

# Correlation of the Cytotoxic and Antioxidant Activities of Moroccan Pomegranate (*Punica Granatum*) with Phenolic and Flavonoid Contents

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**Abstract:** The Pomegranate (*Punica Granatum*), which belongs to the Lythraceae family, has been used for centuries in traditional Greco-Arab and Islamic medicine of its vermifuge properties and also to treat various diseases. The aim of this research was to investigate the cytotoxicity and antioxidant activities of Moroccan Pomegranate (peel, leaves, branches, flowers and corolla). Further, the biological activities were correlated with phytochemical contents of the plant extracts. Methanolic extract from different parts of *Punica Granatum* was assessed for its antiproliferative activity in two human cancer (breast and colon) cells lines (MBA-MD 231 and HT-29), through MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) bioassay using cell viability and cytotoxicity indices. DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was conducted to screen the antioxidant property of the extracts together with its phenolic and flavonoids content were evaluated, as well. The methanolic extract of *Punica Granatum* (peel, leaves, branches, flowers and corolla) showed the highest antiproliferative activity on MBA-MD 231 (IC<sub>50</sub> was 133.53~233.32 µg/mL) and HT-29 (IC<sub>50</sub> was 127.58~203.24 µg/mL) cells. Antioxidants contents are distributed as follows: peel > leaf > flower > corolla > branches. The inhibitory activities required for decreasing initial DPPH by 50% are 8.27, 9.9, 10.06, 11.67 and 13.28 µg/mL, respectively. These results are in correlation with polyphenols content from corolla, peel, leaves, flower and branches are 120.7, 115, 96.65, 90.73 and 64.67 mg GAE/g dw (mg gallic acid equivalents per g dry weight) and flavonoids are 188.8, 221.7, 180.2, 193.7 and 158.5 mg QE/g dw (mg quercetin equivalents per g dry weight). Our results show that the peel, flowers, corolla, leaves and branches of Moroccan Pomegranate may contain a lot of bioactive compounds which are responsible for strong antioxidant and cytotoxicity activities observed here. Our finding indicates the possibility of using the extracts of this plant as source of natural antioxidant and anticancer mainly for its abundant phenolic and flavonoid contents.

**Key words:** *Punica Granatum*, phytochemical screening, polyphenol, flavonoids, antioxidant activity, cytotoxic activity.

## 1. Introduction

The African continent is full of diverse medicinal plants. According to the World Health Organization, more than 80% of African people resort to medicine and traditional medicine to deal with health problems. On roughly 300,000 species of medicinal plants identified on the planet, more than 200,000 live in Africa and have medicinal properties [1].

Nowadays, several research teams are extracting

from medicinal plants substances necessary for the creation of new drugs. However, the potential of plants as sources for the production of new drugs is largely untapped. Indeed, out of an estimated 250,000, only 6% were tested for their biological activity and 15% were assessed on the phytochemical plan [2]. The consumption of a plant-based or phytochemical-rich diet has been associated with a reduced risk of chronic human illnesses such as certain types of cancers, inflammation, cardiovascular and neurodegenerative diseases, hence the current interest for Pomegranate as

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a dietary supplement or a drug because of its enormous compounds with lots of activities and without toxicity [3, 4]. The Pomegranate (*Punica Granatum*) originated in Western Asia, where it is growing spontaneously for over 4,000 years. It belongs to the *Lythraceae* family and it acclimates all-round the Mediterranean. This shrub has been used for centuries in traditional Greco-Arab and Islamic medicine to treat various diseases and it figures prominently in all religions, Judaism, Christianity, Islam, Buddhism and Zoroastrianism [5, 6].

The pericarp is used by Chinese to treat diarrhea, metrorrhagia, metrostaxis and bellyache; The flower is used as a dietary supplement for the treatment of diabetes in Unani medicine; Bark and root are believed to have anthelmintic and vermifugic properties in Ayurvedic medicine; The fruit is employed by South Africa people for the treatment of diarrhea [3]. The potential therapeutic properties of pomegranate are wide-ranging and include treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction and protection from UV (ultraviolet) radiation. Other potential applications include infant brain ischemia, Alzheimer's disease, male infertility, arthritis and obesity [7].

Cancer continues to be a major health issue in terms of morbidity and mortality, despite advances in early detection and improvement in treatment options [8]. Breast cancer is the most commonly diagnosed invasive malignancy and the leading cause of cancer death in women worldwide [9]. Colon cancer, the third leading cause of cancer deaths in the world, has become a common malignant tumor that occurs in the digestive tract [10].

This is the reason that is considerable scientific and commercial interest in discovering new antioxidant and anticancer agents from natural product sources. The potential of using natural products as anticancer agents was recognized in the 1950s by the U.S. NCI (National Cancer Institute) [11].

The main objectives of this research were to evaluate

the protective effects of different extracts of *Punica granatum* against free radical mediated damages under *in vitro* situations. *In vitro* assays were carried on TPC (total phenolic content), TFC (total flavonoid content), DPPH radical scavenging activity and MTT bioassay using cell viability, and cytotoxicity indices on two human cells lines. Further, the biological activities were correlated with phytochemical contents of the plant extracts.

## **2. Materials and Methods**

### *2.1 Plant Material*

Leaves, branches, flowers and corolla were collected in Berrechid, Chaouia-Ouadigha region with GPS coordinates (33°16'12" North, 7°34'59" West).

### *2.2 Sample Preparation*

#### *2.2.1 Sample Preparation for Qualitative Phytochemical Screening*

The samples are harvested, dried, pulverized and sprayed out successively with solvents of increasing polarity. For our study, we used petroleum ether and methanol, according to the protocol developed by Nemlin and Brunel (1995) [12, 13].

#### *2.2.2 Preparation of Methanolic Extract for Quantification of Phenolic Contents and the Evaluation of the Antioxidant and Cytotoxicity Activities*

The plant material (peel, leaves, branches, flowers and corolla), previously dried and pulverized, was soaked for 24 h in 80% methanol with magnetic stirring at room temperature [14] and then filtered through Whatman 0.45 µm pore. The filtrate was evaporated, and the solvent was removed by distillation and last traces of solvent being removed under vacuum [15].

### *2.3 Identification of Phytoconstituents*

Qualitative phytochemical study aims to identify the chemical constituents of groups of pharmacological interest, namely sterols and polyterpenes by Liebermann test, polyphenols by ferric chloride test,

catechic tannins, gallic tannins, flavonoids by Shinoda test, quinoid compounds by Bornstraëger test, alkaloids and leucoanthocyanins contained in extracts. The tests are based on visual observations between the color changes and/or formation of precipitates after addition of specific reagents [2, 13, 16-24].

#### 2.4 Determination of Total Polyphenol Content

The total phenolics of each extract were determined using a modified Folin-Ciocalteu method. The diluted solution of each extract (200 µL) was mixed with 1 mL (1:10 V/V diluted with distilled water) Folin-Ciocalteu reagent, allowed to stand at room temperature for 5 min and then sodium carbonate solution (75 g/L in water, 800 µL) was added. After 1 h of incubation, the absorbance was measured at 760 nm against water blank. A standard calibration curve was plotted using gallic acid (0~100 mg/L). Results were expressed as mg of GAE (gallic acid equivalents) per g of dw [25, 26].

#### 2.5 Determination of Total Flavonoids

The TFC (total flavonoid content) of different parts of *Punica granatum* was determined using the aluminium chloride assay through colorimetry. An aliquot (0.5 mL) of extracts were taken in different test tubes then 2 mL of distilled water was added followed by the addition of 0.15 mL of sodium nitrite (5% NaNO<sub>2</sub>, W/V) and allowed to stand for 6 min. Later 0.15 mL of aluminium trichloride (10% AlCl<sub>3</sub>) was added and incubated for 6 min, followed by the addition of 2 mL of sodium hydroxide (NaOH, 4% W/V). The solution was well vortexed and absorbance was measured against reagent blank at 510 nm. The total flavonoid content (mg/g) was determined from the calibration curve of quercetin and expressed as mg quercetin equivalents [27, 28].

#### 2.6 Evaluation of Antioxidant Activity by the DPPH Test

Antioxidant activity was evaluated by measuring the

radical scavenging activity DPPH (2, 2-diphenyl-1-picrylhydrazyl), where each 1 mL of methanol solution of differing concentrations (5, 7.5, 10, 20 and 50 µg/mL) were mixed with 3 mL of methanolic DPPH solution (0.1 mM). After 30 min incubation in darkness at laboratory temperature 25±2 °C, the radical scavenging activity was measured spectrophotometrically at 517 nm. Inhibition of DPPH free radical by Vitamin C was also analyzed as a positive control. A negative control is one that containing all reagents except the test sample [29].

Inhibition of DPPH free radical in percent (I %) was calculated as follows [30]:

$$\text{DPPH radical scavenging activity (\%)} = \text{I\%} \\ = (A_{\text{blank}} - A_{\text{test}}) / A_{\text{blank}} \times 100$$

where,  $A_{\text{blank}}$  is the absorbance of the control reaction (containing all reagents except the test compound), and  $A_{\text{test}}$  is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC<sub>50</sub>) was calculated from the graph plotting inhibition percentage against extract concentration.

#### 2.7 Evaluation of Cytotoxic Activity

##### 2.7.1 Cell Culture

Cancerous cells were cultured in DMEM media supplemented with 10% heat inactivated fetal calf serum, 1% Glutamine and 1% antibiotics. The cells were grown at 37 °C in a humidified incubator set at 5% CO<sub>2</sub>. Cells were subcultured after they formed a monolayer on the flask. The cells were detached by treating them with PBS and trypsin.

##### 2.7.2 In Vitro Cytotoxicity Assay

The antiproliferative potential of the mixture extract was evaluated against Human collon cancer (HT-29) cell line and Human breast cancer cell line (MBA-MD-231), by the MTT assay [31]. Tumor cell growth was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (WST<sub>1</sub>) to a purple product. Briefly, cells were seeded in a 96-multiwell plates (8 × 10<sup>4</sup> cells/well), and treated

in duplicate with various concentrations of plant extract (15.625~500 µg/mL) for 72 h. Mitomycin was used as positive control. After 72 h of incubation, 10 µL of solution WST<sub>1</sub> was added to each well and incubated at 37 °C for 4 h. Finally, the absorbance was read at 590 nm with a scanning multiwell spectrophotometer.

Data were calculated as percentage viability using the following formula:

$$\text{Cell death (\%)} = (\text{control OD} - \text{sample OD}) / \text{control OD} \times 100$$

$$\text{Cell viability (\%)} = 100 - (\% \text{ cell death})$$

A graph was plotted against the % cell viability against dilution of the extract. The minimum concentration of extract that was toxic to cancer cells was recorded as the effective drug concentration. The IC<sub>50</sub>% values, concentrations which reduce the absorbance of treated cells by 50%, were graphically obtained from the dose response curves.

### 3. Results

#### 3.1 Qualitative Phytochemical Screening

The results of preliminary tests of different phytochemicals extracted from Moroccan Pomegranate revealed the presence of polyphenols, catechic and gallic tannins, flavonoids, alkaloids and leucoanthocyanes in aqueous extract and/or methanol. Contrary to the aqueous extract, methanolic extract

contains traces of quinoid. As to the ether extract, it contains sterols and polyterpenes. All results are reported in Table 1.

#### 3.2 Total Polyphenol and Flavonoids Content

TPC (total phenolic content) was determined from methanolic extract, expressed in mg of gallic acid equivalent per g of dry weight. Values of (TPC), as shown in Fig. 1, varied from 64.67 and 120.7 mg GAE/g dw. Total polyphenol are 120.7, 115, 96.65, 90.73 and 64.67 mg GAE/g dw for corolla, peel, leaves, flowers and branches, respectively.

TFC (total flavonoids content) expressed as mg QE/g dw are highest in peel 221.7, followed by flower 221.7, corolla 188.8, leaves 180.2 and branches 158.5 mg QE/g dw.

#### 3.3 Antioxidant Activity

DPPH is a free radical compound that has been widely used to determine the free radical-scavenging ability of various samples. The free radical scavenging activity determined by DPPH was expressed as the IC<sub>50</sub> value (the inhibitory concentration of extract required to inhibit 50% of the initial DPPH free radical). Results are reported in Fig. 2.

#### 3.4 Cytotoxic Activity

The cytotoxic activity was evaluated on two human

**Table 1** Phytochemical screening of petroleum ether, methanol and aqueous extracts of Moroccan Pomegranate.

Items	Peel			Leaf			Flower			Branches		
	E	M	A	E	M	A	E	M	A	E	M	A
Sterols and polyterpenes	++	-	-	++	-	-	++	-	-	++	-	-
Polyphenols	-	++	++	-	++	++	+	++	++	+	++	++
Tannins	Catechic			-	++	++	-	-	-	-	++	-
	Gallic			-	+	-	-	+	-	-	-	-
Flavonoids	-	+	+	-	+	+	-	++	+	-	++	+
Leucoanthocyanes			+			-			++			-
Quinoid compound	-	+	-	-	-	-	-	+	-	-	-	-
alkaloids	Bouchardat			-	-	-	-	++	-	-	+	
	Dragendorff			-	-	-	-	-	-	-	+	

+ +: present; +: present at trace; -: undetected;

E: petroleum ether extract; M: methanolic extract; A: aqueous extract.

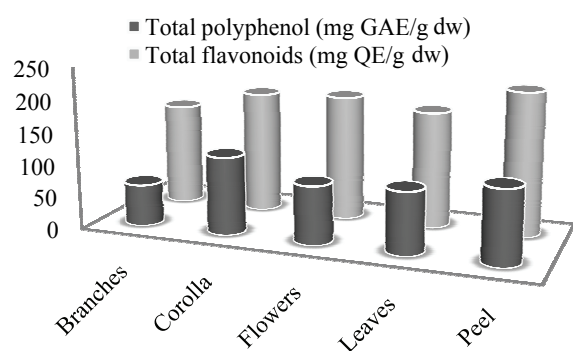


Fig. 1 Total polyphenol and flavonoids content.

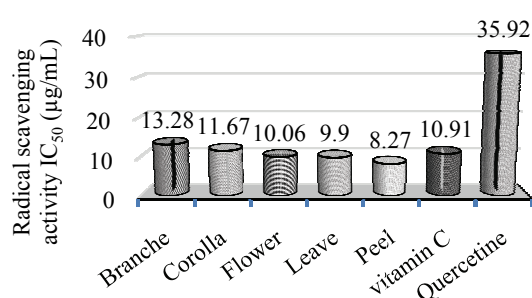


Fig. 2 DPPH radical scavenging activities of pomegranate extracts.

cancer (breast and colon) cell lines (MBA-MD 231 and HT-29). The cytotoxic effect of methanolic pomegranate extracts at different concentrations (15.625, 31.25, 62.5, 125, 250 and 500 µg/mL) on HT-29 and MBA-MD 231 cells line using the MTT assay. The MTT assays data are presented respectively in Figs. 3a and 3b and the corresponding IC<sub>50</sub> are summarized in Table 2.

#### 4. Discussion

The aim of this work was to investigate which parts of pomegranate (peel, leaf, branch, flower and corolla) have best phenolic and flavonoids contents, and evaluate these extracts in terms of antioxidant and antiproliferative capacity.

The results of the phytochemical screening of petroleum ether, methanol and aqueous extracts from different parts of Moroccan Pomegranate, revealed the presence of several bioactive compounds, these results

approve and complement other previous works achieved in Morocco on the aqueous extract of the pomegranates bark, in Tunisia on methanol extract of leaves, bark, seeds and flowers and in China on bioactivity and biosynthesis of the various constituents of this shrub [3, 4, 32].

These phytoconstituents play a significant role in the medicinal properties of many plants. This is manifested by strong radical scavenging activities,

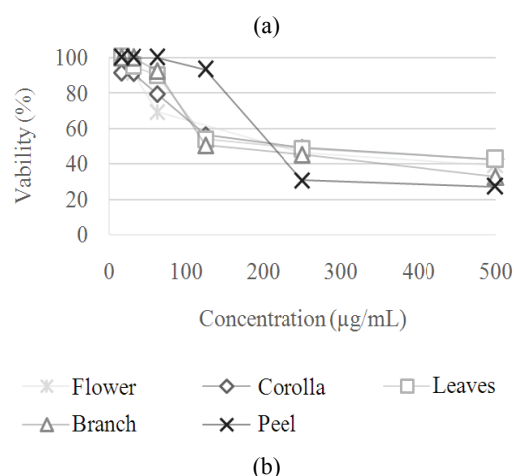
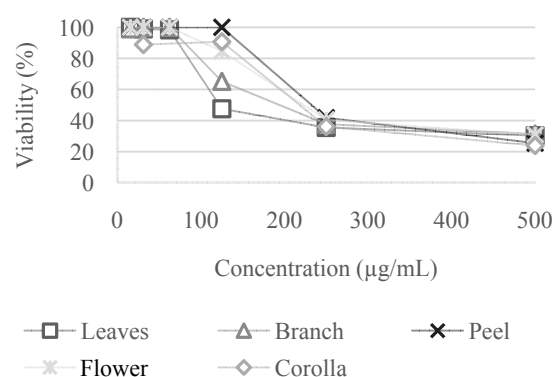


Fig. 3 Cytotoxic activity of methanolic extracts from Pomegranate against: (a) HT-29 cells; (b) MBA-MD 231.

Table 2 IC<sub>50</sub> value of in vitro cytotoxic activity against HT-29 and MBA-MD 231 cell lines.

Parts of shrub	IC <sub>50</sub> (µg/mL)	
	HT-29	MDA-MB-231
Flower	183.18	219.23
Corolla	179.17	233.32
Branches	152.59	133.53
Leaves	127.58	214.02
Peel	203.24	211.51
Mitomycine	2.49	39.81

peel extract are superior to all extracts tested with the lowest IC<sub>50</sub> value of 8.27 µg/mL, leaves and flower have nearly the same value 9.9 and 10.06 µg/mL, followed by corolla 11.67 µg/mL and branches 13.28 µg/mL. We can deduce also that all extracts showed an antioxidant activity lower than ascorbic acid (10.91 µg/mL), except corolla value are comparable with that of reference. Lower antioxidant activity was obtained for methanolic peel extract of Indian Pomegranate approximately 18µg/ml, another research was realized for methanolic flower extract with 1% HCl of Chinese *Punica granatum* (26.23 µg/mL). Antioxidant activity of Gabsi variety of Tunisia obtained for peel, flower, and leaves methanolic extracts are 3.88, 4.55 and 11.44 µg/mL, respectively [4, 29,33].

The role of antioxidants is their interaction depends on oxidative free radicals. The summary of DPPH method is that the antioxidants react with the stable free radical, 2,2-diphenyl-1-picrylhydrazyl (deep violet color) and convert it to 2,2-diphenyl-1-picrylhydrazine with discoloration. The discoloration indicates the scavenging potentials of the sample antioxidant such as phenolic compounds, especially phenolic acids and flavonoids [34]. Phenolic compounds are a much diversified group of phytochemicals that are widely distributed in plants, such as fruits, vegetables, tea and olive oil [35].

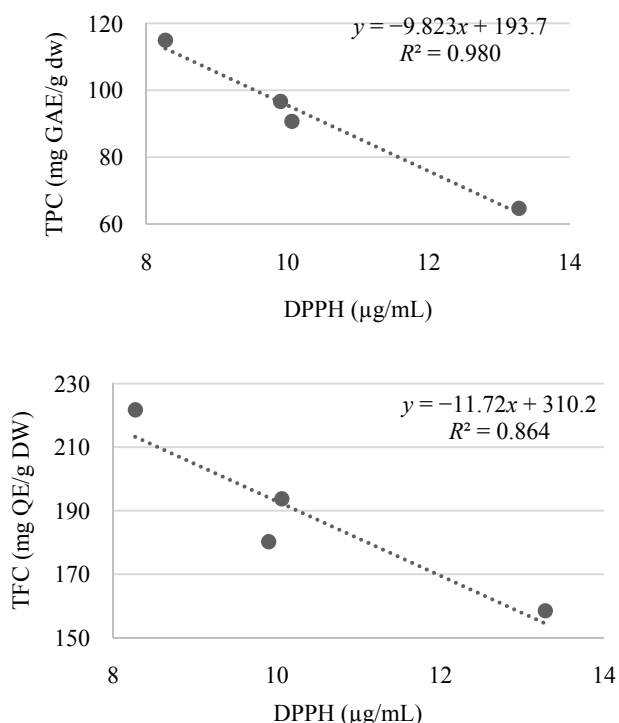
In this study, the total polyphenol expressed as mg GAE/g dw are highest in corolla (120.7 mg GAE/g dw), peel follows closely behind with a value 115 mg GAE/g dw, followed by leaves, flower and branches. Except corolla, we have a strong correlation between antioxidant activity, TPC and TFC with correlation coefficient  $R^2 = 0.98$  and  $R^2 = 0.86$ , respectively (Fig. 4).

The TPC in methanolic extract of Tunisian Pomegranate peel, flower and leaves are 85.6, 66.29 and 14.78 mg GAE/g dw [4]. These results are in agreement with total polyphenol of Chinese pomegranate peel, petal and leaves are 163.74, 110.12 and 91.36 mg GAE/g dw [36]. The TFC of all studied

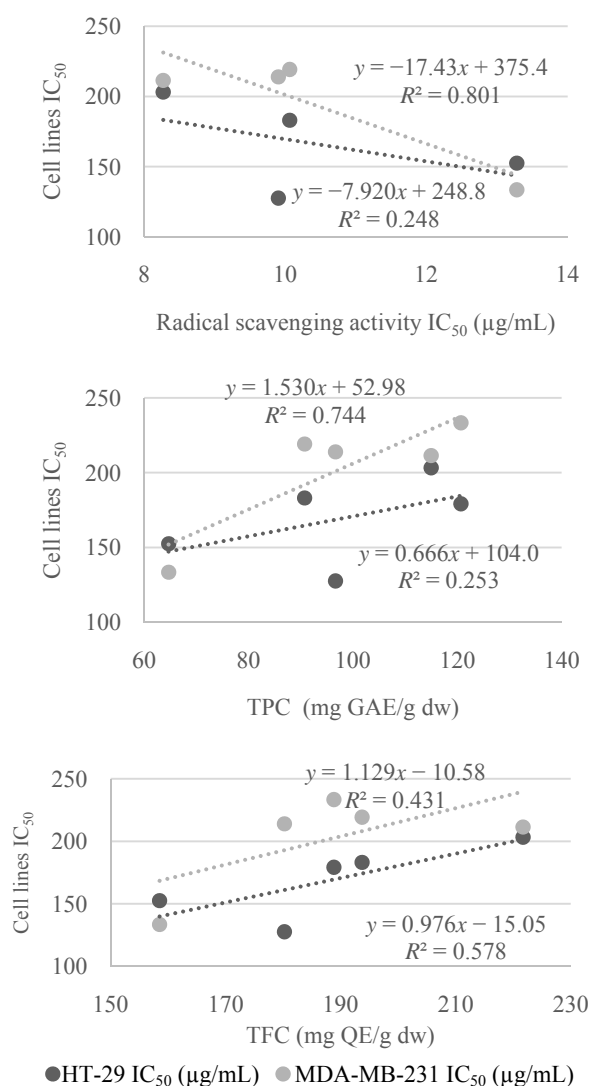
parts are higher than methanolic extract of Gabsi Pomegranate peel, flower and leaves, respectively 51.52, 26.08 and 72.52 mg RE/g dw (mg retin equivalents per g dry weight) [4].

The seed oil, juice, fermented juice, and peel extract of the pomegranate have been shown to exert antiproliferative effects on different human cancer cell lines [37]. The extracts of almost all parts of pomegranate have been demonstrated to interfere with the cytotoxicity of cells in breast and colon human cancer cell lines. As shown in Fig. 5, a higher degree of correlation was observed between antioxidant and antiproliferative activity on MDA-MB-231 cell line.

Furthermore, the correlation coefficient ( $R^2$ ) between the cytotoxic activity and total phenolic content of pomegranate was greatest on MDA-MB-231 cell line, whereas  $R^2$  between the cytotoxic capacities and the flavonoid contents was higher on HT-29 cell line. These results may indicate the synergistic effect of antioxidant compounds on cancer cell properties.



**Fig. 4 Correlation of antioxidant capacity with total phenolic and flavonoid contents of methanolic extract.**



**Fig. 5** Correlation of cytotoxicity with total phenolic and flavonoid contents of methanolic extract.

## 5. Conclusions

The extracts from all examined parts of pomegranate (peel, leaves, branches, flowers and corolla) contained significant amounts of phenols and flavonoids, which play a major regulatory role in oxidation. We confirmed that the extracts demonstrate multidirectional biological activity, such as cytotoxic and antioxidant abilities. The findings of the present study provide an evidence that the peel, leaves, branches, flowers and corolla of methanolic extracts from *Punica granatum* showed to be the potent source of antioxidants which positively correlates with their total phenolic content. Furthermore, the correlation of

cytotoxic activity and total phenolic content of pomegranate was greatest on MDA-MB-231 cell line. Whereas  $R^2$  between the cytotoxic capacities and the flavonoid contents was higher on HT-29 cell line. Therefore, extracts of pomegranate could be used as a readily accessible source of natural antioxidants, in the pharmaceutical industry and for functional foods for the preservation and treatment of cancers.

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