Radio protective effect of bamboo species on ionizing radiation induced genomic damage: An *in vitro* study

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BY

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CERTIFICATE

This is to certify that the thesis entitled "**Radio protective effect of bamboo species on ionizing radiation induced genomic damage: An *in vitro* study"** submitted to the Institute of Science, Nirma University, in partial fulfillment of the requirement for the award of the degree of M.Sc. in Biochemistry and Biotechnology, is a record of research work carried out by Md.Hamzah H Kagzi (14MBT008), Tejashwi Makani (14MBT012) and Darshana Brahmbhatt (14MBT004) under the guidance of Dr. Sonal Bakshi. No part of the thesis has been submitted for any other degree or diploma.

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Dr. Sonal Bakshi
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Declaration:

The above dissertation was carried out jointly by Md.Hamzah H Kagzi (14MBT008), Tejashwi Makani (14MBT012) and Darshana Brahmbhatt (14MBT004) with my guidance.

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Declaration

We declare that the thesis entitled "**Radio protective effect of bamboo species on ionizing radiation induced genomic damage: An *in vitro* study**" has been prepared by us; Md.Hamzah H Kagzi (14MBT008), Tejashwi Makani (14MBT012) and Darshana Brahmbhatt (14MBT004) under the guidance of Dr. Sonal Rajiv Bakshi, Assistant Professor of institute of Science, Nirma University, Ahmedabad. No part of this thesis has formed the basis for the award of any other degree or diploma.

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ABSTRACT

Exposure of radiation in terms of power generation industry, food preservation, space exploration, nuclear war and radiotherapy of cancer affects the normal tissue also apart from the cancer tissue. In order to provide protection against harmful biological effect of radiation, which cause damages to DNA, Lipids and proteins. Exposure to high amount of ionizing radiation causes damages to the hematopoietic system, gastrointestinal system and central nervous system, depending on radiation dose, Hence there is need to search for a compound is still continued, especially from the plant origin. Only one synthetic radioprotector (i.e.Amifostine) is approved by the FDA till date.

Due to its cumulative toxicity, it is limited in use. As a result no safe, ideal radioprotector is available till date hence investigators explored plant kingdom due to its richness in flavonoids, alkaloids, phytosterols, phenolics and any many more phytochemicals responsible for various medicinal values. The bamboo leaf extract which is rich in flavonoids like orientin can be considered as candidate radioprotective agent. We have carried out in vitro assessment of radio-protective effect of bamboo leaf extract, the laboratory end points are: cytokinesis blocked micronucleus assay & COMET assay (Single cell gel electrophoresis) to assess the decrease in DNA damage following exposure to bamboo leaf extracts. These cytogenetic endpoints are as per the standard guidelines of regulatory agencies that monitor methods related to genotoxicity.

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LIST of ABBREVIATIONS

Abbreviation	Full forms
B.aurndinaceae	*Bambusa aurndinaceae*
BN	Binucleated Cells
CBMN	Cytokinesis Blocked Micro-nuclei Assay
CO_2	Carbon dioxide
Conc.	Concentration
Cyt-B	Cytochalasin-B
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
HPBL	Human Peripheral Blood Lymphocytes
Hr	Hour
LAF	Laminar Air Flow
Min	Minutes
mg	Milligram
MNi	Micro-nuclei
OECD	Organization for Economic co-operation and development
RPM	Rotation per minute
RPMI	Roswell Park Memorial Institute
S.D	Standard Deviation
S.E	Standard Error
°C	Degree centigrade
µg	Micro gram
µL	Micro liter
µM	Micro meter

INTRODUCTION

1. INTRODUCTION

1.1 Radiation & Radioprotectors

Ionizing radiation is a type of energy which is released by atoms in the form of electromagnetic waves (gamma or X-rays) or particles (alpha, beta, and gamma). People are exposed to various kind of natural radiation sources present in soil, water and in air. More than 60 naturally occurring radioactive material found in soil.

The naturally occurring gas radon, derived from the rock & soil is the main source of natural radiation source. People exposed to natural radiation from cosmic rays, ultra violet rays from sun. People are also exposed to the lethal radiation through the nuclear explosion & nuclear war. Overexposure to UV rays from the sun is one main reason of people getting skin cancer. UVA & UVB both kinds of UV rays damage the skin & able to cause skin cancer. Radiotherapy is the treatment which is given to the cancer patients. Eighty percent of cancer patients need radiotherapy as a part of their treatment of cancer.

This radiation affects the normal healthy organ and tissues of the body apart from the tumor. In terms of protecting the normal healthy organs and tissues there is a need to develop an effective radio-protector. Till date there is only one synthetic radio-protector (i.e. Amifostine) available approved by FDA, but limited in use due to its cumulative effect.

1.2 Natural radioprotectors: Plants & Herbs

Natural Radio-protectors are the compounds that are designed to reduce genetic damage caused by radiation in normal tissues. They prevent the effect of ionizing radiation on biological system (i.e. Photochemical, herbal extracts, and plants).

List of plants with radio-protective activity or antioxidant activity

Plant species	Scientific names	Component	Activity	Reference
Tomato	*Solanum Lycopersicum*	Carotenoids – lycopenes	Antioxidant	Griffiths et al., 2005; Saraf and Kaur, 2010; Ravichandran et al., 2005
Carrot	*Daucus carota*	β-carotene	Antioxidant	Griffiths et al., 2005; Svobova et al., 2003
Papaya	*Carica papaya*	L-ascorbic acid	Antioxidant and photoprotective	Vile, 1997
Orange	*Citrus sinensis*	L-ascorbic acid	Antioxidant	Cimino et al., 2007
Lemon	*Citrus limon*	L-ascorbic acid	Antioxidant	Apak et al., 2007
Mango	*Mangifera indica*	L-ascorbic acid	antioxidant with anti-inflammatory and immunomodulatory activities.	Song et al., 2013
Celery	*Apium graveolens*	Flavones – 5,7,4'-trihydroxystilibine	antioxidant and ROS scavenger	Griffiths et al., 2005; Svobova et al., 2003
Red clover	*Trifolium pretense*	Isoflavone – Genistein	Inhibit UV induced peroxidase production	Widyarini et al., 2001

There are many other plants whose radioprotective activity is reported are as below:

Curcuma Longa (Haldi), Withania somnifera (Ashwagandha), Ocimum Sanctum (Tulsi), Azadirachta Indica (Neem)Eugenia Jambolana (Jamun), Rajgira (Amarantus Paniculatus), Adhatoda Vasica (Adulsa), Allium Sativum (Garlic), Prunus Avium (Sweet cherry), Myristica Fragrans (Jaiphal), Emblica Officinalis (Amla), Aloe Vera (Gheekumari), Spinacia Oleracea (Palak), Zingiber Officinale (Ginger).

1.3 Properties of ideal Radioprotectors

A substance with immune-modulations, free radical scavenging or anti-stress, anti-inflammatory, antimicrobial, antioxidant, properties may act as a potential radio-protector

- High therapeutic ratio
- Wide window of protection against all types of toxicity
- Preservation of the anti-tumor efficacy of radiation
- High efficacy/toxicity profile(Low intrinsic toxicity profile

1.4 Introduction to plant Bamboo

Bamboo is one of the fastest growing plants on Earth, due to its unique rhizome-dependent system. Certain species of bamboo can grow 91 cm (3 ft) within a 24 hr. bamboo species are found in diverse climates, from cold mountains to hot tropical regions. Bamboo can grow well in hot, humid rain forests and also cold hardy forests having temperature of about 20°C. It can tolerate extreme rainfall ranging between 32 to 50 inches. Bamboo is considered to be a multipurpose plant having 1500 documented uses as medicine, food & fodder, also prevent soil erosion etc. The antioxidants properties found in bamboo leaves. Its shoot has high dietary fiber and thus good for stomach & can cure indigestion as well as diarrhea. Bamboo shoots also help to reduce cholesterol & can control blood pressure. Uses of bamboo plants are not only limited for medicinal purposes but it is also used to make furniture & is an excellent raw material for construction purposes. India has the second largest bamboo reserve after China.

1.5 List of bamboo species in India

Species	States
Bambusa arundinacea	Arunachal Pradesh, Orissa, Maharashtra, Andhra Pradesh, Karnataka, Himachal Pradesh, and Gujarat
Dendrocalamus strictus	Andhra Pradesh, Assam, Gujarat, Maharashtra, Himachal Pradesh, Rajasthan ,Madhya Pradesh, Manipur, Orissa, Karnataka, Uttar Pradesh
Phyllostachys parvifolia	Assam ,Meghalaya, kerela, karnataka, Gujarat
Bambusa balcooa	Mizoram ,Arunachal Pradesh,
Bambusa tulda	Arunachal Pradesh, Assam, Mizoram, Nagaland, Tripura
Polystachia pargracile	Orissa
Melocanna bambusoide	Assam, Meghalaya, Mizoram, Nagaland, Tripura, Manipur
Bambusa pallid	Tripura ,Arunachal Pradesh, Mizoram, Nagaland,
Oxytenanthera nigrociliata	Tripura, Assam
Bambusa polymorpha	Tripura
Pseudostachyus polymorphium	Arunachal Pradesh
Neebenzia balcooa	Nagaland

1.6 Introduction to plant *Bambusa arundinacea*

1.6.1 Classification of Bambusa arundinacea:

Kingdom: Plantae

Order : Poales

Family : Poaceae

Genus : *Bambusa*

Species : *Bambusa arundinacea*

Bambusa arundinacea is the Giant thorny bamboo with stout, short, knotty rhizomes, also called Bamboo manna and in ancient time it is called vamsa in Ayurveda. Found throughout peninsular India, Myanmar (Burma) and Sri Lanka.

1.6.2 Description of plant *Bambusa arundinacea*:

The siliceous substance found near the joint inside is white camphor like crystalline in appearance, slightly sticky to the tongue and sweet in taste. Clums (stems) dense, up to 30 m tall and 5-10 cm in diameter, green, hollow, walls thick, purplish-green when undeveloped, turning golden yellow, leaves linear or linear-lanceolate, 7-18 cm long and 0.2-2 cm wide. It contains 90% silica, iron, potash, lime, alumina, vegetable matter.

1.6.3 Habitat: Plant grows all over India, primarily in forests of western and southern parts of the country. It can grow up to 1500-2000 meters. It is an erect, 15-35 meters tall, thorny tree, with many stems. The leaves are sheathing, linear, 20 cm long and 2 cm broad.

1.6.4 Medicinal uses: *Bambusa arundinacea* has great medicinal value. Bamboo manna is beneficial in different disorders like hyperpiesia, diarrhea, vomiting, heart disease, asthma, fever, cough, tuberculosis etc. the roots are diuretic, tonic, cooling; they are beneficial in skin disorders, burning sensation, general debility and dysuria.

Bambusa arundinacea

A

B

C

D

A www.homeopathyworldcommunity.com/forum/topics/proving-on-bambusa-arundinacea.

B http://www.treeseedsindia.in/bambusa-arundinacea-1218660.html.

C https://commons.wikimedia.org/wiki/File:Bambusa_arundinacea_001.JPG.

D http://www.chhajedgarden.com/bambusa-arundinacea-medicinal-plants-pack-of-2.html.

1.7 Summary

The bamboo leaf extract is rich in flavonoids like orientin can be considered as candidate radioprotective agent.
We carried out in vitro assessment of radio-protective effect of bamboo leaf extract, the laboratory end points were : Cytokinesis Blocked Micronucleus Assay & COMET assay (Single cell gel electrophoresis) to assess the decrease in DNA damage following exposure to bamboo leaf extracts.
These cytogenetic endpoints were as per the standard guidelines of regulatory agencies that monitor methods related to genotoxicity.

MATERIALS AND METHOD

2. Materials and Methods

2.1 Assessment of Radioprotective activity of Bamboo leaf extract by CBMN assay

CBMN method is named as Cytokinesis blocked micronucleus assay. In CBMN assay micronucleus (MNi) can be observed in bi-nucleated (BN) cell. MNi derived from the whole chromosome or chromosomal fragments that lag behind at anaphase during cell division. The cyt-B is responsible for this bi-nucleated appearance of cell, as this cyt-B can deterred the microfilament (i.e. Actin) ring assembly that is required for completion of cytokinesis. With the help of CBMN assay it is possible to measure frequency of chromosomal breakage (MNi), chromosome loss (MNi), chromosome rearrangement e.g. dicentric chromosomes (NPB), Gene amplification (NBUDS), Necrosis and Apoptosis.

2.2 Assessment of Radioprotective effect of Bamboo leaf extract by COMET assay

COMET assay is also called single cell gel electrophoresis (SCGE). This is very useful cytogenetic assay to detect DNA single strand breaks. Cells embedded in agarose gel on a microscope slide, are lysed with detergent and high salt to form nucleoids having DNA linked to the nuclear matrix. When the electrophoresis done at the high pH, it resemble like comets that refers to the pattern of DNA migration through the agarose gel, which is observed under the fluorescence microscopy; The intensity of comet tail in compare to the head shows the number of DNA breaks. It is very useful technique to detect the DNA damage and also for the genotoxicity testing.

2.3 Cytokinesis-Block Micronucleus Assay

2.3.1 Materials

All the materials which were used in this study were of analytical grade and were acquired from SIGMA ALDRICH, HI-MEDIA, MERCK and EMPLURA™**.** The water which was used in the preparation of the solutions was either Distilled water or Double Distilled water. The solutions were stored in appropriate temperature and all the glassware and plasticware were sterilized by autoclaved (at 15psi in liquid cycle at 121°c).

2.3.2 Reagents

Culture Media RPMI-1640: HiKaryoXL™ RPMI-1640 (CAT number AL-165A) (L-Glutamin, FBS, PHA- M, Penicillin, Streptomycin, Sodium Bicarbonate). Test cell culture, Cytochalasin-B : Hi-Media , Potassium Chloride : Merck , Acetic Acide : Emplura , Methanol : Emplura, Giemsa Stain : Hi-Media

- Potato Dextrose media
- N Agar media

2.3.3 Equipments :

- Vacuatte (4.0ml) Sterile vacutainer blood tubes with sodium heparin anticoagulant
- Blood Collection needle [DispoVan®] (10 ml or 7 ml) Sterile syringe
- 0.22µl filter unit syringe driven[Axiva]
- Graduated pipettes [Borosil]

- Benchtop centrifuge [REMI] capable of spinning at 1000g
- Waterbath [Serological] for maintaining hypotonic solution at 37° c
- Microscope Sildes [Super deluxe Microslides, Blue Star®] 75x25 mm, and 1.1mm thick, cleaned with chromic acid
- Microscope [LABOMED VISION 2000 OR NIKON Eclipse 600] Bright field microscope
- Coverslips [Hi-media] 24x60 mm microscope coverslip
- Filterpaper [Whattman]
- Sterile Falcon tube (15 ml)[ABDOS]

2.3.4 Reagent setup

◆ **RPMI-1640 Culture media** (CAT number AL-165A)

Pre-supplemented with HEPES buffer. PHA-M 25 mg, L-Glutamine, 60mg per liter penicillin, 100mg per liter streptomycin, 15% FBS and Sodium Bicarbonate

◆ **Cytochalasin B**

- 5 mg of solid powder Cyto-B dissolved in 8.33 ml of sterile DMSO to give solution of Cyto-B concentration of 600 µg/ml as follow :
- Cyto-B vial was removed from -20°C and it was allowed to attend room temperature. The top seal was sterilized with Ethanol
- 8.33 ml of DMSO was pipetted into 15ml Sterile falcon tube.using a 5ml syringe and sterile needle, 4ml of 8.33 ml DMSO was injected into rubber sealed vial through the 0.22 µm sterile filter

- From the vial 4ml was removed and it was ejected into another 15ml tube labeled as I
- The remaining amount of 4.3 ml of DMSO was aspirated as before into the vial and again ejected into sterile tube I
- The content was mixed properly and 700µl was dispensed into 1.5 ml eppendorfs . The aliquots were then stored at -20° c

◆ Hypotonic solution

- 0.56 mg of KCl was added to 100 ml of DW and mixed well to prepare 0.56% KCl

◆ Fixative

- Acetic acid & Methanol in ratio of 1:3 were mixed to prepare fixative and stored at 4° C

◆ 10% GIEMSA stain

- 4.0 ml of GIEMSA stock was added to Sorrenson's buffer(pH=7) and stored in the coupling jar

2.3.5 Preparation of sterile leaf extract of *Bambusa arundinacea*

An accurate weight of 10.0 gm powdered leaf of was transferred to a 250m RBF, 100ml of ethanol was added to it.

The flask was re-fluxed on heating water bath and filtered through Whattman paper. The procedure was repeated two times for complete extraction from powder.

The powdered extract was reduced to 10ml by evaporation.

The extract solution was first filtered using 0.45µm syringe filter and than by using 0.22µm syringe filter.

The sterility of extract was checked for bacterial and fungal growth by streaking the extract under aseptic on slants of N-Agar media & Potato Dextrose media.

2.4 Methods:

2.4.1 Sampling and blood collection

The blood of healthy non smoking donor of age approximately 23-25 were collected from median cubital vein under proper aseptic condition in sterile heparinised vacutainer and mixed gently to avoid clotting which was later used in setting up culture in sterile condition.

2.4.2 Exposure of blood

The blood was transferred to falcon tube in sterile aseptic condition, the leaf extract was added before 30min of exposure. The blood sample was exposed to 5Gy-γ radiation in gamma radiation chamber 5000 having cobalt 60 as radiation source for irradiation.

2.4.3 Control/Test group

The concentration of extract to be added was selected according to OECD guidelines as reference:

Positive control	Blood + Mitomycin c Blood +Bleomycin Blood + Radiation
Negative control	Blood
Test control	Blood+Extract 3µl Blood+Extract 5µl Blood+Radiation+Extract 3µl Blood+Radiation+Extract 5 µl

All cultures were setup in duplicates in order and flask were coded to avoid biasness in the result.

2.4.4 Culture Procedure

■ Day 1 (0^{th} hour)

1ml whole blood was added to each tube containing complete RPMI-1640 in a culture tube in aseptic sterile condition in LAF. Duplicate culture was setup for each Test sample and Control. The culture tubes were allowed to incubate for 46 hours at 37° C in BOD incubator.

■ Day 3(46th hour)

After 46TH hours of setting up the blood culture 90 µl of Cytochalasin-B was added (6µg/ml) in aseptic sterile condition in LAF to arrest the cytokinesis in each tube. The culture tubes were again allowed to incubate at 37° C for 24-28 hours in BOD incubator.

■ Day 4(72th hour)

Harvesting of culture: After 72 hours of culture setup the tubes were centrifuged at 1000 RPM for 10mins. The supernatant was discarded and the pellet was suspended into 5ml of pre-warmed 37°c hypotonic solution for 1-2 minutes. Approximately 2ml of freshly prepared pre-chilled fixative was added to culture to stop hypotonic treatment.

■ Washing

Culture tubes were centrifuged at 1000 rpm for 10mins, the supernatant was discarded and brown pellet was again suspended into freshly prepared pre-chilled fixative. Fixative wash was given at interval of 10mins until the clear pellet was obtained. After final supernatant was discarded a small amount of fixative was added to pellet maintain cell concentration.

■ Slide preparation

2-3 drops of cell suspension were dropped on grease free pre-chilled slides and was allowed to dry.

■ Staining

The slides were stain in10 % Giemsa stain for 15mins. They were thoroughly rinsed in distilled water and allowed to dry than they were observed under microscope.

2.4.5 Criteria for scoring micronuclei

The criteria for selecting binucleated cell which can be scored for micronuclei frequency :

- The cell should be binucleated.
- Two nuclei in the binucleated cell may be unconnected or might be attached by one or more nucleoplasmic bridge which are not wider than ¼ of nuclear diameter.
- The nuclei in binucleated cell should have intact nuclear membrane and situated within cytoplasmic boundary.
- The two main nuclei in cell may touch each other but should not overlap each other. A cell with two overlapping nuclei can be scored only if nuclear boundaries of either nucleus are distinguishable.
- Thw two nuclei in the binucleated cell must be approximately equal in staining pattern size and staining density.
- The ctyoplasmic boundary or membrane of binucleated cell must be intact and clearly distinguishable from cytoplasmic boundaries of adjacent cells.

Criteria for scoring micronuclei:

- Micronuclei should be morphologically identical and small then main nucleus.
- Micronuclei should not be linked or connected to main nucleus. It may touch but not be overlapping the main nucleus.
- The diameter of micronucleus in human lymphocyte is usually varying between $1/16^{th}$ and $1/3^{rd}$ of mean diameter of main nucleus.
- Ideally micronucleus should have same staining intensity as the main nucleus but occasionally might be more intense.
- It might show no refractility in contrast with nuclear particles.
- Micronuclei should be scored in binucleated cell only with well preserved cytoplasm.

2.5 Comet Assay

The single cell gel electrophoresis also known as comet assay is a Biochemical technique for the detection of DNA single strand breaks (frank strand breaks and incomplete excision repair site) alkali labile site and cross linked with the single cell approach typical of cytogenetic assay.

2.5.1 Preparation of reagents

Materials	Supplier
Dimethylsulfoxide(DMSO)	HiMedia
Disodium EDTA	HiMedia
Ethidium Bromide	HiMedia
Histopaque	HiMedia
Phosphate buffer saline (PBS)	HiMedia
Sodium Chloride (NaCl)	HiMedia
Sodium hydroxide (NaOH)	HiMedia
Triton X-100	HiMedia
Trizma Base	HiMedia

2.5.2 Procedure

◆ PBS(Ca^{++},Mg^{++}free):

Dulbecco's PBS -1 L packet 990ml of dH_2O was added, pH was adjusted to 7.4 q.s. to 1000ml and stored at room temperature.

◆ Lysis solution:

2.5M NaCl weigh 146.1gm, 100mM EDTA weigh 37.2gm and 10mM trizma Base weigh 1.2gm were added 700ml dH_2O ~8gm of NaOH was added and allowed to dissolved. The ph was adjusted to 10.0 using Conc. HCl or NaOH q.s. to 890ml with dH_2O. it was stored at room temperature.

◆ Final Lysis solution:

1% of triton X was added to 10% of DMSO and was refrigerated for at least 30mins prior to slide addition.

◆ Electrophoresis buffer (300mM NaOH/1mM EDTA)

The stock was prepared from 10N NaOH (200g/500 ml dH_2O) and 200mM of EDTA (14.89gm/200ml dH_2O pH 10). Both were stored at room temperature. For 1X buffer 30ml of NaOH and 5.0 ml of EDTA, q.s too 1000ml and was mixed properly. The pH of buffer should be >13 prior to use.

◆ Neutralization buffer :

48.5 gm of 0.4MTris was added to ~800ml dH_2O, the pH was adjusted to 7.5 q.s to 1000ml with dH_2O and was stored at room temperature.

◆ Staining solution :

10gm of Ethidium Bromide was added to 50ml dH_2O and was stored at room temperature. For 1 X stock 1ml was mixed with 9ml of dH_2O.

2.5.3 Preparation of Slides for the Comet assay

Normal melting Agarose	HiMedia
Low melting Agarose	HiMedia
Methanol	HiMedia
Coverslips (no.1 24x 60mm)	HiMedia
Microcentrifuge tubes	Tarson
Micropipettor and tips	Tarson
Micro gel Electrophoresis slides	HiMedia
Coplin jar	Tarson

2.5.4 Preparation of base slides

1% (500mg per 50ml PBS) and 0.5% (250mg per 50ml PBS) LMPA was prepared and 1% (500mg per 50 ml Milli Q water) NMA was prepared. It was microwave or boiled until agarose dissolves. For LMPA, 5ml sample were aliquot into scintillation vials and was refrigerated. LMPA Vial was placed in 37° c water bath to cool and stabilize the temperature.

The Normal melting agarose was dipped in conventional slide up-to 1/3rd frosted area and was removed gently. The slides were placed on tray on flat surface for drying. The slides were stored at room temperature until needed to avoid high humidity conditions.

2.5.5 Cell isolation & Treatment for Comet assay

The blood cultures were setup for controls (Bleomycin, Radiation+blood)and test (blood+5 µl extract, Blood+Radiation+5µl extract) in RPMI media and were incubated at 37° C for 72 hours.

20 µl of whole blood and 1ml of RPMI mixture were taken in microcentrifuge tube and 100µl of Ficoll histopaque was added below the blood/media mixture and it was centrifuges at 2000 x g for 3 mins. 100µl of the bottom of media/top of ficoll layer was removed. 1ml of media was added and mixed to pellet lymphocytes. The supernatant was discarded and pellet was resuspended in 75 µl LMPA.

The slide was coated with the suspension. Coverslip was placed and slide was put on slide tray resting of ice pack until the agarose layer hardens.

The coverslip was removed gently and third agarose layer (80µl LMPA) was added to slide and coverslip was replaced and was rested on slide tray until agarose layer harden.

Remove the coverslip and lower the slide into cold freshly prepared lysis solution. Protect from light and was refrigerated for minimum 2hours.

2.5.6 Electrophoresis of Microgel Slides

The procedure described is for electrophoresis under pH>13 alkaline condition.

After 2 hours at 4° c the slides were removed from lysing solution and slide were placed side by side on the horizontal gel box.

The buffer reservoirs were filled with freshly prepared Electrophoresis buffer pH>13 until liquid level completely covers the slides (avoid bubble over agarose).

The slides were kept in alkaline buffer for 20mins for allowing the unwinding of DNA and the expression of alkali labile damage.

NOTE : The longer the exposure to alkali, the greater the expression of alkali labile damage.

The power supply was turned on to 24volts and the current was adjusted to 300milliamperes by raising or lowering the buffer level. The slides were electrophoresed for 30mins.

The power was turn off and the slides were gently lifted from the buffer and place on drain tray. The slides were coated drop wise with neutralization buffer and allowed to sit for 5mins. The slide was drain and it was repeated 2-3 times.

The slides were stained with 80µl Ethidium Bromide. The slide were dipped in chilled distilled water after 5mins to remove excess of stain. The coverslip was placed over slide and immediately scored or dried before staining as in step 7

Slides were drained and kept in cold 100% ethanol for 20mins for dehydration. The slides were air dried in oven for 30mins abd stored in dry area.

When convenient the slides were re-hydrated with chilled distilled water for 30mins and stained with EtBr as in step 6 and covered with fresh coveslip. The coverslip was removed after scoring rinsed in 100% alcohol to remove the stain and dried for archival purpose.

2.5.7 Evaluations of DNA Damage

For the visualization of DNA damage the observations were made of EtBr-Stained DNA using 40x objective on fluorescent microscope.

The software COMETSCORE was used to analyze the image linked to CCD camera for the assessment of quantitative and qualitative extent of DNA damage in the cell.

RESULT AND DISCUSSION

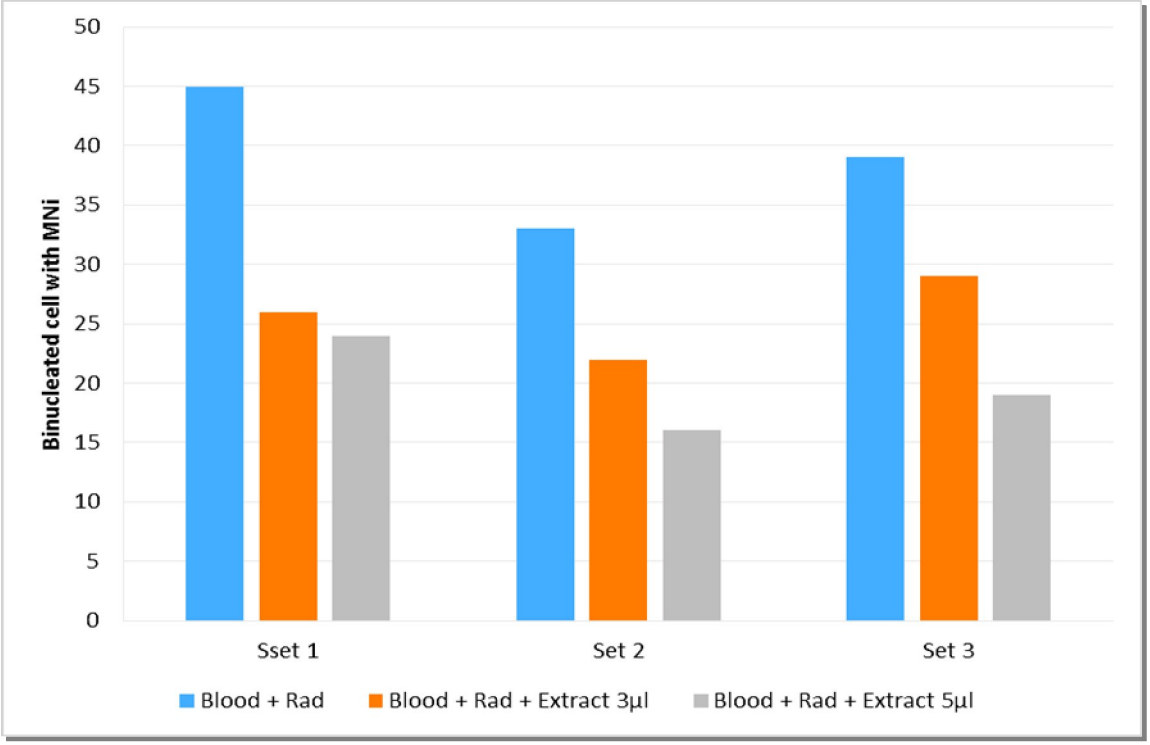
3. Result and Discussion

3.1 CBMN assay

3.1.1 Micronuclei scoring

Micronuclei originate either from acentric chromosome or whole chromosome fragment. Micronuclei can only be counted in binucleated cells because as the cells have finished one nuclear division and so, no mono-nucleated cells were considered.It is faster and reliable assay which requires less skilled experienced person as compared to chromosome aberration assay. .

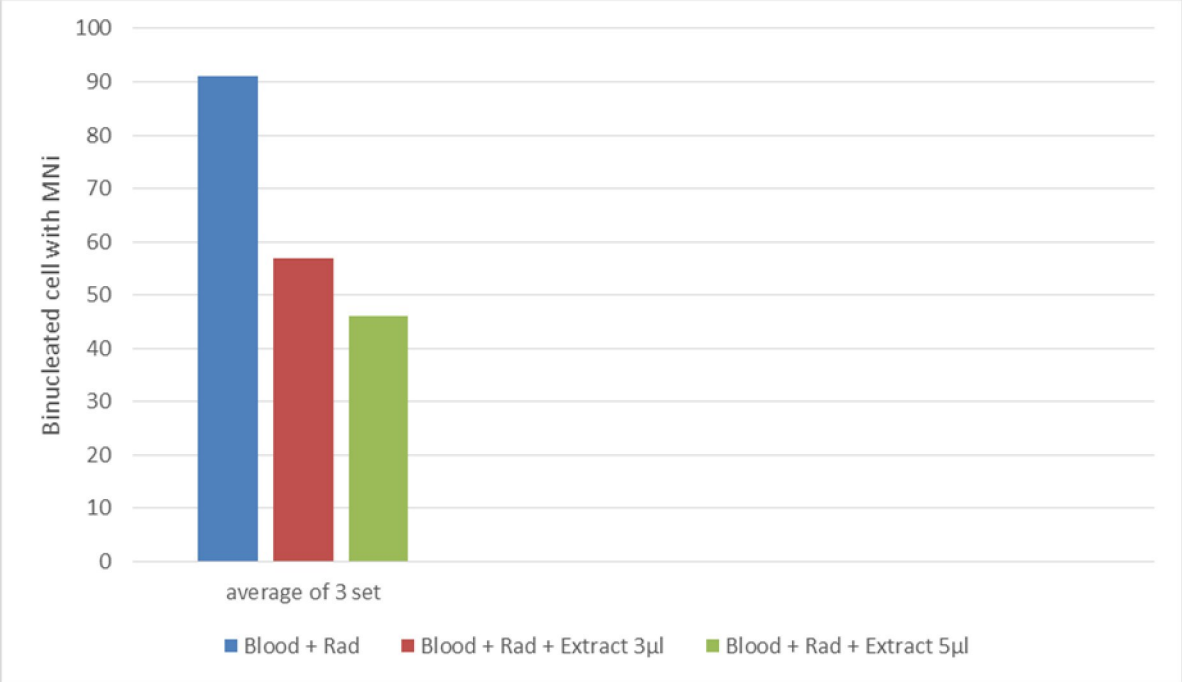
From the entire set of each of the samples total 100 cells were being counted and values were further plotted on graph and statistical analysis was carried out.



The results suggest significant reduction in frequency of radiation induced micronuclei following treatment with extract of Bambusa arundinacea

The positive control shows highest number of micro- nuclei because of being exposed to only radiation and compared to that two test samples of 3µl and 5µl showed decrease in micro-nuclei number in culture sample treated with extract.

Frequency of bi nucleated cells with micronuclei in short term blood cultures treated with Radiation with and without the bamboo extract, as compared to controls

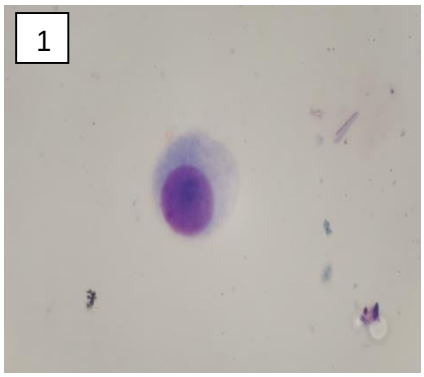


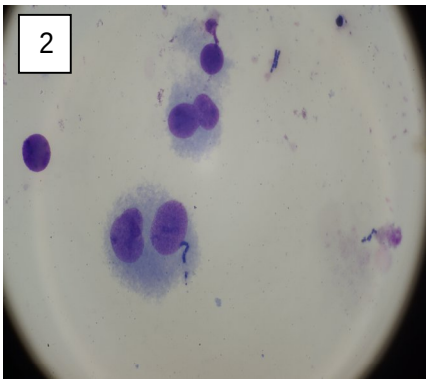
All the three sets were carried out using ***Bambusa aurndinaceae*** as the test sample and each set shows the same decrease in number of micro-nuclei with increase in test sample concentration.

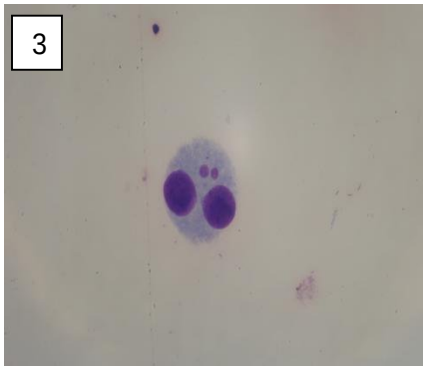
Micro-nuclei scoring sheet for all treatment groups

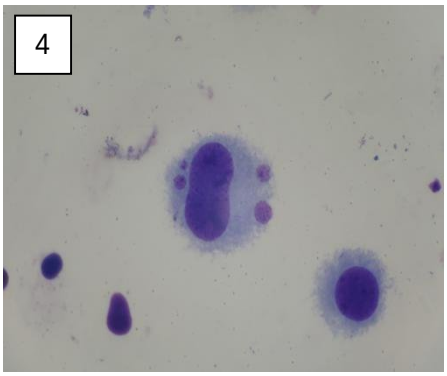
Sr. No.	Samples	Total BN cell count	Bi-nucleated with 1 Micro-nuclei			Bi-nucleated with 2 Micro-nuclei			Bi-nucleated with 3 Micro-nuclei		
			Set 1	Set 2	Set 3	Set 1	Set 2	Set 3	Set 1	Set 2	Set 3
1	**Blood + PHA**	100	0	0	0	0	0	0	0	0	0
2	**Blood + MMC**	100	0	15	2	0	2	0	0	0	0
3	**Blood + Radiation**	100	26	11	29	14	15	8	5	7	1
4	**Blood + Extract (3µl)**	100	0	0	0	0	0	0	0	0	0
5	**Blood + Extract (5µl)**	100	1	0	0	0	0	0	0	0	0
6	**Blood + Radiation + Extract (3µl)**	100	17	17	16	7	4	12	2	1	3
7	**Blood + Radiation + Extract (5µl)**	100	13	11	15	8	5	4	4	0	2

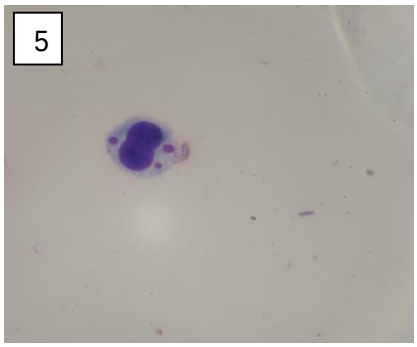
3.1.2 Photomicrographs illustrating micronuclei

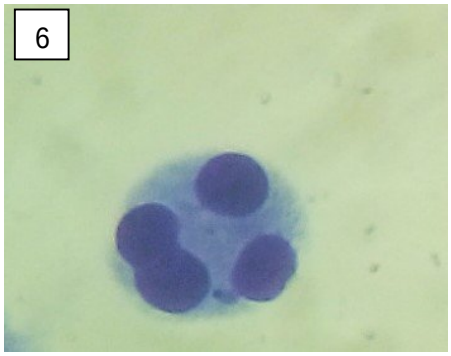












1. Mono-nucleated cell **2. Bi-nucleated**

3. Bi-nucleated with 2 micro-nuclei **4. Bi-nucleated with 4 micro-nuclei**

5. Bi-nucleated with 3 micro-nuclei **6. Tetra-nucleated**

3.1.3 STATISTICAL ANALYSIS

- The table given no.2 shows the mean, S.D and S.E value of each of the three sets and also based on the Student t test the P- values obtained is ≤ 0.001.
- P-value is the number between 0 to 1. It is the probability of getting the observed value of test or more than that.
- Smaller P-value (typically ≤ 0.05) indicates strong evidence against the null hypothesis so, we reject the null hypothesis.
- Larger P-value (typically ≥ 0.05) indicates weak evidence against null hypothesis so fails to reject the null hypothesis.
- Here on the above set of all the treated groups the P- value obtained is ≤ 0.001 and so the values obtained are significant and hence the null hypothesis can be rejected as the value proves the strong evidence against null hypothesis.

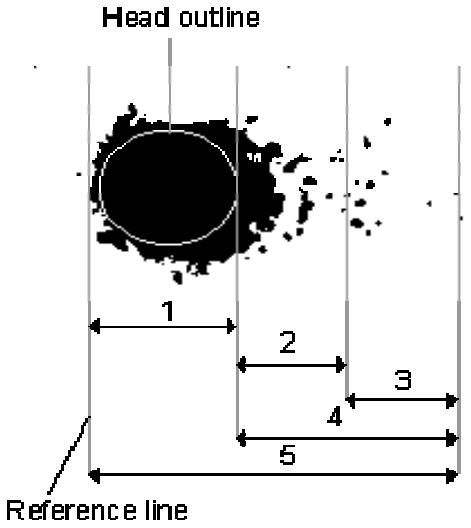
Table no.1

$P \geq 0.10$	Not Significant
$P \leq 0.10$	Marginal
$P \leq 0.05$	Fair
$P \leq 0.01$	Good
$P \leq 0.001$	Excellent

Table no.2

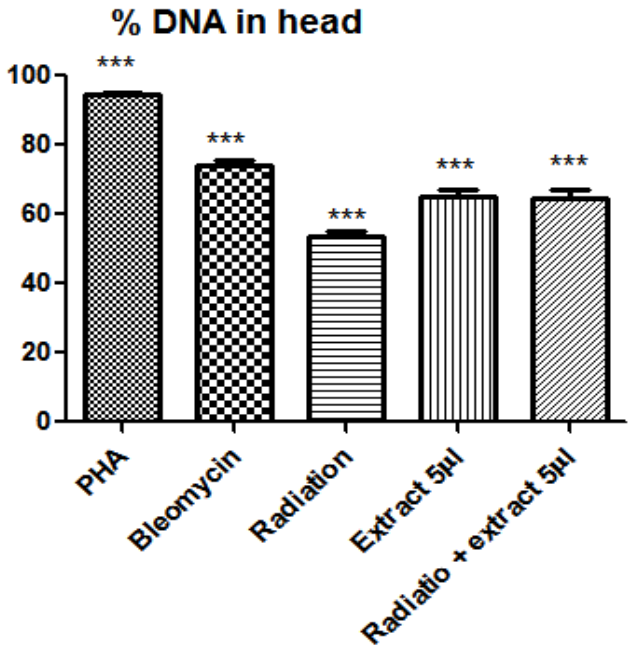
Samples	Mean ± S.D ± S.E	P.Value
SET 1		
Blood + Radiation	0.45 ± 0.497 ± 0.04974	
Blood + Radiation + Extract (3µl)	0.26 ± 0.439 ± 0.04386	p ≤ 0.001
Blood + Radiation + Extract (5µl)	0.24 ± 0.427 ± 0.042708	p ≤ 0.001
SET 2		
Blood + Radiation	0.33 ± 0.4702 ± 0.04702	
Blood + Radiation + Extract (3µl)	0.22 ± 0.4142 ± 0.04142	p ≤ 0.001
Blood + Radiation + Extract (5µl)	0.16 ± 0.3666 ± 0.03666	p ≤0.001
SET 3		
Blood + Radiation	0.39 ± 0.4876 ± 0.04876	
Blood + Radiation + Extract (3µl)	0.29 ± 0.4532 ± 0.04532	p ≤ 0.001
Blood + Radiation + Extract (5µl)	0.19 ± 0.3924 ± 0.03924	p ≤ 0.001

3.2 Results for comet assay

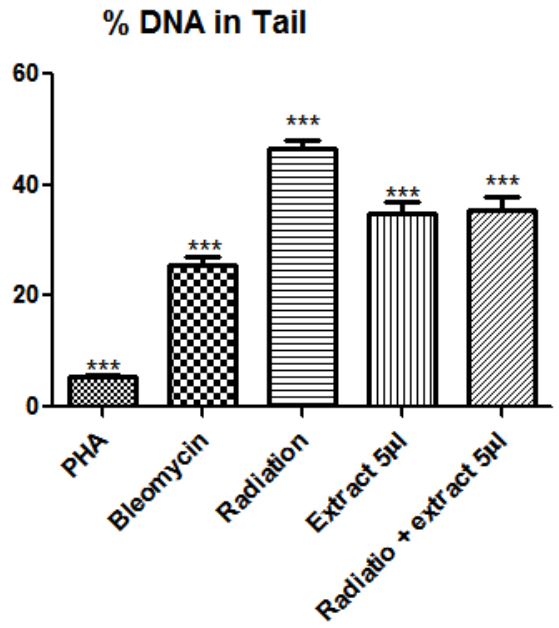


1=Head extent
2=Body extent
3=Tail extent
4=Tail extent without body
5=Comet extent

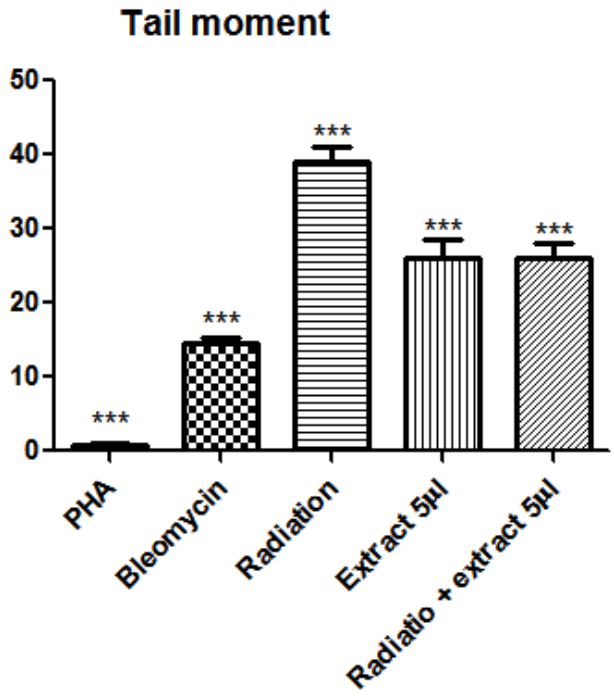
3.2.1 The head % of DNA



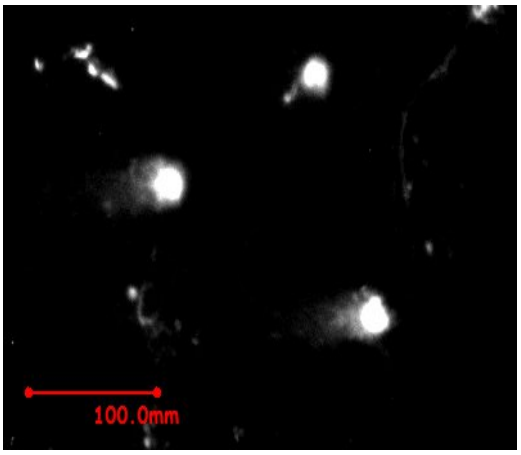
3.2.2 The tail % of DNA



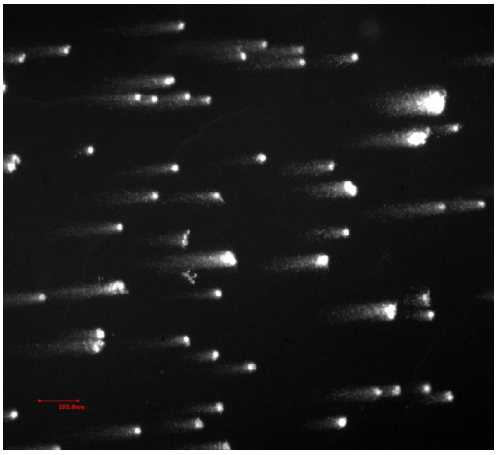
3.2.3 The Tail moment



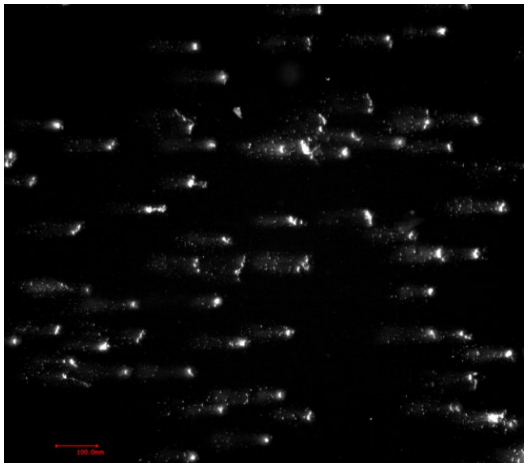
3.2.3 Photomicrographs illustrating DNA damage by COMET assay



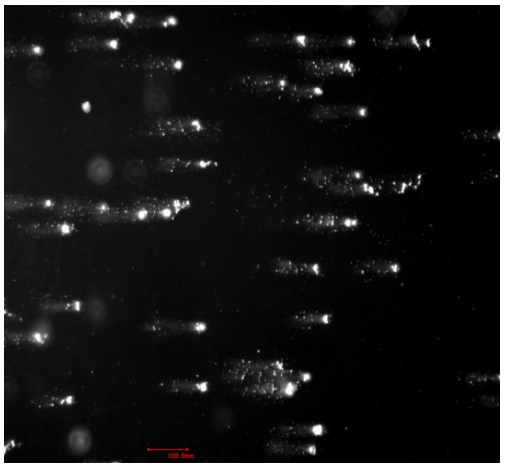
Bleomycin



Radiation

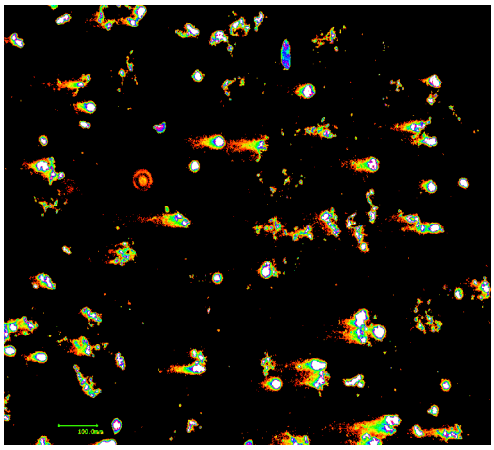


Extract 5µl

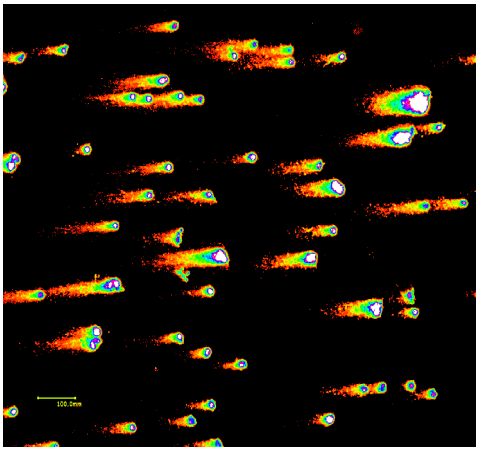


Radiation + Extract 5µl

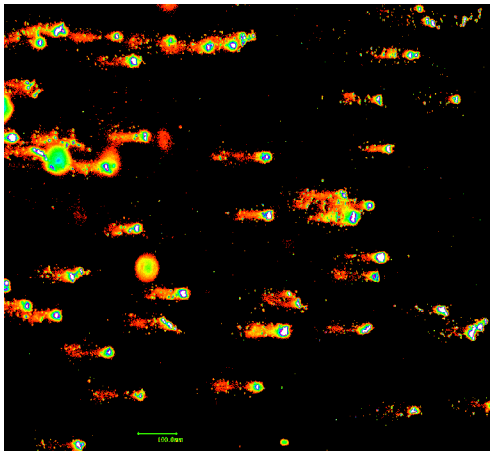
3.2.4 Comet assay images illustrating DNA damage



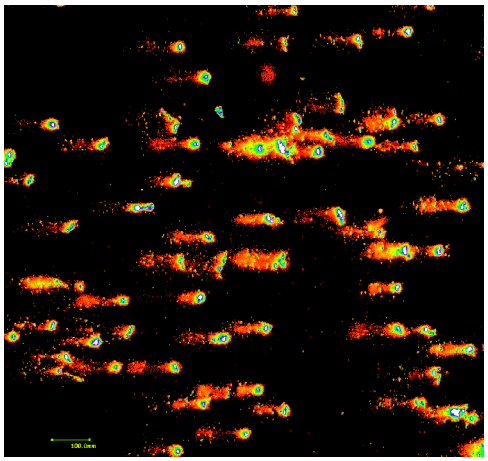
Bleomycin



Radiation



Extract 5µl



Radiation + Extract 5µl

3.3 CONCLUSION

Results of CBMN assay suggest Bambusa aurndinacea can act as a radio-protector as its addition in cultures exposed to radiation shows significant decrease in the frequency of micronuclei by using in vitro short term cultures of human peripheral blood

The COMET assay of ***Bambusa aurndinaceae*** shows increase in the damage with the increase in concentration of extract

SUMMARY AND CONCLUSION

SUMMARY, CONCLUSION AND FUTURE SCOPE

4.1 SUMMARY

- Preliminary Phytochemical screening of the leaves of the Bamboo species showed the presence of active secondary metabolites like flavonoids, tannins, phenolic, saponins, phytosterols and triterpenoids, alkaloids .
- The Total Flavanoid content of ***Bambusa aurindaceae*** was found to be 1.61 %w/v.
- The CBMN Assay of the Leaf Extract of ***Bambusa aurindaceae*** showed significant reduction in micronuclei frequency.
- COMET assay shows decrease in the damage with the extract compared to the positive control.

4.2 CONCLUSION

- The present study suggests the leaf extract of ***Bambusa aurindaceae*** may act as a Radio-protector as it showed significant reduction in frequency of radiation induced micronuclei.

4.3 FUTURE SCOPE

Assessment of the Radioprotective activity can also be performed and validated using other in-vitro methods like cell survival method, assay of free radicals and antioxidant status , Evaluation of lipid peroxidation etc.

Radioprotective assessment can also be carried out for ***Bambusa aurndinacea*** leaf extract in vivo.

Variations in the cell culture method, radiation dose, concentration of leaf extract, concentration of orientin can also be performed.

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- Radiation Recall with Anticancer Agents. Howard A. Burris III, Jane Hurtig, Sarah Cannon Research Institute, Nashville, Tennessee, USA

APPENDIX

1. Incubator

- Switch on the power.
- As per the required temperature press up and down
- PG2 was pressed to set the temperature
- Once the temperature was set, material was placed on the rack
- Close incubation door
- Look once if required
- Once the incubation is done, the racks were cleaned with 70% alcohol
- Main power was than switched off

2. MICROSCOPE

- Remove the dust cover.
- Switch on the microscope.
- Clean-dry the slide if wet, clean the stage.
- Check the optical lens, clean if required with tissue paper only.
- Check Kohler illumination, reset required.
- Start with 4X, move to higher magnification clockwise only.
- When using higher magnification adjust stage height to avoid impact on objective lenses.
- If using 100X objective, use very little amount of oil and avoid 40X touching the oil.
- Clean the stage and optical lenses with tissue paper.
- First lower the light and switch off.
- Put the dust cover back.