International Research Journal of Natural and Applied Sciences
ISSN: (2349-4077)
Impact Factor- 5.46, Volume 5, Issue 3, March 2018
Website- www.aarf.asia, **Email** : editor@aarf.asia , editoraarf@gmail.com

EVALUATION OF ANTIOXIDANT ACTIVITIES AND GENOTOXIC EFFECT OF *LEUCAS ASPERA*

Adhikarimayum Haripyaree
Department of Botany, Imphal College, Imphal, India

Kshetrimayum Guneshwor
Department of Zoology, Ideal Girls' College, Akampat, Imphal-East, India

ABSTRACT

Leucas aspera spreng is a traditional medicine. In the present paper, we are reporting antioxidant and genotoxic activities of this plant. Methanolic extract of plant leaves and flower was evaluated for antioxidant activities by using DPPH and superoxide radical scavenging activity. The plant extract in the reaction mixture shows comparable effect with vitamin C and pyragallol indicating its antioxidant property. In an attempt to determine whether alcoholic extract of the plant interact with genetic material, mammalian cytogenetic assay was used. Mice which received intraperitonial injections of different doses of the extract of Leucas aspera did not induce significantly in chromosomal aberrations, SC damages and micronucleus frequency. Thus, L. aspera shows the capability of using as neutraceoticals.

Key word: Antioxidant activity, DPPH, Superoxide. Clastogenic, Cytogenetic assay, Genotoxicity, Micronucleus, Synaptonemal complex.

Introduction

Herbal medicines are being used by about 80% of the world population, primarily in developing countries for routine health care and are also entering the therapeutics in the developed countries (Kamboj, 2000). These escape toxicity testing before they are marketed as

traditional medicines due to inadequate drug laws. Yet many reports reveal that drugs of plant origin are not free from toxic effects. Hepatic failure and even death following ingestion of herbal medicine have been reported (Dicken *et al.,* 1994). Traditional eye medicines have been linked to childhood blindness in Nigeria (Harries and Cullinan, 1994). Death of 15 persons in USA has been described to a herbal medicine for impotency (Josefson, 1996). Several medicinal plants are mutagenic, clastogenic and carcinogenic (Nandi *et al.,* 1998).

The presence of phytochemicals in medicinal plants have attracted a great deal of attention, concentrated on their role in preventing diseases. The high content of polyphenolic Pcompounds and flavonoids in different parts of various medicinal plants have the antioxidant properties. Different studies have demonstrated that flavonoids present at interesting levels have anti-inflammatory activity (Ferrandiz and Alcaraz, 1991; Prior and Wu, 2005). Besides natural antioxidants, medicinal plants may also provide fibers and other biotic compounds.

Mammalian *in vivo* tests have several advantages over *in vitro* tests because the metabolic activation and detoxification of the chemicals in the intact animal are closer to the human system. *In vivo* mouse chromosome assay has been recognized as one of the sensitive methods to test genotoxicity of plant extracts (Chakrabarti, 2001).The bone marrow micronucleus (BMM) test is one of the least expensive *in vivo* assays for genotoxic effects (Heddle, 1973; Schmid, 1976). However, it has limitations that it only detects chromosome breaks or laggards and not even the mature types of break. Therefore, analysis of traditional chromosome aberration was employed in conjunction with BMM test for comprehensive mutagenicity testing. Synaptonemal complex (SC) analysis holds great promise as an *in vivo* mammalian germ cell assay for resolving effects of chemical exposure to the gonads and evaluating the risk of genetic damage (Allen *et al.,* 1988; Backer *et al*., 1988).

Leucas aspera is widely used medicinal plant and has popular medicinal values (Prajapati *et al.,* 2003; Sinha, 1996). The medicinal uses of the plant include dyspepsia, verminoxis, arthralgia, chronic skin eruption, cough, intermittent fevers and ulcers. This plant contain various compounds namely, lignans and flavonoids (Sadhu *et al.,* 2003), glucoside (Prajapati *et al.,* 2003), and triterpenoid/steroids (Kamat and Singh, 1994). Flavonoids are water extractable organic compounds that are mutagenic in *Salmonella* micro some assay in the strains TA 100 and TA 98 (Porto, 1999). Because of the presence of the above compounds in this medicinal plant some of which are cytotoxic and mutagenic this plant was examined for genotoxicity using

mammalian *in vivo* cytogenetic assay. In present study, we evaluate the antioxidant properties and cytogenetic assay of this medicinal plant in order to determine its medicinal values and the clastogenic potentialities .

Materials and Methods

I Antioxidant Properties:

Chemicals: DPPH (2,2 diphenyl-1-pycryl hydrazyl) was purchased from Sigma Aldrich chemical company (USA), pyragallol from Merck (Mumbai, India), methanol from Qualigens (Mumbai, India), ascorbic acid (Merck), 2 thiobarbituric acid, hypoxanthine, 2-deoxy-D-ribose, nitrotetrazolium blue, xanthine oxidase (Sigma).

Preparation of plant extracts: The plants parts which are used as the herbal food supplements are oven dried at 60^0C. The dried plant parts were crushed into powder form. The powdered plant parts were weighed and crushed with the help of mortar and pestle, with absolute methanol (1gm/10ml). The crude extracts obtained were centrifuged twice and filtered using Whatman No. 1 filter paper, till a clear supernatant was obtained. The supernatant was vacuum evaporated till dryness. The residue obtained was kept at 4^0C for future use.

DPPH assay: The DPPH assay was carried out as described by Cuendet *et al*., 1997 with slight modification. The reaction mixture consisted of 250 μM DPPH in 100% methanol with 50-500 μg/mL of the crude extract of ten herbal plants used as food supplement. Same amount of ascorbic acid and pyragallol are taken for standard solution. After a 30-min incubation period in the dark at room temperature, the absorbance was read against a blank at 517 nm. Percentage inhibition was determined by comparison with a methanol treated control group. The percentage of DPPH decoloration was calculated as follows:

%DPPH decoloration = [1-O.D. sample/O.D. control] X 100

The degree of decoloration indicates the free radical scavenging efficiency of the substances. Values are presented as mean ± standard error of three determinations.

Inhibition of superoxide radical: Superoxide radical generated by the hypoxanthine/xanthine oxidase system was determined spectrophotometrically by monitoring the

product of nitroblue tetrazolium (NBT). Various concentrations of the extracts were added to the reaction containing 100 µl of 30 mM EDTA (pH 7.4), 10 µl of 30 mM hypoxanthine in 50 mM NaOH, 2 ml of 1.42 mM NBT and the final volume of 3ml was made up by 50 mM PO_4 Buffer (pH). After adding 100 µl of 0.5U/ml xanthine oxidase, the reaction mixture was incubated for 30 min at 25^0C. The absorbance was read at 560nm and compared with control samples in which the enzyme, xanthine oxidase, was not included.

The percent inhibition of superoxide radicals was calculated from the optical density of the treated and control samples.

Inhibitory effect (%) = $[(A_{560}$ control $- A_{560}$ sample$)/A_{560}$ control$]X100$

IC_{50} (half of the inhibitory concentration) was calculated for all the methods by using Linear Regression Analysis.

II Cytogenetic assay:

Plant materials were collected from different regions of Manipur and identified based on the vegetative and floral characteristics described in "A hand book of Medicinal plants" (Prajapati *et al.,* 2003). Leaves and flowers of *L. aspera* was air dried, powdered and extracted with 50% aqueous ethanol overnight at room temperature by constant shaking using gyratory shaker. The supernatant was decanted and filtered through Whatman no. 4, stored at 4ºC and used within 24 hours. The concentrations of the extract was expressed as percentage of dry weights of the plant in aqueous ethanol. Inbred Swiss albino mice originated from a single parental pair of sibs were maintained in natural environmental condition with free access food (gram, wheat, milk and bread) and water. Permission of the University Ethics Committee was obtained for animal experiments and the Committee's guidelines for human treatment of animals were strictly followed. Mice weighing about 25 g and 10-12 weeks old were used. Maximum tolerated doses of the plant extract was determined by range test. LD^{50} doses were serially diluted with 50% ethanol until LD^o was achieved. LD^o was taken as MTD. Animals were treated through intraperitonial injections with MTD and half of the MTD of plant extract. Negative control animals received doses of 50% ethanol equivalent to that of plant extract, while positive control animals received EMS at the dose of 240mg/kg body weight dissolved in 1 ml distilled water. Each treatment and control group consisted of 5 animals. The treatment protocol is given in Table 1. Animals were sacrificed after 24 hours of treatment and cytological preparations were made. Metaphase chromosomes were prepared from bone marrow cells using standard

colchicines - hypotonic – spreading – air drying technique. Synaptonemal complexes (SC) were prepared from spermatocytes following the method of Bhagirath and Kundu, 1985. Briefly, one drop of testicular cell suspension in RPMI – 1640 was spread over a large drop of 0.2 M sucrose on a clean slide for 30 seconds, slides were fixed in 4% paraformaldehyde for 5 minutes, washed in 0.2% photoflow for 30 seconds, dried and stained with 70% silver nitrate. SC damages were scored according to Allen *et al*., 1998 and Backer *et al.,* 1988. Micronuclei were prepared from bone marrow cells following the method of Romagna and Staniforth, 1989. Briefly 2-3 drops of bone marrow cell suspension in RPMI-1640 and FCS were put on a clean slide and drawn into a smear with another slide, air dried, fixed in methanol and stored overnight in refrigerator and stained with Giemsa which was filtered through 0.2 µ syringe filter. One slide was prepared from each animal and 1000 PCE were scored from each slide as recommended by two IPCS collaboration studies (Ashby *et al.,* 1983). The results from treated animals were compared with those from control animals using Z- test.

Results

Table 2: Scavanging activity (%) and IC_{50} value of *L. aspera* extract compare with vitamin C and pyragallol on DPPH and superoxide radical inhibition.

Sample Extract	Scavanging Activity(%) (Mean±S.E)	Concentration in µ gm/ml					IC_{50} µ gm/ml
		5	10	15	20	25	
L. aspera	DPPH	6.52±0.12	9.36±0.15	12.61±0.08	24.23±0.03	29.12±0.09	63.42
	Superoxide	4.51±0.25	7.32±0.50	9.15±0.16	11.56±0.10	15.72±0.23	75.81
Ascorbic acid	DPPH	10.11±0.35	17.15±0.32	30.72±0.56	52.71±0.21	65.66±0.27	17.84
	Superoxide	13.57±0.15	17.32±0.16	19.63±0.18	20.37±0.34	39.12±0.15	49.61
Pyragallol	DPPH	15.25±0.22	22.81±0.18	35.06±0.32	55.21±0.25	56.15±0.35	15.90
	Superoxide	31.56±0.20	49.22±0.35	50.80±0.37	53.32±0.33	69.74±0.15	15.67

*Three replicates are taken in each experiment, S.E= Standard Error

The change in colorization from violet to yellow and subsequent fall in absorbance of the stable free radical was measured. The *L. aspera* extract exhibited a dose dependent DPPH radical scavenging activity comparable with pyragallol and ascorbic acid (Table 2). The plant extract also shows relative scavenging activity towards superoxide radicals when compare with two standard compounds vitamin C and pyragallol.

Table 3 presents data on the different types of traditional chromosome aberrations induced by the plant extract. Representative types of traditional chromosomal aberrations are shown in figures 1 to 6. More than 5000 metaphases from five animals were examined. Total number of aberrations of all types were added. Centric fission and centric fusion were the predominant types of abnormality observed in all the treatments. Chromatid break, isochromatid break, ring chromosome, all dot like and fragment were observed at lesser frequency. Frequencies of aberrations in positive and negative control were 31.11% and 5.03% respectively. Frequencies of aberrations in animals treated with *L. aspera* was similar with control value.

Types of synaptonemal complex damages and their frequencies are shown in figures 7 to 12 and Table 4. Types of SC damages are those recommended by Allen *et al*., 1988 and Backer *et al*., 1988. EMS (positive control) induced all types of the recommended damages, while 50% ethanol (negative control) induced only some varying types of damages. Frequency of total damages in mice treated with *L. aspera* and 50% ethanol (negative control) showed almost similar values.

MPCE and their frequencies are shown in figures 13 to 14 and Table 5. NCE having red–orange colour (figure 14) could be differentiated from bluish coloured PCE (figure 13). Proportions of MPCE in mice treated with *L. aspera* show no significant difference among treatments with 50% ethanol.

Table 1— The experimental protocol for the treatment of animals.

Plant (part)	Treatment		Treatment period	No. of animals treated
	Volume in ml/100g body weight (Concentration)			
	MTD	Half of the MTD		
L. aspera (Leaves & flower)	3.2(10%)*	1.6	24 hrs	5

* Extract concentration expressed as plant material dry weight/100 ml 50% ethanol. Extracts of different weights of plant material were subjected to range test for determining MTD. Figures in parenthesis indicate concentration that provided maximum treatment volume of 0.8 ml/animal in MTD range tests.

Table 3 — Chromosomal aberrations in bone marrow cells of mice treated with EMS, 50% ethanol and the plant extract.

Treatment	No. of meta.	Centro. Gap	Centric fusion	Centri c Fission	Chrom. Break	Isochr o. Break	Trans.	Attenu.	Ring Chromo	All dot Like	Frag.	Total Damag e	% of damage ± SE
EMS 24 mg/100g. b. wt (positive control)	5345	175	371	551	65	49	27	192	25	67	141	1663	31.11*± 0.037
50% ethanol (Negative control)	5298	23	57	127	13	0	0	37	10	0	0	267	5.03 ± 0.038
L. aspera													
1.6ml/100g. b. wt	5352	31	69	145	14	0	0	41	13	0	0	313	5.84± 0.018
3.2ml/100g. b. wt	5357	33	71	141	15	0	0	42	14	0	0	316	5.89± 0.015

Abbreviation: Meta.=Metaphase, Centro.=Centromeric, Chrom.=Chromatid, Trans.=Tnanslocation, Attenu.=Attenuation, Frag.=Fragments,
Dam.=Damage. *=P <0.01, SE=Standard Error.

Table 4 — Synaptonemal complex damages in mouse spermatocytes treated with EMS, 50% ethanol and the plant extract.

Treatment	No.of cells scored	Autosome Element Damage							Sex Element Damage							Total Dam	% of dam. ±SE
		SC break	Frag	MAC	Attn	Asyn	Over all Asyn	Auto-Trans	Attn. in X Elem	Attn. in Y Elem	X-Y Sepn	X-A Trans	Y-A Trans	X-fold back Pair	Y-fold Back Pair	SC Break in X	SC Break in Y		
EMS 240mg/100g. b. wt (Positive control)	5335	417	298	35	258	19	21	79	31	28	109	37	17	139	17	35	19	1559	29.22*± 0.077
50% ethanol (Negative control)	5353	151	0	0	47	0	0	21	0	0	27	19	0	21	0	0	0	286	5.34 ± 0.052
L .aspera 1.6ml/100g.b.wt	5503	167	0	0	53	0	0	27	0	0	31	21	0	19	0	0	0	318	5.77± 0.038
3.2ml/100g.b. wt	5528	269	0	0	55	0	0	25	0	0	29	23	0	21	0	0	0	322	5.82± 0.015
									1										

Abrreviation: SC= Synaptonemal complex, Frag= Fragment, MAC= Multi Axial Complex, Attn.= Attenuation, Asyn.= Asynapsis, Auto-trans.= Autosome translocation, Elem.= Element, Sepn.= Separation, Pair.= Pairing, Dam.= Damage, *= P<0.01, SE= Standared Error.

Table 5**.** Frequency of MPCE in bone marrow cells of mice treated with EMS, 50% ethanol and the plant extract.

Treatment	No. of PCE scored	No. of MPCE scored	Ranges of proportion of MPCE (MPCE/PCE)
EMS 240mg/Kg b. wt (Positive control)	5000	107	0.0214*
50% ethanol (Negative control)	5000	19	0.0038
L. aspera 1.6ml/100g. b. wt 3.2ml/100g. b. wt	5000 5000	24 26	0.0048 0.0052

* = P<0.01

Discussion

To maintain antioxidant level in the body, external supplementation is necessary for healthy living. Supplementation of natural antioxidants through a balanced diet could be more effective, and also more economical than supplementation of an individual antioxidant, such as ascorbic acid or Vit E, in protecting the body against oxidative damage under various conditions (Wang *et al.,* 2004). It has been known that several medicinal plants contain active principals possessing antioxidant properties. In Manipur a number of non-conventional and under-used plants based food, particularly belonging to the family Zingiberaceae, possessing rich antioxidant properties are consumed by the people which perhaps may be basis for low incidence of cancers (Chirangini, *et al.,* 2004).

The ranking of the antioxidant activity of the sample may vary with the analysis methods. It is common to evaluate the antioxidant activity of plants using several methods to measure various oxidation products. Many authors strongly suggested that when analyzing the antioxidant activity, it is better to use at least two methods due to differences between the test systems (Schlesier, 2002). Recently it has been appreciated that there is no simple universal method by which antioxidant activity can be measured accurately and quantitatively (Prior *et al.,* 2002). In

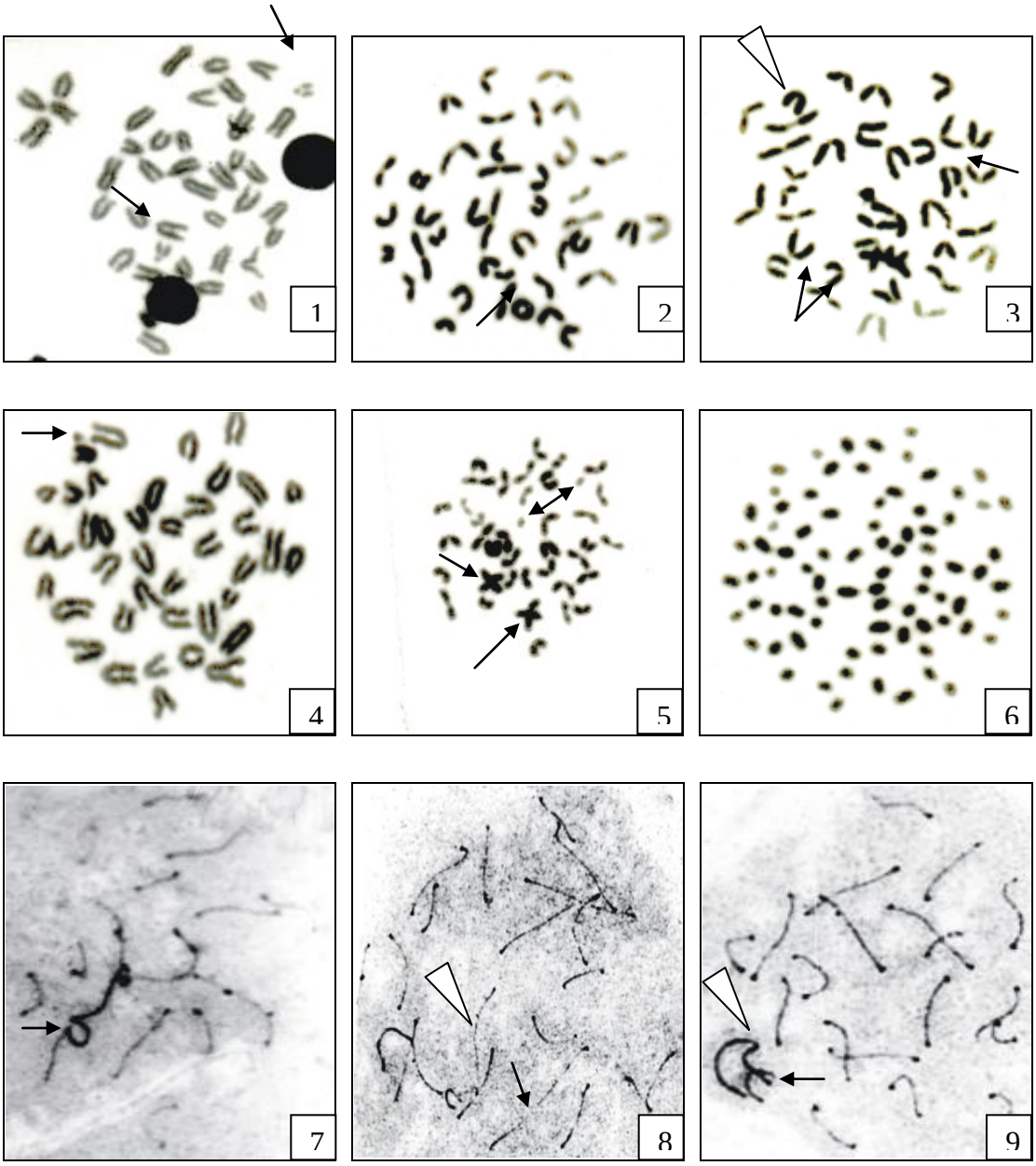
our experiment we used these common methods (DPPH, Hydroxyl radical and Superoxide radical) to analyze the antioxidant activity. The mechanism of each analysis is different. Despite various mechanisms of the methods, combined results of these *in vitro* assays have given an idea of relative antioxidant activity of different herbal vegetables.

In present study, *L. aspera* extract did not showed positive results in all three clastogenic testing protocols based on traditional chromosome aberration analysis, micronucleus assay and SC damages analysis.

Although the plant extract showed negative results in all the genotoxicity testing protocols, further specific and reliable mutagenicity tests need to be done as there are cases of point mutation, frame shift mutation which escape detection by methods employed in the present study. Mutagenicity of certain medicinal plants observed in the earlier studies by other investigators (Nandi *et al*. 1998) compels the necessity of using medicinal plants with specific guidelines, which have a sound basis and relevance to the population concerned.

Conclusion

From the result of investigation it is concluded that *L. aspera* extract can scavenge different free radical which can be shown by using DPPH and superoxide radical scavenging activity. On the other hand the plant extract did not show any positive result in genotoxic testing. Thus *L. aspera* shows capability of using it as neutraceuticals.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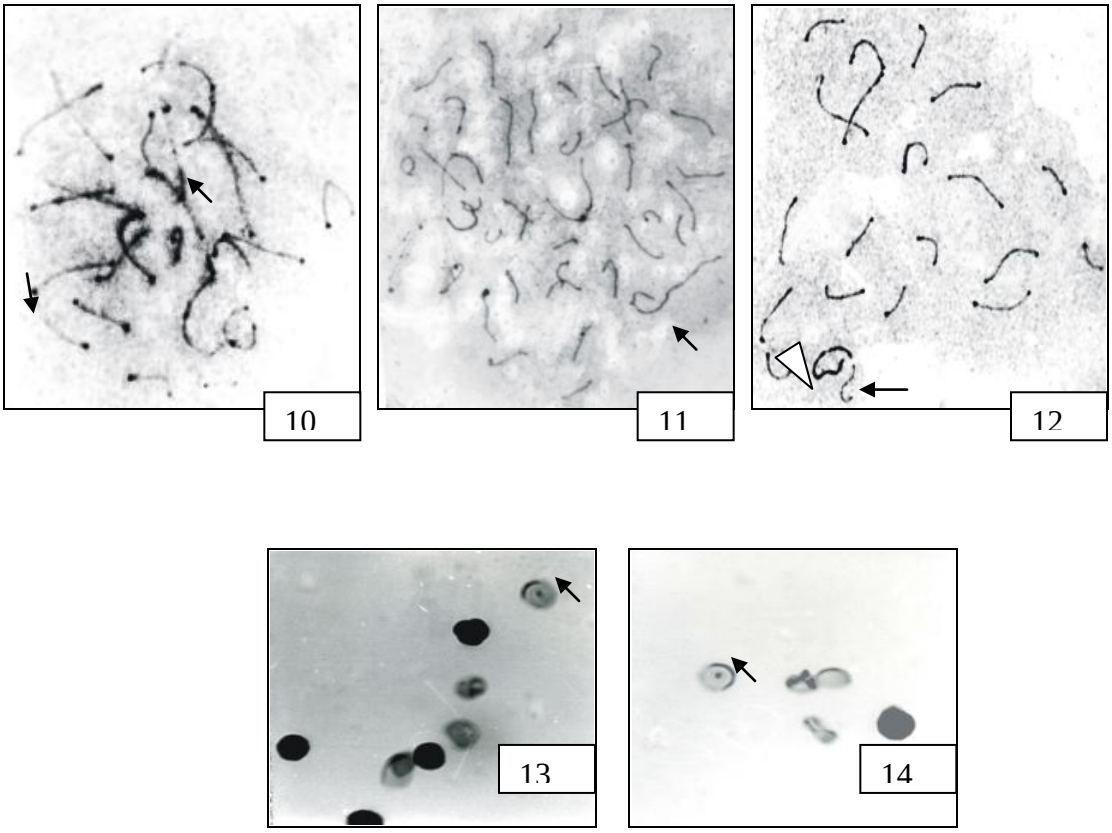

Figure 1-6. Somatic chromosome aberrations. (1) Isochromatid break (solid arrows); (2) Ring chromosome (solid arrow); (3) Chromatid break (solid arrow), Centromeric gap (hollow arrow) and Centric fission (double head arrow); (4) Fragment (solid arrow); (5) Centric fusion (solid arrows), Centric fission (double head arrow); and (6) All dot like.

Figure 7-9. Synaptonemal complex damages. (7) X-A translocation (solid arrow); (8) SC break (solid arrow) and Autosomal attenuation (hollow arrow); (9) X-Y separation (solid arrow) and X-fold back pairing (hollow arrow); (10) Autosome translocation (solid arrows); (11) X-A translocation (solid arrow); and (12) X-Y separation (solid arrow) and X-fold back pairing (hollow arrow).

Figure 13. PCE with single micronucleus (solid arrow).

Figure 14. NCE with single micronucleus (solid arrow).

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