Appraisal of Cell Cycle Delay and Cytotoxicity Inducing Potential of 3-epicaryoptin in *Allium Cepa* L.

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Abstract

Diterpenoid 3-epicaryoptin ($C_{26}H_{36}O_9$) is abundant in leaves of *Clerodendrum inerme*, a traditionally used medicinal plant, having insect antifeedant activities. Here, we aim to explore its cell cycle delay and cytotoxic effects in *Allium cepa* root apical meristem cells. *Allium cepa* roots were treated with 3-epicaryoptin (100, 150 & 200 µg mL^{-1} concentration) and colchicine (200 µg mL^{-1} concentration) for 2, 4, 4+16 h (4 h treatment followed by 16 h recovery) and the induced cell cycle delay and cytotoxic effects were analyzed. The highest metaphase cells frequency for 3-epicaryoptin (150 µg mL^{-1}) and colchicine (200 µg mL^{-1}) treated samples were found to be 66.2±0.58 % and 82.35±0.74 % respectively at 4 h treatment. Treatment of 3-epicaryoptin and colchicine increased the aberrant cells %, CA, MN, c-metaphase, and PP cells. Therefore, indiscriminate use of *C. inerme* in traditional medicine should be restricted and the cell cycle delay and cytotoxicity inducing effects of 3-epicaryoptin needs further exploration for its cancer chemotherapeutic applications.

Keywords: 3-epicaryoptin; *Allium cepa* test; metaphase arrest; chromosomal abnormalities; micronuclei; polyploidy

1. Introduction

3-epicaryoptin, a clerodane diterpenoid, was first isolated and identified by Hosozawa et al. (1974a) from the leaves of *Clerodendrum calamitosum* Maxim (Verbenaceae) (Hosozawa et al., 1974). In the case of natural products, diterpenoids have emerged as one of the most important secondary metabolites given their distinct biological activities and drug-like properties as demonstrated by the success of taxane-type diterpenoids in preclinical studies and clinical treatments. Among the diterpenoid groups, the clerodane diterpenoids comprise a large class of natural products which have been studied more in recent years for their wide range of biological activities like anticancer, anti-inflammatory, antifeedant, *etc.* (Li et al., 2016). Recently we have isolated four clerodane diterpenoids: a) Clerodin, b) 15-hydroxy-14, 15-dihydroclerodin, c) 15-methoxy-14, 15-dihydroclerodin, and d) 14, 15-dihydroclerodin from leaf aqueous extract of *C. viscosum* and shown to have their pro-metaphase arresting, PP, MN, and CA inducing activities (Roy et al., 2020a; Roy et al., 2020b). Earlier the isolated compound 3-epicaryoptin from *C. inerme* had shown to possess potent insect antifeedant activity against the third instar larvae of the tobacco cutworm, *Spodoptera litura* F. and fourth instar nymphs and adults of the potato beetle *Henosepilachna vigntioctopunctata* Fab. (Coleoptera: Coccinellidae) (Govindachari et al., 1999; Hosozawa et al., 1974). In addition to insect antifeedant activity, 3-epicaryoptin reduced the growth and increased the mortality of *Ostrinia nubilalis* (Hubner) (European com borer) larvae (Beninger et al., 1993). It had also inhibited the development of *Musca domestica* and *Culex quinquefasciatus* larvae (Pereira and Gurudutt, 1990). Although the insect antifeedant and larvicidal potential of 3-epicaryoptin has been well documented, yet there is no report available on its cytotoxic effects.

Clerodendrum inerme (L.) Gaertn. (family Lamiaceae) is a perennial shrub, widely distributed in tropical and subtropical regions of the world. The plant is used in ayurvedic

medicine for the treatment of various diseases such as rheumatism, asthma, stomach pains, hepatitis, skin disease, tumours, *etc*. (Kirtikar and Basu, 1975; Muthu et al., 2006) Pharmacological studies have showed that *C. inerme* leaves have analgesic and anti-inflammatory (Amirtharaj et al., 2010; Khanam et al., 2014), antidiabetic (Rajeev et al., 2012, antipyretic (Thirumal et al., 2013), mosquito larvicidal (Kalyanasundaram and Das, 1985, hepatoprotective (Chakraborthy and Verma, 2013), antifungal (Sharma and Verma, 1991), antimicrobial (Anandhi and Ushadevi, 2013; George and Pandalai, 1949), antiviral (Mehdi et al., 1997) and anticancer (Manoharan et al. 2006; Chouhan et al., 2018; Kalavathi et al., 2016) activities. Phytochemical investigation of different parts of the *C. inerme* revealed the presence of glycosides, anthraquinones, proteins, phenolics, flavonoids, saponins, tannins, iridoids, diterpenes, triterpenes, sterols, steroids, carbohydrates, fixed oils, volatile oils, lignin, *etc* (Prasad et al., 2012; Tanu et al., 2011). In our previous study, we have reported colchicine like metaphase (c-metaphase), and cell cycle delay inducing effects of the leaf aqueous extract of *C. inerme* in onion root tip cells (Barman et al., 2020). However, the bioactive compounds responsible for these activities are not determined. Recently we have standardized the liquid-liquid fractionation-based isolation and purification of compound 3-epicaryoptin from leaf aqueous extract of *C. inerme* (Ray et al., 2019), but its cytotoxic effects yet to study. Therefore, the present study designed to investigate its cell cycle delay and cytotoxicity inducing effects on *A. cepa* root apical meristem cells in terms of MI alteration, CA, specifically c-metaphase, MN, and PP induction.

Allium cepa L. is one of the most established plant bioassays for the cytotoxicological assessment due to its kinetic proliferation properties, large chromosomes and low chromosome number ($2n = 16$), easily observed with a light microscope, as validated by the UNEP and the IPCS (Cabrera and Rodriguez, 1999; Gomes et al., 2013). *Allium cepa* test system provides an important information about the possible mechanisms of action of an

agents on the genetic material (such as clastogenic or aneugenic effects) and has a strong correlation with mammalian/cell culture systems and therefore several microtubule targeting chemotherapeutic drug likes paclitaxel, vincristine, vinblastine, podophyllotoxin, has also been studied in *A. cepa* model for monitoring their cytogenotoxicity (Leme and Marin-Morales, 2009; Cragg et al., 1996; Bonfoco et al., 1995; Sehgal et al., 2006). The use of *A. cepa* as a test system was first investigated by Levan demonstrating disturbances in the mitotic spindle apparatus due to the use of colchicine (Levan, 1938). Studies have found that colchicine treatment causes root growth inhibition and swelling effects, increase the frequency of metaphase cells with haphazardly arranged condensed chromosomes by inhibiting mitotic spindle organization. As a result of these, chromatids fail to move to the opposite poles and eventually become enclosed in a new nuclear membrane and proceed into interphase as a doubled chromosomes number of PP cells (Hague and Jones 1987; Ray et al. 2013). Treatment of colchicine in *A. cepa* could also induce an increased frequency of CA and MN cells (Kundu & Ray 2016). In this study, colchicine was used as a standard cytotoxic agent and its effects were compared with the 3-epicaryoptin induced cytotoxic effects in *Allium cepa* root tip cells.

2. Materials and methods

2.1.Chemicals

Glacial acetic acid, orcein, and methanol were purchased from Merck Ltd. Mumbai, India. Colchicine was obtained from Himedia Laboratories Pvt. Ltd. Mumbai, India. 3-epicaryoptin was extracted from *C. inerme* leaf aqueous extract (Ray Sanjib, 2019). The other chemicals were purchased from reputed manufacturers.

*2.2.*Effects of 3-epicaryoptin on *Allium cepa*

Allium cepa L. root apical meristem cells were used as a plant model. 3-epicaryoptin and colchicine induced root growth inhibitory effects, cell cycle kinetics, CA, MN, and PP were analyzed.

2.3.Root growth retardation and swelling effects

The similar sized *A. cepa* bulbs were purchased from a local vegetable store at Golapbag, Burdwan, and allowed for root sprouting as described earlier (Barman et al., 2020; Kundu and Ray, 2017; Ray et al., 2013). For root growth inhibition analysis, the sprouting roots were exposed continuously with the different concentrations of 3-epicaryoptin (12.5, 25, 50, 100, 200 µg mL^{-1}) and colchicine (200 µg mL^{-1}) for 24, 48 and 72 h. The roots length was measured and the root growth inhibition percentage and the EC_{50} value were determined at 24, 48 and 72 h. 3-epicaryoptin (100, 150 and 200 µg mL^{-1}) and standard colchicine (200 µg mL^{-1}) induced morphometric root tip swelling effects were also measured after 4 h treatment followed by16 h recovery in water.

2.4.Study of cell cycle kinetics and chromosomal aberrations

To determine the cell cycle delay-inducing effects of 3-epicaryoptin, the MI, and dividing cell percentage at different phases were analyzed in *A. cepa* root tip cells and for the cytotoxic effects, the CA were analyzed. The onion roots (about 1.5-2 cm) were exposed with the different concentrations (100, 150, and 200 µg mL^{-1}) of 3-epicaryoptin for 2, 4, and 4 h and then allowed for 16 h recovery in water. The different cytotoxic effects were compared with the standard spindle poison, colchicine (200 µg mL^{-1}). In the case of control groups, the onion roots were maintained in distilled water. For root tip squash preparation, the control and treated onion root tips were fixed in aceto-methanol 3(methanol): 1(acetic acid) for 24 h, then hydrolysed in 1N HCl at 60 □ C for 10 minutes, stained with aceto-orcein (2%) and finally squashed in 45% acetic acid (Chaudhuri and Ray, 2015). The squashed root tips were

observed in a bright field light microscope at 400X magnification and the prophase, metaphase, anaphase, telophase cell percentage as well as the MI, and CA were scored.

2.5.Scoring and statistical analysis

Data obtained on MI, (MI (%) = Number of dividing cells/Total number of cells scored X 100), cells percentage at different phase (prophase, metaphase, anaphase and telophase), CA, MN and PP cell frequencies were analyzed by using 2 × 2 contingency $\chi 2$ test and the IC_{50} value for the root growth retardation was calculated with probit analysis in SPSS Version 20. Differences between control and treated groups were considered as significant at $p \leq 0.05$ or 0.01 or 0.001. All the data were expressed as mean ± SEM (standard error of mean). Correlations among dividing phase and among different mitotic abnormalities were calculated using Pearson's bivariate correlations and analyses were done using PAST version 4.05.

3. RESULTS

3.1.Root growth retardation and swelling effects

Treatment of 3-epicaryoptin causes concentration-dependent statistically significant root growth retardation effects in *A. cepa* root apical meristem cells (Figure 1). The maximum root growth retardation (89.77%, $p< 0.001$) effect of 3-epicaryoptin treatment was at 200 µg mL^{-1} concentration at 72 h. The standard colchicine (200 µg mL^{-1}) induced the highest root growth retardation percentage (92.08%, $p< 0.001$) at 72 h. The IC_{50} values were 68.38, 70.50 and 63.96 µg mL^{-1} respectively at 24, 48, and 72 h of 3-epicaryoptin treatment. The root tip swelling phenomenon was also observed in 3-epicaryoptin (100, 150, and 200µg mL^{-1}) and colchicine (200 µg mL^{-1}) treated samples after 4 h treatment followed by 16 h water

recovery. A comparatively better root tip swelling effect was observed at a concentration of 200 µg mL^{-1} of 3-epicaryoptin than colchicine treatments (Figure 1).

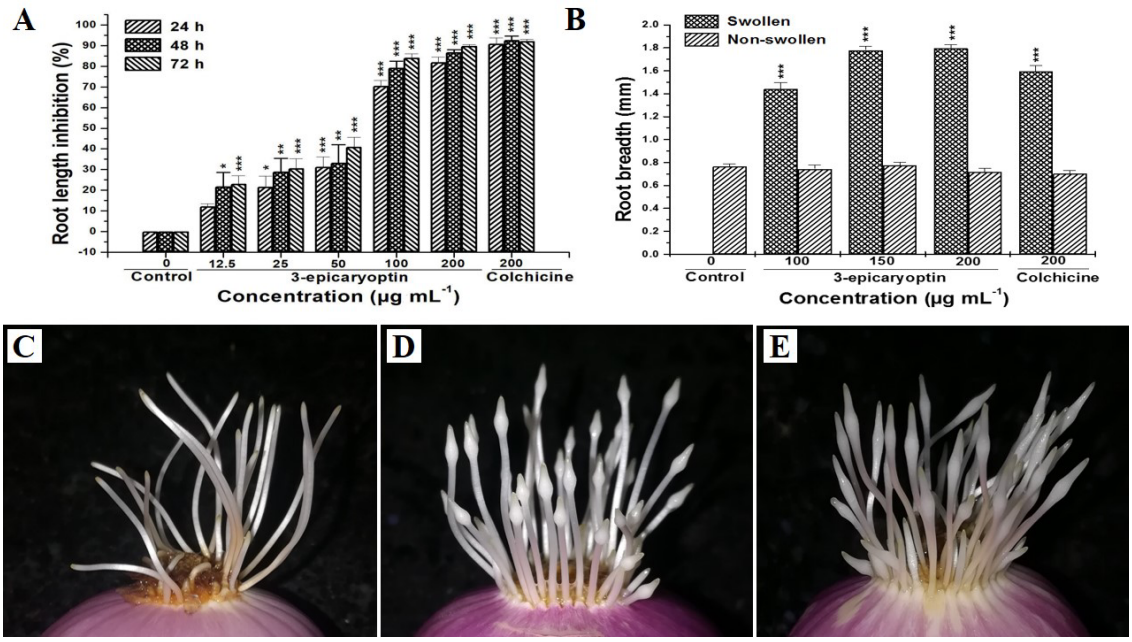

Figure 1. The effects of 3-epicaryoptin and colchicine in the *A. cepa* root length inhibition % after 24, 48, and 72 h continuous treatment (A), and the root tip swelling effects at 16 h water recovery after 4 h treatment (B-E). (B) Measurement the diameter of the *A. cepa* root tips, (C) Control, (D) 200 µg mL^{-1} concentration of 3-epicaryoptin and (E) 200 µg mL^{-1} concentration of colchicine induced *A. cepa* root tip swelling effects.

3.2.Mitotic index and dividing cells percentage

The effects of 3-epicaryoptin and colchicine on the MI% and dividing phases in *A. cepa* root apical meristem cells as presented in Table 1. Data indicate that 3-epicaryoptin (100, 150, and 200 µg mL^{-1}) and colchicine (200 µg mL^{-1}) treatment causes an increase in MI% ($p< 0.001$) at 2 and 4 h treated onion root tip cells. However, in the case of 16 h water recovery treatment, a dose-dependent decrease in MI% was observed as compared to the untreated controls. Here, 3-epicaryoptin induced the highest MI% at 150 µg mL^{-1}(12.29%) followed by 200 µg mL^{-1} (11.97%) at 4 h, whereas at16 h recovery, it was decreased to 4.90% ($p< 0.01$) and 3.33 % ($p< 0.01$) at the respective concentrations of 150 and 200 µg mL^{-1}. In the case of

200 µg mL^{-1} colchicine treatment, a statistically significant increase in MI % (13.18%, $p< 0.001$) was also observed at 4 h and again decreased (3.22%, $p< 0.01$) at 16 h recovery samples. Study of cell cycle kinetic revealed that 3-epicaryoptin (150 and 200µg mL^{-1}) continuous treatment for 4 h causes a significant decrease in prophase % (23.22% and 24.49%) and increase in metaphase % (66.2% and 63.31%) when compared to control (28.84%). The standard colchicine (200µg mL^{-1}) at 4 h also decreased prophase (11.3%) and increased (82.35%) the metaphase cells percentage. Both 3-epicaryoptin and colchicine treatments increased the prophase-metaphase cumulative frequency (PMCF) and the anaphase-telophase cumulative frequency (ATCF) significantly decreased (Figure 2). However, both PMCF and ATCF were always significantly higher in case of colchicine-treated samples. The increasing tendency of PMCF was observed at 4 h and scored as 71.43±0.59%, 83.03±0.59%, and 89.43±0.87%, 87.81±0.26% respectively for 0, 100, 150 and 200 µg $mL^{-1}$of 3-epicaryoptin and 93.66±1.35 % for 200 µg $mL^{-1}$of colchicine treatments. On the other hand, at 4 h treatment, decreasing tendency of ATCFs were scored as 28.78±0.78, 16.95±0.59, 10.55±0.87 and 12.17±0.26% respectively for 0, 100, 150 and 200 µg mL^{-1} of 3-epicaryoptin and 6.28±0.73% for 200 µg $mL^{-1}$of colchicine.

Table 1. Influence of 3-epicaryoptin and colchicine on MI and percentages of the different cell division phases of *A. Cepa* root apical meristem cells.

H	Con. (mg/mL^{-1})	TC	TDC	MI (%)	Pro (%)	Meta (%)	Ana (%)	Telo (%)
2	0	1731	88	5.1±0.40	44.62±3.38	26.65±3.27	15.48±1.60	13.22±2.92
	100	2381	176	7.41±0.44**	38.29±2.75	35.77±0.84	13.97±1.17	11.94±2.41
	150	1588	175	11.04±0.80***	36.96±0.41	52.3±0.91**	5.98±0.48*	4.72±0.11*
	200	1967	167	8.48±0.57***	33.25±0.75	44.61±0.74	14±0.43	8.11±0.46
	200[@]	2364	299	12.64±0.34***	12.77±0.41***	78.59±0.92***	7.29±0.52*	3.51±0.41***
4	0	2044	120	5.88±0.77	42.58±0.16	28.84±0.43	13.67±1.48	15.1±0.71
	100	2403	194	8.07±0.42**	30.05±0.78	51.92±0.49**	10.45±0.27	6.5±0.32*
	150	2196	270	12.29±0.34***	23.22±0.36**	66.2±0.58***	4.29±0.47**	6.26±0.46*
	200	2124	254	11.97±0.34***	24.49±0.16*	63.31±0.32***	4.83±0.19**	7.33±0.14*
	200[@]	2280	301	13.18±0.23***	11.3±0.60***	82.35±0.74***	4.41±0.38***	6.28±0.73***
16[R]	0	2435	124	5.11±0.32	34.21±0.49	31.14±1.25	15.08±1.19	19.54±0.23
	100	1889	104	5.51±0.22	29.97±0.72	54.37±0.90*	7.69±0.32	7.94±0.49*
	150	2227	109	4.90±0.25	39.63±0.36	47.03±0.77	2.39±0.47***	10.92±0.45
	200	2225	74	3.33±0.21**	39.94±0.5	43.11±0.51	6.17±0.82	10.75±0.07
	200[@]	2433	79	3.22±0.12**	18.55±0.82	41.44±0.72	8.39±0.66	11.76±0.89

*Significant at $p < 0.05$.**Significant at $p < 0.01$.***Significant at $p < 0.001$ as compared to their respective control with 2 × 2 contingency $\chi 2$ test with respective df = 1. TC: total cells; TDC: total dividing cells; MI: mitotic index. R; Recovery (4 h treatment followed by recovery).

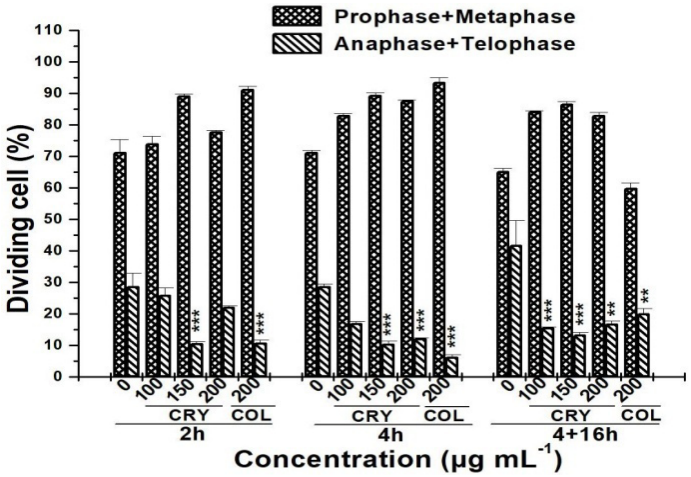

Figure 2: Influence of 3-epicaryoptin (100, 150, 200 µg mL^{-1}) and colchicine (200 µg mL^{-1}) on the 'prophase-metaphase' and 'anaphase-telophase' cumulative frequencies in *A. cepa* root tip cells. *Significant at $p< 0.05$, **$p< 0.01$ and ***$p < 0.001$ with 2 × 2 contingency $\chi 2$ analysis compared to respective control at $df = 1$. Con: Control.

3.3.Chromosomal abnormalities

3-epicaryoptin induced different types CA (anaphase bridge, C-metaphase, vagrant, laggards and sticky chromosome, multipolar anaphase-telophase) in *A. cepa* root meristem cells which are comparable to colchicine effects (Table 2 & Figure 3). The 3-epicaryoptin (100, 150, 200µg mL^{-1}) and colchicine (200µg mL^{-1}) induced statistically significant increased CAs. 150 µg mL^{-1} of 3-epicaryoptin treatment showed significant increase the total CAs% (8.79%, 7.8% and 30.46%) as compared to standard colchicine (13.48%, 15.91% and 33.48%) respectively at 2 h (p <0.001), 4 h (p <0.001), and 16 h water recovery treatment ($p < 0.001$). The most visible CAs induced by 3-epicaryoptin was c-metaphase. The mean percentages of c-metaphase were 29.61±0.81%, 51.56±0.57% and 41.04±0.64% at 2 h ($p < 0.001$) and

40.95±0.87%, 63.39±0.51% and 60.43±0.57 % at 4 h ($p < 0.001$) for the respective concentration of 100, 150, and 200 µg mL^{-1} of 3-epicaryoptin, whereas, the negative control showed 1.01±0.50% and 0.45±0.45% of c-metaphase cells at the respective fixative hours. While 200 µg mL^{-1} colchicine showed the highest c-metaphase frequency (72.65±0.11 % and 77.24±0.10 %) at both 2 h ($p < 0.001$) and 4 h ($p < 0.001$) treated samples (Table 2). 3-epicaryoptin treatment also increased the frequency of vagrant chromosomes. 150 µg mL^{-1} of 3-epicaryoptin treatment was found to be 13.89±0.21% ($p<0.01$), 13.64±0.34% ($p<0.01$), and 15.09±0.49 % ($p<0.001$) of vagrant chromosome after 2 h, 4 h, and 4 h treatment followed by16 h recovery in water. Colchicine (200µg mL^{-1}) induced the highest vagrant chromosome percentage (11.53±0.03%) at 4 h after treatment. A significant frequency ($p < 0.05$) of sticky chromosome was also observed at 150µg mL^{-1} (12.13±0.54 %) and 200µg mL^{-1} (11.27±0.19 %) of 3-epicaryoptin and 200 µg mL^{-1} (15.15±0.29 %) of colchicine treatment after 4 h. Besides the above abnormalities, 3-epicaryoptin and colchicine could increase the frequency of anaphase bridge, lagging chromosome, polar deviation and multipolar anaphase-telophase. Though the frequency was not statistically significant as compared to the control group (Table 2 & Figure 3).

Table 2. Effects of 3-epicaryoptin and colchicine on the frequency of CA in *A. cepa* root apical meristem cells.

Hours	Conc. (mg/ml)	TC	TDC	%of Aberrant cell	Ana-Bridge (%)	C-meta (%)	Vagrant (%)	Cr Sti (%)	Lagging cr (%)	Polar deviation (%)	Multipolar Ana Telo (%)
2	0	1245	66	0.34±0.01	1.01±0.5	1.01±0.50	0.54±0.54	0.46±0.46	0.95±0.47	0	0
	100	1160	78	3.53±0.36***	7.66±0.15	29.61±0.81***	10.63±0.29	8.04±0.48	8.59±0.37	3.08±0.67	3.4±0.27
	150	1175	132	8.79±0.01***	8.28±0.62	51.56±0.57***	13.89±0.21**	10.25±0.33	4.23±0.35	1.37±0.24	3.25±0.36
	200	1356	114	5.25±0.40***	7.08±0.0.34	41.04±0.64***	9.85±0.56	9.16±0.0.48	4.91±0.25	0.78±0.43	4.91±0.25
	200@	1453	185	13.48±0.26***	4.85±0.02	72.65±0.11***	9.70±0.04	11.68±0.30*	1.25±0.17	0.53±0.00	1.61±0.00
4	0	1287	71	0.25±0.02	0.88±0.44	0.45±0.45	0.97±0.48	0.52±0.52	0.88±0.44	0.52±0.52	0

	100	1306	88	4.69±0.09***	7.24±0.53	40.95±0.87***	9.07±0.09	10.26±0.56	11.28±0.6	2.34±0.79	5.35±0.56
	150	1252	127	7.8±0.08***	9.91±0.34	63.39±0.51***	13.64±0.34**	12.13±0.54*	6.79±0.05	1.53±0.36	11.26±0.24
	200	1419	148	8.97±0.61***	4.91±0.27	60.43±0.57***	12.36±0.18**	11.27±0.19*	4.68±0.48	0.88±0.16	6.96±0.15
	200@	1520	211	15.91±0.54***	3.68±0.26	77.24±0.10***	11.53±0.03**	15.15±0.29**	0.47±0.00	0	1.92±0.00
16[R]	0	1176	63	0.27±0.01	0.49±0.49	1.59±0.09	1±0.5	0.59±0.59	0.50±0.50	0	0
	100	1161	64	19.95±0.78***	3.13±0.14	23.36±0.75***	6.75±0.28	6.27±0.28	4.7±0.21	1.56±0.07	6.75±0.28
	150	1085	26	30.46±0.63***	7.54±0.24	35.57±0.29	15.09±0.49	8.38±0.84	0.83±0.83	0.83±0.83	7.54±0.24
	200	1229	34	26.41±0.61***	3.9±0.66	21.37±0.73	11.77±0.50	5.63±1.01	1.73±0.86	0.83±0.83	4.8±0.41
	200@	1505	50	33.48±0.0.44***	1.22±0.62	5.81±0.53	1.47±0.74	2.56±0.36	0	0.54±0.0.5	5.26±0.43

*Significant at $p < 0.05$, **Significant at $p < 0.01$, ***Significant at $p < 0.001$ as compared to their respective control with 2 × 2 contingency $\chi 2$ test with respective df = 1. TC: total cells; TDC: total dividing cells; AC: aberrant cells; Meta: metaphase; Ana: anaphase; Telo: telophase; Cr: Chromosome; Sti: Stickiness. R; Recovery (4 h treatment followed by recovery).

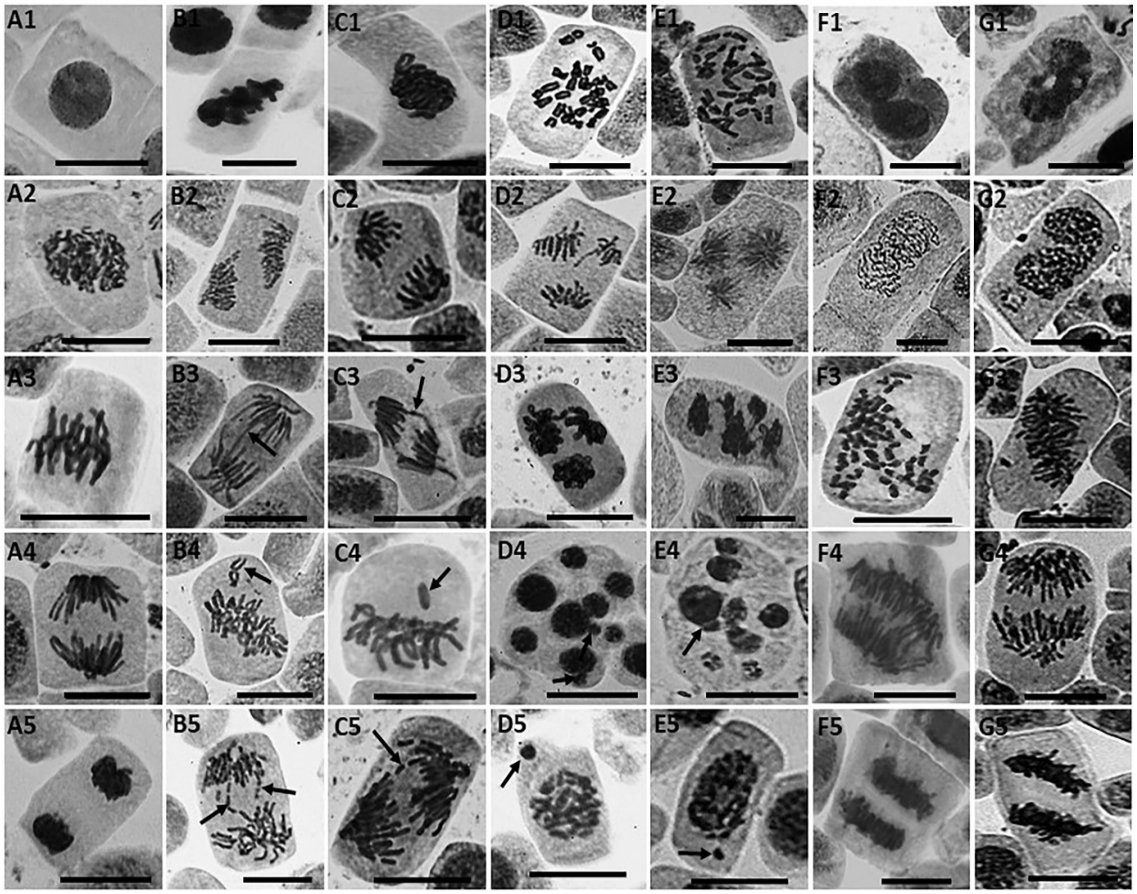

Figure 3. Shows 3-epicaryoptin and colchicine induced formation of different types of CA, MN and PP cells in *A. cepa* root apical meristem cells. (A1-A5) control interphase, prophase, metaphase,

anaphase and telophase; (B1-B5) 3-epicaryoptin induced formation of: (B1) Sticky chromosome at metaphase (150 µg mL^{-1}/4 h), (B2) Polar deviation at anaphase (100 µg mL^{-1}/16 h R), (B3) Anaphase bridge (200 µg mL^{-1}/2 h), (B4) Vagrant chromosome at metaphase (100 µg mL^{-1}/4 h), (B5) Lagging chromosome at anaphase (200 µg mL^{-1}/2 h); (C1-C5) 200 µg mL^{-1} colchicine induced formation of: (C1) Sticky chromosome at metaphase (4 h), (C2) polar deviation at anaphase (2 h), (C3) Anaphase bridge (2 h), (C4) Vagrant chromosome at metaphase (16 h R), (C5) Lagging chromosome at anaphase (4 h); (D1-D2) 3-epicaryoptin induced formation of: (D1) C-metaphase (100 µg mL^{-1}/4 h), (D2) Multipolar anaphase (200 µg mL^{-1}/4 h), (D3) Multipolar telophase (150 µg mL^{-1}/2 h) (D4) cells with multiple MN and nuclear buds (150 µg mL^{-1}/16 h R), (D5) MN at prophase (100 µg mL^{-1}/16 h R); (E1-E5) 200 µg mL^{-1} colchicine induced formation of: (E1) C-metaphase (4 h), (E2) Multipolar anaphase (16 h R), (E3) Multipolar telophase (16 h R), (E4) cells with multiple MN and nuclear buds (16 h R), (E5) MN at prophase (4+16 h); (F1-F5) 3-epicaryoptin induced formation of: (F1) PP interphase (150 µg mL^{-1}/4+16 h) (F2) PP prophase (100 µg mL^{-1}/16 h R (F3) PP metaphase (100 µg mL^{-1}/16 h R), (F4) PP anaphase (200 µg mL^{-1}/16 h R) and (F5) PP telophase (150 µg mL^{-1}); (G1-G5) 200 µg mL^{-1} colchicine induced formation of: (G1) PP interphase (16 h R) (G2) PP prophase (16 h R), (G3) PP metaphase (16 h R), (G4) PP anaphase (16 h R) and (G5) PP telophase (16 h R). R; Recovery (4 h treatment followed by recovery).

3.4.*Micronuclei and polyploidy*

Both 3-epicaryoptin and colchicine treatments induced the formation of MN and PP cells in *A. cepa* root apical meristem (Figure 5 & 6). A significant increase in the frequency of MN and PP cells were observed at 4 h treatment and also at 4 h treatment followed by 16 h water recovery. The MN frequencies were 12.94±0.34, 24.84±0.54, and 22.10±0.73% respectively for 100, 150, and 200µg mL^{-1} of 3-epicaryoptin treated samples after 4 h treatment followed by 16 h ($p< 0.001$) recovery. While, 200 µg mL^{-1} colchicine showed the highest MN percentage, 27.73±0.6%, at 16 h ($p<0.001$) recovery treatment (Figure 5). 3-epicaryoptin treatment also statistically increased ($p<0.001$) the PP cells frequency at 4+16 h.150 µg mL^{-1} concentration of 3-epicaryoptin showed 30.61±0.6% increased the PP cells frequency. Whereas, the standard colchicine (200µg mL^{-1}) induced 32.66±0.79% increase in PP cells frequency (Figure 6).

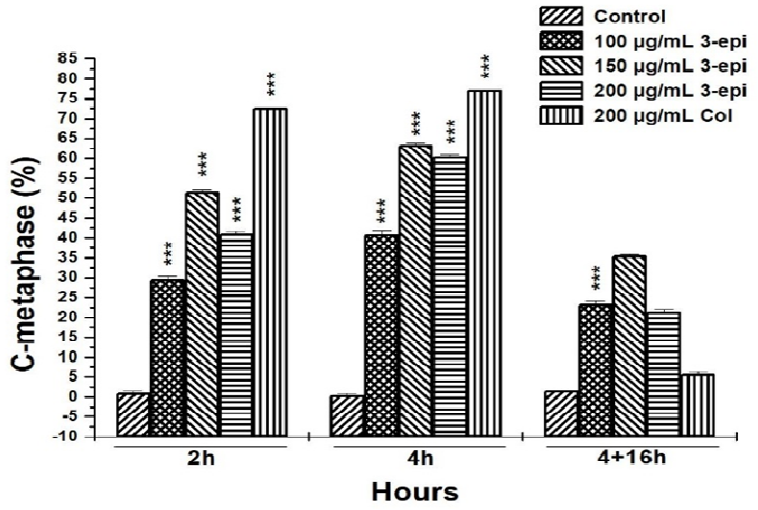

Figure 4: The c-metaphase inducing effect of 3-epicaryoptin and Colchicine on *A. cepa* root tip cells. ***Significant at $p< 0.001$ with 2×2 contingency $\chi 2$ analysis compared to respective control at $df = 1$. 3-epi: 3-epicaryoptin; Col: Colchicine.

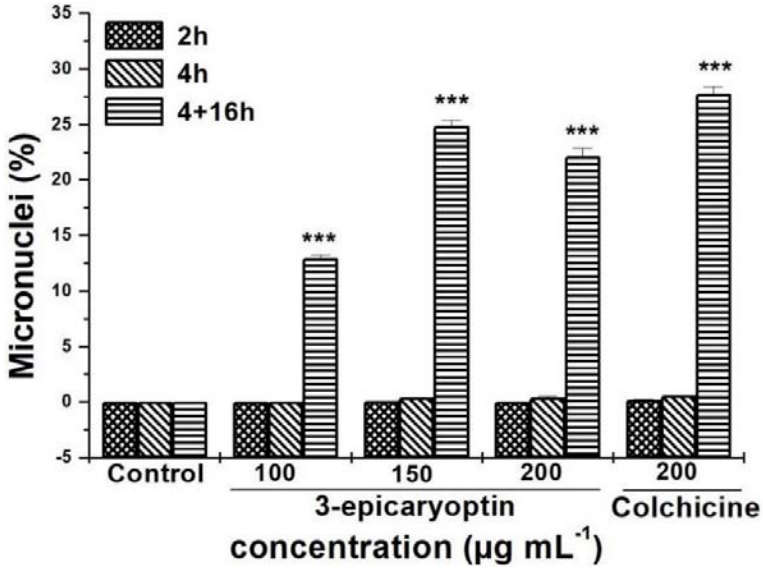

Figure 5: The MN inducing effect of 3-epicaryoptin and Colchicine on *A. cepa* root tip cells. ***Significant at $p< 0.001$ with 2×2 contingency $\chi 2$ analysis compared to respective control at $df = 1$.

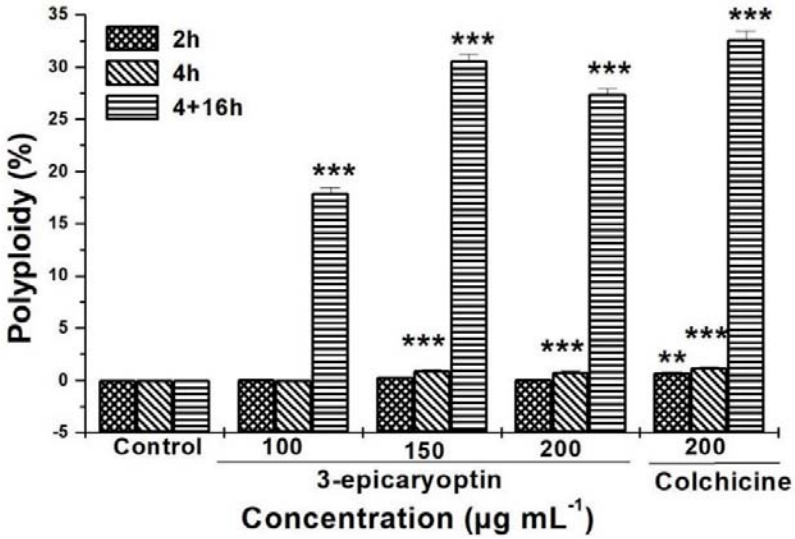

Figure 6: The PP inducing effect of 3-epicaryoptin and Colchicine on *A. cepa* root tip cells. ***Significant at p< 0.001, **Significant at p< 0.01 with 2 × 2 contingency χ2 analysis compared to respective control at *df* = 1.

3.5. Correlation analysis

Pearson's bivariate correlation analyses were performed to determine positive or negative correlation among dividing phase and with MI. After 4 h treatment with 3-epicaryoptin, there was a strong positive correlation of metaphase with MI (r = 0.99227)**.** However, prophase and anaphase had a strong negative correlation (r = -0.99422 & -0.99994), with MI, respectively. Prophase and anaphase also showed a strong negative correlation (r = -0.99986 & -0.99358) with metaphase. During 4+16 h treatment with 3-epicaryoptin, metaphase also showed a strong positive correlation (r = 0.91204) with MI and strong negative correlation (r = -0.9484) with prophase. However, anaphase showed a low positive correlation (r = 0.44018) and telophase showed a strong negative correlation (r = -0.92074) with metaphase (Figure 7 & Table S5).

Pearson's bivariate correlation analyses was showed that after 4 h treatment with 3-epicaryoptin, there was a strong positive correlation of c-metaphase, vagrant chromosome, MN and PP with aberrant cells (AC) (r = 0.92516, 0.85639, 0.98442 & 0.93011). Similarly, vagrant, chromosome stickiness, MN and PP were strongly correlated with c-metaphase (r = 0.98828, 0.93743, 0.97748 & 0.99991, respectively). There was a positive correlation of chromosome stickiness, MN and PP with vagrant chromosome. Moreover, MN showed a strong positive correlation with PP, respectively. After 4+16 h treatment with 3-epicaryoptin, AC showed strong positive correlation with vagrant chromosome, PP, MN and anaphase bridge (r = 0.9999, 0.98926, 0.98553 & 0.88184). While c-metaphase and chromosome stickiness showed moderate positive correlation with AC (r = 0.70739 & 0.63756). However, lagging chromosomes and polar deviation had a strong negative correlation (r = -0.98594 & -0.92416) with AC and was found to be similar to that of 4 h treatment. Vagrant chromosome had showed strong positive correlation (r = 0.98297 & 0.98704) with MN and PP cells and MN showed similar trends with PP cells (r = 0.99972) (Figure7, Table S6).

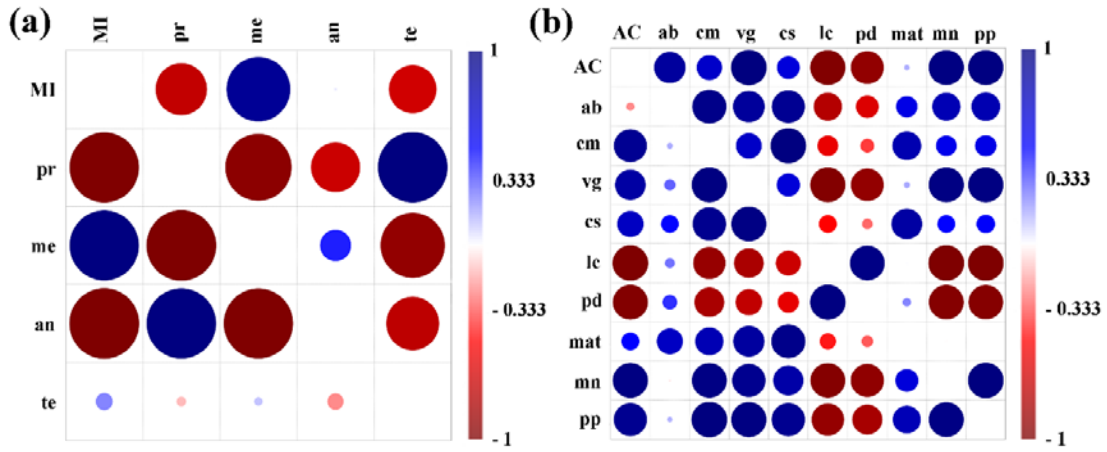

Figure 7. Pearson's correlation analysis for MI, dividing phase (a), and CA (b) after 4 h and 4+16 h of water recovery treatment with 3-epicaryoptin. Here, the lower triangle showed the correlation plot for 4 h and upper triangle showed the correlation plot for 4+16 h of treatment. Mitotic index (MI), prophase (pr), metaphase (me), anaphase (an), telophase (te), aberrant cells (AC), anaphase bridge (ab), c-mitosis (cm), vagrant (vg), chromosome stickiness (cs), laggard (lc), polar deviation (pd), multipolar anaphase-telophase (mat), micronucleus (mi), and polyploid cells (po).

4. Discussion

In the present study *A. cepa* tests system was used for the determination of cell cycle delay and cytotoxic effects of 3-epicaryoptin and was compared with the standard spindle poison compound colchicine. The *A. cepa* assay has been extremely useful methods for the biological monitoring, investigation of environmental pollution, determination of toxicity as well as evaluation of cytotoxic potentials of various chemical substances (Bakare et al., 2012; Bakare et al., 2013; Bakare et al., 2000; Frescura et al., 2012). Here, the antiproliferative and cell cycle delay inducing activities of 3-epicaryoptin was assessed by observing the root length retardation, swelling, MI%, and distribution frequencies of the different mitotic phase percentage (prophase, metaphase, anaphase and telophase). In addition, the types of structural CAs as well as MN and PP cells formation were also noted for the determination of aneugenic and/or clastogenic effects. The data exhibited statistically significant ($p < 0.01\&< 0.001$) *A. cepa* root growth retardation effect of 3-epicaryoptin with the IC_{50} value of 70.5 µg mL^{-1} at 48 h. The IC_{50} values for the root growth inhibition at 48 h is considered as reliable for the assessment of cytotoxic effects of tested compounds (Konuk et al., 2007). Generally, the apical meristematic activity is associated to the root growth retardation, to cell elongation and activation of enzymes, which promote the elongation and relaxing of cell wall during differentiation (Fusconi et al., 2006; Webster and MacLeod, 1996). Over 200 µg mL^{-1} concentration of 3-epicaryoptin treatment in *A. cepa* for 4 h treatment followed by 16 h recovery (4+16 h) showed the highest root tip swelling (club shaped) effects and that was similar to that of colchicine effects.

Several study reports showed that growth inhibitory effect of any chemical compound might have been manifested through its cytotoxic effect. Therefore, the present study was further extended to determine the effect of 3-epicaryoptin on the MI% and division cells percentage and CA on root meristem cells of *A. cepa*. 3-epicaryoptin (100, 150, 200 µg mL^{-1}) and

colchicine (200 µg mL^{-1}) treatment showed an increased frequency of MI at 2 and 4 h and again decreased after 16 h water recovery treatment as compared to control. The observed increased MI% might be due to arrest the cell cycle in metaphase at 2 and 4 h and later water recovery treatment for 16 h, cells revert to the interphase condition. The restitution of nuclei that they formed are PP interphase cells. These restitution causes to a fall in the MI% which was increased due to metaphase arrest during the early hours of treatment by 3-epicaryoptin and colchicine (Davidson et al., 1966). The Pearson's bivariate correlation analysis also indicates a strong positive correlation of increased metaphase with MI% at 4 h treatment. We have also compared the MI and the prophase: metaphase ratio of control, 3-epicaryoptin and colchicine treated samples. In 3-epicaryoptin (150µg mL^{-1}) and colchicine (200µg mL^{-1}) treated roots for 4 h, the mean MI% is 12.29% and 13.18% respectively and that is significantly ($p< 0.001$) different from untreated control roots (5.88 %). Thus, the increased MI% is significant in the early hours of treatment. On the other hand, prophase**:** metaphase ratio for 3-epicaryoptin (150 µg mL-1) is 23.22**:**66.20 and in the case of colchicine (200µg mL^{-1}) treated roots it is 11.3**:**82.35, whereas the untreated controls showed 42.58**:**28.84. These indicate an increased number of cells at metaphases in 3-epicaryoptin and colchicine treated roots may be due to as a results of metaphase arrest (Davidson et al., 1966). Besides these metaphase arresting effects, 3-epicaryoptin and colchicine exposure also induced the various CA like anaphase bridges, c-metaphase, vagrant chromosome, sticky chromosomes, c-mitosis, multipolar anaphase-telophase cells in the root tip cells of *A. cepa.* Study of these chromosome abnormalities has been considered to be a promising test to determine the cytogenotoxic potentials of the applied substances (Caritá and Marin-Morales, 2008). Majority of the observed abnormalities induced by 3-epicaryoptin and colchicine were associated with spindle poisoning effects such as c-metaphase, vagrant and disoriented chromosomes or chromatin dysfunction such as stickiness. Among these CAs, the highest

types of abnormalities induced by 3-epicaryoptin and colchicine was c-metaphase. It may be mainly occurred due to the irregular distribution of spindle apparatus and or microtubule destabilization effects. It is the spindle poisoning effect of colchicine, showed haphazardly arranged condensed chromosomes at metaphase and therefore blocks the cell progression from metaphase to anaphase (Bonciu et al., 2018; Fiskesjö, 1985). The induction of vagrant chromosomes results of precocious movement of chromosome in spindle poles which leads to an unequal number of chromosomes separation in the daughter nuclei and results the formation of daughter cells with unequal sized nuclei at interphase. The chromosome stickiness is considered to be a chromatid type aberration and its occurrence indicates abnormal DNA condensation, irregular chromosome coiling, chromosome fragmentation and formation of bridges at anapahase-telophase stages, and inactivation of the spindles (Yüzbaşioğlu et al., 2003; Badr and Ag, 1987; Klášterská et al., 1976).

In addition to the CAs, cells containing MN and PP were also observed at interphase stage in all the treated concentration (100, 150 & 200 µg mL^{-1}) of 3-epicaryoptin at 4 h treatment followed by 16 h water recovery. Pearson's correlation analysis showed a strong positive correlation of MN with PP. The most effective concentration of 3-epicaryoptin was found to be 150 µg mL^{-1}. Such type of MN cells can be originate by chromosomal break and results in the formation of acentric fragments (clastogenic action) or may be due to the loss of entire chromosomes as a consequence of dysfunction in normal spindle apparatus, therefore, was not incorporated with main nucleus during the course of cell division (aneugenic action) and ultimately may leads to the formation of aneuploid and poliploid cells in subsequent mitotic division (Chauhan et al., 1986; Chauhan and Sundararaman, 1990; Fenech and Crott, 2002; Yi and Meng, 2003). 3-epicaryoptin and colchicine induced the formation of such several MN in the treated cells must probably result from its aneugenic action rather than clastogenic. On the other hand, study of PP cells frequency (30.61±0.6, $p < 0.001$) formed by 3-

epicaryoptin treatment (150µg mL^{-1}) was more or less similar to the colchicine (200µg mL^{-1}) induced PP cells percentage (32.66±0.79, $p < 0.001$). Levan *et al* (1938) concluded that the colchicine induced formation of PP cells in *A. cepa* root tip cells were occurred due to disrupt in the mitotic spindle polymerization, thus prevent the migration of the chromosomes into the poles and remain dispersed throughout the cytoplasm (Levan, 1938). At this stage cytokinesis is not taking place and the chromatids eventually get enclosed with new nuclear membrane and proceed into interphase as a PP cell. Carvalho et al. (2019) also demonstrate that the PP cells arise when the mitotic spindle formation was hindered but the cell cycle was continued and enters into the G1 phase without completing the longitudinal chromosomes segregations, which would normally occur in anaphase and thus became PP (Carvalho et al., 2019). Thus 3-epicaryopptin induced increased metaphase (c-metaphase) cells frequency at early hours (2 and 4 h) and the formation of MN and PP cells at 16 h recovery may be due to its colchicine like microtubule destabilization based aneugenic effects.

5. Conclusions

The compound 3-epicaryoptin induced cell cycle delay, metaphase arrest, and high frequency of colchicine like metaphase (c-metaphase), MN, and PP cells in *A. cepa* root tips cells that are may be due to its colchicine like spindle poisoning effects. The 3-epicaryoptin showed a better persistent and effectiveness over cytotoxic actions of colchicine in *A. cepa* root tip cells; since it has continuously stimulated alterations even at 16 h water recovery. Therefore, to ascertain its chemotherapeutic applications there is urgent need for further therapeutic benefit and cytotoxic risk assessments using the different standard test systems.

Disclosure statement

No conflict of interest was declared.

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