



Phytochemical screening and antitumor potential of *Punica granatum* peel extract

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Abstract

The constant investigation on medicines are vital to resolve growing complication in medical conditions and microbes resistance. Medicinal plants such as *Punica granatum* are storehouse of certain valuable phytochemical substances that might be helpful in medical complications like tumor. The current research was aimed to study the phytochemistry of *P. granatum* peel extract and explore *In vitro* antitumor activity of extracts prepared in different solvents including ethanolic, methanolic, aqueous, ethyl acetate and petroleum ether. Qualitative study for the existence of certain phytochemical compounds like steroids, saponins, alkaloids, carbohydrates, phlobatanins, anthraquinones, glycosides and tannins was accomplished for each extract using standard methods. Most of the phytochemical molecules were present in methanolic, ethanolic and ethyl acetate extracts while least number of molecules were found in aqueous solvent. The antitumor activity was examined by using *Agrobacterium tumefaciens* At 10 strain and potato disc tumor assay technique and assessment were established on the basis of tumor inhibition of several concentration of extracts (10 ppm, 100 ppm, 1000 ppm). Results discovered that different concentrations of *P. granatum* peel extract showed different percentage of inhibition. Increase in percentage inhibition of tumor were observed with increase of peel extract concentration. At 1000 ppm of crude ethanolic extract (CEE) showed maximum inhibition 65%. All the extracts significantly inhibited tumor formation. The obtained results showed antitumor efficacy of *P. granatum* peel extracts.

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Introduction

Plants have been used for centuries, to treat health related abnormalities. In olden days a number of spices and plants parts were used in foodstuffs, not only as a flavoring and preservative agent but also as traditional medication (Shan *et al.*, 2007). Plants are rich source of secondary metabolites such as tannins, flavonoids, alkaloids and terpenoids.

These metabolites have been studied for several health related activities and now several plants received attention as a competent means of controlling many medical problems (AL-Gazaly *et al.*, 2002). Epidemiological studies have proved that fruits and vegetables consumption is accompanying with a negligible rate of cancer, cardiovascular and other medical abnormalities (Sun *et al.*, 2002). However, all fruits have not similar composition and it is very important to consider those fruits having high quantity of bioactive compounds.

Considering this feature, *Punica granatum* (Pomegranate) is an exciting source having high phenolic compounds even three fold more than red wine and green tea (Gilmi *et al.*, 2000). *P. granatum* is a main source punicalagin isomers, ellagic acid derivatives and some other important bioactive molecules like hydrolysable tannins, flavanols and anthocyanin (Gilmi *et al.*, 1995; Gilmi *et al.*, 2000; Garcia-Alonso *et al.*, 2004) phenolic bioactive compounds that hold hepato-protective activity, neuro and anti-inflammatory activity and play important role in cancer and cardiovascular diseases (Larrosa *et al.*, 2006; Faria *et al.*, 2007; Sartippour *et al.*, 2008; Koyama *et al.*, 2010). Several phytochemical ingredients have been detected in different parts of the *P. granatum* making it a best choice in pharmacology (Prakash and Prakash, 2011). Today the high antibiotic resistance property of microbes and complicated medical conditions has led to the progress of new tonic agents that are functional against these microbes and counter these conditions. These agents are obviously the plants bioactive ingredients. Therefore, the current study was aimed with the objectives to evaluate phytochemical

contents and antitumor activity of *P. granatum* peel extract.

Materials and methods

All the chemicals and reagents utilized in present work were of analytical grade and acquired from Sigma-Aldrich Chemical Co.

Plant materials

Fresh *P. granatum* fruits were collected from Nagar gardens Chitral, Pakistan [35°50'46"N 71°47'09"E]. The fruits were peeled manually and collected peels were further processed in Microbiology Research Laboratory at Quaid-I-Azam University Islamabad. Peel samples were washed systematically and white fruit sacs were separated. The prepared peels were air dried for 14 days with continuous nursing to avoid fungal contamination. Air dried peels were milled to fine powder and kept in air tight clean bottle at room temperature for analysis.

Crude Extracts Preparation

The powdered plant material of 1.5 g was extracted with 50 mL of ethanol (99%) for crude ethanolic extract CEE, methanol (80%) for crude methanolic extract CME and double deionized water for crude aqueous extract CAE and incubated in shaking incubator at 25°C with continuous shaking at 150 rpm for 7 days.

All the extracts were filtered by using Whatman filter paper and filtrate was further concentrated in vacuum evaporator at 40°C to evaporate all the solvents. The dried obtained extracts were used for further investigation.

Preparation of soxhlet extract

Ethyl acetate and petroleum ether was used for the preparation of soxhlet extract. The sample (6 g) was extracted with 200 mL ethyl acetate and petroleum ether using Soxhlet extractor for 5 hours at temperature below the boiling point of the solvent (77°C). The ethyl acetate extract (EAE) and petroleum ether extract (PEE) were collected in flask and stored in sterilized tubes. Percentage yield of all the obtained

extracts was calculated by using the following formula

$$\% \text{Age yield} = \frac{\text{Extract weight}}{\text{Dried sample weight}} \times 100$$

Phytochemical assessment of peel extract

Qualitative study for the existence of certain phytochemical compounds like steroids, saponins, alkaloids, carbohydrates, phlobatanins, anthraquinones, glycosides and tannins was accomplished for each crude extract. The dried extracts obtained were dissolved in distilled water for required concentration.

Test for Saponins

The test solution was mixed with water and shaken vigorously and observed for froth formation, the froth that are stable up to 10-15 minutes indicating the Saponins presence.

Test for Glycosides

For the study of glycosides the test solution was mixed with glacial acetic acid and ferric chloride solution and added few drops of concentrated H₂SO₄ and checked for the development of two layers. The reddish brown layer and the upper layer of acetic acid turns bluish green representing glycosides in the sample.

Test for Tannins

Test solution of 2 mL when treated with distilled water and mixed. FeCl₃ solution of few drops were added, green precipitate when formed after well mixing indicates the tannins presence in the solution.

Test for Phlobatanins

2 mL of test solution and 1% HCl each were mixed and boiled. Red precipitates were formed that indicate the presence of phlobatanins in test solution.

Test for Anthraquinones

Test solution of 3 mL and 3 mL of benzene were mixed, shaken well in test tube and filtered. In the filtrate added 5 mL of 10% ammonia solution and mixed well. Absence of red, violet or pink color in the

lower phase indicated the anthraquinones absence in test solution.

Test for Carbohydrates

Test solution of 3 mL was mixed with 2 mL of Molisch' reagent and properly shaken, then carefully added 2 mL concentrated sulfuric acid. Carbohydrate presence was established by the violet ring formation at inter phase.

Test for Amino acids

Boiled test solutions with 0.2% solution of Ninhydrin for 2 minutes in water bath. Absence of blue or blue violet color indicates the absence of amino acid in test solution.

Test for Alkaloids

Few drops of Hager's reagent (saturated picric acid solution) were treated with test solution. Yellow precipitate formation showed positive result for alkaloids.

Test for Steroids

Acetic anhydride of 2 mL was added with test solution with 2 ml sulfuric acid. Transformation of color from violet to blue or green directed the presence of steroids in test solution.

Test for flavonoids

Few drops of aluminum (1%) solution were added with test solution. Appearance of yellow coloration indicates the flavonoids presence in test solution.

Antitumor Activity

To check the antitumor potential of *P. granatum* peel extracts, potato disc method was used. *Agrobacterium tumefaciens* (At10 strain) pure culture was grown at 28°C for 48 hours in shaking incubator. Surface sterilization of red skinned potato was done with 70% ethanol solution for 30 minutes. Cylinders of potatoes were made with the help of sterilized 8 mm cork borer and washed with autoclaved distilled water. Both ends of these cylinders were cut about 1 cm and discarded with the help of sterilized blade. In the petri plates, potato cylinders of 5 mm thick discs were made. These discs

were then washed with 70 % ethanol solution and positioned 5 discs per solidified agar plates. Out of which one was a positive control (vincristine dissolved in DMSO 1mg/mL), one was negative control (simple Dimethyl sulfoxide DMSO) while other three are for their respective concentration (10 ppm, 100 ppm, 1000 ppm) of all extracts dissolved in DMSO. About 20 µL of inoculums was applied on the surface of each potato disc of particular concentration and controls and permitted to diffuse for 15-20 minutes. Petri plates were made airtight by wrapping parafilm around it and placed in incubator at 28°C for 21 days. Each experiment was performed in triplicate.

Staining Procedure

For tumor conformation Lugol's solution (10% KI, 5% I₂) was prepared in distilled water. This solution was applied on each disc and allowed for 10-15 minutes to diffuse. The tumors lack starch so did not get stained and appeared creamy to orange while rest of potato turned dark blue. These stained potato discs were observed under dissecting microscope and outgrowths on discs were add up as tumors. All the experiments were repeated three times.

Calculation of Percentage Inhibition

Numbers of tumors per disc were counted. Then for calculating percentage inhibition following formula was used.

$$\% \text{Inhibition} = \frac{\text{No of tumors in negative control} - \text{No of tumors in sample extract}}{\text{No of tumors in negative control}} \times 100$$

Results

Percent yield of *P. granatum* peel extracts obtained are shown in Fig. 1. The obtained yields are based on the dried weight of sample raw materials.

Among these peel extracts, the highest (6%) and the lowest (3.3%) yields of extraction were observed for Ethanolic and Ethyl acetate respectively. Phytochemical evaluation for qualitative detection of various chemical constituents in *P. granatum* peel extracts was performed. Each extract was screened three times for the existence of key phytochemicals and from the time interval taken by the reaction to start, the amount of phytochemicals were reported (Table 1).

Table 1. Qualitative phytochemical screenings of pomegranate peel extracts.

S.No	Test	CAE	CME	CEE	PEE	EAE
1	Saponins	-	-	-	-	-
2	Glycosides	-	+++	+++	+	++
3	Tannins	+	+++	++	-	+
4	Phlobatanins	-	++	+	-	+
5	Anthraquinones	-	++	+++	++	-
6	Carbohydrates	++	+++	+++	+++	+++
7	Amino acids	-	+	++	-	-
8	Alkaloids	-	++	+	+	++
9	Steroids	-	++	+++	-	++
10	Flavonoids	-	+	++	-	++

Legend: +++ = High amount (test positive within 5 minutes intervals), ++ = moderate amount (Test positive after 5 minutes but within 10 minutes), + = trace amount (Test Positive after 10 minutes but within 15 minutes) and - = complete absence.

Antitumor activity of *P. granatum* peel extracts was determined by potato disc tumor assay method against *Agrobacterium tumefaciens* (At 10 strain) (Fig. 2). Different concentrations of *P. granatum* peel extracts (10 ppm, 100 ppm, 1000 ppm) showed different percentage of inhibition (Fig. 3). Negative

control was used simple DMSO that did not inhibit tumors and positive control was Vincristine that had shown 100% tumor inhibition. Increase in percentage inhibition of tumor were observed with increase of different peel extract concentration (Fig. 4).

Discussion

Since ancient time bioactive molecules of medicinal plants are in use to treat different medical conditions. High resistance pattern of microorganisms, side effects of antibiotics and other health related complications have evolved the medicinal plants importance to be used as an alternative solution. Among these, *Punica granatum* is well known for its

pharmacological properties. In present study *P. granatum* peel was extracted with five different solvents such as water, methanol, ethanol ethyl acetate and petroleum ether. Formerly methanolic (Braga *et al.*, 2005; Dell'Agli *et al.*, 2009), aqueous and polyphenolic (Haidari *et al.*, 2009) extract of *P. granatum* were successfully used for anti-inflammatory and antibacterial activity.

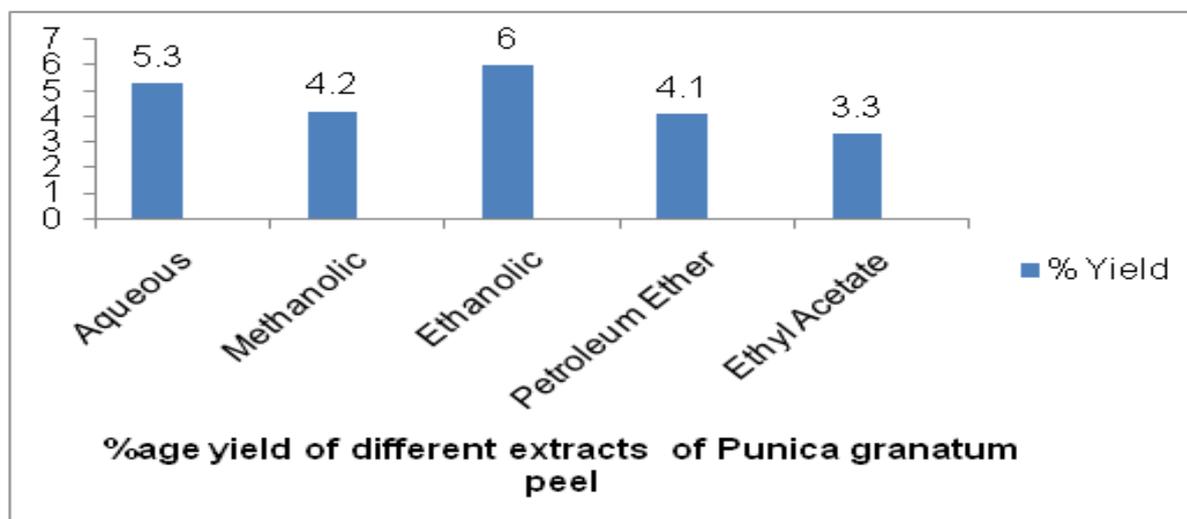


Fig. 1. Percentage yield of different *Punica granatum* peel extracts.

The percent yield of extracts in all solvents were studied comparatively and the highest (6%) and lowest (3.3%) yields were observed for Ethanolic and Ethyl acetate solvents respectively. Sultana *et al.*, (2008) reported the percent yield of dry pomegranate peels after extracting with 80% methanol was 16.4%.

Generally, plants produce several important phytochemical molecules. Therefore, detailed study is

essential on phytochemistry and pharmacology of traditional plant yields because this may lead to discovery of new drug having therapeutic importance and further these studies may assist their quantitative assessment and qualitative separation of pharmaceutically active compounds. Phytochemical assessment in the present study, has shown the presence of steroids, glycosides, flavonoids, tannins and carbohydrate in the peel extract of *P. granatum*.

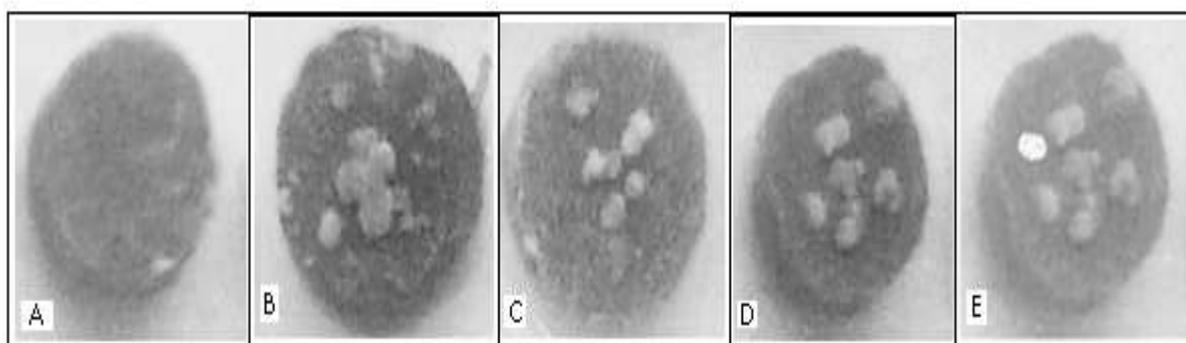


Fig. 2. Results of potato disc assay for peel extracts. The potato disc having no tumor is positive control (A), while negative control (B) have highest numbers of tumors. All other experimental disc (C), (D) and (E) have low number of tumors as compared to negative control.

The comparable results were also described by Uma Maheswar *et al.*, (2012); Hegde *et al.*, (2012); Satheesh Kumar, (2012); Kannaiyan Moorthy, (2013); Amina and Filali, (2013). Further the presence of different phytoconstituents in different extracts may be responsible for the therapeutic properties of pomegranate. Tannins and flavonoids are phenolic compounds and are a major group of plants bioactive compounds that act as primary antioxidants. Since these plant bioactive compounds were found to be the part of extracts, it might be accountable for the effective antioxidant capacity of *P. granatum*. For instance, the presence of flavonoids might be responsible for its use as anti-inflammatory

properties (Jasim *et al.*, 2010). Tannins inhibit the microbial growth by precipitating microbial proteins (Prasad *et al.*, 2008). The secondary metabolites, photochemical and other plants bioactive chemical ingredients of medicinal plants account for their pharmaceutical worth. For example, Saponins have hypotensive and cardio depressant properties. Glycosides are cardio active medications used in the cure of congestive heart failure and cardiac arrhythmia. The presence of Saponins in whole fruit and seeds extract and glycosides in all the extracts might play a role in the cardio protective potential of pomegranate.

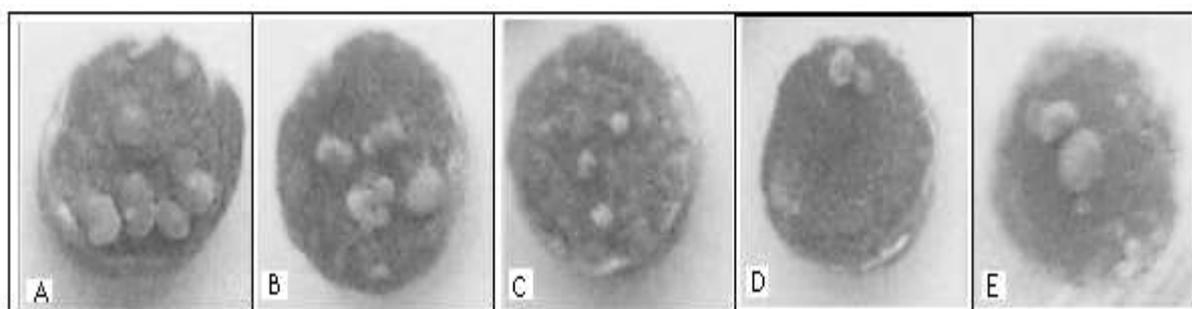


Fig. 3. Results of potato disc assay for peel extracts. The negative control represented highest number of tumor (A), compared to all other experimental discs having extract concentration of 10 ppm (B), 100 ppm (C) and 1000 ppm (D & E).

Presence of several metabolites propose high potential for the plant to use as a useful source of phytochemicals. In general, there was difference in phytochemical amounts and presence depending upon solvents and most of the phytochemical molecules were present in methanolic, ethanolic and ethyl acetate extracts while least number of molecules were found in aqueous solvent in present study. Similarly, Elfalleh *et al.*, (2012) point out that the difference in *P. granatum* phytochemistry is according to the solvent used for extraction. In addition, the peel composition of *P. granatum* depends on several factors such as processing factors, cultivar type, environmental factors and post harvesting (Houston, 2005).

The present study results proposed that the peel extracts of *P. granatum* has high potential of antitumor activity determined by using potato disc

assay method and *Agrobacterium tumefaciens* At 10 strain. Potato disc tumor assay is very simple, fast and low-priced method for antitumor screening used by Wedge and Camper, (2000) for identification of antitumor activity of ellagic acid and fruit extract of *Meliva volkensii*. Different concentrations of peel extract showed different percentage of inhibition. The percentage inhibition of tumor increases with increase different concentration of peel extracts. At 1000 ppm of crude ethanolic extract (CEE) showed maximum inhibition 65%. All the extracts significantly inhibited tumor formation. About 20% and greater than 20% inhibition of tumor is measured as significant value for plant extract (Jurenka, 2008). Jasim *et al.*, (2002) also used *Agrobacterium tumefaciens* At 10 strain and tested the antitumor activity of methanolic extract of *Fagonia cretica*. Extreme percentage inhibition of this plant extract was 77.04% at 1000 ppm concentration. Galsky *et al.*,

(1980) studied the effects of numerous plant extracts and compound on crown gall tumor development and found no effect of compounds on bacterial viability and attachment to tumor inducing site. According to Coker *et al.*, (2003) the action of compounds and

extracts have no effect on microbial viability but it inhibit tumor formation. This may be due to inhibiting the bacterial attachment with the suitable tumor inducing site.

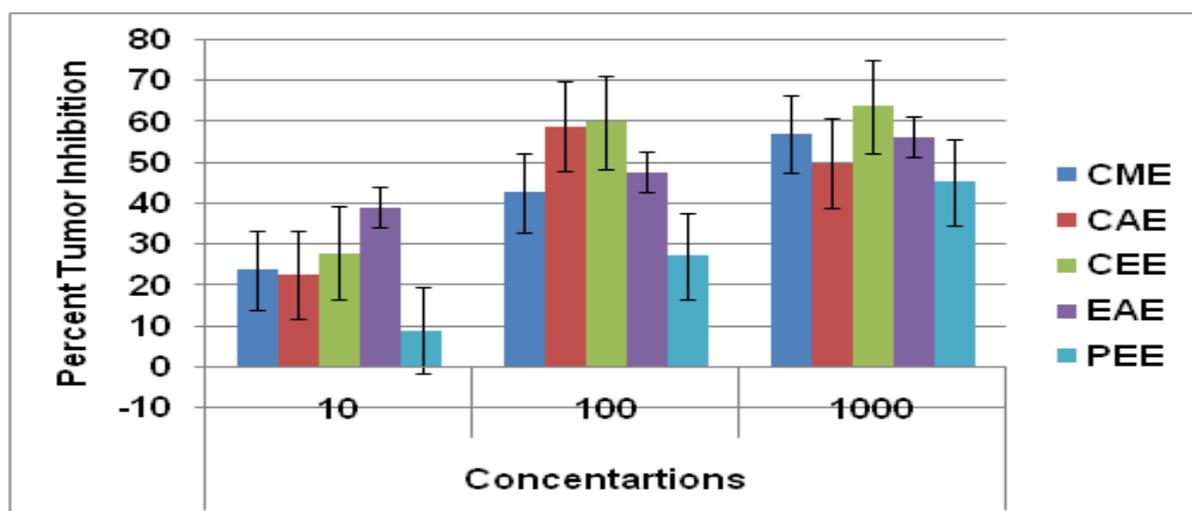


Fig. 4. Percent tumor inhibition activity of different concentration of *P. granatum* peel extracts.

Conclusion

On the basis of the current results it is concluded that *Punica granatum* peel holds an important bioactive compound and secondary metabolite which exhibit antitumor potential against the *Agrobacterium tumefaciens* At 10 strain using potato disc tumor assay. Increase in percentage inhibition of tumor was observed with increase of different concentration of peel extracts. Furthermore, isolation, purification and individual characterization of each bioactive compound may be more potential and significant pharmaceutically. Further study on *In vivo* assessment also required to check antitumor nature *P. granatum* peel extracts.

Recommendation

Due to the existence of valuable bioactive molecules and antitumor activity in *P. granatum* it is strongly recommended that it may be included in the diet for healthy lifestyle.

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