# Anticancer and Antioxidant Activities of Standardized Whole Fruit, Pulp, and Peel Extracts of Egyptian Pomegranate

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**Abstract:** Different parts of the fruit of *Punica granatum* L. family Punicaceae cultivated in Egypt were extracted and standardized to be assessed for their anticancer and antioxidant properties. An HPLC method was modified and validated for standardization using ellagic acid (EA) as a marker. The 50% ethanol was proven to be a better solvent for extraction than the 70% ethanol, as the 50% hydroalcoholic fruit extract was standardized to contain 5.9 ± 0.15 % total polyphenols compared to 4.4 ± 0.35 % in the 70% hydroalcoholic extract. The 50% hydroalcoholic extracts of the whole fruit, pulp and peels were standardized to contain 0.3 ± 0.05, 0.02 ± 0.01 and 1.9 ± 0.1 % of EA, respectively. The peel extract showed the highest antioxidant activity ($IC_{50}$ = 0.50 ± 0.9 mg/ml) compared to the other two extracts, as well as, a pronounced anticancer activity against MCF-7 human breast cancer cells and HCT-116 colon cancer cells with $IC_{50}$ values of 7.7 ± 0.01 and 9.3 ± 0.06 µg/ml, respectively. The standardized peel extract was formulated into capsules. Here we report the possible use of pomegranate peels, a biological waste product, to develop natural pharmaceutical preparations.

**Keywords:** Anticancer, antioxidant, biowaste, ellagic acid, peels, *punica granatum*, validation.

## INTRODUCTION

Carcinogenesis is becoming a big load on families and economies. Fruits consumed in our daily diet could be a solution to this burden by providing a chemoprotective or/and chemotherapeutic remedy. Oxidative stress may initiate molecular events in the cancer process, and reduction of oxidative stress may protect against carcinogenesis [1]. Moreover the fruit waste by-products possessing such activities would be a great support to the economy sparing huge sums of money.

*Punica granatum* L. family Punicaceae is cultivated around the world in subtropical and tropical regions such as in Iran, California, Turkey, Egypt, Italy, India, Chile and Spain. The world pomegranate production amounts to approximately 1,500,000 tons [2], where the peels (pericarp, rind or hull) amounts to approximately 60% of the pomegranate fruit weight [3]. Pomegranate fruit extracts rich in ellagitannins (ETs) proved their efficacy as antioxidant and anticancer agents, especially against breast and colon cancer [3-6].

Pomegranate peel extracts exhibited marked antioxidant capacity in several studies using unsafe solvents such as methanol and a mixture of methanol, acetone, ethyl acetate and water [7-9].

Knowing that no such studies were performed on Pomegranate growing in Egypt, we decided to carry out a comparative study to assess extracts from different Egyptian pomegranate compartments, namely the whole fruit, pulp and peels, with the aim of defining an effective standardized extract having a suitable yield to be incorporated into a pharmaceutical preparation as a dietary supplement. Solvents used for extraction were chosen to be safe and suitable for large scale production afterwards.

ETs are the predominant phenolics in pomegranate peel, mesocarp and arils extracts [10].

Upon consumption these ETs are hydrolyzed releasing EA, which is then converted to urolithin derivatives by gut microflora [11]. EA is known for its antioxidant, anti-inflammatory and anticarcinogenic properties, besides it is considered as a biomarker for human bioavailability studies involving consumption of ETs containing food, as it is detected in human plasma [12]. For these reasons we chose EA, among several other phenolic compounds present [13], as a bioactive marker for standardization of pomegranate extracts investigated in this study. Validation of the HPLC method used was also performed.

## MATERIALS AND METHODS

### Plant Material and Chemicals

Fresh Pomegranate fruit cultivated in Egypt were obtained from Sekem farms, Belbeis, Egypt. All chemical reagents and extraction solvents were of analytical grade, and all analysis solvents were of HPLC grade. Ellagic acid, diphenyl-1-picrylhydrazyl (DPPH) and gallic acid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Preparation of Pomegranate Extracts

Fresh pomegranate fruit were collected in October 2008, cut into pieces, blended in 70% and 50% ethanol solution.

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Pomegranate fruit were peeled manually where the edible pulp (arils) was blended with 50% ethanol. The peels were left to dry in the shade, extracted and macerated in 50% ethanol solution. All extracts were macerated for two days, filtered and the marc macerated for another two days, filtered and concentrated under vacuum at 50 $^{o}$C. The yields of the extracts were 28 ± 2.3, 14 ± 1.5 and 45 ± 3.2 % for the whole fruit, pulp and peels, respectively, based on dry weight.

### Spectrophotometric Determination of Total Polyphenols

Total polyphenols calculated as gallic acid were determined by the Folin-Ciocalteu method according to the European pharmacopoeia [14], using gallic acid as a standard. Folin-Ciocalteu reagent was purchased from Loba Chemie, Mumbai, India.

### Standardization of Pomegranate Extracts by HPLC

An HPLC method, modified after Gudej and Tomczyk [15], was adopted for the determination of the concentration of ellagic acid in pomegranate different extracts. An Agilent 1100 series HPLC was used, equipped with an Agilent G1311A quaternary pump, a G1314A variable wavelength detector and a G1328A manual injector.

Samples were dissolved in methanol, filtered through PTFE 0.45µm syringe filter (Macherey-Nagel, Germany) and injected into a Hypersil 100 RP-18, 5µm, 250 x 4 mm column. The mobile phase was methanol (solvent A) and 0.5% orthophosphoric acid in water (solvent B). Gradient elution was carried out at a flow rate of 1.2 ml/min as follows: 0-5 min 50 to 60% A in B, 5-8 min 60 to 85% A in B and 8-10 min 85 to 50% A in B. Measurements were made with an injection volume of 20 µl and UV detection at 254 nm. A standard calibration curve was prepared using different concentrations of ellagic acid in methanol (1.6, 3.2, 4.8, 6.4 and 8 µg/ml). Three replicate assays were performed for each sample.

### Validation of HPLC Method

Linearity was determined by injecting five different concentrations of EA standard solution (1.6 - 8 µg/ml). The accuracy was calculated as the percent recovery of spiked pomegranate extract samples with EA samples at three concentrations (1.6, 3.2 and 4.8µg/ml). To establish the intra-day and inter-day precision of the method, EA was assayed on one day and three separate days at three different concentrations (1.6, 4.8 and 8 µg/ml). Each was injected in triplicates. Limit of quantitation (LQ) and limit of detection (LD) were determined based on the standard deviation of the response (σ) and the slope of the calibration curve (S) according to the following equations [16]:

$$LQ = 10 (\sigma/S)$$

$$LD = 3.3 (\sigma/S)$$

### Antioxidant Activity

The antioxidant activity was assayed using a modified quantitative DPPH assay [17]. DDPH solution was prepared with an HPLC grade methanol at a concentration of 0.004%. Extracts were dissolved in water at a concentration of 0.4, 0.6, 0.8 and 1 mg/ml, with 100 µl of each test solution added to 3 mL DPPH solution. Blank samples were run using 100 µl water in place of the plant extract. After a 30 min incubation period at room temperature, the absorbance was measured against a blank at 517 nm. Gallic and ellagic acids were used as positive controls at a concentration of 0.02, 0.04 , 0.06, 0.08 and 0.1 mg/ml. Inhibition of free radical in percent (I%) was calculated according to this formula: I%= [ ( $A_0$ - $A_1$)/ $A_0$] X 100. Where $A_0$ is the absorbance of the control reaction (containing all reagents except the extract), and $A_1$ is the absorbance of the extract. Measurements were carried out in triplicates.

### Anticancer Activity

Human breast carcinoma cells (MCF-7) and colon carcinoma cells (HCT-116) obtained from the National Cancer Institute, Cairo, Egypt were used for screening the anticancer activity of the pomegranate extracts using the sulforhodamine B (SRB) colorimetric cytotoxicity assay [18]. The extracts were dissolved in DMSO and tested at concentrations of 1, 2, 2.5, 5 and 10 µg/ml. Doxorubicin was used as a positive control at the same concentration range. Final concentration of DMSO in the culture medium was maintained at 0.1% to avoid solvent toxicity. Cytotoxicity was expressed as the percent of viable cells relative to cells incubated in the presence of 0.1% DMSO vehicle control. The concentration of the plant extract inhibiting cell growth by 50% relative to vehicle control ($IC_{50}$ value) was also determined. Each measurement was performed in triplicate.

### Formulation of the Peel Extract

Pomegranate peel extract was dissolved in least amount of ethanol and loaded on silicon dioxide HDK®N20 (Nünchritz, Germany) in the ratio of 73-75% (native extract 2-3:1) : 25-27% (silicon dioxide). The prepared extract (PE) was dried in an oven at 50$^{o}$C and analyzed. Formulation into a capsule (size 0) was achieved by mixing 400 mg of PE with 65 mg micro-crystalline cellulose 101, 20 mg talc and 15 mg magnesium stearate.

## RESULTS AND DISCUSSION

### HPLC Standardization of Pomegranate Extracts

A 50% and a 70% hydroethanolic extracts of pomegranate whole fruit were prepared and analyzed spectrophotometrically to determine the better solvent for extraction. The 50% hydroalcoholic extract proved to contain 5.9 ± 0.15 % total polyphenols calculated as gallic acid, compared to 4.4 ± 0.35 % in the 70% extract. Hydroalcoholic extracts (50%) of the whole fruit, pulp and peels were prepared and standardized using an HPLC method, modified after Gudej and Tomczyk [15]. Knowing the antioxidant, anti-inflammatory and anticarcinogenic properties of EA, it was chosen as a bioactive marker for standardization of these extracts. EA peak appeared at a retention time of 5.9 min. The peel extract was standardized to contain 1.9 ± 0.01 % of EA, while the fruit and pulp extracts contained 0.3 ± 0.05 and 0.02 ± 0.01 %, respectively Fig. (**1**). The calibration curve showed good linearity for EA in the range of 1.6 - 8 µg/ml, with a correlation co-efficient ($R^2$) of 0.998 Fig. (**2**). The percent recovery was within the range of 98.0 – 102.0 % showing the accuracy of the method. The intra- and inter-day variation of the method was carried out and the low values of relative standard deviation (RSD < 2%) within a day and day to day variations revealed that this method is precise (Table **1**). The minimum

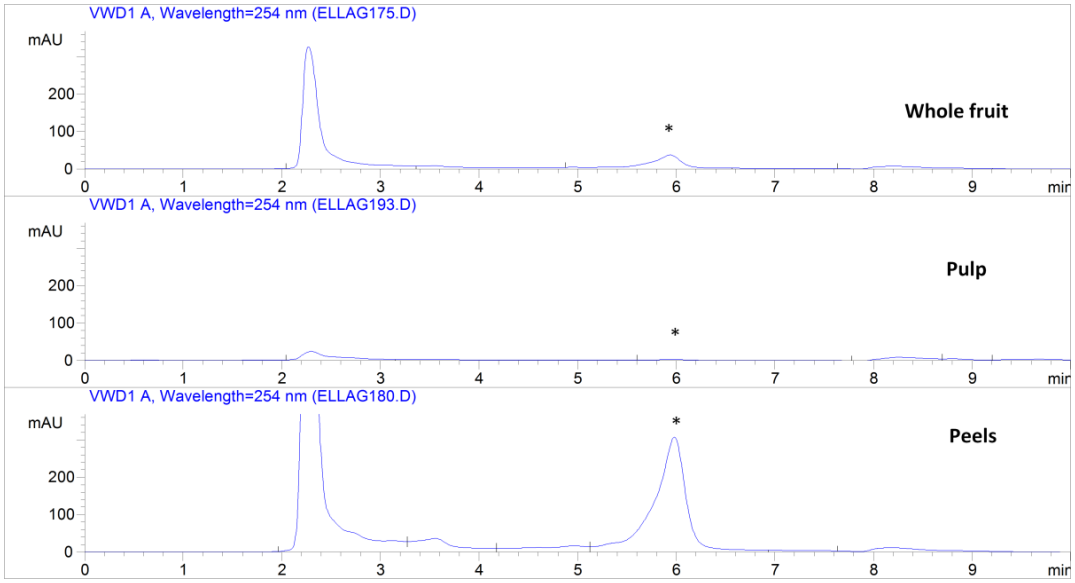


**Fig. (1).** HPLC chromatograms of 50% ethanolic extracts of pomegranate fruit, pulp and peels. Peaks highlighted with an asterisk represent that of ellagic acid (Rt at 5.9 min). Chromatographic conditions are described under "Materials and Methods".

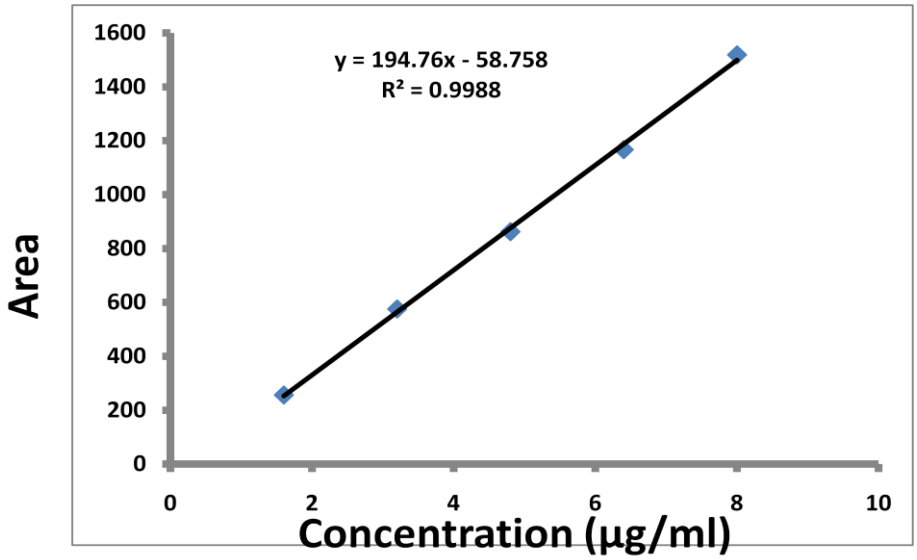


**Fig. (2).** Calibration curve of EA using an HPLC method modified after Gudej and Tomczyk [15].

concentration levels at which the analyte can be reliably detected (LD) and quantified (LQ) were found to be 0.116 and 0.354 µg/ml, respectively (Table **1**). The LD and LQ of an HPLC method, previously developed by Panichayu-pakarananta [9] for standardization of pomegranate peel extract using also EA, were 1.00 and 2.50 µg/ml, respectively. His percent recovery was 98.5% versus 99.57 ± 1.017 % achieved by our method. The EA peak did not interfere with any other peak in the extract samples Fig. (**1**), which indicated the specificity of the proposed method.

### Formulation of Peel Extract

PE was used as the raw material for preparation of pomegranate capsules. It was characterized as a yellowish brown powder, standardized to contain 1.5 ± 0.05% of EA. The prepared capsules were opaque brown hard gelatin capsules having a filling weight of 500 ± 10%, analyzed to contain 1.4 ± 0.07% of EA and 8.5 ± 0.25% of total polyphenols. Disintegration time of the capsules was 3 min.

### Antioxidant Activity of Pomegranate Extracts

Pomegranate standardized extracts were assessed for their capacity to scavenge DDPH free radical along with gallic acid as a positive control. The antioxidant activity data are presented in Table **2**, in terms of $IC_{50}$ ± SD (n=3), which is the concentration in mg/ml causing 50% inhibition of the free radical. The 50% ethanolic extract of the peels standardized to contain the highest percent of EA (1.9 ± 0.1%) exhibited a pronounced antioxidant activity ($IC_{50}$ = 0.50 ± 0.9 mg/ml) compared to the fruit (0.3 ± 0.05% EA) and pulp (0.02 ± 0.01% EA) extracts showing an $IC_{50}$ of 1.09 ± 0.5 mg/ml and 13.6 ± 0.8 mg/ml, respectively. These results show a direct correlation between the EA content in pomegranate extracts and its ability in quenching free

**Table 1. Results of HPLC Method Validation Used for Standardization of Pomegranate Extracts**

| | |
|---|---|
| Range | 1.6 – 8 µg/ml |
| Linearity | correlation coefficient: 0.998<br>slope: 194.75<br>Intercept: -58.7583 |
| Accuracy | Recovery: 99.57 ± 1.017 % |
| Precision | Intra-day RSD 1.72%<br>Inter-day RSD 1.86% |
| Limit of Detection | 0.116 µg/ml |
| Limit of Quantitation | 0.354 µg/ml |
| Specificity | No interference with other peaks |

radicals. The contents of total phenolics in pomegranate peel extract was reported to be 10-fold as much as its content in the pulp extract, which causes its stronger antioxidant ability [19]. It was also mentioned that the EA content and the antioxidant activity of the ethyl acetate fraction separated from the crude peel extract using water and ethyl acetate partition was higher than that of the crude extract [9].

**Table 2. Antioxidant Activity Assayed by DPPH Test of Pomegranate Extracts**

| Extract | $IC_{50}$ (mg/ml) ± SD |
|---|---|
| Whole fruit | 1.09 ± 0.5 |
| Peels | 0.50 ± 0.9 |
| Pulp | 13.6 ± 0.8 |
| Gallic acid | 0.05 ± 0.4 |
| Ellagic acid | 0.08 ± 0.5 |

Activity is expressed as $IC_{50}$ ± SD (n=3), which is the concentration (mg/ml) causing 50% inhibition of the free radical. Whole fruit and peel extracts were tested at 0.4, 0.6, 0.8 and 1mg/ml, pulp extract at 4, 6, 8 and 10 mg/ml while the positive controls (gallic and ellagic acids) at 0.04, 0.06, 0.08 and 0.1 mg/ml.

## Anticancer Activity of Pomegranate Extracts

The anticancer activity of pomegranate fruit, peels and pulp extracts standardized to their EA content was assessed. Cytotoxicity was tested against MCF-7 cell line (human breast carcinoma) and HCT-116 cell line (human colon carcinoma) along with doxorubicin as a positive control. The dose-dependent cytotoxic activity of the three standardized extracts are presented in Figs. (**3** and **4**). The anticancer activity against both cell lines increased by increasing the extract concentrations from 1 to 10 µg/ml for all three extracts. The pomegranate peel extract showed a pronounced cytotoxic activity against MCF-7 cells compared to the other two extracts at a concentration of 10 µg/ml Fig. (**3**), confirmed by the least $IC_{50}$ (7.7 ± 0.01 µg/ml) (Table **3**). On the other hand, the anticancer activity of the fruit extract against HCT-116 colon cells was the most prominent revealed by an $IC_{50}$ of 4.8 ± 0.02 µg/ml followed by the pulp extract ($IC_{50}$ = 6.1 ± 0.04 µg/ml). It was previously reported that the ellagitannins and their intestinal bacterial metabolites, urolithins, released in the colon upon consumption of pomegranate juice could potentially diminish the risk of colon cancer development, by inhibiting cell proliferation and inducing apoptosis [5]. Also pomegranate ET-derived compounds especially urolithin B have potential for the prevention of estrogen-responsive breast cancers by significantly inhibiting testosterone-induced MCF-7 cell proliferation [4]. However according to the presented data, EA content alone does not reflect the potency of the anticancer activity as the pomegranate fruit is rich in several other cytotoxic compounds as sterols, hydroxybenzoic acids, catechins, epicatechins, anthocyanidins and flavonoids which could also contribute to the anticancer activity [3]. Future studies need to address the possible mechanisms of anticancer activities of the different extracts and their corresponding compounds.

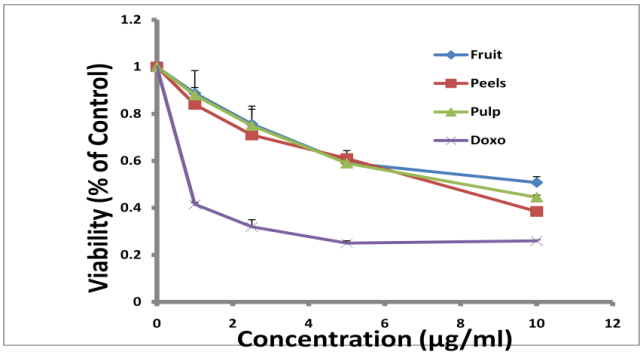


**Fig. (3).** Dose-dependent cytotoxicity of pomegranate extracts on MCF-7 breast cancer cells using the SRB assay. Viability is calculated as a percent of control (cells incubated with 0.1% DMSO). Results are the average of three independent experiments ± SD (n=3). Doxorubicin (Doxo) was used as a positive control.

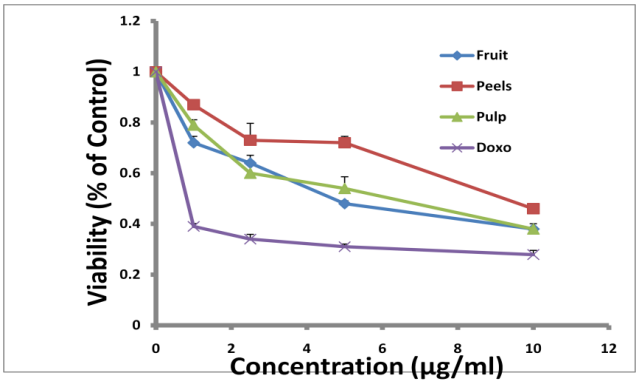


**Fig. (4).** Dose-dependent cytotoxicity of pomegranate extracts on HCT-116 colon cancer cells using the SRB assay. Viability is calculated as a percent of control (cells incubated with 0.1% DMSO). Results are the average of three independent experiments ± SD (n=3). Doxorubicin (Doxo) was used as a positive control.

## CONCLUSION

This study indicates that pomegranate peel extract of Egyptian pomegranate collected in October, standardized to contain 1.9 ± 0.1 % of EA, has a potential as an antioxidant

**Table 3. Cytotoxicity of Pomegranate Extracts on Human MCF-7 and HCT-116 Carcinoma**

| Extract | $IC_{50}$ µg/ml ± SD | |
|---|---|---|
| | MCF-7 | HCT-116 |
| Whole fruit | 9.9 ± 0.03 | 4.8 ± 0.02 |
| Peels | 7.7 ± 0.01 | 9.3 ± 0.06 |
| Pulp | 7.9 ± 0.05 | 6.1 ± 0.04 |
| Doxorubicin | 0.7 ± 0.01 | 0.69 ± 0.01 |

Cytotoxicity is expressed as $IC_{50}$ ± SD (n=3), which is the concentration of the plant extract inhibiting cell growth by 50% relative to cells incubated in the presence of 0.1% DMSO vehicle control.

compared to the whole fruit and pulp extracts. EA could be used as a biomarker for pomegranate dietary supplements used as antioxidants. Further studies are required to assess the EA content of pomegranate peel extract and its corresponding activities at different growing seasons in Egypt.

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