CHAPTER - 1

MATERIALS AND METHODS

*In vitro study- Human lymphocyte cultures*

- Chromosomal Aberration analysis: (CAs).
- Sister Chromatid Exchange analysis: (SCE)
- Cell cycle kinetics (RI)

*In vivo study- Albino mice bone marrow cells.*
INTRODUCTION

Cancer is a dreadful human disease, all cancers are caused by abnormalities in the genetic material of the transformed cells and these abnormalities may be due to the effect of carcinogens such as tobacco, smoke, radiation, chemicals or infectious agents. Nutrition and diet are regarded as major factors contributing to human carcinogenesis (Sugimura 2000). Cancer kills annually about 3500 people per million populations around the world and approximately 47% of all cancers patient are present in the India sub-continent. Various exhaustive studies have been done in previous decades, but desired success is not achieved so for, presently new dimension has been added to cancer research by inclusion of phyto-products for their anti cancerous properties. At least three approaches to the prevention of cancer can be envisioned, foremost is to reduce human exposure to environmental carcinogens through careful monitoring around dwelling and through education to
encourage changes in life style. The second approach is to identify individuals at high risk of cancer development through predisposing genetic or biochemical factors, followed by appropriate clinical follow-up. Third chemoprevention for several reasons has received growing consideration as a means of cancer control. Preventive effects of naturally occurring dietary elements against tumor induction and growth have been demonstrated (Wattenberg, 1996; Kim & Masuda, 1997; Siess et al., 1997; De Marini, 1998; Surh, 1999; Ferguson et al., 2004). Such dietary compounds preferably originating from dietary constituents such as fruit vegetables and shrubs are referred as chemopreventive agents. Chemoprevention is used to lower the rate of cancer incidence (Tanaka, 1997). The eminent fact that fruit and vegetable intake is associated with a reduced risk of cancer (Wargovich, 1997; Siess et al., 1997; Ames, 1998). Although more than 1500 anticancer drugs are in state of active development, with over 500 of the drugs are under clinical trials. Considering the severity of the disease it needed continuous attention at all level of prevention, particularly for search of phytochemicals for chemoprevention. Plants have long been used in the treatment of cancer (Hartwell, 1982). At National Cancer Institute about 35,000 plant samples from 20 countries were collected and 114,000 extracts were screened for
anticancer activity (Shoeb, 2005). Before 1983 approximately 92 anticancer drugs were commercially available in the USA and among world wide approved anticancer drugs between 1983 and 1994, 60% are of natural origin (Cragg et al., 1997). Natural origin is defined as natural products, derivatives of natural products or synthetic pharmaceuticals based on natural product models (Jaspars and Lawton, 1998). In India, there are only limited studies on anticancerous properties of herbal extracts, they are too confined to tomato, garlic and neem extract. The use of coastal plants extracts for anti cancer research is largely ignored.

Coastal plants extracts has very significant role in anticancer drugs. The coastal vegetation is categorized into three major divisions Mangroves, salt-marshes and sand-dunes based on occurrence and adaptations. The mangrove forest constitute mostly trees or shrubs predominantly occuring in muddy soil substrate of inter-tidal areas, lagoons, estuaries and backwaters in tropical and sub tropical countries. The salt mares are plant communities of grasses, herbs or shrubs, which grow in wet soil substrates that are alternately inundated and drained by tidal action. The sand dune vegetation occupies dry sandy area of coast. (Kathiresan K et al., 1997). All the vegetation thrive under extreme coastal environment by virtue of synthesizing stress induced metabolites
belonging to chemical groups like steroids, triterpenoids, saponins, flavonoids, alkaloids and tannins. These coastal plant-derived phytochemicals are mostly unexplored for anticancer property. Thousands of herbal and traditional compounds are being screened worldwide to validate their possible use as anti-cancerous drugs.

Current research indicates that other dietary variables, including total caloric intake, anticarcinogenic substances and exposures to carcinogens that occur naturally in foods or are induced during preparation, may be more relevant determinants in the diet – cancer relationship.

**METHYL METHANE SULPHONATE (MMS)**

Methyl methanesulfonate is an ester of sulfuric acid that exists as a colourless liquid at room temperature. It is soluble in water, dimethyl formamide, and propylene glycol, but only slightly soluble in nonpolar solvents. Methyl methanesulfonate is stable under normal temperatures and pressures, but it forms irritating corrosive compounds or toxic gases in the presence of fire (IARC 1974, Akron 2009). Methyl methanesulfonate is used experimentally as a research chemical and as a solvent catalyst in
polymerization, alkylation, and esterification reactions (IARC 1974, Wyatt and Pittman 2006). It has been tested as a cancer chemotherapeutic agent.

Production of methyl methanesulfonate is limited, because it is used only in research (IARC 1974, 1999). Methyl methanesulfonate is not produced commercially in the United States (IARC 1999, HSDB 2009). Methyl methanesulfonate was tested for carcinogenicity in rats by subcutaneous and intraperitoneal injection, producing local tumors and tumors of the nervous system. Following oral administration to mice, it increased the incidence of lung tumors and of lymphomas. In rats, it produced neurogenic tumors after administration of a single dose as well as following prenatal exposure (IARC, 1974). Methyl methanesulfonate is potential human carcinogen as evident from studies in experimental animals.

Methyl methanesulfonate caused tumors in mice and rats at several different tissue sites when administered through several different routes. Exposer of methyl methanesulfonate through drinking water caused benign lung tumors (adenoma) and lymphoma of the thymus in male mice. In male rats, subcutaneous injection of methyl methanesulfonate caused cancer at the injection site (squamous-cell carcinoma and polymorphic-cell sarcoma), and 1 of 12 rats developed kidney cancer (nephroblastoma). A
single intraperitoneal injection of methyl methanesulfonate caused tumor of the nervous system (malignant neurofibroma, astrocytoma, malignant neurinoma etc.) in adult rats of both sexes and in the offspring of pregnant rats exposed (Clapp et al, 1968, IARC 1974). Since methyl methanesulfonate was listed in the Sixth Annual Report on Carcinogens, additional studies in rodents have been identified. In female mice, subcutaneous injection of methyl methanesulfonate caused cancer at the injection site (sarcoma) (Segal et al, 1987). In male rats exposed of methyl methanesulfonate by inhalation for six weeks causes significantly increase in the incidence of nasal tumors (mainly squamous-cell carcinoma) (IARC 1999).

Methyl methanesulfonate induced mouse germ cell mutations, chromosomal aberrations, DNA damage, micronuclei, sister chromatid exchanges. In somatic cells of rodents under in vivo chromosomal aberrations was observed. In in vitro studies of human and rodent cell cultures, it increased the frequency of DNA damage, gene mutation, sister chromatid exchanges and micronuclei as well as chromosomal aberrations. If released to air, methyl methanesulfonate will exist in the vapour phase and will react slowly with hydroxyl radicals, with a half-life of 69 days. If released to a moist environment, it will hydrolyze
with a half-life of 4.56 hours at 25°C. It is not expected to bioconcentrate in aquatic organisms or volatilize from water (HSDB 2009).

In other studies on lower animals, Methyl methanesulfonate induces somatic and sex-linked mutations in *Drosophila* and DNA damage in *Escherichia coli*.

We considered that methyl methanesulfonate is a direct-acting methylating agent which is mutagenic in a wide range of *in-vivo* and *in-vitro* test systems.

**DEFINITION OF THE PROBLEM AND OBJECTIVES OF THE PRESENT WORK.**

Nowadays man is being exposed to a large number of mutagenic substances through environment and diets. Epidemiologic studies suggest a strong link between cancer and environment that include all contributory factors other than genetic susceptibility, such as exposure to chemical hazards in the workplace, sunlight and ionizing radiation air and water pollutants, reproductive and sexual behaviour, alcohol and tobacco use and diet. Experiments using short-term genotoxicity assays on laboratory animal have uncovered a wide array of food-borne compounds that elicit mutations, transform cell growth characteristics and increase cancer rates.
Such chemicals with potential human concern are encountered in the food supply from a variety of sources.

Some coastal and other anticancerous plants can reduce or completely prevent the risk of chemical carcinogenesis. The role of some plants, vegetables and fruits in reducing the risk of cancer were highlighted in a number of studies. Prescriptive and proscriptive approaches for cancer prevention in relation to some medicinal plants are important to reduce the incidence of cancer. Plants like Morinda citrifolia, Terminalia catappa, Alstonia scholaris, Accanthus illicifolius, and Ceasalpinia bundcella have proved antimutagenic and antigenotoxic potential.

Keeping in view the practical applications of these compounds, antigenotoxic study through a battery of in vivo and in vitro assays have been attempted. Methyl methane sulphonate (MMS) was taken as inducer of genotoxicity (-ve control), where as, Morinda citrifolia, Terminalia catappa, Alstonia scholaris, Accanthus illicifolius, and Ceasalpinia bundcella were used as antigenotoxic agents. These natural plants had been used with a view to study the following

**OBJECTIVES:**

To study the effects of these anticancerous plants, their extract were analysed under *in vivo* i.e. treatment of mice and analysis of C.A. in the bone
marrow of mice have been done.

- To study the anticarcinogenic effects of anticancer plants extracts on human chromosomes.

- The evaluation of the chromosome damage and counter effect of anticancer plants extract have been paralleled with the presence of metabolic activation (S9 mix).

- To study the counter-genotoxic effects of anticancer plants extracts on the frequency of sister chromatid exchange (SCEs).

- The effect on cell growth kinetics was ascertained in the presence and absence of S9 mix.

**MATERIALS AND METHODS**

The *in vivo* and *in vitro* evaluations of the antimutagenic and antigenotoxic potentials of five coastal plants were done using mice bone marrow cells and human blood lymphocytes respectively. The albino mice were healthy and free from any diseases. The adult volunteers were in 20-25 age groups and free from exposure to drugs, alcohol and irradiations. Extract of these coastal plants was tested on the bone marrow cells using chromosomal aberrations and number of aberrant cells as marker. In *in vitro* studies, chromosomal aberrations, sister chromatid exchange and cell cycle kinetics were taken as marker. These experiments were conducted with and without exogenous metabolic activation system.
DESCRIPTION OF TEST PLANTS

In the present study, five coastal medicinal plants namely *Morinda citrifolia*, *Terminalia catappa*, *Alstonia scholaris*, *Acanthus ilicifolius* and *Ceasalpinia bonduc*, are selected as antigenotoxic agents against the Methyl methanesulphonate (MMS) induced genotoxicity.

**METHYL METHANE SULPHONATE.** Methyl methanesulfonate is a laboratory chemical with potential mutagenic properties.

**Physiochemical data**

Methanesulfonic acid, methylester, synoname - MMS.

\[
\text{O} \\
\text{H}_3\text{C} - \text{S} - \text{O} - \text{CH}_3 \\
\text{O}
\]

Discription: --- Colourless liquid.

Molecular formula --- \( \text{C}_2\text{H}_6\text{O}_3\text{S} \)

Molecular weight --- 110.13 g/mol.

Boiling point --- \(202.5^\circ\text{C}\)

Melting point --- \(20^\circ\text{C}\)

Solubility --- Soluble in water, DMSO or Methanol

**Toxicology and Safety data:**

MMS: \(\text{LD}_{50} = 0.4\) mg/kg (Oral, duckling).
MMS is a Methyl methanesulfonate (MMS) is an alkylating agent and a known carcinogen. It is also a suspected reproductive toxicant, and may also be a skin/sense organ toxicant. In 1974 the IARC classified the MMS as carcinogens. Methylmethanesulphonate are potent liver carcinogens and DNA damaging agents. Originally, this action was believed to directly cause double stranded DNA breaks. In the present study extra precaution was taken during the handling of this chemicals, mask and gloves were used and all preparations were done in the laminar flow hood to avoid any direct contact.

**Morinda citrifolia (Noni) Extracts:**

Higher plants are extensively used in traditional medicines, presently these are analysed for their role in modulating the activity of environmental genotoxicants. It is evident from data that out of total chemical structures discovered, a major share was directly derived from higher plants (Kinghorn and Balandrin 1993, Sarkar and Sharma *et al*, 1996) and several of them can be used as antitumour agents (Mukherjee *et al*, 2001).

An edible tropical plant, *Morinda citrifolia (Noni)* is a traditional medicinal plant belonging to the family Rubiaceae. It is a small fruit-bearing shrub mostly found in Southeast Asian region and believed to
have spread across Australia and Pacific Islands (Brown, 2012). The Noni plant is a small evergreen, usually found 1300 ft above the sea level and spotted growing along lava strewn formed by effusive eruption (Wang et al, 2002a). It is characterized by its elongated trunk with 8-10 inches long bright green leaves (Rivera et al, 2012). It bears peculiar “grenade-like” unripe green to ripe yellowish gawky fruit which softens to lucid when ripe. Due to its lousy taste and odour, it is not preferred to be eaten by the people (Su et al, 2005). The ancestors of Polynesians, who are believed to have brought Noni from south Asia, have utilized the whole Noni plant in various combinations (Brown, 2012). The roots, leaves, bark and the fruit are widely used for its medicinal values to treat various ailments including breast cancer and eye problems (Su et al, 2005). The fruit juice is of high demand in unconventional medicine for the treatment of muscle aches, gastric problems, treat blood pressure, headaches and mental depression and in menstrual difficulties (Brown, 2012). Mouse treated with Noni juice showed enhanced immune system activity by stimulating the production of macrophages and lymphocytes (Kirti, 2007). Bushnell et al (1950) reported that Noni was a traditional remedy used to treat broken bones, deep cuts, bruises, sores, and wounds. Today, in United States, both leaves and fruit are sold in health centres and grocery as juice, tablets
and as a tea (Su et al., 2005). The roots of Noni contain natural sedative and anti-malarial activity (Kirti, 2007).

**Chemical properties of Morinda**

A number of phyto compounds have been extracted in the Noni plant such as scopoletin, octoanoic acid, potassium, vitamin C, terpenoids, alkaloids, anthraquinone (nordamnacanthal, morindone, rubiadin, andrubiadin-1-methyl ether, anthraquinone glycoside), ß-sitosterol, carotene, vitamin A, flavones, glycosides, linoleic acid, alizarin, amino acids, acubin, L-asperuloside, caproic acid, caprylic acid, ursolic acid, rutin and a putative proxeronine. (Levand and Larson, 1979; Farine et al., 1996; Peerzada et al., 1990; Budhavari et al., 1989; Moorthy and Reddy, 1970; Daulatabad et al., 1989; Balakrishnan et al., 1961; Legal et al., 1994; Singh and Tiwari, 1976; Simonsen, 1920; Heinicke, 1985). The dominant substances in the fruit are fatty acids, while the roots and bark contain anthraquinone. The seed of Morinda citrifolia contains 16.1% Oil. The main fatty acid components of the oil were linoleic (55%), Oleic (20.5%), Palmitic (12.8%), Ricinoleic (6.8%) and Stearic acids (4.9%) (Dualatabad et al, 1989; Seidemann, 2002).

In the past few years, considerable advancements were made in the natural products endowed with antimutagenic and anticarcinogenic properties. Many natural products considered as dietary chemopreventive
compounds, have a great potential in the fight against cancer by different mechanisms, including antioxidant, antimutagenic activity, enzyme modulation, gene expression, apoptosis etc (De Flora et al. 2001, Webb and Ebeler 2004, Miadokova et al, 2008).

The whole plant of *Morinda* species was collected after proper identification by a competent plant taxonomist from Department of Botany, S.N (PG) College, Azamgarh, (U.P). The whole plant was powdered in a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) in a round bottom flask for 36 hrs at 60°C. The liquid extract was filtered, cooled and concentrated by evaporating its liquid contents in oven and collected. The powdered extract, termed Morinda extract (ME), was re-dissolved in DDW for the oral administration to the mice. The required dose for *in vivo* treatment was prepared by dissolving the extract in DDW in different concentration. A solution was also prepared in DMSO for *in vitro* experiment.

**Terminalia catappa Extracts:**

*Terminalia catappa* is (family–combretaceae) widely grown in tropical regions of the world as an ornamental tree. The phytochemicals of this plant include tannins, flavonoids and triterpenoids. Aqueous and ethanolic
extracts of leaves were reported for their hepatoprotective activity. Various pharmacological studies have reported that the extract of *Terminalia catappa* leaves and fruits have anticancer, antioxidant, anti-inflammatory and antidiabetic activities, (Mohale *et al.*, 2009). Leaves of *Terminalia catappa* were collected from garden; leaves were shade-dried and powdered using kitchen grinder. The powder was then dissolved in ethanol (95% v/v) as a solvent. The extract was dried by evaporation under vacuum evaporator and stored in airtight container at 4°C in refrigerator. Thereafter suitable aqueous concentrations were prepared for *in vivo* and *in vitro* experiments. The whole plant of *Terminalia* species was collected after proper identification by a competent plant taxonomist from Department of Botany, S.N (PG) College, Azamgarh, (U.P). The whole plant was powdered in a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) in a round bottom flask for 36 hrs at 60°C. The liquid extract was filtered, cooled and concentrated by evaporating its liquid contents in oven and collected. The powdered extract, termed Terminalia extract (TE), was re-dissolved in DDW for the oral administration to the mice. The required dose for *in vivo* treatment was prepared by dissolving the
extract in DDW in different concentration. A solution was also prepared in DMSO for in vitro experiment

**Alstonia scholaris Extracts:**

*Alstonia scholaris* belongs to the family Apocynaceae and is native to India. It grows throughout India, in deciduous and evergreen forests and also in plains (Nadkarni et al, 1976). *Alstonia scholaris* is an antimalarial drug and is marketed as Ayurveda preparation Ayush-64, NRDC, India.

The bark is bitter, astringent, acrid, thermogenic, digestive, laxative, anthelmintic, antipyretic, depurative, stomachic, cardiotonic and tonic (Nadkarni et al, 1976). It is useful in fevers, malarial fevers, abdominal disorders, dyspepsia, tumours, chronic and foul ulcers, bronchitis, cardiopathy, helminthiasis, (Kritikar et al, 2002). In folk medicine, milky juice is applied on wounds, ulcers and rheumatic pains, mixed with oil and dropped into ear, it relieves ear ache. The drug is also used in cases of snake-bite (Nadkarni et al, 1976). The Alstonia species are rich in alkaloids, steroids and triterpenoids and these substances may be responsible for the toxicity *Alstonia scholaris*. Alkaloids: alstonidine, alstone, alstovenine, chlorogenic acid, chlorogenine, ditain, ditaine, ditamine, echicaoutchin, echicerin, echiretin, echitamine, echitein,
echitenin, echitin, porphyrine, porphyrosine, reserpine, venenatine, villalstonine pleiocarpamine, O-methylnacralstonine, macralstonine O-acetylnacralstonine, villalstonine, macrocarnamine, corialstonine and corialstonidine, and triterpenoids: lupeol linoleate, lupeol palmitate and alpha-amyrin linoleate were reportedly present in the stem bark of *A. scholaris* (Jagetia *et al*, 2003). The seeds of *Alstonia scholaris* contain hallucinogenic indole alkaloids which are alstovenine, venenatine, chlorogenine, reserpine, ditamine and echitamine. The seeds also contain chlorogenic acid. Chlorogenic acid is a mild bladder and urethra irritant, resulting in increased sensitivity of the genital region.

Three new picrinine-types of monoterpenoid indole alkaloids were isolated from the leaves of *Alstonia scholaris* which are 5-methoxyaspidophylline, picralinal and 5-methoxystrictamine (Xiang *et al*, 2008). Two compounds identified as megastigmane-3β, 4α, 9-triol and 7-megastigmene-3, 6, 9-triol were extracted from the leaves of *Alstonia scholaris* and are know to be C13-norisoprenoids (Yan *et al*, 2009). Alstonic acids were also extracted from leaves of the same plant and two first-known 2, 3- secofernane triterpenoids were identified (Fei *et al*, 2009).
The hydroalcoholic extract of *Alstonia scholaris* extract produced teratogenic effects in mice at doses greater than 240 mg/kg (>20% of the LD50) when exposure occurred at day 11 of gestation. The extract at doses of 60, 120, 180, and 240 mg/kg did not cause mortality, congenital malformations, or alter the normal growth patterns. Doses of 360 or 480 mg/kg caused a dose-dependent increase in mortality, growth retardation and congenital malformations, characterized mainly by bent tails and syndactyly. These doses also significantly delayed fur development, eye opening, pinna detachment and vaginal opening. Doses of 240-480 mg/kg also delayed incisor eruption and testes decent (Jagetia *et al.*, 2003).

Dried powder of the plant leaves (25g) were extracted twice with 250 ml of 80% methanol overnight with continuous stirring. The pooled extracts were dried by evaporation under vacuum. The extract was suspended in DMSO and used in *in vivo* and *in vitro* experiments

*Acanthus ilicifolius* Extracts:

The shade dried parts of the whole plant (Leaf, Flower, Fruit and roots) were coarsely powdered and extracted. The extract of *Acanthus ilicifolius* was prepared by maceration. 100 g of samples were macerated in 300 ml of solvent (acetone 70%, methanol 80%). The maceration
procedure was repeated three times for 24 hours. The extract was then filtered using Whatman filter paper. The filtrate was vacuum-evaporated and freeze dried before it were used. Different optimum doses were prepared in DMSO for in vivo and in vitro studies.

It contain two new cyclolignan glycosides, (+)-lyoniresinol 3a-O-β-D-galactopyranosyl-(1 → 6)-β-D-glucopyranoside and (+)-lyoniresinol 2a-O-β-D-galactopyranosyl-3a-O-β-D-glucopyranoside in the aerial parts of Acanthus ilicifolius (Zhang et al, 2004). A phenylethanoid glycoside (ilicifolioside A) and an aliphatic alcohol glycoside (ilicifolioside B) have been isolated from the aerial parts (Kanchanapoom et al, 2002). Two lignan glucosides, (+)-lyoniresinol 3a-(2-(3, 5-dimethoxy-4-hydroxy)-benzoyl)-O-beta-glucopyranoside, and dihydroxymethyl - bis (3, 5 - dimethoxy – 4 - hydroxyphenyl) tetrahydrofuran-9 (or 9') -O-beta-glucopyranoside have been isolated from the aerial parts (Kanchanapoom et al, 2001). 11-epoxymegastigmame glucoside and megastigmane glucosides (roseoside) have been reported from Acanthus ilicifolius growing in China (Wu et al, 2003). 2 - benzoxazolinone and blepharin have been reported from plant growing in Vietnam (Nagao et al, 2005). A new coumaric acid derivative acancifoliuside, acteoside, isoacteoside, acanthaminoside, (+)-lyoniresinol
3a-O-beta-glucopyranoside, (-)-lyoniresinol, and alpha-amyrin, have been isolated from the methanolic extract of the leaves of *Acanthus ilicifolius* (Huo *et al*, 2008).

**Caesalpinia bonducella Extracts:**

*Caesalpinia bonducella* (Fever nut, Bunduc nut) belongs to family: Caesalpiniaceae and commonly known as Nata Karanja (Hindi), a prickly shrub, found throughout the hotter parts of India, Myanmar and Sri Lanka. The chemical constituents of the plant include flavonoids, triterpenes (Lai *et al.*, 1977; Purushothaman 1982). The leaves of this plant are traditionally used for the treatment of liver disorders, inflammation and tumors (Kirtiker and Basu, 1975). It has also been reported to possess multiple therapeutic properties like antipyretic, antidiuretic, anthelmintic antibacterial, anti-anaphylactic and antidiarrheal, antiviral, antiamebic and anti-estrogenic. The hepatoprotective and antioxidant role of *Caesalpinia bonducella* on paracetamol-induced liver damage in rats and also anti-inflammatory, analgesic and antipyretic activity has been reported (Gupta *et al.*, 2003a, b). Antitumor activity and antioxidant properties of
*Caesalpinia bonducella* against Ehrlich ascites carcinoma in Swiss albino mice was noticed (Gupta *et al.*, 2004).

The seeds of *Caesalpinia bonducella* were shade dried at room temperature. Then the shade dried seeds were powdered, 60g of coarse powder was defatted with petroleum ether and extracted exhaustively with 95% methanol at Temperature 60°C. The extract was dried by vacuum evaporator. Methanolic extract of *Caesalpinia bonducella* was dissolved in DMSO to prepare different optimum concentration for different studies. The whole plant was powdered in a mixture and the hydro-alcoholic extract was prepared by refluxing with the double distilled water (DDW) and alcohol (3:1) in a round bottom flask for 36 hrs at 60°C. The liquid extract was filtered, cooled and concentrated by evaporating its liquid contents in oven and collected. The powdered extract, termed Caesalpinia extract (CE), was re-dissolved in DDW for the oral administration to the mice. The required dose for *in vivo* treatment was prepared by dissolving the extract in DDW in different concentration. A solution was also prepared in DMSO for *in vitro* experiment.

**METHODOLOGY**

**IN VIVO METHODOLOGY**

Albino mice 8-10 weeks old (25-35 gm in weight) were exposed to
different test chemicals by appropriate routes (I.P. injection) and were sacrificed at sequential intervals of 16, 24, and 32 hours of stipulated treatment time. Animals have been treated with each test substance as shown in the tables. Samples have been taken three times after treatment as mentioned above. The central sampling interval was 24 hrs, since cell cycle kinetics can be influenced by the test substances, one earlier and one later sampling interval was adequately spaced within the range of 6 to 48 hrs. The additional dose levels were tested in a subsequent experiment, samples have been taken at the predetermined duration.

**Preparation of slides:** - Immediately after sacrifice, the bone marrow have been obtained by standard procedure and exposed to hypotonic solution and cells were fixed in carnoy’s fixative. Chromosome preparations were made from bone marrow cells following standard procedure as described below. The stained slides were examined and metaphase cells were scored for chromosomal aberrations. Prior to sacrifice, mice were further treated with colchicines, a spindle inhibitor to arrest the cells in C-metaphase. The slides were stained in 10% aqueous Giemsa solution and 100 bone marrow metaphase cells from each animal were scored under code. The types of chromosomal aberrations considered were chromatid and chromosome gaps, breaks, and fragments, exchanges
and pulverization (severely damaged cells). The reduction factors due to test chemicals treatment were calculated using the formula:

\[
\frac{(\text{Aberrant cells in control} - \text{aberrant cells in MMS+ test chemicals})}{\text{(Aberrant cells in control} - \text{aberrant cells in negative control})} \times 100 = \% \text{ Reduction}
\]

Control Groups:

Concurrent positive and negative controls had been included in this study.

Positive controls— A single dose of MMS, a compound known to produce chromosomal aberrations \textit{in vivo}, was used as positive control showing a significant response.

Test Chemicals:

Dose level: - For an initial assessment one dose of the each test chemicals were used. The dose was the highest attainable dose. Additional three doses have been used for the determination of dose-response. The route of administration was intra-peritoneal injection.

\textbf{IN VITRO LYMPHOCYTES CULTURE}

Most of the cytogenetic studies being carried out involving the examination of metaphase chromosomes. The evaluation of chromosomal
damage at metaphase stage gives much precise and detailed picture of the clastogenic agent than anaphase or telophase stage. Human peripheral blood lymphocytes are extremely sensitive indicators of the in vitro assay system. The chromosomal changes (numerical and structural) were utilized for investigation of the genotoxic as well as antigenotoxic potentiality of test chemicals. The parameters studied included chromosomal aberrations (CA), sister chromatid exchanges (SCEs) and cell growth kinetics (RI) both in the presence and in the absence of exogenous metabolic activation system.

**Preparation of S\(_9\) Liver / Microsome Fraction.**

The S\(_9\) liver / microsome fraction, so to say, formed the exogenous metabolic activation system utilized for the present investigation and was prepared as follows.

**Induction of rat liver enzyme.**

For preparing S\(_9\) fraction, the standard procedures as recommended by Ames *et al* (1973) and Moron and Ames (1983) were followed. Swiss albino healthy rats (Wister strain, obtained from Animal house, Biotech, Varanasi, India) each weighing about 200g were given 0.1% (1mg/ml) of Phenobarbital in drinking water for one week for the induction of liver enzymatic activities.
**Removal of livers from the rats.**

The rats were sacrificed through cervical dislocation. In order to ensure the sterility of S₉ preparation, the livers were removed aseptically using sterile surgical tools in laminar flow cabinet. Outmost care was taken not to cut across the esophagus or the intestine, which could otherwise cause the contamination of liver homogenate. The removed livers were immediately placed in 0.15 M KCl chilled solution in culture tube.

**Preparation of liver homogenate fraction**

The whole procedure was carried out at 0-4°C using sterilized solutions and glassware. The livers was washed in chilled KCl several times so as to remove the traces of haemoglobin, which inhibits enzyme activity. The washed livers were transferred to a beaker containing three volumes of 0.15 M KCl (3 ml/g wet liver) and after mincing with sterile scissors, these were homogenized by a tissue homogenizer at 4°C. The homogenate was centrifuged in a refrigeration centrifuge for 10 min at 9000 rpm. The supernatant (S₉ fraction) was decanted and saved as 1ml aliquots in polypropylene storage vials and stored in liquid nitrogen till further use.

**Preparation of S₉ mix**

The S₉ mix from S₉ fraction was prepared as fresh every time for use in the culture. S₉ fraction was complemented with 8 µM NADP, 100 µM
Na$_2$HPO$_4$ of buffer with 7.4 pH, 0.8 ml of S$_9$ mix was added every time along with the test chemicals in the cultures.

**Chromosomal Aberrations**

**Preparation of culture media:** Tissue culture medium RPMI–1640 (Flow Laboratories) with L-glutamine and Hepes buffer without NaHCO$_3$ was prepared in advance and stored at 4$^0$C, but the storage period never lasted longer than a week. About 1.574g of medium was dissolved in 100 ml of double distilled water by gentle shaking. Antibiotics, penicillin (100 IU/ml) and streptomycin (100 IU/ml) (Hoechst) were also added and pH was adjusted from 6.8-7.2 with N/10 NaHCO$_3$ and HCl. The medium was filtered and sterilized using Millipore filtration assembly by 0.45 μm Millipore filters. The filtered medium was then stored in sterilized tightly capped glass bottles.

**Collection of blood samples**

Peripheral blood from the healthy donors was taken fresh every time through veinal puncture under aseptic conditions (disposable needle and disposable syringes, Unitech) and Heparin (500 IU/ ml; Micro Lab) was used as anticoagulant. The tightly capped glass vials were gently mixed and stored at 4$^0$C for half an hour to separate blood cells from plasma.

**Setting of the cultures**
Lymphocyte culture was carried out by adding 0.8 ml of plasma containing white blood cells (WBC) in 4.5 ml of culture medium supplemented with 0.1 ml phytohaemagglutinin–P (PHA–P, Micro lab) and 15% fetal calf serum (Gibco). The culture vials were then tightly capped to avoid CO₂ loss and after gently mixing, culture tubes were incubated at 37°C in dark and colchicine was added 2 hrs prior to harvesting for arresting the cells at metaphase stage.

**Harvesting of the cultures**

After appropriate durations, the cultures were taken out from the incubator and their contents, after gentle shaking, were transferred to a centrifuge tube, the cells were spun down by centrifugation for 10 min, at 1200 rpm. Pellets were saved by discarding the supernatant. Hypotonic treatment (0.075 M KCl) was given for 10-12 minutes at 37°C and the cells were recollected by centrifugation. The cell pellet was suspended in 5 ml freshly prepared chilled fixative (3:1; methanol: acetic acid), which was added drop by drop with a Pasteur pipette with continuous shaking to avoid formation of clots. In order to ensure the proper fixation, the cells were kept suspended in the fixative for a minimum period of one hour but preferably overnight. Two or three changes with fresh fixative were given before preparing the slides.
Slide preparation and staining

After giving final washing in the fixative the cells were resuspended in 0.2 ml of fresh fixative. Two or three drops of cell preparation were dropped on clean, grease free, pre-chilled and wet microscopic slides and air-dried.

One-day-old slides were stained in Giemsa (Sigma) for 15 minutes and rinsed in 95% alcohol and finally in absolute alcohol for proper differentiation, after air-drying these slides were dipped in xylene for 5 minutes before mounting in DPX.

Analysis of the cells

In order to avoid the bias in scoring of the chromosomal anomalies before and after treatment of different test chemicals all slides were coded prior to scoring. A total of 300 well spread metaphase were analyzed for each concentration of the test chemicals and for each time duration to analyzed various chromosome and chromatid type aberrations by using the method as described by Evans (1984).

Chromatid type aberrations

Chromatid gap (g); Chromatid break (b); Chromatid deletion (d), Chromatid fragment (f) and Minute (m).

Chromosome type aberrations
Sister Chromatid Exchange Analysis

Mechanism of detection:

Sister chromatid exchange is a sensitive rapid and objective method of observing reciprocal exchange between sisters chromatid. This method depends upon the phenomenon of 5-bromo-2-deoxyuridine (Brd U) incorporation into DNA in place of thymidine. After two rounds of cell division, the chromatids were labeled with Brd U and consequently differentially stain with Hoechst stain. The BrdU incorporation quenches the fluorescence of 33258 Hoechst. Therefore, the light energy is absorbed but not emitted by such dyes, which results in the reduced staining of chromatid with Giemsa (Latt and Wohllel, 1975; Latt, 1976).

Labeling of chromosomes with BrdU

Sister chromatid exchange analysis was carried out following the standard procedure of Latt et al (1981). The cells in the culture were exposed to nucleoside, BrdU (Sigma) after 24 hrs of culture initiation at the final concentration of 2 μg/ml. The culture vials were tightly capped and covered with almunium foil to avoid light exposure and incubated at
$37^0\text{C}$ for another 48 hrs in dark.

**Slide preparation and staining**

After 2hrs of colchicines treatment, the cultures were harvested and processes following the same procedure as desired for the chromosomal aberration analysis.

For the differential staining of SCEs the methods of Perry and Wolff (1974) and Latt et al (1981) with slight modifications were followed. One-day-old slides were dipped in 0.5 μg/ml of 33258 Hoechst stain (Sigma) dissolved in double distilled water in horizontal coplin Jar. The slides were then put in flat glass dish with the layer of cells facing upwards. These were covered by thick layer (2-3 cm) of phosphate buffer ($p^H$ 6.8) and exposed to UV lamp (15W, 254 μm, Philips) from a distance of 10-15 cm for 30-45 minutes. The slides were taken out from the buffer, washed twice in double distilled water and air-dried. These were then incubated in 2x SSC (0.3 M NaCl, 0.03M Sodium citrate, $p^H$ 7.0) at $65^0\text{C}$ in water both for 90 minutes using vertical coplin jars. The slides were taken out and rinsed in distilled water. The air-dried slides were then stained in Giemsa for 20 minutes and rinsed in 90% alcohol followed by rinsing in absolute alcohol. The dried slides were dipped in Xylene for 5 minutes and mounted in DPX.
**Analysis of the cells**

All slides were coded prior to scoring so as to avoid any ambiguity. Around 50 metaphases (25 metaphases/donor) with differentially stained chromatid were scored for each test chemicals treatment in absence of S9 mix and 50 metaphase were scored for each treatment in the presence of S9 mix. The interstitial exchanges between two sister chromatid were scored as two exchange and the terminal exchanges were scored as a single exchange. Student’s ‘t’ test was applied for calculating the significance of difference between the treated and the controls.

**Cell Cycle Kinetics analysis**

The cells undergoing first (M₁) second (M₂) and third (M₃) divisions were detected by studying the BrdU labeled differentially stained chromosomes, following the method of Tice et al (1976) and Crossen and Morgan (1977). The cells with both the chromatids being darkly stained were scored as M₁ cells, those with one dark and one lightly stained chromatid as M₂ cells and those having mixture of both the differentially stained and uniformly stained chromatids were scored as M₃ metaphase. Around 100 well spread metaphase were scored for each concentration and each treatment durations from each donor in the absence as well as in the presence of S9 mix. The replication index (RI) was calculated according to
the formula of Tice (1979) as given below. The deviation from the controls was determined by using Chi-square ($\chi^2$) test.

\[
R.I = \frac{(M_{1X1}) + (M_{2X2}) + (M_{3X3})}{100}
\]

**Statistical Analysis**

Standard deviation (SD) and standard error (SE) were calculated using the following formula

\[
\text{Mean (} \bar{X} \text{)} = \frac{\sum X}{n}
\]

\[
\text{Variance } \delta^2 = \frac{\sum(X^2n.X^2)}{n-1}
\]

\[
\text{S.D. (} \delta \text{)} = \sqrt{\frac{\sum X^2 - nx^2}{n-1}}
\]

\[
\text{S.E} = \frac{SD}{\sqrt{n}}
\]

Where,

\[
X = \text{Variable}
\]

\[
N = \text{Number of observation}
\]

\[
\sum X^2 = \text{Sum of square of individual variables.}
\]

\[
\bar{x} = \text{Mean of variables}
\]

\[
\delta = \text{Standard deviation}
\]

2 x 3 Chi-Square test $\chi^2$ for homogeneity test of variance was used to
analyses the cell growth kinetics exchange with the normal control. The level of significance was tested from standard statistical tables of Fisher and Yates (1963).

Student two-tailed ‘t’ test was used for calculating the statistical significance in SCE and chromosomal aberration by comparing the effect induced by different test chemicals to the respective control. The following formula were used for this purpose.

\[
t = \frac{\bar{x}_{1\text{(control)}} - \bar{x}_{\text{treated}}}{\sqrt{(S.E_{\text{of control}})^2 + (S.E_{\text{of treated}})^2}}
\]

The statistical significance was calculated from Fisher and Yates table at \((n_1+n_2-2)\) degree of freedom (df) at 0.05% level of significance.
## Table - 1 of chemical concentration

### 1.1- Control

<table>
<thead>
<tr>
<th>Positive and Negative Control</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMS</td>
<td>5 μg/ml</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide (DMSO)</td>
<td>5 μg/ml</td>
</tr>
</tbody>
</table>

### 1.2- In vivo Concentrations of Phyto-Chemicals.

<table>
<thead>
<tr>
<th>Phyto-products.(mg/kg.bw)</th>
<th>1st Dose</th>
<th>2nd Dose</th>
<th>3rd Dose</th>
<th>4th Dose</th>
<th>5th Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morinda Extracts.</td>
<td>MC₁ 100</td>
<td>MC₂ 150</td>
<td>MC₃ 200</td>
<td>MC₄ 250</td>
<td>MC₅ 300</td>
</tr>
<tr>
<td>Terminalia Extracts</td>
<td>TC₁ 250</td>
<td>TC₂ 300</td>
<td>TC₃ 350</td>
<td>TC₄ 400</td>
<td>TC₅ 500</td>
</tr>
<tr>
<td>Alstonia Extracts</td>
<td>AS₁ 75</td>
<td>AS₂ 150</td>
<td>AS₃ 200</td>
<td>AS₄ 275</td>
<td>AS₅ 350</td>
</tr>
<tr>
<td>Acanthus Extracts</td>
<td>AI₁ 50</td>
<td>AI₂ 100</td>
<td>AI₃ 150</td>
<td>AI₄ 200</td>
<td>AI₅ 250</td>
</tr>
<tr>
<td>Caesalpinia Extracts</td>
<td>CB₁ 200</td>
<td>CB₂ 250</td>
<td>CB₃ 300</td>
<td>CB₄ 350</td>
<td>CB₅ 400</td>
</tr>
</tbody>
</table>

### 1.3- In vitro Concentrations of Phyto-Chemicals.

<table>
<thead>
<tr>
<th>Phyto-products. (mg/kg.bw or μg/ml)</th>
<th>1st Dose</th>
<th>2nd Dose</th>
<th>3rd Dose</th>
<th>4th Dose</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th></th>
<th>MC₁ 100</th>
<th>MC₂ 200</th>
<th>MC₂ 250</th>
<th>MC₂ 300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morinda Extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Terminalia Extracts</td>
<td>TC₁ 200</td>
<td>TC₂ 250</td>
<td>TC₃ 300</td>
<td>TC₄ 400</td>
</tr>
<tr>
<td>Alstonia Extracts</td>
<td>AS₁ 100</td>
<td>AS₂ 175</td>
<td>AS₃ 250</td>
<td>AS₄ 300</td>
</tr>
<tr>
<td>Acanthus Extracts</td>
<td>AI₁ 50</td>
<td>AI₂ 150</td>
<td>AI₃ 200</td>
<td>AI₄ 250</td>
</tr>
<tr>
<td>Caesalpinia Extracts</td>
<td>CB₁ 150</td>
<td>CB₂ 200</td>
<td>CB₃ 250</td>
<td>CB₄ 300</td>
</tr>
</tbody>
</table>

**REFERENCES**


Latt, S.A. (1976). Longitudinal and lateral differentiation of metaphase chromosomes based on the detection of DNA synthesis by


Latt, S.A; Allen, J; Bloom, S.E; Carrono, A.V; Falke, E; Kram, D; Schneider, E; Shredk, R; Tice, R; White field, B; and Wolff, S. (1981). Sister Chromatid exchange; a report of the Sister chromatid exchange; a report of the genotoxic programme. Mutat. Res. Vol. 87: pp. 1777-62.


Miadokova, E; Nadova, S; Vickova, V; Duhova, V; Kopaskova, M; Cipak, L.; Rauko, P; Mucaji, P; Grancaj, D. (2008), Antigenotoxic effect of

Mohale, DS; Dewani, AP; Chandewar, AV; Khadse, CD; Tripathi, AS; Agarwal, SS; (2009). *J. Herbal, Medicine & Toxicology*, vol. 3(1): pp. 7-11.


Nadkarni, AK; (1976). Dr. KM Nadkarni’s Indian Materia Medica, Bombay, India. Popular Prakashan. vol. 1: pp. 80-83.


Purushothaman, KK; Kalyani, K; Subramaniam, K; Shanmughanathan, SP; (1982). Structure of bonducellin-A new homoisoflavone from *Caesalpinia bonducella*. Indian J Chem. 21B: 383-386.


Shoeb, M; Celik, S; Jaspars, M.; Kumarasamy, Y; MacManus, S; Nahar, L; Kong, TLP; Sarker, SD; (2005). Isolation, structure elucidation and


Su,BN; Pawlus, AD; Jung,HA; Keller,WJ; McLaughlin,JL; and Kinghorn, AD; (2005) “Chemical constituents of the fruits of Morinda citrifolia (Noni) and their antioxidant activity,” Journal of Natural Products, vol. 68, no. 4, pp. 592–595, 2005.


Tice, R; Schneider, EL; and Rary, JM; (1976). The utilization of BrdU incorporation into DNA for the analysis of cellular Kinetics. Exp. Cell. Res. 102, 232-236.

Wang, MY; Anderson, GL; and Nowicki, (2002) “Preventative effect of Morinda citrifolia (Noni) at the initiation stage of mammary breast carcinogenesis induced by 7,12-dimethylbenzo(a)anthracene (DMBA) in female Sprague-Dawley (SD) rats,” Cancer Epidemiology, Biomarkers & Prevention, vol. 11, pp. 1218S.


