ANTI-CLASTOGENIC ACTIVITY OF ROSELLE (*Hibiscus sabdariffa*) EXTRACT USING A VARIETY OF SHORT-TERM GENOTOXIC BIOASSAYS

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Plants have always played a major role in the treatment of human and animal diseases. Medicinal plants are a therapeutic resource which used by the traditional population of the world especially for the health care. The use of medicinal plants is now increased worldwide (Banatnouny *et al.*, 1999).

It was reported that Roselle (*Hibiscus sabdariffa*) is antiseptic, digestive, diuretic, emollient and purgative (Duke, 1985; Truswell, 1992). Recent scientific research work has established the protective effect of the dried flower extract of *Hibiscus sabdariffa* (Tseng *et al*., 1997); anti-inflammatory activity (Dafallah and Mustapha, 1996), antihypertensive effect of the calyx extract (Adegunloye *et al*., 1996; Onyeneka *et al.*, 1999) and anti-mutagenic activity (Morton, 1987). It was also reported that the cultivation of Roselle as a "recent" crop in Arab-speaking countries is centered more on its pharmaceutical than its food potential. In 1971 this crop was distributed in tropical areas especially in Africa and India. In these countries, it is also cultivated to some extent for the freshly calyx of the flower from which jelly, a kind of tea named karkadeh is extracted. Karkadeh is widely cultivated in Sudan for the extraction of the jelly which is dried and exported to other countries. (Chewonarim *et al*., 1999).

The present work aim to investigate the anti-genotoxic activity of *Hibiscus sabdariffa*. To achieve such a purpose an investigation of cytogenetic effect of syrup extract from calyx and sub-calex, in decreasing chromosomal abnormalities, after treatment of mice, human lymphocytes and *Allium cepa* have been carried out. A variety of short-term *in vivo* and *in vitro* genotoxic bioassays which that recommended by EPA-US. These bioassays are analysis of chromosomal abnormalities in mice bone marrow, analysis of micronucleus in mice bone marrow, estimation of cell proliferation, analysis of primary spermatocyte (diakinesis stage) in mice , analysis of mitotic activity and cell proliferation in *Allium cepa* cells, analysis of chromosomal abnormalities in *Allium cepa* cells and estimation of micronucleus in interphase cells for mice bone marrow.

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MATERIALS AND METHODS

Materials

Cytological analysis

The cytogenetical characterization aim to investigate the potentiality of calyx and sub-calyx extract to play an important role in reducing the clastogenic effect caused by the well known positive controls(sodium nitrite in *Allium cepa*); cyclophosphamide in mice and ethyl methane sulfonate in human lymphocytes.

Experimental models & chemicals

The genetic models used throughout this work were *Allium cepa* (2n = 16), albino mice (*Mus musculus*, (2n = 40) and human lymphocyte chromosomes (2n = 46).

Cold and hot Roselle extracts treatments

1-Cold treatment

Three doses were prepared as follows: 25 g/100 ml; 12.5 g/100 ml and 6.25 g/100 ml (Hirunpanich *et al.*, 2005) were incubated overnight at 37°C and filtered. Each mouse received 100 µl for 60 days.

2- Hot treatment

Three doses were prepared as follows: 25 g/100 ml; 12.5 g/100 ml; and 6.25 g/100 ml were boiled, filtered and mice were treated; each mouse received 100 µl for 60 days.

3- Sodium nitrite, cyclophosphomide and Ethyl methanesulfonate were used as mutagenic substances for positive control group

Experimental design techniques

Three doses i-e., (6-25 g, 12.5 g and 25 g/100 ml) were used in mice treatment for 60 days (Chewonarim, *et al*. 1999). Cyclopho sphamide (50 mg/kg. b.wt) was as a positive control. Technique given by Brusiek (1980) were used for the analysis of metaphase index, chromosomal abnormalities, micronucleus assay given by Schmid (1975) was used for estimation of micronucleated polychromatic erythrocytes. Analysis of human lymph was carried out according to Schwartz (1974).

Analysis of variance, Duncan's multiple range test and chi square were used for MNT, the tables given by Hart and Engberg-Pedersen (1983) were used.

Methods

- Analysis of metaphase index (MTI) and analysis of chromosomal abnormalities in mice bone marrow cells. These analyses were done as that described by Brusick (1980).
- Micronucleated polychromatid erythrocytes were investigated according to Schmid (1975).
- Analysis of mice primary spermatocytes. Five male mice samples were used for each dose. These doses were

orally given for 10 days. The animal samples were killed by decapitation (24 hr) after the last dose. The used procedure basically follows the description given by Oud *et al*. (1979); Adler (1984) and Seehy and Osman (1989).

- Human lymphocyte culture technique. This technique was carried out according to the description given by Schwartz (1974) and the same concentrations of cold and hot extract were added to the culture.

RESULTS AND DISCUSSION

Chromosomal abnormalities

Cytological examination of chromosomal aberrations in mice bone marrow after treatment with *Hibiscus* extract is shown in Tables (1 and 2). Different types of structural and numerical aberrations were obtained (Robertosonian Centric Fusion, gap, fragment and polyploidy), however, positive control gave high percentage of Stickiness and some of this aberrations are given in Figs. (1a-1e). Cyclophosphamide was capable in inducing hyperploidy i.e > 2n (Figs. 1f-1g). Hot extract induced a significant increase of micronucleus (Fig. 1i). Total aberrant of metaphases were found to be 39% after treatment with cyclophosphamide. The results showed that the high concentrations of caused a high degree of stickiness and accordingly high aberrant metaphases were obtained and ranged from 5 to 54%. It probably seems that the high percentages of aberrant metaphases might be caused by cytoplasmic disturbance which induced by high concentrations. Tables (1 and 2) showed the results obtained after treatment with hot extract. Comparing data in these two tables, one can conclude that the cold extract was found to be capable in decreasing the total aberrant metaphases caused by cyclophosphamide.

It is taken for granted that the degree of mutagenic potentiality of environmental pollutants which evaluated in one test system may not be the same in another one, therefore, testing for the induction of DNA lesions and the mutagenicity using a variety of short-term assays has become as an accepted part of the toxicological evaluation of drugs, industrial intermediates, cosmetics, food and feed additives, pesticides, etc……..

According to Brusick (1987) positive controls are included to establish the ability of the analyzers to correctly determine aberrations and to ascertain the expected test-to-test and animal-to-animal variations, and to establish the sensitivity of a particular test. However, cyclophosphamide is a clastogenic agent for various animal species. Chorvatovicova and Sandula (1995) recommended the use of this drug in cytogenetical studies as a positive control.

Micronucleus test

Cyclophosphamide and hot extract were proven to be clastogen, since statistical analysis showed significant increases in micronucleus. Tables (3&4) which illustrated the data obtained from the analysis of micronucleated polychromatic

erythrocytes. It is clear that the cold extract showed anticlastogenic activity.

Analysis of primary spermatocytes:

Tables (5 and 6) showed the results obtained from the analysis of diakinesis stages after treatment of mice with cold and hot extracts. Different types of aberrations at diakinesis such as fragment, Stickiness XY univalent, autosomal univalent and translocation in addition to Stickiness were obtained (Figs. 1b, 1c, 1d, 1e and 1f). These results showed that cyclophosphamide was capable to reach the germinal cells. On the other hand, cold extract was proven to be capable of decreasing the clastogenic effect which caused by cyclophosphamide and indicated cold extract has *in vivo* anticlastogenic activity.

Allium cepa

The analysis of mitotic activity and chromosomal aberrations in cells of adventitious roots of *Allium cepa* are given in Tables (7 and 8).

Mitotic index was 14.8% for the negative control and 6.2% after treatment with sodium nitrite. For cold treatment, it was ranged from 8.1 to 13.4%, while from 4.2 to 12.2% for hot treatment. Figs. (3a-3g) showed the effect of different treatment upon *Allium cepa* genome. Total aberrant metaphases was ranged from 5 to13% after cold extract treatment and it was ranged from 13 to 26% after hot extract treatment.

Data obtained from these genotoxic bioassays revealed that cold extract has anticlastogenic activity upon ethyl methanesulfonate. Which presented strong evidence that cold extract of *hibiscus* has anticlastogenic activity.

Human lymphocyte culture

An attempt was carried out to investigate the *in vitro* effect of *hibiscus* extract upon human chromosomes. Total aberrant metaphases was 3% in the negative control group, 28% after treatment with the positive control (EMS) and it was ranged from 5 to 8% after cold extract treatment and it was ranged from 5 to 9% after hot extract treatment (Figs 2a and 2e) and Tables (9 and 10) showed the effect of cold, hot, and EMS treatments. This result, however, presented evidence that hot extract treatment *in vitro* was positive clastogene, while cold extract was treatment had *in vitro* anti clastogeneic effect.

In conclusion, the present investigation clearly revealed that cold extract treatment of calyx and sub-calyx of *Hibiscus* was proven to decrease the cellular toxicity and clastogenic effect of positive controls (Cyclophosphamide, sodium nitrite and EMS).

Assessing human risk to mutagenic substances represents a formidable task. There is so far no conclusive proof of showing chemical-induced mutation in human germ cells; however, mutagens can alter rodent germ cells and quantitative estimates of induced mutation rates per gene locus or the dose required to double a specific mutation rate which have to be

calculated from results of the *in vivo* - specific-locus or heritable translocation assays. These estimates may be of limited value in calculating human risk or in setting safe exposure levels because they are based on male gametes and, in the case of specific-locus assay, generally on pre meiotic stem cells (spermatogonia). The data do not reflect the risk to later cell stages in spermatogenesis or in female germ cells. Estimates of mutation in postmeiotic sperm and from female gametes will become available; but even so, other important biological variables would interfere with reliable risk estimates and extrapolation between species (Brusick, 1980 & 1987; Abid-Alla, 2007).

The present work recommends that drinking of Roselle calyx syrup (extracted at cold temperature i.e. without boiling) might decrease the genotoxic effect caused by the Sodium nitrite, cyclophosphomide and ethyl methanesulfonate.

Cytological examination and chromosomal abnormalities revealed that cyclophosphamide was found to be effective in inducing significant decreases in cell proliferating rate and giving evidence on its cellular toxicity. Chromosomal abnormalities indicated that it is a strong clastogenic agent which reflects the possible mutagenic activity of cyclophosphamide. DNA damage may be classified into several broad categories based on the nature (presumed mechanism) of the DNA change.

Regarding the micronucleated polychromatic erythrocytes, the micronuclei represent acentric chromosome fragments or whole chromosomes that lost during cellular anaphase. These structures are easy to visualize in erythrocytes and therefore, are often used as a measurement of chromosomal aberrations (Rabello-Gay, 1991).

Exposure to pollutants has been associated with cancers, degenerative neurologic diseases, and altering immune response, but the mechanism of action is unclear. Genotoxic potential is a primary risk factor for long-term health effects such as cancer and reproductive health outcomes. Bolognesi (1997) and Hagmar *et al.* (2001) reviewed the usefulness of cytogenetic biomarkers as intermediate end points in carcinogenesis and concluded that chromosomal aberration (CA) frequency predicts overall cancer risk in healthy subjects, but such associations have not been found for sister-chromatid exchanges and micronuclei (Mn). Although, the genotoxic potential of pesticides is believed to be low, but genotoxic monitoring in farm worker populations could be a useful tool to estimate the genetic risk from exposure to complex pesticide mixtures over extended lengths of time. To date, genotoxic biomarker studies of workers exposed to pesticides have focused on cytogenetic end points including CAs, Mn frequency, and sister-chromatid exchanges.

This conclusion came from the observation that chromosomal aberrations;

micronucleated polychromatic erythrocyte and aberrant diakinesis stages were decreased with the increasing of the plant dose given to the mice beside the data obtained from the analysis of *Allium cepa* genome and human lympthocytes.

SUMMARY

Nowadays, it has been appeared that there are several advantages for the medical use of *hibiscus*, which showed the ability to reduce cholesterol level and lipids in animals at laboratory tests in addition to antibiotic oxidation. Thus, the aim of this research is to study its role as anticlastogenic agent upon the chromosomes damage. The calyx and sub-calyx of the Roselle plant has long been recognized as a source of antioxidants. The objective of this study was to investigate the capability of *Hibiscus sabdariffa* juice to act as anticlastogenic agent by preventing or decreasing chromosomal breaks. In order to achieve such a purpose the genetic material of Mouse (*Mus musculus*, 2n = 40) and roottip cells of Onion (*Allium cepa*, 2n = 16) were selected and used employing a variety of short-term genotoxic bioassays that recommended by EPA-US.The obtained result revealed that Roselle cold extract or syrup treatment had anticlastogenic effect. While hot extract has not. How does this suggested repair system play its role? by activation of cell proliferation, apoptosis; or by interfering with cellular repair system or by all these assumptions. Further research is needed in order to precisely answer this question.

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Table (1): Chromosomal aberrations in mice bone marrow after cold treatment by *Hibiscus* extract.

Treatment	Type of aberrations					Percentage of total aberrant metaphase
	Polyploidy	*RCF	Fragment	Gap	Stickiness	
NC	-	-	1	-	2	3
PC	4	2	6	5	22	39**
6.25	1	2	5	2	7	17**
12.5	4	6	5	8	24	47**
25	8	2	7	6	31	54**
6.25+PC	-	1	-	2	12	15
12.5+PC	1	1	1	-	16	19
25+PC	-	-	-	-	6	6

NC= Negative Contro PC=Positive Control 200 metaphase cells were counted
*Robertosonian Centric Fusion (RCF). ** Significant at 0.01 level of probability.

Table (2): Chromosomal aberrations in mice bone marrow after hot treatment by *Hibiscus* extract.

Treatment	Type of aberrations					Percentage of total aberrant metaphase
	Polyploidy	*RCF	Fragment	Gap	Stickiness	
NC	-	-	1	-	2	3
PC	4	2	6	5	22	39*
6.25	-	4	-	6	9	19*
12.5	5	7	8	10	31	61*
25	10	4	11	13	42	80*
6.25+PC	2	4	2	3	18	29*
12.5+PC	2	-	4	6	21	33*
25+PC	-	2	2	3	21	28*

NC= Negative Contro PC=Positive Control 200 metaphase cells were counted
*Robertosonian Centric Fusion (RCF). * Significant at 0.05 level of probability.

Table (3): Micronucleus test in mice after cold treatment with *Hibiscus* extract.

Treatment	No. cells counted	No. Micronucleated counted	Micronucleated Erythrocytes%
NC	4000	18	0.45
PC	4000	405	10.12*
6.25	4000	18	0.45
12.5	4000	14	0.35
25	4000	14	0.35
6.25+PC	4000	40	1.00
12.5+PC	4000	22	0.55
25+PC	4000	18	0.45

NC= Negative Contro PC=Positive Control
* Significant at 0.05 level of probability. (Hart & Pederson, 1983)

Table (4): Micronucleus test in mice after hot treatment with *Hibiscus* extract.

Treatment	No. cells counted	No. Micronucleatedcounted	Micronucleated Erythecytes %
NC	4000	18	0.45
PC	4000	405	10.12*
6.25	4000	218	5.45*
12.5	4000	229	5.72*
25	4000	232	5.80*
6.25+PC	4000	489	12.22*
12.5+PC	4000	502	12.55*
25+PC	4000	504	12.60*

NC= Negative Contro PC=Positive Control
* Significant at 0.05 level of probability. (Hart & Pederson, 1983)

Table (5): Primary spermatocytes in mice after cold treatment by *Hibiscus* extract.

Treatment	Type of aberrations					Percentage of total aberrant diakinesis
	Stickiness	Fragment	Xy uni-valent	Autosomal	Translocation	
NC	6	1	-	-	-	7
PC	24	10	4	6	-	44*
6.25	2	1	1	1	-	5
12.5	4	1	1	2	1	9
25	8	1	2	1	2	14*
6.25+PC	8	3	-	-	-	11
12.5+PC	6	1	-	1	1	9
25+PC	3	-	1	-	-	4

NC= Negative Contro PC=Positive Control 200 diakineses cells were counte
* Significant at 0.05 level of probability.

Table (6): Primary spermatocytes in mice after hot treatment with *Hibiscus* extract.

Treatment	Type of aberrations					Percentage of total aberrant diakinesis
	Stickiness	Fragment	Xy uni-valent	Autosomal	Translocation	
NC	6	1	-	-	-	7
PC	24	10	4	6	-	44*
6.25	7	1	1	1	2	12*
12.5	11	-	1	1	2	15*
25	13	2	1	1	2	19*
6.25+PC	18	2	-	2	2	24*
12.5+PC	20	3	-	2	3	28*
25+PC	22	2	-	2	2	28*

NC= Negative Contro PC=Positive Control
* Significant at 0.05 level of probability.

Table (7): Mitotic activity and chromosomal aberrations in *Allium cepa* genome after cold treatment by *Hibiscus* extract.

Treatment	MI	Types of aberrations %				
		Stickiness	fragment	Deletion	hyperploidy	Total aberrant metaphases
NC	14.8	4	-	-	-	4
PC	6.2	22	4	2	8	36*
6.25	10.4	8	-	-	-	8
12.5	8.2	4	1	-	-	5
25	8.1	6	2	-	-	8
6.25+PC	11.2	2	3	1	2	8
12.5+PC	12.2	4	-	1	1	6
25+PC	13.4	6	2	2	3	13

NC= Negative Contro PC=Positive Control 200 diakineses cells were counte
* Significant at 0.05 level of probability.

Table (8): Mitotic activity and chromosomal aberrations in *Allium cepa* genome after hot treatment by *Hibiscus* extract.

Ttreatment	MI	Types of aberrations %				
		Stickiness	fragment	Deletion	hyperploidy	Total aberrant metaphases
NC	14.8	4	-	-	-	4
PC	6.2	22	4	2	8	36.5*
6.25	8.2	11	1	2	1	15*
12.5	7.4	6	3	2	2	13*
25	6.3	8	4	4	2	18*
6.25+PC	11.2	4	5	4	2	15*
12.5+PC	12.2	6	6	6	3	21*
25+PC	4.2	8	8	6	4	26*

NC= Negative Control PC=Positive Control
* Significant at 0.05 level of probability.

Table (9): The effect of *Hibiscus* cold extract treatment upon human lymphocyte culture.

Treatment	Types of aberrations %				
	*RCF	fragment	Gap	stickiness	Total aberrant metaphases
NC	-	-	1	2	3
PC (EMS)	6	8	4	10	28**
6.25	-	2	1	-	3
12.5	1	-	-	4	5
25	2	-	-	2	4
6.25+PC	2	-	1	2	5
12.5+PC	3	-	-	4	7
25+PC	2	3	1	2	8

NC= Negative Control PC=Positive Control
** Significant at 0.01 level of probability *Robertosonian Centric Fusion (RCF)

Table (10): The effect of *Hibiscus* hot extract treatment upon human lymphocyte culture.

Conc g/100 ml	Types of aberrations%				
	*RCF	fragment	Gap	stickiness	Total aberrant metaphases
NC	-	-	1	2	3
PC (EMS)	6	8	4	10	28**
6.25	2	1	1	1	5
12.5	2	1	1	1	5
25	3	2	2	3	10**
6.25+PC	-	1	1	4	6
12.5+PC	2	2	-	2	6
25+PC	3	1	1	4	9**

NC= Negative Control PC=Positive Control
* Robertosonian Centric Fusion (RCF 200 metaphases were counted
** Significant at 0.05 level of probability

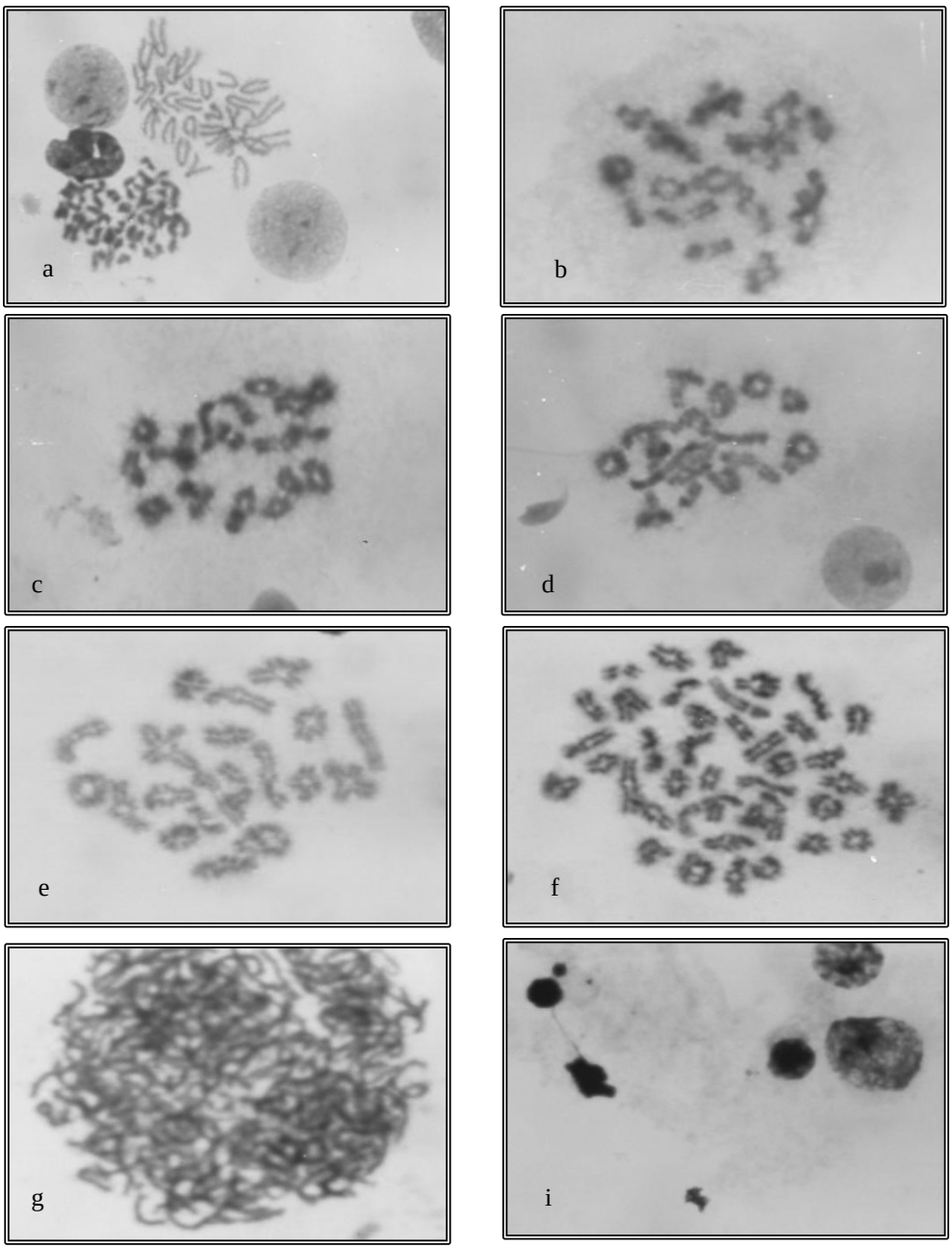

Fig. (1): a) Photomicrograph showing mice chromosomes with high activity. (negative control). b) Photomicrograph showing mice chromosomes with Diplotene stage. (Stickiness after hot treatment). c) Photomicrograph showing mice chromosomes at Diakinesis. (Stickiness after hot treatment). d) Photomicrograph showing mice chromosomes at Diakinesis (exchange after positive control treatment). e) Photomicrograph showing Diakinesis stage with translocation. (hot treatment). f) Photomicrograph showing polyploid at Diakinesis. (cyclophosphomide treatment). g) Photomicrograph showing hyperploidy in mice bone marrow. (cyclophosphomide treatment). i) Photomicrograph showing micronucleus. (hot treatment).

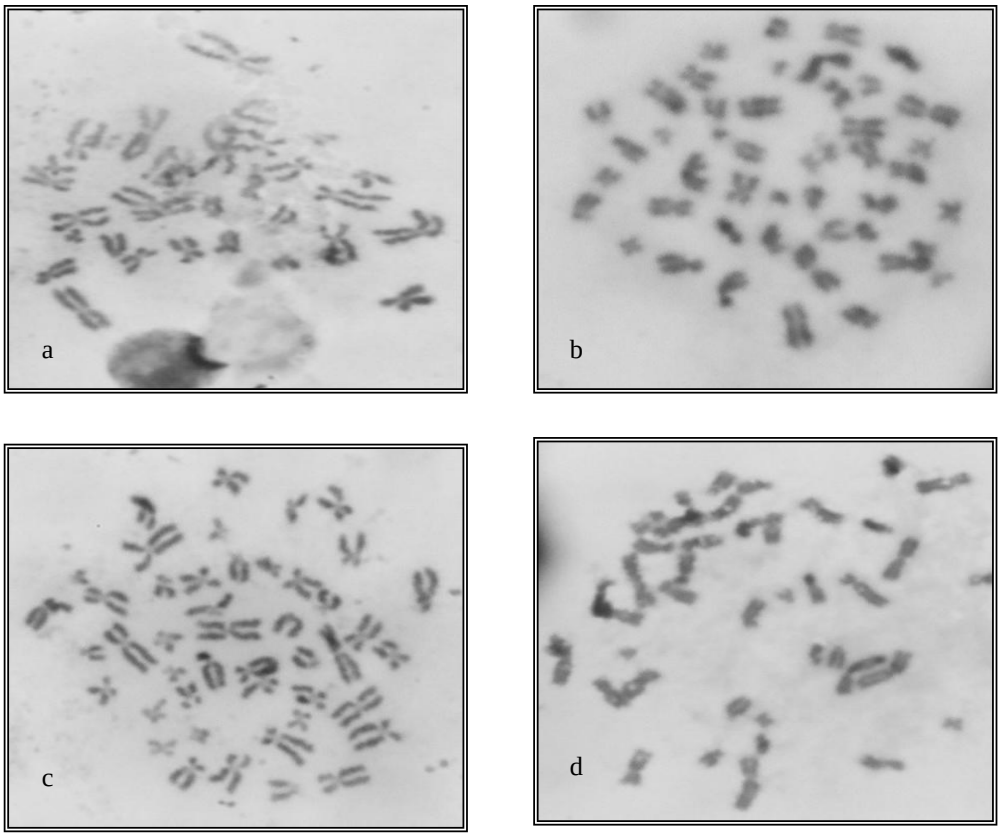

Fig. (2): a) Photomicrograph showing human chromosomes with fragments after treatment with hot extract. b) Photomicrograph showing human chromosomes with chromatid deletion after treatment with hot extract. c) Photomicrograph showing human chromosomes with fragments and chromatid gap after treatment with EMS. d) Photomicrograph showing human chromosomes with fragments and chromatid gap after treatment with hot extract. e) Treatment with cold extracts showing normal chromosomes.

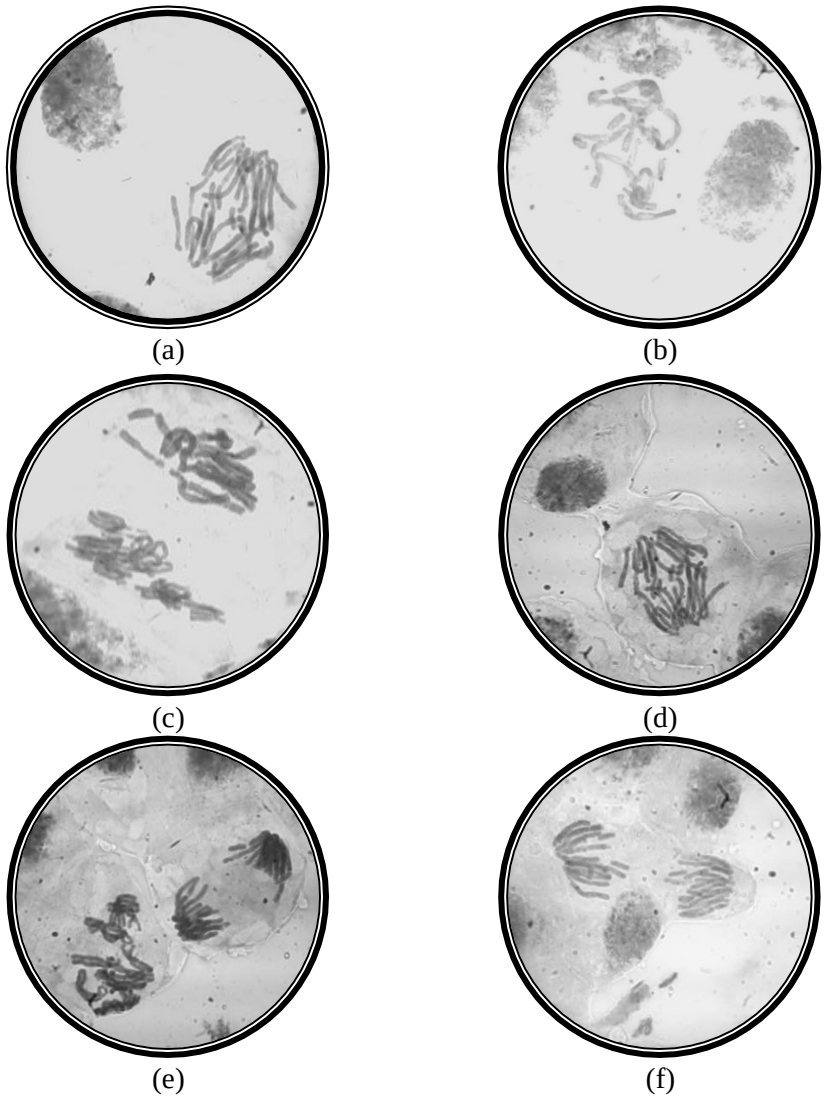

Fig. (3): a) Photomicrograph showing fragment in *Allium cepa*. b) Photomicrograph showing chromosome gap. c) Photomicrograph showing anaphase in *Allium cepa* with stickiness and fragment. d) Photomicrograph showing anaphase stage with sticky ends and fragment in *Allium cepa*. e) Photomicrograph showing late anaphase and metaphase with stickiness. f) Photomicrograph showing anaphase with fragments. g) Photomicrograph showing anaphase with irregular separation.