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ORIGINAL PAPER

Isolation of 24-epibrassinolide from leaves of *Aegle marmelos* and evaluation of its antigenotoxicity employing *Allium cepa* chromosomal aberration assay

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Abstract Brassinosteroids are of universal occurrence in plants. They have been reported to affect plant growth and development through a spectrum of physiological responses. Recently they are reported to confer resistance in plants against a number of biotic and abiotic stresses. In the present study, a brassinosteroid was isolated from *Aegle marmelos* Correa. (Rutaceae) which was characterized to be 24-epibrassinolide (EBL) using various spectroscopic techniques (TLC and ESI-MS analysis). It was evaluated for the antigenotoxicity against maleic hydrazide (MH) induced genotoxicity in *Allium cepa* chromosomal aberration assay. It was shown that the percentage of chromosomal aberrations induced by maleic hydrazide (0.01%) declined significantly with 24-epibrassinolide treatment. EBL (10^{-7} M) proved to be the most effective concentration with 91.8% inhibition. This is the first report on the isolation of 24-epibrassinolide from *Aegle marmelos* and its antigenotoxic effects against MH employing *Allium cepa* chromosomal aberration assay.

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Introduction

Brassinosteroids (BRs) are a class of polyhydroxysteroids which have been recognized as a sixth class of plant growth regulators. They play an essential role in plant growth and development. On application to plants, BRs induce a broad spectrum of cellular responses, such as, stem elongation, xylem differentiation, pollen tube growth and senescence (Tanaka et al. 2003). They also enhance plant resistance to a variety of stresses including drought, extreme temperature, heavy metals, herbicidal injury and salinity (Krishna 2003; Kagale et al. 2007; Hayat et al. 2007; Alam et al. 2007). In addition to plants, BRs also affect physiological processes of insects and mammalian counterparts. Their antiviral effects have been reported against herpes simplex virus type1 (HSV-1) and arena virus in cell culture studies (Wachsman et al. 2002, 2004). Recently their effects in cancer cell lines [human acute lymphoblastic leukemia cell line (CEM) and human myeloma cell line (RPMI 8226)) have also been explored (Swaczynova et al. 2006). In plants they have been isolated and purified from various plant parts through detection at very low concentrations (0.13 ng/kg–120 μg/kg) (Sakurai and Fujioka 1993, 1997). Young growing tissues are

found to contain higher concentrations of BRs than mature tissues (Bajguz and Tretyn 2003).

Aegle marmelos is known for its medicinal importance. It is useful in treating pain, fever, inflammation, respiratory and cardiac disorders, dysentery, diarrhea, diabetes and chemoprotective activity (Murugesa Mudaliar 1988; Singh et al. 2000). The anti-inflammatory, anticancer, antipyretic and analgesic properties of leaves of *Aegle marmelos* have been studied by Veerapan et al. (2005). Venkatesh et al. (2007) observed modulation of doxorubicin-induced genotoxicity by *A. marmelos* and further studied inhibition of doxorubicin-induced clastogenic effects by leaves of *A. marmelos.* The chemical literature survey of *A. marmelos* showed the presence of a number of coumarins, alkaloids, lignan glucosides, triterpenoids, sterols, carbohydrates, anthraquinones and lactones etc. (Ali and Pervez 2004). The presence and role of brassinosteroids in this plant has yet to be studied. With this in mind, our aims are to present a piece of work to investigate the presence of brassinosteroids and evaluation of isolated BR for the antigenotoxicity by employing *Allium cepa* chromosomal aberration assay.

Material and methods

Extraction and purification of brassinosteroids

The study material for the present investigation included fresh leaves of *Aegle marmelos* from the Herbal Garden of Guru Nanak Dev University. Amritsar (Punjab, India). Fresh leaves of *A. marmelos* (2 kg) were homogenized and percolated with 80% methanol (3 × 1000 ml). The combined methanol extract was dried under vacuum using rotary evaporator (Strike 202, Stereoglass, Italy). The methanol extract (129 g) was then partitioned between chloroform and water. The chloroform extract was further partitioned between 80% methanol and hexane. The resulting 80% methanol extract was partitioned between 0.2 M K_2HPO_4 and ethyl acetate. Ethyl acetate fraction (7 g) was dried and subjected to silica gel (60–120 mesh) chromatography with step-gradient elution from $CHCl_3$ to MeOH 0, 1, 2, 3, 4, 5, 6, 7, 10, 15, 20, 50, 100% (each 500–1000 ml). All the fractions were subjected to radish hypocotyl bioassay with an objective to find the bioactive fractions (Fig. 1). Three fractions, AAF1, AAF2 and AAF3, eluted in 5, 50 and 100% methanol/chloroform were found to be active in which AAF3 (1.3 mg) was characterized by TLC and ESI mass spectrometry with authentic compound. The standard BR used was 24-epibrassinolide (EBL) procured from Sigma-Aldrich.

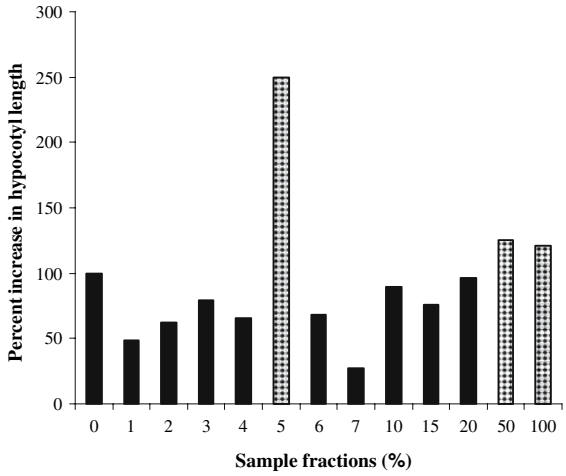

Fig. 1 Biological active fractions of *Aegle marmelos* after silica gel chromatography employing radish hypocotyl bioassay

Thin layer chromatography on silica gel 60 F_{254}, developed with $CHCl_3$:CH_3COOH:CH_3OH:H_2O (64:32:12:8) and detected by spraying Liebermann–Burchard reagent. Electrospray ionization mass spectrometry of AAF3 (ESI-MS) and standard 24-epibrassinolide was carried out by the addition of 10 µl of concentrated aqueous formic acid solution to the sample mixture at a total volume of 1000 µl (i.e., a 0.1% final concentration). ESI-QTOF-MS was performed in positive ionization mode in QTOF Mass Spectrometer (Micromass, Manchester, UK). ESI-MS was performed by direct infusion (source temperature of 280°C, capillary voltage of 2.1 kV and cone voltage of 23 V) with a flow rate of 10 µl min^{-1} using a syringe pump and mass spectra were acquired and accumulated over 60 s. MassLynx 4.0 (Waters, Manchester, UK) was used for data analysis. Tandem mass spectrometry of single molecular ion in the mass spectra was performed by mass-selecting the ion of interest, which was in turn submitted to 15–35 eV collisions with argon in the collision quadrupole. AAF3 fraction and standard 24-epibrassinolide (22R,23R,24R,2α,3α,22,23-tetrahydroxy-24-methyl-B-homo-7-oxa-5α-cholestan-6-one)

was directly subjected to QTOF-MS/MS analysis. AAF3 fraction and standard showed similar mass fragmentation patterns.

Radish hypocotyl bioassay

The bioactivity of isolated fractions was determined using intact plants of *Raphanus sativus* as described by Takatsuto et al. (1983). About 5 days-old seedlings were placed into the test solutions and kept in the dark for 24 h at 25 ± 2°C. After 24 h, the length of hypocotyls was measured and compared to control. Percent increase over control was calculated.

Allium cepa chromosomal aberration assay

24-epibrassinolide isolated from *A. marmelos* was evaluated for antigenotoxic potential against MH (1,2-dihydropyridazine-3, 6-dione). The appropriate non-toxic concentration of MH was determined as per protocol of Fiskesjo (1985). About 0.01% MH was used against which different concentrations of EBL were tested for their anti-genotoxic potential. Anti-genotoxic effects of EBL were tested by analyzing maleic hydrazide (MH) induced chromosomal aberrations in root tip cells of *Allium cepa* with and without the application of EBL. Onion bulbs were placed on Couplin jars filled with tap water until the roots emerged and grew to an average length of 1–2 cm. The roots were treated with different concentrations (10^{-7}, 10^{-8}, 10^{-9} M) of EBL in combination with MH (0.01%) for 2 h. The treatment of roots with 0.01% MH served as a positive and distilled water served as a negative control. After treatment, the bulbs were washed thoroughly under running tap water. The root tips from each bulb were fixed in Farmer's fluid (glacial acetic acid:ethyl alcohol, 1:3) for 24 h, transferred to 70% alcohol and stored at 4°C. For chromosomal analysis, squash preparations were made in which the root tips were hydrolyzed in 1 M HCl at 60°C for 1 min and then these were transferred to a watch glass containing 1 M HCl and aceto-orcein (1:9). They were then heated intermittently for 3–5 min. The tip of the root was cut, placed on a glass slide in a drop of 45% acetic acid and covered with coverslip. The tip was squashed by tapping with matchstick and sealed with DPX. The cells were scored under microscope at different stages of mitotic cycle. Chromosomal aberrations were studied in 1,200 dividing cells from nine root tips for each treatment. Percent aberrant cells and percent inhibition was calculated with and without 24-epibrassinolide.

The antigenotoxic activity of the fraction was expressed as percent decrease of chromosomal aberrations as follows:

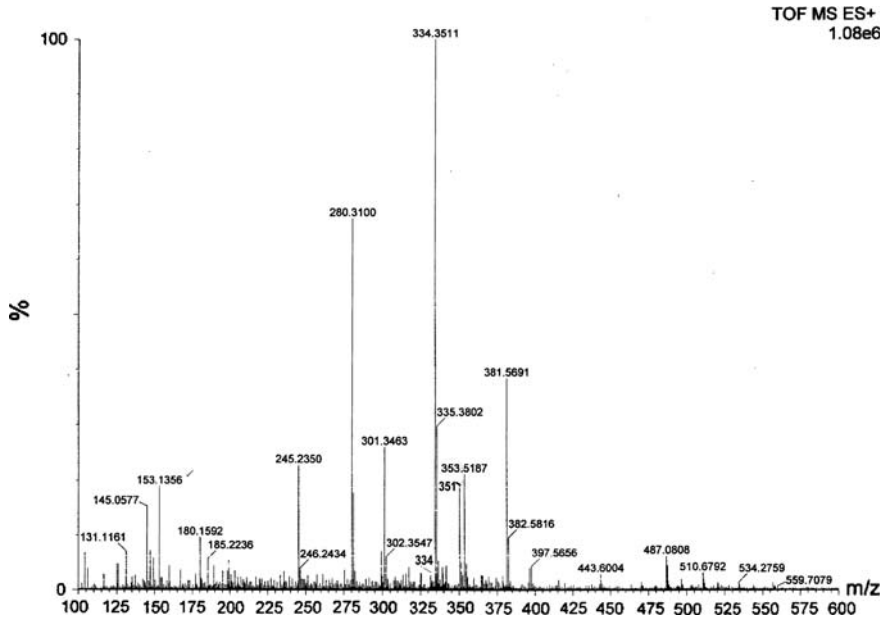

Fig. 2 ESI-QTOF-MS analysis of standard 24-epibrassinolide

$$\text{Inhibitory activity (\%)} = \frac{a - b}{a - c} \times 100$$

a = Number of aberrant cells induced by genotoxicant (MH—positive control).
b = Number of aberrant cells induced by genotoxicant in the presence of fraction.
c = Number of aberrant cells induced in the presence of fraction alone and solvent (negative control).

All values are expressed as means ± standard error. Data were analyzed with one-way analysis of variance (ANOVA) using Tukey's test (Mayers and Grossen 1974).

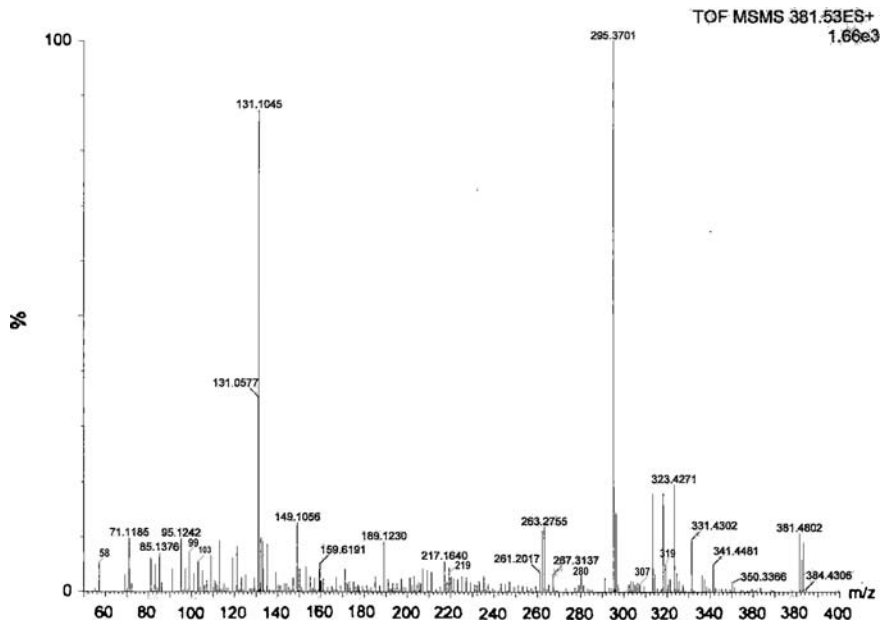

Fig. 3 ESI-QTOF-MS/MS analysis of standard 24-epibrassinolide

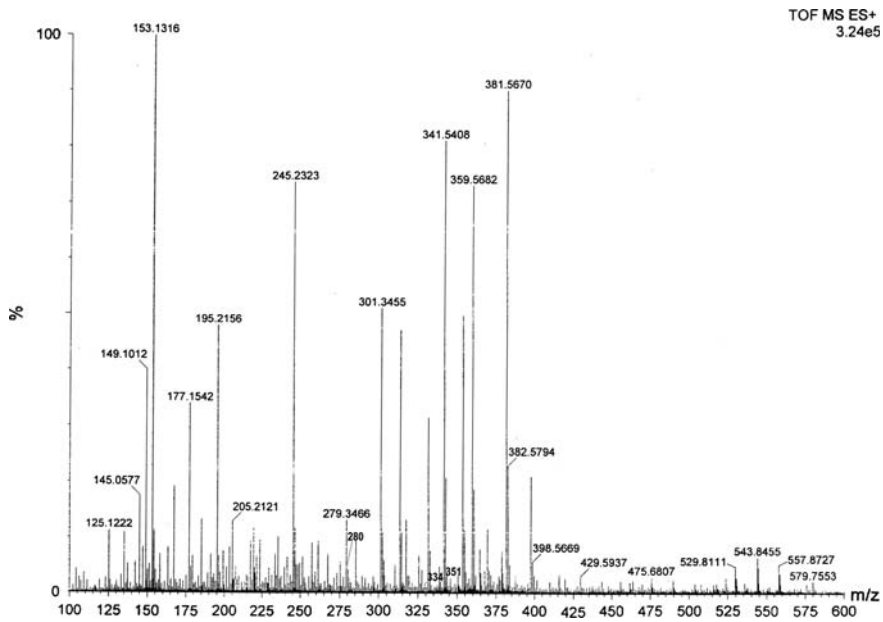

Fig. 4 ESI-QTOF-MS analysis of AAF3 fraction

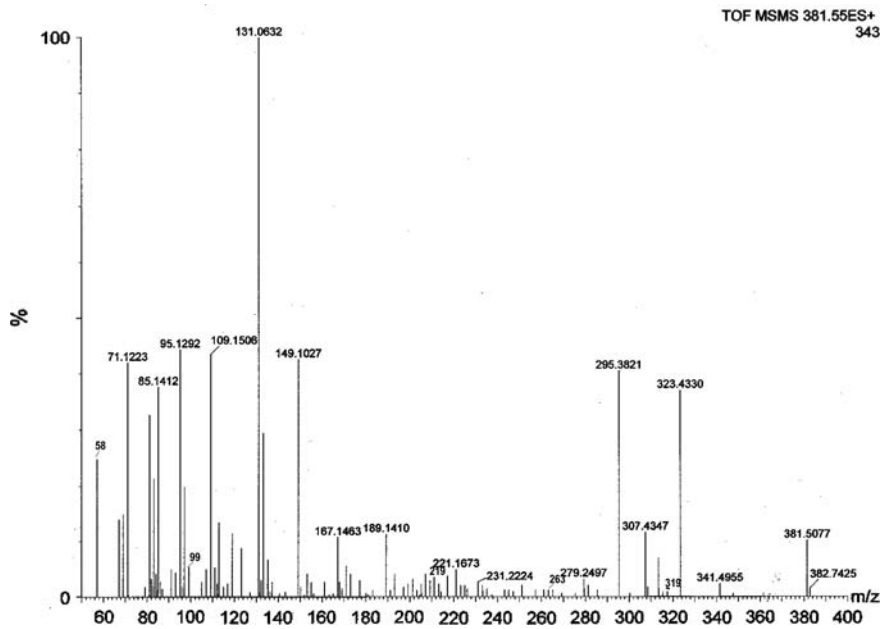

Fig. 5 ESI-QTOF-MS/MS analysis of AAF3 fraction

Results and discussion

We report here for the first time the presence of brassinosteroids (24-epibrassinolide) in *Aegle marmelos* Corr. (Rutaceae) and its antigenotoxic potential against maleic hydrazide (herbicide). In earlier studies, brassinosteroids were isolated and characterized from leaves and seeds of *Camellia sinensis* (L.) O. Kuntze. (Gupta et al. 2004; Bhardwaj et al. 2007). The presence of brassinosteroids in medicinal plants like *Aegle marmelos* suggests a possible medicinal role for brassinosteroids.

AAF3 fraction in *A. marmelos* samples was characterized as 24-epibrasinolide by thin layer chromatography with authentic sample and derivatization with Liebermann–Burchard reagent which revealed a single spot with R*f* (0.6) similar to a known standard. The structure of AAF3 was further confirmed by ESI-QTOF-MS/MS analysis. Electrospray mass spectrometry of standard 24-epibrassinolide (Fig. 2) showed the fragmented mass ion peaks $[M+H]^+$ at *m/z* 381, 351, 334 and 280, however, the molecular ion peak for 24-epibrassinolide (*m/z* 481.3529) was not observed. Further MS/MS of *m/z* 381 showed the product ions at *m/z* 341, 323, 319, 307, 295, 280, 263, 219, 189, 159, 131, 103, 99, 71 and 58 (Fig. 3). Similarly the ESI-MS of AAF3 (Fig. 4) showed fragment ions at *m/z* 381, 351, 334 and 280 and further MS/MS of *m/z* 381 showed the product ions at *m/z* 341, 323, 319, 307, 295, 279, 263, 219, 189, 131, 103, 99, 71 and 58. The peaks at *m/z* 381, 351 and 323 were observed due to the cleavage between C_{22}–C_{23}, C_{20}–C_{22} and C_{17}–C_{20} bonds, respectively (Fig. 5). Three product ions at *m/z* 295, 280 and 103 were observed due to the cleavage between C_{13}–C_{17} and C_{15}–C_{16}, C_{13}–C_{17} and C_{14}–C_{15} and C_2–C_3 and C_6O–C_7 bonds, respectively. The loss of neutral mass units from *m/z* 280 i.e., $[280\text{-}H_2O]^+$ and $[280\text{-}CO_2\text{–}H_2O]^+$ lead to the peaks at *m/z* 263 and 219 respectively. The important

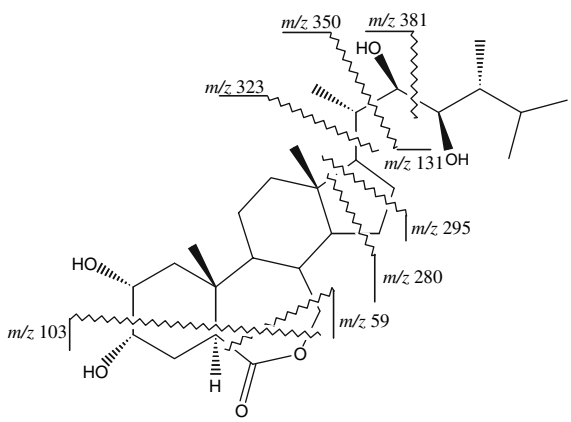

Fig. 6 Structure of 24-epibrassinolide and ESI-QTOF-MS/MS fragmentation pattern

Table 1 Effect of EBL and simultaneous EBL treatments on maleic hydrazide induced mutagenic and genotoxic effects in root tip cells of *Allium cepa*

Type of chromosomal aberrations	Negative control (n = 1,200)		MH (0.01%) (n = 1,200)		10^{-9} M EBL (n = 1,200)		10^{-8} M EBL (n = 1,200)		10^{-7} M EBL (n = 1,200)	
	Aberrant cells	% Aberrant cells (±SE)	Aberrant cells	% Aberrant cells (±SE)	Aberrant cells	% Aberrant cells (±SE)	Aberrant cells	% Aberrant cells (±SE)	Aberrant cells	% Aberrant cells (±SE)
Physiological aberrations										
Stickiness	1	–	243	–	–	–	–	–	3	–
Vagrant chromosome	1	–	48	–	1	–	2	–	–	5
Spindle Inhibition	–	–	7	–	–	–	–	–	–	–
Total physiological aberrations	2	0.16	298	24.83	1	0.08	2	0.16	3	0.25
Clastogenic aberrations										
Chromosomal bridge	1	–	15	–	1	–	1	–	1	–
Chromosomal break	–	–	7	–	–	–	–	–	–	–
Total clastogenic aberrations	1	0.08	22	1.83	–	0.08	1	0.08	1	0.08
Total aberrations	3	0.33 ± 0.07	320	26.91 ± 0.21	2	0.16 ± 0.05	3	0.25 ± 0.04	4	0.33 ± 0.07

	10^{-9} M EBL + MH (n = 1,200)			10^{-8} M EBL + MH (n = 1,200)			10^{-7} M EBL + MH (n = 1,200)		
	Aberrant cells	% Aberrant cells (±SE)	% Inhibition	Aberrant cells	% Aberrant cells (±SE)	% Inhibition	Aberrant cells	% Aberrant cells (±SE)	% Inhibition
Physiological aberrations									
Stickiness	40	–	–	31	–	–	23	–	–
Vagrant chromosome	–	–	12	–	–	2	–	–	
Spindle Inhibition	–	–	–	–	–	1	–	–	
Total physiological aberrations	45	3.75	85.19	43	3.58	86.15	26	2.16	92.20
Clastogenic aberrations									
Chromosomal bridge	1	–	–	2	–	–	2	–	–
Chromosomal break	1	–	–	2	–	–	2	–	–
Total clastogenic aberrations	2	0.16	91.02	4	0.33	85.71	4	0.33	85.71
Total aberrations	47	3.83 ± 0.03*	85.85	47	3.91 ± 0.06*	86.11	30	2.50 ± 0.09*	91.77

The values are significant at $^*P \leq 0.05$ with respect to positive control

n = number of dividing cells observed, −ve control—distilled water, +ve control—0.01% Maleic Hydrazide (MH), EBL—24-epibrassinolide

MS/MS fragmentation pattern of 24-epibrassinolide is shown in (Fig. 6). Thus, on the basis of mass spectral data and TLC with authentic sample, the identity of AAF3 was determined as 24-epibrassinolide.

EBL alone was neither toxic nor clastogenic at 10^{-7}, 10^{-8}, 10^{-9} M concentrations (Table 1). It was interesting to note that when 10^{-7} M, 10^{-8} M and 10^{-9} M 24-epibrassinolide was tested in combination with MH, the percentage of chromosomal aberrations decreased significantly (Table 1). The wide spectrum of genotoxic effects induced by maleic hydrazide includes clastogenic (chromosomal bridges, chromosomal breaks) and physiological (stickiness, spindle inhibition, vagrant, multipolarity) aberrations (Fig. 7). The antimutagenic action of a compound can be defined as the capacity to reduce mutagenic events, such as breakage and translocation of chromosomes and spindle disturbances, caused by mutagen in an organism. It is important to note that 10^{-7}, 10^{-8} and 10^{-9} M concentration of EBL when applied simultaneously with MH showed 91.8, 86.1 and 85.9% inhibition of chromosomal aberrations respectively (Fig. 7). It decreased both the physiological and clastogenic aberrations at all three concentrations (Table 1). Brassinosteroids have been reported to modulate the growth and differentiation of cells at nano to micro-molar concentrations (Clouse and Sasse 1998). They also regulate other activities such as stimulation of cell enlargement and cell division, bending of leaves at pulvinus, and changes in membrane potentials, regulation of gene expression and nucleic acid and proteins. MH is used in agriculture as a phytoregulator to suppress the growth of grass and to slow the growth of trees and shrubs, inhibits sprouting in potatoes, onions, beets and carrots during storage. It also prevents suckering in tobacco, and induces dormancy in citrus. MH induces clastogenic damage in plants by inhibiting the synthesis of nucleic acids and proteins and by affecting cell division through inducing chromosome aberrations and sister-chromatid exchange without interfering with cell extension (Kihlman and Sturelid 1978). With this in mind it is assumed that EBL when applied in combination with MH, it is suggested that nucleic acids and proteins in *Allium cepa* roots are protected from the damage caused by MH. Earlier

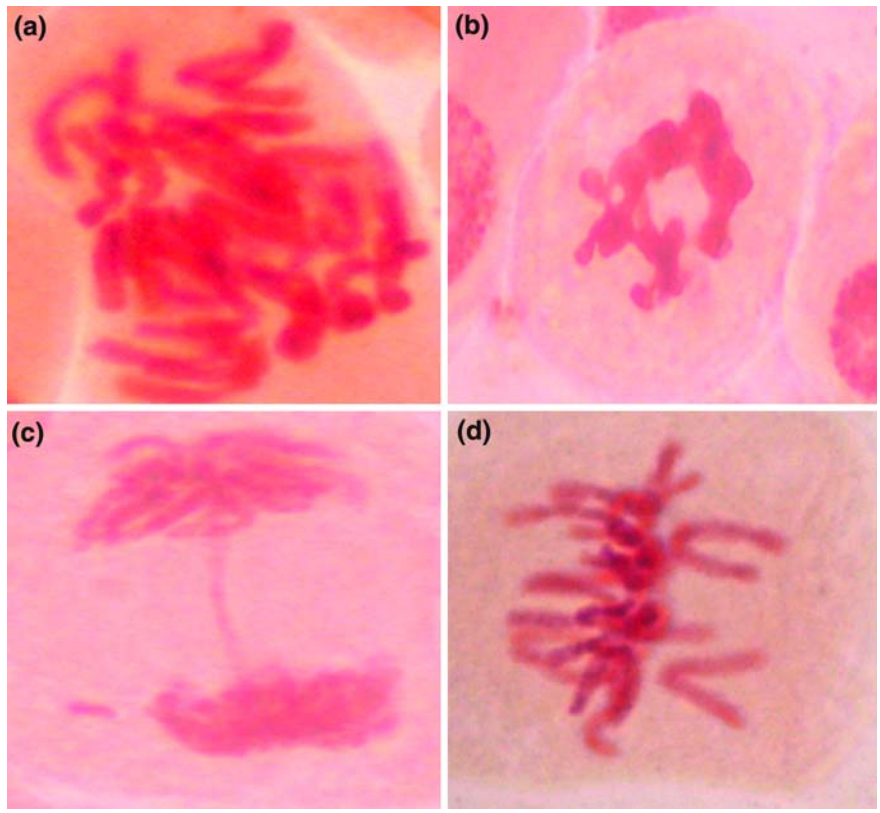

Fig. 7 Maleic hydrazide induced chromosomal aberrations in root tip cells of *A. cepa*. Representative (**a**) spindle inhibition; (**b**) stickiness; (**c**) chromosomal bridge; (**d**) vagrant chromosome

studies on the effects of EBL on root length and the mitotic index showed that 1.04×10^{-7} M concentration of EBL doubled mean root length as well as mitotic index compared to the controls (Howell et al. 2007). Khrustaleva et al. (1991) also studied the effects of EBL on the genetic structure and the processes in cell nuclei of several cultivars of barley. They revealed that EBL caused no aberrations in meiosis, reduced the frequency of chromosomal aberrations, and increased plant survival and fertility. In the anaphase and tetrad phase, EBL decreased the number of aberrations almost twofold when it was applied simultaneously with nitrosourea. Kamuro and Takatsuto (1999) have already suggested an important role for brassinosteroids in protecting the plants against environmental stresses.

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