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Genotoxic effect of *Clerodendrun infortunatum* L. aqueous leaf decoction on root meristem cells of *Allium cepa* L.

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Abstract: ***Clerodendrun infortunatum*** **L, conventionally used as traditional and folk medicine, has scientifically validated pharmacokinetics; nevertheless, an assessment of toxicity and mutagenicity must precede its safe and efficient therapeutic use. Macroscopic (root morphology) and microscopic (Mitotic Index MI, Chromosomal Aberrations CA, Micronuclei Index MN) parameters of *Allium cepa* test were employed to access toxicity of the plant extract. Marked alteration in root growth and form, significant reduction in MI and abundance of CA specially chromosome clumping and presence of micronuclei, at higher concentration and duration of treatment, supported by statistical analyses, indicate dose dependent, severe, lethal genotoxicity of the aqueous leaf extract. Appropriate administration in minimal doses is thus recommended for therapeutic application.**

Index Terms: ***Clerodendrum infortunatum*** **L.,** ***Allium cepa*** **test, macroscopic and microscopic parameters, genotoxicity**

INTRODUCTION

Clerodendrun infortunatum L. (family Lamiaceae) is an indigenous medicinal plant widely used in Ayurvedic, Unani, Chinese, African and other traditional systems of medicine; with a repertoire of pharmacologically active constituents- alkaloids, terpenoids, flavonoids, steroids, saponins, glycosides, tannins, polyphenols and carbohydrates (Haris & Mahmood, 2016; Verma & Gupta, 2014; Wang, *et al* 2018). Aqueous extracts of the plant have been traditionally used to treat cold, asthma, fever, indigestion, pain, dysentery, rheumatism, skin diseases tumor, post-natal complications, leprosy and inflammatory diseases (Ghosh, 2012; Verma & Gupta, 2014; Wang, *et al* 2018). Methanolic extract of leaves have antidiabetic (Das, *et al* 2011) and memory enhancing effects in rat and mice (Gupta & Singh, 2012); ethanolic, ethyl acetate, hexane and chloroform extract show in *vitro* antioxidant (Gouthamchandra, *et al* 2010), antimicrobial (Waliullah, *et al* 2015) and anticancer activities (Haris & Mahmood, 2016).

Aqueous decoctions used in traditional medicine contain complex mixture of biologically active components; synergistic interaction and multifarious effects of the phytoconstituents, lend it therapeutic activity (Gilbert & Alves, 2003). Aqueous extract of *C. infortunatum* contain bioactive constituents such as saponins, flavonoids, tannins (Verma & Gupta, 2014) and terpenoids, but can become toxic (Gadano, *et al* 2006; Paes-Leme, *et al* 2005) and cause chromosomal damage with prolonged use or with overdose (Celik & Aslantürk, 2010; Chaudhury & Ray, 2014; Ping, *et al* 2012). Potential toxicity of the aqueous extracts can be assessed using plant as test system, being easier to perform and adapted by International Program on Plant Bioassay and United Nations Environmental Program (Ma, *et al* 1999; Leme & Marin-Morales, 2009) for monitoring toxicity of environmental and biological samples.

Allium cepa test has been an efficient, sensitive, reliable, *in vivo* bioassay, based on fast response in root growth dynamics and convenient chromosomal features- good chromosome size, low number, stable karyotype (Firbas & Amon, 2014; Fiskesjö, 1985; Leme & Marin-Morales, 2009; Levan, 1938), that allow easy identification of distinct mitotic phases and chromosomal aberrations; with good correlation with mammalian system (Grant & Salamone, 1994). Sensitivity of the test relies upon fast

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response in root growth dynamics and easy identification of genotoxic end points- changes in Mitotic Index (MI), appearance of Chromosomal Aberrations (CA) and Micronuclei Index (MN) (Bonciu, *et al* 2018; Fiskesjö, 1985; Leme & Marin-Morales, 2009).

Earlier studies on cytotoxic effects of the aqueous leaf extracts of *Clerodendrum* on *Allium cepa* L., at different concentrations for 4hours (Kundu & Ray, 2017) and different dilutions for 5 days (Roy & Roy, 2019) have revealed significant reduction in MI and a range of CA at higher concentrations and dilutions, but have not considered the macromorphological aspect of the *Allium cepa* test. Here we investigate the genotoxic potential of the aqueous leaf extract, through a treatment regime based on early changes in root growth morphology (macroscopic parameter) and assessment of genotoxic end points (microscopic parameter) using the *Allium cepa* test.

MATERIALS AND METHODS

Clerodendrum infortunatum L was collected from West Tripura and voucher specimen submitted to Departmental Herbarium. Leaves were separated, surface sterilized, dried, weighed, powdered and the powdered material was kept in airtight vials for experimental use. Fresh healthy bulbs of onion (*Allium cepa* L.) were obtained from local market; equal sized bulbs selected and kept 2-3 days for rooting.

Decoction of powdered leaf in different concentrations (5mg/ml, 10mg/ml and 15mg/ml) were made with tap water and filtered. Tap water was used as negative control. Twenty equal sized *Allium* bulbs were kept with their root zone immersed in water for rooting. A preliminary trial was performed to select concentrations of leaf decoction, wherein root necrosis occurred beyond 20mg/ml, 6hours of treatment. Onion bulbs with 1-2 cm long slender, healthy roots were then exposed to 5mg/ml, 10mg/ml, 15mg/ml for two days, but root necrosis at 15mg/ml, 42 hours limited the exposure duration to 42hours. Observations on root growth, length and morphology were recorded, thereafter roots excised for cytological procedure, at 6 hours interval up to 42 hours. All treatments were performed in triplicate. The excised root tips of *Allium cepa* L. were fixed in 1:3 acetic: alcohol overnight. Fixed root tips were rinsed in distilled water, kept in 45 % acetic acid for 5 minutes, stained with 9:1 aceto-orcein: HCl solution, warmed, kept for an hour and squashed in 45% acetic acid (Sharma & Sharma, 1980). Slides were prepared for cytological observation and photomicrographs obtained under Zeiss AxioScope 1 microscope.

Metric observations include number of cells in different mitotic phases, number of aberrant cells, number of dividing cells and number of cells in microscopic field (at 40X objective). Mitotic Index (MI), Chromosomal Aberration (CA) and Micronuclei Index (MN) calculated as:

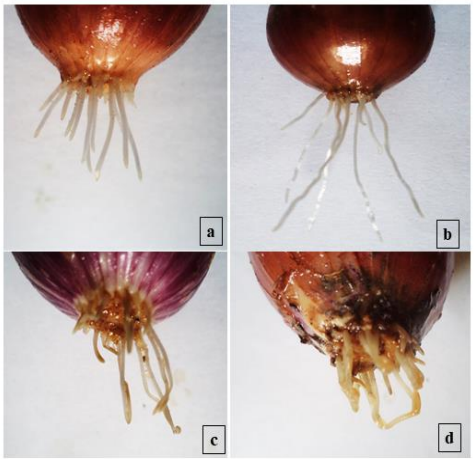
$$MI = \frac{\text{Total number of dividing cells}}{\text{Total number of cells}} \times 100$$

$$CA = \frac{\text{Number of aberrant cells}}{\text{Number of dividing cells}} \times 100$$

$$MN = \frac{\text{Number of Micronuclei}}{\text{Number of nondividing cells}} \times 100$$

At least 2000 cells were scored for each concentration and duration of treatment along with control. The mean values, standard deviation and standard error of mean were calculated for each attribute. Two-way and One-way ANOVA with post hoc Tukey's Test were performed at $p<0.05$ significance level.

Figure 1 Root morphology at tap water treatment (a-6 hours, b-42 hours); root morphology at 15mg/ml aqueous leaf decoction treatment (c-6 hours, d- 42 hours)



RESULTS

Leaf decoction of *Clerodendrum infortunatum* L. impeded root growth of *Allium* (Table I), induced cell division intervention (Table II) and a range of chromosomal and nuclear aberrations in *Allium cepa* root meristem cells (Table3). The genotoxic effect of the leaf extract is evident in dose dependent reduction in root length, MI and abundance of CA, MN. Drastic change in root length, growth and form was noted beyond 10mg/ml, 12hours treatment with leaf extract. Roots got bent, slimy, slackened, necrotic and growth ceased at and beyond 15mg/ml, 36 hours treatment (Fig.1), limiting the treatment duration. Statistically significant reduction in root length at 5mg/ml (69.81%), 10mg/ml (59.91%) and 15mg/ml (59.46%) was noted over control. A general reduction in MI as compared to control and marked reduction in MI at 5mg/ml (15.08%), 10mg/ml (9.34%) and 15mg/ml (6.10%) was noted. Statistical analyses show reduction in MI to be significantly affected by both concentration and duration of treatment. A range of chromosomal aberrations -clumped and sticky chromosomes, c-mitosis, anaphase and telophase bridge, disorientation, polyploid

Table I Root morphology of *Allium cepa* treated with leaf extract of *Clerodendrum infortunatum* as compared to negative control

Control and treatments	**Root length at different hours of treatment with leaf infusion (cm)**								**Form, growth and color**
	6 hours	**12 hours**	**18 hours**	**24 hours**	**30 hours**	**36 hours**	**42 hours**	**Mean ±SEM**	
Control	1.15	1.40	1.70	2.30	2.80	3.40	4.00	2.22±0.34[a]	Straight, fast, white
5mg/ml	1.35	1.50	1.60	1.65	1.68	1.70	1.70	1.55±0.07[b]	Thin, slow, light brown
10mg/ml	1.18	1.27	1.39	1.44	1.45	1.45	1.45	1.33±0.06[b]	Thin, slacken, very slow, brown
15mg/ml	1.18	1.28	1.37	1.38	1.43	1.43	1.43	1.32±0.04[c]	Slacken, slimy, bent, growth arrest, dark brown

Root length at treatment duration taken as average of three readings; similar letters not significantly different at 5% confidence level using Post Hoc Tukey's test

Table II Mitotic Index of *Allium cepa* cells treated with aqueous leaf infusion of *C. infortunatum* and negative control

Control and extract concentrations	**Mitotic Index at different concentration and treatment duration ± SEM**						
	6hours	**12hours**	**18hours**	**24hours**	**30hours**	**36hours**	**42hours**
Control	11.69±0.16[a]	11.68±0.19[a]	9.52±0.16[b]	9.15±0.11[c]	6.94±0.20[e]	8.09±0.16[a]	5.57±0.14[a]
5mg/ml	8.62±0.16[a]	7.87±0.23[a]	5.45±0.25[b]	5.51±0.28[c]	4.41±0.25[e]	2.61±0.23[f]	0.84±0.11[a]
10mg/ml	7.57±0.24[a]	5.66±0.17[a]	6.64±0.20[d]	4.22±0.25[a]	2.23±0.31[c]	0.84±0.19[f]	0.52±0.12[f]
15mg/ml	5.96±0.22[a]	5.04±0.27[a]	1.38±0.30[c]	1.56±0.27[a]	1.00±0.25[a]	0.93±0.21[f]	0.34±0.11[f]

similar letters are not significantly different at 5% confidence level using Post Hoc Tukey's test

Table III Aberrant cell types and Chromosomal Aberration Rate of *Allium cepa* cells treated with aqueous leaf infusion of *C.infortunatum*

Aberrant Cell Type	**Aberrant and dividing cells*at treatment duration**								**Chromosomal Aberration Rate**
	6 hours	**12 hours**	**18 hours**	**24 hours**	**30 hours**	**36 hours**	**42 hours**	**Total**	
Metaphase Clumping	12	17	4	12	8	6	2	**61**	**2.72**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
Sticky Anaphase	7	6	9	6	3	3	0	**34**	**1.51**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
Binucleate cell	11	0	5	4	0	0	0	**20**	**0.89**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
Anaphase disorientation	1	2	2	0	0	0	0	**5**	**0.22**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
C-mitosis	3	5	5	9	1	2	2	**27**	**1.20**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
Bridge	0	3	6	3	4	0	0	**16**	**0.71**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
Vagrant	0	0	3	2	0	0	0	**5**	**0.22**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
Endomitosis	0	0	0	0	3	4	0	**7**	**0.31**
	611*	521*	393*	339*	210*	122*	50*	**2246***	
Micronuclei	0	0	0	4	0	2	6	**12**	**0.53**
	611*	521*	393*	339*	210*	122*	50*	**2246***	

cell, binucleate cell and micronuclei were observed (Fig.2) of which metaphase clumping was most abundant. More aberrant cells (6.42% at 5mg/ml, 7.31% at 10mg/ml, and 13.22 at 15mg/ml) and micronuclei (0.07% at 5mg/ml,10mg/ml and 0.11 at 15mg/ml) were observed at higher concentration and duration of treatment. Reduction in MI, frequency of CA and MN were calculated (Fig.3).

Figure 2 Chromosomal aberrations in *Allium cepa* root meristem cells with leaf extract treatment-a) Telophase Bridge; b), i) Anaphase disorganization; c) Micronuclei; d) Sticky Anaphase; e) Metaphase Clumping; f) Binucleate cell at prophase; g) Anaphase Bridge; h), k) c-mitosis; j) Anaphase laggard; l) endomitosis. Bar=10µm

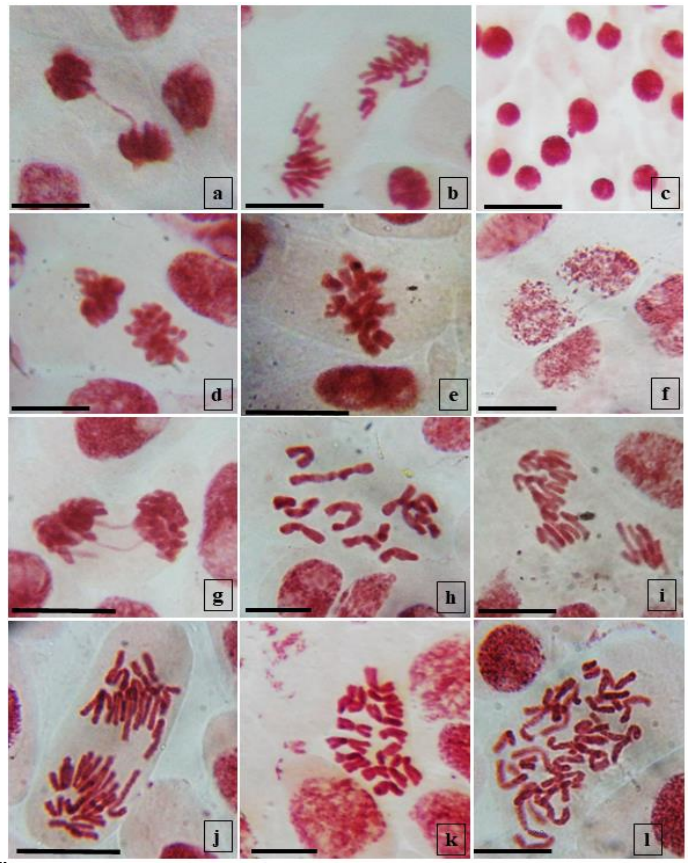
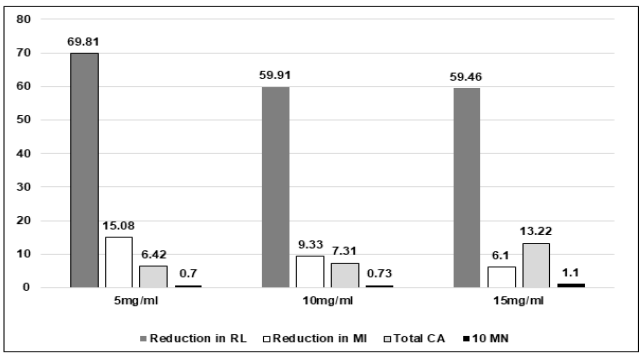


Figure 3 Reduction in MI, CA and MN at treatment concentrations



DISCUSSION

Potential toxicity and mutagenicity of *Clerodendrum infortunatum* aqueous leaf decoction was assessed, employing macroscopic parameter of root growth morphology and microscopic parameter of genotoxic end points in the *Allium cepa* test (Fiskesjö, 1985; Leme & Marin-Morales, 2009). Although macroscopic and microscopic parameters are correlated (Akinboro & Bakare 2007; Fiskesjö, 1985) yet the macroscopic parameters are more sensitive (Fiskesjö, 1985). Standard protocols on routine testing of genotoxicity recommend a preliminary experiment for choosing the highest concentration (Kanaya, *et al* 1994) and inclusion of macroscopic parameter of root growth and form (Bonciu, *et al* 2018; Fiskesjö, 1985) along with the microscopic parameters of genotoxic end points.

In earlier studies on toxicity of *Clerodendrum* leaf extract, 4hours (Kundu & Ray, 2017) and 5days (Roy & Roy, 2019) were chosen as treatment duration, however, no details of preliminary trial or root growth observations were cited. In the present study both macroscopic and microscopic parameters of *Allium cepa* test taken into account, preceded by preliminary experiment to choose highest concentration and treatment duration.

Changes in root growth, turgescence, color and malformation form dependable macroscopic parameters that indicate cytotoxic damage (Firbas & Amon, 2014; Fiskesjö 1985). Slimy, bent, brown and necrotic roots observed at higher concentration and duration of treatment with leaf extract, accompanied by concentration dependent and statistically significant ($p<0.05$) root growth inhibition with an EC_{50} 3.991 indicates severe toxicity.

Deviation from the orderly, directed progression of the cell cycle evident in alteration of MI, appearance of chromosomal and nuclear aberrations upon treatment with leaf extract (Bonciu, *et al* 2018; Leme & Marin-Morales, 2009), indicate genotoxicity. Reduction in MI is a reliable and sensitive indicator of genotoxicity (Leme & Marin-Morales, 2009; Smaka-Kincl, *et al* 1996), a general reduction in MI was observed in treated cells of *Allium*. Average MI of control-8.95 was higher than that recorded after treatment with 5mg/ml (5.04), 10mg/ml (3.95) and 15mg/ml (2.32) leaf extract. Reduction in MI ranging from 6.10-15.08% was observed in the present study; 40% reduction in MI was recorded in an earlier study (Kundu & Ray, 2017). Reduction below 22% of negative control (as observed at all concentrations in this study) can have lethal impact (Antonsie-wiez, 1990) on organism. MI reduction below 50% is referred to as the cytotoxic limit value (Panda & Sahu, 1985; Rank & Nielsen, 1997). Reduction in MI indicates antiproliferative effect (Gadano, *et al* 2002), and may be due to cell cycle arrest, delay, slow progression of cells to mitotic phase, inhibition of DNA synthesis or disruption of spindle function (Kundu & Ray, 2017).

Chromosomal aberrations possibly appear due to DNA, protein, chromosome damage; DNA breaks, inhibition of DNA synthesis and repair, or replication of mutated/ altered DNA (Nefic, *et al* 2013). Leaf decoction treated cells of *Allium* recorded 8.41% of CA in the present study, earlier studies recorded 3.81% (Kundu & Ray, 2017) and 8.36% (Roy & Roy, 2019) of CA. Metaphase clumping and stickiness may appear due to increased chromosome condensation, DNA polymerization and inter chromosomal linkages of chromosome strands, coupled with inappropriate nucleoprotein formation and protein-protein interactions (Ford & Correll, 1992; Kundu & Ray, 2017; Nefic, *et al* 2013). According to Kuras (2004), change in ratio of histones and nucleoproteins leads to atypical metaphase and anaphase, inhibition of cytokinesis and formation of binucleate cells (Fernandes, *et al* 2007). Alteration in spindle function lead to vagrant, precocious movement of chromosomes and anaphase disorganization; spindle poisoning leads to c-mitosis; and inhibition of tubulin polymerization or cytoskeletal protein lead to formation of laggards. Bridges result from exchange between homologous and nonhomologous chromosomes, may be due to intervention of replication machinery, consequence of dicentric chromosome formation and faulty longitudinal separation of sister chromatids during anaphase (Bonciu, *et al* 2018). Polyploid cells usually appear due to segregation defects (Nefic *et al* 2013, Bonciu *et al* 2018). C-mitosis indicates weak toxicity, bridges and laggards indicate moderate toxicity, metaphase clumping and stickiness indicate high, irreversible toxicity associated with cell death (Fiskesjö 1985). Presence of fragmented nuclei, nuclear buds and polynuclear cells are interphase aberrations that indicate cell death process (Nefic *et al* 2013). Micronuclei appear due to DNA rearrangements during chromosomal and nuclear events that fail to incorporate into main nucleus (Nefic *et al* 2013; Bonciu, *et al* 2018; Leme & Marin-Morales, 2009) and can be observed at prophase and interphase. MN range from 0.07% to 0.11% of interphase and 0.20% to 0.85% of prophase in the present study, earlier studies recorded more MN- 0.38% (Kundu & Ray, 2017) and 1.25% to 1.32% (Roy & Roy, 2019). These aberrations are indicators of clastogenic, aneugenic and turbagenic effects of the leaf extract.

Sensitivity and efficiency of the *Allium cepa* test lies in its parameters of toxicity- root growth and form and indicators of genotoxicity -reduction in MI, frequency of CA and MN. Marked alteration in root growth and form indicate severe toxicity. Reduction in MI below 22% indicates lethal effect, 8.41% of CA with high frequency of metaphase clumping and presence of micronuclei indicate high genotoxicity. The present study is partly in accordance with earlier reports (Kundu & Ray, 2017; Roy & Roy, 2019), however, record higher reduction in MI and higher frequency of CA. Statistical analysis through ANOVA and post hoc Tukey's test indicate that the changes in root length were significantly affected by concentration, changes in MI were significantly affected by both concentration and duration of treatment.

CONCLUSION

The results indicate significant, dose dependent toxicity and mutagenicity of the leaf decoction of *Clerodendrum infortunatum* L. Drastic impediment of root growth and malformation at higher concentration indicates severe toxicity, reduction in MI below 22% at all concentrations indicate lethal genotoxic effect, abundance of metaphase clumping (33.34%), stickiness (17.99) and presence of binucleate cells and micronuclei indicate high genotoxicity. Thus, use of the aqueous plant extract in therapeutic applications should be in appropriate and low amounts.

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