MEDICINAL PLANTS:
Phytochemistry, Pharmacology and Therapeutics

– Volume 4 –

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2016
Daya Publishing House®
A Division of
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New Delhi – 110 002

Medicinal Plants: Phytochemistry, Pharmacology and Therapeutics Vol. 4 (2016)
Editors: **V.K. Gupta *et al.***
Published by: **DAYA PUBLISHING HOUSE, NEW DELHI**

Pages **351–373**

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Antigenotoxic Potential of *Punica granatum* in Breast Cancer Patients

K.S. Santhy*[1] and M. Srividya[1]

ABSTRACT

Cancer is a disease that knows no geographic boundaries. Despite decades of basic and clinical research and trials of promising new therapies, cancer remains a major cause of morbidity and mortality. The post genomic era has now opened new avenues in cancer treatment, which is contemplated to be more effective and specific for tumor cells using various methods available for the assessment of DNA damage and repair in cancer patients such as the bacterial Ames test, the scoring of chromosome aberration (CA), micronuclei and sister chromatid exchange (SCE) in proliferating cell populations although the frequency of cells with structural chromosomal aberrations in peripheral blood lymphocytes is the first biomarker for genotoxicity that shows an association with overall cancer risk. The present investigation encompasses Single Cell Gel Electrophoresis (SCGE)/Comet assay as an alternative for cytogenetic test to assess the extent of DNA damage in breast cancer patients undergoing different modalities of treatment viz., chemotherapy, chemotherapy + surgery, chemotherapy + radiotherapy and chemotherapy + surgery + radiotherapy. The present study also comprises the reduction in chromosomal damage by the addition of pomegranate extracts to peripheral blood lymphocyte culture as a biomarker tool for DNA repair capacity of test substance. Therefore, the DNA repair capacity of Pomegranate Fruit Extract (PFE) in cultures of human peripheral blood lymphocytes of breast cancer patients was examined.

Keywords: Antigenotoxic potential, Breast cancer patients, Chromosomal aberrations, Comet assay, Peripheral blood lymphocytes, Pomegranate fruit extract.

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Introduction

Pomegranate (*Punica granatum*), a small tree originating in the Orient, but grown mainly in Iran, India and USA and also in near and far east countries, belongs to the family Puniaceae. Pomegranate also known as Anar has been grown in India since ancient periods as a fruit crop, suitable for arid and semi arid regions, due to versatile adaptability, hardy nature, low maintenance cost and high yield (Bankar and Prasad, 1992). In India, its cultivation is spread all over the country covering about 40,000 hectares of land. The state of Maharashtra alone accounts for 2/3 of total area under cultivation in the country (Anon, 1999) where the annual production of pomegranate is 4.0–4.5 lakh tones.

Pomegranate fruit has a fascinating history of traditional use as food, medicine and cultural icon, dating back thousands of years (Still, 2006). A symbol of fertility and immortality, pomegranates healing properties were discussed in one of the oldest medical texts of *Ebers papyrus* from Egypt (circa 1500 BCE) (Jayaprakasha, 2006). The fruit is mentioned in both Greek and Persian mythology representing life, regeneration and marriage (Langley, 2000). The fruit is also one of the three blessed fruits in Buddhism. In various forms of traditional Asian medicine, pomegranate fruits were recommended as a health tonic and as a treatment for numerous ailments including diarrhea, dysentery and diabetes.

Chemistry of fractions from the peels shows the presence of acids such as citric, malic, lactic, fumaric, tartaric and acetic. The juice also contains numerous pharmacologically more active phenolic compounds such as quinic acid, gallic acid, chlorogenic acid, caffeic acid, ferulic acid and also ellagitannins such as ellagic acid and punicalagin (Gil *et al.*, 2000), most of which occur in high concentrations in the peels (Artik, 1998). The only phenolic compound unique in the juice and not in the peel is anthocyanidins such as delphidin. Further proanthocyanidins, specifically prodelphidins have been discovered in the peels, along with gallocatechins. Thus for all intents and purposes, the chemistry of the peels (including inner membrane) and juice consist of different blends of much of the same active phenolic compounds derived from the flavonoids, tannins, ellagic acids and catechins all of which are strongly antioxidants (Plumb *et al.*, 2002).

Cancer cells exists under a state of oxidative stress, as this increases their survival potential by including mutations (Shinkai *et al.*, 1998), activating redox signaling that may lead to the inactivation of tumour suppressor genes as p53 (Sun and Oberley, 1996) and the activation of pro-survival factors such as NFkB and AP-1 (Riple *et al.*, 1999). Mild levels of reactive oxygen species have been shown to induce proliferation in cancer cells. Therefore, foods rich in antioxidant phytochemicals are important for the prevention of diseases related to oxidant stress such as heart disease and cancer.

In recent years, chemoprevention has received as much attention as chemotherapy in the fight against cancer. The search for new ways to stop cancer before its onset has led investigators to examine a wide variety of natural agents. A recent study at the University of Wisconsin argues that agents capable of intervening at more than one critical pathway in the process of carcinogenesis "will have greater advantage over other single-target agents" (Afaq *et al.*, 2004). The Wisconsin

researchers found that pomegranate fruit extract possesses strong antioxidant and anti-inflammatory properties. In a more recent study focused on the antioxidant effectiveness of plant pigments called bioflavonoids, commonly found in berries, cherries, grapes, and citrus showed that pomegranate juice exhibited three times more antioxidant activity than red wine or green tea (Gil *et al.*, 2000). The active constituent that appears to be responsible is ellagic acid, a naturally occurring polyphenolic compound in pomegranates.

Ellagic acid is active in antimutagenic assays and has been shown to inhibit chemically induced cancer in the lung, liver, skin and oesophagus of rodents (Stoner *et al.*, 1995). Dietary ellagic acid has been shown to reduce the incidence of N-2-fluorenylacetamide induced hepato carcinogenesis in rat and N-nitrosomethyl-benzylamine induced rat oesophageal tumours (Ahn *et al.*, 1996). Larrosa *et al.* (2006) reported that ellagic acid provoked the induction of apoptosis via intrinsic pathway through bcl-xL down-regulation with mitochondrial release of cytochrome-c into the cytosol. Other studies demonstrated that the apoptosis caused by ellagic acid is associated with p53/p21 expression, cell cycle arrest, regulating matrix metalloproteinases, vascular endothelial growth factor expression and platelet-derived growth factor.

Two major classes of polyphenolic compounds present in pomegranate are hydrolysable tannins (HT) and flavonoids, with very small levels of condensed tannins. The HT constitutes predominant polyphenols and includes ellagitannins, gallotannins, and gallagoyl esters. The most abundant HT is gallagyl tannins, punicalagins (anomers A and B) and related tannins, which in turn comprise roughly 63 per cent of the constituent polyphenols. Others reported HT include pedunculagin, punicalin, gallagic and ellagic acid esters of glucose (16.8 per cent), and ellagic acid derivatives (4.9 per cent) (Lansky, 2007).

In various *in vitro* models, the punicalagins have been reported to protect lipids, proteins, and DNA against oxidative damage by several mechanisms: scavenging free radicals, transferring electrons to repair oxidative damaged components, and chelating metal ions (Kulkarni *et al.*, 2007). Similarly Singh *et al.* (2002) reported the high antioxidant activity of methanol extracts of pomegranate peel and seed extracts in various *in vitro* models. Chidambara Murthy *et al.* (2002) reported that rats fed with an extract of pomegranate peel followed by exposure to carbon tetrachloride indicated protected level of the antioxidant enzymes catalase, peroxidase, and superoxide dismutase in the rats. The pomegranate extract also helped to protect the rats' livers from the toxic effects of carbon tetrachloride. Auroma (1994) evaluated nutrition and health aspects of free radicals and antioxidants and reported that synthesis of catalase and peroxidase enzymes were induced by the components of pomegranate peel extracts.

Afaq *et al.* (2004) evaluated pomegranate fruit extract for anti-tumor-promoting effects, specifically involving topical application against skin tumors. The researchers concluded that animals pretreated with pomegranate fruit extract showed 70 per cent less tumor incidence compared to animals that did not receive it. The study authors believe that their results provide "clear evidence that [pomegranate fruit

extract] possesses anti-skin-tumor-promoting effects," and may possess chemopreventive activity "in a wide range of tumor models." These findings support the promising results of a pair of 2003 studies in South Dakota and Japan that explored pomegranate seed oil as a safe and effective agent against skin cancer and colon cancer tumors, respectively (Kohno *et al.*, 2004). A study in 2002 revealed, pomegranate seed oil inhibited the proliferation of human breast cancer cells up to 90 per cent, while polyphenols from fermented pomegranate juice inhibited 47 per cent of cancerous lesion formation in mammary gland cells from mice (Kim *et al.*, 2002).

Cell biology studies have begun to establish that defects in cell-cycle checkpoint controls are fundamental to the accumulation of genetic damage in the mammary epithelial cell, leading to cancer (Deng, 2006). The four cell-cycle transactions, from G1→S, G2→M, spindle formation and functions (Cytokinesis) and daughter cell separation (Karyokinesis) appear to be important points of vulnerability for genetic damage. In normal cells, DNA damage and replication defects may be recognized at these and other points resulting in cell-cycle arrest, DNA repair and programmed cell death (apoptosis). Such DNA damage may be produced by environmental exposure, replicative senescence and by pre-malignancy or malignancy associated cellular changes.

At present a number of options are present to treat cancers among which surgery, radiation therapy and chemotherapy are the established therapies, although, hormone and biological therapies are also in use. Clinically, a doctor may use one of these methods or a combination of methods for treatment as the aim of cancer treatment is to cure the patient and save life. In cases where complete cure is not possible, treatment aims to control the disease and keep the patient normal and comfortable as long as possible. The treatment of each patient is designed to suit an individual and depends on the age of the patient, stage and type of disease.

Although relationship between chromosomal damage and cancer development has been suggested since the beginning of the 20th century, extensive data been gathered on the frequency of chromosomal alterations (CAs) in Peripheral Blood Lymphocyte Culture (PBLC) of humans exposed to known or suspected genotoxic carcinogens are reported after 1960. The idea of causal association between chromosomal alterations and cancer risks are based on the concept that genetic damage in lymphocytes reflects similar damage in cells undergoing carcinogenesis. Peripheral blood lymphocytes are extensively used in bio monitoring of populations exposed to various mutagenic or carcinogenic compounds (Heim and Mitelman, 1987). This is because of the ease of sampling, the possibility of obtaining large number of scorable cells and the documented sensitivity of this system in detecting chromosome damage induced by exposure, particularly ionizing radiation. For carcinogenic processes in the target tissues, structural chromosomal aberrations in peripheral blood lymphocytes have been applied for over 30 years in occupational and environmental settings (including radiation dosimetry) as a biomarker of early effects of genotoxic carcinogens. The frequency of cells with structural chromosomal aberrations in peripheral blood lymphocytes is the first genotoxicity biomarker that has actually shown an association with overall cancer risk.

Breast cancer as the most frequent tumour disease in women is of special relevance. It is currently found that one woman in every eight will be faced with the diagnosis of breast cancer in her lifetime (Incidence and Trends, 2012). Currently in India, the incidence of breast cancer has steadily increased over the years and as many as 100,000 new patients are being detected every year (Zeleniuch and Roy, 2005). The increase reported by the cancer registries is nearly 12 per cent from 1985 to 2001, representing a 57 per cent rise in India's cancer burden (Yip *et al.*, 2006; Farooq and Coleman, 2006).

The trends for increase in breast cancer incidence over time for most of the population in India were found to be statistically significant (Yeole and Kiekue, 2003) and among the six population based cancer registries that were evaluated over the last two decades. In the present study, our focus on peripheral blood lymphocytes of breast cancer patients investigated the potential of Pomegranate Fruit Extract (PFE) as anti-cancer agents by the assessment of the extent of DNA damage in patients undergoing various modalities of treatment *viz.*, chemotherapy, radiotherapy, surgery or combination of these therapies and evaluating the effect on chromosomal aberrations, chromatid type alterations and chromosome type alterations. In the present study, the reduction in chromosomal damage by the addition of PFE to peripheral blood lymphocyte culture is taken as a biomarker tool for DNA repair capacity of test substance. Therefore, the DNA repair capacity of PFE in cultures of human peripheral blood lymphocytes of breast cancer patients was examined.

Materials and Methods

Extent of DNA Damage in Breast Cancer Patients

All the chemicals used were of analytical grade. Normal Melting Agarose, Lower Melting Agarose, Triton X-100 and Tungstosilic acid were purchased from Himedia and the Tris buffer and Di Methyl Sulphoxide (DMSO) were bought from the Merck, Mumbai.

The healthy donor's blood samples (negative samples) were collected from the locale of Coimbatore and the patient's blood samples (Positive samples) were collected from Valavadi Narayanaswamy Cancer Centre, G.Kuppusamy Naidu Memorial Hospital, Coimbatore. IHEC. The group of healthy donors consisted 20 women who were not undergoing any medication at the time of blood collection was selected with no cases of cancer in the family. Among the 40 women with locally advanced breast cancer 12 were undergoing different cycles of chemotherapy alone, 10 had underwent surgery and taking chemotherapy, 8 were with irradiation and chemotherapy and the remaining 10 samples with the combination of all the three therapies, namely, chemotherapy, surgery and radiation therapy.

For the monitoring of DNA damage in the peripheral lymphocytes, about 2-5 ml blood was collected from each donor by venipuncture into heparinzed syringes and brought to the laboratory. The samples were collected from 2007 to 2008. The results of electrophoresis obtained were assessed on the basis of DNA damage categories assigned for the cells. DNA damage was quantified for each cell by measuring the total length (head and tail) according to the criteria adopted by Martin *et al.* (1998).

The data obtained for tumor response studies were subjected to ANOVA. Correlation analysis was performed to assess the relation between age of the patients Vs DNA damage and cycles of chemotherapy Vs DNA damage. Statistical package (SPSS 11.0v) was used for above analysis.

Antimutagenic Activity of Pomegranate

The arils of pomegranate were washed in water and shade dried (Figures 17.2 and 17.3), powdered and separated using an electrical grinder and sieved to obtain a fine powder. Soxhlet extraction of powered arils were carried out to obtain their extracts by using increasing order of polarity of petroleum ether, chloroform, methanol and water. Mc Coy's 5A Medium powder (12 gm) was dissolved in 1000ml of sterile double distilled water. The pH was adjusted to 7.2–7.4 with the addition of 0.22 g sodium bicarbonate. The mixture was filtered using 0.22 µm filter pads in a Seitz filter into a sterile flask and streptopenicillin (0.2 ml/100 ml) was added to the filtrate and stored at –20°C.

About 2.0 ml of venous blood from the experimental subject was drawn into a sterile heparinized syringe and 0.5 ml of the blood was inoculated under aseptic conditions into a culture vial containing 5.0 ml of culture medium, 1.0 ml of ab serum and 0.2 ml of phytohaemagglutinin (PHA). The cultures were incubated at 37°C for a period of 72 hrs and were shaken periodically twice a day in order to facilitate proper mixing of the medium and cells in culture. 48 h old cultures of lymphocytes were treated with 3ml of PFE individually and in binary mixtures and incubated for 24 hours. The dividing cells in 60ml of 0.4 mg/ml working solution were arrested at the metaphase stage by adding 0.05 ml of colchicine solution (0.01 per cent) at 30 minutes before harvesting the culture. 6 ml of pre-warmed hypotonic solution (0.075 m kcl) was added to the test tube after disturbing the pellet obtained after centrifugation at 1000 rpm for 20 minutes was discarded and incubated for 7 minutes1 ml of freshly prepared fixative (methanol and glacial acetic acid (3:1 v/v) was added and centrifuged at 1000 rpm for 10 minutes and two or three changes of the fixative were given to obtain a colourless cell pellet.

A test slide was prepared by placing a drop of the cell suspension on a clean chilled slide and dried immediately at 40°c for a few seconds on a hot plate. The slide was examined under a microscope to see whether the concentration of cells and the spread of the chromosomes enabled detailed examination of metaphases. The rest of the slides were prepared after making suitable dilutions of the cell suspension with fresh fixative. Fifty well spread metaphase plates of each subject were screened under oil immersion lens of the optical microscope and selected metaphases were photographed. The antigenotoxic efficacy of the pomegranate fruit extract was determined by applying ANOVA and the association between total chromosomal alteration and breast cancer risk was analysed using students 't' test.

Results

Extent of DNA Damage

The results pertaining to extent of DNA damage in the peripheral blood

phocytes of breast cancer patients undergoing different modalities of treatments presented under the following headings.

udy Population Profile

The patients were recruited from Valavadi Narayanaswamy Cancer Centre and Kuppusamy Naidu Memorial Hospital, Coimbatore from 2007 to 2009. The clinical ta of the cases and controls are summarized in Table 17.1.

ble 17.1: Clinical Data of the Cases and Controls Studied

Treatment	*Age*	*Sex*	*Cycle*	*Metastasis*	*Parity*
emotherapy (n=12)	39	F	6	–	2
	40	F	5	–	2
	40	F	2	–	2
	42	F	3	Lung Secondaries	2
	53	F	5	–	4
	55	F	2	–	2
	55	F	1	–	4
	56	F	3		2
	57	F	1		2
	57	F	3		2
	60	F	3		2
	66	F	2	–	4
Chemotherapy + Surgery)	33	F	6	–	2
(n=10	35	F	1	–	3
	35	F	2		2
	39	F	6	–	2
	40	F	1		2
	45	F	2		3
	46	F	6		2
	49	F	5		3
	50	F	2		2
	50	F	1	–	–
Chemotherapy +	50	F	2	Lung Secondaries	2
Radiotherapy (n=8)	53	F	1		2
	55	F	2		2
	55	F	5	–	2
	55	F	4	–	2
	55	F	1	–	–
	57	F	4	–	–
	65	F	1	Bone metastasis	2

Contd...

Table 17.1–*Contd...*

Treatment	*Age*	*Sex*	*Cycle*	*Metastasis*	*Parity*
Chemotherapy + Surgery+	38	F	6		2
Radiotherapy (n=10)	43	F	6		2
	46	F	8		–
	47	F	5		2
	48	F	6		2
	52	F	6		2
	53	F	4	Bone metastasis	2
	60	F	6		3
	65	F	6		–
	70	F	6	Lung Secondaries	2
Controls (n=20)	38	F	–	–	2
	33	F	–	–	2
	30	F	–	–	2
	32	F	–	–	1
	37	F	–	–	2
	50	F	–	–	2
	33	F	–	–	–
	30	F	–	–	2
	40	F	–	–	1
	35	F	–	–	2
	45	F	–	–	2
	40	F	–	–	2
	40	F	–	–	3
	50	F	–	–	2
	58	F	–	–	2
	45	F	–	–	–
	40	F	–	–	4
	38	F	–	–	2
	42	F	–	–	3
	39	F	–	–	2

Profile of Breast Cancer Patients

Altogether 40 breast cancer patients were studied for their profile from the data obtained through a questionnaire. The age group of patients varied from early 30 to 70 years, so that almost the population at high risk of onset of neoplasm was included in the study. All the patients in this study were found to have stage III of cancer according to Tumor Nodal Metastasis values; this may be attributed to less awareness regarding the symptoms of malignancy.

At the time of blood collection, the patients undergo different modes of treatments as suggested by their oncologists (Table 17.1). Out of four modes of therapies categorized, there were 12 patients undergoing chemotherapy alone, patients undergoing chemotherapy after surgery (n=10), patients undergoing chemotherapy and radiotherapy together (n=8) and the patients undergoing the chemotherapy, surgery and radiotherapy in combination (n=10). Out of 40 patients, five patients were found to have distant metastasis, two patients had lung metastasis and other three had bone metastasis.

The control samples collected from the same age groups as the patients. None of the controls (n=20) were having any malignancy. Out of the 20, two were found to be nulliparous.

DNA Damage Studies

Data pertaining to comet parameters obtained from blood lymphocytes of breast cancer patients who underwent different modalities of treatments is presented in Table 17.2.

Tail Length

The advanced breast cancer cases that underwent chemotherapy alone showed longest tail length (22.15 μm) which was followed by the cases who underwent chemotherapy + surgery (17.09 μm) and chemotherapy + radiotherapy (14.60μm). The cases who underwent all the three treatments showed significantly reduced tail length (11.08 μm), while the length was found to be minimum in controls.

Number of Damaged Cells

The data on number of damaged cells per 50 cells counted with respect to different treatments showed statistically significant (p=0.05) results. The data indicated maximum values in the patients who underwent chemotherapy alone (41) and chemotherapy + radiotherapy (40.27) which were on par with each other. In control sample, the damaged cell per 50 cells counted was found to be minimum (5.63).

Head-Tail Ratio

Though there was marginal difference among the treatments and controls, the statistical analysis of the data was found to be non significant.

Primary Damages

Interestingly, the number of cells with minimal damages *i.e.*, primary damages were found to increase in the patients with all the three therapies together (13.50/50 cells counted) followed by the patients undergoing radiotherapy and chemotherapy (12.75/50 cells counted)

The cases undergoing chemotherapy (8/50 cells) and chemotherapy + surgery (7.9/50 cells) was almost equal in values showing minimum cells with primary damages, although controls were observed to have a less number of primary damaged cells.

Table 17.2: Extent of Damage to Multi Modalities of Treatment with Reference to Selected Comet Parameters

Treatment	*Tail Length (μm)*	*No. of Damaged Cells/ 50 cells*	*Head/ Tail Ratio*	*Primary Damage/ 50 Cells*	*Secondary Damage/ 50 Cells*	*Tertiary Damage/ 50 Cells*
Chemotherapy alone	22.15	41	0.05	8	22.75	10.13
Chemotherapy + Surgery	17.09	38.87	1.12	7.9	23.63	7.25
Chemotherapy + Radio therapy	14.60	40.27	1.19	12.75	22.63	4.75
Chemotherapy + Surgery + Radio therapy	11.08	34.5	1.39	13.50	17	4.13
Controls	01.49	5.63	1.49	4.13	1.88	0.25
F value	8.473**	19.86 **	1.509^{NS}	5.85**	7.729**	6.735 **
CD	6.09	8.12	0.046	4.49	7.0	3.28
S.E	2.124	1.26	–	1.323	2.636	1.135

**: p = 0.01; NS: Non Significant.

Secondary Damages

When compared with the number of secondary damaged cells per 50 cells counted for each sample in different treatment options, the cases showed a minimum number of secondary damage cells (17/50 cells) who were in the category with all the three treatments (chemotherapy +surgery + radiotherapy).

Tertiary Damages

Statistical analysis revealed that the number of tertiary damaged cells was found to increase in patients with chemotherapy alone (10.13) followed by chemotherapy + surgery (7.25). The tertiary damaged cells decreased significantly in cases with chemotherapy + surgery + radiotherapy (4.13/50 cells), which was on par with the other treatment option *viz.*, chemotherapy + radiotherapy (4.75/50cells) showing a marginal difference, while the controls were found to have a least value of 0.25/50 cells.

Correlation Analysis

Correlation analysis was carried out for finding the relation between DNA damage parameters and age of the patients Table 17.3. A positive correlation was found when the age of the patient was correlated with the selected comet parameters, namely the tail length, head - tail ratio, number of damaged cells and secondary damages per 50 cells counted (r = 0.012, 0.014, 0.10 and 0.197 respectively). The results were showing an increase in the DNA damage parameters with an increasing age. Correlation analysis was done for finding the relation between DNA damage parameters and cycles of chemotherapy the patient underwent. Table 17.4 shows a negative correlation between cycles of chemotherapy and comet parameters, namely the tail length, number of damaged cells, secondary damages and tertiary damages (r = -0.11, -0.088, -0.27 and -0.066 respectively) revealing that as the cycles of chemotherapy increases during the treatment, DNA damage in the lymphocytes decrease simultaneously

Table 17.3: Correlation between Age of the Patients and Extent of DNA Damage

Parameters	*r Value*
Tail length (μm)	+ 0.012
No. of damaged cells/50 cells	+ 0.10
Head - tail ratio	+ 0.014
Primary damage	– 0.326
Secondary damage	+ 0.197
Tertiary damage	0.064

Antigenotoxic Efficacy of PFE

The antigenotoxic potential of PFE (Figure 17.1) in the cultured human lymphocytes of breast cancer patients using chromosome aberration assay has been portrayed below. A total of ten blood samples were collected from breast cancer

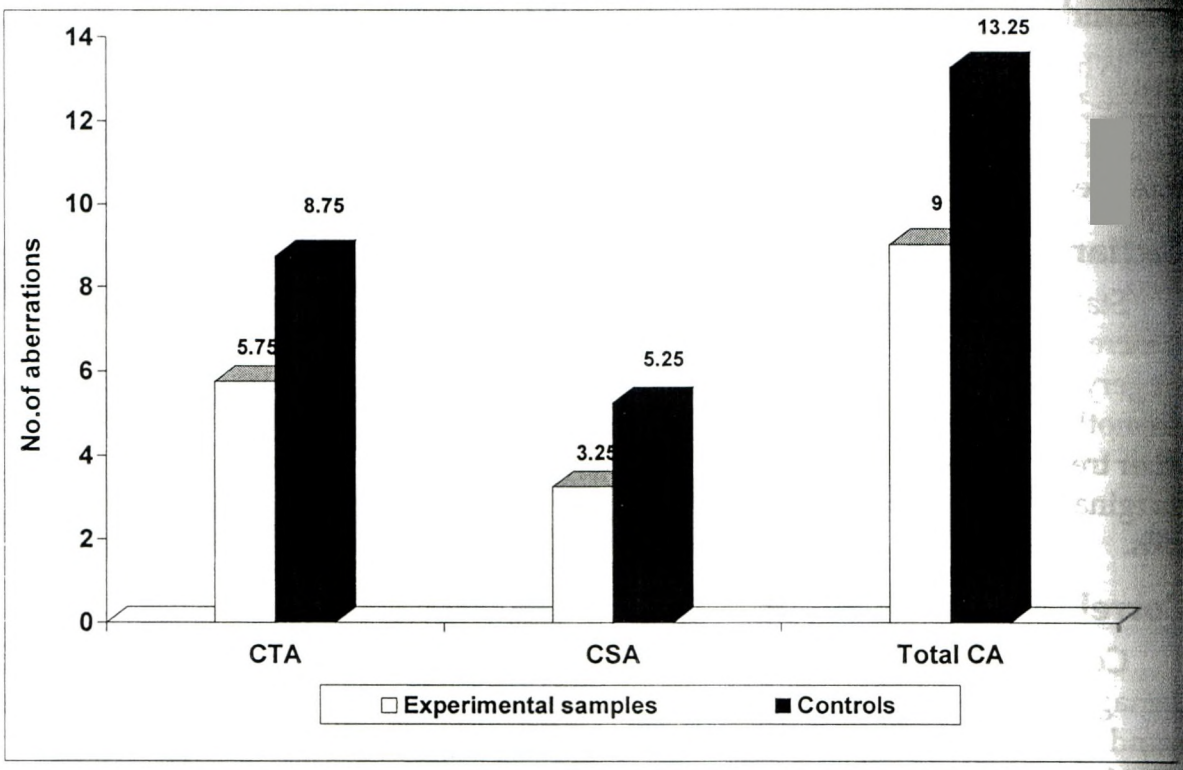

Figure 17.1: Antigenotoxic Potential of Methanol Extract of *Punica granatum* in the Cultured Lymphocytes of Breast Cancer Patient.

patients. Sample treated with PFE were taken as experimental samples and untreated samples were considered as control group of samples. Table 17.5 depicts the profile of the experimental and control group of samples, including age of the patients, stage and chromosomal alteration. Age of the patients ranged from 30 to 70 years. Samples collected were with different stages of disease (breast cancer) namely stage I, stage II, stage III and stage IV. The total chromosomal alteration was found to be higher when the stage of cancer increased. The percentage inhibition of chromosomal alteration after the addition of methanol, chloroform, petroleum ether and water extracts of *Punica granatum* was found to be 20 - 40 per cent.

Table 17.4: Correlation between Cycles of Chemotherapy and Extent of DNA Damage

Parameters	*r Value*
Tail length (μm)	– 0.112
No. of damaged cells/50 cells	– 0.088
Head - tail ratio	+ 0.060
Primary damage	+ 0.327
Secondary damage	– 0.27
Tertiary damage	– 0.066

Treatments with methanol extract inhibited (Table 17.6) chromosomal alteration to 34.31± 4.81 followed by treatment with chloroform extract (5.3±2.02). Treatment with petroleum ether extract showed poor inhibition (2.92±1.03). Treatment with

water extract showed no inhibition in the chromosomal alteration. Of the four treatments, maximum reduction in chromosomal alteration was found in the methanol extract (p=0.05).

Table 17.5: Chromosomal Aberration in Peripheral Blood Lymphocyte Culture Treated with Extract of PFE

Sl.No.	*Age*	*Stage*	*CA of Experimental Samples**		*Total CA of Experimental Samples**	*CA of control Samples*		*Total CA of Control Samples*
			CTA	*CSA*		*CTA*	*CSA*	
1.	37	I	1	0	1	3	1	4
2.	43	II	2	0	2	4	0	4
3.	60	III	4	2	6	6	3	9
4.	53	II	2	1	3	3	2	5
5.	49	I	1	1	2	2	1	3
6.	52	II	3	2	5	3	3	6
7.	51	I	1	0	1	2	1	3
8.	63	IV	5	4	9	7	5	12
9.	56	I	2	1	3	3	2	5
10.	48	II	2	2	4	2	3	5

* Breast cancer samples treated with PFE (Experimental group of samples; TA: Chromatid type of aberration; CSA: Chromosome type of aberration; CA: Chromosomal alteration; Stage: Stage of the disease.

Table 17.6: Frequency of Inhibition of Chromosomal Alteration in Human Lymphocytes of Breast Cancer Patients Treated *in vitro* with *Punica granatum* Extracts

Name of the Extracts	*Frequency of Inhibition (per cent)*
Methanol	34.31±4.81(80.71)
Chloroform	5.3±2.02(12.5)
Petroleum ether	2.9±1.03(6.82)
Water	0±0(0)
SE	0.2039
F	4.006*
CD	0.7116

* Significant at p=0.05.

Table 17.7 illustrates the reduction in CTA, CSA and total CA in the experimental samples treated with methanolic extract of pomegranate arils. Reduction in the number of CTA in the experimental samples was found to be 5.75±1.5 than in the control samples (8.75±3.59). The result was found to be statistically significant at p=0.01. Correspondingly CSA was found to reduce significantly in the experimental samples

treated with methanolic extract (3.25±1.5) than in control samples (5.25±2.06). Alike CTA and CSA, the total chromosomal alteration (CA) was found to decrease in the experimental samples (9±2.45) when compared to that of control samples (13.25 ± 3.40) (p=0.01).

Table 17.7: Effect of Methanol Extract of *Punica granatum* in the Cultured Lymphocytes of Breast Cancer Patient.

	CTA	*CSA*	*Total CA*
Experimental samples	5.75±1.5**	3.25±1.5**	9±2.45
Control	8.75±3.59	5.25±2.06	13.25±3.40

** Significant at 1 per cent level; P = 0.01 per cent.

Discussion

Medicinal plant exerts their chemopreventive potential by interfering with covalent interaction of a carcinogen with DNA, modifying DNA repair process, antioxidant properties and preventing cellular proliferation. Profound evidence has revealed that medicinal plants can reduce genetic damages induced by mutagens and carcinogens (Premkumar *et al.*, 2004). The modulatory effect of natural compounds on the chromosomal aberrations induced by various kinds of chemicals and drugs is well established (Bhattacharya *et al.*, 2004; Siddique and Afzal, 2005; Dutta *et al.*, 2007).

In human cells, both normal metabolic activities and environmental factors such as UV light and radiation can cause DNA damage, resulting in as many as 1 million individual molecular lesions per cell per day (Lodish *et al.*, 2004). Many of these lesions cause structural damage to the DNA molecule and can alter or eliminate the cell's ability to transcribe the gene that the affected DNA encodes. Other lesions induce potentially harmful mutations in the cell's genome, which affect the survival of its daughter cells after it undergoes mitosis. Consequently, the DNA repair process is constantly active, as it responds to damage in the DNA structure. When normal repair processes fail and when cellular apoptosis does not occur, irreparable DNA damage may occur, including double strand breaks and DNA cross linkages (Acharya, 1971; Bjorksten *et al.*, 1971).

DNA damage and defective DNA repair are the underlying molecular events driving the initiation and progression of cancer. In the last few years, the alkaline comet assay has gained increased application in clinical medicine. The comet assay is now widely accepted as a standard method for assessing DNA damage in individual cells. It has been used in a broad variety of applications including human bio-monitoring, genotoxicology, ecological monitoring and as a tool to investigate DNA damage and repair in different cell types in response to a range of DNA damaging agents (Collins, 2004).

Apart from the beneficial actions of chemotherapy, the adverse consequences of its action on normal tissues are well known as antitumour drugs do not selectively damage tumour cell DNA (Bignami *et al.*, 2003) alone. Following *in vivo* exposure to

anti-neoplastic drugs, diverse lesions in DNA are induced. The type of DNA damage produced by antineoplastic agents depends on the stage of cell cycle at the time of treatment. The majority of lesions occur during the DNA synthesis (S) phase, often due to misreplication. Both neoplastic and non-neoplastic cells attempt to repair them. However, if unrepaired, DNA lesions may give rise to chromatid - type aberrations during S-phase and interfere with the transcription and replication of DNA, resulting in cytotoxic and mutagenic effects (Saito, 1993; Colombo *et al.*, 2001).

In the present study, the alkaline comet assay, as a biomarker was employed to assess the DNA damage in peripheral blood lymphocytes of breast cancer patients undergoing different modalities of treatment. Of all the modalities used, chemotherapy recorded the maximum damage with regard to comet parameters *viz.*, tail length, number of damaged cells, head/tail ratio, primary damage and tertiary damage. A similar finding was reported by other authors who studied DNA damage in lymphocytes from breast cancer patients undergoing chemotherapy with cyclophosphamide and other alkylating agents (Hellman *et al.*, 1995; Hartley *et al.*, 1999).

Kopjar *et al.* (2007) studied chemotherapy- induced DNA damage in non-target cells using alkaline comet assay in breast cancer patients and confirmed the efficacy of alkaline comet assay as a sensitive technique to detect significantly elevated DNA migration in blood cells of patients who underwent the first cycle of chemotherapy. The above study corroborates with the present study as comet assay was able to detect significantly maximum damage with regard to comet parameters in patients who underwent different cycles of chemotherapy. The present study also falls in line with the observation of Suarez *et al.* (2008), who studied the DNA damage in peripheral blood lymphocytes in patients undergoing combined chemotherapy for breast cancer and observed a significant increase in DNA damage after 2nd cycle of chemotherapy, which persisted until the end of treatment. Similar findings were observed by Haldan *et al.* (2002) who reported severe degree of damages of DNA at the relapsed stage of post chemotherapy induced patients when little or no immature cells are seen in peripheral blood and bone marrow.

Alford supported the fact that there is harm to DNA by chemotherapy practices alone and suggested the most common agents used for head and neck cancers *viz.*, cirplastin, 5-flucorocracil, bleomycin and methodtrevate have preference of uptake in rapidly dividing cells and have various modes of tumoricidal actions including inhibition of DNA synthesis, formation of free radicals which causes breaks in DNA strands and prevention of repair of sub-lethal damage to DNA.

In the present study, the number of damaged cells, an important comet parameter was found to be high in patients who underwent chemotherapy alone, followed by the patients who underwent chemotherapy + radiotherapy. Similarly Nascimento *et al.* (2001) carried out a study on evaluation of radio induced damage and repair capacity in blood lymphocytes of breast cancer patients and found that basal level of damage and the radio-induced damage were higher in lymphocytes from breast cancer patients than in the lymphocytes from healthy donors. Few more studies (Riguad *et al.*, 1990; Parshad *et al.*, 1991) recorded high sensitivity and a reduced

repair capacity in blood lymphocytes from breast cancer patients when exposed to X-rays, gamma rays and UV lights as evaluated by the determination of chromosome aberrations. Moreover, Bekum and Broerse (1991) examined that ionizing radiation is an etiological agent known to act on the induction of breast cancer but on the other hand, it is a therapeutic modality used in cancer treatment. Durel *et al.* (2004) examined genomic instability in breast cancer patients who underwent surgery before any radiation or chemotherapy as evaluated by comet assay. Similarly in the present study, the patients who underwent chemotherapy + surgery showed higher values for tail length and number of damage/50 cells when compared to the other two combined modalities *viz.*, chemotherapy + radiotherapy and chemotherapy + surgery + radiotherapy.

Inspite of many biomarkers available for the assessment of genetic DNA damage and cancer risks, in the present investigation the alkaline comet assay was used to examine the levels of DNA damage in peripheral blood lymphocytes of breast cancer patients who underwent different modalities of treatments. Based on the observation of other authors (Vaghef *et al.*, 1997; Olinski *et al.*, 1997), this method could efficiently detect the harmful effects of chemotherapy and radiotherapy induced DNA damage.

In the present study an attempt was made to compare the DNA damage profile with age of the patients irrespective of the cycles of chemotherapy. It was found that DNA damage is more in older age patients as compared with younger age patients. The above findings corroborate with the study of Deffaud *et al.* (1997) and Peace and Succop (1999) who observed an increase in the frequency of affected cells with ageing process such as altered cellular metabolism and decrease in the efficiency of DNA repair. An American study by Singh *et al.* (1999) detected 12 per cent increase in the basal level of DNA damage in individuals of age above 60 years compared with individuals of below 60 years, which could be ascribed to a 5-fold higher content of highly damaged cells among older individuals. The results of the present finding throws light on the efficacy of comet assay for the detection of DNA damage in the patients undergoing different modalities of treatment.

Antigenotoxic Efficacy of PFE

The pomegranate juice and extracts have been shown to have potent *in vitro* antioxidant properties (Gil *et al.*, 2000; Kulkarni *et al.*, 2007) attributed to its high content of polyphenols including Ellagitannins (ETs) and Ellagic acid (EA). Recently, there are numerous reports on the *in vitro* and *in vivo* anti-cancer properties of pomegranates (Kim *et al.*, 2002; Albrecht *et al.*, 2004; Kohno *et al.*, 2004). Peripheral blood lymphocytes are extensively used in biomonitoring of populations exposed to various mutagenic or carcinogenic compounds. This is because of the ease of sampling, the possibility of obtaining large number of scorable cells and the documented sensitivity of this system in detecting chromosome damage induced by exposure (Heim and Mitelman, 1987).

According to Sowjanya *et al.* (2009) the chromosomal alteration analysis is one of the widely used parameters for testing the protective effects of natural compounds on the drug and chemical induced toxicity. The conceptual basis for using chromosomal aberrations in peripheral blood lymphocytes as a biomarker is the fact

its bioactive compounds was responsible for the decreased chromosomal alteration in blood lymphocyte culture of the breast cancer patients, proving the synergistic effect of all biocompounds present in the extract rather than single compound like ellagic acid. The present study on goes with Kim *et al.* (2002) who observed that the pomegranate extracts and polyphenols have selective effects against breast cancer. From their results, it was clear that both aqueous and oil fractions of pomegranate fruit extract has multiple *in vitro* suppressive effects on human breast cancer cells. Settheetham and Ishida (1995) in their study found that apoptosis, an early response to cell death is a useful marker for predicting tumour response after anticancer treatment. Aqueous pomegranate peel extract resulted in apoptotic DNA fragmentation and suppression of growth in two human Burkitt's lymphoma cell lines, Raji and P3HR-1.

Antiproliferation effect of ellagic acid has been studied in many kind of cancer cell lines although the exact mechanism has not been well investigated (Seeram *et al.*, 2005). Many investigators (Li *et al.*, 2005; Larrosa *et al.*, 2006) reported that ellagic acid represents the anti-proliferative activity through the induction of cell cycle arrest, apoptosis or antioxidant effects. In the present investigation, the PFE was shown to decrease the chromosomal aberration like CSA and CTA in the peripheral blood lymphocytes of breast cancer patients. Further studies may be carried out to explore the exact mechanism of action.

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