Research Article

Study of *In-vitro* Anti-Cancer and Anti-Oxidative Properties of Aqueous Extract of *Punica Granatum* Flowers

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ABSTRACT

The different parts of *Punica granatum* plant are known to possess phytochemicals with beneficial properties. References for the same can be found even in the ancient systems of medicine. Given the prevalence of cancer, it is imperative to search for newer tools to overcome it. As indicated by independent groups of researchers, pomegranate is a source of phytochemicals with anti-cancer activities such as squalene, gallic acid derivatives, flavanoids, etc. In the present study, the anti-cancer property of the aqueous extract of pomegranate flowers was evaluated on different cancer cell lines *in-vitro* using the MTT assay. All the cell lines were found to be sensitive to the extract with IC_{50} values for the different cell lines ranging from 5-10.6 µg/mL. The anti-oxidant property of the extract was studied using the DPPH radical scavenging assay and was found to be comparable to the ascorbic acid standard. Further phytochemical analysis and *in-vivo* studies need to be performed to ascertain the potential of pomegranate flowers aqueous extract as anti-cancer and anti-oxidative agent.

Keywords: *Punica granatum*, anti-cancer, anti-oxidative.

INTRODUCTION

Punica granatum (family Punicaceae) originated in the Middle Eastern region and as of today over thousand cultivars exist around the globe.[1] It has been revered as a symbol of life, longevity, health, femininity, fecundity, knowledge, morality, immortality and spirituality, if not Divinity.[2] The ancient Indian system of medicine, Ayurveda, considers the pomegranate plant as a "pharmacy unto itself" because of the fact that almost every part of the plant has medicinal properties such as the barks and roots have antihelmenthic and vermifuge properties, the peels are a cure for diarrhea and oral aphthae, while the juice acts a "blood tonic".[3-6] Pomegranate was not only used in ancient medicine, but also has applications in modern day practices. Pomegranate is used, in the treatment of acquired immune deficiency syndrome (AIDS), cardiovascular protection, oral hygiene, in cosmetics and ophthalmic ointment among others.[7-13] Research related to pomegranate has been increasing due to the anti-oxidant, anti-inflammatory, and anti-cancer potential of pomegranate. These three pharmacological activities are inter-related, since anti-oxidant and anti- inflammatory drugs can also serve as chemopreventive and chemotherapeutic agents.[4]

Comprehensive research relates increased amount of reactive oxygen species (ROS) content with disease states such as diabetes, cardiovascular disorders, cancer, etc.[14-17] These highly reactive radicals can interfere with the normal cellular functioning by reacting with most of the available biomolecules. Even though anti-oxidative factors are present and active in the body, they need to be replenished time and again. The main sources of anti-oxidants are fruits, vegetables which contain polyphenolic compounds, flavanoids, alkaloids, beta-carotenoids, ascorbate, etc. Flavanoids have been reported to activate radical scavenging molecules such as enzymes which inhibit oxidases, and reduce radicals.[18,19]

The ROS are known to cause oxidative damage to nucleic acids in the form of serious lesions like single-stranded breaks, double-stranded breaks may lead to mutagenesis which can prove to be carcinogenic.[20] Cancer is one of the most dreaded diseases globally. According to the cancer statistics report by the American Cancer Society, one in every four deaths in the U.S.A. is due to cancer.[21] Breast cancer is the most prevalent form of cancer; while liver cancer is the third-most common cause of cancer death globally. Cancer of the kidney, cervix, liver and breast together make up a substantial proportion of the global cancer burden. The most common forms of cancer i.e. breast, lung and prostate have a high probability to metastasize to the bone thus leading to complications of hypercalcemia, spinal cord compression, pain, etc.[22]

Thus, the combined characteristics of anti-oxidant and anti-proliferative properties of potential drug candidates are highly desired to combat cancer. Keeping this in mind, the reported study was undertaken to study the anti-cancer and anti-oxidant properties of *P. granatum* flower extract *in-vitro*. To test the anti-oxidant activity, DPPH radical scavenging assay was performed. To test the anti-cancer activity, the MTT assay was performed on cancer cell lines derived from the kidney (ACHN), liver (HepG2), breast (MCF-7), bone (LN-18) and cervix (HeLa). These particular cell lines were selected to encompass the major organs or tissues which together make up a majority of cancer cases as well as cancer deaths globally.

Investigation of these anti-cancer and anti-oxidant properties of *P. granatum* flowers extract *in-vitro* using different cell lines makes this study unique.

MATERIALS AND METHODS

P. granatum flowers harvested from a local farm in the month of December. The cancer cell lines HeLa (cervix), HepG2 (liver), MCF-7 (breast), LN-18 (brain) and ACHN (kidney) were procured from National Centre for Cell Science, Pune. The cells were maintained in T-25 flasks (Nunc) containing MEM essential medium (Gibco) or RPMI-1640 medium (Gibco), fetal bovine serum (Gibco), Penicillin-Streptomycin (Gibco). The flasks were incubated in humidified 37 °C 5 percent (%) CO_2 incubator (Thermo Scientific). Other instruments and reagents used were as follows: inverted microscope (Zeiss), haemocytometer (Hausser Scientific), 1X Hanks' Balanced salt solution (HBSS) (Gibco), 12-well plate (Nest), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (Life technologies), 0.25% Trypsin 0.02% EDTA solution (Himedia), dimethyl sulfoxide (DMSO) (Loba Chemicals), spectrophotometer (Shimadzu) and 0.2-µm syringe filter cartridges (Corning), L-Ascorbic acid (SRL), Methanol (Merck), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (SRL).

Extraction of *Punica granatum* flowers

P. granatum flowers were rinsed in distilled water twice and shade-dried. The dried flowers were powdered using a mortar-pestle. 1 gram (g) of powder was added to 20 milliliters (mL) purified water and macerated overnight at room temperature. After maceration, the suspension was filtered using Whatman filter paper separately. The filtrate was then centrifuged at 1500 rpm for 10 minutes. The supernatant of the filtrate was filter sterilized using a 0.2-µm syringe filter under sterile conditions and labeled as aqueous extract. The concentration of aqueous extract was 42.056 µg/mL. This extract was used for further experiments.

Cell line maintenance

The cell lines MCF-7, ACHN, and HeLa were maintained in MEM with 10% fetal bovine serum (FBS), 100 units/mL Penicillin, 100 µg/mL Streptomycin. HepG2 cell line was maintained in RPMI-1640 supplemented with 10% FBS, 100 µg/mL Penicillin, 100 units/mL Streptomycin; whereas, LN-18 was maintained in DMEM supplemented with 10% FBS, 4 mM L-Glutamine, 100 µg/mL Penicillin, 100 units/mL Streptomycin. All the above cell lines were maintained in T-25 culture flasks incubated in a humidified 37°C incubator with 5% CO_2. The growth media was changed 2-3 times weekly and passaged at sub-confluency using Trypsin EDTA solution.

Anti-proliferative assay using MTT

At 70-80% confluency, the cell cultures were treated with 0.5 mL Trypsin EDTA solution and incubated at 37°C with 5% CO_2 for 5-10 minutes till isolated cells had dislodged from the flask surface. 4.5 mL of the respective growth medium was added to the flask and transferred to a 15 mL centrifuge tube to be centrifuged at 800 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended in 5 mL growth medium.

Cell count was measured using the hemocytometer. Accordingly, the cell suspension was prepared using the growth medium and 800 µL of it containing 10^4 cells was added to each well in 12-well plates.

The plates were then incubated in a humidified 37°C incubator with 5% CO_2 for 24 hours (hrs). 50 µL, 25 µL, 12.5 µL, 6.25 µL and 3.13 µL of aqueous extract were added to separate wells in triplicate to get final concentrations of 42.08 µg/mL, 21.04 µg/mL, 10.52 µg/mL, 5.26 µg/mL, 2.21 µg/mL respectively.

50 µL purified water was added to the cell control wells. The plates were again incubated in a humidified 37°C incubator with 5% CO_2 for 24 hrs. The media was discarded and the wells were gently washed with 400 µL 1X HBSS. Fresh 360 µL media was added to each well along with 40 µL 0.5 mg/mL MTT solution and incubated at 37°C with 5% CO_2 for 3 hrs. The solution was discarded and 400 µL DMSO was added to each well and incubated at room temperature in dark for about 30 minutes following which absorbance was measured at 540 nm using a spectrophotometer. The percentage inhibition (% Inhibition) was calculated as follows:

$$\% \text{ Inhibition} = 100 - (A_{sample} / A_{cell\ control} * 100)$$

Where, A_{sample} is the absorbance of the sample at 540 nm.

$A_{cell\ control}$ is the absorbance of the cell control at 540 nm.

DPPH radical scavenging assay

10 mM (17.5 µg/mL) ascorbic acid stock solution was prepared in methanol. Serial dilution of ascorbic acid of 7 µg/mL, 3.5 µg/mL, 1.75 µg/mL, 0.875 µg/mL, 0.438 µg/mL, 0.219 µg/mL, 0.109 µg/mL, 0.055 µg/mL were made up to a total volume of 250 µL using methanol. The aqueous extract was serially diluted from concentrations of 42.056 µg/mL to 0.329 µg/mL to make up a final volume of 250 µL in methanol. 750 µL of 200 µM DPPH solution in methanol was added to each tube. 1000 µL of 200 µM DPPH solution was used as the control and methanol was used as blank solution. The tubes were vortexed and incubated at room temperature in dark for 30 minutes following which absorbance was measured at 517 nm. The scavenging activity was calculated as below:

$$\% \text{ Scavenging} = [(A_{control} - A_{sample})/A_{control}]*100$$

Where, $A_{control}$ is the absorbance of the control at 517 nm.

A_{sample} is the absorbance of the sample at 517 nm.

RESULTS AND DISCUSSION

Anti-oxidative activity

Free radicals are biologically produced in the cells. They are efficiently scavenged by anti-oxidants such as glutathione, ascorbic acid, superoxide dismutase, etc.[23-25]

If these free radicals remain unattended, they spontaneously react with the available biomolecules.[26]

Free radicals are known to cause lipid peroxidation, bonding between nucleotides of DNA which may lead to double-strand breaks.

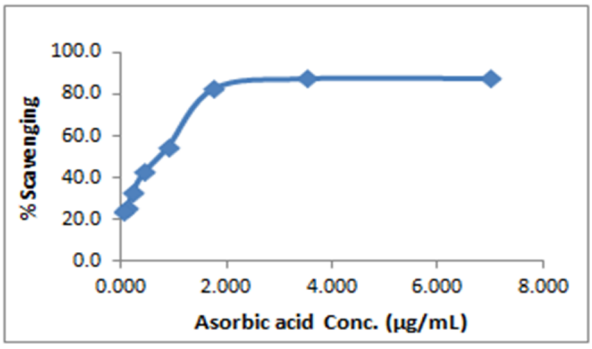
Such instances of DNA damage can cause the cells to turn oncogenic.[27] Hence, anti-oxidative property can prove to be an important tool against cancer.

The DPPH radical scavenging assay is one of the most commonly used assays to study the anti-oxidative property of substances.[28]

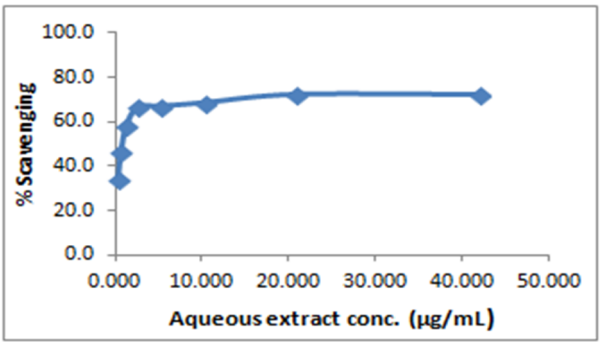
The DPPH radical is a free, stable radical which is neutralized after being reduced by the anti-oxidant. This reduction is detected as a change in absorbance maxima, from violet towards yellow or colorless.

In this study, the anti-oxidative capacity was compared to ascorbic acid under similar conditions.

The IC_{50} values for ascorbic acid and aqueous extract of *P. granatum* petals were calculated as 0.770 µg/mL and 0.915 µg/mL respectively. Fig. 1. The ascorbic acid equivalent of the aqueous extract of *P. granatum* petals was about 1.2. Earlier reports of free radical scavenging activity analysis of plant extracts using DPPH assay state varied results which can be attributed to modified methods being used.[29,30] Polyphenolic compounds present in phytochemicals have been related to the anti-oxidative property of substances both *in-vitro* and *in-vivo*.[31,32]



(a)



(b)

Figure 1: Graph of sample concentration against % free radical scavenging in DPPH assay. (a) plot of the standard ascorbic acid against its percent free radical scavenging; (b) plot of the aqueous extract of *P. granatum* petals concentration against its percent free radical scavenging.

Anti cancer activity

It has been reported in the phytochemical analyses of various parts of *P. granatum* plant, that it contains some components known to have anti- cancer properties such as squalene, gallic acid, etc.[4,33-38] Aqueous extract of other plants have also been reported to contain these water soluble phytoconstituents and have significant anti-cancer properties.[19] For the present study, the MTT assay was performed to study the anti-proliferative activity of the extract. In this assay, the bright yellow colored tetrazolium salt, MTT, is reduced by the mitochondrial enzyme succinate dehydrogenase converting it to dark colored water insoluble formazan crystals.[39] These crystals when dissolved in dimethyl sulfoxide have absorbance maxima at 540 nm and can thus be used to correlate the number of metabolically active or 'live' cells. For this study, cancer cell lines from the different organs of the human body such as brain (LN-18), kidney (ACHN), liver (HepG2), breast (MCF-7) and cervix (HeLa) were used to better evaluate the anti-cancer properties of the aqueous extract. All the cell lines were treated under similar conditions of cell number, incubation and exposure time, concentration of the extract and the mean percent inhibition (% Inhibition) was plotted against the concentration of the aqueous extract. Fig. 2.

From the results of the anti-proliferative MTT assay (Figure 2), it can be concluded that *P. granatum* flower aqueous extract used in this study has anti-activity against all the tested cell lines originating from different organs or tissues.

The approximate half maximal inhibitory concentration (IC_{50}) values, calculated using Microsoft Excel software, for the respective cell line have been stated below in Table 1.

S. No.	Cell Line	IC_{50} (ug/mL)
1	MCF-7	5.047 ± 0.272
2	LN-18	10.614 ± 0.384
3	ACHN	6.363 ± 0.231
4	HepG2	5.134 ± 0.403
5	HeLa	7.144 ± 0.021

Table 1: IC_{50} values calculated for the respective cell lines (Mean ± S.D.)

Similar results of cytotoxic activity of have been reported using the MCF-7 cell line, however, the differences in preparation of the aqueous extract, parameters of cell number per well, confluency of cell culture during harvesting of the cells, exposure to the extract may be attributed to the different IC_{50} values.[40]

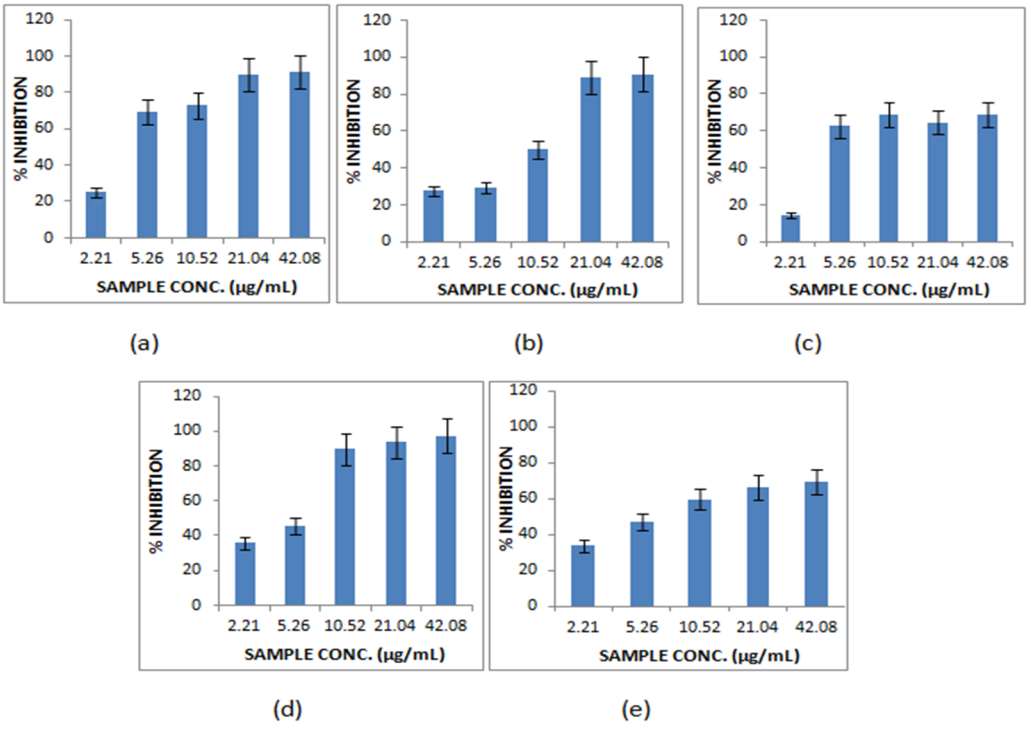


Figure 2: Graph of sample volume of aqueous extract against the percent inhibition using MTT assay for different cell lines; where (a) MCF-7, (b) LN-18, (c) ACHN, (d) HepG2, and (e) HeLa cell lines.

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CONCLUSION

The results mentioned above indicate that the aqueous extract of *P. granatum* flowers contain phytochemicals having anti-cancer and anti-oxidant properties.

Thus, further analysis of the constituent phytochemicals and *in-vivo* studies need to be carried out to explore the potential of pomegranate flowers' extract as a novel source of drug candidates as chemotherapeutic and chemopreventive agents.

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