I. Introduction

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 war against cancer is far from over. The post genomic era has now opened new avenues in cancer treatment, which is contemplated to be more effective and specific for tumour cells. The sequencing of the human genome is likely to speed up the identification of factors involved in cancer pathogenesis and lead to an age of individually tailored anticancer drug therapy. However, about half of the global cancer burden is carried by developing countries that ironically have access to only 5% of the resources available to fight the disease (Pal et al., 2003).

More than one million new patients suffer from breast cancer annually in the world. In developed countries, breast cancer is the most common malignancy diagnosed among women and in developing regions, it ranks second to cervical cancer (Michael and Jemal, 2003). Among American women, breast cancer represents 32% of all new patients of cancer and is the second leading cause of cancer deaths (15%) after lung cancer (Zeleniuch and Roy, 2005). It is estimated that 211,240 patients suffer from invasive breast cancer in a year in the United States. These numbers represent a sharp increase over past 30 years (Hadjiiski et al., 2006). In developed countries the age standardized incidence rates are around 100/100,000 women with mortality rate about 25/100,000. These rates are upto five fold higher than that reported from Asian regions, which have the lowest incidence of breast cancer. Breast cancer incidence is low in India compared with high income countries, but it has increased in recent decades particularly among urban women (Gajalakshmi et al., 2009). 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% from 1985 to 2001, representing a 57% rise in India’s cancer burden (Yip et al., 2006 and Farooq and Coleman, 2006).

The cancer registries also suggest that age - standardized incidence rate are rising even more rapidly in low incidence regime such as Africa and Asia. Probably the socio-economic and life style changes (e.g, late child bearing and dietary changes) and
associated changes in menstrual patterns are responsible for the rise in developing countries. At the same time, improved life expectancy will increase the burden of breast cancer in developing countries as more older women are likely to develop breast cancer than younger women (Parmar and Badwe, 2004 and Althuis et al., 2005).

Breast cancer is rarely found before the age of 25 years. Thereafter, the incidence is reported to increases with age until menopause when the rate of increase is less pronounced. About three quarters of diagnosed cases are in postmenopausal women. Despite extensive research to find the cause of breast cancer, the etiology is largely undetermined (Lacey et al., 2009 and Marsden, 2003).

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). Trends in breast and cervix cancer in six population based cancer registries were evaluated over the last two decades. This approach showed a decreasing trend for cancer of the cervix and increasing trends for cancers of breast throughout the entire period of observation in most of the registries (Yeole, 2008).

Geographical variation in incidence of breast cancer can be attributed to exposure to various risk factors. Among these, the high risk factor are: diet, early menarche, late menopause, late first full term pregnancy, nulliparity, no breast feeding and family history of breast cancer in two or more first degree relatives. The minor risk factors are: obesity in postmenopausal women, hormone replacement therapy, smoking, exposure to low dose radiation and excessive alcohol intake. The present study attempts to find out some of the risk factors of breast cancer among patients in western Tamilnadu covering Coimbatore, Erode, Karur and Salem attending Valavadi Narayanasamy Cancer Centre, G. Kuppusamy Naidu Memorial Hospital in Coimbatore.

Cell biologic 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. For example, direct DNA damage, aberration in DNA methylation, mutation or loss of expression of BRCA 1/2 or P53 genes, deregulated expression of C-Myc or cyclin D1 genes and defects in DNA mismatch repair genes may all cause defects in DNA damage dependent cell-cycle check point controls contributing to onset or progression or both of breast cancer.

There are many methods available for the assessment of DNA damage and repair. Up to now the most commonly used are, the bacterial Ames test, the scoring of chromosome aberration (CA), micronuclei and sister chromatid exchange (SCE) in proliferating cell populations. These methods have been and remain very useful but nevertheless they have a number of important shortcomings, among them is the requirement for proliferating cell populations (Giri et al., 1999).

In view of the above shortcomings and because of the need for more rapid short-term screening tests, further tests were developed in recent years and others are still in development. Single cell gel electrophoresis assay or the so called comet assay is one of the recent advent and considered a very important alternative for the cytogenetic tests. It does not have the above shortcomings and is much less labour intensive, more rapid and less expensive and results can be obtained within a relatively short time after sampling (i.e, within a few hours).

The SCGE / comet assay allows the detection of DNA alteration of diverse kinds such as double strand breaks, single strand breaks, alkali-labile sites, incomplete repair sites and cross links (Tice et al., 2000). In the SCGE assay, cells are embedded in agarose, lysed and electrophoresed under low voltage, so that fragmented and related DNA migrate farther than intact or cross-linked DNA, resembling the image of a comet. The extent of migration of the “tail” of the comet is related to increased DNA damage. These images can be analysed and compared in a cell to cell basis.

At present a number of options are present to treat cancers. Surgery, radiation therapy and chemotherapy are the established therapies. However, hormone and biological therapies are also in use. The doctor may use one of these methods or a combination of methods for treatment. The aim of cancer treatment is to cure the patient and save life. The cases where complete cure is not possible, treatment aims to control the disease and to 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. Hence in the present study, the extent of DNA damage in blood lymphocytes of patients undergoing various modalities of treatment viz., chemotherapy, radiotherapy, surgery or combination of these therapies were assessed.

Pomegranate (*Punica granatum*), a small tree originating in the orient belongs to the family Puniaceae. Pomegranate also known as Anar has been grown in India since ancient periods. This fruit crop is suitable for arid and semi arid regions due to versatile adaptability, hardy nature, low maintenance cost and high yield (Bankar and Prasad, 1992). Pomegranate is grown mainly in Iran, India and USA and also in most near and far east countries. 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). According to trade, the annual production of pomegranate in the country is 4.0 - 4.5 lakh tones.

Chemistry of fractions from the peels shows the presence of acids such as citric, malic, lactic, fumaric, tartaric and even acetic (Malgarejo, 2000). The juice also contains numerous pharmacologically more active phenolic compounds most of which occur in even higher concentrations in the peels (Artik, 1998). These include flavonoids such as quinic acid, gallic acid, chlorogenic acid, caffeic acid, ferulic acid and also ellagittannins such as ellagic acid and punicalagin (Gil et al., 2000). The only phenolic compound unique in the juice and not in the peel is anthocyanin such as delphidin. Further proanthocyanidins, specifically prodelphidins have been discovered in the peels, along with gallocatechins and all of which are strongly antioxidants (Plum et al., 2002). Thus for all intents and purposes, the chemistries 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 these compounds are potent antioxidants.

Oxidation is a metabolic process that leads to energy production necessary for essential cell activities. However, metabolism of oxygen in living cells also leads to the unavoidable production of oxygen-derived free radicals, commonly known as Reactive Oxygen Species (ROS) (McCord, 1994 and Adegoke, 1998) which are involved in the onset of much disease (Ames et al., 1993). These free radicals attack the unsaturated
fatty acids of biomembranes which results in lipid peroxidation and the destruction of proteins and DNA, which cause a series of deteriorative changes in the biological systems leading to cell inactivation. Thus the identification of antioxidants, which can retard the process of lipid peroxidation by blocking the generation of free radical chain reaction, has gained importance in recent years.

Pomegranate juice and extracts have been shown to have potent \textit{in vitro} antioxidant properties, attributed to its high content of polyphenols including elagitannins and ellagic acid (Mi \textit{et al.}, 2000; Kulkarni \textit{et al.}, 2005). Recently, there have also been numerous reports on the \textit{in vitro} and \textit{in vivo} anti cancer properties of pomegranates (Kim \textit{et al.}, 2002; Albrecht \textit{et al.}, 2004; Kawai and Lansky, 2004; Hora \textit{et al.}, 2003 and Kohno \textit{et al.}, 2004). The major pomegranate elagitannin, punicalagin, is reported as the active ingredient responsible for >50% of the juices antioxidative potential (Mi \textit{et al.}, 2000). Ellagic acid is active in antimutagenesis assays and has been shown to inhibit chemically induced cancer in the lung, liver, skin and oesophagus of rodents (Stoner \textit{et al.}, 1995). Dietary ellagic acid has been shown to reduce the incidence of N-2-fluorenylacacetamide induced hepatocarcinogenesis in rat and N-nitrosomethylbenzylamine induced rat oesophageal tumours (Ahn, \textit{et al.}, 1996).

Oxidative damage to DNA promotes mutation and thus enhancing the risk of carcinogenesis. Damage to DNA is likely to be a major cause of cancer and other diseases (Ames, 1971). The Salmonella microsome assay (Ames \textit{et al.}, 1975) along with other short term assays always is being extensively used to survey a variety of substances in our environment for antimutagenic effect. Homogenates of rat liver (or other mammalian tissue) is added to the bacterial suspension as an approximation of mammalian metabolism. Using this system several antimutagens were detected. Hence the present investigation has been conducted to evaluate the antimutagenic activity of pomegranate seed and peel extracts using Salmonella reverse mutation assay.

Human lymphocytes in culture constitute an ideal test system to evaluate the cytogenetic damage induced by environmental factors (Rossner \textit{et al.}, 2005). The use of chromosomal alterations as markers of early biological effects is well established in genotoxicity studies.

A relationship between chromosomal damage and cancer development has been suggested since the beginning of the 20\textsuperscript{th} century, but only since 1960 have 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. The idea of causal association between chromosomal alterations and cancer risks based on the concept that genetic damage in lymphocytes reflects similar damage in cells undergoing carcinogenesis.

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. Chromosomal Aberrations (CA) include chromosomal breaks and exchanges visible in arrested metaphase stage cells and are usually divided into Chromosome Type Aberrations (CSAs) and Chromatid Type Aberrations (CTAs), which differ from each other morphologically. Chromosome type aberrations involve the same locus on both sister chromatids on one or multiple chromosomes whereas CTAs affect one or several sister chromatids of a chromosome or several chromosomes. 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. In the present study, the reduction in chromosomal damage by the addition of pomegranate extracts to peripheral blood lymphocyte culture is taken 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.

In view of the above, the present investigation was undertaken with the following objectives:

- To study the epidemiology of breast cancer among patients in western Tamilnadu covering Coimbatore, Erode, Karur and Salem attending Valavadi Narayanasamy Cancer Centre, G.Kuppusamy Naidu Memorial Hospital, Coimbatore.

- 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.
To evaluate the antimutagenic effect of petroleum ether, chloroform, methanol and water extract of pomegranate peel and seed in Ames Salmonella reverse mutation assay.

To carry out the chromosomal aberration studies using Pomegranate Fruit Extract (PFE) in the peripheral blood lymphocytes of breast cancer patients.

II. Review of literature

The review of literature pertaining to the topic “Assessment of antigenotoxic potential of Punica granatum in the reversal of DNA damage in breast cancer patients” is presented in the following headings.

2.1. Epidemiology of breast cancer

Many studies in the literature have reported that breast cancer is related to the reproductive life of women; such as early menarche, late menopause, nulliparity, late age at first child birth, diet, physical exercise and hormone usage (Ozmen, 2008 and Kuru et al., 2002).

Henderson (1993) and Kelsey (1993) in their study stated that epidemiological risk factors for breast cancer, aside from socio-demographic characteristics such as age, socio-economic level, occupation and marital status include: life style factors such as diet, obesity and sedentary life, genetic factors such as family history of breast cancer and hormonal factors such as early menstruation and late menopause, nulliparity, pregnancy at an advanced age and use of oral contraceptives. Numerous epidemiological studies on risk factors of breast cancer have produced evidence on international variations. Therefore, the epidemiological factors for breast cancer are reviewed under the following headings.

2.1.1. Age

Jemal et al. (2003) highlighted that breast cancer is the most common type of cancer in women and increases in incidence with advancing age. Peace and Succop (1999) observed an increase in the frequency of affected cells with ageing which could
be attributed to the ageing process, such as altered cellular metabolism.

Ferlay et al. (2004) from their study pointed out that ageing is one of the single greatest risk factor for the development of new breast cancer. In the reports of American Cancer Society (2005), it is stated that breast cancer incidence has a distinctive age-specific curve, with an estimated 64% of women over the age of 55 at the time of breast cancer diagnosis. In the USA, the estimated risk of new breast cancer is 1 in 13 for women aged 60 to 79, compared with 1 in 24 for women aged 40 to 59 and 1 in 229 for women aged 39 and younger (American Cancer Society, 2005). According to Honung (2003), the incidence of breast cancer increases from 4/1000 for women aged from 30-39 to 15/1000 in the age group 40-49, 28/1000 in the age group 50-59 and 36/1000 in the age group 60-69.

The risk of second primary breast cancer seemed to be higher for women who were under age 45 at the time of their initial breast cancer diagnosis than it was for older women (Li et al., 2003). Camplejohn et al. (2006) carried out studies on heritability of DNA-damage induced apoptosis and its relationship with age in lymphocytes from female twins and found that, in samples aged 18 - 80 years, apoptotic response of lymphoid cells to DNA damage was significantly reduced with increasing age. Oliwenstein (2005) reported that there are certain cancers, in particular that are linked to ageing. These include colon, rectal, prostate, lung, bladder, stomach and breast cancer. Fenech et al. (1999) reiterated the belief in the link between age related genetic damage and cancer.

Anderson et al. (2006) identified a unique oxidation-induced lesion in the DNA of breast epithelium, myoepithelium and stoma and found that the highest concentrations of this lesion tended to occur in women in 33 to 46 years age group, a bracket that corresponds to a known rise in the incidence of breast cancer. Malins et al. (2006) studied age related concentrations of two similar mutagenic DNA lesions and demonstrated that their occurrence roughly commensurate with the age at which the incidence of female breast cancer rises. Singh et al. (1991) in a study detected a 12% increase in basal level of DNA damage among individuals > 60 years compared with individual < 60 years, which could be ascribed to a 5-fold higher content of highly damaged cells among older individuals. Singh et al. (1990) observed that cells from older individuals have less resistance to DNA damage by ex vivo X-ray.
exposure.

2.1.2. Diet

Giovannucci (1999) stated that, diet is a major environmental risk factor in the prevention and treatment of cancer. Ip (1990) opined that calorie restriction inhibited tumour development because fat intake is highly correlated with energy intake in epidemiological studies that assess associations between dietary fat intake and the risk of breast cancer.

Carroll (1975) and Rose et al. (1986) in different studies proposed a strong positive correlation between per capita fat consumption and mortality from breast cancer. Goodwin and Boyd (1987) reviewed the published results from 14 case - control studies that examined the relationship between the intake of total fat or fat containing foods and risk of breast cancer. Blount et al. (1997) and Jacob et al. (1998) reported a high correlation between dietary fat intake and breast cancer risk in humans. Rohan et al. (1987) noted that in humans, total calories could influence breast cancer risk through its effect on obesity and age at menarche. Potter (1996) suggested that persons interested in reducing their risk of cancer could be advised as a prudent measure to minimize their intake of food high in animal fat, particularly red meat, because this is likely to decrease the risk of prostate and colon cancers. Because milk contains IGF-1 (Insulin Growth Factor), which has an identical amino acid sequence to human IG7- 1 (Honeger and Humbel, 1986), it has been suggested that its consumption may be linked to breast cancer (Outwater et al., 1997 and Epstein, 2001).

Tannenbaum and Silverstone (1953) proved that diets high in fat could promote tumour growth in animal models. Freedman et al. (1990) and Welsch (1992) reported that dietary fat has a clear effect in tumour incidence in many models. Willett (2001) reported a strong correlation between per capita consumption of fat and breast cancer mortality in international comparison studies and animal experiment that showed a high fat diet increased the incidence of chemically induced mammary tumours.

Sonestedt et al. (2008) reported that a high consumption of omega - 6 - polyunsaturated fatty acids, which are found in most types of vegetable oil including
sunflower oil, may increase the likelihood that postmenopausal women may develop breast cancer. Trichopoulou et al. (1995) in their case control studies in Spain and Greece, found that women who used more olive oil had reduced risks of breast cancer. Furthermore Welsch (1992) reported that olive oil has been shown to be protective relative to other source of fats in some animal studies.

Krishnaswamy (1996) in his study stated that spices and condiments which are part of the Indian diet have chemical constituents which have antioxidant, antimutagenic and anticarcinogenic properties. Velie et al. (2005), Ronco et al. (2006) and Marchioni et al. (2007) in different studies proposed that traditional diets appear to be associated with a lower risk of cancer. Ronco et al. (2006) reported that traditional Uruguayan diets (cooked greens, beans and legumes, cabbage, fried fish and chicken) and low intakes of salad dressing diet were associated with a lower risk of breast cancer. As estimated by Mattison et al. (2004), a high intake of dietary fibres has been related to lower risks of breast cancer.

Wargovich (1999) and Wattemburg (1992), have conducted epidemiological and experimental studies and reported that cereals, vegetables, fruits and certain beverages contain a variety of potential cancer preventing substances. As estimated by Wargovich (1999), green leafy vegetables, beans of all varieties, cruciferous vegetables namely cabbage, brussel sprouts, cauliflower and broccoli are rich in anticarcinogens. Umbelliferae vegetables like carrots, celery, parsnip, alliums namely onions, garlic and chives, solanaceous vegetables like potatoes, tomato and brinjal have significant levels of cancer protecting non nutrients. Block et al. (1992) highlighted that all the citrus fruits, grapes, apples, strawberries, plums, pineapple and melons have high levels of protective phytochemicals.

According to Stover (2004), folate is the co-enzyme that carries one carbon unit and is thereby of great importance in the metabolism of aminoacids and nucleotides. The two main mechanisms that links folate deficiencies to cancer development are: a reduced synthesis of S-adenosyl methionine (SAM), which results in aberrations in DNA methylation and a reduced synthesis of the pyrimidine thymidylate which results in the misincorporation of uracil into DNA. Another possible mechanism is impaired purine synthesis and subsequent changes in DNA. High folate intake was shown to be associated with a decreased risk of postmenopausal breast cancer (Lajous et al., 2006
Ames (1983) reported that diet is not only a source of antimutagens/anticarcinogens but also source of mutagens. The carcinogens in the diet may be exogenous in origin or formed as a result of the interaction of components of foods endogenously e.g., heterocyclic amines.

2.1.3. Life style

It is estimated that around 75% of women with breast cancer malignancy have no established risk factors other than age and living in a western society (Fisher et al., 1998, Lopez oten and Diamandil 1998). As estimated by Kelsey (1993) and Kelsey (1996) in population based case-control study of women residing in Marin country, it was recognized that breast cancer risk factors were childhood and adolescent socio-economic factors.

Yaun et al. (1988) reported that women with a higher socio-economic level and more schooling have different life styles which include a diet rich in saturated fat, lower parity and older age at their first pregnancy. Braaten et al. (2004) stated that higher education in women is a conferred risk factor for breast cancer. According to Faggiano et al. (1994), education is a strong surrogate for socio-economic status. The quest for higher education delays age at marriage and age at first child birth which is also associated with reduced parity and these factors have been shown to increase breast cancer risk. Northern California Cancer Centre (1997) reported that the high incidence of breast cancer was associated with the socio demographic characteristics of the country and not with a geographically specific exposure. Prehn (1998) and Northern California Cancer Centre (1997) reported that women living in Marin country have a higher prevalence of some recognized breast cancer risk factors such as higher education and income as well as low and late parity.

According to Bernstein et al. (1992), physical activity is mean of reducing breast cancer risk because of its potential effects on hormone profiles and weight gain. Friendenreich (2001) found a relationship between physical activity and breast cancer risk and over the past decade, physically active women had lower breast cancer risk. Friedenreich et al. (2002) in their epidemiological study observed a reduced risk of breast cancer with increasing levels of physical activity.
Research on Cancer (2002) in its study on weight control and physical activity concluded that “convincing” evidence exists for an inverse association between breast cancer risk and physical activity. Brain et al. (2007) in their study observed a reduction in invasive breast cancer risk with increasing recreational physical activity. However, this reduction in risk was limited to women with no family history of breast cancer. According to Friedenreich (2001), reduction in risk associated with lifetime recreational physical activity, which was limited to women with no family history of breast cancer. Verloop et al. (2000) found that the inverse association between lifetime recreational physical activity and breast cancer risk was stronger in women with a family history of breast cancer. Carpenter et al. (2003) and Berstein et al. (2005) in their different studies found that the reduction in breast cancer risk associated with lifetime exercise was limited to women who had no family history of breast cancer. McTiernan (2003) assessed the effect of physical activity on estrogens and other hormone exposure and other biomarkers of risk would provide valuable insights on the mechanisms of physical activity in reducing breast cancer risk.

2.1.4. Anthropometric parameters

Overweight women might have an increased need for DNA repair in mammary epithelial cells because of an elevated proliferative activity caused by higher concentration of estrogen (McTiernan et al., 2003). Schapira et al. (1994) have reported the importance of central and upper body obesity as a risk factor for breast carcinoma.

Pathak et al. (1992) in an international study reported that in Asian population, both pre and postmenopausal obesity was associated with a higher risk of breast cancer. Vandeloo et al. (2007) in their study indicated that critical pathways for understanding the relation between obesity and breast cancer risk are likely to depend on two points in time: at menarche and menopause, time when some metabolic factors occur and women would experience some physiological (hormonal) changes in their body. Sieri et al. (2004) in their Italian cohort study in premenopausal and postmenopausal women reported lower risk of breast cancer with the consumption of raw vegetables and olive oil, particularly among normal weight (BMI <25) women. Hall et al. (2000) suggested that high current BMI has been linked to a reduced risk of breast cancer in premenopausal women or in women younger than 50-55 years. Hunter et al. (1993) have shown that the heavy body weight increased the risk of breast cancer in postmenopausal women but
decreased the risk in premenopausal women. According to Brandt (2000), weight control may reduce the risk of breast cancer among postmenopausal women. Parazzini et al. (1990) reported an inverse correlation of breast carcinoma risk and height in postmenopausal woman in Italy. As estimated by Michels (2004), height was found to be strong predictor of breast cancer risk with comparable effects in both premenopausal and postmenopausal women. The nurse’s health study also reported that the 60% greater risk for postmenopausal breast cancer associated with overweight and obesity, the study was limited to women who had never used hormone replacement therapy (Morimoto et al., 2002).

2.1.5. Hormonal factor

According to Kroman et al. (1998), the “estrogenic window” that is, early menses and late menopause are the possible independent risk factors of breast cancer. The increased risk associated with the prolonged duration suggests that the number of years of menstrual activity and as a consequence a greater exposure to estrogens, is an important etiological factor for this disease. Wu et al. (1996) suggested that regardless of ethnicity, length of unopposed exposure to estrogen may play a major role in the development of breast carcinoma.

Key and Verkasalo (2001) suggested that hormone levels might be higher throughout reproductive life in women who undergo early menarche than in women with a late occurrence of menarche. Constantino et al. (1999) reported that breast cancer occurrence has been linked with reproductive factors and prolonged lifetime exposure to estrogen, suggesting that estrogen plays a major role in disease development.

According to Russo and Ruso (1995), the high levels of circulating hormones during pregnancy result in differentiation of the Terminal Duct Lobular Unit (TDLU), which is the major site of malignant transformation in the breast. This process of differentiation of TDLU is protective against breast carcinoma development and its effect is permanent. According to Henderson et al. (1988), ovarian hormones particularly estrogens, play a major role in breast cancer development. Key and Pike (1988) studied the association of various reproductive and menstrual characteristics with breast cancer risk in humans, strongly suggested that endogenous hormones are etiologically involved in the cancer development. According to Carpos (2004), aromatase inhibitors, which prevent the
aromatase enzymes catalyzing the final step in estrogen biosynthesis are also successful in the prevention and treatment of breast cancer. Stoll (1997) reported that the increased risk of postmenopausal breast cancer risk may be related to increased aromatization of androgens to estrogens in adipose tissue in addition to lower levels of sex hormone binding globulin that may contribute to higher availability of estrogen in obese postmenopausal women. Pike et al. (1993) concluded that sufficiently large numbers of women who have used Estrogen Replacement Therapy (ERT) for extended period of time found a modest increase in breast cancer risk among exposed women.

Pike et al. (1993) opined that the change in breast cancer incidence rate with respect to age is influenced by the possible inclusion of reproductive hormones in breast cancer etiology. According to McTiernan et al. (1986), the correlation between estrogen receptor status and progesterone receptor status is sufficiently high that it is difficult to separate any independent association they may have with breast cancer risk factors.

Toniolo et al. (2000) and Kaaks et al. (2003) in their different studies found that high insulinemia may increase breast cancer risk by several mechanisms, including an alteration of cell cycle kinetics on inhibition of apoptosis or through a gonadotrophic effect or through metabolic effects on the liver, where insulin inhibits the synthesis of sex hormone binding globulin and IGF-1-binding proteins 1 and 2, thus increasing the bioavailability of both sex hormones and IGF-1.

According to Grodin et al. (1990), women with android obesity and larger waist hip ratio reflect a greater androgenic state. Androgens chiefly androesteredione, through peripheral conversion in adipose tissue act as the main source of estrogens in postmenopausal women. Ries et al. (1990) estimated that until around 1980, most of the increase in breast cancer incidence rates occurred in postmenopausal women, but now increases are also seen in premenopausal women. Grann et al. (2000) concluded that in contrast to sporadic cancers, most BRCA gene associated cancer is estrogen receptor negative.

2.1.6. Genetic and family history

According to Bernstein et al. (2003), in recent years, approximately 5 to 10% of all breast cancer can be accounted for hereditary breast cancer susceptibility disorders. Skeil (2006) suggested that if a women’s blood related relatives on either her mother or
father’s side of the family have had breast cancer, then she is at increased risk for the disease.

As suggested by Stalsberg et al. (1993), all histological types reported among women also occur among men and p53 gene mutation have been reported with the same frequency in the tumour tissue of male and female patients with breast cancer.

According to Zeleniuch (2005), family history has long been recognized as key risk factor for the development of breast cancer. Slattery (1993) and Yang et al. (1998) in their different studies reported that 5-10% of women have a mother or sister with breast cancer, and about twice as many have either a first or second degree relative with breast cancer. Pharoah et al. (1997) from their analysis found a relative risk of breast cancer conferred by a first or second degree relative with breast cancer to be 2.1 (95% CI 2.0-2.2). According to Marchblanks et al. (2002) and Davis et al. (2002), the overall rate of positive first degree family history was 19% among the cases.

According to Harvey (1985), women with a history of primary breast cancer have a three to four fold increase in risk for primary cancer in the contralateral breast. Anderson et al. (1985) and Horn et al. (1988) suggested that the risk of second primary in the breast especially high in women with a positive family history of breast cancer.

Bennett et al. (2000) reported that about 5% of breast cancer is attributable to rate high penetrance mutations in a small number of specific genes e.g. BRCA 1, BRCA 2, ATM, PTEN and TP53. Mutations in BRCA1 and BRCA 2 account for up to 50% of hereditary and familial breast cancer. According to Smith et al. (1996), more than 700 mutations and sequence variations have been detected so far and only a few are recurrent in unrelated families.

2.1.7 Menstrual and reproductive factors

According to Fisher et al. (1993), Kuller, (1995) and Kelsey and Bernstein (1996), characteristics of women’s reproductive history provide the most consistent evidence for the risk of breast cancer. Early onset of menarche, a late menopause, delayed childbirth, nulliparity and low cumulative lactation time are the important risk factors of breast cancer. According to Buchholz et al. (2003), in comparison with women who had menarche before the age of 12 years, later age at menarche was associated with an
approximately 40% reduced risk of breast cancer. Kelsey et al. (1993) and Hasey (2005) reported that early age at menarche is associated with increased risk of breast cancer and there appears to be a 20% decrease in breast cancer risk for each year menarche is delayed. Risk has also been associated with time when “regular” or predictable menstrual cycles are first established.

According to Bruzzi et al. (1988) and Henderson et al. (1985), the earlier the age at menarche, with its associated earlier onset of regular menstrual cycles, the higher the risk of breast cancer, also the later the age at menopause the higher a woman’s risk of breast cancer. Mc.Mahon (1970) reported that the increased risks associated with early age at menarche and late age at menopause suggest that the total number of years of menstrual activity is of etiologic importance. Kelsey et al. (1993) considered early menarche as a modest risk factor for first primary breast cancer.

Joan et al. (2007) in their study found that early age at menarche has utmost modest impact on contralateral breast cancer risk. According to Kelsey et al. (1996), starting menarche at age 11 years or earlier is considered as an established breast cancer risk factor and starting menarche at age 15 years or older is considered as an established protective factor. Henderson et al. (1985) considered age at menarche as an established risk factor for breast cancer and modest elevations in breast cancer risk are associated with younger ages at menarche. They also suggested that age at the onset of menses is not the only aspect of menarche that is important in determining the breast cancer risk.

Ma et al. (2006) and Henderson et al. (2008) in their different studies found that late menopause is a risk factor for breast cancer. According to Kelsey et al. (1993), a shorter menstrual cycle over the age range 20-39 years is associated with increased risk. Tao et al. (1988) and Wang et al. (1985) observed the effect of age at menopause on breast cancer risk in the study conducted in Tianjin, China.

Ewertz et al. (1988) have shown that induced abortion in the first and second trimester of first pregnancy was significantly associated with breast cancer risks and also women with two or more induced abortions before their first full term pregnancy had a breast cancer risk relative to those without induced abortions. Ruso and Ruso (1980) hypothesized that pregnancy interrupted by abortion increase a risk for breast cancer
because breast cells may be left undifferentiated and thus are more prone to oncogenic influences. Daling et al. (1994) found a risk of breast cancer associated with induced abortion among nulliparous women. Rookus et al. (1995) confined their analysis to ever-pregnant women finding elevated risk associated with induced abortion both before and after a full term pregnancy.

According to Paffenbarger et al. (1980), for women who use oral contraceptives at an especially late age and women who use at an especially early age before their first pregnancy there is an increased risk of breast cancer. McPherson et al. (1989) suggested that long term use of oral contraceptives is associated with a higher risk for early onset of cancers, usually those occurring before age 45 years. Pike et al. (1993), Kuller, (1995) and Kelsey and Bernstein (1996) reported that there is small increase in risk of breast cancer associated with long term use of oral contraceptive and Hormone Replacement Therapy (HRT). Prentice and Thomas, (1987) have found that oral contraceptive use does not affect the risk of breast cancer in majority of women regardless of the dose, brand, or type of estrogen or progesterone. Pike et al. (1983) reported that long-term use of oral contraceptives, especially during certain parts of reproductive life is associated with a modest increase in breast cancer risk. This effect may be even stronger when the pregnancy is interrupted at the end of the first trimester or when it is not proceeded by a full term pregnancy.

McTiernan and Thomas (1986) and Siskind et al. (1989) in their different studies found that as the number of months of breast feeding increases, the risk of breast cancer decreases, particularly for premenopausal women. Henderson et al. (1996) from their findings reported that parous women have lower risk of breast cancer than nulliparous women. According to Kelsey and Gamman (2008), Nuns have high risk of breast cancer presumably because of their usual nulliparous status. Pathak et al. (1986), Kvale (1987) and Hayed (1989) noted the protective effect of parity, mainly for breast cancer diagnosed in women of about age 50 than in younger women. Bruzzi et al. (1988) suggested that the increase in risk of breast cancer last about 10 years after the last pregnancy. McMahon et al. (1970) reported that breast cancer of single woman and nulliparous married women were similar and are approximately 1.4 times that of parous married women. Murphy et al. (1990) suggested that levels of sex hormone-binding globulin (which may be inversely related to breast cancer risk) may be higher in mothers of twins than in other parous mothers, thus providing another explanation for the
possible lower proportion of twins born to women who subsequently develop breast cancer. Kvale et al. (1987) found a consistent and highly significant inverse association between high parity and breast cancer risk and the apparent protective effect of high parity was found in all patients among Nordic population. Wu et al. (1996) found an independent protective effect of parity among Asian - American women. According to Okabia et al. (2006), if a woman delivers her first child close to the age at which menopause occurs, her lifetime risk is actually higher than if she was nulliparous.

According to Hunter et al. (1993), a full term pregnancy brings about exposure to high levels of estrogen and progesterone, and thus might be followed for several years by an increased risk for breast cancer until long term protective effects resulting from changes in the susceptibility of breast tissue predominate. Wingo et al. (1997) concluded that breast cancer risk is not associated with an increased number of either spontaneous and induce abortions. Joan et al. (2007) reported that although having a Full Term Pregnancy (FTP) is associated with reduced breast cancer risk among postmenopausal women, women who have their first term pregnancy in their 30's and 40's are at somewhat higher breast cancer risk than nulliparous women. Vaittinen et al. (2000), Cook et al. (1996) and Storm et al. (1992) provided inconsistent report that last age at FTP and low parity or nulliparity are associated with increased risk of contralateral breast cancer. The Pike model predicts that the given age, a woman with a full term pregnancy in the proceeding 5-10 years is at increased risk of breast cancer compared to a nulliparous woman (Pike et al., 1983). According to Kato et al. (1992), early age at first full term pregnancy is not a strong protective factor for breast cancer in young women. Kvale and Heuch (1987) and Kalache et al. (1993) found that age at last delivery was independently associated with breast carcinoma risk.

Soini (1977), Olsson et al. (1983) and Whelan et al. (1992) reported that a shorter menstrual cycle length over the age range 20-30 years is associated with increased risk. According to Kroeman (1980), ovulatory menstrual cycles that are associated with estrogen unopposed by progesterone and with infertility increase the risk of breast cancer in women.

2.2. Extent of DNA damage in breast cancer patients

2.2.1. Genomic instability in breast cancer
According to Chen and Hunter (2009), biomarkers used for molecular epidemiology in cancer can be categorized into different class: marker of exposures, markers of dose, markers of internal dose, markers of biological effective dose, markers of altered structure / function, markers of susceptibility, markers of cancer subtype and markers of prognosis. Mars and Saenders (1990), Mackay *et al.* (1990) and Ahuja and Araga (1997) in their study reported that a number of numerical and structural chromosomal abnormalities and amplification of oncogenes have been associated with breast cancer.

Ponder (1994) highlighted that, two important pre-disposing genes, BRCA1 on chromosome 17q21 and BRCA 2 on chromosome 13q12-13, account for about two third of familial breast cancer cases. Gonzalez *et al.* (1990), Lininger *et al.* (1990) and Katsama *et al.* (2000) in different studies proposed that in the presence of exogenous and endogenous agents, women may develope cytogenetic alterations, such as deletions, amplifications in oncogene and tumor suppressor genes, leading to cellular transformation and neoplasm.

Rajeshwari *et al.* (2000) noted that persistent basal DNA damage may reflect higher exposure to carcinogen and deficient DNA repair. According to Easton *et al.* (1993), mutations in the BRCA1 gene are involved in above 50% of families with a high incidence of breast cancer and in atleast 80% of families with a high rate of early breast and ovarian cancers.

Rahman *et al.* (2000) in their study on the genetics of breast cancer susceptibility proposed that mutations of the BRCA 2 gene cause early breast cancer in woman and also a higher risk in man. Studies of Mallya (1998) and Smith *et al.* (1994) suggested that in addition BRCA 1/2, other tumor suppressor genes such as the p53 and AT genes, which are involved in breast cancer, participate in cell repair process. Miki (1994) and Tavitigian (1996) in linkage analysis of families with a high risk of breast cancer have identified two major susceptibility genes BRCA 1 and BRCA 2. Peto (1999) and Serova (1997) in their studies have shown that as many as 60% of families with site specific female breast cancer cannot be explained by mutations in BRCA 1 and BRCA 2 genes.

DNA occurs spontaneously and constantly throughout the life of an organism and can be enhanced further by exogenous DNA damaging factors viz., environmental pollution, ionizing radiation, UV rays and chemotherapeutic drugs. The most detrimental form of DNA damage is chromosomal double-strand breaks (DSBs), which is lethal to the cell if not repaired. DNA DSBs can be induced by ionizing radiation, DNA replication errors, or cell oxidative metabolism.

According to Khanna and Jackson (2001) and Pierce (2001), two major pathways for the repair of double strand break in mammalian cells include homologous recombination (HR) repair which essentially provides an error free repair by using homologous template and the more error prone non-homologous end joining. Swift et al. (1991) and Olesen et al. (2001) in different studies noted that ATM (Ataxia Telangectasia Mutated) mutations in hetrozygous carriers seem to increase cancer predisposition, particularly breast cancer. Easton et al. (1993) estimated that 50% of women who survive childhood cancer will develop breast cancer by the age of 50 and lifetime penetrance approaches 100%. Oldenburg et al. (2003) in a recent search for breast cancer susceptibility genes among families with no BRCA 1 and BRCA 2 mutation suggested a model in which CHEK 1100 del C interacts with an as yet unknown gene to increase breast cancer risk. In 1999, study of cell cycle check point as therapeutic targets, by Stewart et al., revealed that defects in cell cycles check - point controls are fundamental to the accumulation of genetic damage in the mammary epithelial cell, leading to cancer. DeMichele and Weber (2000) in the evaluation of inherited genetic factors put forward that a general hypothesis in the field of hereditary cancer genetics is the "two hit" hypothesis. According to the hypothesis, a point mutation might be inherited in one allele of a candidate gene at a putative susceptibility locus and that Loss Of Heterozygocity (LOH) or another genetic alteration might occur in the other allele of that locus later in the life, leading to genomic instability and cancer.

Dickson et al. (2006), discussed that gene amplification and resultant over expression of Epidermal Growth Factor Receptor (EGFR)-related HER-2/neu protein occur in approximately 25% human breast cancer cases and over expression of EGFR occurs in the absence of gene amplification in 40% of breast tumors.

According to Dickson et al. (2000), antisense c-Myc oligonucleotides block estrogen induced proliferation in breast cancer cells and amplification of the e-Myc gene
is a common genetic alteration in breast cancer; approximately 1/5th of the breast cancer. Elenbaas et al. (2001) reported that high level of telomerase expression lead to cell immortalization and the catalytic subunit of this enzyme (hTERT), together with three oncogenes-SV40T, SV40t and a mutant c-RasH - convert human mammary epithelial cells to cancer.

Oesterreich et al. (2002) carried out study on human breast cancer cell evasion by cadherin - II and found that, estrogen appears to down regulate expression of E-cadherin in breast cancer cells, potentially contributing to the progression of disease.

Djuric et al. (2001), Hu et al. (2002) and Patel et al. (1997) stated that higher levels of DNA damage and deficient DNA repair may predispose individuals to breast cancer. Vijver et al. (2002) evaluated that high risk early stage breast cancers are distinct in their gene expression profile from lower risk early stage cancer.

2.2.2. DNA damage study using Comet assay

The Single Cell Gel Electrophoresis (SCGE) assay also known as the comet assay is a rapid relatively simple, visual biochemical technique for measuring and quantifying DNA damage. Wong et al. (2005) opined that the comet assay is a highly versatile, reliable and relatively simple and robust method to assess DNA damage, protection and repair.

According to Tice et al. (2000), the SCG/comet assay allows the detection of DNA alternations of diverse kinds, such as double-strand breaks, single strand breaks, alkali-labile sites, incomplete repair sites, and cross links. Yasuhara et al. (2003), Olive et al. (1993) and Reiss et al. (1990) reported that comet assay allows us to distinguish apoptotic from normal and necrotic cells based on the DNA fragmentation pattern. The SCGE electrophoresis allows detection of DNA fragmentation in single cells and was initially used for DNA damage estimation (Bednarek et al., 2006).

According to Eastman and Barry (1992), Kassli et al. (2000) and Bajpayee et al. (2006), chromosomal aberration test and comet assays are found to be sensitive genotoxicity test to investigate DNA damage and repair. Dusinka et al. (2008) suggested that when standardized and validated, the comet assay could provide invaluable information in the areas of hazard identification and risk assessment of environmental
and occupational exposure, diseases linked with oxidative stress, nutrition, monitoring the effectiveness of medical treatment and investigating individual variation in response to DNA damage that may reflect genetic or environmental influences.

Gontijo et al. (2001) elucidated that high levels of cellular DNA damage in non-neoplastic urothelial cells may predispose smokers to urinary bladder cancer as shown by the results of their study to detect primary DNA damage in non-neoplastic urothelial cells of smokers and ex-smokers by comet assay.

Ahuja et al. (1997) suggested that in combination with morphological, biochemical and cytogenetic parameters, the comet assay may serve as a novel tool to detect the cancer and predict the stage of cervical dysplasia. Analysis of DNA damage using the comet assay in infant fed cow's milk by Dundaroż et al. (2003) revealed the fact that non-human milk feeding may increase the risk for cancer or a specific cancer or group of cancers as well as risk for disease such as type-I diabetes mellitus and Crohn's disease. It also suggested that there is some level of DNA damage in the lymphocytes of infants not breast fed and this may lead to malignancy in childhood or later in life.

Andreoli et al. (1997) found increased frequency DNA damage among individual working in gas station. Muller et al. (2000) carried out experiment on genotoxicity activity of five HaloAcetoNitriles (HANs) by the comet assay and Ames assay and suggested that all HANs induce DNA damage in Hela S\textsubscript{3} cells, increased the mean tail moment significantly. Udumudi et al. (1998) conducted study on risk assessment in cervical dysplasia patients by SCGE and analyzed that patient with cervical dysplasia exhibited longer comet tails in their cervical epithelial cells and peripheral blood lymphocytes.

The comet assay appears to have many advantages including allowing relatively high-throughput screening requiring a few cells and facilitating the detection of primary DNA damage in individual cells (Tice et al., 2000).

2.2.3. DNA damage study in breast cancer patients undergoing different modalities of treatment

Jaloszynski et al. (1997) reported a high sensitivity and reduced DNA repair capacity in peripheral blood cells from breast cancer patients treated with bleomycin
using comet assay. Jaloszynski et al. (1999) opined that halothane and isoflurane anesthetic drugs were capable of increasing DNA migration in a dose dependent manner. In their breast cancer risk study, Rajeshwari et al. (2000) have reported elevated levels of DNA damage in breast cancer cases and in woman with family history of breast cancer at base-line after treatment with a mutagene, N-Methylnitroso guanidine and following DNA repair. Romanet et al., (1999) described a direct relationship between DNA damage and repair and drug metabolism in lymphocytes and treatment efficacy.

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 the basal level of damage, and the radio-induced damage were higher in lymphocytes from breast cancer patients than in the lymphocytes from healthy donors. They also proposed that the repair capacity of blood lymphocytes from the patients was slower than that of lymphocytes from healthy donors. Rigaud et al. (1990), Helzsover et al. (1995) and Parshad et al. (1991) found a high sensitivity and a reduced repair capacity in blood lymphocytes from breast cancer patients when exposed to X-rays, Gamma and UV lights as evaluated by the determination of chromosome aberrations. Bekum et al. (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.

Le et al. (1999) and Hannan (2001) in different experiments detected higher level of DNA adducts in normal adjacent breast tissues and observed altered DNA repair in cultured skin fibroblast of breast cancer patients. Smith et al. (2005) conducted studies on DNA damage and breast cancer risk and elucidated that DNA damage increased with increasing BMI in cases, while it decreased with increasing BMI in controls. Fodor et al. (2000) in the study on the impact of radiotherapy and incidence and time of occurrence of local recurrence in early stage breast cancer after breast conserving concluded that radiotherapy has the property of not only preventing but also delaying Ipsilateral Breast Tumour Recurrence (IBTR). Ajanta et al. (2002) in their study revealed that DNA damage of post treatment relapsed cases were more than untreated cases.

Durel et al. (2001) also examined genomic instability in breast cancer patients. They collected lymphocytes after surgery and before any radiation or chemotherapy was
delivered and subjected to varying doses of Co-60γ-radiation and then evaluated by comet assay. They found markedly higher mean tail moment in patients than in controls.

2.3. Antimutagenic activity of pomegranate

2.3.1. Pomegranate - An overview

Pomegranate fruit has a fascinating history of traditional use as food, medicine and cultural icon, dating back thousands of years (Still, 2006). Originating in the Middle East, pomegranates were one of the earliest fruits to be domesticated and their range now includes the Far East, India, the Mediterranean and the Americas. A symbol of fertility and immortality, pomegranates healing properties were discussed in one of the oldest medical texts, the *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 3 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.

In addition to the conventional nutritional compounds noted just above, pomegranate juice was reported to be comprised of 85.4% water, 10.6% total sugars, 1.4% pectin, 0.2-1.0% polyphenols, and organic acids. Other reported minor compounds include fatty acids, sterols, triterpenoids and α-tocopherol (Lansky, 2007). The quantitative profile of pomegranate constituents has been found to vary considerably though, depending upon the cultivar, geoclimatic factors, harvesting, processing, and storage conditions (Gil et al., 2000).

The polyphenols are responsible for the astringent quality of the juice. All parts of the fruit contain polyphenols, including the peel, the inner membranes, and the arils; although the pericarp (peel and membrane) is reported to contain the highest concentrations (Gil et al., 2000).
According to Gil et al. (2000), the pomegranate contains 2 major classes of polyphenolic compounds: hydrolysable tannins (HT) and flavonoids, with very small levels of condensed tannins. The HT are the predominant polyphenols in pomegranate and include ellagitannins, gallotannins, and gallagoyl esters. The most abundant HT is the gallagyl tannins, punicalagins (anomers A and B) and related tannins, which comprise roughly 63% of the constituent polyphenols (Gil et al., 2000). Other reported HT include pedunculagin, punicalin, gallagic and ellagic acid esters of glucose (16.8%), and ellagic acid derivatives (4.9%) (Lansky, 2007 and Gil et al., 2000).

Noda et al. (2002) stated that the flavonoid component consists mainly of anthocyanidins, flavonols, and flavonol glucosides. The anthocyanidins are the pigments which imbue the juice with its ruby red colour and include the compounds cyanidin; cyanidin-3-glucoside; cyaniding 3,5-diglucoside; cyanidin-3-rutinoside; delphinidin; delphinidin- 3-glucoside; delphinidin 3,5-glucoside; pelargonidin-3-glucoside; and pelargonidin-3,5-glucoside (Gil et al., 1995; Of these, cyanidin derivatives appear to be the most abundant, followed by delphinidin glucosides (Rozenberg et al., 2005). Other flavonoid constituents include kaempferol, myricetin, rutin, narigenin, luteolin and luteolin glycosides, quercetin, and quercetin glucosides (Kim et al., 2002 and Elswikja et al., 2004). The main condensed tannins found in the juice are catechin, procyanidin B1, and procyanidin B2 (Artik et al., 1998). Gallocatechin and its derivatives have also been reported (Plumb et al., 2002). Other minor phenolics include gallic acid, protocatechuic acid, catechin, and phloridzin (Poyrazoglu et al., 2002).

2.3.2. Therapeutical action of pomegranate

Pomegranate (Punica granatum L.) has been known to possess considerable pharmacological properties like antimicrobial, antiviral, anticancer, antioxidant and antimitogenic effect (Seeram et al., 2005; Negi et al., 2003). The species Punica granatum L. (Punicaceae) has been widely used by traditional medicine in America, Asia, Africa and Europe for the treatment of different types of diseases, (Gracious, Rose et al., 2001; Kim et al., 2002 and Murthy et al., 2004).

From a pathogenic point of view, Negris et al. (2000) have shown that regular pomegranate juice reduced the expression of oxidation-sensitive genes at the sites of perturbed shear stress and protected against high prone atherosclerotic areas in hyper
cholesterolemic mice.

In order to determine whether proatherogenic conditions induced by turbulent shear stress can be attenuated also by pomegranate fruit extract, Nigris et al. (2007) first studied cultured human coronary artery endothelial cells subjected to high shear stress. Negriss et al. (2007) analysed high prone and low prone atherosclerotic aortic areas and vascular function of hypercholesterolenic mice after chronic oral administration of PFE in comparison to regular pomegranate juice.

In atherosclerotic apolipoprotein E-deficient (E0) mice, 8 weeks of pomegranate supplementation reduced macrophage lipid peroxides by 37%. In contrast, Aviram et al. (2000) reported that in E0 mice, 11 weeks of supplementation with pomegranate juice reduced macrophage LDL oxidation by 90% with an associated reduction in cellular lipid peroxidation and superoxide release. The size of atherosclerotic lesions was reduced by 44% and the number of foam cells declined compared to the controls.

According to Rozenberg et al. (2005) macrophage oxidative stress levels were reduced in both streptozotocin-induced diabetic Balb/C mice and healthy control mice that were fed pomegranate polysaccharides for 10 days.

Regular Pomegranate juice administrated to hypertensive patients caused a significant drop in blood pressure, a reduction in carotid plaque development and an improvement of stress-induced myocardial ischemia in patients who have coronary heart disease. (Aviram et al., 2000, Summer et al., 2005 and Aviram et al., 2004). Gujral et al. (1960) conducted preliminary studies on the antifertility effect of some indigenous drugs and reported that pomegranate hull or root extract used orally or intravaginally prevents fertility. Williaman and Schubert (1961) carried out studies on alkaloid bearing plants and their alkaloid contents and reported that pelletierienes present in pomegranate showed anthelmintic activity.

Anon (1969) observed the effect of pomegranate in the treatment of dysentry, diarrhoea, stomach inflammation and cardiac disorder. Bianchini and Corbetta (1979) conducted studies on health plants of the world and reported the therapeutic uses of pomegranate as vermifugal, taenicidal, astringent, antispasmodic, antihysteric, diuretic and carminative. Lust (1983) conducted studies on herbal plants and reported that the bark of pomegranate has antibacterial, antiviral and astringent properties.
Hasten (1983) examined flavonoids, a class of natural products of high pharmacological potency and reported that flavonoids distributed among photosynthesizing cells of pomegranate has pharmacological activity. Jain and Puri (1984) assessed the traditional use of pomegranate hull and reported that pomegranate hull is used in the treatment of diabetes, burns and leprosy. According to Duke and Ayensu (1985), dried pericarp, roots, barks and juice of pomegranate are employed in the treatment of colic, colitis, diarrhea, dysentery, leucorrhea, paralysis and rectocele. Duke and Ayensu (1985) evaluated the medicinal value of pomegranate flowers and reported that the flowers are used in treating cough, dysentery and stomach ache. Nagaraju and Rao (1990) conducted studies on plant crude drugs and reported that pomegranate hulls in the form of an aqueous decoction were effective in treating dysentery, diarrhea and stomatitis. Investigatory studies on antibacterial, antifungal and anthelmentic properties of medicinal plants by Naovi et al. (1991) revealed that roots of pomegranate have anthelmentic property. Bown (1995) noted that all parts of pomegranate contain unusual alkaloids known as pelletierines which paralyze tapeworms so that they are easily expelled from the body by using a laxative.

Sen and Packer (1996) carried out studies on antioxidant and redox regulation of gene transcription and reported that pomegranate fermented juice has an indirect role in inhibiting inflammation, pathogenesis of more complex disease pattern such as AIDS, carcinogenesis, atherosclerosis and diabetes by its antioxidant effect. Antitumour and antiviral action of polyphenols present in pomegranate peels were evaluated by Mavlyanov et al. (1997) and they concluded that pomegranate hulls have antitumour and antiviral action against the virulent intestinal bacteria-Salmonella typhi and Vibrio cholerae, the parasite - Amoeba and most viruses like Herpes simplex, Poliovirus and Human Immunodeficiency Virus (HIV).

Artik (1998) determined phenolic compounds in pomegranate juice and revealed that peel contains numerous pharmacologically active phenolic compounds. Moneam et al. (1998) observed oestrogen content of pomegranate seeds and reported the presence of coumestrol which was proved to be antimutagenic. Lee and Watson (1998) conducted studies on rich concentration of diverse biflavonoids in pomegranate and its free radical scavenging activity and recommended it as a medicinal article used for the treatment of
AIDS. Jafri et al. (2000) conducted experiments in normal and diabetic rats to find the effect of pomegranate flower extract and reported that flower extract reduced blood sugar levels in rodents.

Aviram et al. (2000) stated that fresh juice of pomegranate inhibit LDL oxidation and formation of atheromatous plaque in rodents and humans. He also found that consumption of concentrated pomegranate juice per day reduced LDL susceptibility and increased the activity of serum paraoxonase. According to Kaplan et al. (2001), pomegranate juice reduced atherosclerotic lesion size in apolipoprotein E- deficient mice.

Aviram and Dronfeld (2001) analysed inhibitory effect of pomegranate juice and reported that consumption of concentrated pomegranate juice decreased the activity of serum angiotensin converting enzyme and reduced the systolic pressure with hypertension. Kim et al. (2002) examined chemopreventive and therapeutic effect of pomegranate and concluded that fraction of pomegranate like fermented and unfermented juice and peel extract exerted antiproliferative effects on human breast cancer cells. Vanelswijk et al. (2004) studied rapid dereplication of estrogenic compounds in pomegranate using online biochemical detection and reported that various parts of the pomegranate juice and peel extract exerted suppressive effects on human cancer cells in vitro. Okomato et al. (2004) conducted studies in menopausal syndrome of ovariectomized mice to find the effect of pomegranate extract and recommended that pomegranate extract is clinically effective for depressive state and bone loss in menopausal syndrome women.

2.3.3. Antioxidant activity of pomegranate

The potent antioxidant capacity of pomegranate and its components has been reported by numerous scientists using multiple in vitro assay systems (Kulkarni et al., 2007; Reddy et al., 2007; Rout and Banerjee, 2007; Sestili et al., 2007; and Shiner et al., 2007). This activity is largely due to the polyphenolic constituents (Rosenblat and Aviram, 2006; Seeram et al., 2005; and Azadroi et al., 2005). Pomegranate juice has both a higher total polyphenolic content and greater antioxidant activity than other
commonly consumed fruit juices, including grape, cranberry, orange, and apple juice among others (Rosenblat and Aviram, 2006).

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. Singh et al. (2002) carried out studies on antioxidant activity of pomegranate peel and seed extracts and showed the high antioxidant activity of methanolic extracts of pomegranate peel in various in vitro models.

Antioxidant and eicosanoid enzyme inhibition properties of pomegranate seed oil and fermented juice flavonoids were studied by Shay et al. (1999) and they concluded that pomegranate fermented juice and cold pressed seed oil has strong antioxidant activity close to that of butylated hydroxyanisole and green tea and greater than that of red wine. Gil et al. (2000) reported the presence of ellagic acid, gallic acid and tert-gallic acid in pomegranate juice while studying the antioxidant activity of pomegranate juice. Rosenberg et al. (2000) evaluated steroid hormone activity of flavonoids and reported that ellagic acid is an extremely potent antioxidant. Antioxidant properties of gallocatechin and prodelphinidins from pomegranate peel were studied by Plumb et al. (2002) to assess strong antioxidant properties of gallocatechin and prodelphinidins present in pomegranate peels.

According to Seeram et al. (2005) and Rosenblat et al. (2006), the antioxidant level in pomegranate juice was found to be higher than in other natural juices such as blueberry, cranberry and orange as well as red wine.

Li et al. (2005) found that pomegranate peel extract obtained by use of a mixture composed of methanol, ethanol and water was significantly higher in FRAP value (a value showing antioxidant efficiency) than those obtained using individual solvent namely using methanol, ethanol or acetone. According to Ghasemian et al. (2006), the peel extracts obtained from the mixture of different solvents is more powerful in recovering antioxidants than are individual solvents.

The potent antioxidant properties of pomegranate juice have been attributed to its high content of punicalagin isomers that can reach levels > 2g/L juice. (Gil et al., 2000; Cerda et al., 2003 and Carda et al., 2003).
Gil et al. (2000) calculated that the punicalagins constituted 62.8% and other hydrolysable tannins 16.8% of the total phenolic content, and together they accounted for 78.5% of the antioxidant activity. These results subsequently led many researchers to focus on the hydrolysable tannins as the primary antioxidant constituents of pomegranate juice. 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 oxidatively damaged components, and chelating metal ions (Kulkarni et al., 2007). Pomegranate juice constituent prodelphinidins, gallocatechin, ellagic acid and gallic acid derivatives, fatty acids, and polysaccharides have also been found to exert significant antioxidant effects (Rout and Banerjee, 2007 and Sestili et al., 2007).

Pomegranate juice possesses potent antioxidant activities that are associated with its antiatherogenic properties in mice and inhibition of cyclooxygenases and lipoxygenases (Schubert et al., 1999; Aviram et al., 2000; Seeram et al., 2005 and Rosenblat et al., 2006).

2.3.4. Anticancerous activity of pomegranate

The anticancer activity of pomegranate tannin fractions and the isolated pure constituents ellagic acid and punicalagins have been reported by a number of investigators (Kulkarni et al., 2007; Lansky et al., 2005; and Larrosa et al., 2006). Heftmann et al. (1966) examined estrone in pomegranate seeds and reported highest concentration of estrone in dried seeds which showed preventive property towards breast cancer.

PFE (70% acetone-water extract) was found to significantly improve survival time in athymic male nude mice xenografted with human A549 non-small cell lung cancer (Khan et al., 2007). Human clinical relevance of the 2 doses assessed (0.1% and 0.2% PFE) was based on the assumption that a typical healthy person (70 kg) would reasonably consume 250 to 500 ml of pomegranate juice per day. In female A/J mice, the pomegranate fruit extract was also found to inhibit the growth and progression of lung tumours induced by 2 chemical carcinogens, NTCU and B(a)P (Khan et al., 2007).

The inhibitory effect of a PFE was evaluated in severe combined immunodeficient mice xenografted with human prostate cancer cells (LAPC-4) (Seeram
et al., 2007). Oral administration of the pomegranate fruit extract significantly inhibited LAPC-4 proliferation, producing a 1.8 cm$^3$ reduction in tumor volume 6 weeks after inoculation. Pomegranate pericarp polyphenol and oil extracts were tested in a PC-3 xenograft model employing athymic nude mice. At a dose of 2ug/g body weight, both fractions reduced tumor volume by 72% compared to controls at 35 days post-inoculation (Albercht et al., 2004).

Ellagic acid and punicalagins were also evaluated in Caco-2 colon cancer and normal colon cells (Larrosa et al., 2006). They dose-dependently inhibited Caco-2 proliferation but their effect was additive, not synergistic. Both compounds significantly increased Caco-2 apoptosis but neither induced apoptosis in the normal colon cells. It appeared that ellagic acid was the actual apoptosis inducer though, as punicalagins treatment did not induce apoptosis until its hydrolysis product, ellagic acid, accumulated in the media. Sudheer et al. (2007) and Rommel et al. (1993) reported that ellagic acid is a proven anti-carcinogen used in alternative medicine and to prevent cancer. It is present in strawberries, cranberries, walnut, pears and pomegranates.

2.3.5. Antimutagenic activity of pomegranate / ellagic acid

Ghasemian et al. (2006) conducted a study on the antimutagenic effect of peel extracts of two Iranian cultivators of pomegranate using Ames assay in TA100 and TA1535 strains and showed that the pomegranate peel extracts have both antioxidant and antimutagenic properties. Teel and Castonguay (1992) investigated the inhibitory effects of five structurally related polyphenolic compounds of pomegranate on the mutagenicity of NNK in Salmonella typhimurium TA 1535 and reported that highest dose of polyphenols inhibited mutagenesis. Negi et al. (2003) carried out experiment on antimutagenic and antioxidant activities of pomegranate peel extracts and reported that the methanolic extract of pomegranate peel was more effective than acetone, ethylacetate and water extract.

Pina et al. (1996) conducted studies on antimutagenic effect of ellagic acid against aflatoxin B 1 using Salmonella Microsuspension Assay and reported that ellagic acid significantly inhibited mutagenicity of all doses of aflatoxin B 1 in both tester strains.
TA 100 and TA 98 with the addition of S9. Negi et al. (2003) also evaluated the inhibitory effect of pomegranate peel extracts against the mutagenicity of sodium azide to *S. typhimurium* TA100 and TA1535. Li et al. (2005) and Polyrazoglu et al. (2002) found that pomegranate peels is rich source of antioxidant, specially polyphenols, such as ellagi acid, quercetin and punicalagin. Presence of these polyphenols in the pomegranate peel may be responsible for antimutagenicity of peel extracts.

Mandal et al. (1987) studied the inhibition of aflatoxin B1 mutagenesis in *Salmonella typhimurium* and DNA damage in cultured rat and human tracheobronchial tissues by ellagic acid and found the inhibitory effect on mutagenicity induced by AFB1 in the Ames test. Smerak et al. (2002) evaluated the antimutagenic effect of ellagic acid and its effect on the immune response in mice using Ames bacterial mutagenicity test in strain TA 98 and TA 100 and deduced that ellagic acid repairs strong immunosuppressive effects of all mutagens applied.

### 2.4. Chromosomal aberration studies using PFE in peripheral blood lymphocytes of breast cancer patients

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. According to Cleary (1991) and Stanbridge (1992), chromosomal rearrangements play an important role in the activation of proto oncogenes and inactivation of tumour suppressor genes. Heim et al. (1989) reported that several types of genetic predisposition to cancer may be associated with constitutional chromosome instability. Heim and Mitelman (1987) and Sandberg (1990) stated that alterations of the karyotype found in all type of neoplastic cells and are often highly specific for particular diagnostic categories.

According to Heim and Mitelman (1987) 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, particularly
ionizing radiation. Hagmar et al. (1994) stated that the conceptual basis for using Chromosomal Aberrations (CA) in peripheral blood lymphocytes as a biomarker is the fact that extent of genetic damage in peripheral blood lymphocytes reflects similar events in the precursor cells for carcinogenic processes in the target tissues.

Schmid, (1973) and Agarwal et al. (1994) reported that the development of cancer is related to genetic damage in somatic cells. Agents that can alter an organism’s genome by causing toxic effect on cellular genetic materials are referred to as mutagenic. Such substances increase the error rate in the replication of the genome and induce mutations by damaging the organism’s DNA. Agarwal et al. (1994) and Gaizev et al. (1996) found an abnormality in chromosomal structure (translocations, deletion and duplication) and chromosomal number (aneuploidy and polyploidy) due to exposure to chemical or physical carcinogen mutagen. According to Guercin et al. (1978) an increase in chromosomal breakage and chromosome loss is associated with increased risk of cancer and in the progression of neoplastic transformation. Hagmar et al. (2001) and Bhuvaneshwari et al. (2004) stated that bone marrow micronucleus test and detection of chromosomal aberrations have been widely used as a tool to indicate carcinogen induced DNA damage as well as to assess the antimutagenic effect of natural and synthetic chemopreventive agents. Rossner et al. (2005) found a strong association between chromosomal aberration frequency in peripheral blood lymphocytes and the risk of stomach cancer.

Carrano and Natarajan (1988) stated that chromosomal aberrations in peripheral blood lymphocytes can be used for assessing the surveillance capacity of healthy individuals exposed to known potential mutagens and carcinogens. In addition, Yunis (1983) and Mitelman (2000) reported that chromosome alterations and chromosomal abnormalities, are typical features of neoplastic cells, and can be used as biomarkers. According to Aitio et al. (1988), although specific chromosome alterations detected in neoplasms are generated during carcinogenesis, it has been hypothesized that the frequency of
chromosomal aberrations in peripheral blood lymphocytes of healthy individuals represents a marker of susceptibility to cancer, on the basis of the concept that genetic damage in peripheral blood lymphocytes reflects similar damages in different target cells undergoing carcinogenesis.

Boffetta et al. (2007) found that the type of chromosomal aberrations occurring in peripheral blood lymphocytes may differ, depending upon the genotoxic agent or mixture of agents acting on the cell cycle as either S-phase-dependent or S-phase-independent agents. Peripheral blood lymphocytes are the tissue of first choice for molecular epidemiological studies that assay DNA repair capacity and the micronucleus in peripheral blood lymphocytes has been validated as a biomarker of chromosomal damage (Deng et al., 2009). A high frequency of chromosome aberrations, sister chromatid exchange or micronucleus in peripheral blood lymphocytes from healthy subject was studied and a positive trend between CA frequency and increased cancer risk was observed (Bonassi et al., 1995 a and b; Bolognesi et al., 1997).

Mitelman et al. (2004), Bonassi et al. (2004) and Mitelman et al. (2007) in their studies reported strongest mechanistic and epidemiological evidence for phenotypic markers for chromosome damage. According to Jyothish et al. (1998) and Jaloskynski et al. (1997) the lymphocytes of breast cancer patients are hypersensitive to bleomycin (a radio material agent) induced chromosome damage. Mars et al. (1990) carried out studies on chromosomal abnormalities in breast cancer and suggested that a number of numerical and structural chromosomal abnormalities are related with the breast cancer. Hall et al. (1990) have verified the association of the magnitude of chromosome damage with cancer risk. O’Connell et al. (1998 and 2003) by different studies highlighted that in breast cancer centrosomal defects appears to pre-dominate in early disease, potentially causing chromosomal type of instability. Felix (2001) reported that DNA topoisomerase II causes chromosomal breakage and translocation in leukemia.

Measurement of Micro Nucleus (MN) frequency in Peripheral Blood Lymphocytes (PBL) is extensively used in molecular epidemiology and cytogenetic study to evaluate the presence and the extent of chromosomal damage in human populations exposed to genotoxic agents bearing a
susceptible genetic profile (Fenech et al., 1999). MN technique has been successfully applied to identify dietary and genetic factors that have a significant impact on genome stability (Kimura et al., 2004).

According to breast cancer and environmental risk factors (1997), breast tissue is particularly sensitive to developing cancer for several reasons. According to Cheng et al. (2009), DNA repair is an essential mechanism for cells to maintain their genomic integrity under endogenous or exogenous assault. Reduced DNA repair capacity is associated with increased risk for several environmentally related cancers.

Elevated levels of Micro Nucleus are indicative of defects in DNA repair and chromosome segregation, which could result in generation of daughter cells with altered gene dosage or deregulation of gene expression that could lead to evolution of the chromosome instability phenotype often seen in cancer (Fenech et al., 1999, Kimura et al., 2004, Fenech 2002; Fenech et al., 2003 and Krisch et al., 2003).

According to Kimura et al. (2004), Rajagopalan et al. (2004) and Fenech et al. (2003), the formation of Micro Nucleus in dividing cells is the result of chromosome breakage due to unrepaired or mis-repaired DNA lesions, or chromosome mal segregation due to mitotic malfunction. These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation machinery. Hagmar et al. (2004) and Rossner et al. (2005) demonstrated that the frequency of CA in peripheral blood lymphocytes of healthy subject is a predictor of cancer risk.

According to Bonassi et al. (2007), the extent of genetic damage measured in lymphocytes reflects the occurrence of early carcinogenic event in the target tissues. Rossner et al. (2005) described a strong association between chromosomal aberration frequency in peripheral blood lymphocyte and the risk of stomach cancer. Elevated micronucleus may be related prospectively with breast cancer risk, for which, there is evidence of an association in case-control studies (Vagra et al., 2006).
2.4.1. Antigenotoxic effect of fruit and vegetables in human lymphocytes

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 and Dutta et al., 2007).

Ratanavalachi et al. (2010) studied the cytotoxic and genotoxic activities of an aqueous extract from Thai noni leaves on human lymphocytes in vitro and reported that the extract at a concentration 0.8 to 20 mg/ml neither induce CA, nor sister chromatid exchange in human lymphocytes and concluded that there is no genotoxic effect for the extract but all concentrations induced cytotoxicity.

Kolanjiappan and Manoharan (2005) demonstrated the anti carcinogenic and antilipidoperoxidative effects of Jasminum grandiflorum L. In DMBA (7, 12-dimethylbenzene (a) anthracene) induced mammary carcinogenesis. Manoharan et al. (2006) studied the effect of Jasminum grandiflorum on DMBA induced chromosomal aberrations in bone marrow of Wistar rats and reported that the flowers and leaves have potent effects in DMBA - induced chromosomal aberrations.

Ratanavalachi et al. (2010) conducted chromosomal aberration assay and sister chromatid exchange assay using human lymphocytes for evaluating the antigenotoxic effect of Thai noni juice and indicated that noni fruit juice at 6 mg/ ml is the optimum dose for cell survival and cell replication.

The effects of high doses of vitamin C and E against induced chromosomal damage in Wistar rat bone marrow cells have been studied by Antunes and Takahashi (1998) and opined that natural antioxidants have the potential to prevent chromosome damage. Silva et al. (2007) assessed the anti genotoxic potential of Fucus vesiculosus extract on cultured human lymphocytes using chromosomal aberration assay and reported that only when lymphocytes were pre treated with reduction, there was a
reduction in doxorubicin induced chromosomal aberration. Alpsoy et al. (2010) conducted experiment on the antimutagenic activities of *Viscum album* fruit ethanolic extract in human lymphocytes using chromosomal aberration analysis and suggested no significant increase in total aberrations against Tri Chloro Ethylene (TCE) - induced genotoxic damage.

Modulatory effect of garlic extract against the cyclophosphamide induced genotoxicity in human lymphocytes *in vitro* was assessed by Sowjanya et al. (2009). The results indicated the significant decrease in the frequency of CA and sister chromatid exchange, suggesting that the garlic extract modulates the cyclophosphamide induced genotoxicity in a dose dependent manner.

Bhattacharya et al. (2004) reported reduction of chryostile asbestos - induced genotoxicity in human peripheral blood lymphocytes by garlic. Siddique and Afzal (2005a) observed the antigenotoxic effect of allicin against methyl methanosulphonate induced genotoxic damage.

Hence in the present study, the antigenotoxic potential of pomegranate, *Punica granatum* was assesses using chromosomal aberration assay in the peripheral blood lymphocytes of breast cancer patients.
III. Materials and methods

The materials used and methods adopted in the present study entitled “Assessment of antigenotoxic potential of pomegranate in the reversal of DNA damage in breast cancer patients” are furnished below:

Phase - I

3.1. Epidemiology of breast cancer

A total of 200 breast cancer patients who attended Valavadi Narayansamy Cancer Centre, G. Kuppusamy Naidu Memorial Hospital, Coimbatore, were selected as the study participants. The age group of the patients varied from early 30 to 70 years. Age matched apparently healthy individuals were selected as control group participants. Both control and experimental group participants were administered a questionnaire to obtain information related to their age, lifestyle, dietary habits, anthropometric parameters, medical and genetic history and menstrual and reproductive history.

3.1.1. Data collection

Standardized structured questionnaires were developed. The questionnaire was designed to gather socio-demographic data including age, marital status, anthropometric factors like BMI, reproductive history including age at menarche, age at marriage, age at first child birth, parity, intake of oral contraceptive, number of abortions and menopause status.
3.1.2. Data analysis

All questionnaires were reviewed for missing or incorrect data. All data entry and analysis were conducted using STATA. Descriptive analysis was carried out to categorize the demographic variables of the study participants. For conditional logistic regression, the variables were categorized as follows: socio demographic data (<40 yrs, 40 - 50 yrs, >50 yrs), education > high school (yes/no), marital status (single / married), monthly household income (1000 - 5000, 5000 - 10000, >10000), BMI (>25/<25), dietary factors (veg, non-veg), frequency (daily, weekly, monthly), junk food (yes/no), fried food (yes/no), type of oil (sunflower oil, groundnut oil, gingerly oil, palm oil), snack chips (yes/no). Medical and genetic history, diabetes (yes/no), hypertension (yes/no), existing family history of cancer (yes/no), menstrual and reproductive history- age at menarche (>13 years/< 13 years), regular periods (yes/no), age at marriage (18-24, 25-29, >29, ever married), Oral Contraceptive (yes/no), nulliparous (yes/no), age at 1st child birth (>29 years/<29 years), number of pregnancy (1-3, 4-6), parity (>3/<3), age at last child birth (<35 years/ >35 years), menopause age (<50 years/>50 years), and number of abortions (1, 2, >3).

Percentages for categorical variables were calculated. The chi-square test was used in the statistical analyses to evaluate the significant factors associated with breast cancer risk by estimating the odds ratio (OR) and 95% Confidence Intervals (CI). The level of significance was set at p < 0.05 for all hypothesis tests in the study. Crude Odds Ratios (OR) for variables in the model were drawn from simple conditional logistic regression. The OR were estimated with 95% CI.

Phase - II

3.2. Extent of DNA damage in breast cancer patients

3.2.1. Collection of samples

For the monitoring of DNA damage in the peripheral lymphocytes, about 2-5 ml blood was collected from each donor by venipuncture into heparinized syringes and brought to the laboratory. The samples were collected from 2007 to 2008.

3.2.2. Healthy donors
The group of healthy donors consisted 20 women who were not undergoing any medication at the time of blood collection were selected. None of them had cases of cancer in the family.

3.2.3. Breast cancer patients

The group consisted of 40 women with locally advanced breast cancer. The samples were collected randomly. Out of these 40 patients 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.

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 Narayanswamy Cancer Centre, G.Kuppusamy Naidu Memorial Hospital, Coimbatore. A questionnaire was prepared to collect information related to their life style such as dietary habits, reproductive history and medical history, exposure to the chemicals and physical agents and consanguinity.

3.2.4. Chemicals

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. All the chemicals used were of analytical grade.

3.2.5. Instruments

Electrophoresis tank

Horizontal electrophoresis tank from Genie (Bangalore) was used, with power pack adjusted for needed 25 V.

Microscope

Transmission microscope, Hund Wilovert S (Germany) was used to score the slides with a fixed ocular micrometer. Nikon Coolpix 4500 was used to take the pictures of comet cells for the study.
Conventional glass microscope slides and 22 x 55 mm cover slips from blue star have given the reliable and reproducible comet assay results.

3.2.6. Experimental design

The randomly collected samples were divided into four groups according to the mode of treatment they underwent, on the basis of information filled in the questionnaire. The treatment given were,

1. Chemotherapy alone
2. Chemotherapy and surgery
3. Chemotherapy and radio therapy
4. Chemotherapy, surgery and radiotherapy

The blood samples were lysed and electrophoresed to assess the degree of DNA migration in different modes of treatment.

3.2.7. Single Cell Gel Electrophoresis

The Single Cell Gel Electrophoresis also knows as the Comet Assay is the technique employed to detect the damage and repair at the level of single cell. This is one of advanced technique in the oncology.

The relevance of the single cell gel assay lies in its requirement for a reduced number of cell samples and its ability to evaluate DNA damage in non-proliferating: In contrast to cytogenetic bio-monitoring studies which are mainly done with lymphocytes, the single cell gel assay can be used with any cell population which constitute an advantage for these kind of studies. The SCGE is a novel approach for the assessment of DNA strand breakage in single cell (Fig.1).

3.2.7.1. Principle
The single cell gel assay allows the detection of DNA alteration of diverse kinds such as double strand breaks, single strand breaks, alkali-labile site, incomplete repair sites and cross links. In the single cell gel assay the individual cells are embedded in agarose and lysed to unwind their DNA. The DNA is then subjected to a gel electrophoresis procedure under low voltage so that fragmented and relaxed DNA migrate further than intact or cross linked DNA, resembling the image of a 'comet'. The formation of comet...
Fig.1. Schematic representation of steps involved in alkaline comet assay
like image is due to the migration of negatively charged DNA fragments towards the positive pole. The extent of migration of the "tail" of the comet is related to the increased DNA damage.

These DNA comets can be visualized after silver staining under any transmission microscope. These images can be analyzed and compared in a cell-to-cell basis. The head length and tail length parameters can be used to measure the DNA damage individual cells. The assay was performed as described by Singh et al. (2000) with minor modifications.

3.2.7.2. Preparation of slides

For the comet assay the gels were prepared in the phosphate buffered saline.

Preparation of NMPA

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>-</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>-</td>
<td>0.288 g</td>
</tr>
<tr>
<td>KCl</td>
<td>-</td>
<td>0.05 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>-</td>
<td>0.05 g</td>
</tr>
</tbody>
</table>

To the above contents added 25ml of distilled water and mixed well. To this, 195mg NMPA was added and melted in the microwave oven. Divided them in separate aliquots and stored in cool place (4°).

Preparation of LMPA

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>-</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>-</td>
<td>0.288 g</td>
</tr>
<tr>
<td>KCl</td>
<td>-</td>
<td>0.005 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>-</td>
<td>0.005 g</td>
</tr>
</tbody>
</table>
The ingredients were dissolved in 25 ml of double distilled water and mixed well. To this PBS, added 125 mg of LMPA and melted it in the microwave. Separated in aliquots and store at low temperature.

**Slide layering**

The ultimate goal of slide preparation was to obtain uniform gel sufficiently stable to survive throughout the data collection as well as to ensure visualized comet with minimal background noise. The slides were labeled prior to the layering. The numbers of agarose layer used per slide are three in the following manner

**First layer / Basal layer**

When the NMPA was hot 90 ml of agarose was taken and smeared for equal and uniform layer on the slide. This was the basal layer or frosted layer. The slides were left on a flat surface to dry.

**Second layer**

After drying, to the coated / frosted slide added another 90ml of normal melting point agarose and placed coverslips (22x50 ml) on the slides for equal layering. The slides were kept in the refrigerator (4°C) until the gel hardened (20-30 min).

**Third layer / Casting gel with cells on the slide**

After the gel layer hardened at lower temperature, the cover slip was taken out and a third layer of 70ml LMPA mixed with ~10,000 cells in 15 µl blood was added. The coverslip was replaced immediately and placed the slide on the slide tray, allowing the layer to settle down by keeping it back into the refrigerator (4°C). The concentrations of cell in agarose as well as the concentration of agarose were important parameter for ensuring a successful analysis.

**3.2.7.3. Lysing**

**Preparation of lysing stock**

- **NaCl**: -102.27 g
- **EDTA**: -26.04 g
<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>-0.84 g</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>-8.4 g</td>
</tr>
<tr>
<td>Sodium lauryl sulphate</td>
<td>-7 g</td>
</tr>
</tbody>
</table>

The above ingredients were added to about 700 ml ddH$_2$O and NaOH pellets were added. The solution was made up to 890 ml with ddH$_2$O and stored at room temperature.

**Working solution**

To the lysing stock solution, added 10% DMSO and 1% Triton X-100.

Coverslip was removed and slide was lowered slowly into the cold, freshly made lysing solution. For lysing, the slides were kept overnight at low temperature. The chilled lysing solution was used to maintain the stability of the agarose gel.

3.2.7.4. **Neutralization**

After overnight lysis of the lymphocytes, the slides were removed gently from the lysing solution. Rinsed slide carefully in Tris buffer (3x5 min) to remove detergents and salt. The neutralization buffer was dropped over the slides and rinsed carefully.

3.2.7.5. **Alkali unwinding**

Prior to electrophoresis, incubated the slides in the alkaline (pH>13) electrophoresis buffer for atleast 30 minutes to produce single strand DNA breaks and to express alkali labile sites.

Preparation of alkaline solution.

10N NaOH - (100 g/ 250 ml ddH$_2$O)

200mM EDTA - (7.445 g/100 ml ddH$_2$O)

Stock solution was prepared and stored at the room temperature.

For 1X buffer, 15 ml NaOH and 2.5ml EDTA was added, made up to 500 ml and mixed well.

3.2.7.6. **Electrophoresis**
After the alkali unwinding, the single stranded DNA in the gels was electrophoresed under alkaline conditions to produce comets. The alkaline buffer used during electrophoresis was the same (pH>13) buffer used during alkali unwinding. The slides were kept in the electrophoresis tank as close as possible and the bubbles over the gels were avoided. The power supply of 25 Volts was adjusted and the slides were electrophoresed for 15 min.

3.2.7.7. Neutralization

Turned off the power supply. Gently lifted the slides from the buffer and placed on a drain tray. Dropwise coated the slides with neutralization buffer, let it for at least 5 minutes. Drained the slides and repeated two more times. However, increased rinsing may be useful in situations where a high background was seen during scoring.

3.2.7.8. Silver staining

Preparation of solutions

Fixing solution

- Trichloroacetic acid - 37.5 g
- Zinc sulphate-7-hydrate - 12.5 g
- Glycerol - 12.5 ml

Mix the above contents with 250 ml ddH₂O.

Staining solution A

Dissolved 25g of Na₂CO₃ in 500 ml of ddH₂O and make a clear solution.

Staining solution B

- Ammonium Nitrate - 100 mg
- Silver Nitrate - 100 mg
- Tungstosilic acid - 500 mg
- Formaldehyde - 250 μl

Mixed the above ingredients in 500 ml ddH₂O in the given order and stir lightly to make a clear solution.
**Stopping solution**

0.5 ml glacial acetic acid was added in 45 ml of distilled water.

**Working staining solution**

Prepared fresh solution by adding 68 ml of solution B to the 32 ml of solution A.

**Fixing**

After draining the neutralizing solution, the slides were fixed by pouring the fixing solution dropwise. The slides were drained 2 more times, as in the neutralization.

After fixing, the slides were washed with H₂O several times to ensure the removal of excess fixative. The slides were then allowed to dry at room temperature for at least 1 hour. The fixed slide then left at 37°C overnight or until stained.

**Staining of the slides**

The freshly prepared staining solution was poured over the dried gel slides. The slides were stained for at least 45 min with gentle agitation and stained until a grayish colour developed on the slides.

The slides were then washed twice by dipping them in the distilled water and then transferred to the stopping solution (1% glacial acetic acid). The slides were taken out from stop solution after two minutes and again rinsed in the dd H₂O. The slides were kept for drying and as the silver stain does not fade on storage, the slides were stored in a dust free environment for long periods.

**3.2.7.9. Evaluation of DNA damage**

The stained slides were scored by observing them under transmission microscope. The cells were scored according to the degree of damage or the migration of DNA from cells after electrophoresis. The proportion of damage profile of DNA was assessed by visual scoring of total length and the head length of the cells (i.e. from the beginning of the head to the end point of the tail).

For each sample at least 50 cells were counted randomly and the slides were observed at the 40x objective magnification. The metric used for the length of DNA
migration was in microns using ocular micrometer in the microscope eyepiece and stage micrometer.

The frequency of round cells, cells with short tails, cells with medium long tails and cells with long tails were counted among 50 cells and analyzed. And cells according to their tail length were categorized into undamaged, primary, secondary and tertiary damaged.

Categorizing cells as undamaged (i.e., no migration), short migration, long migration and complete migration (i.e., no nucleus remaining), helped to calculate the viability of the cells.

3.2.7.10. Interpretation

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).

3.2.8. Statistical analysis

The data obtained for tumour 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.

Phase - III

3.3. Antimutagenic activity of pomegranate

3.3.1. Collection of the samples

Pomegranate fruits (Fig.2) were procured from a local market.

3.3.2. Preparation of the peel and seed powder

The peels and seeds of pomegranate were washed in water and shade dried. Dried peels and seeds separated from fruits were powdered separately using an electrical grinder. Fine powder (Fig.3) was obtained by sieving.
3.3.3. Soxhlet extraction

10g of the peel and seed powders were weighed using an electrical balance (Denver 210) and made into 8 packets using xerohaze filter paper (10 A grade SD's). Soxhlet extraction (Fig.4) of powdered peels and seeds were carried out to obtain their extracts. Petroleum ether, chloroform, methanol and water were used as solvents for soxhlet extraction in the increasing order of polarity. The distillation process was carried out at a low temperature of 40°C. After evaporation of solvents, corresponding residues were obtained and stored in the refrigerator for further use.

3.3.4. Preparation of extracts

100 mg of soxhlet extract was dissolved in 2 ml of DMSO (Dimethy1 sulfoxide) and then mixed with 100 ml distilled water and this formed 1000 ppm solution. From the stock solution, solutions of required concentration were prepared and used in this study.
Fig. 2. A view of pomegranate tree with fruits

Fig. 3. Pomegranate peel and seed powders

Fig. 4. Soxhlet apparatus
3.3.5. Bacterial tester strains and chemicals

*Salmonella typhimurium* TA100 and TA98 tester strains were generously donated by Professor B.N. Ames (University of California, Berkeley, U.S.A). All the chemicals used are of analytical grade from Himedia and Qualigens Fine chemicals, India.

3.3.6. Ames Salmonella microsome assay (Maron and Ames, 1983)

Ames Salmonella microsome assay was carried out for pomegranate peel and seed extracts for determining the mutagenicity / antimutagenicity.

### Principle

A set of histidine requiring strains is used for mutagenicity / antimutagenicity testing. These strains are incapable of growth in the absence of histidine in the growth medium. When a mutagen is added to the culture, the strains are mutated back, thereby losing the histidine dependence for its growth. The number of revertant colonies resulting after the actions of mutagens depends upon the potency of the compound. The type of mutation in the histidine operon in the strains are different, thereby enabling the identification of frame shift mutagens, those which are mutagenic towards strain TA98, base pair substituting mutagens, those which are mutagenic towards strain TA100.

### Spontaneous reversion

Spontaneous reversion of the tester strains of histidine independence is measured routinely in mutagenicity / antimutagenicity experiments and is expressed as the number of Spontaneous Revertants per plate. Each tester strain reverts spontaneously at a frequency that is characteristic of the strain.

Each mutagenicity / antimutagenicity assay conducted included control plates without the test compound to assess the Spontaneous Revertants(SR) frequency.

### Determination of the number of bacteria
The bacterial culture was diluted 1:200,000. 100 ml of this diluted culture and 500 ml of histidine enriched KCl were added to 2 ml molten top agar, mixed and poured onto minimal basal agar. Incubated at 37°C overnight and the colonies were counted. The properties and genotypes of the tester strains of *Salmonella typhimurium* is given in Appendix-III.

3.3.7 Preparation of solutions for the mutagenesis/antimutagenesis assay minimal glucose (basal agar) plates

For the mutagenesis/antimutagenesis assay the minimal glucose agar medium was prepared as follows.

**Solution A**

2.625 l water in water bath
citric acid 190.48 g/l
\[ \text{K}_2\text{HPO}_41247.62 \text{ g/l} \]
\[ \text{NaNH}_4\text{PO}_4333.3 \text{ g/l} \]

The substances were best soluble when added in the given order. Made sure that one is completely dissolved before adding the next. Filled screw-capped bottles in 400 ml portions and autoclaved. Solution could be kept any length of time. Risk of contamination by opening of bottles was minimal, since bacteria cannot grow well in concentrated salt solution.

**Solution B**

Dissolved 20 g MgSO$_4$. 7H$_2$O (MW 249.48) in 1000 ml water, filled in 200ml portions and autoclaved. Solution can be kept indefinitely at room temperature. Opened bottles were discarded, since there is a risk of contamination.

**Solution C**

In a 2 liter flask, autoclaved 30g of Bactoagar and 1500 ml water containing a stirring rod.

**Solution D**
Autoclaved 40 g glucose and 500 ml water. Allowed the solution to cool for 20-40 minutes.

**Basal agar**

Combined solution C and D and added 40 ml of solution A and 10 ml of solution B. Mixed well and poured into sterile petriplates @ 25 ml/plate.

**Nutrient broth for culturing of bacteria**

Mixed 25 g nutrient broth in 1 liter water. Filled in 20-40 ml portions, autoclaved immediately. It could be stored for any length of time.

**Histidine-Biotin solution**

(450 ml Histidine, 512 ml biotin, 250 mM sodium phosphate buffer)

350 mg/1-histidine, HCl, 625 mg D-Biotin

Dissolved in about 3 liters of 250 mM sodium phosphate buffer, pH 7.4.

**Histidine-Tryptophan enriched KCl**

Dissolved 2.8 mg histidine, 2.8 mg tryptophan per ml KCl solution and filtered sterile.

**Top Agar**

30g Bacto agar 0.6%

30g NaCl 0.6%

5 liters water

Autoclaved the solution

**HB-TopAgar**

Allowed the top agar to cool a bit. To 10 volumes of top agar, added 1 volume HB solution and mixed well. Distributed into 2 ml portions in sterile tubes and held at 45°C to 50°C.
3.3.8. Effect of samples on the mutagenicity / antimutagenicity of *Salmonella typhimurium* TA100 and TA 98

The mutagenicity/antimutagenicity of the samples were carried out in the absence of metabolic mixture. The testing can be done as follows.

The Standard Mutagen i.e., Sodium azide for TA 100 and Daunomycin for TA 98 in the absence of S9 mixture were plated along with the samples.

The concentration of Standard Mutagen used was as follows.

- Sodium azide: 10 µg/plate
- Daunomycin: 6µg/plate

The results of the Ames test are expressed as number of revertants / plate.

**Summary of the assay**

HB-top agar was distributed into 2 ml portions and held at 45°C. To the top agar, depending on the test group, the following were added in succession.

- A 100µl of overnight grown culture
- B 10µl of sample
- C 10µl standard Mutagen

**Spontaneous Revertants (SR):** A alone
**Mutagenicity / antimutagenicinity of compound:** A and B
**Standard Mutagen (SM):** A and C
**Mutagenicity / antimutagenicity of the sample:** A, B and C

**Culturing the bacteria**

The night before the mutagenicity / antimutagenicity experiment, inoculated nutrient broth with the cells scrapped from the master plates. Incubated overnight in a shaker incubator at 37°C.
3.3.9. Mammalian microsome assay (Metabolic Activation System)

The S9 fraction of the liver homogenate from mouse induced with Phenobarbital along with co-factors was used as the metabolic activation system.

3.3.10. Induction of drug-metabolizing enzyme in mouse

Most carcinogens are inactive when present in the environment. Upon entering the system they are converted to active metabolites by the carcinogen metabolizing enzymes. The enzymes are of 2 major types (1) the activating enzymes and (2) the detoerifying enzymes. The components of the drug metabolizing system include ary1 hydrocarbon (benzo (a) pyrene) hydroxylase (AHH), cytochrome b5 and cytochrome P amino pyrene N-demethylase (APNID), ethoxy resorufin-o-dithylase (ERRD), epoxide hydroxylase (EH), glutathione-s-transferase (GST) and reduced glutathione. Many chemicals are used to induce the drug metabolizing enzymes. The most commonly used inducers include poly chloro bi-phenyl mixture (Aroclor, 1254) and Phenobarbitone (PB). In the present investigation, PB was used according to the methods reported earlier (Kunz et al., 1987).

Swiss Laca male mouse 8-10 weeks old weighing about 25 g was injected with PB at a dose of 1 mg/g body weight intra peritoneally for three consecutive days.

Preparation of S9 fraction

After PB administration for 3 consecutive days, on the 4th day the animal was killed by cervical dislocation, following overnight fasting. The liver was quickly excised using sterile surgical tools, washed with cold isotonic Kcl blotted dry between sterile filter paper folds and weighed. A 20% homogenate was prepared in cold isotonic Kcl. The homogenate was spun at 9000 xg in a refrigerated centrifuge at 4°C for 15 minutes. The supernatant (S9 fraction) was distributed into sterile 2 ml vials after adjusting the protein concentration to 40 mg / ml.

Protein estimation by Lowry’s method

Protein was estimated according to the method of Lowry et al. (1951).

Principle
The protein sample was suspended in 1ml of 1N NaOH at 100ºC for 4-5 minutes. 5 ml of alkaline copper reagent was added to it and the mixture was allowed to stand at room temperature for 10 minutes. 0.5 ml of Folin-ciocalteu reagent was added rapidly and the contents in the tube was mixed thoroughly.

The amount of protein in the sample was calculated with a standard curve prepared using bovine serum albumin. The tube was mixed thoroughly.

The amount of protein in the sample was calculated with a standard curve prepared using bovine serum albumin.

**Preparation of the co-factors and the S9 mixture**

The cofactors used are MgCl₂, KCl, Glucose-6-phosphate, NADP and phosphate buffer.

The S9 mixture was prepared fresh just prior to each as follows.

- **S9** - 0.1 ml
- **MgCl₂** - 0.8 m moles
- **KCl** - 33.0 m moles
- **G-6-PO₄** - 5.0 m moles
- **NADP** - 4.0 m moles
- **0.1 M phosphate buffer** - 100 m moles
- **Phenobarbitone** - 1mg/plate

The S9 fraction was stored in deep freezer (-20ºC).

The stock solution of the cofactors was prepared and stored as described by Maron and Ames (1983). The S9 mixture prepared fresh was held on ice throughout the assay. The sterility was tested by plating the S9 mixture on the minimal glucose plate. The use of optimum amount of S9 mixture is important in testing the mutagenicity / antimutagenicity of a compound. In the present assay, 50 µl of S9 mix per plate was found to be optimum and hence the concentration was used throughout the assay.

**Statistical analysis**
The data observed with respect to different studies were scrutinized and subjected to student 't' test.

Phase - IV

3.4. Chromosomal aberration studies using PFE in peripheral blood lymphocytes of breast cancer patients

3.4.1. Preparation of the Pomegranate Fruit Extract (PFE)

The arils of pomegranate were washed in water and shade dried (Fig 5,6). Dried arils were powdered separated using an electrical grinder. Fine powder was obtained by sieving.

Soxhlet extraction of powered arils were carried out to obtain their extracts. Petroleum ether, chloroform, methanol and water were used as solvent for soxhlet extraction in the increasing order of polarity.

3.4.2. CHEMICALS AND CULTURE MEDIUM

The medium used in this study was mc coy's 5a. medium powder (12 gms) was dissolved in one litre of sterile double distilled water.
Fig. 5. Pomegranate, *Punica granatum* fruits

Fig. 6. Pomegranate arils used for extraction
Fig. 7. Human peripheral blood lymphocyte culture - experimental setup

Fig. 8. Human blood lymphocyte culture treated with PFE - 48 hours
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 filterpads in a seitz filter into a sterile flask. streptopenicillin (0.2 ml / 100 ml) was added to the filtrate to prevent fungal growth and the filtrate was stored at -20°C.

fetal bovine serum

commercially available fetal bovine serum (fbs) (sigma) was used for the leukocyte culture.

phytohaemagglutinin (pha)

the mitogen pha was prepared in the laboratory using red kidney bean - *phaseolus vulgaris*. 25 g of the seeds were surface sterilized in boiling water for few minutes and soaked overnight in 100 ml of sterile ringer's solution in a conical flask. the seeds were ground well for 15 minutes and the paste obtained was centrifuged at 1000 rpm for 20 minutes. the supernatant was collected and spun again. to this, 10 to 20 mg of fungizone (mycostatin) was added. this served as the stock solution and was stored in the deep freezer. 1 ml of the stock solution was diluted with 9 ml of sterile distilled water to give the working solution.

giemsa stock and working solutions

giemsa powder (1.0 gm) was dissolved in 53 ml of glycerol which was mixed thoroughly for 2 hrs over a magnetic stirrer kept at 56°C. the solution was allowed to cool until it reached the
room temperature and then 83 ml of methanol was added and mixed well overnight using the magnetic stirrer. It was filtered using whatman no.1 filter paper and was stored at 4°C. Working stain solution was prepared by adding 2.0 ml of stock solution and 2.0 ml of di-sodium hydrogen orthophosphate (10% solution) to 46.0 ml of double distilled water (pH 6.8).

3.4.3. Establishment of the culture

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 (about 30 drops) 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 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 (fig. 7).

3.4.4. Treatment details

Forty eight hours cultures of lymphocytes were treated with 3 µl of pfe individually and in binary mixtures and incubated for further 24 hours. After 72 hours, the culture of 60ml of 0.4 mg/ml working solution of colchines to arrest the dividing cells (fig. 8).

3.4.5. Harvesting of culture

The dividing cells were arrested at the metaphase stage by adding 0.05 ml of colchicine solution (0.01 %) at 30 minutes
before harvesting the culture. The contents of the vials were centrifuged at 1000 rpm for 20 minutes at the end of colchicine treatment. The supernatant was discarded and 6 ml of pre-warmed hypotonic solution (0.075 m kcl) was added to the test tube after disturbing the cell bottom. The contents of the test tubes were incubated for 7 minutes. After incubation, 1 ml of freshly prepared fixative (methanol and glacial acetic acid (3:1 v/v) was added and centrifuged at 1000 rpm for 10 minutes. Later, the supernatant was discarded and two or three changes of the fixative were given to obtain a colourless cell pellet.

3.4.6. Cleaning and preparation of slides for culture

New slides were allowed to remain in chromic acid overnight. These slides were then kept in a horizontal coupling jar under running tap water for 2-3 hr. The slides were finally rinsed in double distilled water and stored in 70 % ethyl alcohol. Before using the slides for spreading, they were wiped and dried.

3.4.7. Preparation of chromosome slides

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.

3.4.8. Chromosomal analysis

Fifty well spread metaphase plates of each subject were screened under oil immersion lens of the optical microscope and selected metaphases were photographed.

3.4.9. Statistical analysis

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.