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Full Length Research Article

Phytochemical Screening of *Punica granatum* Peel in Antioxidant and Antitumor Activity in Oral Cancer (Kb) Cell Line

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Abstract

Pomegranate are a rich source of natural molecules, representing considerable biodiversity due to climate variations between the Northern, Southern, Eastern, and Western regions of the country. (PMG; *Punica granatum*) feel possess a well-balanced phytochemical composition, and anticancer, with proven adjuvant benefits in experimental oral cancer chemotherapy; Here, traditional medicinal approaches have been used for hundreds of years. Healthy lifestyles, low levels of stress and microbial infections, and dependence on flora and herbal medicine might in combination explain why the burden of oral cancer is lower in some regions than in others, such bioactivity could be affected by PMGs. which could be related to their high content of phytochemicals. In comparison with single herb treatment, The chemical and phytochemical screening composition, antioxidant capacity, and anti-oral cancer potential [in vitro (MTT assay) in (kb) cells. However, there in vitro cytotoxicity increased sharply (from 32 ± 8.5% to 55 ± 1.9%) upon 24 h incubation with the peel extract at 50–300 µg/ml at 24 h against normal (retinal) and oral cancer (KB) cell lines. The anti-oral cancer potential of the PMG feel is phenogenotype-specific, although it could be more effective in nutraceutical formulations (concentrates). The present study is aimed at determining phytochemical constituents, the most potent high antioxidant, and wound healing activities for the methanol fraction of pomegranate, cytotoxic activities were identified for the anticancer activities of some species, such as anti-oral cancer activities and the most potent cytotoxic activities were identified for the families for a crude extract of the feel mode of treatment.

***Key words*:** *Punica granatum*, Phytochemicals screening, Oral cancer, MTT assay, Antioxidant, Antitumor activity

Cancer is one of the most devastating human ailments in both developed and developing countries and is the second leading cause of death after cardiovascular diseases [1-2]. The World Health Organization (WHO) and the American Cancer Society (ACS) reported that globally the second growing fatal disease is cancer, claiming approximately 9.6 million mortalities in 2018. Around 1 in 6 demises worldwide is caused by malignancy and roughly 70% of these mortalities transpire in low- and middle-revenue nations. According to the ACS 2018 statistics in the United States (USA), an approximated 1,735,350 latest cancer cases in various hospitals/institutions and 609,640 cancer demises are witnessed [3]. The most effective way of reducing cancer risk is targeting preventable risk factors (e.g., unhealthy nutrition, smoking) and correct diagnosis/control of non-preventable ones (e.g., genetic factors and primordial inflammatory response), Phytotherapy is highly preferred by the general population worldwide, and wound dressing with certain herbs is a prehistoric tradition. Many healing effects of botanical extracts are well studied and accepted by modern medicine. It has been established that anti-inflammatory, antimicrobial, and anesthetics agents, in addition to protective modalities, will be helpful in promoting the healing cascade.

The fruit and bark of pomegranate are used against intestinal parasites, dysentery, and diarrhea. The juice and seeds are considered a tonic for the throat and heart. It is used to stop nose and gum bleeds and treats hemorrhoids. The peel is tough and leathery, about 2–5 inches in width, and its color ranges from yellow to deep pink/red. The peel makes almost 50% of the entire mass of the product (fruit) [4].

An antioxidant is a compound that, when added to a food product, delays the process of lipid peroxidation or the oxidation of some other biomolecule and although there are synthetic antioxidants such as butylated hydroxyanisole (BHA) it has restricted use since they can have adverse effects on the health [5]. For over thousands of years now, natural plants have been seen as a valuable source of medicinal agents with the proven potential of treating infectious diseases and with lesser

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side effects compared to the synthetic drug agents. Among the main components reported in the peel, flavonoids of (luteolin) and flavones can be mentioned as glycosylated (naringin), anthocyanins (delphinidin, cyanidin, pelargonidin, as well as their glycosylated forms) being Tanaka the latter responsible for the colorings of the fruit [5]. As for ellagitannins, punicalagin and punicalin have also been identified being specific for pomegranate, as well as the presence of corilagin, casuarinine, pedunculagina, telimagrandin, granatin A and B, punicafoline [6]. Cell viability was quantified by MTT assay. Apoptotic cells were determined by qualitative (staining methods) and quantitative analysis (Annexin-propidium iodide-based flow cytometry). To our knowledge, the comprehensive [in vitro (several cell lines, high-throughput chemical characterization) plus in silico (cheminformatics)] screening for the genotype-specific anticancer potential of PMG peel is here reported for the first time; from the computational point of view, the application to the informatics methods involved in food chemical research (food informatics) is innovative.

MATERIALS AND METHODS

Collection of the fruit peel material

Pomegranate peel (*Punica granatum*) was collected from the local market of Cuddalore district, Tamil Nadu. It was washed with tap water to clean the fruit peel. Then the cleaned peel material was sun-dried and powdered by using a mechanical grinder. The peel powder was stored in a plastic container for further study.

Source of chemical and reagents

All other chemicals used were of analytical grade, purchased from Hi Media Laboratories Pvt. Ltd., India, and Sigma Aldrich Chemicals Pvt. Ltd (India).

Preparation of extract

The *Punica granatum* peel powder simple extraction method that, although valuable. This procedure was carried out by Kushwaha [7], method with slight modifications. In this procedure, the 50 g powdered raw material was soaked in the 600 ml ethanolic as a solvent at room temp for at least three days with frequent agitation. After the extractions, the solvent is removed from the mixture, frequently by the open dish evaporation method to concentrate the product at 50 –55 degrees C. The concentrated water extract was 5g weighed and stored by using an electronic balance to determine the extractive yield. Then the ethanolic extract was stored in a clean empty plastic container for further use [7].

Qualitative methods: The *Punica granatum* peel powder exacts were analyzed for alkaloids, carbohydrates, glycosides, saponins phytosterols, phenol, tannins, protein and amino acid flavonoids, and diterpenes. Extracts were dissolved individually in hydrochloric acid and filtered.

Mayer's test: Filtrates were treated with Mayer's reagent (potassium Mercuric iodide solution) are added to the sides of the test tube. The appearance of a white creamy precipitate indicates the presence of alkaloids.

Hager' s test: The extract is treated with Hager's test (saturated picric acid solution) presence of alkaloids is confirmed by the presence of a yellow precipitate.

Test of carbohydrates: *Punica granatum* peel extracts were dissolved individually in 5 ml of distilled water and filtered. The filtrates were used to test for the presence of carbohydrates.

Molisch's test: Filtrates were treated with 2 drops of alcoholic ∝ -naphthol solution in a test tube. The formation of the violet ring at the junction indicates the presence of carbohydrates.

Benedict's test: Filtrates were treated with Benedict's reagent and heated gently. Orange-red precipitate indicates the presence of reducing sugar.

Feelings test: Filtrates were treated with Fehling's solutions A and B. A prominent yellow precipitate confirms the test is positive.

Test of glycosides: *Punica granatum* peel extracts were hydrolyzed with dil. Hydrochloric acid, and the subject to the test for glycosides.

Modified borntrage test: The *Punica granatum* peel extracts were treated with ferric chloride solution and immersed in a boiling water bath for about 5 minutes. The mixture was cooled and extracted with an equal volume of benzene layer was separated and treated with ammonia solution. The formation of rose to pink color in the ammonia layer indicates the presence of ethanol glycosides.

Test of saponins

1) *Forth test*: The *Punica granatum* peel extracts were diluted water to 2 ml and this was shaken in a graduated cylinder for 15 minutes. The formation of a 1 cm layer of foam indicates the presence of saponins.

2) *Foam test*: 0.5g of Punica *granatum* peel extract was shaken with 2 ml of water. If the foam produced persists for 10 minutes, it indicates the presence of saponins.

Test of phytosterols

Salkowski's test: Extracts were treated with chloroform and filtrated. The filtrate was treated with a few drops of conc. sulphuric acid, shaken well, and allowed to stand. The appearance of golden yellow color indicates the presence of triterpenes.

Test of phenols

1). *Ferric chloride test*: *The Punica granatum* peel extracts were treated with 3-4 drops of ferric chloride solution. The formation of bluish-black color indicates the presence of phenols.

2). *Test of tannins*: The *Punica granatum* peel extract was treated with 10% alcoholic ferric chloride solution and the formation of a blue or greenish color solution indicated the presence of tannins.

Test of flavonoids

1) *Alkaline reagent test*: The *Punica granatum* peel extract was treated with a few drops of sodium hydroxide solution. The formation of intense yellow color, which becomes colorless with the addition of dilute acid, indicates the presence of flavonoids.

2) *Lead acetate test*: The *Punica granatum* peel extract was treated with a few drops of lead acetate solution. The formation of a yellow color precipitate indicates the presence of flavonoids.

Test of proteins and amino acids

1). *Xanthoprotein test*: The *Punica granatum* peel extracts were treated with a few drops of concentrated nitric acid. The formation of yellow color indicates the presence of proteins.

2). *Ninhydrin test*: The *Punica granatum* peel extract, 2.25% w/v ninhydrin reagent was added and boiled for a few minutes. The formation of blue colour indicates the presence of amino acids.

Test of diterpenes

1). *Copper acetate test*: The extract was treated with 3-4 drops of copper acetate solution. Formation *granatum* peel extract of emerald green color indicates the presence of deterrence.

2). *Phytochemical screening*: The *Punica granatum* peel extracts were analyzed for preliminary phytochemical screening to check the presence of photochemical stock solution of the extracts with a concentration of 1mg/ ml was prepared and used for the screening.

Quantitative of total alkaloids: The (5g) of plant powder was added to a 250 ml conical flask. Into this, added 200ml of acetic acid (10%) in ethanol and allowed to stand for 4 hours. The solution was filtered and concentrated to one-fourth of its original volume using a water bath maintained at 80 °C. Concentrated ammonium hydroxide was added in drops to the filtered solution until the precipitation was complete. Later, the entire solution was settled, filtered and the residue obtained was weighed. The number of alkaloids was expressed as mg/g of the sample.

Quantitative of total phenol: About 100µl of plant extract was mixed with ethanol and Follin-ciocalteu reagent, and the volume was made up to 3 ml. The mixture was added with sodium carbonate (20%) and incubated in a dark room for 30 °C for 4 minutes after gentle mixing. The absorbance was measured at 725 nm using a spectrophotometer against a reagent blank. A standard calibration curve was constructed using different concentrations (30-150µg/ml) of Gallic acid and the total phenolic content was expressed as mg catechol equivalent/g.

Quantification of secondary metabolites

1). *Quantification of total flavonoids content*: The aluminum chloride colorimetric assay was used for total flavonoids determination as described by [8]. 100 ul (200mg/ml) of the extract was mixed with 2.5 ml of distilled water and 300ul of 5% sodium nitrate then it was incubated at room temperature for 5 minutes and 300ul of 10% aluminum chloride, 2 ml of 1m sodium hydroxide and 1ml of distilled water were added. Then absorbance of the reaction mixture was measured at 512 nm along with the standard quercetin and blank the total flavonoids content was determined as microgram quercetin equivalent by using the standard, quercetin graph obtained by comparing the calibration curve prepared from a reference solution containing quercetin (10-300ug/ml).

2). *Quantitative of total saponins*: Total saponins were estimated using gravimetric methods of obadont and ochuko, with slight modifications. After the addition of ethanol to a known amount of peel powder, the conical flask was shaken and maintained at 55 °C. After filtration, the residue was re-extracted with ethanol, and the extracts were pooled. The volume of the pooled extract was reduced to 50ml using a water bath set for 90 °C. The concentrate was mixed with diethyl ether in a separating funnel and Shaked vigorously. The aqueous layer was recovered, while the ether layer was discarded. Repeated the procedure thrice and butane was added finally to the pooled extract. The total saponins content was expressed as mg/g sample.

3). *Evaluation of in vitro antioxidant activity*: The antioxidant activity of the ethanol extract was determined by the phosphor molybdenum method as described by [9] briefly 0.3ml of extract was mixed with 3ml of reagent solution (0.6m) sulphuric acid 28nm sodium phosphate and 4mm ammonium molybdate. The reaction mixture was incubated at 95 °C for amino acid cooled to room temperature. Finally, absorbance was measured at 695nm using a spectrophotometer (Shimadzu) UV-1609).

4). *Nitric oxide scavenging activity*: The 1ml of the peel extract was taken as a test solution. 2ml of sodium nitroprusside was mixed and incubated at 23 °C for 2 hours (150 minutes). After the incubation period, 1 ml of the Griess reagent was added. The absorbance of the chromophore that formed was read at 550 nm different concentrations of Ascorbic acid(20-100µg) were taken in the test tubes. 2ml of sodium nitroprusside was mixed and incubate at 23 °C for 2 hours (150 minutes). After the incubation period, 1 ml of Griess reagent was added. The absorbance of the chromophore that formed was read at 550nm [10].

In vitro anticancer activity

1). *Cell culture maintenance*: Oral cancer KB cell lines were procured from the cell repository of the National Centre for Cell Sciences (NCCS), Pune, India. Dulbecco`s Modified Eagle Media (DMEM) was used for maintaining the cell line, which was supplemented with 10% Fetal Bovine Serum (FBS). Penicillin (100 U/ml), and streptomycin (100 µg/ml) was added to the medium to prevent bacterial contamination. The medium with cell lines was maintained in a humidified environment with 5% CO_2.

2). *MTT assay*: Cell viability assay, KB viable cells were harvested and counted using a hemocytometer diluted in DMEM medium to a density of 1×10^4 cells/were seeded in 96 well plates for each well, and incubated for 24 h to allow attachment. After KB cells were treated with control and the containing different concentrations of *Punica granatum* 50 to 300 µg/ml was applied to each well. KB cells were incubated at 37 °C in a humidified 95% air and 5% CO_2 incubator for 24 h. After incubation, the drug-containing cells wash with fresh culture medium, and the MTT (5 mg/ml in PBS) dye was added to each well, followed by incubation for another 4 h at 37 °C. The purple precipitated formazan formed was dissolved in 100 µl of concentrated DMSO and the cell viability was absorbance and measured 540 nm using a multi-well plate reader. The results were expressed at the percentage of stable cells with respect to the control. The half maximal inhibitory concentration (IC_{50}) values were calculated and the optimum doses were analysed at different time period.

$$\text{Inhibitory of cell proliferation (\%)} = \frac{\text{Mean absorbance of the control} - \text{Mean absorbance of the sample}}{\text{Mean absorbance of the control}} \times 100$$

The IC_{50} values were determined from the sample *Punica granatum* does responsive curve where inhibition of 50% cytotoxicity compares to vehicle control cell. All experiments were performed at list three times in triplicate.

RESULTS AND DISCUSSION

Secondary metabolites afford imperative pharmaceutical properties for human health [11]. Compounds belonging to the terpenoids, alkaloids and flavonoids are used as drugs or as dietary supplements to heal or prevent various diseases and in particular some of these compounds seem to be competent in preventing and inhibiting various types of cancer [12]. (Table 1, Fig 1) lists the phytochemical constituents of *Punica granatum* peel. All the phytochemical constituents tested were present in phenolic extract of *P. granatum* peel except glycosides and anthocyanin. It was noted that ethanolic peel extract of *Punica granatum* showed the presence of all phytochemical constituents except tannins, glycosides and anthocyanin.

Table 1 Phytochemical screening

S. No	Test	Observation	Interference	Intensity
1	Carbohydrates			
	i). Benedicts test	Red precipitate	Presence reducing sugar	+
	ii). Fehling test	Brick red precipitate	Presence reducing sugar	++
2	Protein			
	i). Biuret test	Green colour formation	Absence of protein	+
	ii). Millon's test	Yellow precipitate	Absence of protein	+
3	Flavonoids			
	Lead acetate test	Yellow precipitate	Presence of flavonoids	+
4	Tannins			
	i). Ferric chloride test	Dark green colour	Presence of tannis	-
	ii). Acetic acid test	Pale yellow	Absence of tannis	-
5	Terpenoids			
	i). Hirshorm	Green under precipitate	Absence of terpenoids	-
	ii). Lieberman starch morasky test	Light yellow	Presence of terpenoids	+
6	Phenolic compounds			
	i). Ferric chloride test	Deep brown colour	Absence of phenolic compound	-
7	Steroid test	Precipitate yellow	Absence of steroid	-
8	Amino acid test			
	i). Ninhydrin test	Brownish yellow	Absence of amino acid	-
9	Saponin	Vigorous with water	Presence of saponin	+
	i). Foam test	persistent		

++ = Presence of high constituents; + = Presence of low constituents; - = Absence of high constituents

The phytochemical screening of various extracts shows the presence of certain important components such as phenols, glycosides, flavonoids, terpenoids, carbohydrates, proteins and amino acids. Phytochemical constituents afford imperative pharmaceutical properties for human health. These compounds can be used as drugs or as dietary supplements to heal or to prevent various diseases.

Fig 1 The phytochemical screening

Antioxidant potential of Punica granutum

Phosphomolybdenum assay: Total antioxidant activity (TAC) was used to measure in phosphomolybdenum assay and found extremely significant ($p<0.0001$) in phenolic extracts as well as ascorbic acid. Increase in total antioxidant capacity was directly proportional to concentration of all extracts (Fig 2) and ascorbic acid. Further, TAC in standard ascorbic acid was found to be highly significant ($p<0.0001$) when compared to *P. granatum* increasing concentrations.

Fig 2 The antioxidant screening

The absorbance of ascorbic acid standard and all the peel extracts reflects directly the reducing power which is nothing but the total antioxidant capacity.

Anti-tumour activity of Punica granatum: Effects of *Punica granatum* L. var. spinosa extract on cell death of oral cancer cell line (KB cells) (Fig 3). As determined by MTT assay, peel extract at 50, 100, 200, and 300µg/ml was chosen for KB cell line in cell death detection ELISA.

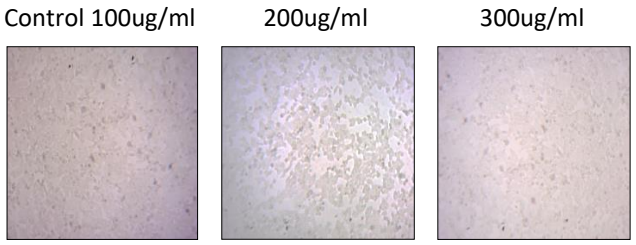

Fig 3 The morphological changes in control and ethanol extract treated oral cancer (kb) cells for 24 h.

The proportion of dead KB cells increased sharply (from 32±8.5%, to 55 ± 1.9%) upon 24 h incubation with the peel extract at 50–300 µg/mL at 24 h. These results suggested that the apoptotic response of KB ell lines should be evaluated at different concentration points.

CONCLUSION

Nowadays herbs are extensively used for the research purpose and it possesses more than one chemical entity so it has been widely used for the research investigations. The peel-based compounds have the effective dosage when *Punica granatum* peel contains more constituents it can be considered beneficial for further investigation. The numerous natural compounds were studied scientifically for their anticancer activity against various cancers, which may lead to the development of promising anticancer agents. Eighty percent ethanol was a better solvent for extracting active constituents from peels. Phenolic from peel extract exhibited a potent antioxidant activity as evaluated by reduction tests. *Punica granatum* extracts and purified fractions from pomegranate peels could provide health benefits to humans and may be employed in food perseveration and pharmaceutical purposes. The results of the antioxidant activity showed that the ethanol extract showed a good antioxidant activity. In the present study it is shown that aqueous ethanol *Punica granatum* extract is potential anticancer agent with high selective for malignant cells compared to normal cell line. The result reveals that *Punica granatum* extract exhibits good inhibitory effects on tested cancer cell lines. MTT assay commonly used technique that was carried out to assess the cytotoxic activity of agents, live cells. The cytoxic effects of *Punica granatum* were observed in KB cells which indicate the *Punica granutum* extract possessed increased cytotoxicity by the oral cancer KB cells. However, furthers studies are suggested to determine their structures and the possible mechanisms of action. Therefore, *Punica granatum* peel can be exploited in functional as well a therapeutic agent on a regular basis new antioxidant lacking those effects are being searched all over the world as alternative to the results of the study stated that the ethanol extract acted as a activity in a dependent nature. Further, highlighting the synergistic multi–component effects of *Punica granatum* peel on biological functions would be a recommendation for further studies, as well as studies of the mechanism of action and new biomarkers to prove the effectiveness of bioactive compound in preventing and treating several symptoms and pathologies. Further researches will be required to confirm the bioactivities of these potential.

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