Chapter 17

An Insight into *Ampelocissus latifolia* as a Green Alternative to Chemical Herbicides with its Allelopathic and Cell Cycle Modulatory Activities

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ABSTRACT

Herbicides, used to kill unwanted plants in agricultural fields, usually function as branched chain amino acid inhibitors, growth regulators, root growth inhibitors, cell membrane disruptors, pigment synthesis inhibitors etc. Rampant use of chemical herbicides imposes serious environmental impact like biomagnification, having profound effect on human and animal health, causing side effects ranging from mild to deadly. Recently, the search for biological herbicides of plant origin is gaining renewed interest. The present study investigated the allelopathic and cell cycle modulatory effects of aerial parts' aqueous extract of *Ampelocissus latifolia* (AAEAL) on radicle growth inhibition and cyto-genotoxicity induction in some plants like wheat, onion, moong beans and also on the inhibition of budding and proliferation of the aquatic plant lesser duckweed fronds. Wheat seeds, onions, moong beans and lesser duckweeds were exposed to a wide range of concentrations (0.5-6 mg/mL) of AAEAL for different time periods (15 days for lesser duckweed bioassay and 2-96 h for other plants) for morphometric bioassays, study of morphological and cytological alterations, genotoxic stress induction and lesser duckweed frond budding bio-assays. After 24-96 h seedling lengths were measured. Cyto-genotoxic effect was analyzed on onion root apical meristem cells by scoring mitotic index and percentages of cellular and chromosomal aberration using light microscope. Ethidium bromide and acridine orange staining was done for fluorescence microscopic analysis of phytotoxicity. Results indicated that AAEAL inhibited ($p<0.001$) the growth of the seedlings in a concentration dependent manner, with concomitant decrease in mitotic index ($p<0.001$) and increased cyto-genotoxicity ($p<0.001$) in treated root tip cells. Interphase nuclear condensation, c-metaphase, vagrant chromosome, chromosomal bridge, sticky chromosome were the commonest types of aberrations found. Decrease in frond number and the increased number of dead fronds of AAEAL treated lesser duckweeds also suggested allelopathic potential of the extract. Thus allelochemicals from *A. latifolia* possess a strong inhibitory activity against other plant species (both terrestrial and aquatic), directing its good future prospect for the formulation of cost effective and eco-friendly herbicidal agents.

Keywords: AAEAL, Chromosomal abnormalities, Cyto-genotoxicity, Fluorescence microscope, Mitotic index

INTRODUCTION

Plant allelopathy refers to any beneficial or harmful effects of one plant on another plants, both crop and weed species (Smith and Martin, 1994). Allelopathic activity can be exerted by the release of phytochemicals, known as allelochemicals, from plant parts by root exudation, leaching, volatilization and other processes in both natural and agricultural systems. Allelochemicals are a subset of secondary metabolites, not directly required for the growth and development of the allelopathic organism (Smith and Martin, 1994). This phenomenon of allelopathic activity can be implemented in the formulation of bio-herbicides i.e. the herbicides from biological sources. Rampant use of chemical herbicides in the agricultural fields imposes serious environmental impact like biomagnification, having profound effect on human and animal health, causing side effects ranging from mild to deadly. Recently, the search for biological herbicides of plant origin is gaining renewed interest. Various phytochemicals like steroids, terpenoids, glycosides, carbohydrates, alkaloids, flavonoids, anthraquinones, saponins, tannins etc. possess allelopathic activity (Barnes and Putnam, 1987).

If an agent (or group of agents) possesses traditional therapeutic values on one hand and serves as a biodegradable bio-herbicide on the other hand, it will be a boon in the society regarding both the agricultural and animal health aspects after proper exploration of its properties.

Keeping this in view the present study was undertaken to explore the allelopathic and cell cycle modulatory effects of aerial parts' aqueous extract of *Ampelocissus latifolia* (AAEAL). For this purpose, radicle growth inhibition and cyto-genotoxicity induction on wheat, onion, moong bean seedlings and also budding and proliferation inhibition of the aquatic plant: lesser duckweed fronds, were studied.

The plant *Ampelocissus latifolia* is used traditionally to treat a variety of ailments like gout, fractured bone, dental troubles, ulcers, dysentery (Mishra and Billore, 1983; Patil and Patil, 2012) dyspepsia, indigestion and tuberculosis (Prusti and Behera, 2007; Swarnkar and Katewa, 2008). It is used as an antidote for snake bite, applied on wounds, abscess and for easy labour and delivery of a baby (Patil and Patil, 2005). Several scientific studies have explored the chemical composition and various therapeutic properties of this plant. *A. latifolia* is rich in various phytochemicals like carbohydrates, phenolics, tannins, flavonoids, anthraquinones, terpenoids, saponins etc. (Tamilarashi *et. al*., 2000). Recently antibacterial, antioxidant (Pednekar and Raman, 2013) and anti-inflammatory (Tamilarashi *et. al*., 2000; Patel *et. al*., 2013) activities of *A. latifolia* have been reported, signifying good therapeutic potentials of this plant. In the present study we have tried to explore the anti-mitotic and cell cycle modulatory activities of AAEAL as the basis of allelopathic mode of action using simple bench-top bio assays. Bench-top assays are considered as simple, cheap and reliable assays for the preliminary tests. Plant-based assays using moong bean and wheat seeds, onion bulbs, lesser duckweed fronds form the basis of toxicity assessments and allelopathic potentiality determination. There are study reports showing exogenous agent-induced cessation of *Triticum aestivum* L. (wheat) root elongation and swelling of the root tips leading to altered microtubule polymerization and alteration of stability by interaction with microtubule-associated proteins and/or microtubule organizing centers (Armbruster *et. al*., 1991). *Allium* test is considered as one of the best-established test systems to determine the toxicity in the laboratory condition (Fiskesjo, 1985; Grant, 1992; Konuk *et. al*., 2007; Liman *et. al*., 2011; Saxena *et. al*., 2005). This system is widely accepted as a model to study environmental parameters due to its availability, high sensitivity and easily observable macroscopic and microscopic parameters (Yildiz *et. al*., 2009). Moreover, the cytotoxicity levels of an agent can be determined by the increase or decrease in the Mitotic index (MI) (%). The mitotic index is used as an indicator of cell proliferation (Gadano *et. al*., 2002). MI (%) less than the negative control may indicate that the growth and development of exposed organisms have been affected by test compounds (Fernandes *et. al*., 2007). Yadav (1986) and Vyuyan (2002) reported that mitotic index can be disrupted by inhibiting the process of cell division and inhibition of DNA synthesis, by an accumulation of cells at the interphase stage, by disturbing the normal functioning of the mitotic spindle and by producing chromosomal abnormalities which lead to mitotic index reduction. Thus MI (%) is an important parameter for growth modulatory activity assessment of any agent. The use of *Allium cepa* genetic system for cyto-genotoxicity study is validated by the International Programme on Chemical Safety (IPCS) and The United Nations Environmental Programme (UNEP), as an efficient test for the analysis and monitoring of *in situ* genotoxicity assessment of environmental substances (Cabrera and Rodriguez, 1999; Silva *et. al*., 2004; Teixeira *et. al*., 2003; Vicentini *et. al*., 2001). The methanol extract of dried latex and the crude dried latex of *Calotropsis procera* demonstrated anti-mitotic activity in the *Allium cepa* model (Sehgal *et. al*., 2006). Another plant model used in our study, the moong bean seeds was also found to be responsive to exogenous agent exposure. Kumar and Singhal (2009) have reported that germinating moong bean seeds can be used as a model for preliminary evaluation of the cytotoxic effect of drugs. They evaluated the significant reduction in mitotic index in the root tip meristematic tissue of moong seedlings after exposure of several drugs and plant extracts. Ray *et. al*. (2013) also demonstrated *Synedrella nodiflora* induced growth retardation and altered root morphology of moong bean seeds. Study of various chromosomal abnormalities in plant models after plant extract exposure is an important way for toxicity evaluation. Chromosomal aberrations provide important information and may be considered an efficient test to investigate the genotoxic potential of the substance analyzed (Carita and Marin-Morales, 2008).

Thus, based on the literature survey and strong utility of the plant models for the initial screening of allelopathic potential of phytochemicals, the present study finds it to be relevant to explore allelopathic activity in the light of cyto-genotoxicity and cell cycle modulation.

MATERIALS AND METHODS

1. Chemicals:
Glacial acetic acid, orcein and methanol were obtained from BDH Chemicals Ltd., UK. EDTA was procured from Gibco, Grand Island, N.Y., USA. DMSO was obtained from Thermo Fisher Scientific Pvt. Ltd., Mumbai, India. RNase A was purchased from Merck, India. Agarose powder for gel electrophoresis was obtained from Promega Corporation, Madison, USA. Ethidium bromide was obtained from Sigma, St. Louis, M.O., USA and acridine orange was purchased from S.D. Fine-Chem. Ltd., Mumbai, India. Other chemicals used in this study were of analytical grade from reputed manufacturers.

2. Collection of aerial parts of *A. latifolia*, processing and storage:
Fresh aerial parts of *A. latifolia* were collected in large scale from Burdwan University campus, West Bengal, India in August 2011. This plant species was taxonomically identified by Dr. Ambarish Mukherjee (Taxonomist), Professor, Department of Botany, The University of Burdwan. The voucher specimen (No.BUGBAC012) is maintained in the department for future reference. Collected plant materials were washed in tap water, shade dried, directly crushed into small pieces and followed to pulverize using an electric grinder (Philips Mixer Grinder HL1605). Ground powder was stored in airtight container for future use.

3. Preparation of Crude aqueous extract:
Water is the best choice to extract polar compounds present in the plant material. For the preparation of a crude aqueous extract of *A. latifolia*, 20 g of dried powdered plant material was extracted with 400 mL of distilled water for 6 h at low heat (50°C) in a water bath and after every 2 h of the extraction, the extract was filtered through No. 1 Whatman® filter paper. The extract was vacuum dried and stored at -20°C for future use. The process was repeated to get a bulk amount of extract. The extract was coded as AAEAL for Aerial Parts' Aqueous Extract of *Ampelocissus latifolia*. For determining the extract concentration 5x3 mL of the extract was evaporated to dryness in hot air oven.

4. Experimental plants:
Moong bean (*Vigna radiata* L.), wheat (*Triticum aestivum L.*), onion (*Allium cepa* L.) and lesser duckweed fronds (*Lemna minor* L.) were used as experimental plant models. The allelopathic and cell cycle modulatory effects of AAEAL were analyzed in terms of root growth retardation, mitodepression, cyto-genotoxicity, fluorescence microscopic analysis of phytotoxicity and DNA degradation assays.

5. AAEAL induced allelopathy in terms of seedling growth retardation:

On moong bean and wheat seedlings: Moong beans and wheat seeds were surface sterilized with 1% sodium hypochlorite solution for 2 minutes and washed with distilled water vigorously for 10 minutes. The seeds were allowed to germinate in an environmental chamber at 23±2°C on wet filter paper in glass Petri dishes. After 24 h, seedlings were exposed to different concentrations (0.5, 2 and 4 mg/mL) of AAEAL. The experiment was set in triplicate each with 10 seeds. Root lengths were recorded after 96 h. Distilled water was used as culture medium for the untreated control seedlings (Ray *et. al*., 2013).

On onion bulbs: Similar sized onion bulbs were purchased from Burdwan University farm. Before initiation of the experiment, the outer scales of the bulbs and the dry bottom plates were removed without destroying the root primordia, washed well and allowed for root sprouting in test tubes containing distilled water. Initially, a series of 20 to 30 bulbs were placed in distilled water for germination. After 48 h, the best growing bulbs having nearly equal sized roots were exposed to different concentrations (0.5, 2 and 4 mg/mL) of AAEAL for 96 h. The whole experimental setup was kept in an environmental chamber maintained at 23±2°C. Root lengths were recorded after 96 h for morphometric analysis (Ray *et. al*., 2013).

6. AAEAL induced allelopathy in terms of cell cycle modulation, cyto-genotoxicity and DNA degradation:

Treatment and preparation of mitotic phases from onion root apical meristem cells and analysis of cyto-genotoxicity: For the cytological assay, 48 h aged onion root meristem cells were exposed to two different concentrations (0.5 and 2 mg/mL) of AAEAL for 4 and 24 h, simultaneously maintaining the control group in distilled water. After that root tips were cut, fixed in aceto-methanol (3 parts methanol : 1 part glacial acetic acid) for 24 h, hydrolyzed for 2 minutes in 1 N HCl at 60°C, stained with 2% aceto-orcein and squashed in 45% glacial acetic acid for each treatment. Slides were randomly coded and for each set of experiment at least five slides were studied under brightfield light microscope with 40X objective lens. Mitotic index percentages were calculated using the formula: [Number of cells present in dividing phases (prophase, metaphase, anaphase and telophase)/ Total number of cells] X 100.

Additionally, the frequencies of chromosomal abnormalities were scored and abnormalities were expressed per cell basis (Ray *et. al*., 2013).

Lesser duckweed (Lemna minor* L.*) bioassay: The lesser duckweed bioassay was used to study the phytotoxic activity of AAEAL. Twenty plants of *Lemna minor* L. having a rosette of two fronds were added to each flask containing 0.25, 1 and 2 mg/mL concentrations of AAEAL using Hoagland solution as the culture medium. The control group was maintained in Hoagland's solution. The experiment was done in quadruplet. Experimental setup was kept in a growth cabinet for 15 days. On the 15th day, the numbers of fronds per flask were counted (Azhar *et. al*., 2009). Interpretations of results were made by analyzing the percentage of growth inhibition after extract exposure, with reference to the negative control. Additionally, toxicity percentage was calculated by considering the percentages (%) of dead fronds. Simultaneously, acridine orange-ethidium bromide staining was also done for fluorescence microscopic analysis of phytotoxicity.

Fluorescence microscopic analysis of AAEAL induced phytotoxicity in wheat and lesser duckweed root cells: Phytotoxicity and apoptosis induction in wheat and onion root apical meristem cells were analyzed after staining with acridine orange and ethidium bromide and studying under the fluorescence microscope. Acridine orange (AO) can penetrate in both the live and dead cells while ethidium bromide (EB) penetrates only in dead cells and they stain differentially. Blue filter excitation on living cells stained with acridine orange gives green colour, early apoptotic cells allowing limited penetration of ethidium bromide possess green to yellowish nuclei with perinuclear chromatin condensation while late apoptotic cells coloured in dark red possess fragmented or condensed chromatin. Germinated wheat and onion roots were exposed to 0.5-2 mg/mL of AAEAL for 48 h, while maintaining the negative control groups in distilled water For staining, equal concentration (0.01%) of acridine orange (AO) and ethidium bromide (EB) solutions were mixed just before the use. Treated and untreated roots were stained for 2 minutes, washed in distilled water thoroughly, squashed on a glass slide under cover slip and the colour patterns were observed with the Leica fluorescence microscope (10X objective lens).

Analysis of AAEAL induced DNA degradation on wheat root apical meristem cells: Genomic DNA was extracted from the treated and untreated root apical meristem cells. 24 h germinated wheat seedlings were treated with 0.5-4 mg/mL concentrations of AAEAL for 24 h. DNA was isolated following the extraction procedure as described by Hameed *et. al*. (2004), and DNA degradation pattern was analyzed by agarose gel electrophoresis.

Reagents and Buffers

1. Extraction Buffer (Total Volume 100 mL)

1.	100 mM Tris base	1.21 g
2.	1.5 M NaCl	8.766 g
3.	0.1% SDS	1 g
4.	β–mercaptoethanol.	0.2% (v/v) (Added immediately before use)
	Added 100 mL double distilled water.	

2. Chloroform : Iso-amyl alcohol (24:1)

3. 96% ethanol

4. TE Buffer (Total Volume 100 mL)

1.	10 mM Tris base	0.121g
2.	1 mM EDTA	0.037224 g
	Added 100 mL double distilled water.	

5. Ribonuclease-A (50 µg/mL)

6. 50X TAE Buffer (Total Volume 1000 mL)

1.	Tris base	242 g
2.	Glacial acetic acid	57.1 mL
3.	0.5 M EDTA (pH 8.0)	100 mL
4.	Autoclaved Millipore water	842.9 mL

Process of DNA Extraction

Samples were collected and kept at -20°C for 24 h. After 24 h, samples were homogenized in 600 µL pre warmed (at water bath maintained at 65°C) extraction buffer in labelled 1.5 mL autoclaved Eppendorf tubes. DNA was extracted following the method of Hameed *et. al*. (2004). Eppendorf tubes were then placed in a water bath at 65°C for 10 minutes with frequent gentle mixing after every 2 minutes for 5 times and were taken out from the water bath and then left for 2 minutes at room temperature. To each tube, 600 µL of chloroform-isoamyl alcohol mix (24:1) was added and mixed gently. Samples were centrifuged at 4,000 rpm for 5 minutes. The upper aqueous phase was then collected and two volumes of ice-cold 96% ethanol was added to each tube and mixed gently for 6 to 12 times to precipitate the DNA. Samples were then centrifuged at 10,000 rpm for 10 minutes to pellet the DNA. The pellet was dissolved in 40 µL TE buffer. Following that isolated DNA samples were treated with DNase-free Ribonuclease A (50 µg/mL) for 25 minutes at 37°C. DNA was again precipitated with addition of three volumes of 96% ethanol and samples were centrifuged at 10,000 rpm for 10 minutes to pellet the DNA which was dissolved in 40 µL TE buffer with gentle agitation. For quantification and purity of DNA, absorbance was measured at 260 nm and at 280 nm respectively. A solution with an OD 1.0 at 260 nm contains 50 µg/mL of DNA.

Agarose gel electrophoresis of isolated DNA

Following isolation and quantification, the DNA samples were subjected to electrophoresis in 1.5% agarose gel (1X TAE was used as gel preparing and running buffer) at constant 60 volts for 3 h. During loading, equal amounts (15-20 µg/well) of DNA preparations were loaded. The gel was stained with ethidium bromide and observed and photographed using UVP Gel Documentation- Imaging System.

SCORING AND STATISTICAL ANALYSIS

Seedlings growth was recorded and the growth retardation percentages were calculated. Root lengths were expressed as Mean±SEM. The difference between the untreated and treated groups for the seedling lengths was analyzed with the Student's t-test. The effects on cell cycle kinetics were determined by scoring mitotic index (MI) % and individual cell phase frequencies. MI % was calculated as No. of cells in dividing phase/The Total No. of cells scored x 100. Individual cell phase frequencies were calculated as Number of cells in particular phase/The total number of dividing cells x 100. The statistical significance of the difference between the control and treated groups for MI % and cell phase frequency were analysed using 2x2 contingency χ^2-test. Differences between controls and treatments were considered statistically significant at $p<0.05$, $p<0.01$, $p<0.001$.

RESULTS

1. AAEAL induced allelopathy in terms of seedling growth retardation:

Morphometric bioassay for root growth retardation of moong bean, wheat and onion seedlings

Moong bean seedlings: Data indicated concentration-dependent root growth retardation of moong bean seedlings after 96 h of AAEAL exposure. Minimum root length was observed after treatment with 4 mg/mL concentration ($p<0.01$, Student's t-test) as compared to the control group maintained in distilled water. The root growth inhibitions were calculated as 29.3, 39.5 and 52.9 % respectively for the concentrations of 0.5, 2 and 4 mg/mL of AAEAL at 96 h (Figure 1, 2).

Wheat seedlings: AAEAL showed concentration-dependent growth retardation effect on wheat roots. Root lengths were calculated as 0.82±0.16 ($p<0.001$), 0.62±0.08 ($p<0.001$) and 0.45±0.08 ($p<0.001$) as compared to the control group (2.85±0.10). Additionally, growth inhibition was calculated as 51, 59 and 80% at 96 h for the concentrations of 0.5, 2 and 4 mg/mL respectively (Figure 1, 2).

Onion seedlings: Results indicated that AAEAL caused growth retardation of onion roots in a concentration-dependent manner. In the present study, the maximum root length (2.85±0.10 cm) was recorded from the untreated groups of onion, while the minimum length (0.45±0.08 cm) was recorded from the highest concentration (4 mg/mL) of AAEAL (Figure 1, 2).

2. AAEAL induced allelopathy in terms of cell cycle modulation, cyto-genotoxicity and DNA degradation:

Mitotic index depression and cyto-genotoxicity induction in onion root apical meristem cells

Data indicated the trend of mitodepression in AAEAL treated onion root tip cells as compared to the untreated control roots. Concentration-dependent reduction in mitotic index was observed in AAEAL treated samples (Table 1). Significant ($p<0.001$) differences in mitotic index were seen between treated and untreated root tip cells. Mitotic index percentages were calculated as 2.55±0.23 ($p<0.001$) and 0.80±0.09 ($p<0.001$) after treatment with 0.5 and 2 mg/mL (of AAEAL) respectively for 4 h as compared to the control for which MI % was calculated as 4.85±0.46. After the exposure of AAEAL (0.5 mg/mL) for 24 h, the percentage of MI was found to be 2.35±0.21 ($p<0.001$), whereas treatment with 2 mg/mL of AAEAL for 24 h resulted in all the cells to be arrested at interphase stage signifying the incidence of delay in cell cycle kinetics.

Cytogenetic analysis on onion root tip cells revealed increased number of cytological and chromosomal aberrations in treated root apical meristem cells in concentration dependent manner (Figure 3; Table 1). There were 264, 332 and 405.26% increase in chromosomal abnormalities per cell after treatment with 0.5 (4 h), 2 (4 h) and 2 (24 h) mg/mL of AAEAL respectively. Presence of different chromosomal and cytological aberrations like sticky and clumped chromosome, anaphase and telophase bridge, delayed and laggard chromosome, c-metaphase, vagrant chromosome, chromosome loss, micronucleus, interphase nucleus condensation, binucleate cells point towards the possible mechanisms involved in the root growth inhibition in treated root tip cells.

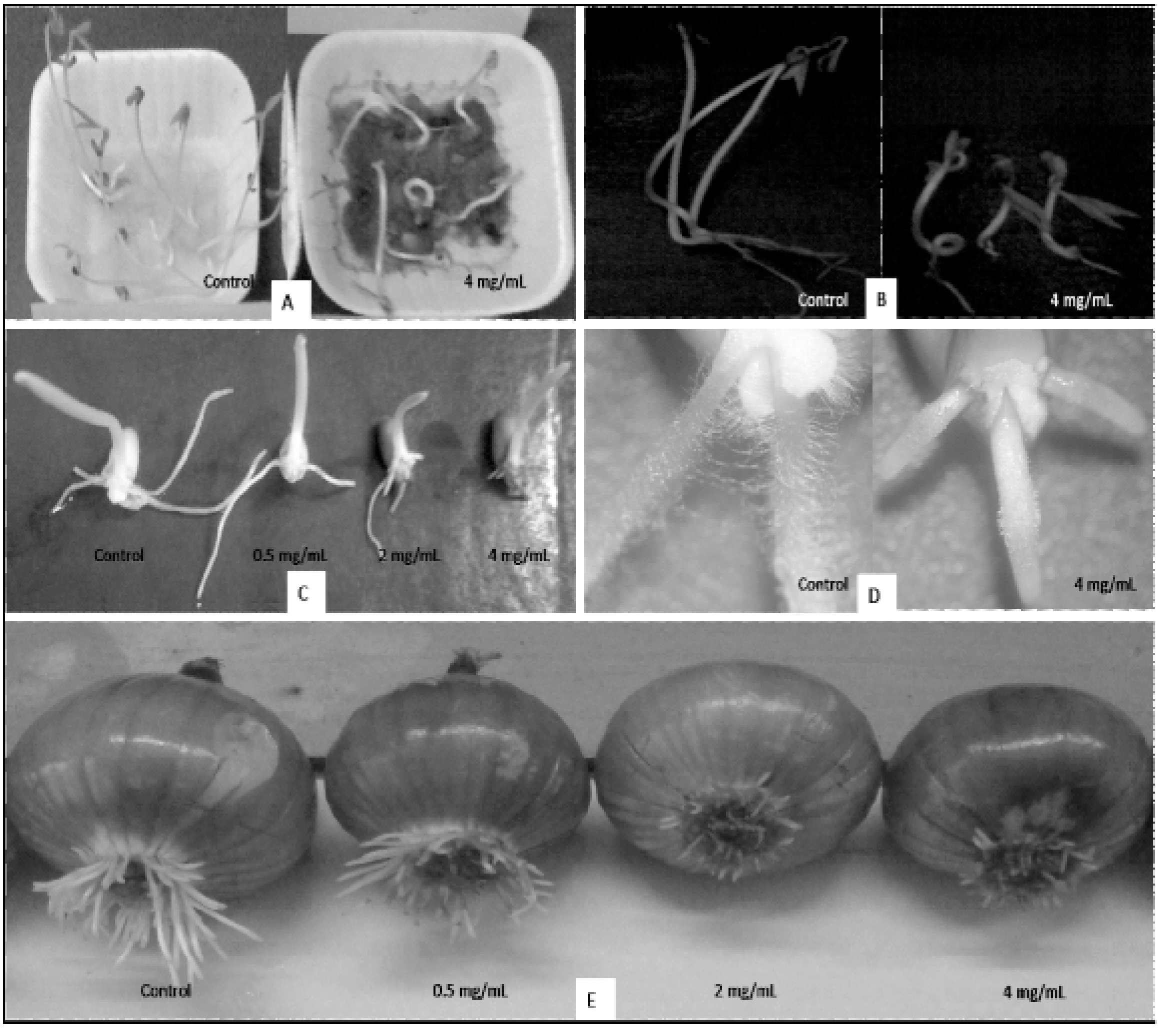

Fig. 1. Root growth retardation effect of AAEAL on moong bean, wheat and onion seedlings after 96 h of extract exposure in a concentration dependent manner. Photographs A and B: untreated and treated moong bean seedlings, C: root lengths of wheat seedlings after AAEAL treatment, D: stereomicroscopic view of untreated and treated wheat roots, E: root lengths of onion seedlings after AAEAL treatment

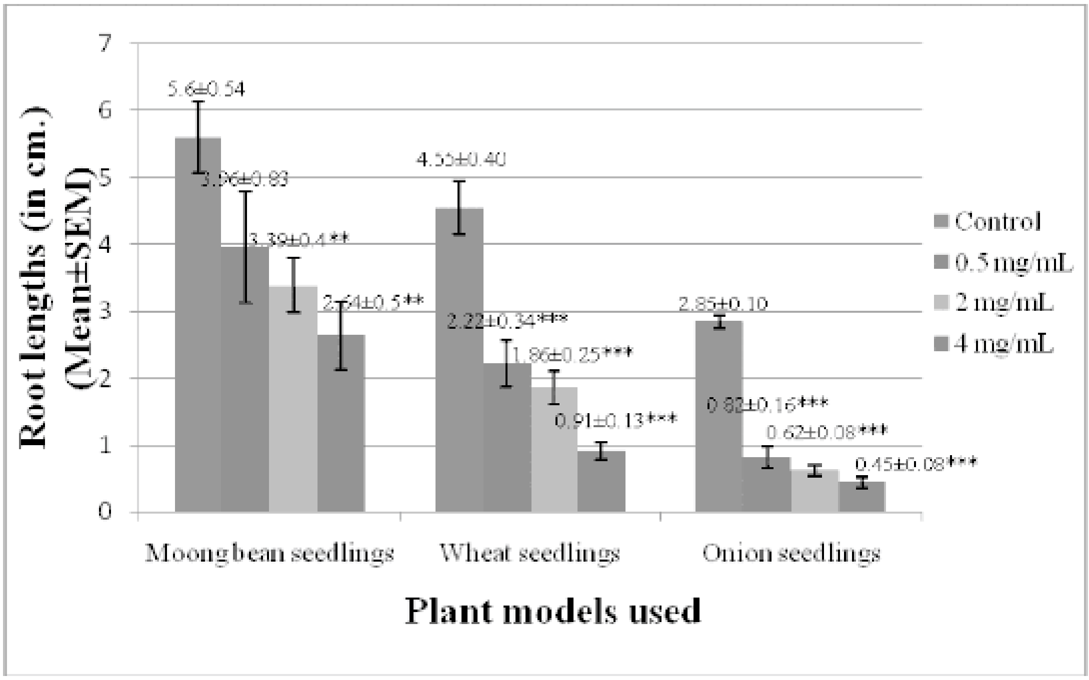

Fig. 2. Root lengths of moong bean, wheat and onion seedlings after 96 h of treatment with 0.5, 2 and 4 mg/mL of AAEAL. Experiments were done in triplicate and data were represented as Mean±SEM. ***Significant at *p<0.001* as compared to the control by Student's t-test

Table 1: Pooled data showing AAEAL induced delay in cell cycle kinetics and increase in cytotoxic stress in onion root apical meristem cells

h	Conc. (mg/mL)	TC	IC (%)	MI (%) Mean±SEM (% Reduction)	No. of chromosomal abnormalities scored	Abnormalities/cell Mean±SEM (% Increase)
4	00	3717	95.37	4.85±0.46	39	0.25±0.03 (0)
	0.5	4937	97.53	2.55±0.23[a] (47.42)	100	0.91±0.12[a] (264)
	2	6265	99.20	0.80±0.09[a] (83.50)	48	1.08±0.19[a] (332)
24	00	2400	94.08	5.93±0.30	28	0.19±0.02
	0.5	5888	97.64	2.35±0.21[a] (60.37)	120	0.96±0.12[a] (405.26)
	2	4208	100	0 (100)	-	

[a]Significant at *p<0.001* as compared to the respective controls by 2x2 contingency χ^2-test (d.f.=1). h: hours, Conc.: concentrations, TC: Total Cells scored, IC: Interphase cells, MI: Mitotic index.

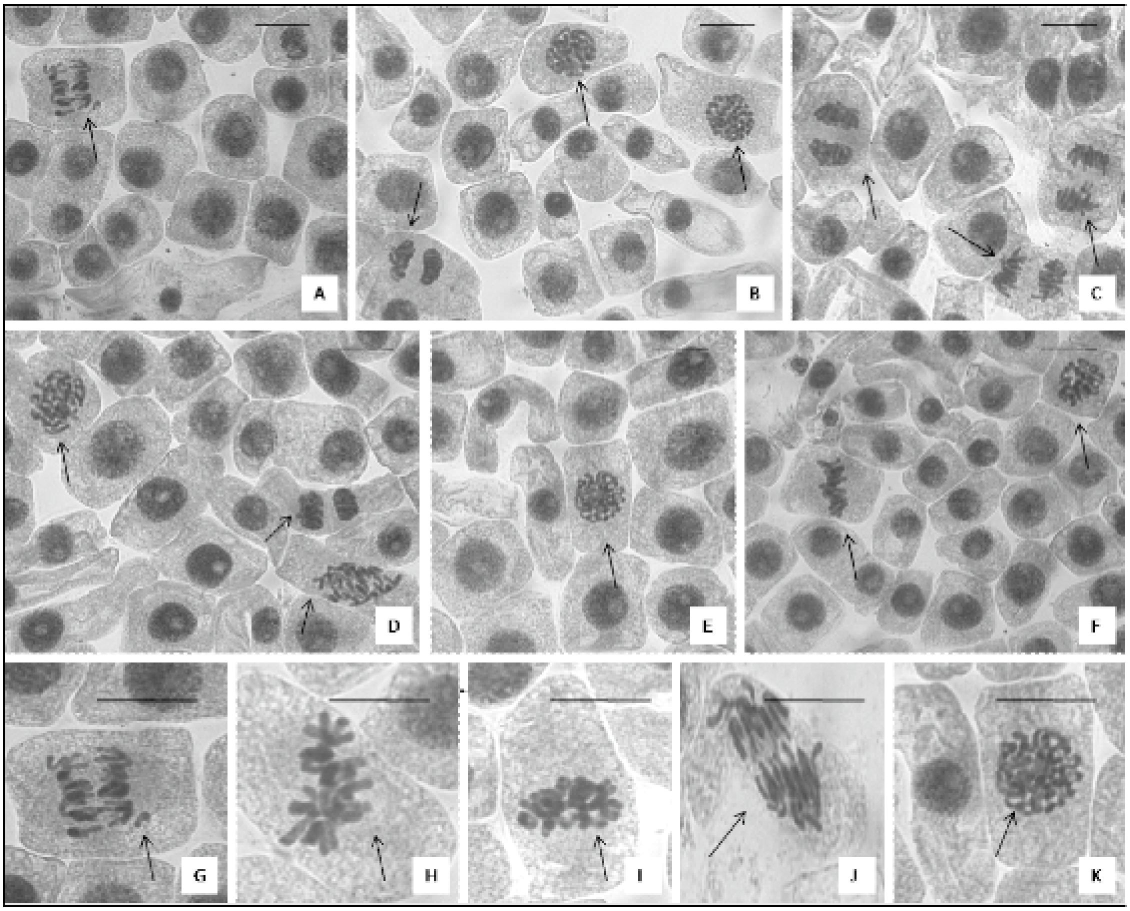

Fig. 3. Chromosomal abnormalities observed in onion root apical meristem cells after different hours of AAEAL treatment at concentrations of 0.5 and 2 mg/mL. A- chromosome loss in anaphase, B and E- chromosomal condensation and clumping, C- vagrant chromosome at anaphase and chromosomal clumping at telophase, D- ring formation and anaphase bridge, E- sticky or clumped chromosome at metaphase, F- metaphase plate deviation and ball metaphase, G- chromosome loss and anaphase bridge, H- c metaphase, I- chromosome clumping, stickiness and ring formation at metaphase, J- anaphase bridge with vagrant chromosome, K- clumped chromosome. Photomicrographs (A-F) (200X) were magnified (2x) using Microsoft PowerPoint. Some of the cells (Photomicrographs G-K) were further enlarged (2x) to highlight chromosomal abnormalities

Table 2: Pooled data showing phytotoxic activity of AAEAL on lesser duckweed (*Lemna minor*) fronds

Treatment	Conc. (mg/mL)	Initial number of fronds	Number of fronds after 15 days		Number of dead fronds among total number of fronds
			Total number	% Increase	
Control	00	80	92	15	1
AAEAL	0.25	80	84	5	7
	1	80	81	1.25	18
	2	80	80	0	37

conc.:concentration

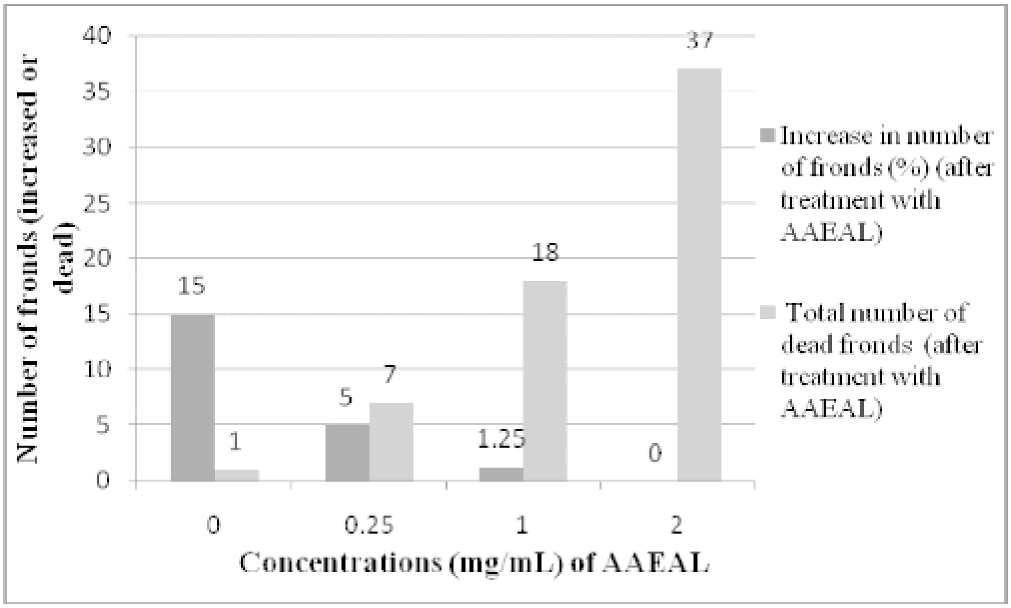

Fig. 4. Assessment of phytotoxic activity of AAEAL (0.25, 1 and 2 mg/mL treatment for 15 days) on *Lemna minor* fronds as compared to the control

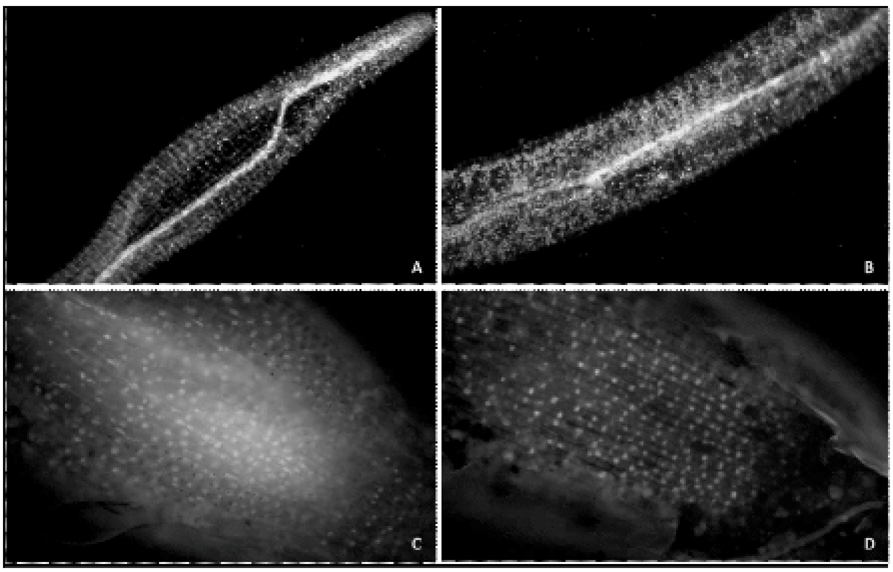

Fig. 5. Fluorescence microscopic photomicrographs showing AAEAL induced phytotoxicity in lesser duckweed and wheat root cells, as compared to the untreated control; damaged cells showed ethidium bromide fluorescence (red) and untreated control, showed acridine orange fluorescence (green). Photomicrographs A and C denotes the control samples of lesser duckweed and wheat respectively; B and D denotes AAEAL (2 mg/control, 48 h) treated lesser duckweed and wheat root cells respectively. Photomicrographs (100X) were further magnified (4x) using Microsoft PowerPoint

Lesser duckweed bioassay for the assessment of cytotoxicity

The lesser duckweed bioassay was used to study the phytotoxic activity AAEAL. Data indicated trends of decreased frond growth or budding after extract exposure (Table 2; Figure 4). In the case of an untreated control group, there was 15% increase in the number of fronds, whereas, a concentration-dependent decrease in frond numbers was observed after exposure of the extracts for 15 days. Increase in frond numbers were calculated as 5%, 1.25% and 0% after exposure of 0.25, 1 and 2 mg/mL of AAEAL. Simultaneously, numbers of dead fronds were also calculated. The total number of dead fronds increased from 7 to 18 to 37 after treatment with 0.25, 1 and 2 mg/mL concentrations, which was also evidenced by increased number of ethidium bromide stained (red fluorescence) cells of the fronds.

Fluorescence microscopic detection of AAEAL-induced phytotoxicity in wheat and lesser duckweed root cells

Acridine orange-ethidium bromide double staining for fluorescence microscopic analysis of cytotoxicity on wheat and **lesser duckweed** roots treated with different concentrations of AAEAL (0.5-2 mg/mL), revealed concentration dependent increase in toxicity and characteristic colour patterns. Here damaged cells showed ethidium bromide fluorescence (red) and untreated controls showed acridine orange fluorescence (green), thus differentiating dead cells from the live cells (Figure 5).

Analysis of AAEAL induced DNA degradation on wheat root apical meristem cells

Results of agarose gel electrophoresis indicated increased DNA fragmentation of wheat root apical meristem cells after treatment with AAEAL after 24 h (Figure 6). There was no difference as such in DNA degradation pattern in case of untreated and 0.5 mg/mL AAEAL treated wheat DNA. Gradual degradation of DNA was found from 1 mg/mL treatment and DNA degradation effect became prominent after exposure of 2 and 4 mg/mL concentrations, indicating induced phytotoxicity and apoptosis in wheat root apical meristem cells after 24 h of exposure.

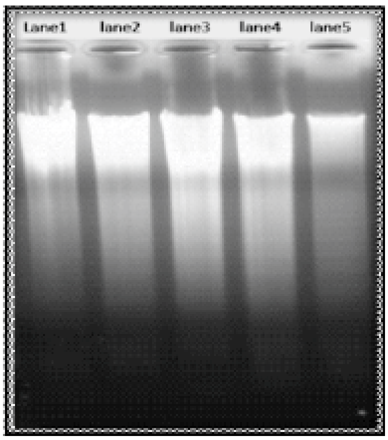

Fig. 6. DNA degradation pattern after AAEAL exposure (lane 1-5: 00, 0.5, 1, 2 and 4 mg/mL respectively) on wheat root apical meristem cells

DISCUSSION

Plant-based bioassays are considered as useful, simple, reliable and convenient methods for the preliminary assessment of allelopathic activity and are used for the detection of fungal toxins, plant extract toxicity, heavy metals, pesticide toxicity etc. (Angayarkanni *et. al*., 2007; Camparoto *et. al*., 2002). The present study was aimed to investigate the allelopathic and cell cycle modulatory activities of aerial parts' aqueous extract of *Ampelocissus latifolia* (AAEAL) using apical meristem cells.

Root apical meristem cells are considered as the simplest and cheapest models to assess genotoxicity of toxicants as they exhibit high sensitivity to exogenous agents like drugs, pollutants, chemicals (Fiskesjo, 1985). In the present study, allelopathic and cell cycle modulatory activities of AAEAL were investigated by morphometric bioassays considering the root length and root morphology changes of moong bean, wheat and onion seedlings and cytogenetic analysis of onion root apical meristem cells for mitotic abnormalities, nuclear condensation, mitotic index depression and delay in cell cycle kinetics analysis. Experiments were also done on the budding of lesser duckweed fronds to test extract induced phytotoxicity. Moreover, fluorescence microscopic and DNA degradation analysis were also performed for the assessment of extract induced cytotoxicity/phytotoxicity. In this study, we have used more than one plant models for screening purposes in order to accumulate in depth knowledge regarding the allelopathic activity of the extract.

Data indicated increased percentages of root growth inhibition of moong bean seedlings after AAEAL exposures (Figure 1, 2). In the present study, root growth inhibitions were calculated as 29.3, 39.5 and 52.9 % respectively for the concentrations of 0.5, 2 and 4 mg/mL of AAEAL at 96 h. The minimum length (2.64±0.5 cm) was recorded from the highest concentration (4 mg/mL) of AAEAL at 96 h**.** A number of earlier studies have shown that the level of growth inhibition increases with the increasing extract concentrations (Ray *et. al*., 2013) and roots are more sensitive towards the extracts than the shoots, which may be due to the fact that roots remain under direct exposures of the extracts. A similar trend of increased percentages of wheat root growth inhibition occurred after AAEAL (Figure 1, 2) exposure. Like moong beans and wheat seeds, onion roots were also found to be sensitive to AAEAL (Figure 1, 2). These results are in agreement with previous studies, showing the allelopathic effect of crude aqueous extracts on other plants (Siddiqui, 2007). Tannic acid, a standard polyphenolic compound was also reported to influence plant development by interacting with auxin and gibberellic acid (Corcoran, 1972) and causing plant growth retardation. Growth retardation is a result of the suppression of cell division and induction of chromosomal aberrations, which ultimately leads to cell death (Fiskesjo, 1985). The present study revealed that the extract may contain bioactive compound(s) that might have interacted with the cell cycle machineries for exerting allelopathic activity. Narciclasine 1, isolated from *Narcissus* bulbs was found to inhibit the growth of wheat grain radicles (Ceriotti, 1967).

Light microscopic and fluorescence microscopic studies of allelopathic, antimitotic and cytogenotoxic activities of AAEAL revealed extract induced cell cycle delay and induction of cyto-genotoxic stress in treated cells (Figure 3, 5; Table 1).

Our results are in accordance with the previous study reports showing extract induced toxicity at the level of root growth retardation and chromosomal alteration (Pugliesi *et. al*., 2007). *A. cepa* assay enables the assessment of different genetic endpoints, mitotic index and chromosome aberrations. Mitotic index is used as an indicator of cell proliferation which measures the proportion of cells in the mitotic phase of the cell cycle. Mitotic index (MI) % measures the proportion of cells in different cell cycle phases and reduction in the mitotic index can be considered as delay in cell cycle kinetics (Rojas *et. al*., 1993). Hence, the decrease in the mitotic index of *A. cepa* meristematic cells could be interpreted as a delay in cell cycle kinetics. Data showed treatment with AAEAL (0.5 and 2 mg/mL) to root tip cells produced significant ($p<0.001$) reduction in the mitotic indices in onion root apical meristem cells (Figure 3, Table 1). AAEAL treatment on *A. cepa* root at a concentration of 2 mg/mL for 4 h led to the reduction of the Mitotic index (MI) by 83.50%, with concomitant rise in chromosomal abnormalities upto 332%. The reduction of the mitotic index might be explained as being due to the obstruction of the onset of prophase, the arrest of one or more mitotic phases, or the slowing of the rate of cell progression through mitosis (Ray *et. al*., 2013). There are also many reports where mitotic index depression was considered as an indicator of cell cycle delay (Fachinetto, 2007; Levan, 1938; Ray *et. al*., 2013; Salam, 1993). These depressions of mitotic index indicated distinct allelopathic activity of AAEAL. Moreover, the increased number of chromosomal and nuclear abnormalities such as c-metaphase, sticky and clumped chromosome, delayed and laggard chromosome, anaphase and telophase bridge, vagrant chromosome, chromosome loss, micronucleus, interphase nucleus condensation, binucleate cells etc. in the treated onion root tip cells indicated cyto-genotoxicity as the underlying cause behind extract-induced allelopathy. In the case of AAEAL treatment, the percentage of the number of abnormalities per cell was also increased in concentrations and time-dependent manners, excepting the case for 2 mg/mL at 24 h. It can be explained by the fact that with the longer time of exposure to the highest concentration, the existence of dividing cells had diminished drastically and the cells became arrested at the interphase stage. Such a concentration-dependent reduction in mitotic index percentage with an increase of chromosomal abnormalities suggested that the exposure of AAEAL to root apical meristem cells led to cytotoxic stress, reduction in cell numbers entering into the mitotic cycle and altogether increased interphase cell frequency. Such a reduction in the mitotic index suggests that the extract exposure led to cell cycle disturbances and decrease in cell number entering into the mitotic division. It may be due to a variety of reasons like cell cycle arrest or delay, slow progression of cells from the S phase to the M phase, the inhibition of DNA synthesis, preventing the cells from entering in mitosis or due to spindle disruption that may ultimately lead to cell death which are also interpreted as direct toxic effects of AAEAL. AAEAL induced high frequency of c-metaphase in onion root tip cells. This phenomenon might have occurred due to the microtubule disruption. Microtubule-disrupting agents arrest the cells at metaphase by triggering activation of a mitotic checkpoint, which ensures accurate attachment of chromosomes to the mitotic spindle, before entering into anaphase. When drug treatment causes microtubules to fail to attach to the kinetochores, mitotic checkpoint continues to generate signals that inhibit metaphase to anaphase transition leading to metaphase arrest and induction of c-metaphase (Amon, 1999; Burke, 2000).

Relation between allelopathic activity and the presence of various other chromosomal abnormalities in treated onion root tip cells is also in accordance to the previous study reports. Chromosome without telomeres become “sticky” and may fuse with other broken chromosome ends. According to Fiskesjo (1985), sticky chromosomes indicate a highly toxic, irreversible effect, probably leading to cell death. Abnormal metaphases and anaphases are indicators of microtubule malformation effect of the extracts (Hayashi and Karlseder, 2013). Laggard and vagrant chromosomes also could be the result of spindle disturbances (Fiskesjo, 1985; Leme and Marin-Morales, 2009). Vagrant chromosomes occur due to the inhibition or failure of the spindle fiber formation and these reversible mitotic abnormalities are established as the low genotoxic influences (Fiskesjo, 1985). Anaphase chromosomes with fragments and bridges are indicators of induced clastogenic effects. The presence of dicentric chromosomes and unequally exchanged chromatids undergoing translocation are responsible for bridge formation (Yi and Meng, 2003). Formation of bridges and fragments in anaphase and telophase resulting from chromosomal breakage indicated the clastogenic potential of extracts tested (Leme and Marin-Morales, 2009). We observed decreased MI (%) and increased chromosome aberration frequencies in the treated samples. The decrease in MI (%) can also explain the reduction of root size considering that the cell division is directly associated with root growth (Campos *et. al*., 2008). There are similar types of reports on mitotic index depressions (Fachinetto, 2007; Levan, 1938; Ray *et. al*., 2013; Salam, 1993). Our results are in agreement with the previous study reports showing plant extract induced toxicity at the level of root growth retardation and chromosomal alteration (Pugliesi *et. al*., 2007). *A. cepa* root tip cells are accepted as an admirable genetic model to evaluate genotoxic effects such as chromosome aberrations and disturbances in the mitotic cycle. Levan first introduced *A. cepa* root tip assay and later it was proposed as a standard method to study genotoxicity (Angayarkanni *et. al*., 2007; Camparoto, 2002; Fachinetto e*t. al*., 2007; Fiskesjo, 1985; Levan, 1938). Results of the present study also reflected the utility of root tips of *A. cepa* for monitoring the genotoxic effects of plant extracts. Growth retardation, changes in the mitotic index, micronuclei formation and chromosome aberrations are important cytogenetic endpoints that are normally used in cyto-genotoxicity evaluation (Krishna *et. al*., 1991; Pugliesi *et. al*., 2007). Cytogenetic tests are advantageous for identifying the damaging effects of substances in various concentrations under different exposure times for evaluation of their influence on living organisms (Al-Sabti and Kurelec, 1985; Chauhan *et. al*., 1999).

The lesser duckweed bioassay was used to study the phytotoxic activity of AAEAL. Data indicated trends of decreased frond growth or budding after extract exposure (Figure 4; Table 2). Fluorescence microscopic analysis after staining with acridine orange and ethidium bromide indicated concentration-dependent phytotoxicity and characteristic colour patterns (Figure 5) in treated wheat and lesser duckweed roots after AAEAL exposure, where damaged cells showed ethidium bromide fluorescence (red) and untreated controls showed the acridine orange fluorescence (green). Agarose gel electrophoresis of DNA of wheat root tip cells also revealed concentration-dependent degradation indicating prominent cytotoxic activity (Figure 6).

Thus, *A. latifolia* with its strong allelopathic and cell cycle modulatory activities may possess good future prospect as natural environment friendly, bio-herbicides in the field of agriculture.

CONCLUSION

In conclusion, allelochemicals from aerial parts' aqueous extract of *A. latifolia* exert their effect of allelopathic activity by causing cell cycle delay and cyto-genotoxicity induction on the experimental plant species. However, detailed structural and molecular investigation on the allelopathic phytoconstituents, their mode of action and large scale field studies are required for its formulation as eco-friendly bio-herbicides.

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