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Mutational and Carcinogenic Potential of Amaltas Fruit Via Oxidation

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Abstract

Long term intake of plant-based medicines without knowing their toxicities and mutagenic potentials is common globally. Therefore, current work is an extension of cancer patient survey at Nuclear Institute of Medicine and Radiotherapy (NIMRA), for long term utilization of medicinal plant. Genotoxic potential Amaltas fruit (AF) was tested by *Cicer arietinum* L. and *Allium cepa* L plant assay. Abnormality index (A.I.), types of abnormalities and oxidative damages were the major parameters of genotoxicity. Analysis of variance (ANOVA) revealed statistically significant differences (LSD) at $p \leq 0.05$ for A.I. and oxidative damage in both assay plants, except negative control. Both A.I. and oxidative damages revealed incubation dependent increase. In both assay plant the major chromosomal aberration induced was fragmentation. AF induced polyploid cells, apoptotic cells and elongated cells only in *Cicer arietinum* L. assay. Oxidative damages in the form of nuclear membrane and cell membrane damage were observed in aberrant cells along with ghost cells. It is concluded that AF is capable to cause genotoxic effect incubation dependent manner. High rate of fragmented cells reflects its mutagenicity and carcinogenicity mediated oxidation of DNA and membranes in both assay plants. Induction of more ghost cells and less frequent apoptosis reflects activation of oncogene. Development of Pilus like projections in ghost cells depicts proliferation potential of defected cells. Prolonged utilization of AF was the cause of cancer in surveyed patients. *Allium cepa* L. was more sensitive as assay plant to the genotoxin. It is recommended that AF must be used occasionally.

Key words: Mutation and Carcinogenesis, *Cassia fistula* L., Oxidation

Introduction

Cancer is second leading cause of deaths globally. Although currently people are aware of known cancer risks and try to avoid them but still cancer prevalence is increasing drastically in Pakistan. Therefore, it is inevitable to screen for the unknown cancer risks targeting young to old. 18 medicinal plants has been consumed for more than 10 years for intestinal disorders by cancer patients registered at cancer hospital NIMRA Jamshoro. *Cassia fistula* (Amaltas) fruit was one of those plant parts (Seema, 2018). Amaltas fruit (AF) is also used to Some other disorders for with AF is utilized are diabetes, pruritus hematemesis and leukoderma and (Neelam *et al*., 2011; Thirumal *et al*., 2012). It is still not screened for dose and time dependent genotoxic effects. Usually, anti-tumor plant extracts are potent cytotoxins commonly used as nuclear medicine after advent of cancer to kill damaged cells to avoid proliferation of rotted cells. However, the same dose may cause mutation and double stranded DNA damages to healthy cells. As cytotoxic MP has ability to stops mitosis so all the human body components will not be produced. Therefore, the use of alternative medicinal plant parts with genotoxic potential must be avoided.

Amaltas fruit is reported cytotoxic in number of studies for as anti-fungal, antibacterial, laxative, anti-inflammatory and anti-tumor activities. Liver damage in Albino rats is reported due to Amaltas fruit extract in take (Das *et al*., 2008). A well-known alkaloid of AF pyrrolizidine and *N*-oxide derivatives is found cytotoxic (Roeder *et al.,* 2009). Anthraquinone, glycosides and Rhein constituent of Amaltas has been cyto-genotoxic in mice and guinea-pig ileum (Mukhopadhyaya *et al.,* 1998). The assessment of toxic properties of *C. fistula has been* declared extremely important for public health protection to avoid DNA damaging effects leading to caner in consumers (Jothy *et al*., 2011; Bakare *et al*., 2023).

Therefore, an indirect approach was attempted in plant assay to predict possible dose dependent genomic damages to humans. Current work will contribute in dose administration of AF leading to reduction in cancer incidence.

Material and Method

Assay plants and experimental design: To carry plant chromosomal aberration assay as an indicator of mutational and carcinogenic potential of Amaltas fruit two higher plants viz. Chickpea (*Cicer arietinum* L.)

and Onion (*Allium cepa* L.) were used for comparative efficiency and sensitivity. The experiment was carried out using Complete Randomized Design (CRD) with three replications at 25 ^{o}C room temperature.

Treatments used: AF Aqueous 5% solution was used for genotoxicity testing. Depending on recommended root germination methods and difference in treated part, *Cicer arietinum* L. seed were treated for 30 and 90 minutes prior germination of roots (Qureshi *et al*., 2014) and *Allium cepa* L. bulbs were incubated for 24 and 48 hours after initial germination of roots in dH_2O (Firbas and Amon, 2014). Positive control for plant assays 0.2% EMS was as positive control and untreated roots grown in water as negative control. Respective controls were kept for same periods as Amaltas fruit aqueous extract treatment.

Extract preparation and root recovery: AF pulp was initially broken in to small pieces with pestle motor followed by grinding in electric machine. AF powder was soaked overnight in dH_2O. After 24 hours samples were filtered with Wittman filter paper. 20 healthy seeds were used for each treatment and sown into three sand posts for root germination, whereas, three onion bulbs were used to get roots in beakers with dH_2O.

Cytological slide preparation: Slides were prepared by squash method (Dille and King, 1983), and stained with 2% acetocarmine (in 45% glacial acetic acid). Means of six slide per treatment were used to score chromosomal aberrations and oxidative with the help of Inverted microscope (Olympus 51x) at 400 magnification. Digital camera (USB-2.0) Dino eye was used for photography.

Abnormality index (A.I.): The abnormality index was calculated by the method of (Racuciu, 2009) according to the following formula:

$$\mathbf{A.I.} = \frac{\textbf{Total abnormal dividing cells}}{\textbf{Total dividing cells}} \times \mathbf{100}$$

Types of Chromosomal aberrations: Sometimes scoring aberrant cells is not enough to estimate level of genotoxicity therefore, abnormal cells were further investigated for type of chromosomal anomalies in each cell cycle stage. Means were used to compare the treatments for Level of DNA damages.

Oxidative damages: Oxidative damages a common mechanism of damaging heredity material by most of medicinal plants used as alternative medicine. Therefore, oxidative damages were scored by cytology as suggested by (Firbas and Amon, 2014; Qureshi *et al*. 2017). Mean number of cells were used to record, nuclear membrane, cell membrane damage and ghost cells.

Statistical analysis: The mean data of abnormality index and oxidative damages was authenticated through Two-way Analysis of Variance (ANOVA), followed by the Least Square Difference (LSD) test at $p \geq 0.05$ for Abnormality index and Oxidative Damages with the help of computer software Statistics 8.1.

RESULT

Abnormality index (A.I.): The major determinant of genotoxicity is percentage of abnormal dividing cells or Abnormality index. ANOVA analysis followed by mean comparison by LSD $p \leq 0.05$ revealed significant difference A.I. for the two assay plants. In *Cicer arietinum* assay low percentage of abnormal cells (61.4 and 67.7 %) was found as compare to *Allium cepa* assay (76.8 and 69.67%) that was closely similar to its positive control (76 and 80.3%) (Table. 1). Incubation dependent increase was observed in *Cicer arietinum* assay whereas in *Allium cepa* assay random effects were recorded (Figure. 1)

Table 1. Abnormality index of *Cicer arietinum* and *Allium cepa* root tip affected by varying incubations of Amaltas fruit extract (LSD p≤0.05)

S. No.	Name of treatment	C. A.		Total	A. C.		Total
		30M	90M		24H	48H	
1	**-ve cont.**	0^{h}	0^{h}	**0**	0^{h}	0^{h}	**0**
2	**AF**	61.4^{g}	67.7^{f}	**129.1**	76.8^{f}	69.67^{e}	**146.47**
3	**+ve**	73.7^{d}	76.4^{c}	**150.1**	76.4^{c}	80.3^{a}	**156.7**

(C.A. = *Cicer arietinum*; A.C. = *Allium cepa*; M= Minutes; H= Hours; -ve cont. = Negative control; AF= Amaltas Fruit; +ve cont. = Positive control; Means with the same alphabets are non-significantly different from each other and with different alphabets are significantly different at ($p \leq 0.05$))

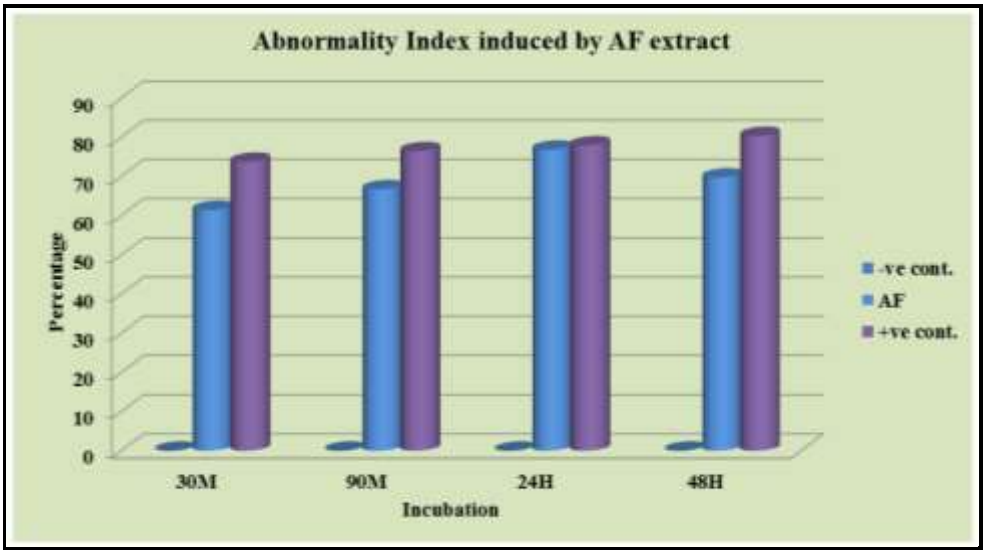

Figure 1. Abnormality index affected by varying incubations of AF extract

Types of abnormalities: Results of *Cicer arietinum* and *Allium cepa* assay revealed fragmentation as major DNA damage for all incubations of AF and positive control (Table. 2 & 3) (Figure.2). The other chromosomal abnormalities observed at different stages of cell cycle were scattered nuclei, sticky chromosomes and translocation ring, laggards, anaphase bridges, multipolar cells, micronuclei, di-nuclei and multi nuclei (Figure. 3).

Polyploid, apoptic, and elongated cells: In *Cicer arietinum* assay polyploid cells with sticky chromosomes were only witnessed in 30M incubation of AF (20 cells), while apoptosis (13 cells) and elongated cells (1.75 cells) (Figure. 4). in 90M incubation. All the epigenetic changes recorded in *Cicer arietinum* were not recorded in *Allium cepa* Assay.

Table 2. Chromosomal aberrations mediated by negative (dH_20) and positive control (.2% EMS) in *Cicer arietinum* and *Allium cepa*

S. No.	Stages	Abnormalities	C. A.				A. C.			
			30M		9M		24H		48H	
			-ve cont.	+ve cont.	-ve cont.	+ve cont.	-ve cont.	+ve cont.	-ve cont.	+ve cont.
1	Metaphase	Scattered nuclei	0	27.2	0	32.2	0	22.5	0	12
		Sticky metaphase	0	23.7	0	43.7	0	98	0	115.5
		Translocation ring	0	7.25	0	39.2	0	0	0	3.75
2	Anaphase	Laggard	0	131.5	0	71.2	0	0.75	0	88
		Anaphase bridges	0	46.7	0	4	0	0	0	0
		Multipolar	0	0	0	0.25	0	0	0	0
		Fragmentation	0	194.2	0	111.5	0	152.7	0	176
3	Interphase	Micronuclei	0	28.2	0	1.5	0	2	0	3.5
		Di-nuclei	0	14.5	0	71	0	0.5	0	2
		Multinuclei	0	16	0	12.7	0	0.5	0	0.75
Grand total			0	498.5	0	387.5	0	275	0	401.5

(C.A. = *Cicer arietinum*; A. C. = *Allium cepa*; M= Minutes; H= Hours; -ve cont. = Negative control; +ve cont. = Positive control)

Table 3. Chromosomal aberrations mediated by Amaltas fruit extract in *Cicer arietinum* and *Allium cepa* root tip cells

S. No.	Stages	Abnormalities	C.A.		A.C.	
			30M	90M	24H	48H
1	Metaphase	Scattered nuclei	23	34.25	20.25	36.5
		Sticky metaphase	23.75	0	3.75	7.5
		Translocation ring	8.25	0	6	14.75
	Anaphase	Laggard	23	18.5	55.25	99.5
		Anaphase bridges	17.5	16.25	7.75	15.5
		Multipolar	0	0	7.25	15.75
		Fragmentation	25.25	151.25	163.5	324
3	Interphase	Micronuclei	21.5	0	2	0.25
		Di-nuclei	2.5	10.25	1.75	0.75
		Multinuclei	23.5	15.25	1	3.5
Grand Total			168.25	245.75	429	357.5

(C.A. = *Cicer arietinum*; A. C. = *Allium cepa*; M= Minutes; H= Hours)

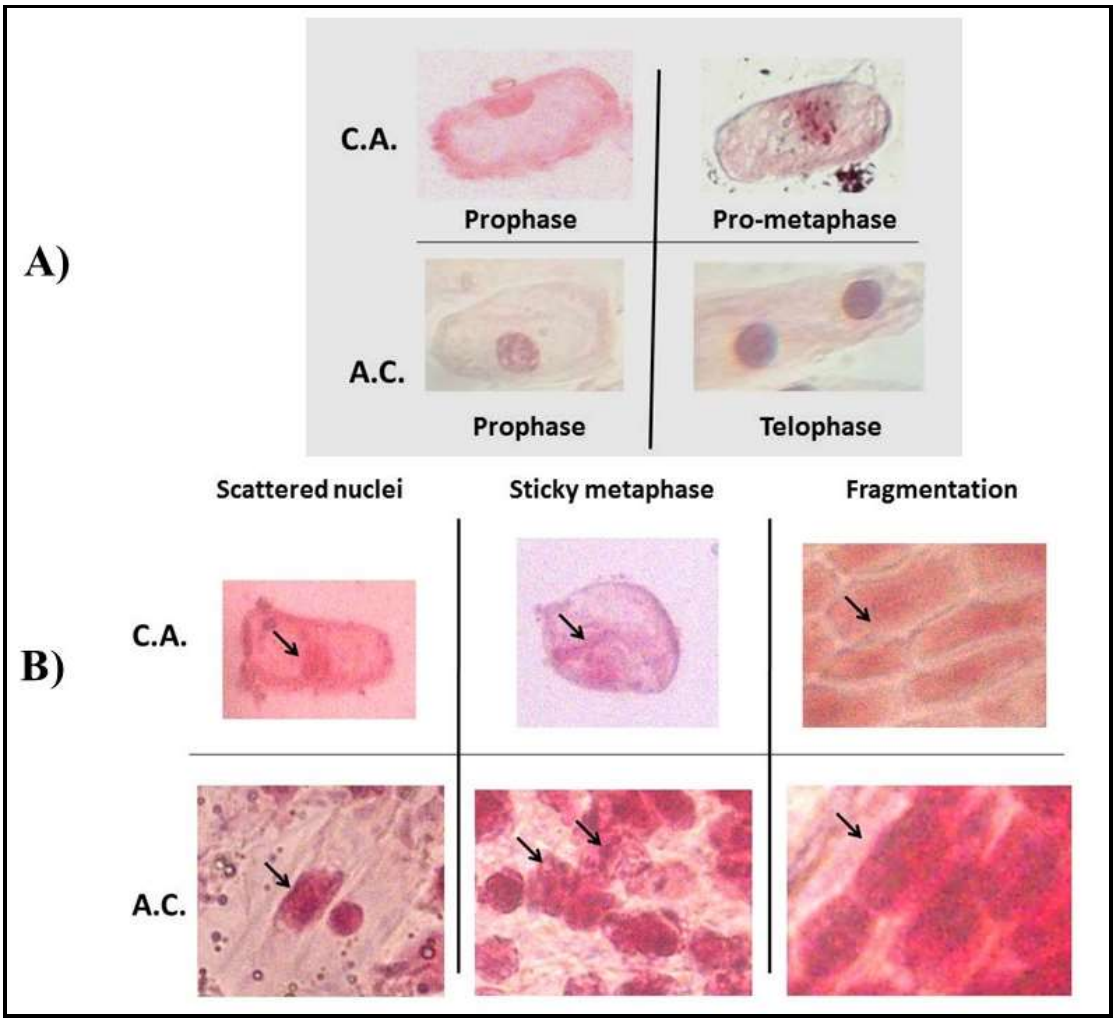

Figure 2. Normal mitotic cells (A) in negative control and chromosomal aberrations (B) in positive control (indicated by arrow) witnessed in *Cicer arietinum L. (CA)* and *Allium cepa* L. (AC)

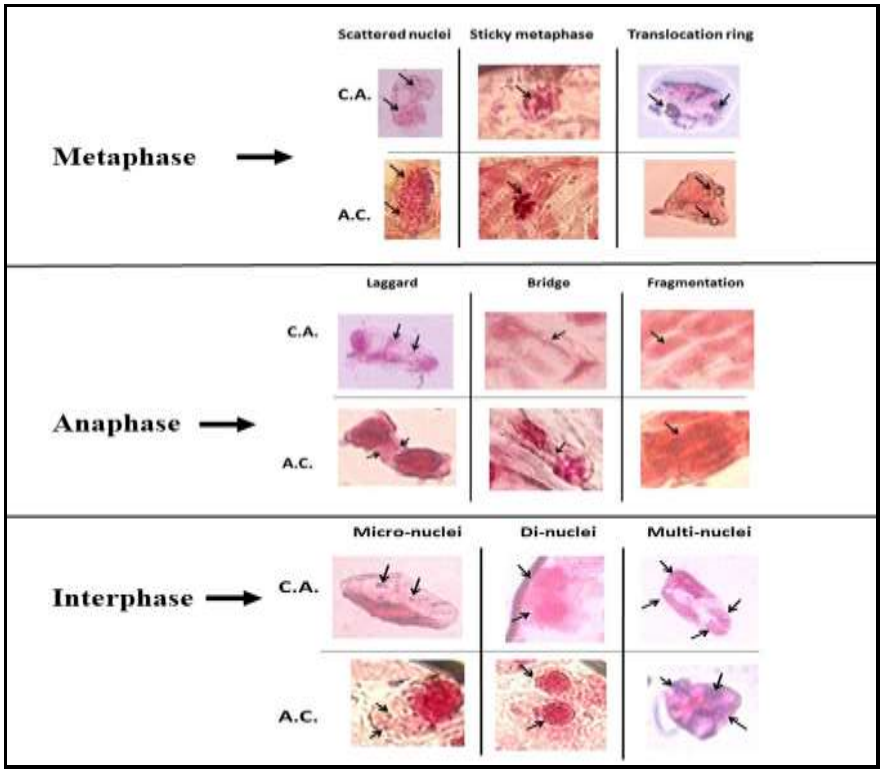

Figure.3. Amaltas fruit induced Chromosomal aberrations (indicated by arrow) in stages of cell cycle witnessed in *Cicer arietinum L. (CA)* and *Allium cepa L. (AC)*

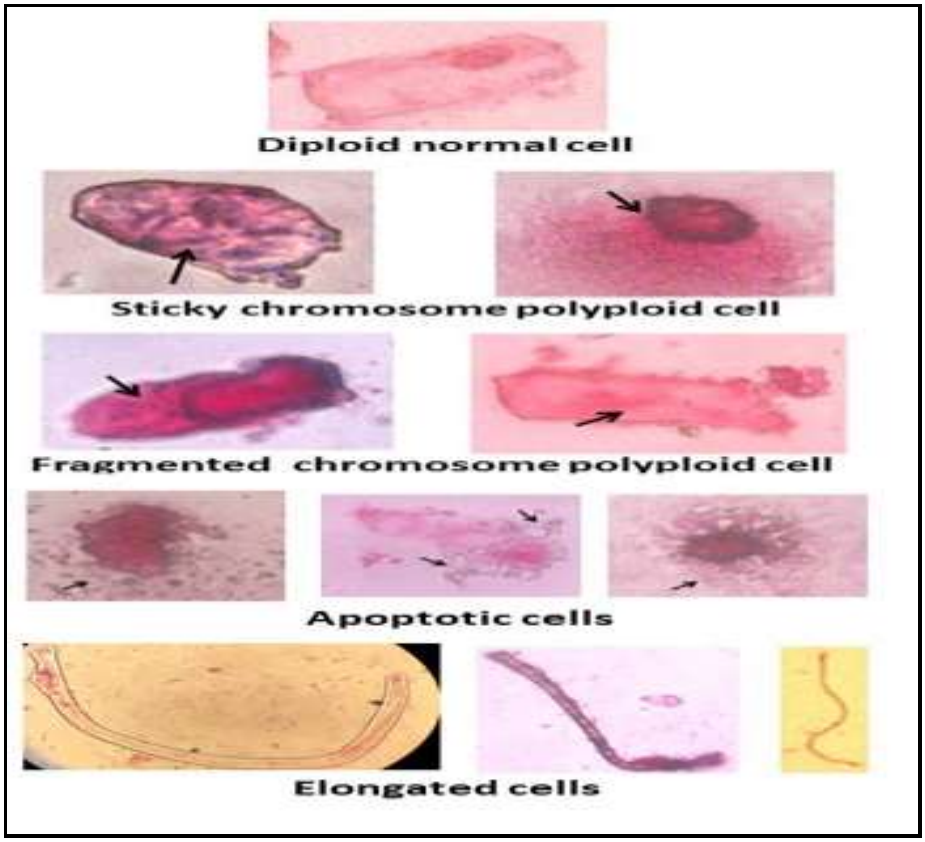

Figure 4. Amaltas fruit induced Polyploid, Apoptotic (indicated by arrow) and Elongated cells witnessed in *Cicer arietinum L.*

Oxidative damages induced by medicinal plant extract: Significantly varying number of **ghost cells** were scored in both assays for all AF incubation period except negative and positive control (LSD p≤0.05) (Table. 4). Results of both plant assays revealed increase in ghost cells with increasing incubation period (Fig. 5 & 6). Unique future of proliferating ghost cell is cytoplasmic membrane projection similar to pilus of bacterial cells used to infect surrounding injured as well as healthy cells were observed in *Cicer arietinum assay* (Figure. 7). In *Cicer arietinum* and *Allium cepa* assay both incubation period of AF extract was significantly different for **cell-membrane damages** (Table. 4). The negative control showed no oxidative damage of any kind in either of the two incubations in contrast to the positive control, which caused more cell-membrane damage (63.2, 65.5 cells), (Figure. 8 & 9). In the *Cicer arietinum* L. and *Allium cepa* assay both incubation period of AF extract was significantly different for nuclear-membrane damages (Table. 4). The cytology revealed increase in cells with broken irregular membrane with an increase in incubation period with AF in both assay plants (Figure. 10 &11).

Table 4. Ghost cells, cell membrane and nuclear membrane damages induced by Amaltas fruit extract in C*icer arietinum* and *Allium cepa* root tip cells (LSD p≤0.05)

Ghost cells							
S.No.	**Name of treatment**	**C. A.**		**Total**	**A. C.**		**Total**
		30M	**90M**		**24H**	**48H**	
1	**-ve cont.**	0^{k}	0^{k}	**0**	0^{k}	0^{k}	**0**
2	**AF**	15^{j}	17^{i}	**32**	61.2^{c}	76^{b}	**137.2**
4	**+ve cont.**	41.7^{f}	46.5^{e}	**88.25**	80.25^{a}	81.2^{a}	**161.5**
Cell membrane damages							
S.No.	**Name of treatment**	**C. A.**		**Total**	**C. A.**		**Total**
		30M	**90M**		**24H**	**48H**	
1	**-ve cont.**	0^{k}	0^{k}	**0**	0^{k}	0	0
2	**AF**	7.25^{j}	18.75^{i}	**26**	38^{h}	50.7^{e}	88.7
3	**+ ve cont.**	63.2^{c}	65.5^{b}	**128.7**	59.7^{d}	70.5^{a}	130.2
Nuclear membrane damage							
S.No.	**Name of treatment**	**C. A.**		**Total**	**C. A.**		**Total**
		24H	**48H**		**24H**	**48H**	
1	**-ve cont.**	0	0	**0**	0	0	**0**
2	**AF**	12^{j}	17.7^{i}	**29.7**	31^{g}	38^{f}	**69**
3	**+ ve cont.**	56.5^{d}	64^{b}	**120.5**	62.5^{c}	63.2^{bc}	**125.7**

(C. A. =Cicer arietinum; A. C. =Allium cepa; M= Minutes; H= Hours; -ve cont. = Negative control; AF= Amaltas Fruit; +ve cont. = Positive control; Means with same alphabets are non-significantly different from each other and with different alphabets are significantly different at (p≤0.01))

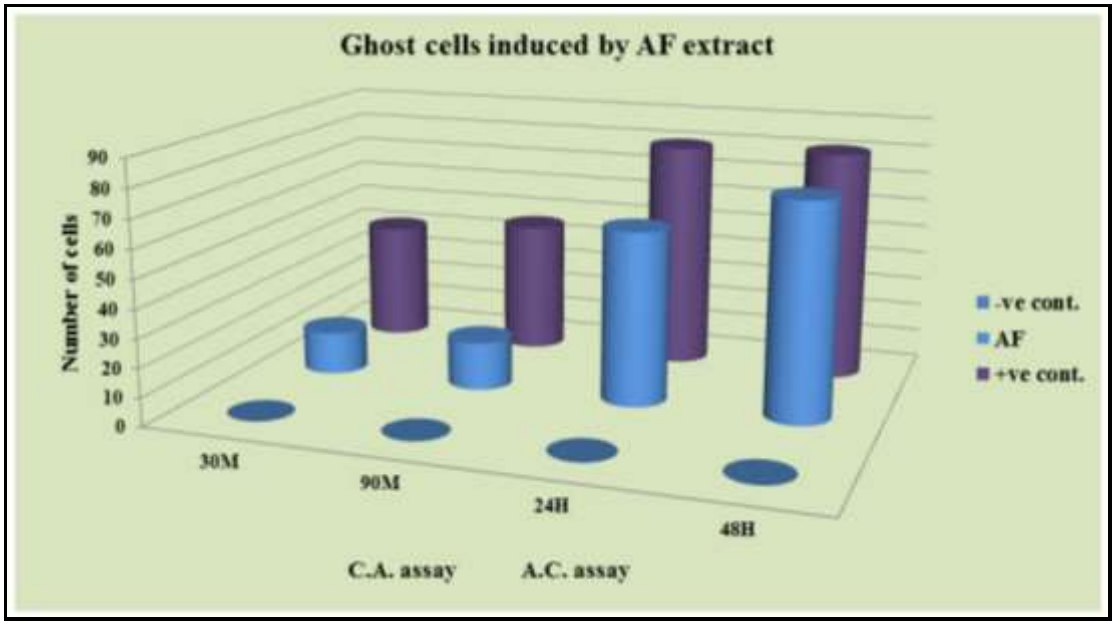

Figure 5. Ghost cells affected by varying incubations of AF extract

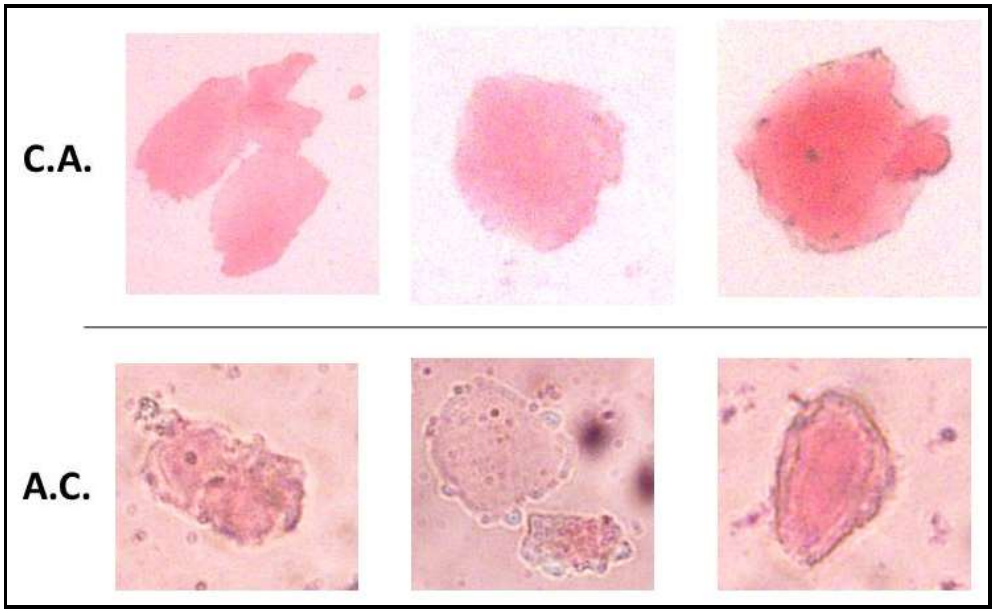

Figure 6. Ghost cells induced by AF witnessed in *Cicer arietinum* and *Allium cepa*

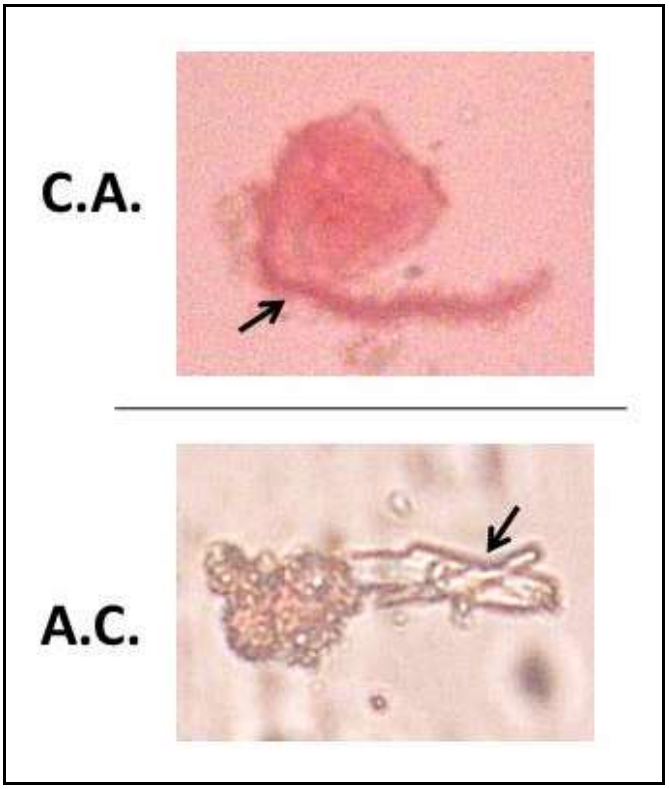

Figure 7. Pilus like projection (indicated by arrow) induced by AF witnessed in *Cicer arietinum*

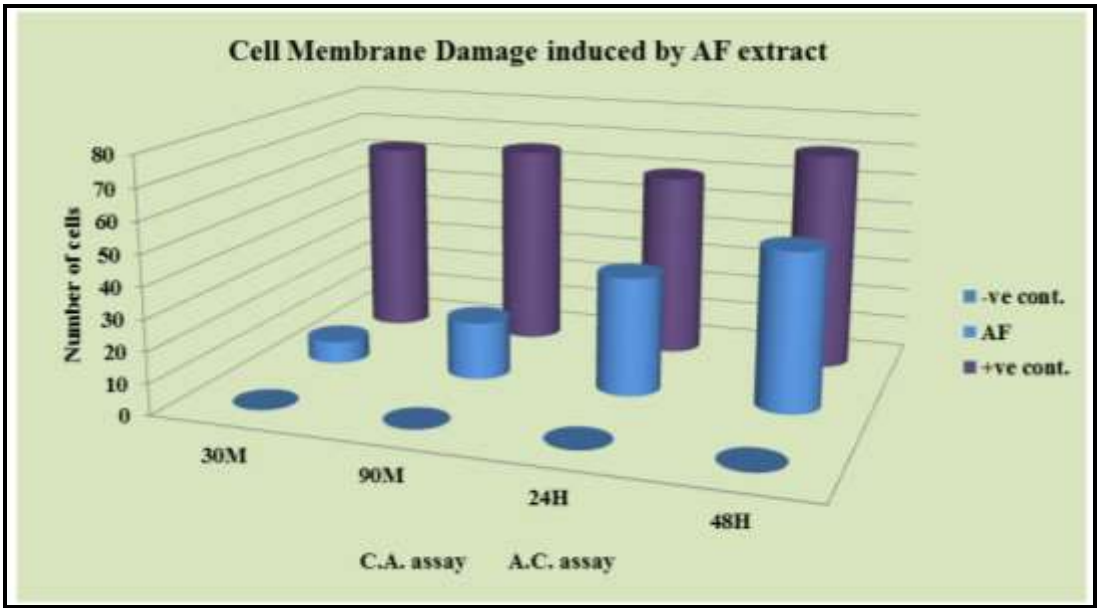

Figure 8. Cell membrane affected by varying incubations of AF extract

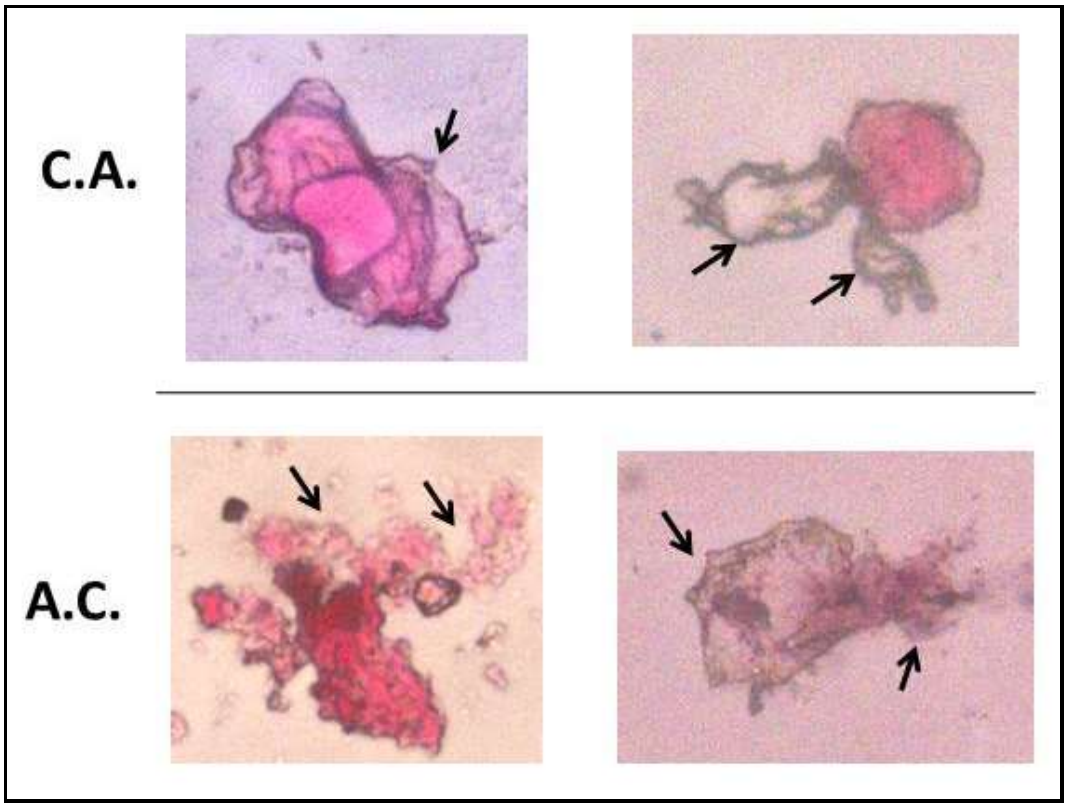

Figure 9. Cell membrane damage (indicated by arrow) witnessed in *Cicer arietinum* and *Allium cepa*

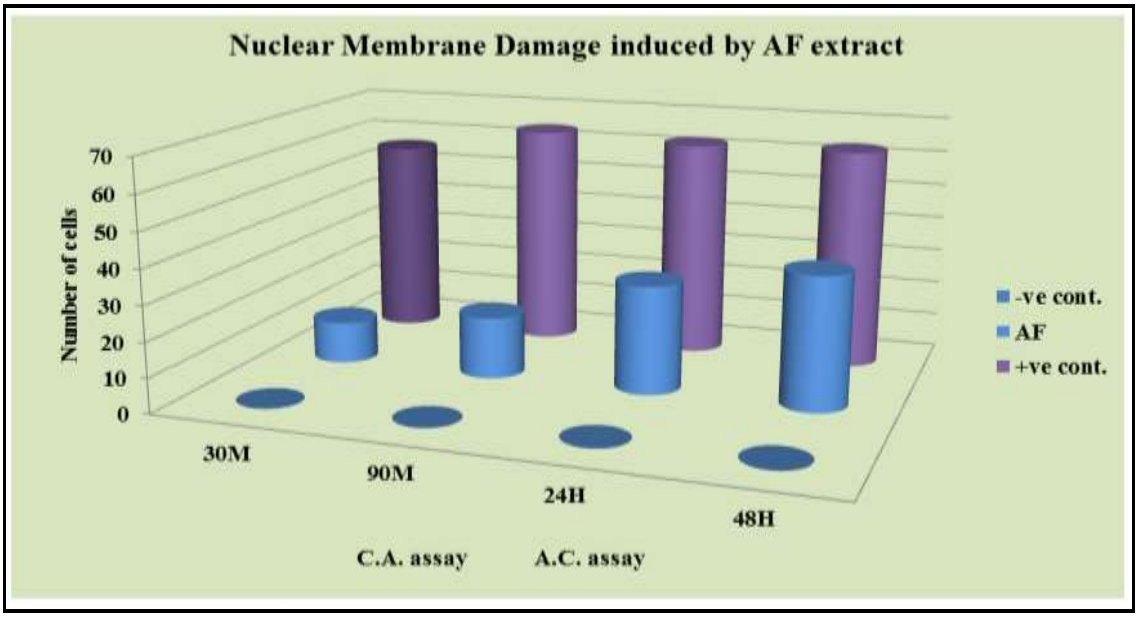

Figure.10. Nuclear membrane damages induced by varying incubations of medicinal plant extracts

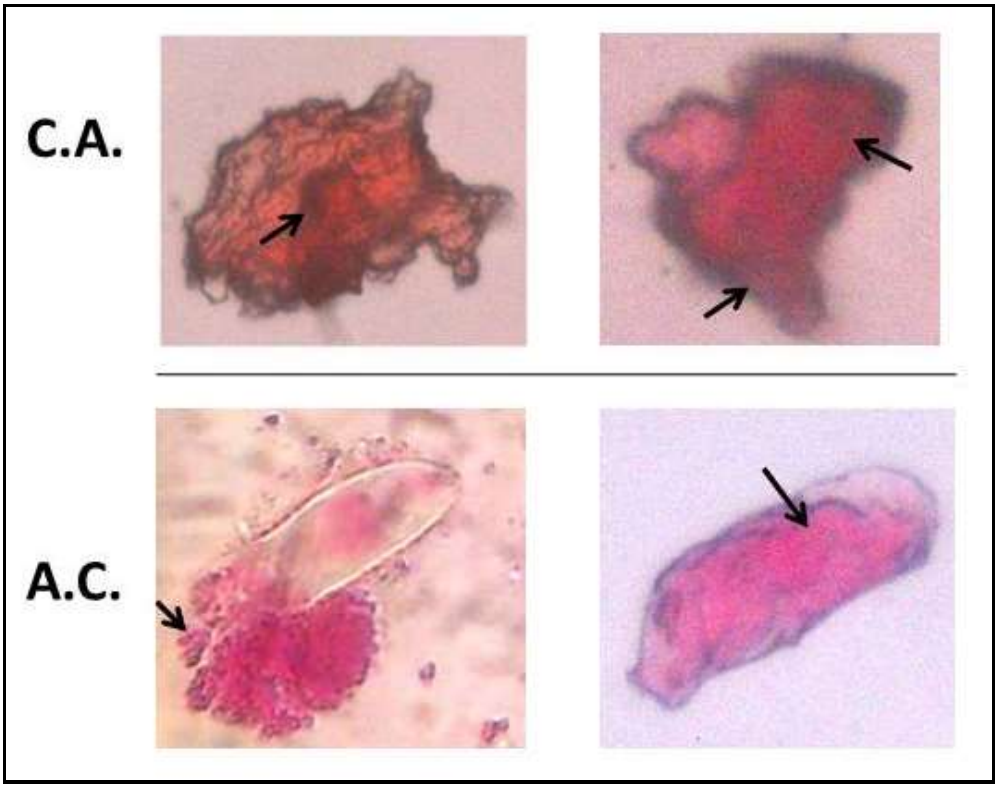

Figure 11. Nuclear membrane damage (indicated by arrow) witnessed in *Cicer arietinum* and *Allium cepa*

Discussion

Increased sticky metaphase chromosomes might be due to the denaturing of topoisomerase II by higher Zinc and Mn content of AF (data not shown here), leading to restriction of chromosome segregation. In addition to this, DNA double stranded breaks observed in current study is mediated oxidation of DNA by the trace metals. Current results are consistent with the findings of Kumari et al. (2011). They witnessed dose dependent increase chromosomal aberration index and micronuclei by Zinc nanoparticles. They recommend zinc oxides as clastogenic/genotoxic and cytotoxic agent. As AF is rich in Zinc, so increased concentrations might provoke zinc oxide formation. Mn is also reported as threat to genomic integrity moderating oxidation of DNA (Nicolai, *et al.* 2021).

Increase in abnormal cells with increase in incubation period exhibited by AF extract in *Allium cepa* assay is in accordance with previous medicinal plant based studies in insecticidal plants famous for cytotoxicity (Mondal *et al*., 2006; Pankaj *et al.*, 2014; Dhulgande *et al*., 2015; Qureshi *et al.*, 2015;

Wijeyaratne and Wadasinghe, 2019). Studies on house hold detergents, shampoos and plant species also reported large number of fragmented cells as found in current work (Mondal *et al*., 2006; Qureshi *et al*., 2014; Dhulgande *et al*., 2015). The result reflects carcinogenic potential AF as deletion is major cause found in most of cancers (Qin, 2002). Researchers corelates unfinished or disrepair of DNA with fragmentation (Ping *et al.,* 2011). The leads to The Cells with deletions are termed as laggards in anaphase observed in present cytology are generated because of disturbance in RNA metabolism consequent lack protein synthesis (Darlington and Cour *et al*., 1976). Chromosome bridge formation can result through adherence or by the breakdown and reunion of a dicentric chromosome (Jabee *et al*., 2008). Induction of micronuclei in AF treated roots tip cells have been reported in many human and animal studies (Dave et al., 1991; Roy et al.., 1999; Roberts, 1997; Sulkowska et al.,2003; Moutasim *et al*., 2011; Aniket *et al*., 2013).

Micro-nuclei are formed when many double stranded breaks of same size occur in chromosome by genotoxins making hard for repair protein to recognize and pest at correct chromosome (Luzhna *et al*., 2013). Cytogenetic abnormalities revealed by this work, is a characteristic attribute of cancer cells, the influence of chromosome abnormalities in tumor progression ranges from altering the expression level of oncogenes to fostering proliferation, metastasis, and drug resistance. Chromosome amplification and deletion are the most common structural chromosome abnormalities, which occur in 88% of cancer samples (Taylor *et al*., 2018).

Large number of cancer studies declared generation of polyploidy cells as another chief characteristics of carcinogenesis (Honma *et al*., 2010; Mosieniak and Sikora, 2010; Honma *et al*., 2012; Beyaz *et al*., 2013; Kumar 2013; Taylor *et al*, 2018; Kou, *et al*., 2020). It has been reported that triploid, diploid, and tetraploid cells coexist and cause whole-genome rearrangement in cancer cell lines (Salmina, *et al*., 2019) Therefore, occurrence of polyploid cells in our work revealed carcinogenic potential of AF have. This may due to spindle inhibition by the pyrrolizidine alkaloid found tested medicinal plant (Roeder, 2009). Current phenomenon is supported by other researchers who suspected alkaloids to induce aneuploidy and/or polyploidy (Yue *et al*., 2010). Induction of apoptic cells by AF in agreement with previously reported work on *Inula viscosa* leaf extracts on the root tip cells of *Allium cepa* (Celik and Aslanturk, 2010). The possible mechanism behind apoptosis are defects in cell cycles G2 phase permitting damaged cell to undergo programmed cell death (Dipola, 2002). Presence of elongated cells in AF treated root tip cells in agreement with findings of previously by *Cassia occidentals* extract (Arora, 2013). Present findings are in agreement with those of Geno-toxicologist from the same eco-geographical region (Kabooro, 2018). Defects in the nuclear and cell membranes, as well as the detection of ghost cells observed during current studies were also observed for aqueous extracts of alternative medicinal plants used for arthrius, and gastrointestinal problems by various researchers (Çelik and Aslantürk, 2010; Qureshi *et al.*, 2016; Parveen, 2016; Bhand, 2019; Junejo, 2019, Kaboroo, 2019). Observed oxidative damages can be correlated with high concentration of Magnease in AF. Mn in higher concentration is reported to loss of oxygen from covalent bonds (Bornhorst, *et al*., 2014; Milatovic *et al*., 2009), and formation of secondary super oxides causing subsequent more DNA segment breaks (Lindahl, 1993; Poetsch, 2020). Overall results of oxidative damages suggests that Free radicals can also break phosphodiester bond by removing oxygen that led to double strand breaks. Errors in repair of chromosome double stranded break cause gene mutations leading to carcinogenesis (Pizzino *et al*., 2017).

Conclusion

It is concluded that AF is capable to cause genotoxic effect incubation dependent manner. High rate of fragmented cells reflects its mutagenicity and carcinogenicity mediated oxidation of DNA and membranes in both assay plants. Induction of more ghost cells and less frequent apoptosis reflects activation of oncogene. Development of Pilus like projections in ghost cells depicts proliferation potential of defected cells. Prolonged utilization of AF is the cause of cancer in surveyed patients. *Allium cepa* L. was more sensitive as assay plant to the genotoxin. It is recommended that AF must be used occasionally.

Authors Contribution

Saida conducted the research, S.T, Qureshi planned and managed the experiments, AN Soomro supported for writing the manuscript and statistical analysis, S. Malik helped in writing the manuscript. Z. Punar helped in data recording.

Authors Conflict

All the authors have no conflict on this publication.

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