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Research Article

Polyphenol Content and Bioactivity of Pomegranate Peel and Their Cultivar and Environment Dependencies

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Abstract: Phenolics contents and *in vitro* bioactivities of 10 pomegranate peels and their cultivar and environment dependencies were investigated. The results showed total free polyphenol contents of the peels ranged in 104.17-200.91 mg GAE/gDW, which were 10-12 folds more than total bound ones and punicalagins B was predominant component of them. Total free phenolic content of pomegranate peel were significantly different among some cultivars and all produce regions which have significant different environment, significantly positively correlated with total sunlight time and negatively with total precipitation and average temperature in fruit mature period. They were significantly positively correlate with DPPH· scavenging capacity, $ABTS^{+\cdot}$ scavenging capacity, total reducing power and total antioxidant capacity of the peels. The purified phenolic extracts could induce Caco-2 cells apoptosis in vitro in dose dependent manner and through multiple cell signaling pathways. In conclusion, phenolic content of pomegranate peel depend on both cultivar and meteorological condition. Antioxidant and antiproliferative activities of pomegranate peel extract positively correlate with its total phenolic content.

Keywords: Antioxidant activity, antiproliferative activity, environmental influence, phenolic content, pomegranate peel

INTRODUCTION

If reactive oxygen species conn't be balanced by antioxidant defenses in human body, they can damage to lipids, proteins and nucleic acids (McCord, 2000; Devasagayam *et al*., 2004) and cause cardiovascular disease, cancer, diabetes mellitus, neurodegenerative disorders, rheumatoid arthritis and ageing (Valko *et al*., 2007; Mahantesh *et al*., 2012). Therefore, antioxidant nutraceutical has been developping to strengthen antioxidant defenses.

Pomegranate polyphenols, very rich in pomegranate peel and found having antioxidant, antibacterial, anticarcinogenic, anti-atherogenic, antiinflammatory, anti-allergic, antiparasitic and anti-diabetes activities (Jurenka, 2008; Viuda-Martos *et al*., 2010; Ismail *et al*., 2012) have been extracted from pomegranate peel to produce antioxidant nutraceuticals. Pomegranate polyphenol supplement is one of them popular in market (Espín *et al*., 2007; Madrigal-Carballo *et al*., 2009; Tehranifar *et al*., 2011). However, the phenolic content, composition and bioactivities of the goods are inconsistent (Madrigal-Carballo *et al*., 2009; Zhang *et al*., 2009), due to diverse pomegranate cultivars and extraction technology (Shiban *et al*., 2012; Saad *et al*., 2012).

In this study, polyphenols were extracted from peels of 10 pomegranate cultivars collected from 4 regions of China. The phenolic content and antioxidant and antiproliferative activities of the extracts were investigated and compared. The results showed that not only cultivar but also local meteorological condition during fruit mature period significantly effected on the polyphenol contents and bioactivities of the extracts.

Highlights:

- Pomegranate peel's polyphenol content existed cultivar and regional differences
- Meteorological condition of mature strongly effected on peel's polyphenol content
- Free tannins are main phenolic component and antioxidants of pomegranate peel extract
- The phenolics could induce cells apoptosis through multiple cell signaling pathways.

MATERIALS AND METHODS

Pomegranate peel powder preparation: The tested pomegranate cultivars include *cv*. Kashisuan (KSS), *cv*.

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Kashitian (KST), *cv.* Dahongpisuan (ZZS), *cv.* Damaya (DMY), *cv*. Suanlvzi (MZS), *cv*. Tianlvzi (MZL), *cv.* Tianshazi (MZSA), *cv.* Lintongsuan (LTS), *cv.* Jingpitian (JPT) and *cv.* Sanbaitian (SBT). Among them, KSS, ZZS, MZS and LTS are sour pomegranate and the others are sweet one. KSS and KST were collected from Kashi, Xinjiang and coded as XJ-1 and XJ-2; ZZS and DMY from Zaozhuang, Shandong and as SD-1 and SD-2; MZS, MZL and MZSA from Mengzi, Yunnan and as YN-1, YN-2 and YN-3; LTS, JPT and SBT from Lintong, Shaanxi and as SX-1, SX-2, SX-3.

The fruits were harvested and transported back to our laboratory by Express Delivery in early October, 2011. After being cleaned with water, the peel was separated from the fruit manually, dried at 50°C for 24 h and smashed into powders of 60 mesh. These peel powders were stored in glass bottles for late use.

Chemicals and cells: Folin-Ciocalteu and Folin-Dennis reagents, 2, 2`-azino-bis -3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazoliumbromide (MTT) were purchased from Sigma-Aldrich Co.. Phenolic compound standards were purchased from Vick's biological Inc. Primary antibodies against tBID, P53 and GAPDH were obtained from Santa Cruz Biotechnology. PARP and cleaved-Caspase-3 antibody were obtained from Cell Signaling Technology. The BCA protein assay kit and ECL were obtained from Thermo Scientific Inc. Acridine Orange (AO), Ethidium Bromide (EB), 4, 6-diamidino-2-phenylindole (DAPI) and DNA ladder extraction kit were purchased from Beyotime Institute of Bio-technology. Caco-2 cell was purchased from Collection of Cell Cultures of the Fourth Military Medical University of PLC. Mem-High Glucose culture medium and Fetal Bovine Serum (FBS) were purchased from Gibco.

Phenolics extraction:

Free phenolics extraction: Two grams of peel powder was extracted with 40 mL petroleum ether and diethyl ether mixture (3/2(*v/v*)) to remove lipid. Then the powder was extracted with 40 mL of 60% aqueous ethanol and 40 kHZ of ultrasonic aid for 30 min at room temperature. After centrifugal at 4000 r/min for 10 min, the supernatant was collected and the residue was extracted again in same way. The two supernatants were combined together and diluted to 100 mL with 60% aqueous ethanol and then stored at -20°C for late use. The final residue was used in the next step.

Bound phenolics extraction: Based on the method described by Oboh and Ademosun (2012), the residue mentioned above was mixed with 40 mL of 4 N NaOH. After being shaken for 1.5 h, the mixture was adjusted pH 2 by HCl and diluted to 100 mL with distilled water. After centrifugal at 4000 r/min for 10 min, the supernatant was stored at -20°C for late use.

Phenolics content determination: Total polyphenols content was determined with Folin-Ciocalteu method and expressed as milligram of gallic acid equivalent per gram of dry peel powder (mg GAE/gDW). Total flavonoids content was determined with the aluminum chloride colorimetric method described by Lin and Tang (2007) and expressed as milligram of quercetin equivalent per gram of dry peel powder (mg QUE/gDW). Total tannin content was determined with the Folin-Denis method and expressed as milligram of tannin acid equivalents per gram of dry peel powder (mg TAE/gDW).

Phenolic composition analysis: Fifty milliliters of the phenolic extract sample was infused into the D-101 macroporous resin column in the speed of 5 bed volumes per hour (5 BV/h). After equilibrium for 1 h, distilled water was passed through the column at speed of 5 BV/h to remove the impurity. Then the phenolics were eluted out by aqueous ethanol (70%, v/v) at speed of 2 BV/h. The elution was concentrated to almost dry at 40°C in a rotary evaporator and the residue was re-dispersed in 5 mL of 30% aqueous ethanol and kept -20°C for late use.

The purified phenolic sample was diluted with aqueous methanol (50%, *v/v*) to the final total polyphenol concentration of 1 mg/mL and filtrated through a 0.22 μm filter. After 15 μL of the filtrate being injected in HPLC system (C18 reversed phase column of 250×21.1 mm i.d., 5 μm particle size; 12.5 nm pore size), the HPLC was carried out in condition of column temperature: 30°C, detector wavelength: 280 nm, mobile phase A: 1% acetic acid, mobile phase B: methanol, total mobile phase flow rate: 0.8 mL/min and gradient elution procedure: 15% B to 25% B in 15 min-25% B for 10 min-25% B to 75% B in 40 min-75% B to 15% B in 15 min-15% B for 5 min. The phenolic compound was identified and quantified by comparing retention time and peak area with that of the standard. The result was expressed as micrograms per milliliter of the purified phenolic extract solution (μg/mL).

***In vitro* antioxidant activity assay:** DPPH Radical Scavenging Capacity (DRSC) was assayed with the method described by Locatelli *et al*. (2009). ABTS Radical Scavenging Capacity (ARSC) was assayed with the method described by Re *et al*. (1999). Total Reducing Power (TRC) was assayed with the method described by Amarowicz *et al*. (2010). Total Antioxidant Capacity (TAC) was assayed with the method described by Prieto *et al*. (1999). All results

were expressed as gram of gallic acid equivalent per gram of dry peel powder (mg GAE/gDW).

Anti proliferative activity assay:

Cell culture and special media preparation: Caco-2 cells were normally cultured in Mem-High Glucose medium with 12.5% Fetal Bovine Serum (FBS) and 2% penicillin/streptomycin at 37°C and 5% (v/v) CO_2. The purified phenolic sample was dissolved in PBS and diluted with serum-free culture medium to the final total polyphenol concentration of 100 mg/mL and further diluted with serum-free culture medium to be a serial special media with total polyphenol concentrations of 10, 20, 50, 100, 200 and 500 μg/mL, respectively.

Cytotoxic effect assay: Caco-2 cells were seeded at 1×10^5 cells/mL in a 96 wells polystyrene culture plate. After adding a special medium into specified well, the cells were cultured at 37°C and 5% CO_2 for 48 h. Then, the medium was removed and the cells were incubated with 5 mg/mL of MTT at 37°C for 4 h. Finally, 100 μL of DMSO was added into the well to dissolve formazan crystals and the absorbance of the solution was measured at 490 nm with a microplate reader (Bio Rad Laboratories Ltd., China). Meanwhile, control test (use serum-free culture medium instead of the special medium) was done. The result was expressed as cell viability remaining rate, which was defined as the absorbance quotient between test and control test (% of control).

AO/EB double staining and DAPI staining: Caco-2 cells were cultured in the special medium having total polyphenols concentration of 100 μL/mL for 24 h. The collected cells were fixed with 4% formaldehyde for 10 min and stained with 60 μL AO/EB solution (100 μg/mL AO and 100 μg/mL EB) or DAPI solution (10 μg/mL) at room temperature for 10 min. The morphological change of the cells, including reduction in volume and nuclear chromatin condensation, were observed under a fluorescence microscope (Olympus, IX71, Japan).

Western blot analysis: After being cultived in a special medium for 48 h, the cells were harvested and lysed in cell lysis buffer (P0013, Beyotime Institute of Biotechnology, Jiangsu, China) for 10 min on ice. Then the sample was centrifuged at 15,000 g for 10 min at 4°C to get supernatant. The total protein concentration of the supernatant was determined by BCA Protein Kit (Thermo Fisher, Shanghai, China). And the supernatant was treated with SDS buffer at 95°C for 5 min. The proteins in it were separated by SDS-PAGE and electro-transferred onto a polyvinylidene fluoride membrane (0.45 μm, Millipore) by using a semidry transfer apparatus (Bio Rad, Shanghai, China). Blocking was performed for 2 h in 5% of nonfat dry milk dispersed in TBST (20 mM Tris, 166 mM NaCl and 0.05% Tween 20, pH 7.5). Then, the membrane was washed by TBST for 3 times and then immersed in the TBST buffer diluted primary antibody solution. After standing overnight at 4°C and being washed by TBST for another 3 times, the membrane was immersed in the secondary antibodies solution and incubated at 25°C for 2 h. After the membrane was washed with TBST again, the blots were developed by adding chemiluminescent substrate (Thermo Fisher, China) and exposed in a Molecular Imager Chemidoc XRS System (BioRad, Shanghai, China).

Table 1: Meteorological data of the 4 regions during pomegranate mature time

Region	TP (mm)	AP (hPa)	AT (°C)	A H (%)	TS (h)
SX-LT	1078	970.15	17.4	71	308.2
YN-MZ	1012	870.55	21.25	69	324.8
XJ-KS	90	872.5	16.85	41	541
SD-ZZ	668	1010.35	17.5	79	443.6

SX-LT, Lintong, Shaanxi; YN-MZ, Mengzi, Yunnan; XJ-KS, Kashi, Xinjiang; SD-ZZ, Zaozhuang, Shandong; TP, total precipitation in the period of pomegranate mature time. (i.e., 10/08/2011~10/10/2011); AP, average atmospheric pressure in the period; AT, average temperature in the period; AH, average relative humidity in the period; TS, total sunlight time in the period

Environment information: Kashi is located in the southwestern of Xinjiang (east longitude: 79°59′, northern latitude: 39°28′). Lintong is located in middle of Shaanxi (109°12′, 34°22′). Zaozhang is located in the southern of Shandong (117°32′, 34°30′). And Mengzi is located in the southeastern of Yunnan (103°23′, 23°23′). The meteorological data during pomegranate maturity period of the 4 regions were provided by China Meteorological Data Sharing Service System and listed in Table 1.

Statistical analysis: Every test was performed in triplicates and the result was expressed as mean ± SD. One-way Analysis of Variance (ANOVA) and correlation analysis were carried out by DPS 6.55 statistical program software.

RESULTS AND DISCUSSION

Phenolic content of the pomegranate peel: As Table 2A shown, Total Free Polyphenol Content (TFPC) of the peel (104.17-200.91 mg GAE/gDW) was about 12 times more than Total Bound Polyphenol Content (TBPC) (7.35-15.94 mg GAE/gDW). Different regional peels had significantly different ($p<0.05$) TFPC and TBPC, but same regional peels had similar TFPC in most cases. YN peels had the lowest TFPC, while XJ and SD peels had much higher TFPC and TBPC. Similarly, Total Free Flavonoid Content (TFFC) (23.12-54.58 mg QUE/gDW) of the peel was about 10 times more than total bound flavonoid content (TBFC) (2.89-5.17 mg QUE/gDW) and Total Free Tannin

Table 2: Phenolic content, composition of pomegranate peel and relationship between the content and local meteorology

Table 2A: Total free and bound phenolic contents of pomegranate peels

Culivar code	Cultivar	TFPC (mg GAE/g DW)	TBPC (mgGAE/g DW)	TFFC (mg QUE/g DW)	TBFC (mg QUE/g DW)	TFTC (mg TAE/g DW)	TBTC (mg TAE/g DW)
SX-1	JPT	153.74±4.27c	12.97±0.51c	44.32±1.29bc	4.40±0.21ab	223.98±4.12d	14.74±0.25d
SX-2	SBT	148.43±1.74c	7.35±0.48e	41.30±1.28c	3.65±0.30c	224.66±1.58d	9.01±0.22g
SX-3	LTS	145.06±6.03c	10.34±0.83d	36.17±0.70d	2.89±0.24d	196.62±7.10e	11.79±0.52f
YN-1	MZL	114.06±5.25d	12.99±0.99c	25.13±2.04e	3.84±0.26bc	156.83±3.53f	14.91±0.20d
YN-2	MZSA	113.58±1.98d	13.74±0.24bc	25.02±1.06e	4.04±0.17abc	164.74±6.79f	15.5±0.39bcd
YN-3	MZS	104.17±3.81d	11.45±0.28d	23.12±2.03e	3.10±0.22d	155.02±4.83f	13.28±0.52e
XJ-1	KST	182.69±5.72b	12.78±0.48c	41.53±1.43c	4.39±0.05ab	263.09±2.93c	15.77±0.21abc
XJ-2	KSS	200.91±5.29a	14.97±0.54ab	52.46±1.23a	4.62±0.40a	299.95±1.85a	15.94±0.39ab
SD-1	DMY	184.26±5.32b	15.94±0.29a	45.77±1.07b	4.43±0.21a	278.92±1.00b	16.37±0.12a
SD-2	ZZS	199.10±6.32a	14.08±0.71bc	54.58±0.80 a	4.19±0.19abc	292.49±4.63a	15.03±0.29cd

TFPC, total free polyphenol content; TFFC, total free flavonoid content; TFTC, total free tannin content; TBPC, total bound polyphenol content; TBFC, total bound flavonoid content ; TBTC, total bound tannin content. Data were expressed as means ± SD. In same column, data followed by different letters are significantly different ($p<0.05$) from each other.

Table 2B: Correlation between phenolic content and meteorological parameter

	TFPC	TFFC	TFTC	TBPC	TBFC	TBTC
TP (mm)	-0.74**	-0.55	-0.73*	-0.32	-0.35	-0.48
AP (hPa)	0.44	0.58	0.44	0.27	0.19	-0.01
AT (°C)	-0.86**	-0.88**	-0.84**	-0.28	-0.46	-0.21
AH (%)	-0.31	-0.15	-0.29	0.02	-0.21	-0.24
TS (h)	0.80**	0.62*	0.80**	0.39	0.37	0.51

*, significant correlation ($p<0.05$); **, highly significant correlation ($p<0.01$); TFPC and TBPC, total free and bound polyphenol contents; TFFC and TBFC,, total free and bound flavonoid contents; TFTC and TBTC, total free and bound tannin content; TP and TS, total precipitation and total sunlight time in the period (10/08/2011~10/10/2011); AP, AT and AH, average atmospheric pressure, average temperature and average relative humidity in the period

Table 2C: Phenolic constituent of purified polyphenol sample from pomegranate peel

Phenolic compound	Cultivar code	Production region	FPC (μg/mL)	FPRC (%)	BPC (μg/mL)	BPRC (%)
Punicalagin A	SX-1	SX-LT	299.17	5.43	27.98	3.87
	YN-1	YN-MZ	178.32	5.47	56.03	5.58
	XJ-2	XJ-KS	301.32	5.72	28.08	6.26
	SD-1	SD-ZZ	199.16	5.24	44.96	7.00
Gallic acid	SX-1	SX-LT	87.86	1.60	3.60	0.50
	YN-1	YN-MZ	159.79	4.91	31.39	3.13
	XJ-2	XJ-KS	152.01	2.89	0.57	0.13
	SD-1	SD-ZZ	102.41	2.69	4.99	0.78
Punicalagin B	SX-1	SX-LT	5006.79	90.96	624.48	86.32
	YN-1	YN-MZ	2825.54	86.75	862.81	85.94
	XJ-2	XJ-KS	4649.50	88.33	366.45	81.70
	SD-1	SD-ZZ	3388.18	89.14	530.46	82.64
Catechine	SX-1	SX-LT	85.44	1.55	4.65	0.64
	YN-1	YN-MZ	72.63	2.23	2.09	0.21
	XJ-2	XJ-KS	130.09	2.47	4.03	0.90
	SD-1	SD-ZZ	76.31	2.01	3.26	0.51
Chlorogenic acid	SX-1	SX-LT	2.79	0.05	1.08	0.15
	YN-1	YN-MZ	1.35	0.04	0.42	0.04
	XJ-2	XJ-KS	1.57	0.03	0.62	0.14
	SD-1	SD-ZZ	0.80	0.02	1.46	0.23
Caffeic acid	SX-1	SX-LT	0.44	0.01	1.08	0.15
	YN-1	YN-MZ	0.43	0.01	0.60	0.06
	XJ-2	XJ-KS	0.60	0.01	0.57	0.13
	SD-1	SD-ZZ	0.50	0.01	1.21	0.19
Epicatechine	SX-1	SX-LT	1.37	0.02	17.74	2.45
	YN-1	YN-MZ	1.96	0.06	0.54	0.05
	XJ-2	XJ-KS	0.77	0.01	16.08	3.59
	SD-1	SD-ZZ	1.70	0.04	0.40	0.06
Ferulic acid	SX-1	SX-LT	5.06	0.09	0.09	0.01
	YN-1	YN-MZ	0.26	0.01	0.15	0.01
	XJ-2	XJ-KS	0.53	0.01	0.41	0.09
	SD-1	SD-ZZ	7.12	0.19	0.20	0.03
Ellagic acid	SX-1	SX-LT	5.74	0.10	33.51	4.63
	YN-1	YN-MZ	11.29	0.35	39.30	3.91
	XJ-2	XJ-KS	20.00	0.38	23.58	5.26
	SD-1	SD-ZZ	11.58	0.30	39.07	6.09
Quercetin	SX-1	SX-LT	4.86	0.09	2.45	0.34

Table 2C: Continue

Phenolic compound	Cultivar code	Production region	FPC (μg/mL)	FPRC (%)	BPC (μg/mL)	BPRC (%)
	YN-1	YN-MZ	2.35	0.07	2.41	0.24
	XJ-2	XJ-KS	2.38	0.05	ND	0.00
	SD-1	SD-ZZ	6.82	0.18	2.51	0.39
Keampferol	SX-1	SX-LT	5.12	0.09	6.74	0.93
	YN-1	YN-MZ	3.10	0.10	8.22	0.82
	XJ-2	XJ-KS	5.02	0.10	8.15	1.82
	SD-1	SD-ZZ	6.59	0.17	13.38	2.08
All detected	SX-1	SX-LT	5504.64	100.00	723.41	100.00
	YN-1	YN-MZ	3257.02	100.00	1003.96	100.00
	XJ-2	XJ-KS	5263.78	100.00	448.53	100.00
	SD-1	SD-ZZ	3801.17	100.00	641.89	100.00

FPC, free phenolic compound content of the purified free phenolics sample; FPRC, relative content of the free phenolic compound compared to total detected free phenolic compounds; BPC, bound phenolic compound content of the purified bound phenolics sample; BPRC, relative content of the bound phenolic compound compared to total detected bound phenolic compounds; SX-LT, Lintong, Shaanxi; YN-MZ, Mengzi, Yunnan; XJ-KS, Kashi, Xinjiang; SD-ZZ, Zaozhuang, Shandong.

Content (TFTC) (155.02-299.95 mg TAE/gDW) was about 12 times more than Total Bound Tannin Content (TBTC) (9.01-16.37 mg TAE/gDW). Different regional peels had significantly different ($p<0.05$) TFFC and TFTC, but same regional peels had similar TFFC and TFTC in most cases. YN peels had the lowest TFFC and TFTC, while XJ and SD peels had much higher TFFC and TFTC. These results suggest that produce environment may strongly effect on TFPC, TFFC and TFTC of pomegranate peel.

There are very limited researches having compared phenolic contents of different regional pomegranate peels in China. Chen *et al*. (2011) reported peel TPC of 6 Xinjiang and 6 other province pomegranates ranged in 172.2-279.3 and 122.3-147.5 GAE/gDW (converted data) respectively, which also showed XJ pomegranate peels had much higher TPC than others.

Relationship between peel phenolic content and meteorology in mature period: As Table 2B shown, total precipitation was significantly negatively correlated with both TFPC ($p<0.01$) and TFTC ($p<0.05$). Average temperature was significantly negatively correlated with TFPC ($p<0.01$), TFFC ($p<0.01$) and TFTC ($p<0.01$). And total sunlight time was significantly positively correlated with TFPC ($p<0.01$), TFFC ($p<0.05$) and TFTC ($p<0.01$). While, both average atmospheric pressure and average relative humidity were not significantly correlated with any kinds of phenolic contents. These results suggest that lower total precipitation, lower average temperature and higher total sunlight time during the fruit mature time are conducive to increase TFPC generally and the significant difference of TFPC among different regional pomegranate is at least partly due to environmental difference.

There is very little information available in literature about the effect of produce environment on phenolic content and bioactivity of pomegranate peel as we known. One possible reason is the reported phenolic content data strongly related to the sampling, determination and result expression methods (Ismail *et al*., 2012; Calín-Sánchez *et al*., 2013), which usually inconsistent among different researches. Another reason may be most researchers had neglected to collect the environment information.

In the present research, extraction and determination methods kept consistent, the environmental data of the 4 regions, collected from reliable source, were obviously different from each other; and all tested pomegranates belong to different cultivars, Therefore, phenolic content difference between peels is must due to cultivar or environment differences, or both of them. According to the results mentioned above, it is reasonable to believe that mature environment has played more important effect on phenolic content of pomegranate peel than cultivar.

Phenolic compositon of pomegranate peel: As Table 2C shown, 11 phenolic compounds were identified in the purified peel phenolic samples. They were punicalagins A&B, gallic acid, catechine, epicatechine, chlorogenic acid, caffeic acid, ferulic acid, ellagic acid, quercetin and keampferol. Amoung them, punicalagins B accounted for 81.7~91.0% of the whole and punicalagins A for 3.9~7.0%. Although the absolute contents of these compounds in these pomegranate peel extracts existed certain difference, their relative contents were similar from each other. These results suggest that all pomegranate peels have similar phenolic elementary composition.

Seeram *et al*. (2005a) reported that purified aqueous extract of pomegranate peel mainly contained punicalagin (80-85% w/w), ellagic acid (EA; 1.3% w/w) and unquantified amounts of punicalin and EA-glycosides. Qu *et al*. (2012) reported the gallic acid, punicalagin A, punicalagin B and ellagic acid concentrations of the pomegranate (*c.v.* Wonderful) peel water extract were 1.63, 2.02, 2.18 and 4.91 mg/g respectively. These data also suggest punicalagin is the predominant phenolic compound in pomegranate peel.

Antioxidant capacities of pomegranate peel phenolics: As Table 3A shown, DRSC of Peel's Free

Table 3: *In vitro* antioxidant capacity of pomegranate peel extract and its relationship with phenolic content
Table 3A: *In vitro* antioxidant capacity of pomegranate peel phenolic extract

Peel (Cultivar Name)	DRSC (mg GAE/gDW)		ARSC (mg GAE/gDW)		TRP (mg GAE/gDW)		TAC (mg GAE/gDW)	
	Free P	Bound P	Free P	Bound P	Free P	Bound P	Free P	Bound P
JPT	88.24±0.49d	5.51±0.15d	106.40±0.33bc	5.97±0.29c	151.07±1.25bc	10.02±0.38b	199.60±5.22b	17.24±0.63b
SBT	92.27±2.37cd	4.86±0.08e	103.56±0.76c	3.96±0.16d	145.37±5.41c	4.68±0.05d	198.59±5.69b	12.19±1.30d
LTS	71.36±3.17e	4.71±0.22e	94.53±0.78d	6.20±0.05c	131.05±5.51c	8.75±0.23c	185.17±0.95c	14.31±0.98c
MZL	59.26±4.78f	5.72±0.12cd	77.72±2.04f	6.85±0.25b	98.12±1.65d	10.45±0.15b	160.67±2.37d	17.92±0.62b
MZSA	61.54±6.46f	6.13±0.18ab	79.12±1.53f	7.10±0.15ab	95.22±2.43d	11.53±0.31a	153.62±3.80d	18.52±1.42b
MZS	55.13±3.09f	5.49±0.10d	69.60±1.06g	5.90±0.18c	87.91±5.15d	8.78±0.26c	136.85±7.59e	15.40±0.55c
KST	99.59±4.99bc	6.09±0.11ab	88.17±5.40e	7.50±0.51a	131.85±18.70c	12.00±0.27a	160.84±3.56d	19.12±1.19ab
KSS	105.55±3.02ab	6.30±0.21ab	107.66±1.11bc	6.83±0.28b	140.66±30.70c	11.38±0.99a	197.75±2.61b	18.66±1.28b
DMY	109.89±3.46a	6.37±0.11a	118.91±1.99a	7.41±0.18a	170.09±13.20ab	12.14±0.22a	275.44±0.47a	20.55±1.23a
ZZS	107.47±4.54ab	5.98±0.16bc	108.96±5.16b	7.29±0.12a	177.60±4.42a	12.20±0.74a	269.56±1.19a	18.60±0.18b

DRSC, DPPC$^{\bullet}$ scavenging capacity; ARSC, ABTS$^{+\bullet}$ scavenging capacity; TRP, total reducing power; TAC, total antioxidant capacity; P, phenolics. Data were expressed as means ± standard deviations. In same column, data followed by different letters indicate their mean values are significantly different (p<0.05) from each other.

Table 3B: Correlation between free phenolic content and antioxidant activity

	TFPC	TFFC	TFTC	DRSC	ARSC	TRC
TFFC	0.96**					
TFTC	0.99**	0.96**				
DRSC	0.96**	0.95**	0.98**			
ARSC	0.81**	0.89**	0.83**	0.88**		
TRP	0.85**	0.92**	0.86**	0.90**	0.95**	
TAC	0.72*	0.78**	0.74**	0.78**	0.89**	0.92**

*: Significant correlation (p<0.05); **: Highly significant correlation (p<0.01); TFPC, Total Free Polyphenol Content; TFFC: Total Free Flavonoid Content; TFTC: Total Free Tannin Content; DRSC: DPPC Radical Scavenging Capacity; ARSC, ABTS^{+} radical scavenging capacity; TRP: Total Reducing Power; TAC: Total Antioxidant Capacity

Table 3C: Correlations between bound phenolic content and antioxidant activity

	TBPC	TBFC	TBTC	DRSC	ARSC	TRP
TBFC	0.68*					
TTC	0.96**	0.69*				
DRSC	0.90**	0.79**	0.91**			
ARSC	0.87**	0.47	0.91**	0.79**		
TRP	0.93**	0.60*	0.96**	0.85**	0.98**	
TAC	0.96**	0.74**	0.97**	0.93**	0.91**	0.96**

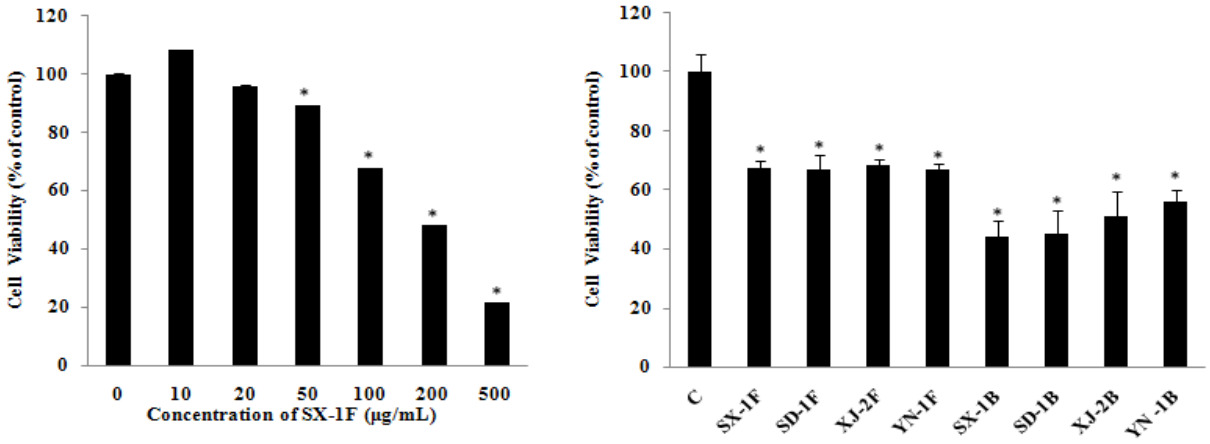
*: Significant correlation (p<0.05); **: Highly significant correlation (p<0.01); TBPC: Total Bound Polyphenol Content; TBFC: Total Bound Flavonoid Content; TBTC: Total Bound Tannin Content; DRSC, DPPC radical scavenging capacity; ARSC, ABTS^{+} radical scavenging capacity; TRP: Total Reducing Power; TAC: Total Antioxidant Capacity

Phenolics (PFP) ranged in 55.13-109.89 mg GAE/gDW and DRSC of Peel's Bound Phenolics (PBP) range in 4.71-6.37 mg GAE/gDW. ARSC of PFP and PBP ranged in 77.72-118.91 and 3.96-7.50 mg GAE/gDW. TRC of PFP and PBP ranged in 87.91-177.60 and 4.68-12.20 mg GAE/gDW. TAC of PFP and PBP ranged in 136.85-275.44 and 14.31-21.30 mg GAE/gDW. All of the antioxidant capacities were significantly different among different regional pomegranate peels, but similar within same regional pomegranate peels in most cases. SD peels had the highest DRSC, ARSC, TRC and TAC, while YN peels had the lowest. These results suggest that pomegranate peel's antioxidant ability closely dependents on its total phenolic content, especially TFTC and environmental condition.

Some literatures also reported pomegranate peel extracts of some cultivars had significantly different antioxidant capacity. Hajimahmoodi *et al*. (2008) reported the peel extract of Sweet white cultivar had more antioxidant potential than other cultivars. Shams Ardekani *et al*. (2011) reported peel extracts of Sour summer, Sweet saveh malas and Black cultivars had higher antioxidant activity than other cultivars. Fawole *et al*. (2012) reported radical scavenging activities of pomegranate peel extracts of Arakta, Ganesh and Ruby cultivars were significantly higher than other cultivars. However, these literatures had not analyzed environmental effect on polyphenol content and antioxidant capacity of peel.

Relationship between antioxidant capacity and phenolic content: As Table 3B shown, DRSC, ARSC, TRC and TAC were all significantly positively correlated with TFPC, TFFC and TFTC (p<0.01). As Table 3C shown, DRSC, ARSC, TRC and TAC were also significantly positively correlate with TBPC, TBPC and TBTC (p<0.01 or p<0.05). These results suggest that all of pomegranate peel phenolics are antioxidants and the free tannins are the main antioxidants followed by free flavonoids. They are consistent with previous researches (Reddy *et al*.,2007; Viuda-Martos *et al*., 2010; Tehranifar *et al*., 2011).

Antiproliferative activity of the purified peel phenolics: As Fig. 1a shown, treatment with the free phenolic sample purified from SX-1 peel decreased the



(a) (b)

Fig. 1: Effect of pomegranate peel phenolics on viability of Caco-2 cells
(a): Viabilities of Caco-2 cells treated by free phenolics purified from SX-1 peel at different concentrations; (b): Viabilities of Caco-2 cells treated by phenolics purified from other pomegranate peels at 100 μg/mL; Error bars were drawn in accordance with SD. *, indicate the mean value of the test was significant ($p<0.01$) different with that of the control test; SX-1F, SD-1F, XJ-2F, and YN-1F, the free phenolic samples purified from SX-1, SD-1, XJ-2, and YN-1 pomegranate peels respectively; SX-1B, SD-1B, XJ-2B, and YN-1B, the bound phenolic samples purified from SX-1, SD-1, XJ-2, and YN-1 pomegranate peels respectively

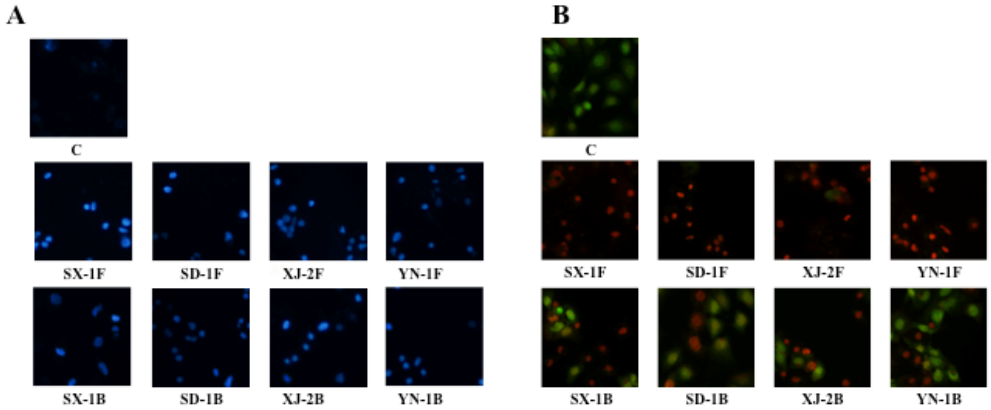


Fig. 2: Caco-2 cells apoptosis induced by purified pomegranate peel phenolic extracts
A: Images of DAPI staining experiment; B: Images of AO/EB double staining experiment SX-1F, SD-1F, XJ-2F and YN-1F, the free phenolic samples purified from SX-1, SD-1, XJ-2 and YN-1 pomegranate peels respectively; SX-1B, SD-1B, XJ-2B and YN-1B, the bound phenolic samples purified from SX-1, SD-1, XJ-2 and YN-1 pomegranate peels respectively

viability of Caco-2 cells in a dose-dependent manner and the viability had dropped to 60.7% through 48 h treatment at 100 μg/mL. As Fig. 1b shown, treatment with phenolic samples purified from other peels had similar efficacy. These results suggest that all phenolic samples purifed from any kind of pomegranate peel have cytotoxicity on Caco-2 cells and the efficacy is dose-dependent. This is agreed with the previous research (Orgil *et al*., 2014) which found there were positive relationships between high levels of TPC, punicalagin and gallagic acid treatment and MCF-7 proliferation inhibitory activities.

As Fig. 2 shown, the cells' morphology changed to spheroid shape, accompanied by chromatin condensation, indicating the cells had apoptosis in certain degree due to phenolics induced toxicity. In Fig. 2A, we can see 90% of Caco-2 cells' chromatin condensed after the cells exposed to the phenolics at 100 μg/mL for 24 h. In Fig. 2B, we can see purified free phenolics had induced more cells into late apoptosis state than purified bound phenolics. These results suggest that PFP are more effective to induce apoptosis than PBP at same dosage and the phenolics' cytotoxicity is mainly performed by apoptosis effect.

As Fig. 3A shown, when Caco-2 cells were exposed to pomegranate peel phenolics, their caspase-8, caspase-3, p53 and the ratio of tBid/Bid all increased and their PARP content also had a little change. These results suggest that the phenolics induced cell apoptosis through a cascade caspases-dependent pathway and p53 apoptotic pathway also involved.

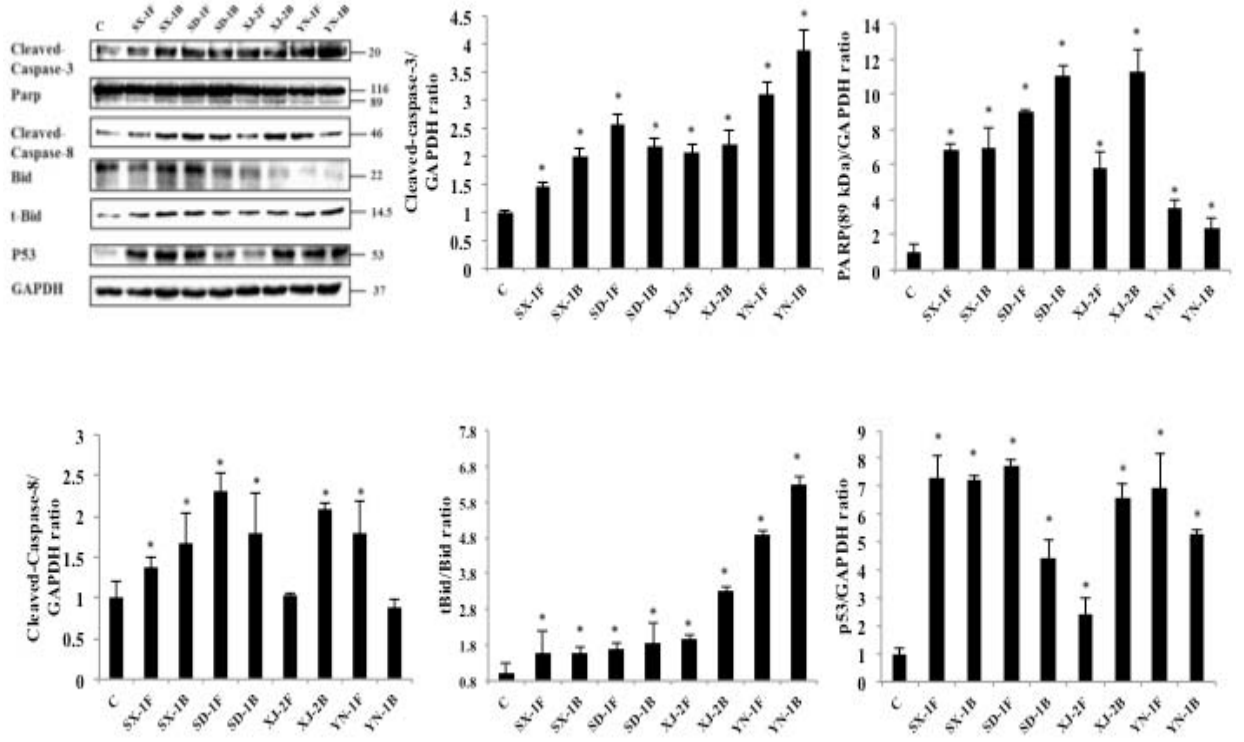


Fig. 3: Western Blot analysis results
A: Images of the monitored proteins in the Western Blot analysis experiment; B, C, D, E and F: Expression degree ratio of apoptosis-related proteins after Caco-2 cells were treated by phenolic samples purified from different pomegranate peels. The monitored proteins include cleaved-caspase-3, PARP, cleaved-caspase-8, Bid, tBid, p53 and GAPDH; Error bars were drawn in accordance with SD; *, indicate the mean value was significantly ($p<0.01$) different with that of control; C, control; SX-1F, SD-1F, XJ-2F and YN-1F, the free phenolic samples purified from SX-1, SD-1, XJ-2 and YN-1 pomegranate peels respectively; SX-1B, SD-1B, XJ-2B and YN-1B, the bound phenolic samples purified from SX-1, SD-1, XJ-2 and YN-1 pomegranate peels respectively

These results are consistent with the previous studies which showed that punicalagin exhibited strong anti-proliferative activity against human lung, breast and cervical cancer cell lines (Aqil *et al*., 2012); punicalagin, elligic acid and total pomegranate tannins all had apoptotic effect on cells at 100 μg/mL (Seeram *et al*., 2005b); and caspase-independent apoptotic pathway (Fadeel and Orrenius, 2005), caspases-dependent apoptotic pathway (Chen *et al*., 2004; Zou *et al*., 2011) and p53 apoptotic pathway (Hofmann and Sonenshein, 2003) all worked.

Antiproliferative activity difference of different phenolic samples: From Fig. 3B to 3F we can see that, different purified phenolics samples at same dosage induced Caco-2 cells to express apoptosis-related proteins with a little different degree, which indicate the modes of apoptosis in these treatments were slightly different from each other. This result suggests that the purified phenolic samples from different pomegranates only had slightly different antiproliferative potential due to their phenolic compositions only having slight difference.

CONCLUSION

This research revealed that free tannins were main components of peel extract and punicalagins B was predominate compound of purified peel phenolic extract for all 10 tested pomegranates. TFPC of pomegranate peel depend on both cultivar and meteorological condition in pomegranate mature period. Antioxidant capacity of the peel extract was significantly positively correlated with TFPC. All of purified peel phenolic samples had similar elementary composition and could induce Caco-2 cells apoptosis with similar efficiency through multiple cell signaling pathways.

Abbreviations and nomenclature:

TFPC	:	Total free polyphenols content
TBPC	:	Total bound polyphenols content
TFFC	:	Total free flavonoids content
TBFC	:	Total bound flavonoids content
TFTC	:	Total free tannins content
TBTC	:	Total bound tannins content
PFP	:	Peel's free phenolics
PBP	:	Peel's bound phenolics
ARSC	:	$ABTS^{+}$ radical scavenging capacity

DRSC : DPPH radical scavenging capacity
TRC : Total reducing capacity
TAC : Total antioxidant capacity

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Author contributions: Linwei Liu led the study. Tian Yuan designed the study, collected test data and drafted the manuscript. Xuan Li, Xu Meng and Zhongmei Gao helped the data, collection and analysis. The authors declare that there is no conflict of interest.

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