

Evaluation of the Secondary Metabolites of the Waste Pomegranate Rind and its Cytotoxicity against Oral Cancer (KB 3-1)

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Abstract

Cancer is life threatening disease which decrease the quality of the human life and the available medicine to treat the cancer has adverse side effects. The current investigation is to uncover natural products to treat cancer without any side effects. *Punica granatum L.* (Pomegranate) belongs to the family fruit-bearing deciduous small plants with unique flavour, taste, nutritionally rich and popular in health promoting characteristics. *P. granatum L.* peels were collected and extracted for phytochemicals analysis using solvent ethanol and the preliminary analysis revealed the presence of phytochemicals. The analytical GC-MS was carried out to confirm the phytochemicals with pharmacologically and biologically active compounds. The anticancer activity of the extract was carried out against oral cancer cell line (KB 3-1) and this peel extract exhibited promising anticancer property. The MTT assay showed 94.53% inhibition on the oral cancer cell line and clonogenic assay showed decrease in the colonies after treating with the peel extract. These showed positive and encouraging results with efficient anticancer properties and further studies can help identify novel compounds with pharmacological properties for effective cancer treatment.

Keywords: *Punica granatum L.*, phytochemicals, GC-MS, MTT, clonogenic assay, pharmacological.

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(Received: 03 June 2019; accepted: 27 July 2019)

Citation: Jesse Joel T., Jagadish Kumar Suluvoy and Jency Varghese, Evaluation of the Secondary Metabolites of the Waste Pomegranate Rind and its Cytotoxicity against Oral Cancer (KB 3-1), *J Pure Appl Microbiol.*, 2019; **13**(3): 1667-1672. <https://doi.org/10.22207/JPAM.13.3.40>

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INTRODUCTION

Cancer is the group of abnormal cells which divide unusually by overcoming the normal rules of the cell growth and escape the mechanism of apoptosis. Cancer cells (tumors) can be carried through blood vessels to lymph nodes and cause secondary tumors and effect the other place in the body which is called as metastasis¹. Due to its unpredicted nature and more than hundred different varieties of cancers with different mechanism in each type of cancer, it is difficult to identify the medication for the cancer. The current available medication for the cancer is surgery, chemotherapy and radiation, which are having different side effects like fever, nausea, xerostomia hair loss, anxiety and weakness in the body². People began using complimentary and alternate medicine to treat cancer, in which 58 therapies were recognized as prophylactic methods for the treatment of oncogenic ailments with no adverse effects and in this herbal and plant based medicine is the best to treat cancer^{3,4}. Fruits play pivotal role in the human nutrition by providing supplements (vitamins) and dietary roughage and raw materials. Transparent scientific proof indicate that the regular consumption of fruits can prevent cervical, stomach, pancreatic, bladder cancers, high diet of fruit intake will prevent 20% of cancers^{5,6}.

P. granatum L. have antioxidant⁷, anticancer⁸ (2006) anti-proliferative⁹ and the complete *P. granatum L.* content (peel, seed, leaf and flower) have the free radical scavenging activity. *P. granatum L.* extract exhibited strong antibacterial resistance and high antioxidant activity and enhanced liver and kidney functions¹⁰. The *P. granatum L.* having anti-llergic condition against the release of b-hexosaminidase from antigen-stimulated rat basophilic leukemia (RBL-2H3) cells and anti-inflammatory effect on human keratinocyte cells¹¹. *P. granatum L.* has well-known for its ethnomedical properties with beneficial biological features, the biomolecules available in this fruit is used for as substitutes for synthetic food additives, as nutraceuticals and chemo-preventive agents¹². *P. granatum L.* showed in vivo anti-inflammatory activity in mice, it inhibited the neutrophil MPO activity and attenuates LPS-induced lung inflammation in mice¹³. This *P. granatum L.* inhibited type 2 diabetes¹⁴ and it can fight against atherosclerosis

and cardiovascular diseases¹⁵. Other than this biological activity *P. granatum L.* can be utilized as biosorbent to remove nickel from water and the maximum adsorption potential of peel adsorbent for nickel removal was Ca.52 mg g⁻¹.

Based on the established report on the *P. granatum L.* benefits and ayurvedic therapies utilization of this fruit has increased. The current aim of the study is to extract the phytochemicals from the *P. granatum L.* peels for the phytochemical analysis, GC-MS and anti-cancer properties against oral cancer (Human) cell line. The available phytochemicals through GC-MS can be done further research to give biologically important and useful compounds for the human welfare and finding the advantageous compounds for the treatment of cancer.

MATERIALS AND METHODS

Collection of Plant material

The *P. granatum L.* (Pomegranate) fruit peels were collected from Coimbatore district Tamil Nadu region India. The plant was authenticated by the Botanical Survey of India, Coimbatore, Tamil Nadu, and the authentication number is (BSI/SRC/5/23/2017/Tech.). The *P. granatum L.* peels were washed thoroughly under running tap water and shade dried at room temperature.

Cell line

The KB 3-1 oncogenic cell culture was obtained from the National Cancer Institute (Maharashtra, India). Their concentration of cells was determined using a haemocytometer. The obtained cell line was sub-cultured and kept for further use.

Chemicals

The chemicals used in this experiments were purchased from Hi-Media (Mumbai, India). All other chemicals used for the experiments were of analytical reagent grade.

Preparation of *P. granatum L.* rind extract

The dried peels of *P. granatum L.* was grinded into powder and extracted with ethanol by using continuous hot extraction method. The obtained product was concentrated using vacuum rotary evaporator and the extract was preserved in the freezer (4°C) for further use.

Phytochemical analysis

Test for Alkaloids

The crude extracts were heated with

2% Hydrochloric acid on a heated liquid bath and chilled, sieved then treated with the Mayer's reagent, presence of yellow precipitation or turbidity confirmed the presence of Alkaloids¹⁷.

Test for phenols

Crude extract +2% solution of FeCl_3 = A blue-green or black coloration¹⁸.

Test for flavonoids

A few aliquots of 1% aluminium solution were suspended into a part of the aqueous plant extract. A yellow coloration specifies that the flavonoids are present¹⁹.

Test for Tanins

Crude extract + Gelatine solution + sodium chloride = white precipitate²⁰.

Test for Saponins

Distilled water + crude extract = Foam formation²¹.

Test for steroids

2ml of chloroform + Conc. H_2SO_4 + Crude extract + Acetic = Greenish coloration²¹.

GC-MS

The *P. granatum L.* peel extract administrated for Gas Chromatography- Mass Spectroscopy (GC-MS) analysis to categorize the phytochemicals present in it. The temperature of the column was set to 80-250°C at 8°C/min rate. Helium act as a transporter gas at a flow rate of 1.0 ml/min. The Thermo GC-Trace Ultra VER: 5.0 (Bremen, Germany) and Mass Spectroscopy (MS) MS DSQ II electron ionization mode with ionization energy of 70 eV was employed. 1µl of sample was used for analysis. Using the retention time and mass fragmentation patterns, a comprehensive analysis of the fruit extract components were established. The National Institute of Standards and Technology (NIST) and Wiley 9.0 library helped identify and correlate the various compounds present in the sample.

MTT Assay

The anti-cancer property of ethanolic *P. granatum L.* peel extract was analysed by the MTT assay using KB 3-1 (Human) cancer cell line. Yellow soluble MTT substrate was reduced by the formazan by the living cells. 0.1ml of KB 3-1 (Human) cancer cell line were (1.5×10^6 cells/well) seeded in ELISA plate and incubated for 24 hours in CO_2 incubator at 37°C, after 24 hours incubation cells were treated with the *P. granatum L.* extract at different concentrations of 50µg, 100µg, 150µg,

200µg, 250µg, 300µg and 350µg was added to the wells, and then plate was kept for incubation for 24 hrs. Then, the cell culture was removed and the plate was washed with PBS. 90 µl of fresh media was added to each well and incubated for three hours. Then 10 µl of MTT (5mg/ml) was added to each well and the plate was kept for 2 hours incubation. 50µl of DMSO was added in each well to stop the mechanism and readings were taken at 570 nm by ELISA plate reader. The percentage of inhibition was calculated by the formulae,

$$\% \text{ Inhibition} = \frac{\text{OD of the Control} - \text{OD of the Test}}{\text{OD of the Control}} * 100$$

RESULTS AND DISCUSSION

The second most dangerous disease in the world is cancer and in accordance with the World Health Organisation (WHO) there are apparently 14 million established oncological cases with a mortality rate of 8.2 million in 2012. Major drawback in curing the disease is lack of proper medicine and the available medicine is having the adverse side effects. Humans started getting medicine from nature and this nature is embedded with inestimable treasure called plants which are more beneficial to human's life. These plants are used as traditional medicine against different kind of diseases from ancient days. Although several medicine available to cure diseases, it has many side effects which will decrease the quality of the human life²³. This herbal medicine once again flourishing in world for the human welfare to cure diseases with any side effects and according to WHO survey (2008) 1/8th of ecosphere's population depend on the ecological health and balance for medicines. *P. granatum L.* is known as an ornamental plant and it also produce

Table 1. The presence of different phytochemicals in the *P. granatum L.* peel extract and it's observed that the absence of steroids and saponins and the presence of phenols, Alkaloids, Flavonoids and Tanins

NO.	Phytochemicals	Presence
1	Alkaloids	-ve
2	Phenols	+ve,+ve
3	Flavonoids	+ve
5	Tannins	+ve
6	Saponins	-ve
7	Steroids	-ve

Table 2. Phytochemicals observed with more than 25% probability by GC-MS analysis

No.	Compound Name	Probability Formula	Molecular Weight	Molecular
1.	2-Furancarboxaldehyde (CAS)	38.2	C ₅ H ₄ O ₂	96
2.	Furfural	38.20	C ₅ H ₄ O ₂	96
3.	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	83.27	C ₆ H ₈ O ₄	144
4.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl(flavinoid)	83.27	C ₆ H ₈ O ₄	144
5.	2-Furancarboxaldehyde, 5-(hydroxymethyl)- (CAS) (saponin)	68.88	C ₆ H ₆ O ₃	126
6.	5-Hydroxymethylfurfural	68.88	C ₆ H ₆ O ₃	126
7.	α-D-Glucopyranoside, α-D-fructofuranosyl (CAS)	39.59	C ₁₂ H ₂₃ O ₁₁	343
8.	Hexadecanoic acid, methyl ester	64.77	C ₁₇ H ₃₄ O ₂	270
9.	di(isityl)di[phosphino]silane	72.42	C ₃₀ H ₅₀ P ₂ Si	500
10.	1-Propyl-3,6-diazahomoadamantan-9-ol	34.06	C ₁₂ H ₂₂ N ₂ O	210

edible fruit with rich source of antioxidants. All the parts of a pomegranate fruit have the free radical scavenging activity (antioxidant property). The study shows that peel has highest antioxidant property from other parts of the fruit²⁴ Wang *et al.*, (2011). Antioxidants contents were as follows: seed < leaf < flower < peel²⁵ Elfalleh *et al.*, (2012). Moreover, another study proved that the peel of the pomegranate showed maximum antioxidant property among 28 others²⁶. Pomegranate peel consists of high number of phytochemicals that are necessary to fight against cancer cells and also this pomegranate peel have antibacterial, anti-allergic activities, anti microbial, tyrosinase inhibition, anti-hyperglycemic, Anti-hepatoprotective, anti-proliferation, hepato protective, and antihyperlipidemic properties¹⁰ Ibrahim, (2010).

In the present study by the preliminary phytochemicals analysis *P. granatum L.* peels were collected, dried, powdered and the phytochemicals were extracted by soxhlet extraction²⁷ process using ethanol as a solvent and it was found out that it is rich source of Phenols, flavonoids, Alkaloids and Tannins were present. This presence of phytochemicals were further tested by the GC-MS and it showed the presence of biological active compounds present in the *P. granatum L.* peel extract. The *P. granatum L.*

showed anticancer properties against human oral cancer cell line (KB3-1). The extracts was used for testing anticancer properties by MTT Assay²⁸. A positive result was obtained and the percentage of inhibition was found and we concluded that as the concentration of extract increases the cell viability decreases. i.e 350µg/ml it showed 80.88 % of inhibition. Therefore the pomegranate peel extract showed an encouraging and positive result against KB3-1, which further can used for the analysis of compound against KB3-1 oral cancer cell line.

Table 3. Inhibition of KB 3-1 (Human) oral cancer cell line by the increase in the *P.granatum L.* peel extract concentration

No.	Concentration (µg/ml)	Inhibition (%)
1.	50	23.36
2.	100	27.25
3.	150	32.09
4.	200	49.55
5.	250	61.94
6.	300	71.85
7.	350	80.88

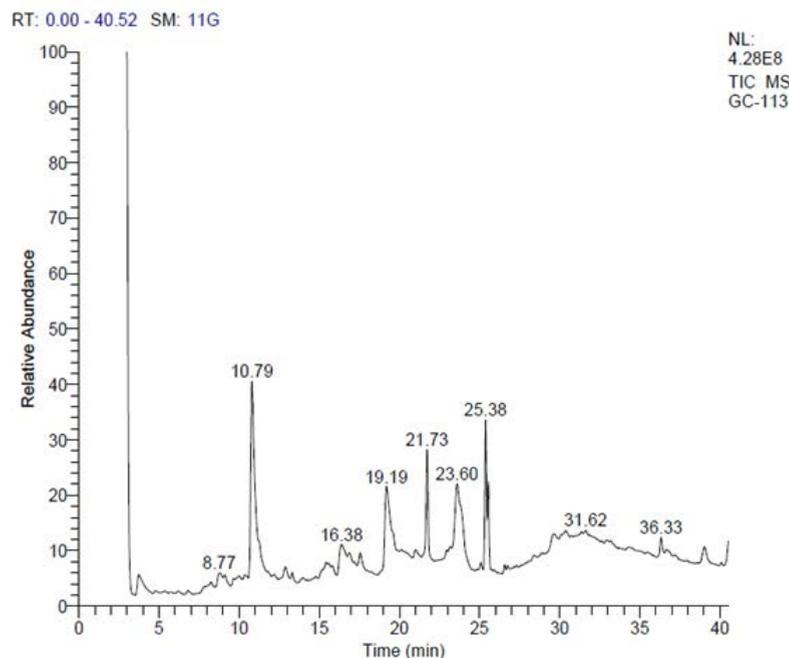


Fig. 1. Chromatogram of the *P.granatum L.* peel extract showing the peaks at different time intervals and with relative abundance.

ACKNOWLEDGEMENTS

We would like to thank the Department of Biotechnology for giving us the Research Lab facilities to carry out the work.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors have made substantial contribution to the work and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All data generated during the study are included in the manuscript.

ETHICS STATEMENT

The study was undertaken after obtaining approval from the Institutional Research and Ethics Committee.

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