Evaluation of Egyptian pomegranate cultivars for antioxidant activity, phenolic and flavonoid contents

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Aim
The aim of the present study was to evaluate total phenolic and flavonoid contents as well as antioxidant activity of the peel, juice and seed extracts of pomegranate fruit from different Egyptian cultivars. The selected cultivar was subjected for evaluation of the effect of peel homogenate on volatile compounds in juice supplemented with this homogenate.

Background
Pomegranate fruit is a rich source of natural antioxidants; it has wide applications in food and pharmaceutical industry.

Materials and methods
Five cultivars of Egyptian pomegranate were subjected to a comparative study of phenolic and flavonoid contents as well as its antioxidant activity. The phenolic compounds were determined through high-performance liquid chromatography, and the volatile compounds of selected cultivar peel were added to juice through gas chromatography and gas chromatography–mass spectrometry.

Results
While the total phenolic content varied between 5.21 mg gallic acid equivalent/g in PG4 fruit juice and 17.24 mg gallic acid equivalent/g in PG1 peel, the total flavonoid content ranged from 9.64 in PG2 juice to 34.28 mg rutin/g in the peel of PG1. All peel, juice and seeds extracts exhibited high antioxidant activities, evaluated using 1,1'-diphenyl-2-picrylhydrazyl and β-carotene assays. Gallic acid, chlorogenic acid and caffeic acid were the predominant phenolic compounds of the pomegranate cultivars. A total of 17 volatile compounds were identified, including six monoterpenes, three monoterpenoids, three aldehydes, three esters, and two alcohols.

Conclusion
Peel, juice and seed of Egyptian pomegranate cultivars contain significant amounts of phenolics and flavonoids contents. However, the peel contains higher contents compared with juice and seeds. Peel homogenate of the selected cultivar showed a remarkable effect on volatile compounds when used for fortification of the pomegranate juice.

Keywords:
antioxidant activity, juice, peel homogenate, pomegranate cultivars, volatile compounds

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Introduction

Nowadays the increased demand for dietary antioxidants has prompted research in the field of pharmaceutical and dietary supplement. The pomegranate fruits are one of the red fruits considered as good sources of the natural antioxidants. There are many commercial beverages containing high contents of polyphenols, which base their marketing strategies on antiradical activity. Recent investigations have recommended the intake of red fruits and their juices for the prevention and reduction of coronary heart disease, cancers and ageing , [1]. It has been reported that pomegranate juice is one of the important sources of anthocyanins (cyanidin, delphinidin and pelargonidin) and some of the phenolics and tannins (such as punicalin, punicalagin and ellagic acid) [2]. Consumption of pomegranate fruit has nutritional and medical benefits, such as reduction in oxidative stress, and platelet aggregation, as well as anticancer, antibacterial, antiviral, and atherogenic modifications to low-density lipoprotein [3].

Pomegranate fruit has wide applications such as in flavouring and colouring, healthcare and cosmetic products. The peel and kernels are the main by-products of pomegranate juice processing and can be used as a garnish for salads and desserts [4].

Recent studies revealed that artificial antioxidants currently used have been found to show several health effects such as teratogenic and carcinogenic effects [5].

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Therefore, the growing interest for natural ingredients has received much attention as sources of biologi cally active substances as antioxidants, antimutagens and anticarcinogens [6]. Several industries producing pomegranate juice, as well as pharmaceutical companies extracting health beneficial compounds from the fruit, have been developed [7]. The major waste from pomegranate processing is the peel, which contains high concentration of phenolic compounds, such as anthocyanins, phenolic acids and tannins. Grooves [8] found that pomegranate peel extract had a strong biological activity and also enhanced liver and kidney functions in animal models.

Among the major problems found in pomegranate juice studies is the low concentration of volatile compounds, leading to low intensities of both odour and aroma [9]. The present study aimed to evaluate the cultivars' variation on the phytochemical contents, phenolic compounds and antiradical activity of some Egyptian pomegranate juices, compared with their peel and seed extracts. Furthermore, this study aimed to evaluate the flavour changes in selected fruit juice after addition of peel homogenate.

Materials and methods

Chemicals

Folin–Ciocalteu, gallic acid, chlorogenic acid, caffeic acid, catechol, vanillic, *P*-coumaric acid, 1,1'-diphenyl-2-picrylhydrazyl (DPPH°), β-carotene, linoleic acid, butylated hydroxyl anisol (BHA) and *tert*-butylated hydroxyl quinone (TBHQ) were purchased from Sigma. All solvents were of analytical grade. The study on plant fruit did not include any human experimentation.

Plant material

Five cultivars of pomegranate, namely wonderful100–1 'PG1', wonderful128–29 'PG2', Mule head 121–22 'PG3', Mule head 118–19 'PG4' and 128–29 'PG5', were obtained from the research field of National Agriculture Research Center, Giza, Egypt. Cultivars were grown under the same agricultural and crop conditions during 2014–2015.

Preparation of peel, juice and seed extracts

Pomegranate fruits were washed and cut into four pieces; the seeds were separated manually and ground in a mixer for 30 s and then filtered through cheese cloth to obtain the fruit juice. The seeds and peels were washed with excess water for removal of sugars as well as adhering materials from seeds, and then were sun-dried. Peels were directly sun-dried. Both peels and seeds were separately powdered in a grinder to get 40-mesh size powder. Finely powdered peels or seeds (5 g) were separately blended for 2 min (Waring blender) with 300 ml of distilled water. Each mixture was then left in the dark at room temperature for 1 h before filtration (Whatman No. 1) and centrifuged at 865*g* for 10 min at 5°C. The extracts were separately concentrated to dryness under reduced pressure at 40°C. Then all were kept at −20°C until analysis.

Determination of total phenolics

Total phenolics in the cultivar extracts were determined according to the method proposed by Ben Nasr *et al.* [10]; the phenolics were expressed as gallic acid equivalents (GAE). The estimation of phenolic content in the extracts was carried out in triplicate and then averaged.

Estimation of total flavonoid

The total flavonoid content of the extracts was measured by applying a colourimetric method proposed by Ibrahim [11]. The flavonoid content was calculated and expressed as rutin equivalents.

High-performance liquid chromatography analysis

Each sample was centrifuged in an Eppendorf tube (4 min at 5000*g*) and then the centrifuged supernatant was allowed to pass through a Millipore filter of 0.45 μm.

High-performance liquid chromatography conditions

The filtrate was subjected to separation by high-performance liquid chromatography with the following condition: flow rate 1 ml/min; Agilent 1100 series (Agilent Technologies/Hewlett Packard, Waldbronn, Germany), quaternary pump (G1311A), degasser (G1322A), thermostated autosamples (G1329A), variable wave length detector (G1314A); and column: Zorbax 300SB C_{18} column (Agilent Technologies, USA; made in Netherland). Injection was carried out at wave lengths 280 nm for separation. The solvent system consisted of methanol with 0.1% formic acid (solvent A); and acetonitrile with 0.1% of formic acid (solvent B). The gradient system was programmed as follows: starting at 30% solvent B, increasing to 60% over 10 min, increasing to 100% over 5 min and then returning to 30% over 5 min. The injection was carried out under ambient temperature. The identification of the components in the sample was done by comparing their retention time and UV spectra with those of external standards.

Antioxidant activity measurement

DPPH° radical scavenging activity

The ability to scavenge 1,1'-diphenyl 1–2-picrylhydrazyl (DPPH°) radical by tested samples was estimated by the method proposed by Fawole and Opara [12].

The scavenging activity was calculated using the following equation:

$$\text{Scavenging activity}\,(\%) = \frac{(\text{Absorbance}_{\text{Blank}} - \text{Absorbance}_{\text{Sample}})}{\text{Absorbance}_{\text{Blank}}} \times 100.$$

BHA and TBHQ were used as positive controls. The values were presented as the mean of triplicate analyses.

β-Carotene bleaching assay

Antioxidant activity of the aqueous solution was determined using a β-carotene/linoleic acid system, as described by Nuncio-Jáuregui *et al.* [13]. Antioxidant activity was calculated as follows:

$$AA = \left[1 - \left(A_{s(0)} - A_{s(120)}\right)/\left(A_{b(0)} - A_{b(120)}\right)\right] \times 100,$$

where AA is the antioxidant activity, $A_{S(0)}$ is the absorbance of sample at 0 min, $A_{S(120)}$ is the absorbance of sample at 120 min, $A_{b(0)}$ is the absorbance of blank at 0 min and $A_{b(120)}$ is the absorbance of blank at 120 min.

Preparation of peel homogenate for juice supplementation

The peel of the selected pomegranate cultivar was grated to obtain 'pomegranate peel homogenate'. The peel was blended in a food processor and frozen (−20°C) until juice preparation.

Pomegranate juice supplemented with peel homogenate

Fruits were harvested when fully mature according to commercial practice and immediately transported to the laboratory. Pomegranates with defects (sunburn, crack, bruise and cut in the husk) were discarded. Fruits were washed under cold tap water and drained. They were manually cut-up and the outer skin, which encloses hundreds of fleshy sacs, was removed, and then their juice localized in the sacs was extracted using a domestic blender; the resulting juice was filtered through cheesecloth to remove particles. At the same time, the sample was prepared by straining juice (1.5 g/100 ml) for 12 h after the addition of peel homogenate. After straining using a strainer (mesh size<1 mm) to remove rind particles, the juice volatile compounds were extracted and analysed.

Aroma volatiles composition

Extraction of volatile compounds

The aroma volatiles of juice from pomegranate fruit were isolated using a dynamic headspace system. The samples were purged for ~3 h with nitrogen gas (grade of N_2>99.99%) at a flow rate 100 ml/min. The headspace volatiles were swept into cold traps containing diethyl ether and pentane (1 : 1, v/v), and held at −10°C. The solvents containing the volatiles were dried over anhydrous sodium sulfate for 1 h. The volatiles were obtained through evaporation of the solvents under reduced pressure.

Gas chromatography analysis

Gas chromatography analysis was carried out using the Perkin Elmer (PerkinElmer, North Carolina) Auto system equipped with flame ionization detector. A fused silica capillary column DB-5 (60 m×0.32 mm i.d.) was used. The oven temperature was maintained initially at 50°C for 10 min, and then programmed from 50 to 180°C at a rate of 3°C/min. Helium was used as the carrier gas, at a flow rate 1.0 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The retention indices [Kovats index (KI)] of the separated volatile components were calculated with hydrocarbons (C_8–C_{22}) as references.

Gas chromatography–mass spectrometry analysis

The analysis was carried out using a gas chromatography–mass spectrometry under the conditions listed in Table 1.

Compounds identification

The linear retention index (KI) values for unknowns were determined on the basis of retention time data obtained by analysing a series of normal alkanes (C_8–C_{22}). Volatile components were positively identified by matching their KI values and mass spectra with those of standards, and were also run under identical chromatographic conditions in the laboratory [14].

Statistical analysis

Results were given as mean±SD of three independent determinations. One-way analysis of variance and least significant difference was performed using SPSS for (IBM, Chicago) Windows version 16.0 to determine

Table 1 GC–MS analysis conditions

Apparatus	Hewlett-Packard (Canada) (5890)/mass spectrometry
Column	DB-5
Length	30 m
Internal diameter	250×10^{-6} m
Carrier gas	Helium
Total flow	1 ml/min
Mode	Pulsed split (1 : 10)
Injection temperature	220°C
Detector temperature	250°C
Oven temperature	Initially at 50°C for 10 min 50–180°C at a rate of 3°C/min; final temperature 230°C
Ionization voltage	70 eV
Mass range	m/z 39–400 mu

GC–MS, gas chromatography–mass spectrometry.

any significant difference among various treatments applied to compare the means. Differences were considered to be significant at P value less than 0.05 [15].

Results and discussion

Total phenolics and flavonoid contents

In the present study, five Egyptian cultivars were subjected for evaluation of the phenolic and flavonoid contents. The obtained data are compiled in Table 2.

The obtained data showed statistically significant ($P \leq 0.05$) variation between the studied cultivars in total phenolic and flavonoid contents. While total phenolic content varied between 5.21 and 17.24 mg/g GAE for juice and peel of PG4 and PG1, respectively, the total flavonoid content ranged between 9.64 and 34.28 mg/g rutin equivalents in PG2 juice and PG1 peel, respectively (Table 2).

The results tabulated in Table 2 revealed that PG1 peel exhibited the highest concentration of total phenolic and flavonoid content compared with the other cultivars. These results were in agreement with those of a study conducted by Poyrazoglu *et al*. [16] on the Spanish pomegranate [17]. It was found that total phenolic content in juices varied from 144 to 10 086 mg GAE/l; the variation in total phenolic and flavonoid contents in pomegranate juice was correlated with the processing and preparation technology. The consumption of about 250 ml PG1 cultivar juice can cover the recommended daily intake of phenolic compounds, as mentioned by Gil *et al*. [18]. Table 2 showed that the total phenolic content of peel extract was higher than that of juice and seed of all the Egyptian cultivars under investigation, which explain that peel extract had higher antioxidant activity than those of the seed, and juice extracts. The obtained results were in agreement with those reported by Antolovich *et al*. [19].

The phenolic compounds of pomegranate peel, juice and seed extracts were studied by high-performance liquid chromatography on reversed phase column. The results in Table 3 illustrate that phenolic compounds of pomegranate parts consisted of six phenolic acids and catechol.

The obtained data showed that gallic acid and chlorogenic acid followed by caffeic acid were the predominant compounds for pomegranate cultivars. Moreover, gallic acid and chlorogenic acid were higher in peel extracts than in juice and seed extracts. Whereas caffeic acid, catechol, vanillic acid and *P*-coumaric acids compounds were relatively higher in juice extract than in the peel one (Table 3).

The obtained results were in agreement with the fact that pomegranate is very rich in polyphenols [18]. In several studies, the phenolic components extracted from pomegranate have been shown to possess antioxidant activity [20,21]. The antimutagenicity of pomegranate peel may be due to its high phenolic content [22].

Table 2 Total phenolic and total flavonoid contents of some Egyptian pomegranate cultivars peel, juice and seeds

Variety	Total phenolic (GAE mg/g FW)			Total flavonoid (RE mg/g FW)		
	Peel	Seeds	Juice	Peel	Seeds	Juice
PG1	17.24±1.11[c]	12.37±1.35	7.24±0.22	34.28±1.47	26.45±0.29	12.31±0.91[a]
PG2	13.58±0.92	9.56±1.17[a]	6.37±1.16[a]	29.65±0.59[a]	23.92±1.34[a]	9.64±0.25
PG3	9.86±1.13[a]	10.91±0.68	6.43±0.29[a]	21.72±0.38	22.59±1.22	10.38±1.34[b]
PG4	10.29±1.28	9.28±0.59[a]	5.21±0.18[b]	30.29±1.29[a]	24.23±0.95[a]	10.68±1.63[b]
PG5	9.58± 1.19[a]	8.67±1.26	5.34±0.32[b]	26.35±1.16	19.84±1.37	12.91±0.88[a]

The same letter within the same column is not significant ($P \leq 0.05$). FW, fresh weight; GAE, gallic acid equivalent; RE, rutinequivalents.
[c]Values are expressed as mean±SD ($n=3$). Significance of letters from statistical analysis.

Table 3 Phenolic composition (mg/g) of different pomegranate cultivars peel, juice and seeds as GAE

	PG1			PG2			PG3			PG4			PG5		
Cultivars	Peel	Juice	Seed	Peel	Juice	Seed	Peel	Juice	Seed	Peel	Juice	Seed	Peel	Juice	Seed
Phenolic compounds															
Gallic acid	5.43	3.25	2.29	5.37	3.12	1.37	4.56	2.85	0.95	3.67	2.72	1.12	2.65	1.91	0.42
Chlorogenic acid	3.52	2.97	0.73	3.46	2.83	0.29	3.31	1.93	0.18	2.35	1.76	0.04	1.42	1.53	0.13
Caffeic acid	0.01	1.95	0.51	0.04	1.85	0.46	0.08	1.72	0.32	0.17	0.94	0.26	0.71	0.84	0.05
Catechol	0.02	0.64	0.62	n.d.	0.54	0.52	n.d.	0.43	0.41	0.13	0.59	0.18	n.d.	0.46	0.07
Vanillic acid	n.d.	0.75	0.04	0.02	0.69	0.03	0.32	0.48	0.05	0.34	0.37	0.21	0.25	0.33	0.03
P-coumaric acid	0.03	0.95	0.01	0.42	0.81	0.05	0.09	0.71	0.02	0.2	0.67	0.04	0.11	0.14	0.02

GAE, gallic acid equivalent; n.d., not detected.

Evaluation of antioxidant activity

The antioxidant activity of natural products can be determined through different procedures [23], due to many reactions and mechanisms underlying the antioxidative processes [24]. In the present study, the antioxidant activity of fruit peel, juice and seed extracts was evaluated using the DPPH° and β-carotene assays.

The scavenging effects of pomegranate juice using DPPH° radicals and β-carotene methods at different concentrations are shown in Fig. 1. DPPH radicals are widely used to investigate the scavenging activity of natural products [25]. The obtained results in Fig. 1 showed a positive relationship between the increase in concentration of pomegranate juice and the increase in the radical scavenging activity. A similar trend had been reported by Kaplan *et al.* [26]. The radical scavenging activity of pomegranate juice based on DPPH or β-carotene assays explain the antimutagenic and anticarcinogenic activity of pomegranate juice [27]. Data in Fig. 2 showed the effect of pomegranate peel and seed extracts on antioxidant activity measured by DPPH° and β-carotene assays compared with BHA and TBHQ. The obtained results indicated that antioxidant activity of the peel extract was higher than that of seeds, which was similar to the results reported by Styger *et al.* [28].

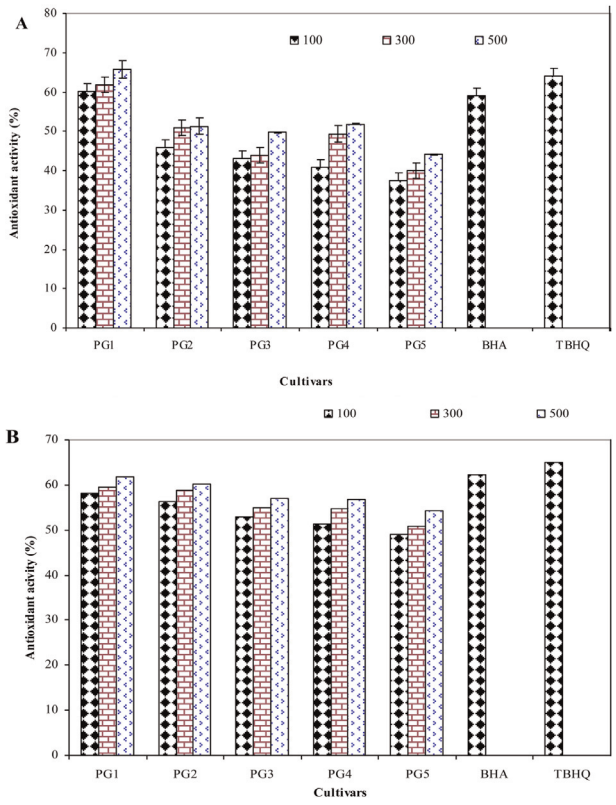
The higher antioxidant activity of pomegranate peel than seed may be attributed to the higher phenolic content [11]. Screening the literature, the antimutagenic activity, antiatherogenic activity in atherosclerotic mice and humans and antioxidant of pomegranate peel were strongly correlated with polyphenol content [29,30].

A strong relationship between the antioxidant activity and total phenolic contents of Egyptian cultivars peel, juice and seeds was found (R^2=0.93, 0.91, and 0.895, respectively). These values were higher in peel extract than juice and seeds, which can be attributed to the higher phenolic content in peel than in juice and seeds.

Effect of peel homogenate on volatile compounds

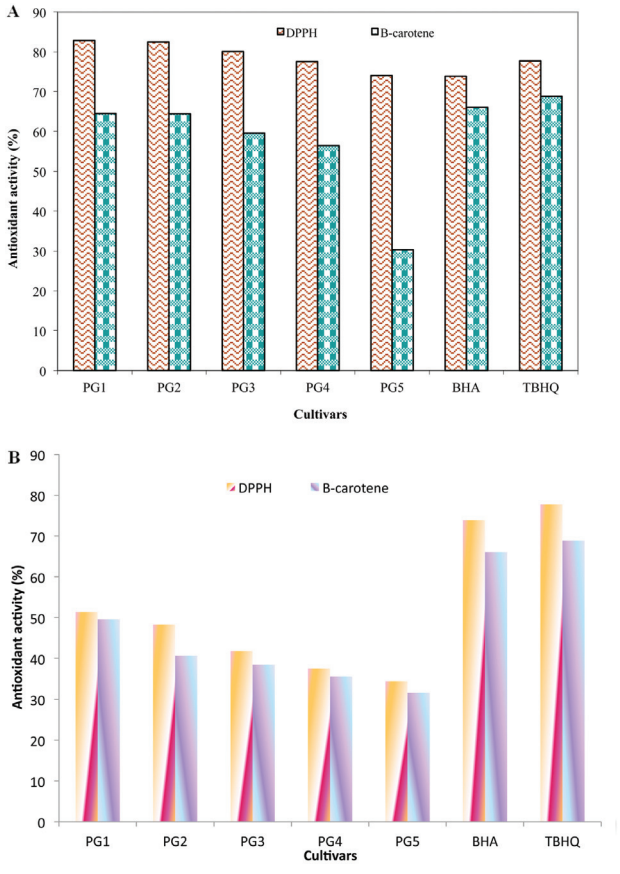
A total of 17 volatile compounds were found in the aroma profile of pomegranate fresh and supplemented with peel homogenate juices (Table 4). The identified volatile compounds included monoterpene hydrocarbons, monoterpenoids, aldehydes, esters and alcohols. Similar

Figure 1



Antioxidant scavenging activity of juice of various pomegranate cultivars as measured by DPPH° (a) and *β*-carotene (b) assays in comparison with BHA and TBHQ at concentration of 100, 300 and 500 µl. BHA, butylated hydroxyl anisol; DPPH°, 1,1'-diphenyl-2-picrylhydrazyl; TBHQ, *tert*-butylated hydroxyl quinone.

Figure 2



Antioxidant activity of different pomegranate peel (a) and seed (b) extracts measured by DPPH° and β-carotene assays. DPPH°, 1,1'-diphenyl-2-picrylhydrazyl.

Table 4 Volatile composition of pomegranate juices

Volatile compounds	KIs	Control juice	Supplemented juice	Identification method[b]
Monoterpenes				
α-Pinene	859[a]	6.12[a]	7.35	ST, MS, KI
β-Pinene	931	4.79	3.82	ST, MS, KI
β-Myrcene	985	8.31	9.65	MS, KI
Limonene	1031	9.62	13.82	ST, MS, KI
γ-Terpinene	1057	2.58	5.71	MS, KI
β-Caryophyllene	1416	0.67	3.28	MS, KI
Monoterpenoids				
Fenchone	1080	4.19	5.39	MS, KI
Camphor	1139	5.74	2.67	MS, KI
α-Terpineol	1186	2.63	4.21	MS, KI
Aldehydes				
Hexanal	791	9.52	3.27	ST, MS, KI
Octanal	998	3.61	2.84	MS, KI
Nonanal	1100	0.76	0.18	ST, MS, KI
Esters				
Ethyl acetate	648	4.72	3.95	MS, KI
Ethyl hexanoate	997	6.49	5.74	MS, KI
Hexyl acetate	1997	1.79	0.81	ST, MS, KI
Alcohols				
3-Hexen-1-ol	856	3.62	4.85	MS, KI
1-Hexanol	876	4.38	6.94	MS, KI

GC–MS, gaschromatography–mass spectrometry; KI, Kovats index. [a]Values are expressed as relative areapercentage to the total identified volatile compounds. [b]Compounds identified by GC–MS(MS)and/or by comparison of MS and RI of standard compound run under similar conditions.

profiles have been previously reported [31]. The identified aroma constituents in the investigated samples (Table 4) can be grouped in five classes: monoterpenes hydrocarbons (α-pinene, β-pinene), their concentrations in fresh juice 6.12 and 4.79%, respectively. While an increase in α-pinene and 7.35% decrease in β-pinene to 3.82% was observed in the supplemented sample. Monoterpenoids included fenchone, camphor and α-terpineol. Aldehydes included hexanal, nonanal and octanal. Esters included ethyl acetate, ethyl hexanoate and hexyl acetate. Alcohols used were 3-hexen-1-ol and 1-hexanol. Generally, a low concentration of aroma compounds in the selected cultivar as well as the supplemented juice was observed. The concentration of volatile compounds depends on several factors such as climatic conditions, maturity and technological factors; as well as storage conditions [32].

The most abundant volatiles in both juices were monoterpenoids especially limonene, which exhibited 13.82 and 9.62% in supplemented and control juice (Table 4), respectively.

The low threshold of a volatile compound gives its importance in flavour of food. Therefore, the high threshold of limonene may reduce its sharing in the volatiles of pomegranate juices compared with α-pinene and β-myrcene. Esters play an important role in fruit and their juice's flavour, its formation through β-oxidation or amino acid pathway [33]. Ethyl hexanoate, ethyl acetate were the predominant esters in studied cultivar juice and supplemented sample with concentration of 6.49, 5.74 and 4.72%, 3.95% in fresh and supplemented juice, respectively. Our result values are lower than the values reported by Granato *et al.* [33].

In general, the addition of fruit peel homogenate increase the total amount of volatile compounds especially monoterpenoids and alcohols and the reduction in aldehydes content (Table 4). These results are in agreement with those of Jung [34] who mentioned that alcohols, which represented 40.76%, followed by terpenoids, are considered as the major compounds in the aroma of pomegranate peel.

The fortification of pomegranate juice with peel homogenate results in a significant increase in monoterpenes, especially limonene, which increased from 9.62% in fresh juice to 13.82% in supplemented juice. In contrast, a decrease in aldehydes was occurred – for example, hexanal (grassy and green odour) was reduced from 9.52 to 3.27% in fresh and supplemented juice, respectively (Table 4).

The low intensity of aroma volatile compounds in studied pomegranate cultivar, even after supplementation with peel homogenate, may result in dislike of pomegranate juice in Egyptian market. The mix technology between

the selected sample and other fruit juices such as apple or orange may be solutions to overcome this sourness of the juice and to increase the marketability of this healthy product.

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Conflicts of interest
There are no conflicts of interest.

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