Antigenotoxic Evaluation of Rhizhophora *Mucronata* Stilt Root Extract Against Cyclophosphamide Induced Toxicity

Anu Tresa Mathew[1], M.Syed Ali*[2], V.Anuradha[3], N. Yogananth[2]
[1]Nehad Agronomy Services, P.O.Box- 288 , PC- 315, Suwaiq, Sultanate of Oman.
[2]PG and Research Department of Biotechnology, Mohamed Sathak college of Arts and Science, Sholinganallur, Chennai-600119
[3]PG and Research Department of Biochemistry, Mohamed Sathak college of Arts and Science, Sholinganallur, Chennai-600119
*Corresponding Author
Syedmicro555@gmail.com

ABSTRACT

In the present study *Rhizophora mucronata* was investigated for its antigenotoxic property. R. *mucronata* is one of the mangrove plants with strong medicinal properties. Stilt root of this plant is known for its radical scavenging property. In this study antigenotoxicity of *R. mucronata* was studied against cyclophosphamide induced genotoxicity using *invitro* and *invivo* methods. Chromosomal aberration assay and Micronucleus Assay were done for *invitro* and *invivo* respectively. Treatment of human leukocytes with *R.mucronata* stilt root extract alone at different doses showed no increase in number of CA and significant decrease in MI were observed when compared to untreated [water]. Meanwhile, when Wistar rats were given *R.mucronata* stilt root extract [i.p] for 5 consecutive days followed by CP injection [i.p]. Results indicate that there was no change in the number of CA in the animals treated with extract alone. However, animals co administered with 40 mg/kg extract and CP(40 mg/kg) showed significant decrease in number of CA and increase in mitotic index when compared to animals treated with CP alone. For micronucleus Assay in Wistar Rat bone marrow cells. The animals treated with extract showed no significant increase in the number of MN when compared to that of control. Animals which received simultaneous treatment of *R.mucronata stilt root* extract (40 mg/kg b.w) and cyclophosphamide showed significant reduction in the number of micronucleus when compared to that of cyclophosphamide treated animals. Analysis of the present results indicated that the extract which had higher enzyme activity (GST and CAT) protected the cells against the toxicity caused by Cyclophosphamide. Moreover the results of histopathology remained positive thus proving the protective role of extract as a potential antigenotoxicant.

KEYWORDS- Cyclophosphamide, *Rhizophora mucronata*, stilt root, antigenotoxicty, Gluthathione-S-transferase , Catalase.

1. Introduction

Cancer is a generic term used for a large group of diseases that can affect any part of the body. Cancer has become one of the ten leading cases. Over 7 lakh new cases and 3 lakh deaths occur annually due to cancer. Nearly 15 lakh patients require facilities for diagnosis, treatment and follow up at a given time. But most of the currently used chemotherapeutic agents induce genotoxicity in normal cells too and cause deleterious effects. Genotoxicity is the property possessed by substances that harms the genetic material. Though there are many mechanisms by which genotoxicity can affect genetic information, one of the most common mechanisms involves the formation of strong chemical bonds between the genotoxins and the molecules that compose

genetic information, such as DNA and RNA. These bonds do not strongly affect the existing genetic data. They do, however, prevent the proper replication of the genetic information. Such changes in the process of genetic replication can cause myriad problems. Chemotherapeutic agents and gamma radiations are major genotoxic agents. Cyclophosphamide (CP) is a commonly used cytotoxic alkylating agent with antitumor and immune suppressant properties. Cyclophosphamide remains inactive until metabolised by the liver CYP3 monooxygenase system. The drug interacts with the mitotic cycle and kills the cells, it can also cause intermitotic cell injury and death. Cyclophosphamide can inhibit both T and B cell immunity.

Antigenotoxic compounds from medicinal and aromatic plants are used over several years. The studies of this type are aimed at understanding the protective mechanisms which may be relevant for the primary prevention of cancer and other mutation related diseases. Plant-derived phenolic compounds exert anti genotoxic property. Antigenotoxic plant extracts can encounter or prevent the adverse effects caused by DNA damaging chemicals. This is due to the fact that dietary intake of phytochemicals has shown protective effect against the dreaded disease. Exposure to genotoxic chemicals that are present in food, environment and also in medical treatments can alter the genetic material permanently and thus may lead to cancer. Mangrove unlike common terrestrial plants, they can withstand high salt concentration, can remain submerged in water, and maintain an efficient nutrient retention mechanism. Mangroves and related species, are known to have many metabolites possessing antibacterial and antifungal, [1],antiviral, antidiarrhoeal, hepatoprotective, antifeedant , larvicidal [2],cytotoxicity and antiplasmodial Properties [3]. It is also reported that, mangrove is a folk remedy for angina, diabetes, diarrhea, dysentery, hematuria and haemorrhage. Mangrove plants are well-known for their flavonoids, alkaloids, coumarins, polyphenols and are a rich source of tannins [4]. Of the various plant parts of *Rhizophora mucronata*, stilt root is well known for its free radical scavenging property [5]. The present study was an attempt to investigate antigenotoxic effect of *Rhizophora mucronata* stilt root extract against cyclophosphamide induced genotoxicity.

2. Materials and Methods.

2.1.Collection of plant materials and preparation of Extract

Fresh stilt root samples of *R. mucronata* was collected from the Mandapam area of Gulf of Mannar region (latitude 9° 17 ' N and longitude 79° 8' E) in the Ramanathpuram district on the South East coast of India. Stilt root samples of *R. mucronata* were subjected for percolation by soaking in ethanol/water (3:1 v/v). The ethanolic extract was then concentrated by using rotary flash evaporator (Superfit, INDIA) and further lyophilised (BENCHTOP 2K) to remove the excess organic residues.

2.2 *IN VITRO* ASSAY

2.2.1. Requirements

RPMI-1640 (HiMedia laboratories), giemsa stain, Whatman No.1 filter paper, methanol , acetic acid, *Rhizhophora mucronata* stilt root extract, antibiotic-antimycotic mixture.

2.2.2. Chromosomal aberration assay in cultured human leukocytes

Blood samples were drawn into a sterile, heparinised syringe by vein puncture from healthy individuals. 0.5ml of the blood was inoculated into each culture vial containing 5ml of culture medium. The cultures were incubated at 37°C for 72 hours. The cultures were treated with three doses of *R. mucronata* stilt root extract *viz*., 2 mg/ml, 4 mg/ml, and 6 mg/ml . Of the three doses 4mg/ml was tested for its genoprotectivity. A single dose of cyclophosphamide (*i.e*.100 µg/ml) was used as a positive control. Also 4 mg/ml of *R. mucronata* stilt root extract and 100 µg/ml of cyclophosphamide were added in a separate culture vial. A minimum of two vials were put up for each treatment.

2.2.3. Harvesting of culture

At the 69th hour of incubation period, 0.05 ml of 0.001% colchicine solution was added and incubated for 40 minutes at 37°C. The cells were centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and 6ml of pre warmed (37°C) hypertonic solution (0.075M KCl) was added to the cells. They were incubated for 5minutes at 37°C and then centrifuged. The cells were fixed in freshly prepared fixative (3 parts of methanol and 1 part of glacial acetic acid). Two more changes of the fixative were given at each 45 minutes interval. Slides were prepared and stained by 4% giemsa stain. A minimum of 50 metaphases were counted. The mitotic index and protection % was also calculated.

$$MI = \frac{\text{Number of dividing cells}}{\text{Total number of cells}} \times 100$$

$$\text{Protection \%} = \frac{\text{Control- Test}}{\text{Control}} \times 100$$

2.3 *IN VIVO* ASSAY

Ten to Twelve week old male Wistar rats weighing 25-30g was used for the present study. The animals were maintained at normal room temperature with humidity of 55±5%. All animals were fed with pellet diet obtained from Mohamed Sathak AJ college of Pharmacy, Chennai, and potable water *ad libitum* throughout the experimental period. The animals were acclimatized to laboratory conditions before experimental procedures were started. All the animals were weighed and numbered. The animals used in the present study were maintained in accordance with the guidelines (OECD,1997)of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and approved by the Institute's ethical committee. The experimental animals were administered [i.p] with the *R. mucronata* stilt root extract (20, 40 and 60 mg/kg body weight,) separately for five consecutive days. Of the three doses 40 mg/kg was tested for its genoprotectivity. The genotoxin, cyclophosphamide (40 mg/kg bw) [LD_{50} =160mg/kg b.w] was dissolved in sterile distilled water and injected intraperitoneally (10 ml/kg) 2h after the final pre-treatment to group treated with *R. mucronata* stilt root extract [40mg/kg bw]. The animals were sacrificed 24 hours after injecting the genotoxin. Control animals received same volume of distilled water. Each pre-treatment group consisted of six rats.

In order to arrest the metaphase stage, colchicine (0.004%) was injected intraperitoneally 2 hours before the animals were sacrificed by cervical dislocation. The femurs were stripped proximally, and the bone marrow was aspirated in 4 ml of 0.9% NaCl (37 °C). The suspension was centrifuged for 5 min at 1000 rpm, and the bone marrow pellet was resuspended in 0.075M KCl at 37 °C for 25 min and then centrifuged for 5 min at 1000 2.2 rpm. Later the pellet was fixed in cold methanol-glacial acetic acid (3:1) for 20 min at room temperature. The treatment with fixative was repeated 2 times. Then the cells were spread on glass slides and air-dried. The slides were stained with Giemsa (4%) for 10 min and 100 well-spread metaphases per animal was scored and the MI was determined.

2.4 Micronucleus test

The methodology of experimental animals is as mentioned in 2.3. The experimental animals were administered [i.p] with the *R. mucronata* stilt root extract (20 mg/kg, 40 mg/kg and 60 mg/kg body weight,) separately for five consecutive days. Of the three doses 40 mg/kg was tested for its genoprotectivity. The genotoxin, cyclophosphamide (40 mg/kg bw) [LD_{50} =160 mg/kg b.w] was dissolved in sterile distilled water and injected intraperitoneally (10 ml/kg) 2h after the final pre-

treatment to group treated with *R. mucronata* stilt root extract [40 mg/kg bw]. The animals were sacrificed 24h after injecting the genotoxin. Control animals received same volume of distilled water. Each pretreatment group consisted of six rats. Genotoxic effects were evaluated in the mouse bone marrow micronucleus test, which was carried out according to Vijaya, 2014 [6]. The bone marrow cells from both femurs were flushed in the form of a fine suspension into a centrifuge tube containing human AB serum. This cell suspension was centrifuged at 2000 rpm for 10 min, and the pellet was resuspended in a drop of serum before being used for preparing slides. Air dried slides were stained with May-Grunewald and Giemsa as described by Schmid, 1975. For each experimental point, six rats were used and 5000 polychromatic erythrocytes (PCEs) were scored per animal to determine the frequency of micro nucleated polychromatic erythrocytes (Mn PCEs).

2.5. STATISTICAL ANALYSIS

The Mean ± standard deviation was calculated for each parameter. Two way analysis of Variance was used for the analysis of chromosomal aberrations and micronucleus assay.

3. RESULTS

3.1. Chromosomal Aberration assay in cultured human leukocytes

When human leukocytes were treated with *R.mucronata* stilt root extract alone at different doses; 2,4,6 mg/ml, no increase in number of CA and significant decrease in MI were observed when compared to untreated [water]. Statistical significant reduction in the number of CA induced (Table 2, Fig 1).

3.2. Chromosomal Aberration assay in rat bone marrow cells

Wistar rats were given *R.mucronata* stilt root extract [i.p] (20, 40 and 60 mg/kg b.w) for 5 consecutive days followed by CP injection [i.p]. Results indicate that there was no change in the number of CA in the animals treated with extract alone. However, animals co administered with40 mg/kg extract and CP (40 mg/kg) showed significant decrease in number of CA and increase in mitotic index when compared to animals treated with CP alone. (Table 3, Fig 2)

3.3.Micronucleus Assay in Wistar Rat bone marrow cells

Table 4 and Fig 3 lists the number of MN/5000 PCEs in rat bone marrow cells. The animals treated with extract (20, 40, 60 mg/kg b.w) showed no significant increase in the number of MN when compared to that of control. Animals which received simultaneous treatment of *R.mucronata stilt root* extract (40 mg/kg b.w) and cyclophosphamide showed significant reduction in the number of micronucleus when compared to that of cyclophosphamide treated animals.

5. DISCUSSION

The use of plant based natural products as chemopreventive agents is drawing a lot of attention and considered to be practically beneficial in certain cell/tissue based systems and animal model systems. It is necessary to provide scientific proof to justify the use of a plant or its active principles for medicinal purposes [7]. Modern drugs, plants and plant extracts must be characterized after their pharmacological screening for their pharmacokinetic and pharmacodynamic properties, including toxicity. A large number of potential chemopreventive agents have been identified, and they function by mechanisms directed at all major stages of carcinogenesis [8-10]. Short term tests such as the mouse bone marrow micronucleus assay, chromosomal aberration analysis, assessment

of antioxidant status and reactive oxygen species (ROS)-induced lipid peroxidation, are widely used in the detection and evaluation of antimutagens and anticarcinogens [11-12].

In human peripheral leukocytes, three doses (2,4, 6mg/ml) of *R.mucronata* stilt root extract were tested for their antigenotoxicity against cyclophosphamide induced toxicity . Administration of the cyclophosphamide alone to the cultures significantly [$p<0.05$] induced chromosomal aberrations [CA] and there was decrease in mitotic index [MI], when compared to that of control. On the other hand, co-administration of the extract and cyclophosphamide significantly reduced the number of chromosomal aberrations and there was increase in MI. Similar to previous work by Lakshmi *et al.,* 2009 [13] were the modulatory effects exerted by the extract of garlic [2,4,6mg/ml] against cyclophosphamide induced genotoxicity in the human lymphocyte cultures *in vitro* by using three different doses of garlic extract and there was a significant decrease in the frequency of chromosomal aberrations and sister chromatid exchanges suggesting that the garlic extract modulates cyclophosphamide induced genotoxicity in a dose dependent manner.

R.mucronata stilt root extract *in vivo* also caused corresponding decrease in the frequency of chromosomal aberration and micronuclei. The animals treated with cyclophosphamide alone showed significant [$p<0.05$] increase in chromosomal aberration and micronuclei. Micronucleus assay is widely used to indicate genotoxic potential of clastogenic agents [14-15]. Co treatment of animals with 40mg/kg extract and cyclophosphamide revealed reduction in chromosomal aberration and micronuclei. The protective effect of *Ocimum.sanctum leaf extract* [200µg/ml] against MMC induced DNA strand break, chromosomal aberrations and micronuclei formation *in vitro* were found to be 67% and 63% protection against DNA strand break, 68% and 59% protection against chromosomal aberrations and 63% and 68% protection against micronuclei formation (Dipanwita Dutta *et al.*, 2007) similarly the protective effect of *R.mucronata* stilt root extract (4mg/ml) against cyclophosphamide induced chromosomal aberrations and micronuclei formation were found to be 70% [*in vitro*] and 58% [*in vivo*] for chromosomal aberrations, [16] in their work on antigenotoxicty of *Ginkgo biloba* extract against cyclophosphamide induced toxicity *in vivo* proved that *G.biloba* extract [200mg/kg] conferred 71% protection when compared to *R.mucronata* stilt root extract [40mg/kg] which gave 94.2% [*in vivo*] for micronuclei formation in the present study.These inferred from the present study that the extract from *R. mucronata* stilt root can be explored as a potential source of safe and efficacious antigenotoxicity and protectively.

Acknowledgements

This work was supported by all staff and crew of Biotechnology Department of Mohammed sathak college of Arts and Science for their timely help and kind cooperation in all stages of work.

Reference

1. Ravikumar, S, Gnanadesigan M, Suganthi P, Ramalakshmi S., Antibacterial potential of chosen mangrove plants against isolated urinary tract infections bacterial pathogens. *Int J Med Med Sci*, 2010, 2(3):94–99.
2. Syed Ali, M, Ravikumar, S, Margaret Beula J., Spatial and temporal distribution of mosquito larvicidal compounds in mangroves, *Asian Pacific Journal of TropicalDisease*, 2012, 401-404.
3. Jacob Inbaneson S, Ravikumar S, Suganthi P, Gnanadesigan M., *In vitro* antiplasmodial activity of ethanolic extracts of mangrove plants from South East coast of India against chloroquine-sensitive *Plasmodium falciparum*. *Parasitol Res.* 2010, doi:10.1007/s00436-010-2128-z
4. Lin, Y. M, P Xiang, P Lin., Studies on Tannins of Mangroves-A Review. *Mar. Sci.* 2005, 29:59-63.

5. Ravikumar, Murugesan Gnanadesigan., Hepatoprotective and Antioxidant Properties of *Rhizophora mucronata* Mangrove Plant in CCl4 Intoxicated Rats, *J.Exp Clin Med* 2012, 4(1):66-72
6. Vijaya, PP., Rekha, L., Anu Tersa Mathew, Syed Ali, M., Yogananth,N., Anuradha,V., and Kalitha Parveen, P. Antigenotoxic effect of green-synthesised silver nanoparticles from *Ocimum sanctum* leaf extract against cyclophosphamide induced genotoxicity in human lymphocytes-*Invitro*. *J. Appl Nanosci*, 2014, 4:415-420.
7. Ammon H.P., M.A Wahl., Pharmacology of *Curcuma longa*. *Planta Med. 1996*, 57 : 1-7.
8. Morse,E.C, Stoner,G., Cancer chemoprevention principles and prospects. J. *Carcinogenesis*, 1996, 14:1737–1746.
9. Tanaka,T. Cancer chemoprevention by natural products. *Oncol. Rep.*, 1996, 1: 1139–1155.
10. Pezzuto,J.M., Plant-derived anticancer agents. Biochem. *Pharmacol.*, 1996, 53:121–133.
11. Locigno,R., CastronovoV., 2001. Reduced glutathione system: role in cancer development, prevention and treatment (review). *Int. J. Oncol*, 2001, 19: 221–236.
12. Ohyama,W., Gonda, M, Miyajima H., Collaborative validation study of the in vivo micronucleus test using mouse colonic epithelial cells. *Mutat. Res.*, 2002, 518:39–45.
13. Lakshmi, B, Sowjanya , K. Rudrama Devi , D. Madhavi Modulatory, M., 2009. Effects of garlic extract against the cyclophosphamide induced genotoxicity in human lymphocytes *in vitro*. J.*Enviro. Biol*, 2009, 30(5): 663-666
14. Hayashi,M., Tice, RR, Macgregor, T, Anderson, D., In vivo rodent erythrocyte micronucleus assay. *Mutat. Res*., 1996, 312: 293–304.
15. Hayashi,M., Mac Gregor, J., *In vivo* rodent erythrocyte micronucleus assay. II some aspects of protocol design including repeat treatments, integration with toxicity testing and automated scoring. *Environ. Mol. Mutagen*, 2000, 35: 234–252.
16. Vilar, K.R. Leite, L, Chen Chen,L., Antimutagenicity protection of *Ginkgo biloba* extract (Egb 761) against mitomycin C and cyclophosphamide in mouse bone marrow. *Gene and Mole. Res*, 2009,8 (1): 328-333

Table 1: Group specification

Group	Animals		Name of the plant species	Drug dose
	Male	***Female***		
I	3	3	Acacia suspension	2%
II	3	3	*R.mucronata* stilt root	250 $mg.kg^{-1}$
III	3	3	Cyclophosphamide	40mg/kg

Table 2. Antigenotoxic effect of *R.mucronata* stilt root extract on human leukocytes against Cyclophosphamide induced toxicity

Sample	Number of aberrations [Mean ± SD]	MI±SD	% Protection
Water	3 ± 2	10 ± 3.51	No induction
CP[100µg/ml]	50.3 ± 2.52[a]	4.1 ± 3	Nil
R.mucronata stilt root extract [2mg/ml]	4.3 ± 3.05	9 ± 2.52	No induction
R.mucronata stilt root extract [4mg/ml]	7.3 ± 3.06	8.8 ± 0.35	No induction
R.mucronata stilt root extract [6mg/ml]	10.6 ± 3.8	8.3 ± 0.36	No induction
R.mucronata stilt root extract [4mg/ml]+ CP[100µg/ml]	14.3 ± 3.05[b]	8.1 ± 0.25	70% protectivity

NOTE- The results are average of five sets of experiments,.a- P<0.05 compared to untreated, b- P<0.05 compared to CP

Table 3. Antigenotoxic effect of *R.mucronata* stilt root extract on rat bone marrow cells against Cyclophosphamide [CP] induced toxicity

Sample	Number of aberrations [Mean ± SD]	MI±SD	% Protection
Water	2.33 ± 1.53	6 ± 3.51	No induction
CP[40mg/kg]	42 ± 3[a]	2.5 ± 0.3	Nil
R.mucronata stilt root extract [20mg/kg]	3.3 ± 2.5	5.4 ± 0.25	No induction
R.mucronata stilt root extract [40mg/kg]	6 ± 2.51	5.3 ± 0.35	No induction
R.mucronata stilt root extract [60mg/kg]	7.3 ± 2.52	5 ± 3.05	No induction
R.mucronata stilt root extract[40mg/kg]+ CP[40mg/Kg]	17.67 ± 2.52[b]	4.9 ± 0.26	58% protectivity

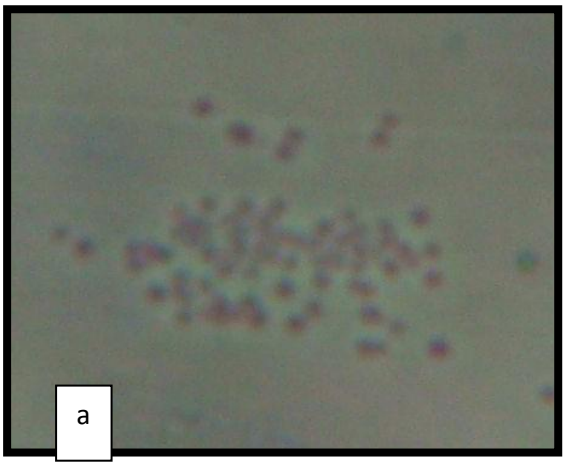
NOTE- The results are average of five sets of experiments. a- P<0.05 compared to untreated,b- P<0.05 compared to CP

Table 4. Protective effect of simultaneous treatment of *R.mucronata* stilt root extract against Micronucleus formation induced by Cyclophosphamide.

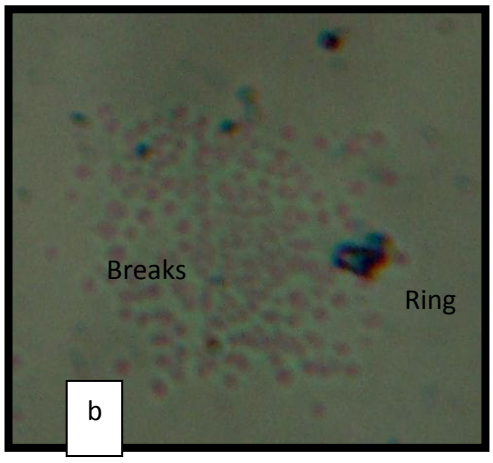
Sample	MN PCE/5000 PCE	PCE/NCE	% Protection
Water	15 ± 3.06	1.085	No induction
CP[40mg/kg]	885 ± 4.62[a]	1.25	Nil
R.mucronata stilt root extract [20mg/kg]	21 ± 4	1.11	No induction
R.mucronata stilt root extract [40mg/kg]	27 ± 4.04	1.04	No induction
R.mucronata stilt root extract [60mg/kg]	33 ± 2.52	1.17	No induction
R.mucronata stilt root extract [40mg/kg]+ CP[40mg/kg]	51 ± 2.05[b]	1.08	94.2% protectivity

NOTE- The results are average of five sets of experiments.a- P<0.05 compared to untreated,b- P<0.05 compared to CP

Fig 1: Microphotographs of metaphase plates of rat bonemarrow cells

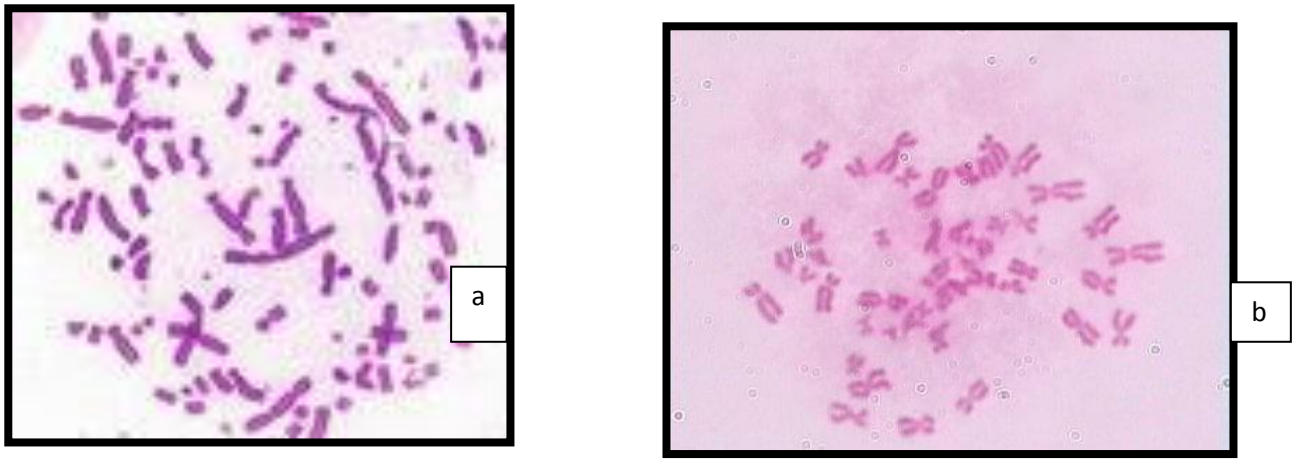


a) Normal well spread metaphase plates



b) polyploid chromosomes with rings and chromatid breaks

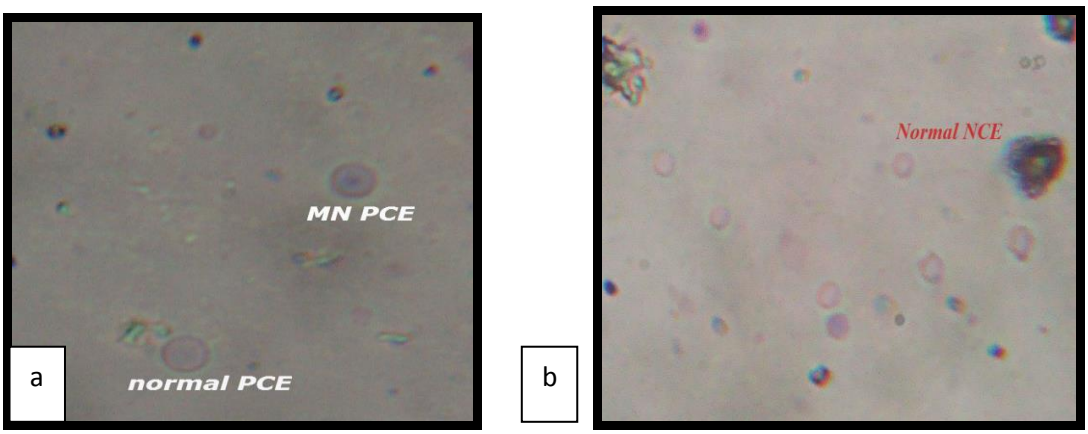
Fig 2: Microphotographs of metaphase plates of human chromosomes



a) Normal well spread metaphase plates

b) chromosomes with rings and chromatid breaks

Fig 3 : Microphotographs of rat bone marrow cells



a) Normal PCEs and micronuclei PCEs

b) normal NCE