Gene expression profiling and fruit quality during ripening stages of Prickly pear (*Opuntia ficus-indica*) in Taif.

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Abstract: Prickly pear (*Opuntia ficus-indica*) is a very important economic plant because it is used as fruit, food, making juice, and food supplements. Ethylene plays a central function in the regulation of the ripening processes in climacteric fruits. The last two steps of ethylene production pathway are controlled by 1-aminocyclopropane-1-carboxylase (ACC) synthase (*Opaccs-1*) from *Opuntia sp.* and 1-aminocyclopropane-1-carboxylase (ACC) oxidase (*Opacco-1*) genes. The juice of full mature fruits was used to evaluate biochemical, nutritional and antioxidant activity for both Shafawi and Toti prickly pear fruits of Taif region. Sucrose was not detected in both cultivars, whereas glucose and fructose content was about twice in Toti compared to Shafawi juice. In addition, nine flavonoids (Cyanidine chloride, Myricetin, Quercetin, Chrysin, Caffeic acid, Delphinidin chloride, Malvidin chloride, Naringenin, Galangin) were estimated in the juice of the full mature fruits. All nine standards were detected in the Toti samples, whereas only Chrysin and Galangin were detected in Shafawi samples. Total flavonoids of these nine chemicals were present in Toti at much higher concentration compared to the Shafawi samples, about 19 fold. Real time PCR was used to estimate the difference in gene expression pattern of *Opaccs-1* and *Opacco-1* genes in both cultivars. In this stage, *opacco-1* expression was higher in both cultivars indicated in the Ct cycles for both genes as 23 and 32 cycles respectively. The expression of the *Opacco-1* and *Opaccs-1* ripening genes showed correlation with the high content of sugars and flavonoids contents of Saudi prickly pear cultivars. On the other hand, the Shafawi juice showed higher antioxidant activity using the scavenging activity of the DPPH. These high content sugars and flavonoids and expression of *Opacco-1* as well as *Opaccs-1* represent good molecular markers of ripening stages in prickly pear. Also, these markers can be used in the case of similar fruits.
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Abbreviations:
ACC: 1-aminocyclopropane-1-carboxylase, .*opacco-1*: 1-aminocyclopropane-1-carboxylase (ACC) oxidase. *opaccs-1*: 1-aminocyclopropane-1-carboxylase (ACC) synthase.

1. Introduction

The prickly pear is the fruit of the genus *Opuntia*, which belongs to the Cactaceae family (El-kossori *et al*., 1998; S´aenz, 2000). On the basis of full fruit development from anthesis to physiological maturity, there are three stages of development namely early, intermediate, and full-full mature (Pimienta-Barrios, 1994). Prickly Pear (*Opuntia ficus-indica*) is a very important economic plant. Its fruit is used as fruit and its leaves are used fresh as food, making juice, used for food supplements. Fresh or processed fruits are considered as an important source of vitamins, minerals, and carbohydrates (Rosas-Cardenas *et al*., 2007).

Fruit full mature includes changes that enhance appearance, flavor, and texture. In most fruits, all the major cell wall polysaccharides appear to be modified during the softening that occurs in the course of full mature (Carrillo-L´opez *et al*., 2002). Cell wall softening is likely to result, at least in part, from the action of enzyme-mediated hydrolysis of cell wall components (Huber, 1983; Seymour and Gross, 1996).

Chemical analysis showed that the prickly pear fruits contain 12–15% sugars (mostly glucose), 0.6% protein, and 0.1% lipids; minerals including calcium, potassium, and magnesium (490, 2200, and 850 ppm, respectively). Its use as human food fortifies the nutritional contribution in the arid and semi-arid zones where it is cultivated (El-kossori *et al*., 1998).

Biochemical and nutritional changes were studied during the full mature process of three

Opuntia sp. morphospecies with different full mature behavior: Naranjona (*O. ficus-indica*), Blanca Cristalina (*Opuntia sp.*), and Esmeralda (*Opuntia sp.*) of early, early-intermediate, and intermediate late full mature, respectively. High value of loss of fresh weight was found in Naranjona, whereas no significant differences were found among morphospecies in soluble solids, total titratable acidity and pH during the postharvest days. Blanca Cristalina and Esmeralda showed higher content of carotenoids. The cell wall enzymes evaluation showed particular behaviors during the full mature of each species suggesting high biochemical coordination between fruit softening and enzyme activity. This study partially provided information about prickly pear full mature which could enhance our understanding about its full mature and improving its shelf life. (Hernandez-Perez *et al*., 2005)

Prickly pear fruit's pulp has a high pH value (5.6 to 6.5) and total soluble solids content ranging from 11 to 17°Brix (Felker *et al*., 2005; El-Samahy *et al*., 2006). Because of its high content of bioactive compounds such as vitamin C, flavonols, phenolic acids, and betalains (Moussa-Ayoub *et al*., 2011), pear fruits could serve as potential products for enhance human health against degenerative diseases such as cancer, diabetes, or cardiovascular diseases (Jacob *et al*., 2008, Lampila *et al*., 2009).

The process of fruit full mature is a very metabolically active stage of plant life cycle. It involves many different phenomena such as softening, chemical and biochemical changes, nutritional changes. These processes occur as a result of the activity of various enzymes leading to the production of active molecules in plant protection and fruit qualities. This leads to the postharvest deterioration of fruit crops (Tucker, 1993) through changes in cell wall composition by cell wall modifying enzymes (Fischer and Bennett 1991; Barnavon *et al*., 2001).

Ethylene plays an important role in several different plant metabolic processes. Its production is linked to various biotic/abiotic stresses. It plays a central function in the regulation of the full mature processes in climacteric fruits, especially the expression pattern of the two genes which control the last two steps of ethylene production pathway: 1-aminocyclopropane-1-carboxylase (ACC) synthase (*Opaccs-1*) from *Opuntia sp.* And 1-aminocyclopropane-1-carboxylase (ACC) oxidase. These two enzymes are encoded by a gene family and have been isolated and characterized from many nonclimacteric full mature fruits. (Fluhr and Mattoo, 1996; Bleecker and Kende, 2000; Jiang and Fu, 2000; Collazo-Siques *et al*., 2003). The expression of the two enzymes was studied at the different fruit full mature stages of prickly pears (*Opuntia ficus-indica*). Also, their cDNAs were isolated and sequenced using degenerate primers with polymerase chain reaction (PCR). The corresponding genes from prickly pears (*Opuntia ficus-indica*) were named *Opaccs-1* (1-aminocyclopropane-1-carboxylase (ACC) synthase) and *Opacco-1* (1-aminocyclopropane-1-carboxylase (ACC) oxidase) for both genes from *Opuntia ficus-indica* (Collazo-Siques *et al*., 2003). At least one copy of both genes was confirmed by southern blot analysis beside other related homologous sequences in the *Opuntia sp.* genome. Analysis of gene expression using northern blotting showed enhancement of gene expression in full mature fruit tissues of *opaccs-1* gene, whereas *opacco-1* gene expression was highly induced in full mature fruit tissues compared to the green ones (Collazo-Siques *et al*., 2003).

Taif is known for the cultivation of roses and fruits particularly: honey-sweet figs, grapes, prickly pear and pomegranates. In Saudi Arabia, efforts are currently under way to enhance the prickly pear production and to increase its introduction into various common foods. This has increased the need to evaluate the nutritional and pharmacological properties of prickly pear fruit. In this report, we studied the gene expression pattern of *Opaccs-1*and *Opacco-1* genes in *Opuntia ficus-indica* cultivars from Taif governorate, Saudi Arabia. The expression of these full mature genes was studied and linked to other fruit quality characteristics including chemical, biochemical, nutritional, and antioxidant qualities. The results of this study shed light on these values and qualities of Saudi prickly pear cultivars and will help to detect molecular markers of full mature stages.

2. Material and Methods

Preparation of prickly pears samples

Two Prickly pear (*Opuntia*) cultivars were used in this study: Shafawi (early ripening) and Toti (intermediate-late ripening). These materials were collected from Taif governorate, Shafa region. Fruit color is yellow (Shafawi) and red (Toti). Samples were collected at three different stages of ripening after 60 days of flowering: immature stage, mature stage, and the full mature stage (0, 14, and 21 days from ripening starting date). Fruits were washed two minutes under tap water with a nailbrush. Skin was removed from the fruits manually after removing of uncolored sides (top and bottom). Fruits were separated into skin and pulp and frozen until used at -80ºC. For RNA isolation, the pulp of mature stage was lyophilized (freeze-dried), ground into fine powder in a coffee grinder, and stored at -80ºC until

used. For other assays, pulp (containing seeds) was cut into small pieces, ground, for 2 minutes, filtered, and stored at −80˚C until analysis.

Design of PCR primers

DNA primers were designed on the cDNA sequence of two major full mature genes; 1-aminocyclopropane-1-carboxylase (ACC) synthase (*Opaccs-1*) from *Opuntia* sp. and 1-aminocyclopropane-1-carboxylase (ACC) oxidase from *Opuntia* sp (*Opacco-1*) (Collazo-Siques *et al*., 2003). Primers were designed using software on the website of Macrogen company (Macrogen, http://dna.macrogen.com). Table (1) summarizes the primer sequences that were used in this study.

Table (1): Sequence of DNA primers that were used in cDNA synthesis and real time PCR.

Primer	Sequence 5' → 3'
Opaccs1F	GGTTCCGAGTGGGGATTATT
Opaccs1R	TGAAATGGTGGTGCCTGTAA
Opacco1F	AGTGGCCTTCAGCTTCTCAA
Opacco1R	CCCGGGTTGTAGAATGAGG

Biochemical and nutritional analysis

Total soluble solids (TSS, expressed as ˚Brix), titratable acidity (expressed as percentage of citric acid), and pH was analyzed. TSS was measured in the juice of the fruit pulp of the full mature stage using table refract meter (Model N-50E; Atago, Tokyo, Japan) and expressed as ˚Brix at 20°C, according to AOAC (1994). Titratable acidity was evaluated according to AOAC (1994) and the pH was determined in the fruit juice of the full mature stage using a microprocessor pH meter (Model: pH 211, HANNA instruments). The results were the average of at least three replicates. Finally, the ˚Brix to acid ratio for each sample was calculated by dividing the ˚Brix value by % of titratable acidity.

Acidity

Acidity was measured by two methods; the first method included using of pH meter at room temperature according to the method described by AOAC (1994). In the second method, titratable acid content was determined by titrating 10 ml juice sample with a solution of 0.1 N NaOH to an end point of pH 8.2 using a digital pH meter and the results were expressed as percentage of citric acid (gm/100ml). The titrated volume of 0.1 N NaOH was recorded and percent titratable acidity (gm citric acid per 100 ml juice) was calculated using the following formula: Titratable acidity (100%) = 0.075 * titrated volume of NaOH (ml) * 0.1 (normality of NaOH) x 100/volume of prickly pear juice (Jayasena and Cameron, 2009). Finally, the ◦Brix to acid ratio for each sample was calculated by dividing the ◦Brix value by % titratable acidity.

Total anthocyanins

Anthocyanin content was measured based on the methods described by Iland *et al.* (1996, 2000). Prickly pear juice (200 µl) was added to 3.8 ml 1 M HCl and incubated for three hours at room temperature. Absorbance of the solution was measured using spectrophotometer at 520 nm using 1 M HCl as a blank. The total anthocyanin was calculated from the following equation:

Anthocyanins (mg/ml) = A520 X DF X 1000^a / 500^b X 100^c.

A520 = Absorbance at 520 nm.

DF = dilution factor of juice in 1 M HCl.

a = 1000 for mg.

b = absorbance of 1% malvidin.

c = df of malvidin.

Phenolic compounds content

Three groups of phenolic compounds were measured in the juice of prickly fruit pulp of the full mature stage. Total flavonol and flavone content, total flavanone and dihydroflavonol content, and total polyphenolics content were measured according to (Kosalec *et al*., 2004). The spectrophotometric assay was applied for quantification of total flavones/flavonols and expressed as quercetin equivalent. For the quantification of flavanones and dihydroflavonols of prickly pear, 2, 4-dinitrophenylhydrazine method (Popova *et al*., 2004) was used. Total polyphenolics content was measured by the Folin–Ciocalteau procedure.

HPLC analysis of sugars (carbohydrates)

Hewlett-Packard APS-2 Hypersil column (4.6 x 250 mm, 5µm particle size) column was used for quantitative analysis of sugars (sucrose, glucose, and Fructose). Acetonitrile with sodium dihydrogen phosphate was used for elution. The elution program was started from 0 to 100% of the eluent for 12 minute duration with flow rate 1.5 ml/min was used at temperature of 35ºC. Injection volume was 20 µl and sugars were detected with Refractive Index.

Antioxidant and DPPH scavenging activity

Scavinging effect of prickly pear samples corresponding to the quenching activity of 1, 1-diphenlyl-2-picrylhidrazyl (DPPH) was estimated as described by (Abd El Hady and Hegazi, 2002; Lahouel *et al*., 2004). Juice samples (500 µl) were mixed with the same volume of 60 µM of DPPH solution and were incubated in dark for 30 min at room temperature. The absorbance was measured at 517 nm. The percentage of scavenging effect was determined by comparing the absorbance solution containing the test sample to that of blank sample as follows: % DPPH Scavenging activity = $(A_0-A_1)/A_0$ x100%; Where, A_0 measurement of the blank, A_1 measurement of the sample.

HPLC analysis of flavonoids

Prickly pear juice samples were assayed on HPLC Agilent Model 1100 system on a Hewlett-Packard Phenomenex Luna C18 column (4.6 x 250 mm, 10µm partical size), separated by using a 13-min linear gradient from 100% 100 mM ammonium acetate (pH 5.5) to 100% methanol at a flow rate of 1.5 mL/ min with oven temperature of 35ºC and injection volume of 20 µl. The nine standards were monitored by A260 nm. For calibration, authentic compounds were dissolved in ethanol and used as standards. Peak areas were converted to micrograms.

Isolation of RNA

Total RNA was isolated from pulp samples from the mature stage of *Opuntia ficus – indica* cultivars using TriReagent solution (MRC, USA) according to manufacturer instructions. RNA was fractionated on 1% agarose gel to check its quality and their concentration was measured by spectrophotometry.

cDNA synthesis and Real time PCR

Opaccs-1 and *Opacco-1* transcripts was determined using one step QuantiTect Cyber Green RT-PCR kit (Qiagen) according to the manufacturer instructions and the iCycler iQ Real Time PCR (Biorad, USA). This includes the reverse transcription for 30 min at 50ºC, initial activation step at 95ºC for 15 min, 45 cycles of denaturation for 15 s at 95ºC, annealing for 30 s at 55ºC, and extension for 30 s at 72ºC. Ten nanogram of total RNA (1×10^{-3} diluted) were included as template for *opacco-1* or *opaccs-1* transcripts. Serial dilutions (1×10^{-2}, 1×10^{-4}, 1×10^{-6}, 1×10^{-8}, and 1×10^{-10}) of total RNA were prepared for evaluation of 18S transcript for the standard curve and were included on each plate (El-Shehawi *et al*., 2007; Van Dijk *et al*., 2009). The fold increase in gene expression was estimated according to the equation: fold in gene expression = $2^{\Delta Ct}$, where ΔCt is the change in threshold cycles (Livak and Sthmittgen, 2001).

Statistical Analysis

One-way ANOVA was used to assess the statistical significance of changes in all indices with the level of significant difference set at $p \leq 0.05$. Statistical analysis software (SPSS 16.0.0 release; SPSS Inc., Chicago, IL) was used for all analyses.

3. Results

Collection and preparation of prickly pear samples

Prickly pear fruit samples were collected at the three different ripening stages. The main morphological features of the fruits at the mature and full mature stages of the two cultivars are shown in Figures (1). No difference in the appearance of immature and the mature stages, therefore the first was excluded.

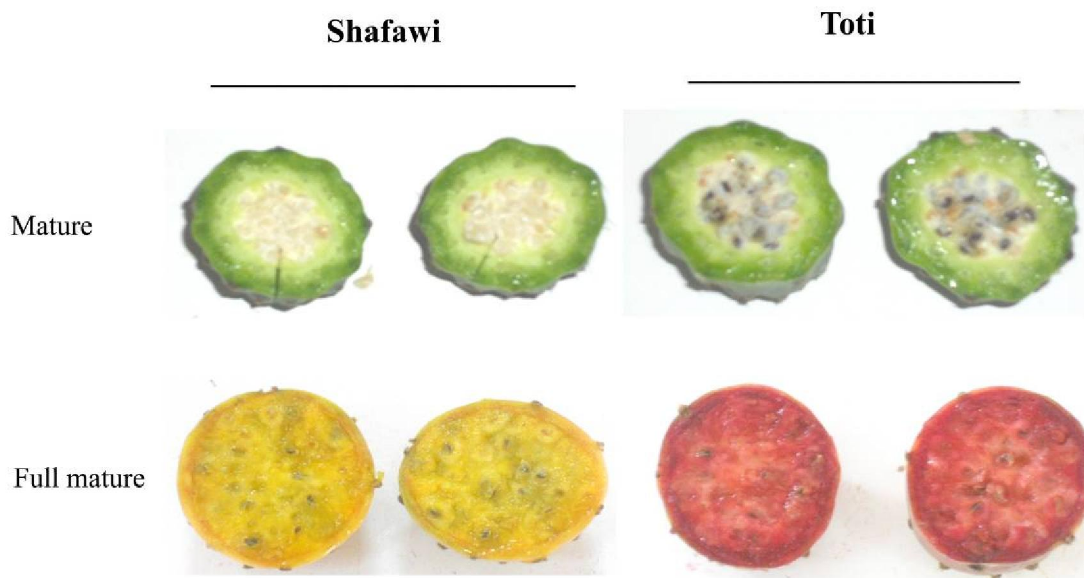

Figure (1): Cross section in prickly pear fruits (Shafawi and Toti) at the mature and full mature stages.

Biochemical and nutritional analysis

The collected fruit samples of prickly pear were used to prepare fruit juice. It was difficult to obtain juice from the immature and mature fruits; therefore measurements were limited to the full mature stage. The results of the biochemical analyses (TSS, titratable acidity, and pH) of full mature fruit samples of the two prickly pear cultivars are presented in

Table (2). Total soluble solids were 8.6±1.71 and 12.6±2.08 ºBrix for Shafawi and Toti juice, respectively. These results were between 9–10.5 ºBrix intervals which is consistent with the interval reported by Sáenz *et al*. (1998) for total soluble solids in prickly pear. Titratable acidity was measured to estimate the organic acid content in the fruit (Stintzing, 1999). Titratable acidity was low in the Shafawi juice (0.057 citric acid) compared to the Toti juice (0.115 citric acid). No significant differences were found between the two cultivars in the pH value of the juice (Table 2). The pH values were in the range already described in other prickly pear cultivars, confirming that this fruit is a low acidic food (Sep´ulveda and S´aenz, 1990; Stintzing *et al.,* 1999) (Table 2). The flavone content in the Shafawi cultivar was 3 µg/ml juice and 4 µg/ml juice in the Toti cultivar (Table 3), whereas the flavonone content was 0.01 and 0.08 mg/ml juice in the Shafawi and Toti cultivars respectively. Total phenolics were 0.276 mg/ml juice for Shafawi and 0.408 mg/ml juice for Toti (Table 3).

Table (2): Biochemical measurements of prickly pear fruit juice at the full mature stage.

Samples	TSS °Brix (%)	Acidity		°Brix/acid ratio (%)
		pH	Titratable acidity Citric acid (gm/100 ml)	
Shafawi	8.6[b]	5.90 [b]	0.057 [a]	150.87 [a]
Toti	12.6[a]	6.23 [a]	0.115 [a]	109.56 [b]

TSS: Total Soluble Solids (°Brix) as percent of sugar (sucrose).
Values within a column followed by the same letter (s) are not significantly different at the $p \leq 0.05$ level according to the one way ANOVA analysis.

Table (3): Flavonoids and anthocyanine content of prickly pear (*Opuntia ficus-indica*) cultivars at the full mature stage.

Samples	Flavone µg/ml juice	Flavanone mg/ml juice	Total phenolic mg/ml juice	Anthocyanin content mg/100 ml juice
Shafawi	3[a]	0.01[b]	0.276[b]	1.92[b]
Toti	4[a]	0.08[a]	0.408[a]	4.6[b]

Flavone (expressed as Quercetine Equivalent, QE), Flavonone (expressed as Naringenin Equivalent, NE), Anthocyanine and total phenolic (expressed as Gallic Acid Equivalent, GAE).

Values within a column followed by the same letter (s) are not significantly different at the $p \leq 0.05$ level according to the one way ANOVA analysis.

DPPH scavenging activity was used to estimate the antioxidant activity of fruit juice of the two prickly pear cultivars. Although Toti showed higher levels of total phenolics, anthocyanins, flavones and flavonones compared to the Shafawi cultivar, the Toti capacity to scavenge the DPPH (28%) was lower than that of the Shafawi one (42.9%). This result indicates that the Shafawi has higher antioxidant activity compared with the Toti juice.

Antioxidant activity of fruit extracts.

Table (4): Antioxidant activities (expressed as DPPH free radical scavenging activity) of prickly pear (*Opuntia ficus-indica*) cultivars.

Samples	Inhibition of DPPH %
Shafawi	42.9[a]
Toti	28.0[b]

Values within a column followed by the same letter (s) are not significantly different at the $p \leq 0.05$ level according to the one way ANOVA analysis.

HPLC analysis of fruit juice

HPLC analysis of juice sugars

The juice content of the three main sugars was estimated using HPLC. Sucrose was not detected in the juice of both cultivars. Toti gave higher concentrations of fructose (11.25 mg/ml) and glucose (20.25 mg/ml) compared to the juice of Shafawi cultivar (fructose 5.37 mg/ml and glucose 11.59 mg/ml) (Table 5, Figure 2).

Table (5): Concentrations of the three main sugars in the juice of Shafawi and Toti cultivars.

Sample	Fructose	Glucose	Sucrose	Total (mg/ml)
Shafawi	5.37[b]	11.59[b]	ND	16.96[b]
Toti	11.25[a]	20.25[a]	ND	31.50[a]

ND: not detected
Values within a column followed by the same letter (s) are not significantly different at the $p \leq 0.05$ level according to the one way ANOVA analysis.

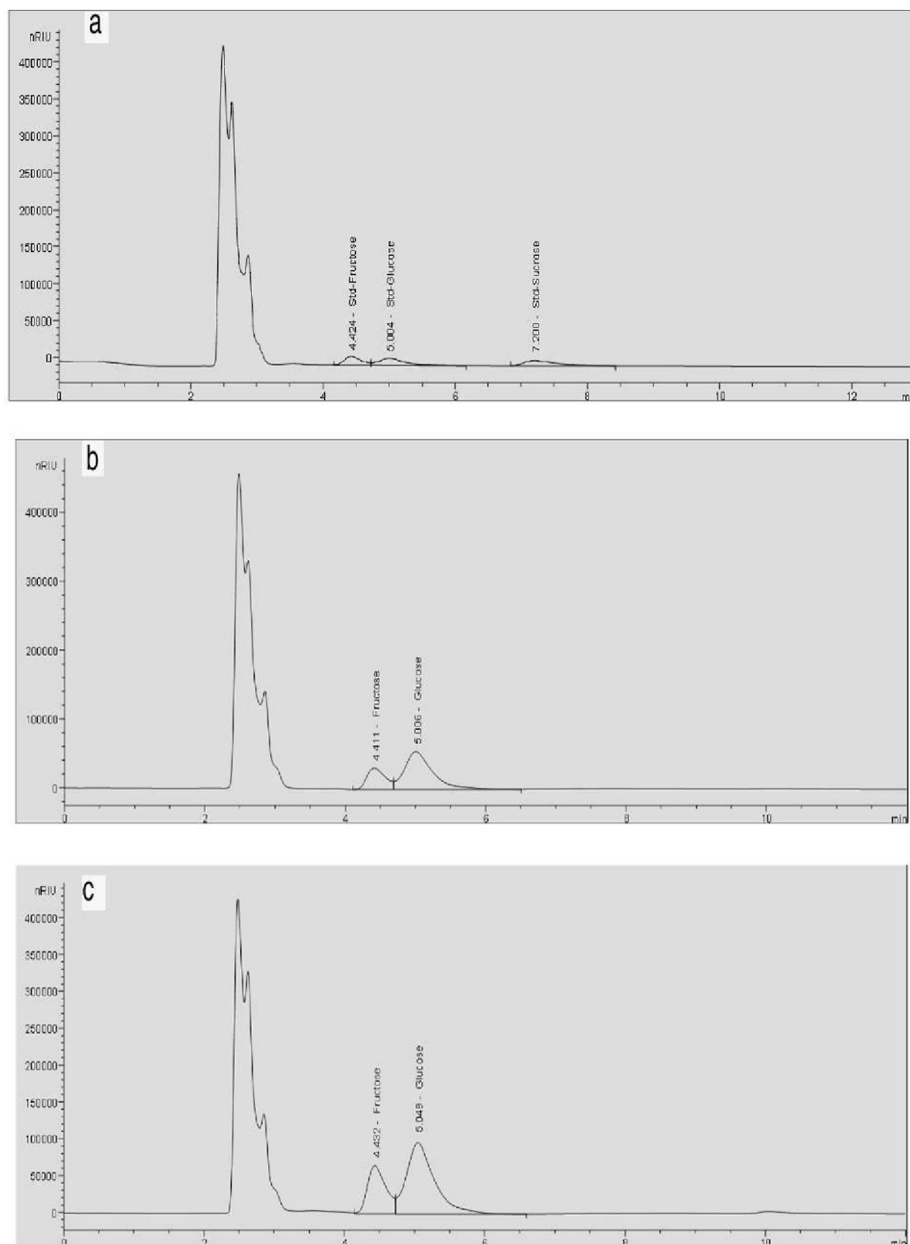

Figure (2): Sugar content using refractive index of Shafawi and Toti cultivars. **a:** Standard mix, **b:** Shafawi juice, **c:** Toti juice.

HPLC analysis of flavonoids

Samples from the full mature stage of Shafawi and Toti prickly pears were HPLC analyzed against nine different standards of flavonoids. The nine standards were divided into two mixes for better separation on the chromatogram. Mix I included Cyanidine chloride, Myricetin, Quercetin, and Chrysin. MixII included Caffeic acid, Delphinidin chloride, Malvidin chloride, Naringenin, and Galangin. All nine standards were detected in the Toti samples, whereas only Chrysin and Galangin were detedcted in the Shafawi samples. Delphinidine showed the highest concentration (15.17 µg/ml) among the nine standards in the Toti samples, while it was not detected in the Shafawi samples. Total flavonoids of these nine chemicals were present in Toti at much higher concentration compared to the Shafawi samples, about 19 fold (Table 6, Figure 3).

Table (6): Concentration of flavonoids (µg/ml) in the Shafawi and Toti prickly pear juice samples measured by HPLC.

Sample	Cyanidine chloride	Myricetin	Quercetin	Chrysin	Caffeic acid	Delphinidine chloride	Malvidine chloride	Naringenin	Galangin	**Total**
Shafawi	0	0	0	0.31	0	0	0	0	0.65	**0.98**
Toti	0.54	0.10	0.12	0.02	0.28422	15.17	1.11	0.44	1.27	**19.05**

cDNA synthesis and Real time PCR

RNA was isolated from the pulp of the mature stage of the two cultivars of prickly pear (Shafawi and Toti) and used for analysis of ***opaccs-1*** and ***opacco-1*** gene expression using real time PCR. Prickly pear pulp ripens faster than the peel. At the mature stage, the ***opacco-1*** is highly expressed in the flesh compared to the ***opaccs-1*** gene. The same pattern is found at the full mature stage, but the expression is much lower for both genes (Collazo-siques *et al.*, 2003). ***Opacco-1*** expression was restricted to the mature and full mature pulp and peel, whereas ***opaccs-1*** was low expressed in mature cladodes, mature green fruit pulp, and mature green fruit peels. Therefore, we restricted our study to compare the expression of ***opaccs-1*** and ***opacco-1*** in the pulp at the mature stage because it is the stage that showed differential expression of the two genes in the pulp (Table 7, Fig. 4). *Opacco-1* expression was higher than the expression of *opaccs-1*; the threshold cycle (Ct) for the first was 23, whereas the Ct for the second was 32 for both cultivars. The fold increase in gene expression was estimated according to the equation: fold increase in gene expression = $2^{\Delta Ct}$. According to this equation *opacco-1* is expressed 2^9 times higher than the *opaccs-1* gene (Livak and Sthmittgen, 2001). *Opaccs-1* works upstream of *opacco-1* in the ethylene production pathway. This means that *opaccs-1* could be more efficient in the enzyme activity because its transcript level is lower (Figure 4).

Table (7): Summary of the threshold cycles (Ct) of *opacco-1* and *opaccs-1* genes at the mature stage of the Shafawi and the Toti prickly pear cultivars.

Cultivar	Opacco-1	Opaccs-1	fold increase in gene expression
Shafawi	23	32	512
Toti	23	32	512

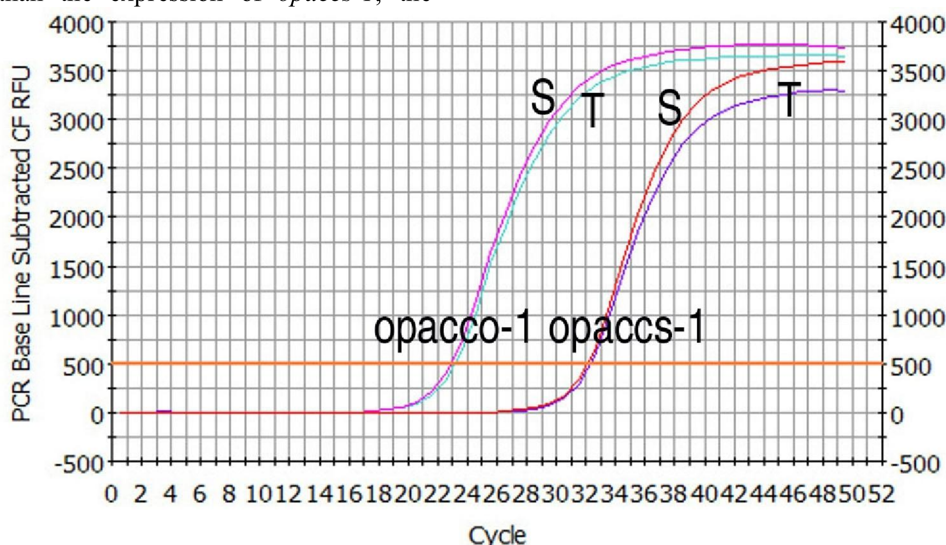

Figure (4): Estimation of gene expression of *opacco-1* and *opaccs-1* genes at the mature stage of the Shafawi (S) and the Toti (T) prickly pear cultivars.

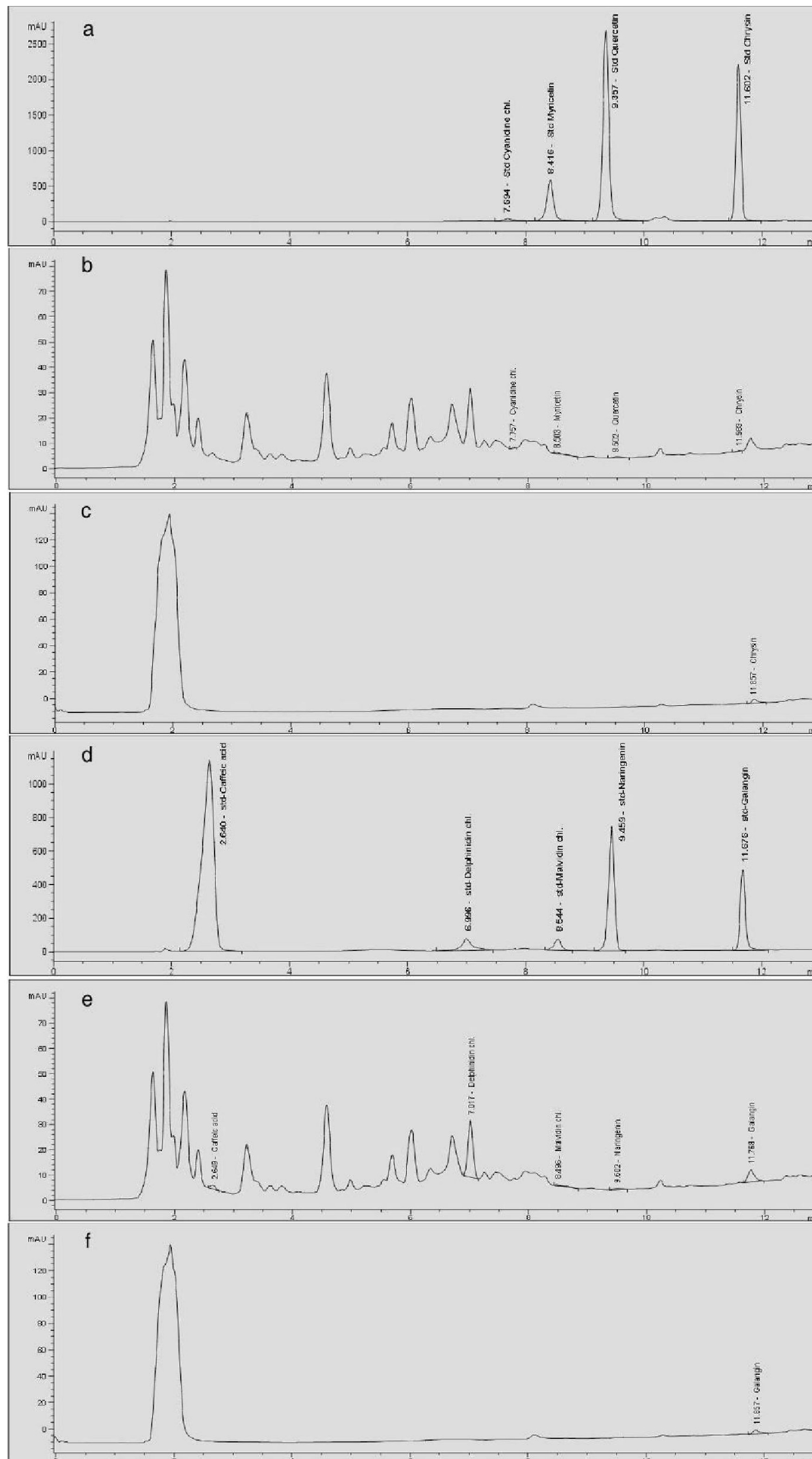

Figure (3): HPLC analysis of flavonoids of Shafawi and Toti prickly pear fruit juice. **a**. Chromatogram of MixI (Cyanidine chloride, Myricetin, Quercetin, and Chrysin), **b.** Chromatogram of the Shafawi juice against mixI, **c**. Chromatogram of the Toti juice against mix I, **d**. Chromatogram of MixII (Caffeic acid, Delphinidin chloride, Malvidin chloride, Naringenin, and Galangin), **e**. Chromatogram of the Shafawi juice against mixII, **f**. Chromatogram of the Toti juice against mixII.

4. Discussion

Samples of prickly pear cultivars (Shafawi and Toti) were collected from Taif governorate. The nutritional value of the pulp of the two cultivars was evaluated. The results indicated that the Toti cultivar seems to be higher in its nutritional value, especially the bioactive components like anthocyanines and flavonoids. Also, the Toti showed higher concentrations of sugars; glucose and fructose. Phenolic compounds are very important secondary metabolites of plants since they are the most abundant constituents present in most fruit juices. They represent a group of structurally diverse substances that are present in various amounts.

Nine flavonoids and anthocyanines standards (Cyanidine chloride, Myricetin, Chrysin, Quercetin, Delphinidine chloride, Malvidine chloride, Naringenin, Galangin and Caffeic acid) were used to measure their concentrations in the two prickly pear cultivars under study. The complete separation of all the nine standard flavonoids by HPLC method was very difficult because phenols contain a large number of hydroxyl groups and there are many isomers. Due to the overlapping of the standards, they were divided into two groups. Standard mixI which included Cyanidine chloride, Myrecitine, Quercetine and Chrysine and standard mix II which included Caffeic acid, Delphinidine chloride, Malvidine chloride, Naringenine and Galangin. The HPLC study of these nine standards indicated that the differences among the two cultivars for the total of nine phenolic compounds were significant. At the same time, significant differences among the two cultivars were obtained for each compound.

In this study, the Toti represented two fold of total phenolics and anthocyanines higher than the Shafawi cultivar (Table 3). Similarly, the Toti showed 19 fold higher than the Shafawi in some selected flavonoids and anthocyanines (Table 6). Most of this difference is mainly due to Delphinidine, an anthocyanines because the Toti juice has 15.17 μg/ml compared to the Shafawi juice in which this compound was not detected (Table 6). When we exclude the Delphenidine, the Toti has about four folds of the other selected flavonoids and anthocyanines (Table 6).

The main difference in the content of total phenols depended on several factors such as cultivar, climatic and ecological factors and harvesting method (Klepacka *et al*., 2011). Different fruit juices, analyzed by (Sautter *et al*., 2005), showed variations in the averages of total phenolics among cultivars. One of the most important factors that affect the differences among cultivars may be due to the different genetic background of these cultivars. Other researchers confirm that the content of phenolic compounds may depend on relevant factors including the cultivar, the method applied for extracting the compounds, and the storage conditions. According to Klepacka *et al.* (2011) the amount of phenolic compounds vary according to factors such as, climate, soil condition, cultivar, ripeness, and pH.

DPPH is a stable free radical that is dissolved in methanol and its purple color shows a characteristic absorption at 517 nm. Antioxidant molecules scavenge the free radical by hydrogen donation and the color from the DPPH assay solution becomes light yellow resulting in a decrease in absorbance. Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation (Hatano *et al*., 1989). Although the Toti cultivar represented higher concentrations of flavones, flavonones, total phenolics, anthocyanines (Table 3), specific flavonoids and anthocyanines (Table 6), it exerted a law antioxidant activity represented in the quenching activity of the DPPH (Table 4). The antioxidant activity of prickly pear cultivars was estimated using the quenching activity of DPPH. The Shafawi juice was able to quench 42.9 of the DPPH, whereas the Toti quenching activity was 28.0. This contradiction could be due to the presence of certain antioxidant compounds which have high antioxidant activity. Similar results were obtained by El-shehawi *et al*. (2011), Fahmi *et al*. (2013). The scavenging activity of DPPH radicals has been widely used to determine the free radical-scavenging activity of different matrices (Pereira *et al*., 2006; Oliveira *et al*., 2008; Fahmi *et al*., 2011; Fahmi *et al*., 2013). In this report, results are expressed as the ratio percentage of the absorbance decrease of DPPH radical solution in the presence of extract at 517 nm to the absorbance of DPPH radical solution at the same wavelength. Although, it is well established that the phenols are the main compounds that are responsible for antioxidant activity in juices (Xia *et al*., 2010), the present result showed no correlation between the total phenols and the antioxidant activity. The relationship between phenolic compounds and antioxidant capacity was inconsistent among the results from different studies. Pereira *et al.* (2006) and Sousa *et al.* (2008) proved that antioxidant activity values were statistically correlated with total phenols content in their analyzed samples. In one study, malvidin-3-glucoside showed the highest antioxidant capacity in grape anthocyanins (Rivero-Perez *et al*., 2008). Although total phenolic index was lower in grape flesh than in grape skin because anthocyanins were absent in the flesh, they possessed equal amounts of reactivity to hydroxyl radicals (Falchi *et al*., 2006). In another study, the results also showed that the anti-radical activity was due to the flavanols, rather than

anthocyanins (Arnous *et al*., 2002). The result suggested that perhaps the antioxidant capacity of phenolics has a concentration saturation limit and above this limit, the activity could not increase further with the concentration (Dani *et al*., 2012). The mechanism was mainly speculated to react directly to generate phenoxyl radicals (Yoshimura *et al*., 2003), which was stable and cuts off the reaction chains. The chemical functional group and structure is OH for antioxidant capacity of phenolic compounds. When the OH added onto the flavonoid nucleus, the activity enhanced, OCH3 groups, the activity diminished. The results were proved by (Majo *et al*., 2005). The o-diphenoxyl groups in resveratol were determined to exhibit higher antioxidant activity than other compositions (Qian *et al*., 2009).

The expression of two fruit ripening related genes (*opaccs-1 and opacco-1*) was analyzed by real-time PCR. Together, they are responsible for the production of ethylene and repining of the fruits. The results of this study revealed that the transcription activity of these repining genes was higher at the mature stage. Similar results were obtained by Collazo-Siques *et al*. (2003). This is logically accepted since transcription precedes translation and the production of enzymes and consequently production of secondary metabolites related to ripeness. The obtained results do not give a direct relationship between the nutritional value of fruits and the difference of gene expression. On the other hand, *Opacco-1* expression was higher than the expression of *opaccs-1*; the threshold cycle for the first was 23, whereas the threshold cycle for the second was 32 for both cultivars. *Opaccs-1* works upstream of *opacco-1* which means that *opaccs-1* could have more efficient enzyme activity and/or higher translation rate that compromise its law transcript level (Table 7, Figure 4). These molecular differences could be due to some molecular differences such as, differences in the combination of cis/trans acting elements in the promoters of these genes, differences in full mature signal transduction pathway in both cultivars, or differences in the interaction of biotic and abiotic factors with the secondary messengers on the expression of these full mature genes.

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