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Quantitative Diversity of Phenolic Content in Peels of Some Selected Egyptian Pomegranate Cultivars Correlated to Antioxidant and Anticancer Effects

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ABSTRACT

Quantitative estimation of total phenolic, flavonoid, proanthocyanidin and hydrolysable tannin content of aqueous and methanol peel extracts of five selected Egyptian pomegranate cultivars was carried out, the gained results revealed that they ranged from 246.37±4.61 to 197.30±4.82 mg GAE g^{-1}, 123.72±2.01 to 96.89±2.59 mg QE g^{-1}, 318.40±2.39 to 80.92±2.41 µg CE g^{-1} and 779.28±28.10 to 519.10±26.30 µg TAE g^{-1} respectively for their aqueous extracts while methanol extracts exhibited varied values ranged from 214.91±3.29 to 148.13±2.99 mg GAE g^{-1}, 136.72± 2.74 to 106.89±2.81 mg QE g^{-1}, 339.80±3.52 to 85.05±2.16 µg CE g^{-1} and 762.90±28.04 to 506.40±25.85 µg TAE g^{-1} respectively. *In vitro* screening of their antioxidant effect revealed that the maximum free radical scavenging percent were observed in Wardy, Assuity and Manfalouty cultivars with calculated values of 98.80±2.02, 97.30±2.86 and 96.82±3.16% for aqueous extracts and 97.30±2.94, 96.74±2.55 and 95.93±2.96% for methanol extracts respectively at dose levels comparatively less than the dose of the reference standard butylated hydroxytoluene (BHT), moreover, the results of screening of their anticancer effect using Hepg2 and U251 cell lines were matched with the gained results for screening of their antioxidant effect as maximum efficacies were demonstrated for the same cultivars at dose levels comparatively less than the dose of the reference standard Cisplatin.

Kew words: Pomegranate, Polyphenols, Flavonoids, Proanthocyanidins, hydrolysable tannins, Antioxidant, Anticancer.

Introduction

Pomegranate, *Punica granatum* L., is an ancient, mystical, unique fruit borne on a small, long-living tree cultivated throughout the Mediterranean region used in folk medicine for the treatment of various diseases (Ajaikumar *et al.*, 2005; Hassan *et al.*, 2012), it has gained popularity in recent years due to its multifunctional nature and nutritional benefit in human diet, the fruit is rich in tannins and polyphenol compounds, which have been reported to reduce disease risks (Martınez *et al.*, 2006; Jaiswal *et al.*, 2010). Fruit peel constitutes about 50% of its total weight (Al-Said *et al.*, 2009) and is often discarded as waste in spite of being containing higher amounts of polyphenol compounds compared to other plant parts, hence possess more potent biological activities (Li *et al.*, 2006; Hajimahmoodi *et al.*, 2008; Gözlekçi *et al.*, 2011).

The unique biochemistry of the pomegranate peel is quite intriguing by virtue of its high content of antioxidant-rich tannins and flavonoids i.e. ellagitannins such as punicalagin and its isomers (2,3-hexahydroxydiphenoyl-4, 6-gallagyl-glucose), as well as punicalin (4, 6-gallagyl-glucose), gallic acid, ellagic acid, and ellagic acid glycosides (Gil *et al.*, 2000; Cerdá *et al.*, 2003; Mousavinejada *et al.*, 2009; Aviram and Rosenblat 2013), in addition to anthocyanins, catechin, proanthocyanidins and flavonoids (Machado *et al.*, 2002; Wang *et al.*, 2010) these polyphenols have been implicated in many pharmacological activities referred to pomegranate peel (Lansky *et al.*, 2007; Althunibat *et al.*, 2010; Viuda-Martos *et al.*, 2010), however, their levels may vary among cultivars resulting in different levels of the same biological effect (Holland *et al.*, 2009).

Pomegranate peel have been reported to possess a wide range of biological effects including anti-cancer (Ackland *et al.*, 2005; Kowalski *et al.*, 2005; Adhami *et al.*, 2006), anti-atherosclerotic (Rajan *et al.*, 2011), anti-inflammatory (Seerama *et al.*, 2005), anti-diabetic (Althunibat *et al.*, 2010), antimicrobial (Al-Zoreky 2009; Endo *et al.*, 2010), antidiarrheal (Olapour *et al.*, 2009) and anti-tyrosinase (Yoshimura *et al*, 2005) activities.

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Material and Methods

Plant Material:

Samples of pomegranate cultivars used for this study were collected from Assuit and El-Badary, Upper Egypt, Egypt on September 2011 and were kindly identified by Prof. Dr. Moneer Abdel-Ghany, Prof. of Plant Taxonomy, Faculty of Science, Cairo, University. Fruits for each cultivar were manually peeled; the peels were separately air-dried, powdered and kept in tightly closed amber coloured glass containers, protected from light at low temperature as possible. Voucher specimens are kept in herbarium, Pharmacognosy Department, Faculty of Pharmacy Al -Azhar University, Cairo, Egypt.

Chemicals:

Folin-Ciocalteu's reagent, vanillin and 1,1-Diphenyl-2-picryl-hydrazil (DPPH) were purchased from Sigma Chemical Co., Saint Louis, MO, USA. Gallic acid, quercetin, aluminum chloride, catechin, tannic acid and Silica gel 60 F254 were purchased from E. Merck, Darmstadt, Germany while all the solvents used were of analytical grade.

Apparatus:

Soxhlet, rotatory evaporator (BUCHI Rotavapor ® R-210/R-215, Germany), chromatographic glass jars, 96 MicroWell™ Plates, Conical Wells**,** Thermo Fisher Scientific USA and Genesys Spectrophotometer (Milton Roy, INC., Rochester, NY).

Preparation of peel extracts:

For each cultivar, 15 g each finely-powdered peel sample were extracted separately with 150 ml of 80% (v/v) methanol and distilled water for one hour to yield the methanol and aqueous extracts respectively, the extracts were filtered under vacuum through Whatmann No.1 filter paper, the residue was re-extracted following the same procedure two more times, extracts collected were dried under vacuum at 40^0 C.

Determination of total phenolic content:

The total phenolic content in aqueous and methanol extracts of each cultivar were determined spectrophotometrically using the Folin-Ciocalteu's reagent where standard curve was done using different concentrations of gallic acid in methanol. The concentrated extracts of the tested plants were dissolved each in least methanol volume then completed to 10ml, 100µl of these extracts were separately diluted with 8 ml distilled water, to each sample 0.5 ml of 50% Folin-Ciocalteu's reagent was added and left 8 min, and then 1.5 ml of 5% sodium carbonate was added, mixed and allowed to stand for 60 min. protected from light. Their absorbance was measured at 725 nm using methanol as blank and the concentration of the total phenolic content of extracts was calculated as mg gallic acid equivalents per g dry weight (mg GAE g^{-1}) (Zhou and Yu 2006).

Determination of total flavonoid content:

Determination of the total flavonoid content in the each tested extract was done colourimetrically using aluminum chloride solution where standard curve was done using different concentrations of quercetin in methanol, 100µl were added to a 96 Micro-well plate and then 100µl of 2% aluminum chloride solution in methanol were added, after 10 min, their absorbance was measured at 415 nm using methanol as blank and the concentration of total flavonoids was calculated as mg quercetin equivalent per g dry weight (mg QE g^{-1}) (Djeridane *et al.,* 2006).

Determination of proanthocyanidin content:

Determination of the total proantho-cyanidin content each of tested extract was done colourimetrically using vanillin-methanol reagent where standard curve was done using different concentrations of catechin in methanol, 0.05 g of dried extract was dissolved in 5 ml methanol or the filtrates made up to 50 ml were used directly, 1 ml of the solution was mixed with 3 ml of 4% vanillin-methanol solution and 1.5 ml hydrochloric acid, allowed to stand for 15 min at room temperature, then the absorbance was measured at 500 nm and the proanthocyanidin contents were expressed as µg catechin equivalents per g dry weight (µg CE g^{-1}) (Kelm *et al.,* 2005).

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Determination of hydrolysable tannins content:

Determination of the hydrolysable content in the each tested extract was done colourimetrically using 2.5% potassium chlorate solution reagent where standard curve was done using different concentrations of tannic acid in methanol, 1 ml of 10-fold diluted extracts and 5 ml of 2.5% potassium chlorate solution were added into a vial and mixed for 10 seconds, the absorbance of the red colored mixture was measured at 550 nm versus the prepared water blank noting that the optimum reaction defined as the time to gain maximum absorbance value, was determined to be 2 min for pomegranate peel extracts and 4 min for standard solutions of tannic acid. Total hydrolysable tannin contents were expressed as µg tannic acid equivalent per g dry weight (µg TAE g^{-1}) (Çam and Hişil 2010).

Screening of antioxidant effect and determination of percent DPPH free radical scavenging:

Determination of antioxidant effect of the tested extracts was done according to the stable DPPH radical technique both qualitatively using thin layer chromatography (TLC) and quantitatively using spectrophotometric methods.

- *TLC assay:*

20 µl aliquot of each extract was spotted on silica gel plates and developed using butanol: acetic acid: water (4:1:5) as a mobile phase, after development, the dried TLC plates were sprayed with 0.2% DPPH solution in methanol and examined after 30 min. where active extracts as antioxidants appeared as yellow spots against purple background (Cavin *et al.,* 1998).

- *Spectrophotometric assay:*

the test was carried out on 96 Micro-Well plate where a standard curve was done using different concentrations of ascorbic acid in methanol (7serial 2fold dilutions to give final range of 100 to 5 µm). 50 µl of a 0.022% DPPH solution in methanol was added to a range solution of different concentrations (7 serial -3 fold solutions to give final range of 1000 to 1.3 µg /ml) of the extracts and (7 serial 2 fold dilutions to give final range of 100 to 5 µm) of compounds to be tested in methanol (230 µl) and their absorbance was measured at 515 nm after 30 min., the percent radical scavenging activities were calculated (Gálvez *et al.,* 2005).

Screening of anticancer effect:

the anticancer effect against U251 and Hepg2 which are corresponding to brain and liver cancer cell lines respectively were performed on the tested extracts where the cells were plated in a 96-multiwell plate (104 cells/well), for 24 h, before treatment with the extracts to allow attachment of cells to the wall of the plate. Different concentrations of the extracts (0, 1, 2.5, 5 & 10 µg/ml in DMSO) were added to the cell monolayer; triplicate wells were prepared for each concentration. Monolayer cells were incubated with the tested samples for 48 h at 37°C, in an atmosphere of 5% CO2. After 48 h, the cells were fixed, washed and stained with sulphorhodamine-B stain, the excess stain was washed with acetic acid and attached stain was recovered with Tris EDTA buffer, the colour intensity was measured in an ELISA reader and the relation between surviving fraction and the extracts′ concentration is plotted to get the survival curve of each tumor cell line after treatment with screened extracts. Cisplatin was used as a reference standard drug, data fitting and graphics were performed by means of the Prism 3.1 computer program (Graph Pad software, USA) (Skehan *et al.,* 1990). Screening of the cytotoxic effect of the tested extracts was carried out in the National Cancer Institute, Cairo University.

Statistical analysis:

The statistical analysis of the outcome data was carried out using one way analysis of variance (ANOVA) followed by student t-test, P value <0.05 were considered as significant (Elliott and Woodward 2007).

Results and Discussion

Pomegranate (*Punica granatum* L.) is an important fruit of tropical and subtropical regions which has been used for centuries in ancient cultures for its medicinal purposes, recent studies revealed that the peel has very generous collection of phenolic compounds (Rather *et al.,* 2010) the major of which are tannin compounds to whom a tremendous number of biological effects have been referred. Tannin compounds are implicated in

antimicrobial (Miguel *et al,* 2010), antioxidant, anticancer and anti-proliferative effects (Ibrahim *et al.,* 2010, Abdel-Hady *et al.*, 2011, Abdel-Motaal *et al.*, 2011, Eghdami *et al.*, 2011, El-Hela *et al.*, 2013).

In recent years there have been many reports concerning the role of polyphenol compounds in counteracting negative effects of oxygen and nitrogen reactive species (ROS/RNS), maintaining the redox homeostasis of biological fluids and preventing human disease such as cardiovascular diseases, atherosclerosis, and other degenerative pathologies such as cancer, diabetes, Alzheimer's and Parkinson's diseases (Tzulker *et al.,* 2007; Jahangir *et al.,* 2009; Cartea *et al.,* 2011; Avarim *et al.,* 2013).

Quantitative estimation of the total phenolic content of the aqueous and methanol extracts of the tested cultivars using Folin-Ciocalteu's reagent table 1, figure 1 showed that the aqueous and methanol extracts of Wardy cultivar contains the highest percent of phenolic compounds (246.37±4.61 and 214.91±3.29) followed by Assuity (224.62±4.84 and 190.44±3.02); Nab El-Gamal (193.39±3.79 and 148.13±2.99); Manfalouty (189.18±3.07 and 163.70± 3.47) and Balady (179.30±4.82 and 158.30±4.15) mg GAE g^{-1} respectively, the total phenols were measured by in terms of gallic acid equivalent (the standard curve equation is y = 0.05 X±0.0545, r^2= 0.9873); while quantitative estimation of total flavonoids of each of the tested cultivars using aluminum chloride reagent and quercetin as standard revealed that the highest percent present in the methanol and aqueous extracts of Wardy cultivar (136.72± 2.74 and 123.72±2.01) followed by Assuity (128.48± 2.53 and 111.48 ± 1.90); Manfalouty (120.68±2.58 and 105.68±1.88); Nab El-Gamal (106.91±2.81 and 99.91±2.10) and Balady (106.89±2.92 and 96.89±2.59) mg QE g^{-1} respectively, the total flavonoid contents of the extracts in terms of quercetin equivalent (the standard curve equation is y = 0.0067X ± 0.0132, r^2 = 0.999).

Quantitative estimation of the total proanthocyanidin content of the aqueous and methanol extracts of the tested cultivars table 1, showed that the methanol and aqueous extracts of Assuity cultivar contains the highest percent of proanthocyanidins (305.60±2.02 and 297.26 ±1.92) followed by Nab El-Gamal (339.80±3.52 and 318.40±2.39); Manfalouty (227.20± 2.02 and 199.49±2.04); Wardy (89.20±1.32 and 82.07±1.33) and Balady (85.05±2.16 and 80.92±2.41) µg CE g^{-1}, the total phenols were measured by in terms of catechin equivalent (the standard curve equation is y = 0.005 X±0.01472, r2= 0.9950); while quantitative estimation of total hydrolysable tannin content of each of the tested cultivars using potassium chlorate reagent and tannic acid as standard revealed that the highest percent present in the aqueous and methanol extracts of Manfalouty cultivar (779.28±28.10 and 762.90±28.04) followed by Wardy (730.19±29.80 and 693.10±28.27); Assuity (691.40±28.35 and 682.52±28.04); Balady (579.30±30.62 and 565.73±28.40) and Nab El-Gamal (519.10±26.30 and 506.40±25.85) µg TAE g^{-1} respectively, the total hydrolysable tannin contents of the extracts in terms of tannic acid equivalent (the standard curve equation is y = 0.0038 X±0.01608, r2= 0.9703).

Table 1: Total phenolic, flavonoid, proanthocyanidin and hydrolysable tannin contents of aqueous and methanol extracts of five selected Egyptian pomegranate cultivars:

Cultivar	Total Phenolic content (mg GAE g^{-1})		Total Flavonoid content (mg QE g^{-1})		Total Proanthocyanidin content (µg CE g^{-1})		Total Hydrolysable tannin content (µg TAE g^{-1})	
	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract
Assuity	224.62 ±4.84	190.44 ± 3.02	111.48 ± 1.90	128.48 ± 2.53	297.26 ± 1.92	305.60 ± 2.02	691.40 ±28.35	682.52 ±28.04
Balady	179.30 ± 4.82	158.30 ± 4.15	96.89 ± 2.59	106.89 ± 2.92	80.92 ± 2.41	85.05 ± 2.16	579.30 ±30.62	565.73 ±28.40
Manfalouty	189.18 ± 3.07	163.70 ± 3.47	105.68 ± 1.88	120.68 ± 2.58	199.49 ±2.04	227.20 ± 2.02	779.28 ±28.10	762.90 ±28.04
Nab-El Gamal	193.39 ± 3.79	148.13 ± 2.99	99.91 ± 2.10	106.91 ± 2.81	318.40 ± 2.39	339.80 ± 3.52	519.10 ±26.30	506.40 ±25.85
Wardy	246.37 ± 4.61	214.91 ± 3.29	123.72 ± 2.01	136.72 ± 2.74	82.07 ± 1.33	89.20 ± 1.32	730.19 ±29.80	693.10 ±28.27

The tabulated values are means ± S.D. where n=3.

Qualitative TLC-DPPH assay of the tested extracts showed that they all are active compounds as DPPH scavengers appearing as zones with different R_f values at in the chromatogram, these results directed the research to quantitative estimation of the antioxidant capacity of each extract individually.

Spectrophotometric quantitative estimation using DPPH method of the antioxidant potential of the aqueous and methanol extracts at three dose levels 500, 750 and 1000µg ml^{-1} revealed that they possesses significant free radical scavenging potential at all tested dose levels and the most effective dose is 1000 µg ml^{-1} which is proven to be more potent when compared with the reference synthetic antioxidant butylated hydroxytoluene (BHT), the most significant free radical scavenging effect indicated as percent free radical scavenging table 2, figure 1 which proved that the higher potency was recorded for the aqueous and methanol extracts of Assuity (98.20±1.93 and 94.37±1.81) followed by Wardy (95.28±1.73 and 92.30±1.92), Manfalouty (90.15±1.70 and 88.03±1.96), Nab El-Gamal (89.72±1.62 and 87.30±1.87) and Balady (85.19±1.52 and 82.04±1.30) respectively

and these extracts have highest calculated percent of total flavonoids i.e. 66, 85.8 & 88.7mg % & of total phenolic compounds i.e. 140.43, 86.17 and 88.70 mg % respectively.

Table 2: Antioxidant effect of aqueous and methanol extracts of five selected Egyptian pomegranate cultivars:

Cultivar Medicinal Plant	% Scavenging*					
	1000 µgml^{-1}		750 µg ml^{-1}		500 µgml^{-1}	
	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract
Assuity	98.20 ± 0.93	92.37 ± 1.01	82.16 ± 0.71	89.93 ± 0.70	61.90 ± 0.62	69.21 ± 0.60
Balady	85.19 ± 0.92	82.04 ± 0.90	65.20 ± 0.60	73.90 ± 0.62	65.73 ± 0.50	62.80 ± 0.52
Manfalouty	90.15 ± 1.00	88.03 ± 0.96	71.44 ± 0.78	79.47 ± 0.73	58.38 ± 0.59	66.10 ± 0.57
Nab- El Gamal	89.72 ± 1.02	87.30 ± 1.01	68.10 ± 0.72	85.03 ± 0.68	56.70 ± 0.60	65.83 ± 0.54
Wardy	95.28 ± 1.03	94.30 ± 1.02	85.40 ± 0.80	83.18 ± 0.82	66.18 ± 0.67	75.30 ± 0.62

*EC_{50} for Butyl hydroxyl toluene (BHT) is 400 µg ml^{-1} equivalent to 93±0.50 % scavenging

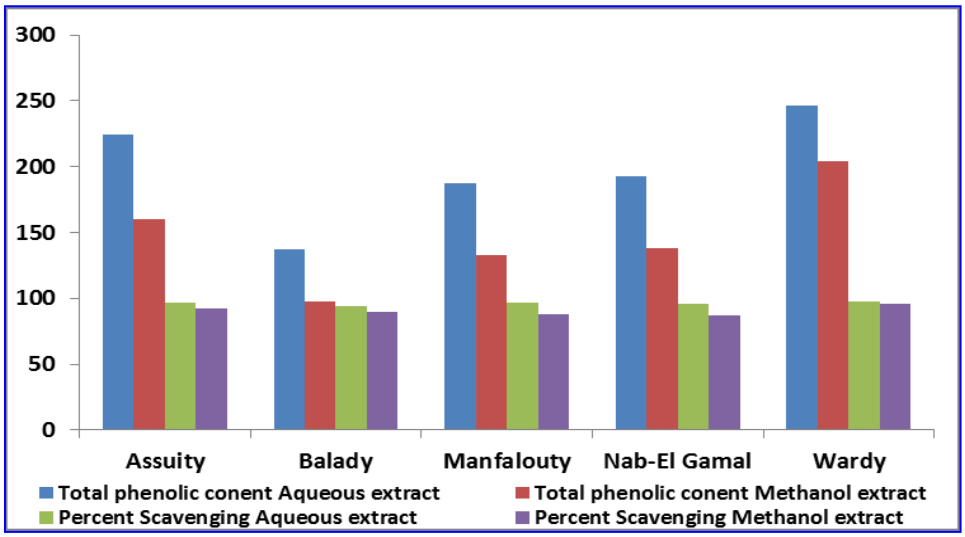

Fig. 1: Total phenolic content and percent DPPH radical scavenging effect in aqueous and methanol extracts of five selected Egyptian pomegranate cultivars.

Fortunately, the anticancer screening of the tested extracts on Hepg 2 and U251 cell lines table 3, figure 2 revealed that the most potent extracts expressed as the least IC_{50} compared to the anticancer standard Cisplatin were the aqueous and methanol extracts of Wardy cultivar (0.34±0.05 and 0.40±0.18), Assuity (0.36±0.02 and 0.50±0.01), Manfalouty (0.42±0.02 and 0.60±0.15), Nab El Gamal (0.43±0.02 and 0.60±0.16) and Balady (0.61±0.03 and 0.85±0.18) µg ml^{-1} for Hepg 2 cell line, while for U251 cell line the most active were the methanol and aqueous extracts of Wardy cultivar (0.40±0.15 and 0.42±0.12), Assuity (0.45±0.20 and 0.56±0.19), Manfalouty (0.51± 0.02 and 0.55±0.17), Nab El Gamal (0.57±0.19 and 0.59±0.22) and Balady (0.59±0.19 and 0.64±0.24) µg ml^{-1} .

Table 3: Anticancer effect of aqueous and methanol extracts of the selected Egyptian pomegranate cultivars:

Cultivar Medicinal Plant	Cytotoxic Effect* IC_{50} (µ g ml^{-1})			
	Hepg2		U 251	
	Aqueous extract	Methanol extract	Aqueous extract	Methanol extract
Assuity	0.36±0.02	0.50±0.10	0. 56±0.20	0.45±0.19
Balady	0.61±0.03	0.85±0.19	0. 64±0.19	0.59±0.24
Manfalouty	0.42±0.02	0.60±0.15	0.55±0.17	0.51±0.18
Nab- El Gamal	0.43±0.02	0.60±0.16	0.59±0.19	0.57±0.22
Wardy	0.34±0.05	0.40±0.18	0.42±0.15	0.40±0.12

*IC_{50} for Cisplatin was 0.87 for Hepg2 and 0.56 µg ml^{-1} for U251.

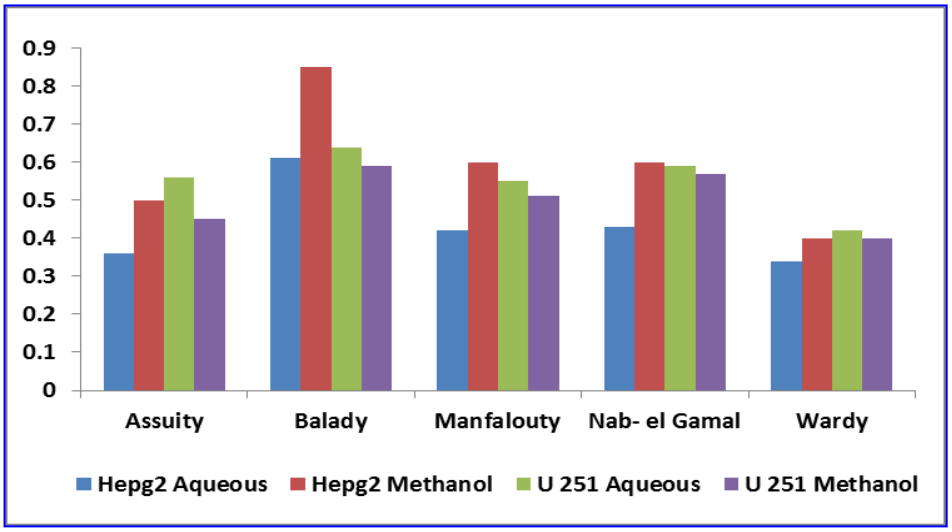

Fig. 2: Anticancer effect of aqueous and methanol extracts of five selected Egyptian pomegranate cultivars.

The anticancer effect can be attributed to the moderate the existence of the total phenolic and flavonoid content in the tested extracts meanwhile the antioxidant effect is mainly referred to their flavonoid content mainly as evidenced by the gained data.

This study presented a comprehensive comparison among the most widely consumed five pomegranate cultivars in Egypt on the basis of their total phenolic, flavonoid, proanthocyanidin and hydrolysable tannin content as well as their antioxidant and anticancer effects, in conclusion, the study revealed that the Wardy cultivar is the most generous source of polyphenol compounds as it was monitored by its highest phenolic content and also can serve as powerful natural antioxidant and anticancer against both Hepg2 and U251 in both its aqueous and methanol extracts, followed by the Assuity and Nab-Elgamal cultivars respectively which exhibited close results.

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