"In vitro assessment of effect on genetic material (genotoxicity) of Caesalpinia crista plant seed extract on cultured human peripheral blood lymphocytes using Chromosomal Aberration Assay and Cytokinesis Blocked Micronuclei Assay"

A Dissertation Project
Submitted to
Nirma University
In Partial fulfillment of requirement for the

The Degree of
Master of Science
In
Biochemistry

Submitted by
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(10MBC008)

Under the guidance of
Dr. Sonal Bakshi

.....Dedicated to
My parents

ACKNOWLEDGMENTS

I would like to express sincere thanks to my thesis advisor who has become an inspiration in my life to achieve successes and goals: an Assistant Professor ***Dr. Sonal Bakshi,*** *for her invaluable guidance, encouragement, suggestion and advice during the entire period of my dissertation period. I am very thankful to her for always helping me and believing in my capabilities.*

I wish to express my deep sense of gratitude to ***Prof. Sarat Dalai,*** *Director, INSTITUTE OF SCIENCE, for his able guidance, useful suggestions and informative discussions on various scientific issues which helped me in upgrading my scientific knowledge*

I am very grateful to the other faculty members ***Dr. Vijay Kothari, Dr. Shalini Rajkumar, Dr. Mili Das, Dr. Sriram Seshadri, Dr. Amee Nair, Dr. Nasreen Munshi*** *for their valuable time to provide us deep knowledge and to improve our abilities in the field of life science.*

I wish to extend my deepest gratitude to our project co-investigator ***Dr. Niyati Acharya*** *from INSTITUTE OF PHARMACY, NIRMA UNIVERSITY for giving me an opportunity to work on the plant extract prepared by her and also* ***NIRMA EDUCATION & RESEARCH FOUNDATION*** *for the financial support.*

I am thankful to all the PhD Scholars specially Arpan Bhatt, Suhani Palkhiwala, Mahendra Pal Singh, Rahul Jog, for their continuous support, motivation, warm hospitality, sharing happiness and helping me with my doubts and difficulties during the entire study.

I specially thank to Mr. Darshan Valani from Zydus Research Centre, Ahmedabad for his kind help.

I would like to thank NIS non teaching staff members: Hasitbhai, Sachinbhai, Bharatbhai and library staff Mrs. Shwetal, and Ms. Jayshree for their constant support.

I will always cherish the memories of time we had together in my life with beloved friends: Vidhi, Astha, Gunjan, Rimanshu, Arpan, Suhani, Mitali, Naisargee.

I would also like to thank Jay, Palak, Rimanshu, Gunjan, and other labmates who gave me their help and shared time during the study, thanks you all.

I deeply and sincerely thanks to my parents for bringing me into the world and reaching upto this level. Special thanks to my little brother and sister who have always made me laugh and helped become strong in any situation of life. Finally my appreciation is devoted to all the members in my family for their love, blessings, encouragement and moral support in my study.

I am thankful to God for giving me such wonderful opportunities in life.

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LIST OF ABBREVIATIONS

µm:	micrometer
^{0}c:	Celsius
BN:	Binucleated cells
BOD:	Biological Oxygen Demand
C.crista:	*Caesalpinia crista*
CA:	Chromosome Aberrations
CBMN:	Cytokinesis Blocked Micronuclei Assay
CO2:	Carbon Dioxide
Cyt-B:	Cytochalasin-B
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DPX:	mixture of distyrene, a plasticizer, and xylene
FBS:	Fetal Bovine Serum
Hrs:	Hours
kcl:	Potassium Chloride
LAF:	Laminar Air Flow
mg:	milligram
MI:	Mitotic Index
min.:	minutes
mm:	millimeter
mM:	millimolar

MNBN: Micronuclei in binucleated cell

MNi: Micronuclei

No.: Number

OECD: Organization for Economic Co-operation and Development

PBL: Peripheral Blood Lymphocytes

PHA: Phytoheamagglutinin

Rpm: rotation per minute

RPMI: Roswell Park Memorial Institute

SCE: Sister Chromatid Exchange

SD: Standard Deviation

Std.: standard

ABSTRACT

Medicinal plants continue to be an important therapeutic aid for alleviating the ailments of human kind. *Caesalpinia Crista* of family *Fabaceae* is a moderately sized deciduous tree, growing wild throughout India especially in the coastal area, forests, and hills. The extracts of various other parts of *Caesalpinia crista* are reported to have anthelmintic, anti-amyloidogenic, immunomodulatory, analgesic, antipyretic, anti-inflammatory, memory enhancing, antidiabetic activity and also antitumor and antioxidant activity. The plant parts have been widely used in medicines for its unique and diverse pharmacological properties. The literature has revealed that seeds and leaves of *Caesalpinia crista* contain around fourteen compounds. The isolated compounds are cassane-and norcassane-type diterpenes and other flavonoids and triterpenoides. Chemical investigation on Caesalpinia crista revealed two diterpenoids (6b-cinnamoyloxy-7b-acetoxyvouacapen-5a-ol and 6b,7b-dibenzoyloxyvouacapen-5a-ol), which have been reported to be cytotoxic to two different cancer cell lines. The phytoconstituents of the plant and its protective effect as antioxidant and antitumor activity in various pathologies triggered our interest to investigate the genotoxic effect of the methanolic extract of *Caesalpinia crista* seeds on cultured human peripheral blood lymphocytes. Our literature survey revealed that, there is little information about genotoxicity of this plant extract. This was undertaken for two aspects: 1) to check if the reported anti-tumor activity of this extract is due to DNA-affecting activity or not, which can be evident if increased DNA-damage is observed in terms of genotoxicity parameters. This is envisaged due to the fact that most of the anti-cancer compounds are DNA-damaging agents. 2) Any effect on chromosome aberration levels following treatment with extract would indicate its mode of action i.e. clastogenic (reflected as chromosome aberration per cell frequency) or aneugenic (reflected as CBMN frequency).

The study was carried out in the presence and absence of metabolic activation system to check if the extract contains a direct acting DNA damaging compound(s) or liver enzyme activation dependent compound(s).

The assessment of genotoxic potential of *Caesalpinia crista* plant seed extract was done using in vitro genotoxicity endpoints, in terms of frequency of chromosome aberrations (CA), and %age of Cytokinesis blocked binucleated cells with micronucleus (CBMN) assay. This study was carried out in compliance with the Test Guidelines of the Organization for Economic Cooperation and Development (OECD, 1997a, b, 2004).

Experiments were done by treating the in vitro short-term cultured human blood lymphocytes at various concentrations of plant extract dissolved in DMSO (vehicle) in the absence and presence of metabolic activation system for long term and short term exposure time respectively.

Results demonstrated significant induction of structural chromosomal aberrations. The methanolic extract of *Caesalpinia crista* plant seeds significantly decreased the mitotic index value at the highest tested concentration indicating its cytotoxicity. The results of chromosomal aberrations indicated that methanolic extract of *Caesalpinia crista* plant seeds had clastogenic activity. Whereas, the results of CBMN assay following treatment with extract did not reveal significant aneugenic activity. The effect of incubation with S9 fraction on the experiment revealed that the methanolic extract of *Caesalpinia crista* plant seeds contains direct acting DNA-damaging compound(s), the activity of which is not significantly affected following metabolic activation.

The present study describes a novel approach for the study of mechanism of action of a complex mixture with potential anti-cancer activity employing endpoints of *in vitro* genotoxicity assessment in order to assess the DNA-damaging activity.

1.INTRODUCTION

1.1 Caesalpinia Crista **plant**:

Medicinal plants continue to be an important therapeutic aid for alleviating the ailments of human kind. Today, there is a renewed interest in traditional medicine and an increasing demand for more drugs from plant sources. This revival of interest in plant derived drugs is mainly due to the current wide spread belief that "green medicine" is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects. However, most of the information available on many medicinal herbs has no supporting scientific data and their use as medicaments is based solely on traditional folk usage that has been perpetuated down the generations (Lopes L.C. *et al*.)

Caesalpinia Crista of family *Fabaceae* is a moderately size deciduous tree, growing wild throughout India especially in the coastal area, forests, and hills. It is frequently found around the marshy and plain land. The plant parts have been widely used in medicines for its unique and diverse pharmacological properties (Suryawanshi *et al*.2011)

TAXANOMY

Kingdom*: Plantae*

Phylum*: Magnoliophyta*

Class*: Angiospermae*

Order*: Fabales*

Family*: Fabaceae*

Genus*: Caesalpinia*

Species*: Caesalpinia crista*

Synonyms: *Caesalpinia paniculata, Guilandina paniculata,*

Guilandina semina Lour, Caesalpinia bonducella.

Fig.1: *Caesalpinia crista* plant **Fig.2: *Caesalpinia crista* plant seeds**

VERNACULAR NAMES

English name: Teri pods, Fever nut

Hindi: Katuk Ranja, Karanjava

Sanskrit: Putrakaranj

Gujarati: Kanchaki, Kankachia

There are around 14 compounds reported from the seeds and leaves of *Caesalpinia crista*. The isolated compounds are cassane-and norcassane-type diterpenes. From the Methanolic extract of seed kernels of *Caesalpinia crista* from Myanmar, five new cassane-type diterpenes caesalpinins MA-ME, and three new norcassane-type diterpenes, norcaesalpinin MA-MC, have been isolated, together with 12 known cassane-type diterpenes, 14(17)-dehydrocaesalmin F, caesaldekarinE, caesalmin B, caesalmin C, caesalmin E, 2-acetoxy-3-deacetoxycaesaldekarine, 2-acetoxycaesaldekarine, caesalpinin C, 7-acetoxybonducellpin C, caesalpinin E, norcaesalpinin B, and 6-acetoxy-3-deacetoxycaesaldekarine (Suryawanshi *et al*,2011). A number of reports on flavonoids, triterpenoids, and steroids indicate that they exert multiple biological effects due to their anti-oxidant and free radical scavenging

abilities. These phytoconstituents produced protective effects against tumors, heart disease, and different pathologies (Gupta M. *et al.2003*)

Plant derived natural products such as flavonoids, terpenes, alkaloids (Osawa T *et al.* 1990) and several other plant products have been tested for anticancer activity and some of them like vincristine, taxol are now available as a drug of choice. Because of this property plant has received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemopreventive effects (Gupta M.*et al.* 2004).

The extracts of various other parts of *Caesalpinia crista* have been reported to have anthelmintic, anti-amyloidogenic, immunomodulatory, analgesic, memory enhancing, antipyretic, anti-inflammatory, antidiabetic activity and also antitumor, antioxidant activity (Suryawanshi *et al*, 2011).

Chemical investigation on *Caesalpinia crista* found two diterpenoids (6b-cinnamoyloxy-7b-acetoxyvouacapen-5a-ol and 6b, 7b-dibenzoyloxyvouacapen-5a-ol) has been reported cytotoxic to two different cancer cell lines (Das B *et al*.2010).

The anticancer activity of root bark of *Caesalpinia crista* was evaluated against the Ehrlich Ascites Carcinoma (EAC) Tumor model. The activity was assessed by using tumor volume, packed cell volume, mean survival time, increase in life span and hematological studies. Oral administration of alcoholic extract of root bark of *Caesalpinia crista* increased survival time. Hematological parameters which were altered by tumor inoculation were restored. Solid tumor mass was also significantly reduced (Bodakhe S. H. 2011).

Further investigation of Ethanolic extract of *Caesalpinia crista seeds* for antioxidant activity by 1,1-diphenyl-2-picryl hydrazyl and hydrogen peroxide methods and anti inflammatory by Carrageenan induced paw edema and analgesic activity by writhing reflexes and by tail immersion method in mice. The extract showed potent antioxidant activity by 1,1-diphenyl-2-picryl hydrazyl and hydrogen peroxide method as compared to the standard ,ascorbic acid (Naresh Singh *et al*.2012).

Studies have shown that Methanolic extract of *Caesalpinia bonducella* leaves increased the life span of EAC-tumor bearing mice and decreased the lipid peroxidation and thereby augmented the endogenous antioxidant enzymes in the liver. The above parameters were responsible for the antitumor and anti-oxidant activities of *Caesalpinia bonducella* (Gupta M *et al*.2004).

The reports of antitumor activity by phyto-constituents of this plant triggered our interest to evaluate the genotoxic effect of the methanolic extract of *Caesalpinia crista* seeds on cultured human peripheral blood lymphocytes.

Our literature survey revealed that, there is little information about genotoxicity of this plant extract. Hence, we report here the study of genotoxic potential of *Caesalpinia crista* plant seed extract in terms of chromosome aberrations (CA) and micronucleus (MN) assay. This study was carried out in compliance with the Test Guidelines of the Organization for Economic Cooperation and Development (OECD- 478, 473-2004,1997).

1.2: GENOTOXICITY ASSESMENT:

Genotoxicity is a property of a substance that makes it harmful to the genetic material contained in organisms. While there are many different factors that can affect DNA, RNA, and other accessory proteins of nucleic acid replication and repair, the genotoxicity implies structural damage to the genetic material. The substances that affect cancer-suppressing genes are considered carcinogenic. Cancer is the uncontrolled growth of cells within the body, and often has genetic causes. Substances with genotoxicity can cause mutations in cells that cause them to divide and grow uncontrollably. They can also have damaging effects on various proteins and other substances that normally prevent such uncontrolled cell growth. Earlier *in vitro* and *in vivo* studies reveal that the plant extracts from various parts of the plant play a modulating role in xenobiotic effects. In defining *in vitro* tests for detecting carcinogens the goal has been to identify and quantify alterations in the molecular pathways associated with the generation and fixation of mutations. The two major categories of chemical carcinogens are DNA-reactive i.e. genotoxic carcinogens and non-genotoxic carcinogens that alter DNA indirectly through other effects on the cell. (Sudin Bhattacharya *et al.1996*).

In vitro genetic toxicology tests are in fact some of the most refined sets of tools available to toxicologists to study carcinogenesis in terms of the toxicity pathways that may precede it; i.e., mutagenicity, clastogenicity, and aneugenicity (Tucker *et al.* 1996)

To assess genotoxicity many short term assays are developed. Assays that measure gene mutation are those that detect the substitution, addition, or deletion of nucleotides within a gene. Assays that measure chromosomal mutation are those that detect breaks or chromosomal rearrangements involving one or more chromosomes. Assays that measure genetic mutation are those that detect changes in the number of chromosomes i.e. aneuploidy. Chromosome alterations are of two types:

1. Structural chromosomal aberrations (chromosome or chromatid breaks, rearrangements)

2. Numerical chromosomal aberrations (aneuploidy, polyploidy).

Cytogenetic assays are divided into two test types:

1. *In vivo* test: Study of chromosomal aberrations in rodent bone marrow cells (Metaphase analysis) in which rats are used as model system or the mouse bone marrow micronucleus test in which mice are used as model system.

2. *In vitro* test: Mammalian cells in culture

Various methods for assessment of genotoxicity: The conventional methods for evaluating genetic damage include **chromosomal aberration (CA), cytokinesis blocked micronuclei assay (CBMN)**, sister chromatid exchange(SCE) frequency, and comet assay.

1.2.1: Cytokinesis blocked micronuclei assay (CBMN):

Micronucleus (MN) is a small additional nucleus and is readily identifiable by light microscopy. Biologically, micronuclei are the chromosome fragments or whole chromosomes that lag behind at anaphase during nuclear division. MN scoring is the indicator of the genetic damage. Therefore MN scoring can be used in various clinical aspects such as to check genotoxicity, biomonitoring of diseases, screening of preneoplastic diseases and identification of high risk patients (Samanta.S *et al*.2012)

Mechanism of Origin of MN:

There are two predominant mechanisms leading to the formation of MN in a mitotic cell:

(1) Chromosomal breakage and

(2) Dysfunction of the mitotic apparatus.

Clastogens induce chromosome breaks and yield acentric fragments. These chromosomal fragments are directly included into micronuclei. In the other mechanism, aneugenic agents prevent the formation of the spindle apparatus during mitosis. As a result, the whole chromosomes lag behind at anaphase. The chromosome is surrounded by the nuclear envelope, forming micronuclei. Therefore the daughter cells have micronuclei containing whole chromosomes. Besides these two important mechanisms, MN may be formed due to broken anaphase bridges. This may be because of dicentric chromatids, intermingled ring chromosomes, or union of sister chromatids.

DNA double strands break is a physiological phenomenon when a cell commits from G0 to G1 of the cell cycle and may occur in normal healthy cells. So MN may also be noted in normal healthy individual but the numbers of micronucleated cells may be less.(Bonassi S *et al*.2001).It has been shown from various studies that the prevalence and frequency of spontaneous occurrence of MN in human lymphocytes increases with age The micronucleation of human lymphocytes is nonrandom and there is increased frequency of age dependent micronucleation of X and Y chromosomes (Norppa H *et al*.2003).The higher micronucleation of X chromosome in female may be due to inactive X chromosome.

Cytokinesis blocked micronuclei (CBMN):

The use of micronuclei (MNi) as a measure of chromosome damage in peripheral blood lymphocytes (PBL) was first proposed by Countryman and Heddle (1976) and subsequently developments of the cytokinesis-block micronucleus (CBMN) method were reported (Fenech and Morley, 1985b).

It became evident after scoring hundreds of slides of lymphocyte cultures for MNi that the ideal stage to score MNi was the binucleated telophase stage (Fenech and Morley, 1985a). The development of the cytokinesis-block (CB) technique has made the human lymphocyte micronucleus assay (MN) a reliable and precise method for assessing chromosome damage.

It is important to count MNi in binucleated cells (BNC) for several reasons:

(i) Cells have to pass through one cell cycle and mitosis after treatment in order to form MNi;

(ii) MNi may be lost, or

(iii) New MNi may arise when passing from second to the third cycle.

To block cells in this stage after completing one nuclear division and to observe binucleated cells cytochalasin-B (Cyt-B) is added (Fenech and Morley, 1985 a,b). Cells that complete nuclear division are then accumulated as binucleated cells because Cyt-B can inhibit cytokinesis without interfering with nuclear division. Scoring of MNi in cytokinesis-blocked binucleated BN cells has since become a standard procedure in genetic toxicology.

1.2.2: Chromosomal Aberration:

The chromosomal aberration assay in human lymphocyte is a valuable assay. This test system uses primary cultures of lymphocytes from freshly drawn blood. These primary cells have a normal chromosome number and reliably detect clastogenic activity. Structural aberrations may be of two types, chromosome or chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type, but chromosome type aberrations also occur. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. Chromosome mutations and related events are the cause of many human genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumor suppressor genes of somatic cells are involved in cancer induction in humans and experimental animals. This assay is used to screen for possible mammalian mutagens and carcinogens. Many compounds that are positive in this assay are mammalian carcinogens; however, carcinogenicity is not by only this mode of action. Correlation is dependent on chemical class and there is increasing evidence that there are carcinogens that are not detected by this test because they appear to act through mechanisms other than direct DNA damage. (OECD 473 guideline, S(2)R)

Mitotic index

Mitotic index is a measure of the proliferation of cell population. It is defined as the ratio of the number of cells in mitosis and the total number of cells scored.

The mitotic index is only an indirect measure of cytotoxicity.

The main objective of this work was analysis of effects of the crude plant extract of *Caesalpinia crista* (methanolic seed extract) on genetic material (genotoxicity) of cultured human peripheral blood cells as the antitumor activity of this has been reported earlier. The plant extract was provided by INSTITUTE OF PHARMACY, NIRMA UNIVERSITY. The following major steps were performed for the current study.

2.1 To define minimum three exposure levels (concentrations) for further genotoxicity testing: The concentration was determined using relative mitotic index (RMI) method.

2.2 To check the aneugenic effects of plant extract: For this CBMN assay is the more relevant assay. CBMN was done to check aneugenic effect.

2.3 To check clastogenic effects of plant extract: To study clastogenic effect of the compounds present in extract Chromosomal aberration assay (CA) was done. Cells were treated with plant extract for short term (4hrs) and continuous (22hrs) treatment.

2.4 To check the genotoxic effect of crude plant extract in presence and absence of metabolic activation system: The effect of various compounds of crude plant extract may vary depending on the absence and presence of metabolic activation system on genetic material, hence, effect on cultured peripheral blood cells was checked by treating the cells with plant extract with and without S9 mix in terms of both the endpoints; Chromosomal aberration (CA) assay and Cytokinesis-blocked binucleated cell with micronuclei assay (CBMN).

2: MATERIALS AND METHODS

2.1 REAGENTS:

REAGENT	**COMPANY**
RPMI – 1640	HIMEDIA
Trypsin	s.d fine chem.Limited
Kcl	MERCK
Sodium hypochloride	s.d fine chem.Limited
DPX	s.d fine chem.Limited
Sodium Heparin	C.D RICH
Methanol	MERCK
Acetic acid	MERCK
Colchicine	HIMEDIA
Phytoheamagglutinin (PHA-M)	HIMEDIA
Giemsa stain	SIGMA
Chromic acid	MERCK
DMSO	SRL
Cytochalasin–B	HIMEDIA
Mitomycin-C (MMC)	MP BIOMEDICALS
Cychlophosphamide(CPA)	HIMEDIA
Anionic detergent for glassware washing	Luxbro

2.2 EQUIPMENTS

EQUIPMENTS	**COMPANY**
Glassware	BOROSIL
Syringe holder	GRIENER bio-one
Multisample Syringe Needles	VACUETTE
Gloves	KALTEX
Aluminium foil	FRESH-WRAP
Micropipette	EPPENDORF
Vacuatte(sodium heparin)	C.D RICH® (3 ML)
Coverslip	BLUE STAR
Microscope slides	AXIVA
0.22µm filter units	AXYGEN
Laminar Air Flow	NOVA
Incubator	EIE INSTRUMENTS
Centrifuge	NURE
Digital pH meter	E1
Freezer	NOVA
Microscope	LABOMED VISION 2000 / Nikon Eclipse 600
Water bath	EIE serological water bath
Bench top centrifuge	CM 101 REMI
Weighing balance	SHIMADZU
Cooling centrifuge	REMI

2.3 REAGENT SETUP:

- Culture Media: RPMI-1640 supplemented with 15% fetal calf serum, L-alanyl-L-Glutamine, HEPES buffer, Penicillin (60 mg/ litre), streptomycin (100 mg/litre), and 2% $NaHCO_3$.
- KCl (Hypotonic): 0.56 gm of KCl was dissolved in 100 ml of sterile distilled water.
- Fixative: Methanol and acetic acid were freshly added in 3:1 ratio respectively and chilled.
- Stock Giemsa stain: 1 gm Giemsa powder was added in 54 ml glycerol mixed and kept at 60 ºC water bath for overnight, 84 ml of methanol was added, filtered, and kept in dark bottle.
- Working Giemsa stain: 4 ml Giemsa stain was added in 25 ml Sorenson's buffer with pH 7 and 25ml water, mixed well and prepared freshly whenever required.
- Sorenson's buffer: 0.345 gm of monobasic sodium phosphate (NaH_2PO_4) and 0.454 gm dibasic sodium phosphate (Na_2HPO_4) was added in 250 ml of sterile distil water for obtaining pH 7.
- Chromic acid: 10 % $K_2Cr_2O_7$ was added with 25 % of H_2SO_4 for slide washing.
- Colchicine: 10 mg absolute powder was dissolved in 10 ml of autoclaved mili-Q water and aliquots were dispensed (1mg/ml). It was used to block the cells at metaphase stage.
- Phytoheamagglutinin (PHA): 25 mg of absolute powder of PHA was added to 25 ml autoclaved mili-Q water and aliquots were dispensed (1mg/ml). Phytohemaglutinin (PHA), derived from extracts of *Phaseolus vulgaris* seeds, on account of its twin properties of causing erythroagglutination and of stimulating progressive lymphocyte mitosis is used as mitotic inducer in the culture. PHA-M stimulates T-lymphocytes.
- Cytochalasin-B: 5 mg of solid was dissolved in 8.33 ml DMSO to give a Cyt-B solution concentration of 600µg/ml as follows:

- Cyt-B vial was removed from -20 ºC and allowed to reach room temperature. Top of the rubber seal was sterilized with ethanol. 8.33 ml of DMSO was pipette into a 50 ml sterile falcon tube. Using a 5 ml sterile syringe and another needle, 4 ml of 8.3 ml DMSO was injected into the vial through the seal using 0.22µm sterile filter.
- 4 ml was removed from the vial and ejected into another sterile 15 ml tube labeled as '1'.
- Remaining 4.3 ml of DMSO was aspirated as before into the vial and again ejected into the sterile tube labeled as '1'.
- All the contents were mixed properly and 500µl was dispensed into sterile 1.5 ml eppendorfs. Aliquots were then stored at -20ºC.

- MMC mitomycin–C: 2mg of solid was dissolved in 2 ml of mili-Q water to give a mitomycin-C solution concentration of 1 mg/ml.
- CPA Cyclophosphamide : CPA was use at std. concentration of 5 µg/ml

2.4 PLANT EXTRACT:

The *Caesalpinia crista* plant extract (methanolic seed extract) was provided by INSTITUTE OF PHARMACY, NIRMA UNIVERSITY.

PHYSICAL PROPERTIES:

- Solubility : soluble in DMSO, water, methanol
- Color: brownish red color
- Sterility testing:

• N-agar	• No contamination
• Potato dextrose agar	• No contamination

- Extraction Efficiency: 10%
- Viscosity: Semisolid, viscous
- Stability: Stable at room temperature

2.5 METABOLIC ACTIVATION SYSTEM (S9 MIX):

Cells were exposed to the plant extract both in the presence and absence of an appropriate metabolic activation system. Mammalian liver is the principal organ responsible for the drugs and other foreign compound (xenobiotic) metabolism. The most commonly used system is a co-factor supplemented post-mitochondrial fraction (S9) prepared from the livers of rodents treated with enzyme-inducing agents such as Aroclor 1254 or a combination of phenobarbitone and ß-naphthoflavone. The post-mitochondrial fraction is usually used at concentrations in the range from 1-10% v/v in the final test medium (OECD 473 GUIDLINE). S9 is comprised of microsomes which are principally derived from the membranes of the endoplasmic reticulum and soluble proteins or cytosol (Mammalian Liver S9 Guidelines)

In many cases the compound as such is not mutagenic, but is converted to a mutagen following its metabolic activation. Based on this phenomenon the crude plant extract was placed in presence of rat liver enzymes to mimic the normal activation process of metabolism (metabolic activation system).The S9 mix was used at concentration of 500 µl/culture For the culture tested without metabolic activation, same amount of phosphate buffer was used instead of S9 mix, at the same concentration.

2.6 EXPOSURE CONCENTRATION/DOSE LEVELS:

For the test finding concentration the dose levels were selected on the basis of mitotic index count. The maximum recommended concentration is 5 µl/ml, 5 mg/ml, or 0.01M whichever is the lowest (OECD 473).A minimum of three descending sequences of dose levels i.e.; 5 mg/ml to 1 mg/ml concentration were selected. Out of these, three final concentrations selected were 4mg/ml, 3mg/ml and 1mg/ml.

2.7 SOLVENT/VEHICLE CONTROL:

DMSO was used as the vehicle control, which does not give any chemical reaction with the test substance and is compatible with the survival of the cells and the S9 activity (OECD 473 GUIDELINE).

2.8 FORMULATON PREPARATION:

The methanolic extract of seeds of *Caesalpinia crista* plant contained the 16.72 gms of solids in waxy form which was soluble in DMSO. 500 gm of seeds were constituted in 1500 ml of methanol, finally 50gms extract was obtained, and hence the extraction efficiency was considered as 10%.

The plant extract was initially dissolved in DMSO to get 500mg/ml, from which various working concentrations of 5mg/ml, 4mg/ml, 3mg/ml, 2mg/ml, 1mg/ml were used for range finding assay. All the formulations were performed under a laminar air flow hood. The stock solutions were filtered sterilized with DMSO-resistant filters. All preparations were performed freshly on the day.

2.9 REFERENCE/CONTROL SYSTEM:

Concurrent positive and negative (solvent or vehicle) controls both with and without metabolic activation were included in each experiment. When metabolic activation is used, the positive control chemical should be the one that requires activation to give a mutagenic response. Positive controls should employ a known clastogen at exposure levels expected to give producible and detectable increase over background which demonstrates the sensitivity of the test system. Positive control concentration was chosen as per the guidelines.

The following positive controls were used:

POSITIVE CONTROL	METABOLIC ACTIVATION SYSTEM(S9)	CONCENTRATION
Mitomycin C (MMC)	-	0.3 µg/ml
Cyclophosphamide(CPA)	+	5 µg/ml

- Mitomycin C (MMC): Mitomycin-C is an anti-cancer ("antineoplastic" or "cytotoxic") chemotherapy drug. It is used for the treatment of adenocarcinoma of the stomach, pancreas, anal, bladder, breast, cervical, colorectal, head and neck, and non-small cell lung cancer.

- Cyclophosphamide (CPA): Cyclophosphamide is a chemotherapy drug usually given to treat lymphomas, leukemia, lung cancer and breast cancer. It may also be used to treat many other types of cancer.

TABLE A: 2.10 CULTURE MEDIUM CONSTITUENTS:

CONSTITUENTS	AMOUNT
RPMI -1640 Medium with 15% fetal bovine serum	8 ml
Phytoheamagglutnin(PHA)	300µl
Whole blood-Human	500 µl

METHODOLOGY:

Sampling and blood collection: Blood from healthy donor was collected using venipuncture taking proper aseptic precautions in sterile heparinised vacutainer and mixed gently to avoid clotting, which was used to set up cultures in sterile conditions.

I. MITOTIC INDEX (MI)

At 0 hr:

Culture tubes containing 8ml RPMI-1640 were taken and kept in the incubator to set 37^0c temperature. PHA was added at a final concentration of 30µl/ml to each of the culture tubes, with 0.5 ml of whole blood was added in sterile condition under laminar air flow (LAF). Culture tubes were allowed to incubate for 48 hrs at 37^0c in BOD/ CO_2 incubator.

At 48 hrs:

After 48 hrs of culture set up plant extract was added in various concentrations i.e. 1mg/ml, 2mg/ml, 3mg/ml, 4mg/ml and 5mg/ml with one culture tube untreated as control and vehicle control. The culture tubes were allowed to incubate for 22 hrs at 37^0C in BOD/ CO_2 incubator.

Harvesting of cultures:

At 70 hours:

The culture tubes were harvested after 22 hrs from starting of culture treatment. Addition of colchicine was done before 2 hrs of harvesting at concentration of 3µg/ml in each culture tube and kept for 2 hrs at 37^0C in a water bath.

The culture tubes were centrifuged at 2500-3000 rpm for 10 min after 2 hrs of colchicine treatment. The supernatant medium was discarded. The cells in the pellet were suspended in 7 ml of pre-warmed 37^0C hypotonic solution and kept for 20 min at 37^0C in water bath. After 20 min 4 ml of chilled fixative (3:1- methanol: glacial acetic acid) was added to each

culture tubes and centrifuged at 2500-3000 rpm for 10 min. Supernatant was discarded and the cells in the pellet (brown-black in color) were re-suspended in fresh pre-chilled fixative(7 ml). Fixative wash at an interval of 10 min were given (until clear pellet obtained). After the final change, supernatant was discarded and small amount of fixative was added to the pellet to adjust the cell concentration.

Slide Preparation and staining:

Two to three drops of cell suspension were dropped on grease free pre-chilled cleaned slides and were allowed to dry. Slides were stained in 4% Giemsa stain for 10 min. They were thoroughly rinsed in distilled water in a coplin jar and allowed to dry. The slides were then mounted with DPX and observed under the microscope.

Scoring for mitotic index:

Well spread metaphases were scored with cells in non dividing stage. The mitotic index was calculated as the number of metaphases per 1000 cells analyzed per culture for each treatment.

Calculation: [Cells in metaphase/total no. of cell count]

II. CHROMOSOMAL ABERRATIONS:

Scoring of chromosomal aberrations was done for short term and long term culture treatment to check the effect of *Caesalpinia crista* plant seed extract on cultured human peripheral blood lymphocytes with and without metabolic activation system.

For continuous treatment:

At 0 hr:

Culture tubes containing 8ml RPMI-1640 were kept in incubator before 30 min of culture set up to bring culture at 37^0c temperature. PHA was added at a final concentration of 30µl/ml to each of the culture tubes, with 0.5 ml of whole blood was added in sterile condition under laminar air flow (LAF). Culture tubes were allowed to incubate for 48 hrs at 37^0c in BOD/ CO_2 incubator.

At 48 hrs:

The plant extract was added in various concentrations of 1mg/ml, 3mg/ml, 4mg/ml with one culture tube as negative control, and a vehicle control was set by treating with DMSO (solvent) 80µl and positive control was set by treating with MMC (Mitomycin-C) at 0.3µg/ml. Culture tube were allowed to incubate for 22 hrs at 37^0c in BOD/ CO_2 incubator.

At 70 hrs:

The culture tubes were harvested after 22hrs from the initiation of culture treatment.

For short term treatment:

At 0 hr:

Culture tubes containing 8ml RPMI-1640 were kept in incubator before 30min of culture set up to bring culture at 37^0c temperature taken. PHA was added at a final concentration of 30µl/ml to each of the culture tubes, with 0.5 ml of whole blood was added in sterile condition under laminar air

flow (LAF). Culture tubes were allowed to incubate for 48hrs at 37^0c in BOD/ CO_2 incubator.

At 48 hrs:

In absence of metabolic activation system (S9 mix):

After 48 hrs of culture set up plant extract was added in various concentrations of 1mg/ml, 3mg/ml, 4mg/ml with a negative control and a vehicle control. The positive control was set by treating with MMC (Mitomycin -C) at 0.3µg/ml. Culture tube were allowed to incubate for 4 hrs at 37^0c in BOD/ CO_2 incubator.

In presence of metabolic activation system (S9 mix):

After 48 hrs of culture setting, 0.5ml of media was removed and 0.5ml of S9 mix was added to each tube. Plant extract was added in various concentrations of 1mg/ml, 3mg/ml, and 4mg/ml with one culture tube untreated as negative control, vehicle control was set by treating with DMSO (solvent) 80µl and positive control was set by treating with CPA (cyclophosphamide) at 5µg/ml. Culture tubes were allowed to incubate for 4 hrs at 37^0c in BOD/ CO2 incubator.

At 52 hrs:

After 4 hrs of culture treatment culture tubes were centrifuged at 2500-3000 rpm for 10 min and supernatants were removed and fresh 8ml of RPMI media was added to each tube. culture tube were incubated at 37^0c in BOD/ CO_2 incubator for 18 hrs.

At 70 hrs:

The culture tubes were harvested after 22 hrs from the initiation of culture treatment (or after 18 hrs of incubation after treatment).

The harvesting and slide preparation were performed as same as mentioned above.

Summary of treatment for chromosomal aberration assay:

Treatment	S9	Duration of treatment(hours)	Harvest time (hours after start of treatment)
Short term(4+ 18)	Absent	4	22
	Present	4	22
Continuous (22)	Absent	22	22

Slide scoring:

At least 100 well spread metaphases were scored for the chromosomal aberration per treatment group. Only a well spread metaphase containing number of chromosomes equal to 46 were considered. Cells with more than or less than 46 chromosomes, with chromosomal break & gap, with chromatid gap & break, fragments ring were recorded on score sheet.

Evaluation and Interpretation criteria:

There were several criteria for determining a positive result, such as a concentration-related increase or a reproducible increase in the number of cells with chromosomal aberrations at a single test concentration. Statistical methods were used as an aid in evaluating the test results.

An increase in the number of polyploid cells may indicate that the test substance has the potential to interfere with the mitotic processes and to induce numerical chromosomal aberrations.

Positive results from the *in vitro* chromosomal aberration test indicate that the test substance induces structural chromosomal aberrations in cultured mammalian somatic cells. Negative results indicate that, under the test conditions, the test substance does not induce chromosomal aberrations in cultured mammalian somatic cells.

III. CYTOKINESIS BLOCKED MICRONUCLEI ASSAY(CBMN)

At 0 hr:

Culture tube containing 8ml RPMI-1640 were kept in incubator before 30min of culture set up to bring culture at 37^0c temperature. PHA was added at a final concentration of 30µl/ml to each of the culture tubes, 0.5 ml of whole blood was added in sterile condition under laminar air flow (LAF). Culture tubes were allowed to incubate for 48hrs at 37^0c in BOD/ CO_2 incubator.

At 48 hrs:

In absence of metabolic activation system (S9):

After 48 hrs of culture set up plant extract was added in various concentrations of 1mg/ml, 3mg/ml, 4mg/ml with one culture tube untreated as control, vehicle control was set by treating with DMSO (solvent) 80µl and positive control was set by treating with MMC (mitomycin -C) at 0.3µg/ml. Culture tubes were allowed to incubate for 6 hrs at 37^0c in BOD/ CO_2 incubator.

In presents of metabolic activation system (S9):

After 48 hrs of culture setup 0.5ml of media was removed 0.5ml of S9 mix was added to each tube. Plant extract was added in various concentrations of 1mg/ml, 3mg/ml, 4mg/ml with one culture tube untreated as control, vehicle control was set by treating with DMSO (solvent) 80µl and positive control was set by treating with CPA (cyclophosphamide) at 5µg/ml. Culture tubes were allowed to incubate for 6 hrs at 37^0c in BOD/ CO_2 incubator.

At 54 hrs:

After 6hrs of culture treatment culture tube were centrifuged at 2500-3000 rpm for 10 min and supernatant was removed, 90 µl Cyt-B was added and 8ml of RPMI media was added to each tube. Culture tubes were incubated at 37^0c in BOD/ CO_2 incubator for 18 hrs.

At 78 hours:

The culture tubes were harvested after 24 hrs from the initiation of culture treatment. The culture tubes were centrifuged at 2500-3000 rpm for 10 min. The supernatant medium was discarded. The cells in the pellet were suspended in 5 ml of pre-warmed 37^0C hypotonic solution for 1-2 min. Approximately 1 ml of fixative was added to stop the hypotonic treatment and was stored at 4^0C for 2 hrs.

The culture tubes were centrifuged at 2500-3000 rpm for 10 min after 2 hrs. The supernatant medium was discarded and the cells in the pellet (brown-black in color) were re-suspended in fresh pre-chilled fixative (7 ml). Fixative wash at an interval of 10 min were given (until clear pellet obtained). After the final change of supernatant was discarded and small amount of fixative was added to the pellet to adjust the cell concentration.

Slide Preparation staining:

Slide preparation was performed same as mentioned above.

Scoring of micronuclei:

Total 1000 cells in binucleated and binucleated with micronuclei stage were counted per treatment and locations of binucleated cells with micronuclei were noted separately.

Criteria for selecting binucleated cells which can be scored for micronucleus frequency:

1. Cells should be binucleated.

2 The two nuclei in binucleated cell should have intact nuclear membranes and situated within the cytoplasmic boundary.

3. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity.

4. The two nuclei within a binucleated cell may be unconnected or may be attached by one or more fine nucleoplasmic bridges, which are not wider than 1/4th of the nuclear diameter.

5. The two main nuclei in binucleated cell may touch but should not ideally overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of either nucleus are distinguishable.

6. The cytoplasmic boundary or membrane of binucleated cell should be intact and clearly distinguishable from the cytoplasmic boundaries of adjacent cells.

Criteria for scoring Micronuclei:

1. Micronuclei should be morphologically identical and smaller than the main nuclei.

2. The diameter of micronuclei in human lymphocytes usually varies between $1/16^{th}$ and$1/3^{rd}$ of the mean diameter of the main nuclei.

3. Micronuclei should be scored in binucleated cells with well preserved cytoplasm.

4. It must show no refractility in contrast with nuclear particles.

5. Micronuclei should not be linked or connected to the main nuclei. It may touch but should not overlap the main nuclei.

6. Ideally micronuclei should have the same staining intensity as the main nuclei but occasionally may be more intense.

STATISTICAL ANALYSIS:

Statistical evaluation of the result was performed by counting differences between the treated and control groups were analyzed using the one way analysis of variance (ANOVA). The result of the statistical evaluation was regarded as significant when the p value <0.05.

3. RESULT AND DISCUSSION

Table 1: Results of Mitotic Index (MI) scoring:

No.	Treatment Groups	Total cells scored	Total No. of metaphase	Mitotic Index (MI=No. of cells in metaphase/total No. of cells counted)	MI (%)	% Reduction in MI
1	Control	1000	23	0.023	100	0.00
2	Vehicle control	1000	44	0.044	191	-91
3	5mg/ml	1000	7	0.007	30.43	69.57
4	4mg/ml	1000	10	0.010	43.47	56.53
5	3mg/ml	1000	14	0.014	60.86	39.14
6	2mg/ml	1000	17	0.017	73.91	26.09
7	1mg/ml	1000	22	0.022	95.65	4.35

Graph 1: Mitotic index (%) of various treatment groups

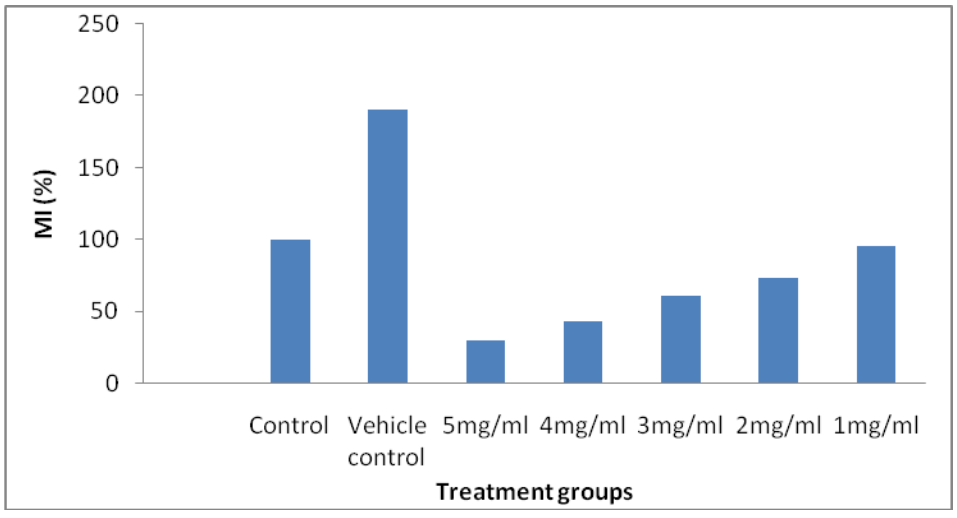


Table 2: Results of chromosomal aberration assay for long term exposure with only plant extract without metabolic activation

No.	Treated Group	No. of well spread metaphase scored	No. of cells observed with aberration	Mean ± SE
1	Control	100	0	0
2	Vehicle control	100	1	0.01 ± 0.01
3	Positive control	100	7	0.07 ± 0.025
4	5mg/ml	100	4	0.04 ± 0.019
5	4mg/ml	100	6	0.06 ± 0.023
6	3mg/ml	100	3	0.03 ± 0.017
7	2mg/ml	100	3	0.03 ± 0.017
8	1mg/ml	100	2	0.02 ± 0.014

Graph 2: Results of chromosomal aberration assay for long term exposure in absence of metabolic activation

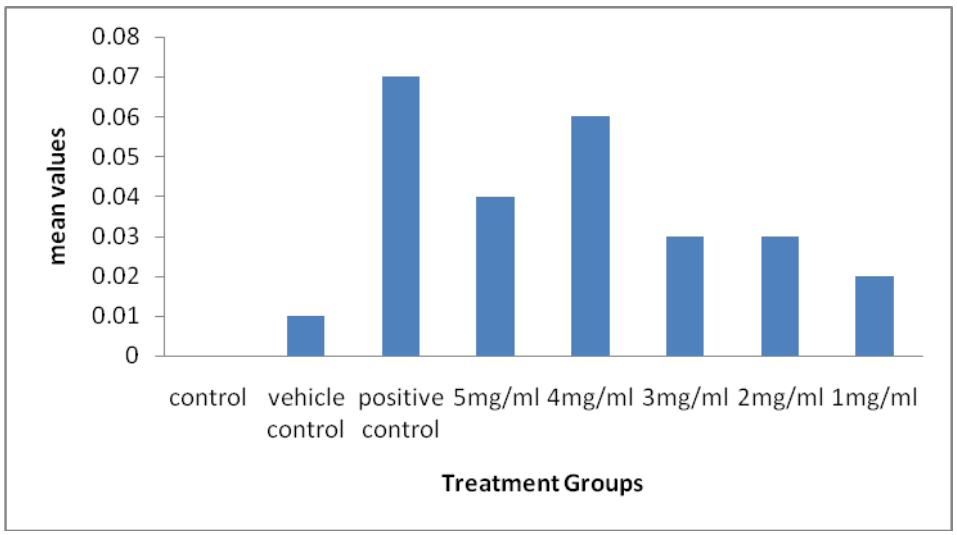
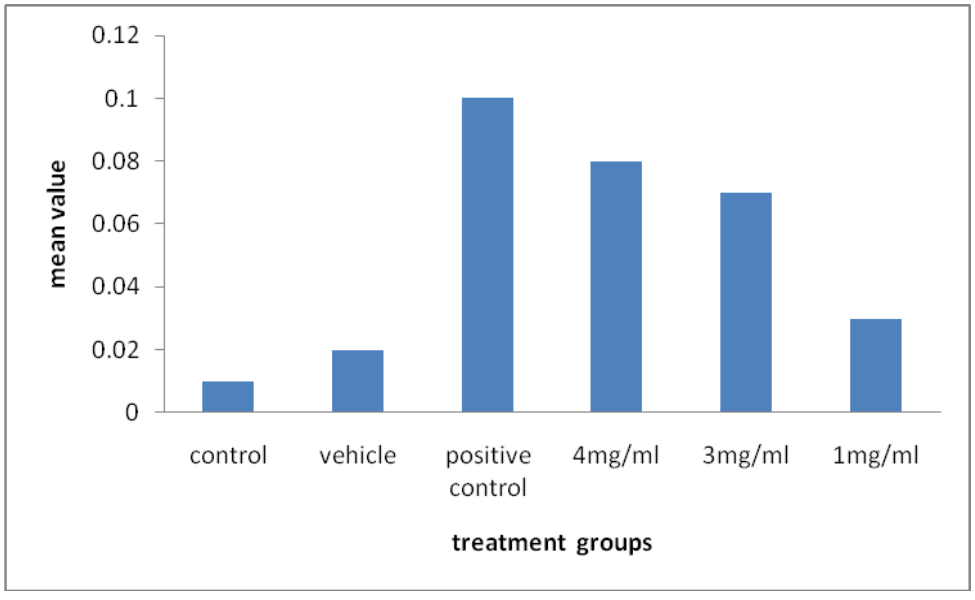


Table 3: Results of Chromosomal Aberration in various plant extract concentration treated in presence of metabolic activation

No.	Treated Group	No. of well spread metaphase scored	No. of cells observed with aberration	Mean ± SE
1	Control	100	1	0.01 ± 0.01
2	Vehicle control	100	2	0.02 ± 0.014
3	Positive control	100	10	0.10 ± 0.03
4	4mg/ml	100	8	0.08 ± 0.027
5	3mg/ml	100	7	0.07 ± 0.025
6	1mg/ml	100	3	0.03 ± 0.017

Graph 3: Results of mean values of various groups for chromosomal aberration assay in presence of metabolic activation



Results4: Results of Chromosomal aberration in various plant extract concentration treated in absence of metabolic activation system for short term exposure

No.	Treated Group	No. of well spread metaphase scored	No. of cells observed with aberration	Mean ± SE
1	Control	100	0	0
2	Vehicle control	100	2	0.02 ± 0.014
3	Positive control	100	9	0.09 ± 0.028
4	5mg/ml	100	8	0.08 ± 0.027
5	4mg/ml	100	10	0.1 ± 0.03
6	3mg/ml	100	8	0.08 ± 0.027
7	2mg/ml	100	2	0.02 ± 0.014
8	1mg/ml	100	0	0

Graph 4: Results of mean values of various groups for chromosomal aberration assay treated for short term exposure time in absence of metabolic activation

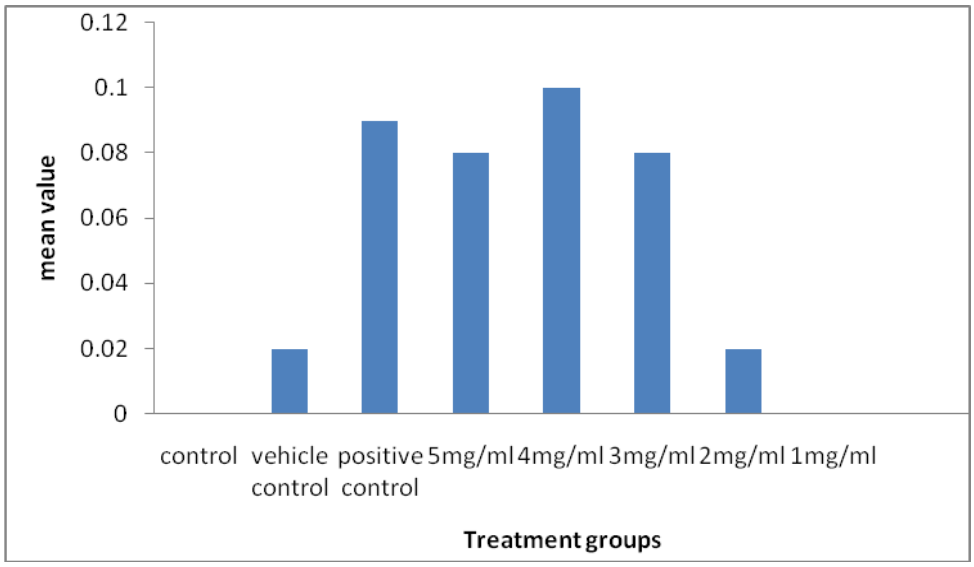


Table B: Statistical analysis of chromosomal aberration

	No.	GROUPS	ANOVA test	p-value
Chromosomal Aberration (CA) assay for short term exposure time in absence of metabolic activation system (S9 mix)	1	Control vs. vehicle control DMSO	0.156	Not significant
	2	Vehicle control DMSO vs. positive control (MMC)	0.029	Significant
	3	control vs. positive control (MMC)	0.002	Significant
	4	control vs. 5mg/ml	0.0037	Significant
	5	control vs. 4mg/ml	0.0010	Significant
	6	control vs. 3mg/ml	0.00374	Significant
	7	control vs. 2mg/ml	0.1567	Not significant
	8	control vs. 1mg/ml	0	Significant
Chromosomal Aberration (CA) assay for long term exposure time in absence of metabolic activation system (S9 mix)	1	Control- vehicle control DMSO	0.318	Not significant
	2	Vehicle control DMSO- positive control (MMC)	0.030	Significant
	3	control-positive control (MMC)	0.0069	Significant
	4	control-5mg/ml	0.043	Significant
	5	control-4mg/ml	0.0127	Significant
	6	control-3mg/ml	0.081	Not significant
	7	control-2mg/ml	0.081	Not significant
	8	control-1mg/ml	0.156	Not significant
Chromosomal Aberration (CA) assay for short term exposure time in presence of metabolic activation system (S9 mix)	1	Control- vehicle control DMSO	1	Not significant
	2	control-vehicle control DMSO(with s9)	0.56	Not significant
	3	control-positive control (CPA)	0.005	Significant
	4	control-4mg/ml	0.0168	Significant
	5	control-3mg/ml	0.0304	Significant
	6	control-1mg/ml	0.3148	Not significant

Table 5: Results of CBMN Assay in absence of metabolic activation

No.	Treatment groups	% of BNC with MNi
1	Control	0.9
2	Vehicle control	2.94
3	Positive control	12.28
4	5mg/ml	4.365
5	4mg/ml	5.97
6	3mg/ml	3.06
7	2mg/ml	2.65
8	1mg/ml	2.41

Graph 5: Results of CBMN-% of BNC with MNi

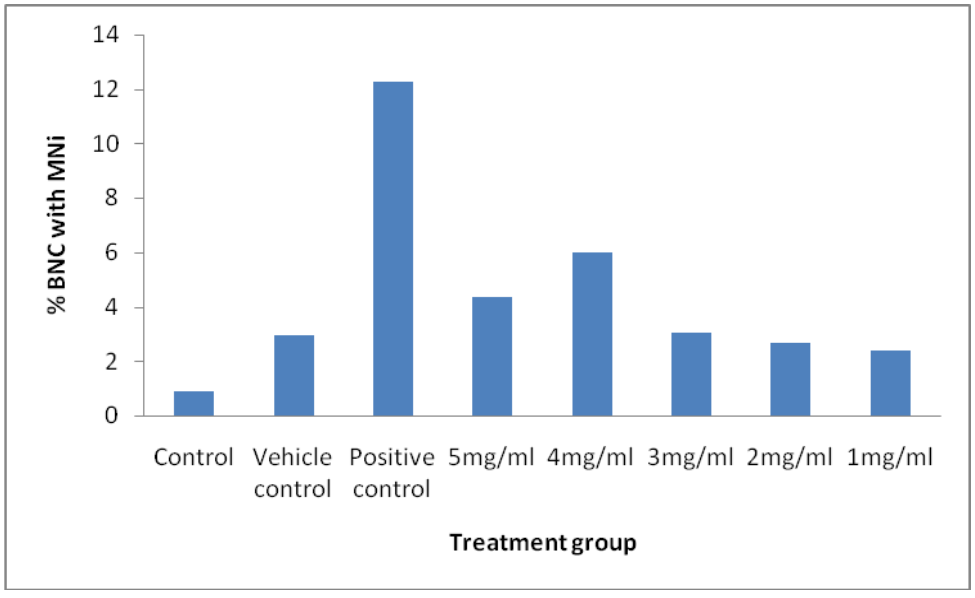


Table 6: Results of CBMN Assay in presence of metabolic activation system:

No.	Treatment groups	% of BNC with MNi
1	Control	2
2	Vehicle control	2.56
3	Positive control	4.69
4	4mg/ml	6.25
5	3mg/ml	5
6	1mg/ml	3.5

Graph 6: Results of CBMN assay in presence of S9 mix-%BNC with MNi

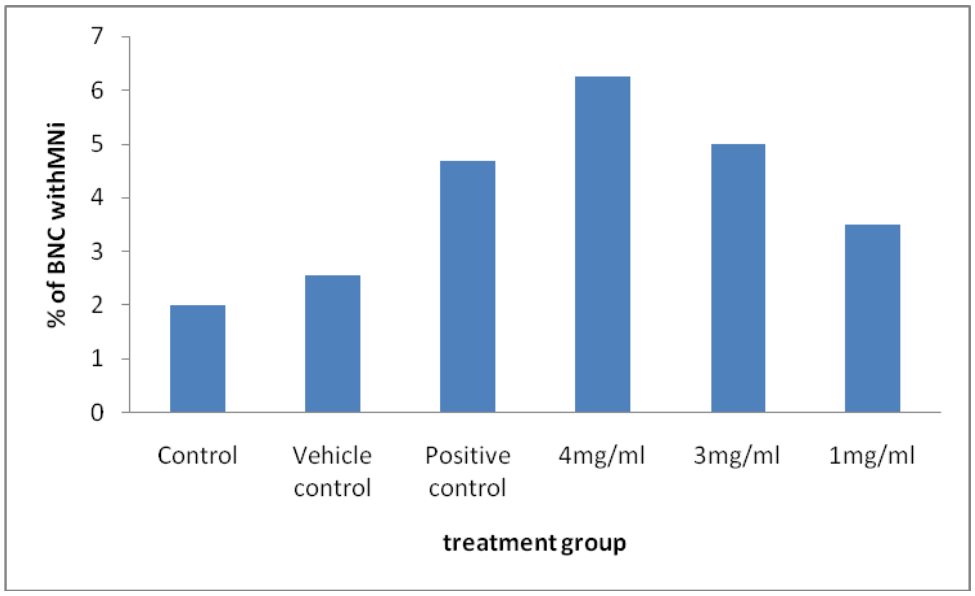


Table C: Statistical analysis of CBMN frequencies:

	No.	GROUPS	P value	
CBMN assay in presence of metabolic activation system(S9)	1	Control vs. positive control	0.40	Not significant
	2	control vs. vehicle control	1	Not significant
	3	Positive control vs. vehicle control	0.40	Not significant
	4	control vs. 4mg/ml	0.150	Not significant
	5	control vs. 3mg/ml	0.250	Not significant
	6	control vs. 1mg/ml	0.65	Not significant
CBMN assay in absence of metabolic activation system(S9)	1	control vs. positive control	0.0008	Significant
	2	control vs. vehicle control	0.563	Not significant
	3	Positive control vs. vehicle control	0.003	Not significant
	4	control vs. 5mg/ml	0.1759	Not significant
	5	control vs. 4mg/ml	0.098	Not significant
	6	control vs. 3mg/ml	0.1759	Not significant
	7	control vs.2mg/ml	0.314	Not significant
	8	control vs.1mg/ml	0.314	Not significant

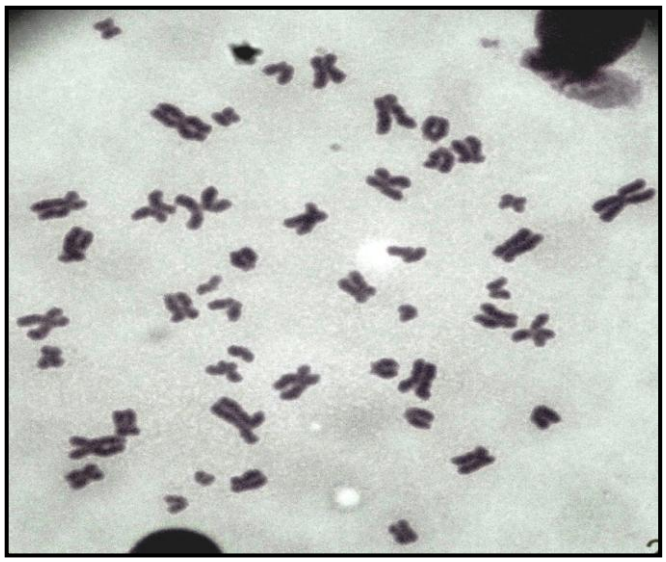


Fig 3: A Giemsa-stained normal metaphase

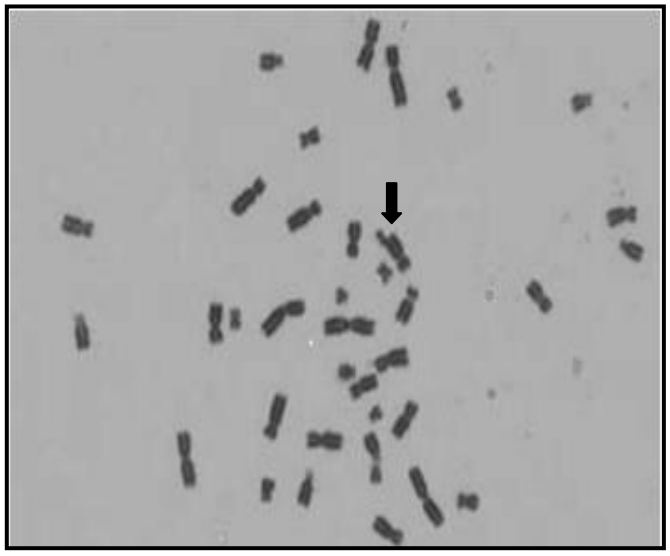


Fig 4: Arrow depicts a chromatid break in Giemsa stained partial metaphase plate

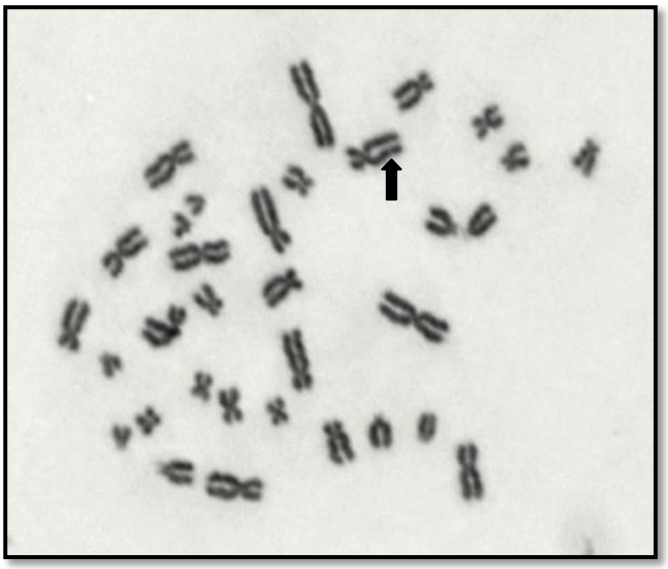


Fig.5:Arrow depicts a fragment in Giemsa stained partial metaphase plate

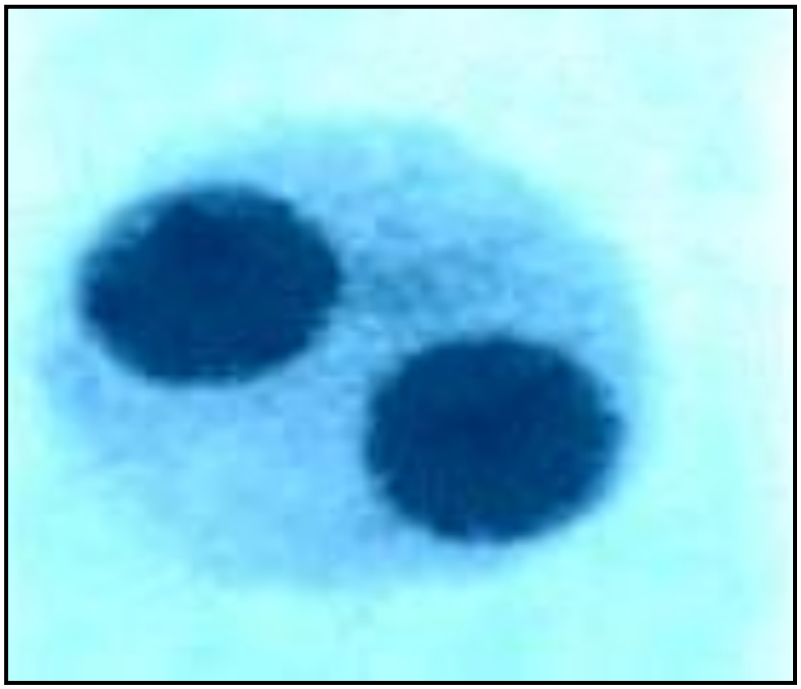


Fig.6: Giemsa stained Cytokinesis blocked binucleated cell

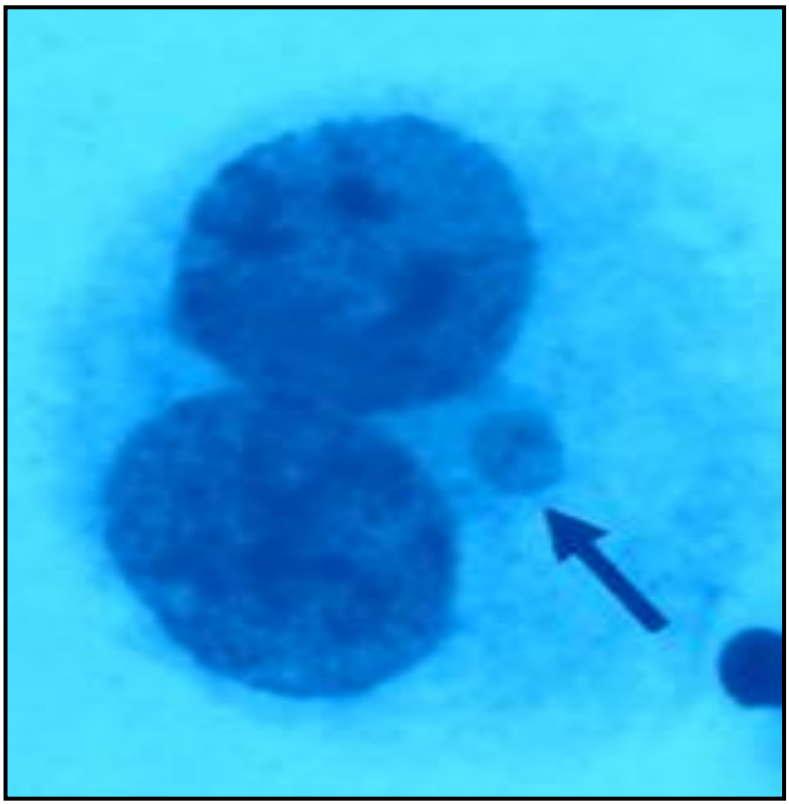


Fig7: Arrow depicts Cytokinesis blocked binucleated cell with micronuclei

In vitro chromosome aberration analysis on cultured human blood lymphocytes:

Possible clastogenic effect of methanolic extract of *Caesalpinia crista* plant seeds was evaluated using mammalian chromosome aberration test using cultured human blood lymphocytes and the results are given in the Tables (2-4) and graphs no. (2-4). 4 and 18 h treatment with different concentration of methanolic extract of *Caesalpinia crista* plant seeds both in presence and absence of S9 mix induced significant number of structural chromosomal aberrations(Table A). The aberration rates at all the tested concentrations of plant extract were higher than the control and the DMSO vehicle control. Treatment with DMSO only (vehicle control) also resulted in chromosomal aberration incidences was nearly similar to those from control data. Positive controls MMC (both 4 and 18 h exposure, absence of S9 mix) and CPA (4h exposure, presence of S9 mix) demonstrated significant induction of structural chromosomal aberrations indicative of the validity of the experiments. Mitotic Index is a measure for the proliferation status of a cell population. Mitotic index was analyzed and results obtained are given in table 1 and graph 1. Methanolic extract of *Caesalpinia crista* plant seeds significantly decreased the mitotic index value at the highest tested concentration indicating its cytotoxicity. Overall, results indicate that methanolic extract of *Caesalpinia crista* plant seeds was clastogenic.

In vitro micronucleus analysis on cultured human blood lymphocytes:

The frequency of binucleated cells and binucleated cells with micronuclei obtained in micronucleus test is shown in Tables and graphs no.(5-6) at different concentration of methanolic extract of *Caesalpinia crista* plant seeds both in presence and absence of metabolic activation system (S9 mix). Results revealed (as shown in table B) that methanolic extract of *Caesalpinia crista* plant seeds did not produce significant aneugenic activity after treatment with any of the tested concentrations when compared to the concurrent with control (untreated group) vehicle control cells ($p < 0.05$) both with and without metabolic activation. Therefore, our results indicate that methanolic extract of *Caesalpinia crista* plant seeds is not aneugenic.

DISCUSSION

Use of herbal remedies is an increasing trend in the field of complementary and alternative medicine. The use of herbal plants has significantly contributed to healthcare through the isolation of bioactive compounds for direct use in medicine. Natural products, as either pure compounds or standardized plant extracts, provide variety of therapeutic opportunities. *C.crista* plant has a long history of use as a remedy in the treatment for a wide range of ailments. It is one of the oldest and most popular herbal medicines in India. Many studies have reported the pharmacological efficiency and benefits of *C.crista* plant, The extracts of various other parts of are reported to have anthelmintic, anti-amyloidogenic, immunomodulatory, analgesic, antipyretic, anti-inflammatory, memory enhancing, antidiabetic activity and also antitumor and antioxidant activity. But little information has been reported about its genotoxicity. Several plant products have been tested for anticancer activity and some of them like vincristine, taxol are now used as a drug of choice. The seeds and leaves of *Caesalpinia crista* plant has been reported to have around fourteen compounds. The isolated compounds are cassane-and norcassane-type diterpenes and other flavonoids and triterpenoides.

The use as home remedy is high among Aadivasi tribe in Gujarat as anthelmintic. The parts like seeds are sometimes used in necklaces are considered febrifugal, tonic, and vesicant. They are used to treat colic, convulsions, leprosy, and palsy. The oil from the seeds is said to soften the skin and remove pimples. The bark is antiperiodic, rubefacient and plant to counteract toothache. A leaf decoction is as collyrium. The different parts such as leaves, seed, root, bark is also used in colic fever, intermittent fever, malaria, menstrual complaints, pneumonia, skin diseases, swelling, tonic, pulmonary tuberculosis and as a uterine stimulant, to cleanse the uterus. It also alleviates the fever, edema and abdominal pain during this period.

Besides the possible use of *C.crista* plant as a therapeutic agent, knowledge about its genotoxic potential is also of interest from the point of view of its use as herbal remedy in addition to investigate the mode of action of ant tumor activity. The present study was carried out to check

genotoxic potential of plant seed extract using *in vitro* assays like chromosome aberration test and micronucleus test. Chromosomal aberration assay and CBMN assay have been commonly used to evaluate cytogenetic effects viz. clastogenic and aneugenic respectively in responses to chemical exposure and an excellent dose response relationship has been established for hundred of chemicals in a wide variety of *in vivo* and *in vitro* short-term experiments. Chromosomal aberrations are changes in chromosome structure resulting from a break and other events involving single or both the chromatids. In micronucleus assay formation of micronuclei is due to chromosomes(s) or fragments with or without the centromere which lag behind during anaphase. Tests were performed using OECD guidelines.

The results of the study reveal that the selected dosages of the plant extract were genotoxic on human lymphocytes *in vitro*. In our study, doses of plant extract (5, 4, 3, 2 and 1 mg/ml) were studied both in presence and absence of metabolic activation system. Extract was found to have genotoxicity at high concentrations both in presence and absence of metabolic activation system. The present study regarding *in vitro* plant extract induced chromosomal aberrations and BNC with MNi frequency both in presence and absence of metabolic activation system on cultured human blood lymphocytes indicates that the anti-tumor activity of the extract can be due to the presence of genotoxic compound(s) that are not dependent on metabolic activation.

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