like dietary flavonoids, an essential part of life linked to reducing a risk of the primary chronic diseases, provide numerous health benefits to human body including the highest activity of antioxidants (De-Moraes Barros et al. 2012) anti-inflammatory, anti-tumor activity (Tomás-Barberán and Clifford 2000; Gattuso et al. 2007). The proper solvent extraction is required for extraction of

bioactive compound to avoid crystallization process during extraction (He et al. 2011). Several scientists worked on the extraction of bioactive compounds from the medicinal plants through solvent extraction methods, but extraction methods regarding bioactive compounds from fruits are not standardized (Vallverdú-Queralt et al. 2011). In the current study, the juice of *C. paradisi* (grapefruit), a rich source of polyphenols, dietary flavonoids, and essential flavonoids, subjected for extraction to acquire a pure form of these constituents through various solvents using HPLC–DAD technique.

Analysis of polyphenols

The identification of several polyphenols accomplished by comparison of their retention time values with the standard substances about the storage period. The maximum contents of various polyphenols extracted in a methanol solvent in comparison with other solvents (Table 1). The chromatogram presented in Fig. 1a showed nine peaks of individual polyphenols (gallic acid, chlorogenic, acid sinapic-acid, ferulic acid, myricetin, quercetin kaempferol, apigenin, and luteolin). The highest peak of myricetin measured in *C. paradisi* Shamber juice in comparison of all others eight peaks through several solvent extractions. It observed that gallic acid, chlorogenic acid, sinapic acid, ferulic acid, myricetin, quercetin, and kaempferol were remained unaffected after 30 days of storage periods over storage periods of 60 and 90 days (Table 1).

Analysis of dietary flavonoids

The quantification of various dietary flavonoids conducted, related to a storage period of *C. paradisi* (Shamber). The chromatogram of four different dietary flavonoids including naringin, hesperidin, total flavones and glycosyl flavone in the juice of *C. paradisi* (Shamber) presented in Fig. 1b while identification of these compounds achieved by comparison of their retention time values with the standard substances. The chromatogram showed the highest peak of Glycosyl flavone in the juice of *C. paradisi* (Shamber) (Fig. 1b). Higher contents of glycosyl flavone and naringin (105 mg/l, 36 mg/l) were prominent in methanol solvent extraction as compared to other solvent exactions methods while crude extraction showed minimum contents. Ether extraction method was best for Hesperidin 221 mg/l in comparison with methanol extraction methods followed by lower in acetone as well as in crude extractions. It observed that total flavones (55 mg/l) contents were higher in methanol and ether extractions while lower in crude extractions. It suggested that crude extraction methods proved to be deprived extraction method for dietary flavonoids of *C. paradisi* (Shamber). It established that the

Table 1 The identification of polyphenolic compounds using different solvent through HPLC–DAD at different Storage periods

Phenolic compounds (mg/l)	Solvent extraction methods				Storage periods		
	Methanol extraction	Ether extraction	Acetone extraction	Crude extraction	30 DAS	60 DAS	90 DAS
Gallic acid	12c	13b	14.5a	11d	12c	11d	10e
Chlorogenic acid	15a	12d	13c	10e	14b	13c	12d
Sinapic acid	16a	15b	14c	13d	14c	13d	12e
Ferulic acid	17a	16b	15c	14d	16b	15c	14d
Myricetin acid	16a	15b	14c	12e	15b	14c	13d
Quercetin	13a	12b	11c	10d	12b	11c	10d
Kaempferol	16a	15b	14c	13d	15b	14c	13d
Apigenin	14a	13b	12c	11d	14a	12c	10ef
Luteolin	12a	11b	10c	9d	12a	10c	9d

Mean sharing same letter in row or column showed significant difference at 5% probability level (LSD) LSD value = 0.001

Fig. 1 **a** Chromatogram of different extracts of grapefruit obtained by HPLC–DAD at 130 nm. **a** (1) Gallic acid; (2) chlorogenic acid; (3) sinapic acid; (4) ferulic acid; (5) myricetin; (6) quercetin; (7) kaempferol; (8) apigenin (9) luteolin. **b** (1) Naringin; (2) hesperidin;

(3) Total flavones; (4) glycosylflavone at 10 nm. **c** Chromatogram of different extracts of grapefruit obtained by HPLC–DAD at 50 nm) (1) naringin; (2) nobilentin; (3) sinensetin; (4) tangeritin; (5) tetra-O-methylscutellarein; (6) heptamethoxyflavone

order of solvent efficiency by the HPLC–DAD method was as methanol > ether > acetone > cured extraction. Storage period is a factor to maintain these vital compounds during the storing process (Chen et al. 2012). The results of

proper solvents extraction in the juice of *C. paradisi* display the higher range of naringin and hesperidin (36 and 220 mg/l or 2.20 mg/100 g) while literature reports the lower range of diverse dietary flavonoids including total

flavanones (94, 140 (mg/l), naringin (17.7–45.4 mg/l) and hesperidin (123.3–206.7 mg/l) in the hand-pressed juice of grapefruit (Nakajima and Ouchi 2014) respectively. On the other hand, the total flavonoids in the Cocktail of (*C. paradisi*) reported by Simirgiotis et al. (2010) in the range of 76.5–130 mg/l, while Wanwimolruk and Marquez (2006) and Peterson et al. (2006) perceived 40–48 mg/l. The current research is in accordance to the previous report of Ko et al. (2010) who reported dietary flavonoids in the peel of oranges and sweet lime which is the vital component of human diets (Nakajima and Ouchi 2014). Similarly, the dietary flavonoid of polyethoxylated flavones previously reported in the citrus parts like flavedo, peel whereas total flavonoids extracted in the present investigation in the range of 32–220 mg/l for the first time in citrus fruit juice via different solvent extractions which were higher than reported work of Ghanim et al. (2007) and Pereira et al. (2014). It was found that naringin, hesperidin, total flavones, and glycosyl flavone were higher in 30 days storage period over 60 and 90 days which was similar to the work of Zhang et al. (2012).

Analysis of essential flavonoids

The essential flavonoids compounds such as narirutin, nobiletin, sinensetin, tangeritin, tetra-O-methylscutellarein, heptamethoxy flavone conducted and reported in Table 2. The methanolic extraction was the leading extraction in comparison with other extraction methods in which higher contents of the above compounds were reported (Table 2). The results reported in the Table 2 showed that 30 days storage periods were dominant over 60 and 90 periods for hesperidin, sinensetin, narirutin, sinensetin, tetra-O-methylscutellarein, heptamethoxy flavone and nobiletin also while minor changes observed in 60 and 90 days storage periods. The chromatogram of the methanol extract of *C. paradisi* (Shmaber) showed essential flavonoids (Fig. 1c) where six peaks detected with numeric numbers of (1) narirutin; (2) nobiletin; (3) sinensetin; (4) tangeritin;

(5) tetra-O-methylscutellarein; (6) heptamethoxyflavone. It observed that the *C. paradisi* (Shmaber) contain higher contents of glycosyl flavone (Fig. 1c). In this study tetra-O-methylscutellarein, isolated for the first time from *C. paradisi* (Shmaber) ensuring potential health benefits which earlier reported in the peel of oranges and sweet lime by Ko et al. (2010). The solvent extraction methods showed that purified dietary flavonoids in *C. paradisi* (Shmaber) which are lipid soluble and lump peroxy-mediated chain could be obtained (Boligon and Athayde 2014). Results reported in Table 2 revealed that methanol was the suitable solvent for extraction of the highest amount of these flavonoids as the majority of flavonoids did not hydrolyze.

The prolonged period of storage showed variations that may be due to alteration in enzymes in the juice of *C. paradisi* (Brito et al. 2014) which was similar to chemical changes in the peel of lemon as reported earlier by De-Moraes et al. (2012) and Nagy et al. (2011). The results reported in Table 2 showed that our results are in accordance to those of Domínguez-Vázquez and Castro-Ramírez (2002) who observed that fresh juice contains high concentrations of essential flavonoids (mainly antioxidant compounds) and possesses the strong antioxidant ability to scavenge the free radicals (Gil et al. 2012). Polyphenol profiles of citrus juices are also known to be a fingerprint for many quality control laboratories. That characterizes by varieties, contamination, and added substances (Buachan et al. 2014). Dahmoune et al. (2013) reported the change in the number of dietary and essential flavonoids in citrus fruits is due to its cytol variations in the cell. The results provided essential clues to understand the polyphenols transport mechanisms in plant cells (Liu et al. 2013). Although the association of flavonoid enzymes with the ER membrane has not thoroughly established which affect the changes in the dietary as well as essential flavonoids (Gil et al. 2012).

The study exposed that the extraction method is significant for purification of polyphenolic compounds due to higher residual ability after extractions (De-Moraes et al.

Table 2 Identification of essential flavonoids using different solvent through HPLC–DAD during storage periods

Essential flavonoids (mg/l)	Solvent extraction methods				Storage periods		
	Methanol extraction	Ether extraction	Acetone extraction	Crude extraction	30 DAS	60 DAS	90 DAS
Narirutin	72a	71b	70c	69d	70c	69d	68e
Nobiletin	1.22a	1.21b	1.10c	0.9d	1.21b	1.10c	0.8e
Sinensetin	2.21a	2.20b	2.11c	2.01d	2.20b	2.11c	2.0e
Tangeritin	1.9a	1.7b	1.6c	1.3d	1.9a	1.7b	1.6c
Tetra-O-methylscutellarein	1.05a	1.03b	1.02c	1.01d	1.02c	1.01d	1.02c
Heptamethoxyflavone	1.25a	1.22b	1.11c	1.1d	1.22b	1.11c	1.0e

Mean sharing same letter in row or column showed significant difference at 5% probability level (LSD) LSD value = 0.001

2012). Therefore, diverse extraction methods in the current study provide the excellent source of collection of different dietary, essential flavonoids and phenolic compounds from *C. paradisi* (Shmaber) (Table 1) (Tomás-Barberán and Clifford 2000; Albishi et al. 2013). While literature showed minuscule information regarding the impact of storage on these essential compounds related to their natural resources (Albishi et al. 2013). It suggests that either the technological treatments and extraction method or the characteristics of the citrus fruits, used for juice production (cultivar, environmental conditions, postharvest storage, etc.) can affect the precipitation of flavones (Nagy et al. 2011). No isomerization regarding storage treatment demonstrated that these changes might link in the symmetrical di-C-glycoside linkage of these compounds (Simirgiotis et al. 2013).

More work is required for developing the fast kinetic method for better understanding of the antioxidant ability of these essential flavonoids compound like the activities of anti-DPPH, anti-hydrogen peroxide, and anti-superoxide anion radicals activities (Staudacher et al. 2013).

Conclusion

It concluded that the various solvents extraction methods for analysis of dietary and essential flavonoids prove as an essential factor in determining a pure and reliable quantity. The phenolic profile of *C. paradisi* (Shamber) suggests that methanolic extraction was the best method for extracting the higher contents of the phenolic profile including, dietary and essential flavonoids from fresh *C. paradisi* (Shamber) fruits. The diverse solvent extraction suggests that methanolic extraction was more acceptable among all other solvents with the storage period of 30 days which presents the maximum amount of these dietary, essential flavonoids and phenolic profile. However, minor changes recorded in 60 and 90 days stored fruits. The current analytical techniques in this study desire more attention of scientist to work and explore more health benefits ingredients from citrus fruits for the advantages of the consumer in fresh and storage fruits.

Acknowledgements The Authors are profoundly thankful to CRI and HEC Pakistan for providing the facilities for the compilation of this project.

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