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17. Wild Grape Plants: A Natural Resource of Bio-Herbicides in Terms of Allelopathic and Cell Cycle Kinetics Modulating Activities

Anwesa Chaudhuri*[1], Ananya Chaudhuri[2], Saumabha Chatterjee[3], Sanjib Ray#[1]
[1]Molecular Biology and Genetics Unit, Department of Zoology, the University of Burdwan, Burdwan, West Bengal.
*Presenting and Corresponding Author, Former Doctoral Fellow,
#Associate Professor, Department of Zoology.
[2]Research Scholar, Department of Chemistry, SKBU, Purulia, West Bengal.
[3]Assistant Professor of Zoology (WBES), Durgapur Govt. College, Durgapur, West Bengal.

Abstract

Indiscriminate use of chemical herbicides leads to a serious environmental impact resulting in hazardous consequences on human and animal health. Recently, the search for biological herbicides of plant origin is gaining renewed interest. The present study was undertaken to investigate the allelopathic and cell cycle modulating effects of the hydro-methanolic extract of aerial parts of wild grape plants (HMEWGP) [*Ampelocissus latifolia* (Roxb.) Planch.] in terms of morphometric growth retardation and mitotic index depression in moong beans (*Vigna mungo* L.) and onions (*Allium cepa* L.). 24 h germinated seedlings were exposed to HMEWGP (0.5-6 mg/mL) for 48 h. After 24 and 48 h, root lengths and branch root numbers were measured. For the analysis of cell cycle kinetics, onion roots were exposed to HMEWGP (0.5 and 2 mg/mL, for 2-24 h); mitotic index and different types of cyto-nuclear abnormalities were analyzed from the squashed root tip cells under the light microscope. Results indicated that HMEWGP induced root morphological alteration, concentration-dependent root growth retardation, branch root growth inhibition ($p<0.001$), decrease in mitotic index ($p<0.001$) and increased cyto-nuclear aberrations (interphase nuclear condensation, c-metaphase, chromosomal bridge, sticky chromosome, vagrant chromosome etc.) in treated cells. Thus, allelochemicals from HMEWGP possess a strong inhibitory activity directing its excellent future prospect for the formulation of cost-effective, eco-friendly herbicides.

Keywords: *Allium cepa*, Cyto-nuclear abnormalities, Mitotic index, *Vigna mungo*, Wild grapes.

1. Introduction

Plant allelopathy refers to any stimulatory or inhibitory effects of one plant on another plant by the release of phytochemicals from root exudation, leaching, volatilization, and other processes in both natural and agricultural systems (Smith and Martin, 1994). Allelochemicals are secondary metabolites, not directly required for the growth and development of the organism. This phenomenon of allelopathic (inhibitory) activity can be exploited in the formulation of biodegradable bio-herbicides i.e. the herbicides of biological origin. Indiscriminate use of chemical herbicides leads to serious environmental impacts like biomagnification, bioaccumulation etc. resulting in hazardous consequences on human and animal health. Recently, the search for biological herbicides of plant origin is gaining renewed interest. Various phytochemicals like steroids, terpenoids, alkaloids, flavonoids, anthraquinones, tannins, glycosides, carbohydrates, saponins 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. Plant crude extract is a mixture of various chemicals which act in concert to exert different activities in the field of both biomedicine and agriculture.

Keeping this in view, the present study was undertaken to explore the allelopathic and cell cycle modulating effects of the hydro-methanolic extract of aerial parts of the wild grape plant [*Ampelocissus latifolia* (Roxb.) Planch. Family: Vitaceae] (HMEWGP). For this purpose, root and branch root growth inhibition, mitotic index depression, and cyto-nuclear aberration induction were studied on moong bean seedlings and onion bulbs.

This plant is used traditionally to treat gout, dental troubles, ulcers, fractured bone, dysentery (Patil and Patil, 2012) dyspepsia, indigestion and tuberculosis (Prusti and Behera, 2007; Swarnkar and Katewa, 2008). Several scientific studies have explored the chemical composition of this plant. Wild grape is rich in various phytochemicals like carbohydrates, phenolics, tannins, flavonoids, anthraquinones, terpenoids, saponins etc. (Tamilarashi *et. al.*, 2000). In the present study, we have tried to explore the anti-mitotic and cell cycle modulating activities of HMEWGP 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 form the basis of toxicity assessments and allelopathic potentiality determination. The moong bean seeds were 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 apical meristem cells of moong seedlings after exposure to several drugs and plant extracts. Ray *et. al.* (2013) also demonstrated *Synedrella nodiflora* induced growth retardation and altered root morphology of moong bean seeds. Onion is considered as one of the best-established test systems to determine the toxicity in the laboratory condition (Fiskesjo, 1985; Liman *et. al.*, 2011). 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). The use of *Allium cepa* genetic system for the cyto-genotoxicity study is validated by the International Programme on Chemical Safety (IPCS) and The United Nations Environmental Program (UNEP) as an efficient test for the analysis and monitoring of *in situ* genotoxicity assessment for environmental substances (Teixeira *et. al.*, 2003). The cytotoxicity levels of an agent can be determined by scoring the mitotic index (MI) (%) and chromosomal abnormalities in the treatment models (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 cell cycle modulation.

2. Materials and Methods

2.1. Chemicals

Glacial acetic acid, orcein, and methanol were obtained from BDH Chemicals Pvt. Ltd., UK. DMSO was obtained from Thermo Fisher Scientific Pvt. Ltd., Mumbai, India. Other chemicals used in this study were of analytical grade from reputed manufacturers.

2.2. Collection of plant products, storage, and extract preparation

Aerial parts of the plant materials were collected from Burdwan University campus, West Bengal and taxonomically identified by Dr. Ambarish Mukherjee (Taxonomist), Professor, Department of Botany, the University of Burdwan. Collected plant materials were washed; shade-dried, and pulverized using an electric grinder (Philips Mixer Grinder HL1605). 50 g of dried powdered plant material was extracted in 1 L of 70% hydro-methanol at 60-70°C for 72 h using Soxhlet apparatus. The extract was filtered, evaporated to dryness, and stored at -20°C. The dried extract was dissolved in DMSO before application.

2.3. Experimental plants

Moong beans (*Vigna mungo* L.) and onions (*Allium cepa* L.) were used as experimental plant models for morphometric bioassay, light microscopic analysis of cell cycle kinetics, and cyto-nuclear aberration analysis.

2.3.1. Culture of* moong beans (*Vigna mungo* L.) *for morphometric bioassay:

Moong beans (*Vigna mungo* L.) were surface sterilized with 1% sodium hypo-chlorite and allowed to germinate in an environmental chamber at 25±2°C (Chaudhuri *et. al.*, 2015). After 24 h, germinated seeds were exposed to 0.5, 1, 2 and 4 mg/mL of HMEWGP, dissolved in DMSO (1%) and covered with another Petri dish. Root lengths and branch root numbers were recorded after 48 h. Experiments were done in triplicate and DMSO was used as culture medium for untreated control seedlings.

2.3.2. Culture of onion (Allium cepa* L.) *bulbs for morphometric bioassay:

Onion bulbs were allowed for root sprouting in distilled water (Ray *et. al.*, 2013). After 48 h, bulbs having nearly equal sized roots were exposed to 0.5, 1, 2 and 4 mg/mL of HMEWGP and compared to the negative control (1% DMSO). Experimental set up was kept in an environmental chamber at 23±2°C. Root lengths were recorded after 24 and 48 h, represented as Mean±SEM and growth retardation percentages were calculated.

2.3.3. Scoring of mitotic index and cyto-nuclear aberrations from onion root apical meristem cells:

24 h aged onion roots were exposed to 0.5 and 2 mg/mL of HMEWGP for 4 and 24 h. 1% DMSO was used as negative control. 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. Nearly 3000 cells were scored under bright field light microscope with 40X objective lens for each group and each hour. Individual cell phase frequencies, mitotic index (MI), and percentages of cyto-nuclear aberrations were scored from the squashed root tip cells.

3. Scoring and Statistical Analysis

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) % as (No. of cells in dividing phase/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 analyzed using 2x2 contingency χ^2-test. Differences between controls and treatments were considered statistically significant at $p<0.05$, $p<0.01$, $p<0.001$.

4. Results

4.1. HMEWGP induced allelopathy in terms of seedlings' root growth retardation:

4.1.1. Moong bean seedlings

Data indicated concentration-dependent root growth retardation of moong bean seedlings after 48 h of HMEWGP exposure. In the present study, the maximum root length (7.28±0.46 cm) was recorded from the untreated roots and the minimum length (1.02±0.22 cm) was recorded from the highest concentration (4 mg/mL) of HMEWGP at 48 h (level of significance was considered at $p<0.001$). Branch root numbers also decreased significantly ($p<0.001$) in a concentration-dependent way, where also the maximum number of branch roots (7.89±0.91) was recorded from the untreated seedlings and the minimum number (3.17±0.66) was recorded from the highest concentration (4 mg/mL) of HMEWGP.

Table 1. Pooled data showing root growth retardation effect of different concentrations of HMEWGP on moong bean seedlings

Conc. in (mg/mL)	Root lengths			Branch Roots		
	Range (cm)	(Mean±SEM)	Inhibition %	Range (cm)	Number (Mean±SEM)	Inhibition %
0.00	2.5-9.9	7.28±0.46	00	4-14	7.89±091	00
0.50	1.6-10.5	7.23±0.55[a]	6.87	0-11	7.22±0.6[a]	8.49
2.00	3.1-9.5	6.63±0.39[a]	8.93	0-12	4.78±0.83[a]	39.42
4.00	0.1-2.8	1.02±0.22[a]	85.99	0-8	3.17±0.66[a]	59.82

Experiments were done in triplicate and root lengths were expressed as Mean±SEM.

[a]Significant at $p<0.001$ by Student's t-test. SEM: The Standard error of mean. Conc.: Concentration.

4.1.2. Onion bulbs

Results indicated that HMEWGP caused growth retardation of onion roots in a concentration-dependent manner. In the present study, the maximum root length (1.29±0.06 cm) was recorded from the control (1% DMSO) group while the minimum length (0.61±0.03 cm)

was recorded from 4 mg/mL of HMEWGP at 48 h (Figure 2). IC_{50} value was calculated as 0.8 mg/mL at 48 h.

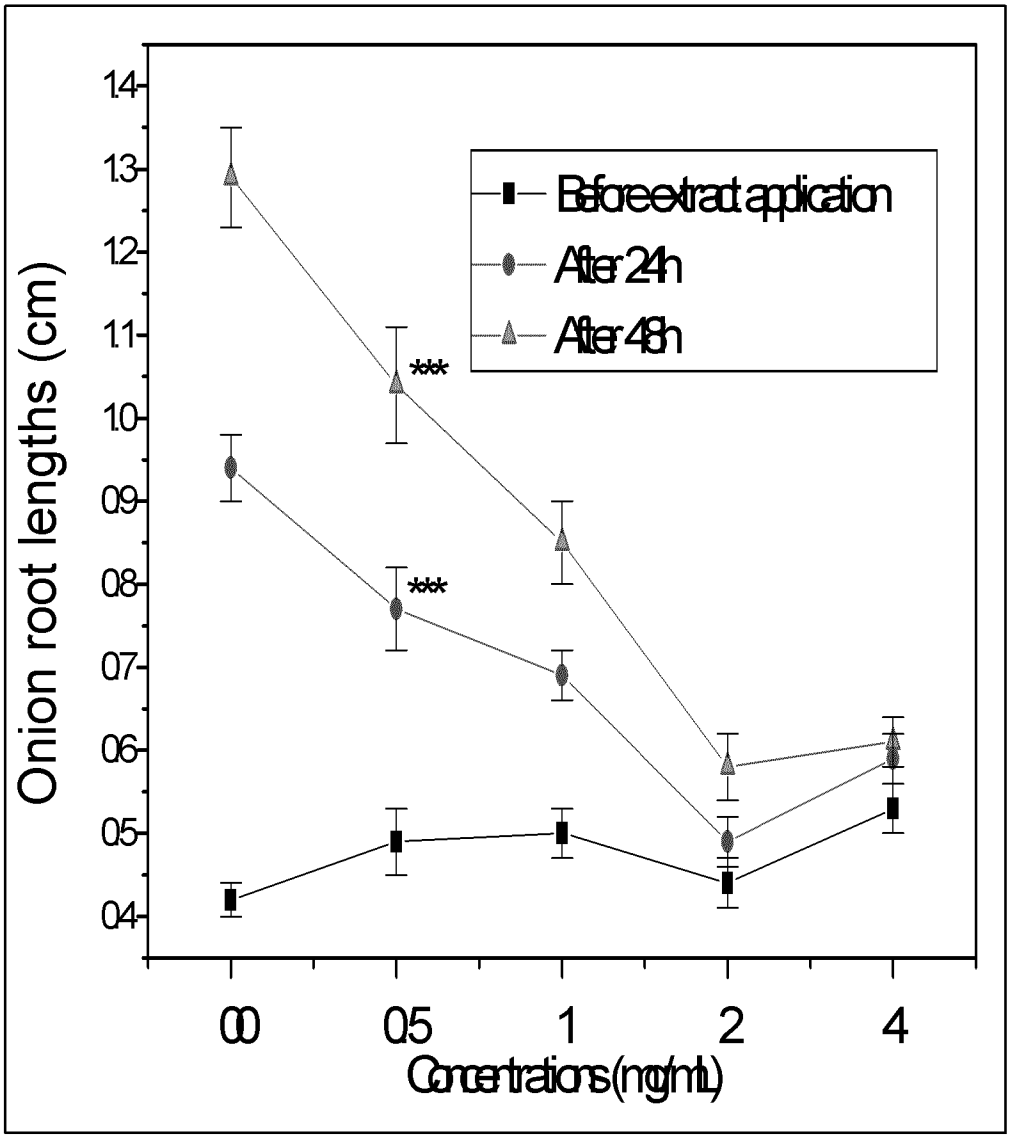

Figure 1. Onion root lengths after HMEWGP exposure at 24 and 48 h. Each data point is expressed as Mean±SEM for the triplicate set of experiments. ***Significant at $p<0.001$ with Student's t-test.

4.2. HMEWGP induced cell cycle kinetics modulation in terms of mitotic index depression and induction of cyto-nuclear aberration on onion root apical meristem cells:

Data indicated the trend of mitodepression in HMEWGP treated onion root tip cells as compared to the control. Concentration-dependent reduction in mitotic index was observed in HMEWGP treated samples (Table 2). Significant ($p<0.001$) differences in the mitotic index were

seen between treated and untreated root tip cells. Mitotic index percentages were calculated as 0.46±0.06 ($p<0.001$) and 0.39±0.07 ($p<0.001$) after treatment with 0.5 and 2 mg/mL (of HMEWGP) respectively for 4 h as compared to the control for which MI % was calculated as 3.76±0.42. After the exposure of 0.5 and 2 mg/mL of HMEWGP for 24 h, the percentages of MI was found to be 0.44±0.08 ($p<0.001$), and 0.13±0.06 ($p<0.001$) respectively as compared to the control for which MI % was calculated as 3.68±0.15 signifying the incidence of delay in cell cycle kinetics.

Cytogenetic analysis on onion root tip cells revealed an increased number of cytological and chromosomal aberrations in treated root apical meristem cells in a concentration-dependent manner (Table 2; Figure 2). There were 111.11, 633.33, 113.64 and 277.27% increase in chromosomal abnormalities per cell after treatment with 0.5 (4 h), 2 (4 h), 0.5 (24 h) and 2 (24 h) mg/mL of HMEWGP respectively. Moreover, the percentages of abnormal interphase cells (condensed nucleus, bi-nucleate cells, nuclear fragmentation, giant chromosomes, micronucleus) also increased in both time and concentration-dependent manners (Table 2; Figure 2). Presence of different chromosomal and cytological aberrations like sticky and clumped chromosome, delayed and laggard chromosome, anaphase and telophase bridge, c-metaphase, vagrant chromosome, chromosome loss, micronucleus, interphase nucleus condensation, binucleate cells etc. point towards the possible mechanisms involved in the root growth inhibition in treated cells (Figure 2).

Table 2. Pooled data showing HMEWGP 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/ dividing cell Mean±SEM (% Increase)	% Abnormal interphase cells (% Increase)
4	00	2438	95.98	3.76±0.42	9	0.09±0.01	1.24±0.15
	0.5	4542	99.52	0.46±0.06[a] (87.77)	4	0.19±0.02[a] (111.11)	7.25±0.42[a] (484.68)
	2	5132	99.59	0.39±0.07[a] (89.63)	14	0.66±0.01[a] (633.33)	12.68±0.12[a] (922.58)
24	00	3693	96.32	3.68±0.15	30	0.22±0.02	1.13±0.17[a]
	0.5	7757	99.56	0.44±0.08[a] (88.04)	16	0.47±0.08[a] (113.64)	14.46±0.61[a] (1179.65)
	2	3050	99.87	0.13±0.06[a] (96.47)	3	0.83±0.17[a] (277.27)	22.93±0.44[a] (1929.20)

[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.

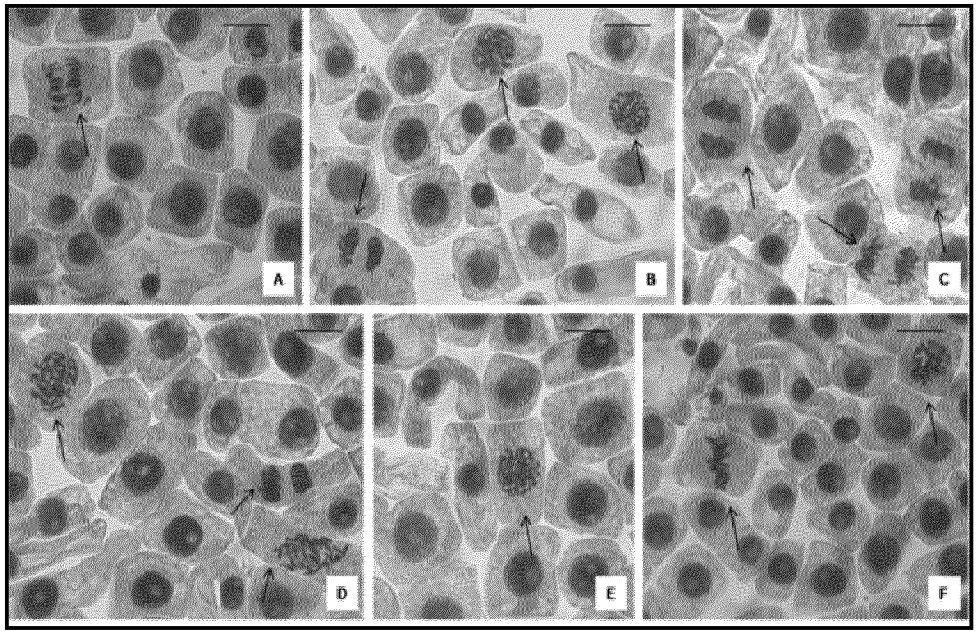

Figure 2. Chromosomal abnormalities observed in onion root apical meristem cells after different hours of HMEWGP treatment at concentrations of 0.5 and 2 mg/mL. A-chromosome loss in anaphase, B and E-chromosomal condensation and clumping, C-vagrant chromosome at anaphase and chromosomal clumping at telophase, D-ring formation and anaphase bridge, E-sticky or clumped chromosome, F-metaphase plate deviation and ball metaphase. Photomicrographs (A-F) (200X) were further magnified (2x) using Microsoft PowerPoint.

5. 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. (Camparoto *et. al.*, 2002). The present study was aimed to investigate the allelopathic and cell cycle kinetics modulating activities of the hydro-methanolic extract of aerial parts of wild grape plants (HMEWGP) using apical meristem cells.

In the present study, allelopathic and cell cycle modulating activities of HMEWGP were investigated by morphometric bioassays considering the root length and root morphology changes of moong bean and onion seedlings and cytogenetic analysis on onion root apical

meristem cells for mitotic index depression and delay in cell cycle kinetics analysis. Data indicated increased percentages of root growth inhibition of moong bean and onion seedlings after HMEWGP exposure (Table 1; Figure 1). A number of earlier studies have shown that the level of growth inhibition increases with the increasing extract concentrations (Chaudhuri and Ray, 2014, 2015 a, b; Ray *et. al.*, 2013). These results are in agreement with the 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 and causing plant growth retardation (Chaudhuri and Ray, 2016; Corcoran, 1972). 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 machinery for exerting allelopathic activity. Narciclasine 1, isolated from *Narcissus* bulbs was found to inhibit the growth of wheat grain radicles (Ceriotti, 1967). Light microscopic study revealed extract induced delay in cell cycle kinetics and induction of cyto-genotoxic stress in treated cells (Table 2; Figure 2). Our results are in accordance with the previous study reports showing extract induced toxicity at the level of root growth retardation and chromosomal alteration (Fiskesjo, 1985). *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 and the reduction in the mitotic index can be considered as delay in cell cycle kinetics (Rojas *et. al.*, 1993). Data showed that treatment with HMEWGP (0.5 and 2 mg/mL) on root tip cells produced significant ($p<0.001$) reduction in the mitotic indices in onion root apical meristem cells (Table 2). HMEWGP treatment on *A. cepa* root at a concentration of 2 mg/mL for 24 h led to the reduction of the Mitotic index (MI) by 96.47%, with concomitant rise in chromosomal abnormalities (277.27%) and abnormal interphase cells (1929.20%). This 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). 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.

Such a concentration-dependent reduction in mitotic index percentage with an increase in chromosomal abnormalities suggested that the exposure of HMEWGP to root apical meristem cells led to cytotoxic stress, reduction in cell numbers entering into the mitotic cycle and altogether increased interphase cell frequency. HMEWGP induced a high frequency of c-metaphase in onion root tip cells which might have occurred due to the microtubule disruption. 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). 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). 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). Results of the present study also reflected the utility of plant root tips for monitoring the genotoxic effects of plant extracts in terms of growth retardation, changes in the mitotic index, micronuclei formation and chromosome aberrations which are considered as important cytogenetic endpoints (Pugliesi *et. al.*, 2007). Thus, *A. latifolia* with its strong allelopathic and cell cycle modulating activities may possess good future prospect as natural and environment-friendly bio-herbicides in the field of agriculture.

6. Conclusion

In conclusion, allelochemicals from the hydro-methanolic extract of aerial parts of wild grape plants (HMEWGP) exert 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.

Conflict Of Interest

No conflict of interest was declared by the authors.

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