Plant Archives Vol. 19 No. 2, 2019 pp. 2719-2725 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ASSESSMENT OF 'CYTOTHREAT' OF THREE CHEMOTHERAPEUTIC DRUGS USING ALLIUM TEST AND ITS AMELIORATION BY AQUEOUS PLANT EXTRACTS

Sudha Gupta, Animesh Kumar Datta*, Ankita Pramanik and Suvendu Dey

Department of Botany, University of Kalyani, Kalyani - 741235, West Bengal, India.

Abstract

Allium test is used to evaluate 'Cyto Threat' of three chemotherapeutic drugs namely, cisplatin, imatinib and harmine (concentrations used: 0.050%, 0.075% and 0.100%) with an objective to assess the extent of cytological damages as those drugs may induce in non-targeted cells of host (human beings) as well as in other biological organisms in ecosystem on exposure to the environment. Results suggest the followings: 1) the drugs affect DNA synthesis as well as induce cytological abnormalities in both dividing and resting cells and aberration frequency is found dose dependent; 2) imatinib and harmine are clastogenic in nature whereas all three drugs are found affecting cellular metabolism and 3) harmine is found to induce enhanced mitotic aberrations than the other studied drugs. Results detect the CytoThreat of the employed drugs and therefore risk monitoring of the drugs on environmental exposure is required apart from selecting unique dose for chemotherapy. Further, the present study encompasses the significance of aqueous plant extracts (seed extract of *Nigella sativa* and rhizome extract of *Curcuma longa*) in amelioration of cytotoxicity induced by the chemotherapeutic agents. The aqueous extracts may be helpful for bioremediation.

Key words: Allium test, Amelioration, Aqueous plant extracts, Bioremediation, Chemotherapeutic drugs, Cytotoxicity.

Introduction

Cisplatin (Cis-diamminedichloroplatinum (II); CIS-DDP), a platinum based anticancerous drug (Rosenberg, 1980; Desoize and Madoulet, 2002; Cepeda *et al.,* 2007; Florea and Büsselberg, 2011) is used worldwide as a potent chemotherapeutic agent (Kartalou and Essigmann, 2001; Fuertes *et al.,* 2003; Basu and Krishnamurthy, 2010). However, it is often accompanied by toxic side effects and secondary malignancies (Chen *et al.,* 2009). Imatinib (also known as "Glevec" or "Glivec"), called as "magical bullet", is a tyrosine kinase inhibitor and is especially used for treatment of chronic myeloid leukaemia–CML (Deininger *et al.,* 2005; Iqbal and Iqbal, 2014), gastrointestinal stromal tumors (GISTS) and other malignancies (Goswami *et al.,* 2016). Besides the synthetic chemotherapeutic drugs cisplatin and imatinib, a plant based (*Peganum harmala* L., Family: Zygophyllaceae) chemotherapeutic agent harmine (a natural β carboline alkaloid-Li *et al.,* 2017) is also a potent inhibitor of tumor development (Jiménez *et al.,* 2008). As the drugs are administered to human system, it is of utmost significance to assess their cytotoxicity as non-targeted cells of the host are also exposed to them.

Heath *et al.,* (2016) categorized the chemotherapeutic drugs as hazardous compounds, and are toxic to reproductive system. Apart from the concern of the drugs affecting non-targeted cells in host (as none of them are site specific), the residual amount excreted through faeces and urine and through improper handling can also induce detrimental effects on different components of ecosystem. Kosjek and Heath, (2011) highlighted the necessity to investigate "CytoThreat" (project funded by the European Community's 7th Framework Programme, 2011-2014, agreement n-265264) of chemotherapeutic drugs (parent compounds, metabolites and transformation products) for monitoring of environmental risk assessment. With the view to it, the present paper

***Author for correspondence :** E-mail : dattaanimesh@gmail.com

evaluates the cytotoxic effects of three chemotherapeutic drugs namely, cisplatin, imatinib and harmine in root tip meristematic cells of *Allium cepa* L. Allium test is used as it is simple, cost effective and an efficient method for assessment of cytotoxicity (Bellani *et al.,* 1991; Abu and Mba, 2011), and the results obtained mostly corroborate with other test organisms (Fiskesjö and Levan, 1993; Verma and Srivastava, 2018) including mammalian system (Teixeira *et al.,* 2003). Further, the study also encompasses whether or not there is any protective roles of aqueous plant extracts (seed extract of black cumin–*Nigella sativa* L. and rhizome extract of *Curcuma longa* L.) against cytotoxicity induced by the environmental pollutants (cisplatin, imatinib and harmine). The aqueous plant extract can be administered with relative ease by expending minimum cost. Seed extract of *N. sativa* (Majdalawieh and Fayyad, 2016; Mollazadeh *et al.,* 2017) and rhizome of *C. longa* (Sa *et al.,* 2010) are reported to possess potent cancer ameliorating effects.

Meterials and Methods

Chemotherapeutic Drugs

Cisplatin (Cytoplatin–50 Aqueous, Cipla; 50 mg/L injection dose), imatinib (Ibatkin–400 tablet, Oncocare) and harmine (Sigma) were the drugs studied for their cytotoxicity using Allium test. The concentrations used for the purpose were 0.100, 0.075 and 0.050%. For cisplatin, 50 mg was dissolved in 50 mL of double distilled water (ddH_2O) as source stock solution (0.100%); while each tablet of imatinib was 400 mg and it was dissolved in 40 mL of water (ddH_2O) to make 0.100%. Similarly, 0.100% harmine concentration was also prepared by dissolving 4.5 mg of the drug in 4.5 mL of ddH_2O. It is significant to note that 0.100% dose of cisplatin and imatinib are generally used in each chemotherapeutic treatment as referred by oncologists. Subsequent dilutions (0.075% and 0.050%) of the drugs were made in ddH_2O with an objective to assess cytotoxicity, if any, under low potency as residual effects.

Preparation of Plant Extracts

Seeds (2 g) of *Nigella sativa* L. (black cumin; Family Ranunculaceae) and shade dried (72 h) rhizomes (2 g) of *Curcuma longa* L. (Curcumin, Family: Zingiberaceae) were crushed to powdered samples by using liquid nitrogen (-80^0C). Each of the powdered sample was dissolved thoroughly in 25 mL of ddH_2O using a magnetic stirrer and subsequently filtered using Whatman No. 1 filter papers. In each case, 1 mL filtrate was taken and the volume of aqueous extract was made up to 100 mL (1.0%).

Treatments

Onion bulbs (*A. cepa* var. *aggregatum*, procured from farmers) were sprouted in sand-saw dust (1:1) trays and dipped in different concentrations (0.100%, 0.075% and 0.050%) of cisplatin, imatinib and harmine for 24 h durations. In each concentration (excepting harmine where 2 sprouted bulbs were treated in each concentration due to lesser amount of solution), 6 sprouted onion bulbs were dipped. Treatments were performed in Petri plates. Following 24 h treatment, 6 roots (2 from each of the 3 onion bulbs and in case of harmine 3 roots from each bulb) were cut, fixed in acetic-ethanol (1:1) for 30 mins and preserved in 70% ethanol for further uses. The treated onion bulbs were then dipped in aqueous extracts (3 bulbs in each extract following cisplatin and imatinib treatments; while one bulb each in case of harmine) of *N. sativa* and *C. longa* for 24 h duration, and following treatments 6 roots from each set were cut, fixed (acetic-ethanol in 1:1 ratio) and preserved (70% ethanol) under refrigeration ($16^0\pm1^0$C).

A control set was maintained ($24^0\pm1^0$C) under uniform laboratory condition(s) following treatment with ddH_2O for 24 h duration. The same stock of *A. cepa* bulbs were used throughout the experiments with 3 replicas for each set.

Assessment of Cytotoxicity

Cisplatin, imatinib, and harmine treated roots (including control roots) as well as roots concomitantly treated with aqueous extracts were cytologically evaluated following staining in 2% aceto-orcein in HCl (9:1) mixture and squashing in 45% acetic acid. For each set, 3 slides (each slide considering as replica) were prepared (2 root tips were squashed in each slide) and observed under Leitz Laborlux S compound microscope with Leica E3 scientific camera attached to it.

Mitotic index (number of dividing cells/total cells scored×100) and, aberration types recorded both in dividing and resting cells and their frequencies were estimated in relation to control.

Ameliorative potentiality (attributes studied: mitotic index and total aberration frequency in dividing and resting cells) of the aqueous plant extracts was also assessed with fold increase (+) or decrease (-) and in percentage in relation to respective control considering the measured values of each toxicant in each concentration.

Statistical Analysis

Data procured for dividing cell frequency and total aberration frequency in dividing and resting cells including untreated control were statistically analyzed to determine

significant variations, if any, between/among doses of treatment using one-way ANOVA (analysis of variance) and computation of CD (critical difference) at 0.05 level. Further, ameliorative potentialities between/among doses of treatments including toxicants were also assessed by similar statistical tool.

Results and Discussion

Cytotoxicity Induced by Cisplatin, Imatinib and Harmine

Mitotic index and types of cytological aberrations (Fig. 1a–l) detected in dividing and resting cells of untreated control and in chemotherapeutic drugs are presented in table 1.

Mitotic index

In relation to control, dose dependent significant ($p < 0.05$) reduction in dividing cell frequency is noted in treatments with cisplatin and harmine whereas both significant ($p < 0.05$) reduction (conc. - 0.05%) and enhancement (conc. - 0.10%) are studied following the drug imatinib. Thus, the chemotherapeutic drugs are found to alter cell cycle dynamics. Reduction in mitotic index by the drugs contributes to their significance in chemotherapy. Compared to control, mitotic index is found relatively more affected in cisplatin treatments than the other two drugs.

Basu and Krishnamurthy, (2010) opined that cisplatin kills cancer cells by interacting with DNA and inhibits its synthesis. Siddik, (2002) suggests that cisplatin induce DNA cross-links as well as DNA-protein cross links interfering with cell division. Non target specific interaction of cisplatin with DNA and cellular proteins (Cepeda *et al.,* 2007) is reported to inhibit DNA replication and cell division (Hartley, 1985; Farrel, 1989). Imatinib mesylate is found to induce acute toxic effects in mitotic cell division of *A. cepa* (Pichler *et al.,* 2014).

Aberration types and frequencies

Untreated control show 2n = 16 chromosome at metaphase (Fig. 1a). The only aberration studied in control is sticky and clumped configuration of chromosomes in 3.81% cells. Aberrations encountered in chemothera-peutic drugs treated mitotic cells are sticky and clumped configuration of chromosomes (Fig.1b), pseudo-chiasma formation (Fig.1c), chromosomal groupings (Fig. 1d), chromosomal fragments (Fig. 1e), rings (Fig. 1f), laggards (Fig. 1g) and bridges (Figs. 1h-i) in dividing cells and micronuclei (Fig. 1j), giant (Fig. 1k), binucleate (Fig. 1l) and anucleate cells formation in resting cells. Most of the giant cells observed in all treatments are with cellular shape

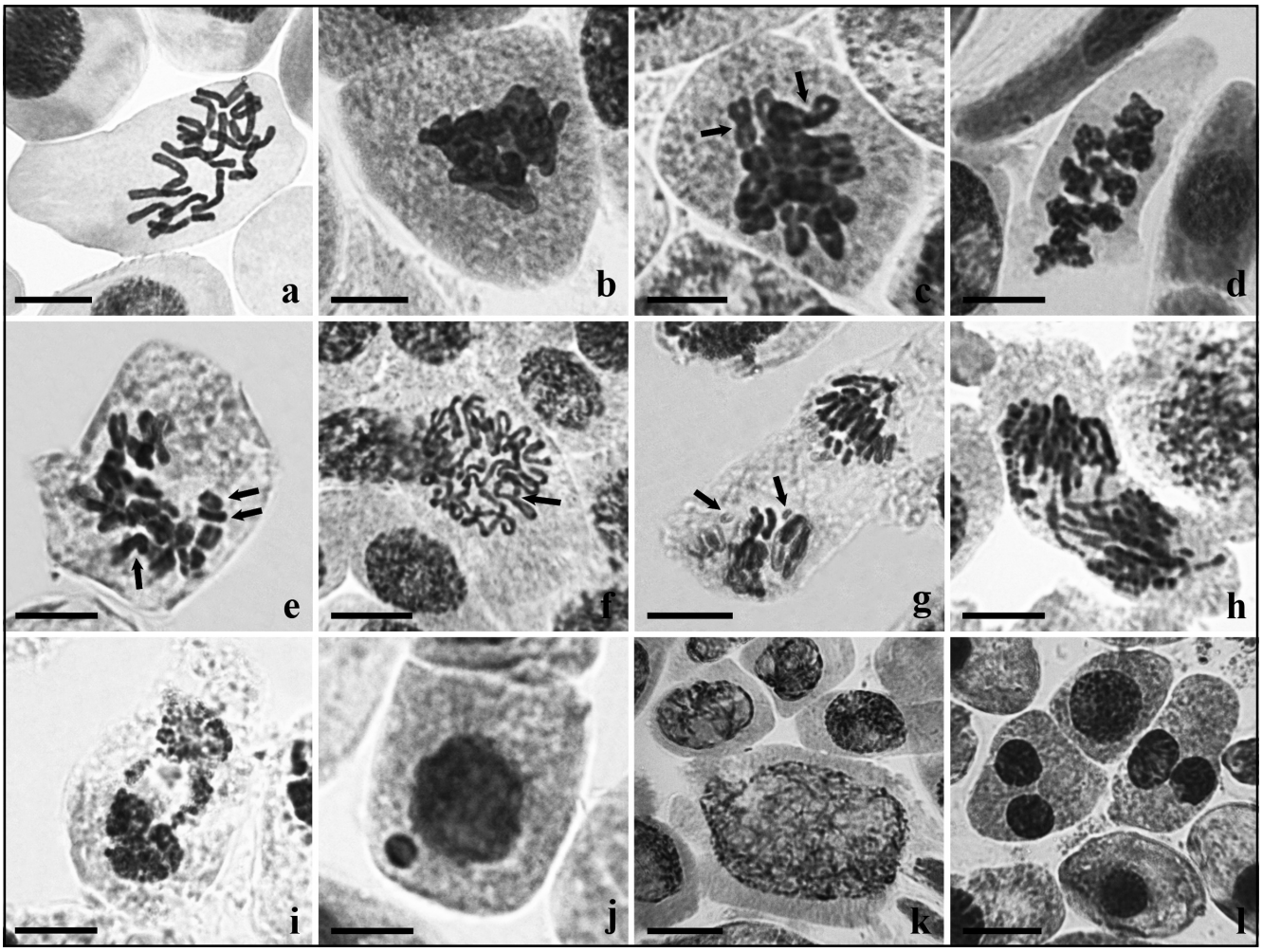
Table 1: Assessment of cytotoxicity of three chemotherapeutic drugs in root tip meristem cells of *A. cepa*.

Treatments	Doses (%)	Total no. of cells scored	Total no. of diving cells	Mitotic index (%)	Abnormality in dividing cells (%)							Frequency of abnormal-dividing cells (%)	Total Resting cell	Abnormality in resting cells (%)				Frequency of abnorma-lresting cells (%)
					Sticky and Clumping	Pseudochiasma	Grouping	Ring	Fragment(s)	Laggard(s)	Bridge(s)			Micronuclei	Anucleate	Giant	Binucleate	
Control	0	2245	289	12.87	3.81	0.00	0.00	0.00	0.00	0.00	0.00	3.81	1956	0.00	0.00	0.00	0.00	0.00
Cisplatin	0.050	2437	242	9.93	4.96	0.83	0.83	0.00	0.00	0.41	0.00	7.02	2195	0.00	0.00	2.28	0.00	2.28
	0.075	2244	173	7.71	13.87	0.58	2.31	0.00	0.00	0.58	0.00	17.34	2071	0.00	0.00	3.61	0.39	4.00
	0.100	9625	427	4.44	20.37	0.70	0.70	0.00	0.00	0.00	0.00	21.78	9198	0.00	0.63	2.70	0.58	3.91
Imatinib	0.050	2436	198	8.13	3.54	0.00	0.00	0.00	0.51	0.00	0.51	4.55	2238	0.54	0.00	6.67	0.63	7.84
	0.075	2417	321	13.28	6.85	0.00	0.00	0.00	0.93	0.00	0.62	8.41	2096	0.91	0.00	10.02	1.77	12.70
	0.100	1876	363	19.35	7.71	0.00	2.75	1.10	1.65	0.00	0.00	13.22	1513	0.93	0.59	14.14	1.85	17.51
Harmine	0.050	1838	210	11.43	7.14	0.95	2.86	1.43	6.19	3.81	4.29	26.67	1628	0.74	0.00	8.11	1.41	10.26
	0.075	2120	203	9.58	22.17	0.99	2.46	0.99	4.93	3.94	2.46	37.93	1917	0.73	0.00	13.20	2.03	15.96
	0.100	1845	134	7.26	31.34	0.75	4.48	0.75	1.49	0.75	0.75	40.30	1711	1.17	2.28	15.30	0.64	19.39
CD at 5% level				2.26								2.96						2.29

Table 2: Ameliorative potentiality of two aqueous plant extracts against cytotoxicity studied in root tip cells of *A. cepa*.

Treatment with doses (%)	Total cells scored	Mitotic Index			Frequency of abnormal dividing cell (%)			Total resting cells	Frequency of abnormal resting cell (%)		
		Observed Value	Fold decrement/ increment	Ameliorating effect (%)	Observed Value	Fold decrement	Ameliorating effect (%)		Observed Value	Fold decrement	Ameliorating effect (%)
Cisplatin 0.050	**2437**	**9.93**	—	—	**7.02**	—	—	**2195**	**2.28**	—	—
Cisplatin 0.050 + *N. sativa*	1738	4.78	-5.15	+51.86	0.00	-7.02	-100.00	1655	2.05	-0.23	-10.09
Cisplatin 0.050 + *C. longa*	2723	10.98	+1.05	+10.57	4.01	-3.01	-42.88	2424	2.15	-0.13	-5.70
Cisplatin 0.075	**2244**	**7.71**	—	—	**17.34**	—	—	**2071**	**4.00**	—	—
Cisplatin 0.075+ *N. sativa*	1689	8.76	+1.05	+13.62	0.00	-17.34	-100.00	1541	3.70	-0.30	-7.50
Cisplatin 0.075 + *C. longa*	2364	12.10	+4.39	+56.94	6.64	-10.70	-61.71	2078	3.46	-0.54	-13.50
Cisplatin 0.100	**9625**	**4.44**	—	—	**21.78**	—	—	**9198**	**3.91**	—	—
Cisplatin 0.100 + *N. sativa*	3010	10.50	+6.06	+136.49	4.75	-17.03	-78.19	2694	3.04	-0.87	-22.25
Cisplatin 0.100 + *C. longa*	3120	10.03	+5.59	+125.90	7.35	-14.43	-66.25	2807	2.71	-1.20	-30.69
CD value at 5% level		1.42			1.16				1.09		
Imatinib 0.050	**2436**	**8.13**	—	—	**4.55**	—	—	**2238**	**7.84**	—	—
Imatinib 0.050 + *N. sativa*	2009	14.88	+6.75	+83.03	3.68	-0.87	-19.12	1710	4.33	-3.51	-44.77
Imatinib 0.050 + *C. longa*	1933	13.66	+5.53	+68.02	3.79	-0.76	-16.70	1669	5.15	-2.69	-34.31
Imatinib 0.075	**2417**	**13.28**	—	—	**8.41**	—	—	**2096**	**12.70**	—	—
Imatinib 0.075+ *N. sativa*	2004	9.53	-3.75	-28.24	0.00	-8.41	-100.00	1813	5.52	-7.18	-56.54
Imatinib 0.075 + *C. longa*	2042	9.21	-4.07	-30.65	0.00	-8.41	-100.00	1854	3.83	-8.87	-69.84
Imatinib 0.100	**1876**	**19.35**	—	—	**13.22**	—	—	**1513**	**17.51**	—	—
Imatinib 0.100 + *N. sativa*	2003	9.19	-10.16	-52.51	11.41	-1.81	-13.69	1819	4.67	-12.84	-73.33
Imatinib 0.100 + *C. longa*	1890	9.05	-10.30	-53.23	0.00	-13.22	-100.00	1719	3.20	-14.31	-81.72
CD value at 5% level		1.59			0.72				1.68		
Harmine 0.050	**1838**	**11.43**	—	—	**26.67**	—	—	**1628**	**10.26**	—	—
Harmine n 0.050 + *N. sativa*	1690	9.88	1.55	13.56	0.00	-26.67	-100.00	1523	6.30	-3.96	-38.60
Harmine 0.050 + *C. longa*	1960	8.42	3.01	26.33	0.00	-26.67	-100.00	1795	4.29	-5.97	-58.19
Harmine 0.075	**2120**	**9.58**	—	—	**37.93**	—	—	**1917**	**15.96**	—	—
Harmine 0.075+ *N. sativa*	2110	11.47	-1.89	-19.73	16.94	-20.99	-55.34	1868	3.75	-12.21	-76.50
Harmine 0.075 + *C. longa*	2134	8.34	1.24	12.94	0.00	-37.93	-100.00	1956	5.06	-10.90	-68.30
Harmine 0.100	**1845**	**7.26**	—	—	**40.30**	—	—	**1711**	**19.39**	—	—
Harmine 0.100 + *N. sativa*	1894	11.03	-3.77	-51.93	16.75	-23.55	-58.44	1685	6.11	-13.28	-68.49
Harmine 0.100 + *C. longa*	1887	14.26	-7.00	-96.42	11.90	-28.40	-70.47	1618	6.37	-13.02	-67.15
CD value at 5% level		0.79			2.92				1.75		

Bold value represents toxicant



Figs. 1a–l: Mitosis in control **(a)** and in anticancerous drugs treated cells **(b-l)** at pro-metaphase and metaphase (a-f), anaphase (g-h), telophase (i) and resting (j-l) stages of *Allium cepa*. **(a)** 2n=16, **(b)** Clumped and stickiness of chromosomes, **(c)** Pseudochiasma formation, **(d)** Chromosomal groupings, **(e)** Fragments (arrows), **(f)** Rings (arrow), **(g)** Laggards (arrows), **(h-i)** Bridges, **(j)** Micronuclei, **(k)** Giant cells, **(l)** Binucleate cells. *Scale bar = 10 μm*

deformity. However, chromosomal fragments, rings, bridges and micronuclei are not observed in cisplatin treatments. Anucleate cells are only observed in 0.10% doses of the drugs. Clumping and stickiness of chromosomes (cisplatin: 0.51% to 1.83%; imatinib: 3.54% to 7.71%; harmine: 7.14% to 31.34%) and giant (cisplatin: 2.28% to 3.61%; imatinib: 6.67% to 14.14%; harmine: 8.11% to 15.30%) and binucleate (cisplatin: 0.00% to 0.58%; imatinib: 0.63% to 1.85%; harmine: 0.64% to 2.03%) cells are the predominant aberrations noted following treatments with the studied drugs.

In relation to control, cisplatin, imatinib and harmine induced mitotic abnormalities in both dividing and resting cells are enhanced significantly ($p < 0.05$) and it is mostly dose dependent (excepting: 0.10% conc. in resting cells). Harmine is found to induce higher cytotoxicity in meristematic cells of *A. cepa* than the other two drugs, and it may possibly be attributed to the crude nature of harmine used in the present investigation. Assessment of cytotoxicity reveals that both imatinib and harmine are clastogenic in nature as they can induce chromosomal breakages (fragments, rings, bridges and micronuclei) whereas all the studied drugs can affect chromosomal DNA (clumping and stickiness) and cellular metabolism (formation of giant, binucleate and anucleate cells). However, it is reported that most of the chemotherapeutic drugs can induce apoptosis through different signalling pathways reducing DNA damages and eliminating necrotic cells from the system (Kartalou and Essigmann, 2001; Li *et al.,* 2017).

Cisplatin is reported to be cytotoxic in human breast and cervical cancer cells (Lanza *et al.,* 2004). The drug can interact with chromosomal DNA (Sherman *et al.,* 1985) causing DNA damages but also possess the ability to response to DNA repair mechanism (Kerr *et al.,* 1994). Russo *et al.,* (2018) opined that imatinib mesylate

can induce DNA damage in crustacean *Daphnia magna* even at low concentrations. Cytotoxicity of harmine is evaluated in four different human cell lines (CBMN, Hela, X33A and Sw 480) using micronuclei assay, and the result suggests that the drug is unable to induce micronuclei levels above that of control levels in a wide range of doses administered (Jiménez *et al.,* 2008). This report is rather contrary to the result obtained with harmine in the present investigation.

Ameliorative potentiality of aqueous plant extracts

Data (dividing cell frequency and total aberration frequency in dividing and resting cells) relating to aqueous plant extracts (extracts of *N. sativa* seeds and *C. longa* rhizome) inducing amelioration in cytotoxicity caused due to chemotherapeutic drugs treatments is presented in table 2. Compared to toxicants (represented as control) at different doses, treatment with aqueous plant extracts show significant ($p < 0.05$) decrement (-) in total cytotoxicity assessed in both dividing and resting cells at variable folds. However, dividing cell frequency manifests significant ($p < 0.05$) increment (+) as well as decrement (cisplatin and imatinib) in relation to toxicants; although, only decrement (significant at $p < 0.05$ level) is noted with harmine treatments. Results suggest that both the aqueous extract possess significant ameliorative potentiality and are effective in reduction of cytotoxicity. Aqueous extracts of *N. sativa* and *C. longa* demonstrate differential ameliorative responses in relation to the attributes and drugs studied. Aqueous plant extracts are reported to be protective against cytotoxicity assessed in root tip cells of *A. cepa* following H_2O_2 (Prajitha and Thoppil 2016; use of leaf extract of *Amaranthus spinosus*) and arsenic trioxide and metanil yellow (Basu *et al.,* 2019; use of seed extract of *N. sativa* and leaf extracts of *Coriandrum sativum*, *Ocimum tenuiflorum* and *Pteris vittata*) treatments.

Present investigations highlight the followings: 1) compared to control, the drugs induce differential responses in relation to dividing cell frequency. Cisplatin and harmine reduce mitotic index dose dependently, and it is in accordance with the efficacy of anticancerous drugs; however, imatinib shows both increase as well as decrease in mitotic index, 2) chemotherapeutic dose (0.100%) in cisplatin and imatinib as well as that of harmine are found to induce cytotoxicity in both dividing and resting cells thereby suggesting the drugs can be of 'CytoThreat' to non-targeted cells of host (human beings) if proper repair mechanism does not prevail. Further, lower doses of the drugs (degradable amount) are also found cytotoxic, a major concern to eco-system, 3) employed aqueous plant extracts are ameliorative in relation to cytotoxicity and can be significant for bioremediation. Therefore, it is suggested that aqueous plant extracts of *N. sativa* (seed) and *C. longa* (rhizome) may be taken together with chemotherapeutic drugs as preventive measures.

Acknowledgment

The authors are thankful to DST-PURSE programme, Kalyani University (Kalyani University (Award No. SR/PURSE Phase 2/37 (G) dated 2nd November 2017) for providing necessary funding and facilities. Thanks are also extended to Dr. Tapas Majumder and his research students Shri Monaj Karar, Department of Chemistry, Kalyani University for generous supply of the chemotherapeutic drug harmine. Authors are also thankful to Ms. Sharmila Khatun for providing required variety of onions from farmer's house to carry out the experiment.

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