Cytotoxicity and chromosomal aberrations induced by methanolic extract of *Cuscuta reflexa* and its pure compounds on meristematic cells of *Allium* species

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Abstract: *Cuscuta reflexa* (Convolvulaceae), is commonly known as amarbel or akashbel. In Bangladesh and Nepal some of the tribes use *C. reflexa* against edema, body ache, cancer, skin infections and liver disorders. Despite its traditional uses there is no information regarding genotoxic effects of either the plant extract or its pure compounds. Methanolic extract of *C. reflexa* (MECR) and pure compounds derived from it namely, odoroside H, neritaloside, and strospeside , were evaluated in *Allium cepa* L. and *A. sativum* L. for their effects on root growth, root apical meristem mitotic index (MI) , and chromosomal aberrations (CAs). In this study, we adopted a new method of calculating percent change in root length. MECR caused a concentration- and time- dependent inhibition in root length at 100 - 10000µg/ml in *A. cepa* root. It was accompanied by a subsequent decline in MI which is an indicative of its cytotoxic effect. On the contrary, at low concentrations a significant rise in root length was noticeable. In *A. sativum*, MECR also reduced the root length having IC 50 values ~8 x and 4.3 x lower than *A. cepa*. A variety of CAs were evident in both Allium systems after treatment with MECR, odoroside H and neritaloside. Thus in MECR, cardenolides glycosides, i.e. odoroside H and neritaloside could be accountable for its genotoxicity.

Keywords: Genotoxicity Mitotic index (MI) Chromosomal aberrations (CAs)

INTRODUCTION

Cuscuta reflexa, commonly known as amarbel or akashbel belongs to the family Convolvulaceae. It is a golden yellow leafless, rootless parasitic twiner (Nadkarni, 1976) grows on *Zizyphus* and on other shrubs and trees worldwide including Pakistan where it grows on *Alhagi*, *Populus* and on the hedges and bushes by the road side in the provinces of Sindh, Balochistan and Punjab (Dawson, 1990). This parasitic plant could induce changes in DNA methylation that may play regulatory role and cause changes in replication and transcription of host DNA (Ashwani *et al.,* 2012). In Bangladesh and Nepal some of the tribes use *C. reflexa* against oedema, body ache, cancer, skin infections and liver disorders (Siwakoti, 1999; Hossan *et al.,* 2009). Pharmacological properties including anti-cancer (Suresh *et al.,* 2011), anti-steroidogenic, anti-viral, anti-convulsant (Gupta *et al.,* 2003), psychopharmacological (Pal *et al.,* 2003), anti-spasmodic (Gilani and Aftab, 1992) and anti-hypertensive (Singh and Garg, 1973) have been associated with it. Three new compound, reflexin, 5-hydroxy-7-methoxy-6-(2,3-epoxy-3-methylbutyl)-flavanone (Tripathi et al., 2005), 7'- (3',4'-dihydroxyphenyl) -N- [(4-methoxyphenyl) ethyl] propenamide and 7'- (4'-hydroxy,3'-methoxyphenyl) -N- [(4-butylphenyl) ethyl] propenamide have been isolated from *C. reflexa* (Anis *et al.,* 2002). More recently, cardenolide glycosides *viz* odoroside H, neritaloside and strospeside, isolated from the methanolic extract of *C. reflexa* (MECR) were also reported to possess anti-cancer activity (Versiani, 2004). Generally, cardenolides are reputed for their cardiovascular effect and other biological activities mainly cardiotonic and cytotoxic due to the presence of a conjugated system (e.g. α, β- unsaturated butyrolactone ring) at C-17 (Wu *et al.,* 2010; Sannomiya *et al.,* 2007). Despite its traditional uses and several pharmacological properties, as mentioned above, there is no information regarding genotoxic effects of either plant extract or its pure compounds.

Indeed, genotoxic testing of new drug entities is a prerequisite for their safety assessment prior to phase I/II clinical trials (Grant, 1994) and recently, it has been realized that traditionally used natural products should also be evaluated for their mutagenecity prior to their in-depth pharmacological investigations (Soliman, 2001). There are many *in vitro* (Ames test, sister chromatid exchange assay and mouse lymphoma test etc.) and *in vivo* assays (mouse spot test, transgenic rodent, somatic mutation and recombination tests etc.) available for the evaluation of cytotoxic and/or genotoxicity of extracts

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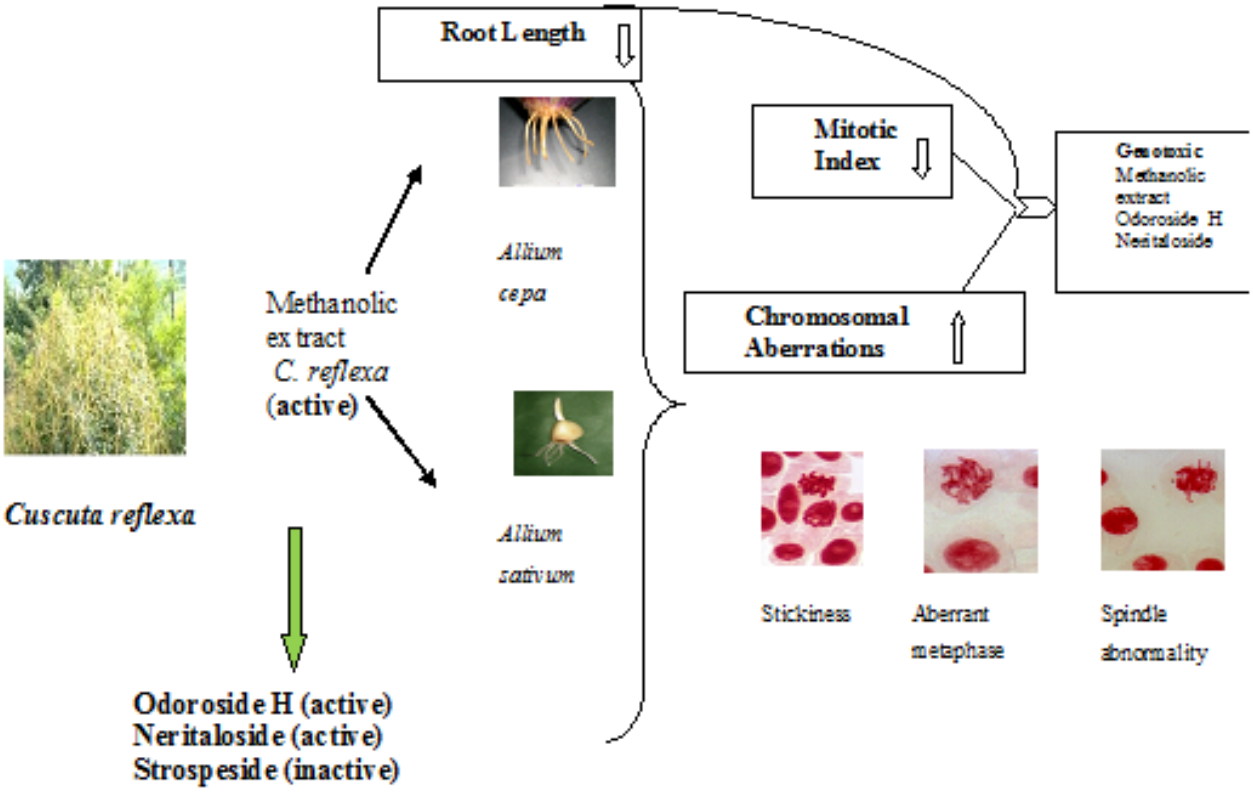
Graphical abstract



and compounds (Fiskesjo, 1985; Unyayar, 2006). Among such assays, inhibition of mitotic cell division and an increase in CAs is widely accepted as one of the most informative indicators of both cytotoxicity and genotoxicity. The *Allium* species appears to be a most valuable tool for evaluating toxicity of compounds by root growth inhibition, while their mutagenecity is identified as chromosomal damages involving spindle apparatus (aneugenic) or DNA damaging (clastogenic) effects (Sehgal *et al.*, 2006). Thus in the present study *A. cepa* (onion) and *A. sativum* (garlic) bulbs were used to assess the genotoxicity of MECR and pure compounds derived from it.

MATERIALS AND METHOD

C. reflexa plant collection

C. reflexa, growing as a parasite over *Nerium oleander* was collected from Karachi in the month of January (winter), 2006 and was authenticated by Dr. Rubina Dawar Department of Botany, University of Karachi, Pakistan with a voucher specimen (No. 66855 KUH) and submitted in the herbarium of the same department.

Preparation of methanolic extract of C. reflexa (MECR)

Fresh, undried, uncrushed plant of *C. reflexa* (3.5 kg) was extracted with methanol at room temperature (four times). The combined extracts were freed of the solvent in *vacuo* to afford thick residue (31.65g), referred as methanolic extract of *C. reflexa* (MECR) and partitioned between aqueous, and ethyl acetate phases. The yellow powder (2.31gm) was obtained from ethyl acetate phase by using solvent separation techniques which afforded odoroside H (1, 90 mg), neritaloside (3, 105 mg) and strospeside (4, 57 mg) by subjecting to vacuum liquid chromatography (VLC) followed by flash column chromatography. The structures of these compounds were elucidated and identified earlier (fig. 1) through extensive spectroscopic methods (Versiani, 2004).

HPLC analysis

The HPLC system was equipped with four LPG-3400A reciprocating pumps and variable wave length (Dionex, VQDD 3100Thermo Scientific, USA), UV-VIS detector and injector with a loop size of 20µl (Rheodyne Model 8125). Reverse phase chromatography was carried out at 25^{o}C. Samples, extract (0.68mg/ml) and odoroside H, neritaloside and strospeside (0.46mg/ml) were prepared in methanol and filtered through an ultra membrane filter (pore size 0.45µm, Millipore, DURAPORE, Ireland) prior to injection in the sample loop.

Chemicals

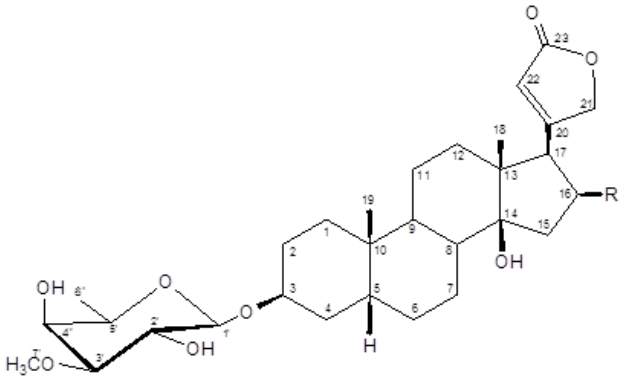
Bleomycin hydrochloride (Nippon, Japan), ethanol (Tedia, USA.), gelatin (Fluka AG, Switzerland), glacial acetic acid (Labscan, Thailand), orcein (W.S. Simpson, London), sodium azide (Ogawa Seiki, Japan), vinblastine sulphate (Pharmedia Laboratories, Pakistan). Dimethyl sulfoxide (DMSO) and all other chemicals of highest purity were used (Sigma, USA).

A. cepa and A. sativum assays

The genotoxic effect of MECR, pure compounds and other test agents were evaluated according to the method described earlier (Sehgal et al., 2006; Rank and Nielsen, 1994) with slight modifications. Since, onion seeds showed poor viability therefore onion bulbs were used that required large amounts of plant extract solution (60 ml/onion bulb). Owing to limited amounts of pure compounds available their genotoxicity was assessed using garlic bulbs, another useful member of *Allium* species widely used for such assays requiring only 15 ml solution/clove. Both *A. cepa* and *A. sativum* were purchased from the local market and stored at room temperature and handled as described below:

Onion bulbs

The outer scales of the healthy onion bulbs (24-34g) and their dried bottom plates were carefully removed without damaging the root primordia and placed in tap water (150 ml) for 3-4 days till roots reached ~2-3 cm in length. Five healthy bulbs were selected for each group : 1) control (distilled water), 2) vehicle control (DMSO, 2%), 3) different concentrations of MECR (1, 10, 100, 1000, 5000 and 10000µg/ml), 4) known mutagen, sodium azide (0.001, 0.01, 0.1, 1 and µM) and 5) anticancer drugs bleomycin (1, 10, 20, 30, 50 and 100µM) and vinblastine (1, 5, 10, 15, 20, 30 and 50µM).



		R
Odoroside H	(1)	H
Neritaloside	(3)	$OCOCH_3$
Strospeside	(4)	OH

Fig. 1: Structures of cardenolides (1, 3 and 4)

Garlic bulbs

After removal of outer dry scales of the bulbs, healthy and equal-sized garlic cloves (n=50) were selected and placed individually in glass vials containing tap water (15 ml) for 4 days to produce roots (1-2cm long), bulbs showing poor root growth were discarded. For each concentration three bulbs were used and cloves with healthy garlic roots (5-7 per clove) were selected for the study while the remaining roots were removed with a surgical blade. Initial root lengths (0h) were recorded and each clove was transferred in separate glass vials containing distilled water (control) or DMSO (2%, vehicle control) or MECR (100 and 1000 µg/ml) or pure compounds derived from it i.e. odoroside H, neritaloside and strospeside at 1 and 10µM and sodium azide (1 µM, IC_{50} as obtained from onion roots).

Root growth inhibition in onions and garlic

After 24 h and 48 h, 5-7 roots per onion/garlic from each group were measured (cm). A modified method was adopted for calculating percent change in root length by measuring the differences in root lengths. The increase in root length was obtained by subtracting the root lengths at 24 h and 48 h from that at 0 h. This approach provided the exact measure of root length increase at these time periods which correlated with the visual observations. The percent change in root length was calculated by:

$$\text{Percent change at 24h or 48h} = \frac{\text{Root length of treatment (b - a) or (b´-a)}}{\text{Root length of control (b - a) or (b´-a)}} \times 100$$

Where, time of root length observations were: a) 0 h, b) 24 h and b´) 48h the percent root growth inhibition was obtained to determine IC_{50} of test agents (the concentration causing 50% inhibition) (Sehgal *et al.,* 2006).

Mitotic index and chromosomal aberrations

The root tips (2-3mm) from various groups were separately fixed in ethanol:acetic acid mixture (3:1) for a period of 24 h at 4°C followed by hydrolysis in 1 N HCl (10 min). After rinsing with distilled water twice, the root tips were stained with aceto-orcein stain (2%) for a period of 30 min. The root tips were placed onto a glass slide containing two drops of glacial acetic acid (45%) and individually teased with a needle. A cover slip was carefully placed to avoid air bubbles and squashed gently. To observe CAs and MI, 3000 cells of three separate bulbs were counted for each treatment and control group. Each experiment was run with three replicates. The number of cells in various stages of mitosis (prophase, metaphase, anaphase and telophase) and Interphase (non-dividing cells) were noted in the control and treated groups. The MI (Leme and Marine-Morels, 2009) was calculated as follows:

$$\text{Mitotic Index} = \frac{\text{Number of dividing cells}}{\text{Total number of cells counted}} \times 100$$

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 12 software package by analysis of variance (ANOVA) followed by post hoc test with Duncan's multiple range test p value of <0.05 was considered statistically significant.

RESULTS

HPLC profile of MECR and cardenolides

The chromatogram of MECR exhibited one major peak with a slight shoulder at a retention time of ~10.0 - 11.5 minutes which coincided with the peak of cardenolides

Table 1: Effect of methanolic extract of *C. reflexa* on *A. cepa* root length at 24 and 48 h

MECR (µg/ml)	Root length (cm)						
	0 h	24 h			48 h		
	(a)	(b)	(b-a)	Change (%)	(b´)	(b´-a)	Change (%)
1	$2.53^{c,2}$±0.07	$3.27^{b,1,2}$±0.06	$0.73^{1,2}$±0.03	14.07^{2}±2.17	$3.94^{a,2}$±0.09	1.42^{2}±0.07	20.34^{2}±1.60
10	$2.63^{c,1}$±0.06	$3.39^{b,1}$±0.07	0.77^{1}±0.04	20.32^{1}±2.98	$4.54^{a,1}$±0.08	1.93^{1}±0.12	63.56^{1}±3.21
100	$2.33^{c,3}$±0.02	$2.92^{b,4}$±0.01	0.60^{3}±0.03	-6.24^{3}±1.39	$3.32^{a,4}$±0.05	0.99^{4}±0.04	-16.12^{3}±1.46
1000	$2.51^{c,2}$±0.05	$3.09^{b,3}$±0.03	0.59^{3}±0.03	-7.80^{3}±3.37	$3.27^{a,4}$± 0.07	0.76^{5}±0.02	-35.60^{4}±2.77
5000	$2.56^{c,1,2}$±0.02	$2.68^{b,5}$±0.03	0.13^{4}±0.01	-79.69^{4}±1.26	$2.73^{a,5}$± 0.05	0.18^{6}±0.03	-84.76^{5}±2.92
10000	$2.53^{a,2}$±0.02	$2.59^{a,5}$±0.01	0.05^{4}±0.02	-92.19^{5}±2.93	$2.54^{a,6}$± 0.01	0.01^{6}±0.02	-99.16^{6}±1.26
Control	$2.59^{c,1,2}$±0.01	$3.23^{b,2}$±0.03	$0.64^{2,3}$±0.03	0	$3.77^{a,3}$±0.06	1.18^{3}±0.06	0
IC_{50}		3300±115.5			1350±312.25		

Distilled water and vehicle control (2% DMSO) gave similar results therefore the data was pooled and referred as control.
Methanolic extract of *C. reflexa* = MECR
Values represent mean ± SEM of root length (cm)
Onion (n) = 60 (control) and 15 (for each treatment)
Time of root length observations: a) 0 h, b) 24 h and b´) 48 h
Difference of root length: (b - a) =24 hr – 0 hr and (b´- a) = 48 hr-0 hr
The negative (-) and all other (+) values represent reduction and enhancement in the root length, respectively.
Homogenous means are represented by similar alphabetical superscripts (a, b and c) in rows and similar numerical superscripts (1-5) in columns. The dissimilar alphabets and numerals are significantly different ($P < 0.05$) from each other
IC_{50} = Concentrations causing 50% growth inhibition

Table 2: Chromosomal aberrations induced by methanolic extract of *C. reflexa*, odoroside H, neritaloside and strospeside in *A. sativum*

Test Agents	Chromosomal aberrations (%)				Total chromosomal aberrations (%)	Mitotic index (%)
	Disturbed metaphase	Fragment	Spindle abnormality	Sticky chromosome		
MECR (µg/ml)						
100	$0.78^{1,2}$±0.22	0.11^{1}±0.11	$0.67^{1,2}$±0.19	$0.22^{2,3}$±0.11	$1.78^{2,3}$±0.48	$4.78^{2,3,4}$±0.29
1000	0	0	0.11^{2}±0.11	$0.33^{1,2,3}$±0.33	0.44^{4} ±0.44	1.11^{5}±1.11
Pure compounds (µM)						
Odoroside H 1	1.11^{1}±0.29	0.11^{1}±0.11	0.89^{1} ± 0.22	$0.44^{1,2,3}$±0.29	3.56^{1}±0.22	$4.33^{3,4}$±0.69
10	$0.78^{1,2}$±0.29	0	$0.22^{1,2}$±0.22	$0.11^{2,3}$±0.11	$1.11^{3,4}$±0.62	$2.78^{4,5}$±1.06
Neritaloside 1	$0.67^{1,2,3}$±0.19	0	$0.44^{1,2}$±0.11	0.89^{1}±0.22	$2.00^{2,3}$±0.33	$3.22^{4,5}$±0.73
10	1.11^{1}±0.22	0	$0.33^{1,2}$±0.19	$0.44^{1,2,3}$±0.11	$1.89^{2,3}$±0.40	$2.67^{4,5}$±0.38
Strospeside 1	$0.11^{3,4}$±0.11	0	0	$0.22^{2,3}$ ±0.22	0.33^{4}±0.19	$6.78^{1,2}$±0.56
10	$0.22^{2,3,4}$±0.11	0	0.11^{2}±0.11	$0.11^{2,3}$±0.11	0.44^{4}±0.11	$6.22^{1,2,3}$±0.68
Sodium azide1	1.11^{1}±0.11	0	$0.67^{1,2}$±0.51	$0.67^{1,2}$±0.19	$2.67^{1,2}$±0.77	$6.44^{1,2,3}$±0.40
Control	$0.22^{2,3,4}$±0.11	0	0	0	0.33^{4}±0.19	7.13^{1}±0.45

Since, control and vehicle control (2% DMSO) results were similar therefore the data was pooled
Each value represents percent mean ± SEM of different chromosomal aberrations and other abnormalities
The dissimilar numerical superscripts (1-5) in columns are significantly ($P < 0.05$) different from each other, whereas, homogenous means are represented by similar numerical superscripts

(odoroside H, neritaloside and strospeside). [fig. 2 (a, b)]. The percentage of cardenolides in extract (1.136%) was calculated as follows:

Peak area of sample/ peak area of standard × weight of standard/ dilution of standard × dilution of sample/ weight of sample × 100.

18.255/ 1086.815 × 0.46 mg/ 1.0 ml × 1.0 ml/ 0.68 mg × 100 = 1.136

Root length inhibition

A. cepa and A. sativum

MECR induced concentration (100-10000µg/ml) dependent reduction (6.24%-92.19%) in root length at 24 h, with maximum reduction reaching to ~99% at 48 h. On the contrary, low concentrations (1 and 10 µg/ml) caused an increase (~17%) in root length which increased further to 2.5x at 48 h (table 1). Similar experiments with sodium azide, bleomycin and vinblastine showed IC_{50} values of 1.05±0.53, 75.33±9.60, 20.17±2.62µM at 24 h and 1.04± 0.45, 49.0±3.79, 13.23±0.93µM at 48 h, respectively.

Thus the potency order of root length inhibition is: sodium azide > vinblastine > bleomycin >>> MECR. In *A. sativum*, MECR also caused a significant dose dependent (100 and 1000µg/ml) inhibition of root length, which was 8x and 4x greater at 24h and 48 h respectively,

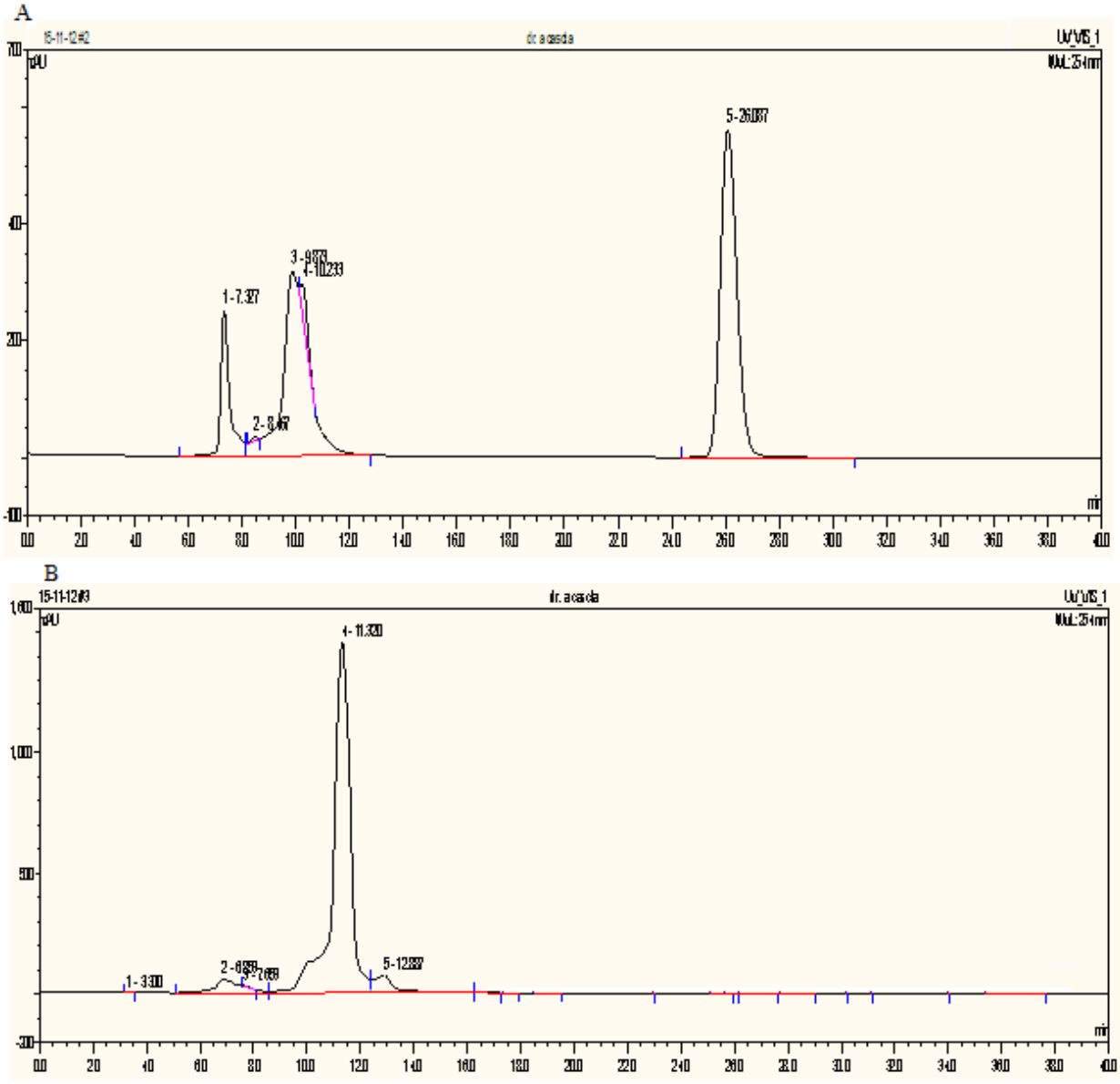
as compared to *A. cepa* described above. The odoroside H and neritaloside (1 and 10µM) also caused root length inhibition (~13% -26%) at 24h with further reduction of 1.4x and 2.1x at 48h, whereas strospeside (1 and 10µM) also showed significant root length inhibition (~4 and 8%) but only at 48 h (data not shown).

Mitotic index and chromosomal aberrations in A. cepa and A. sativum

The normal stages of mitotic cell division of *A. cepa* (prophase, metaphase, anaphase and telophase stages) are presented in fig. 3 (a-d). Each cell stage was counted to obtain MI. In control, the MI was 7.2%, whereas MECR (100-5000 µg/ml) induced a significant concentration dependent decline in it (5.4%-0.5%). Sodium azide (1 µM) and bleomycin (50µM) significantly reduced the MI with similar magnitude (~5%), whereas, vinblastine caused a significant rise by 1.4x as compared to control.

In *A. sativum*, MECR (100 and 1000 µg/ml) also caused significant inhibition in MI (4.78% and 1.11%) which was reduced (1.5-6.4x) as compared to control (MI = 7). In the presence of odoroside H and neritaloside (1 and 10µM) MI was also reduced to about 1.6x whereas; strospeside had no effect (table 2).

In order to obtain a reliable measure of CAs in *A. cepa*, IC_{50} values for root length inhibition induced by sodium



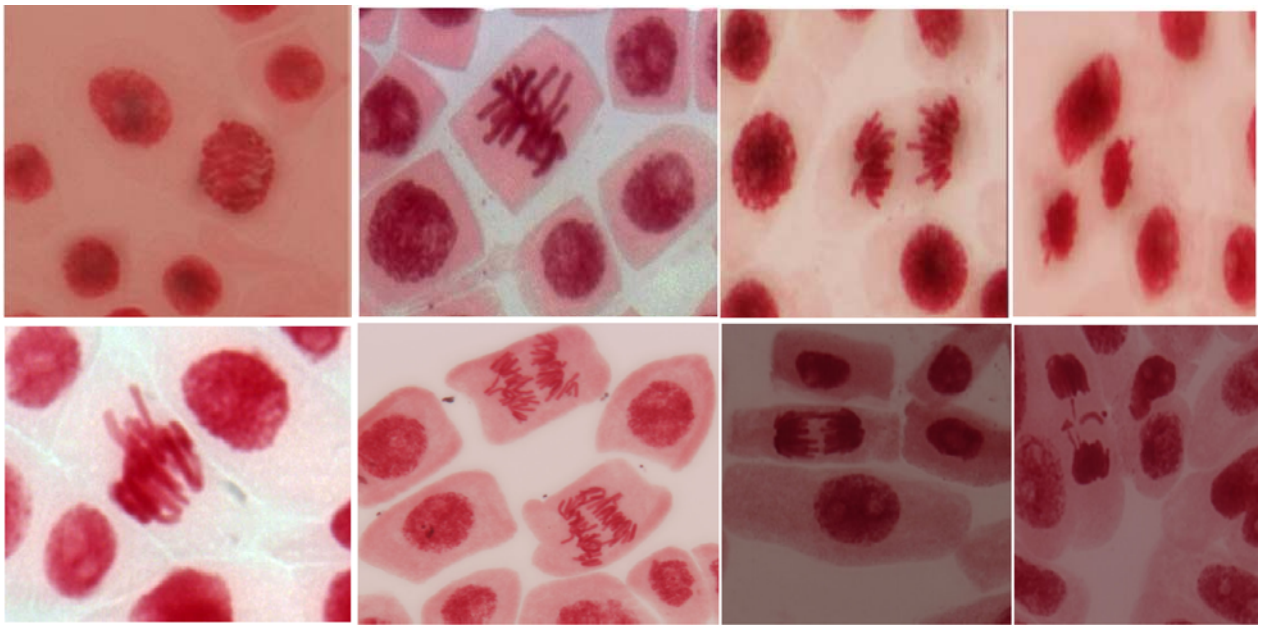
Methanolic extract of *C. reflexa* (0.68 mg /ml) and (b) Cardenolide, odoroside H (0.46 mg/ml). Chromatographic conditions: Lichrocart C-18 column, mobile phase acetonitrile/water (8.5:1.5) with flow rate 0.20 ml/min. and detected at 254 nm.

Fig. 2: HPLC chromatogram of *Cuscuta reflexa* and its cardenolides

Table 3: Chromosomal aberrations induced by methanolic extract of *C.* reflexa, sodium azide, bleomycin and vinblastine in *A. cepa*

Test Agents	Chromosomal aberrations (%)						Total CAs (%)	MI (%)
	Disturbed metaphase	Bridge	Fragment	Vagrant	Spindle abnormality	Sticky chromosome		
MECR (µg/ml)								
1	$0.32^{3,4}$±0.14	$0.03^{3,4,5}$±0.03	0	$0.02^{2,3}$±0.02	$0.11^{3,4}$±0.06	0.13^{4}±0.04	$0.62^{4,5}$±0.18	7.21^{2}±0.29
10	$0.34^{3,4}$±0.05	$0.04^{3,4,5}$±0.04	0	$0.03^{2,3}$±0.03	0.08^{4}±0.01	$0.29^{2,3}$±0.12	$0.79^{3,4}$±0.16	8.08^{2}±0.77
100	0.80^{2}±0.13	$0.08^{2,3,4,5}$±0.04	0.02^{2}±0.02	$0.02^{2,3}$±0.02	0.41^{1}±0.02	0.69^{1}±0.16	2.02^{2}±0.34	5.35^{3}±0.31
1000	$0.61^{2,3}$±0.15	$0.12^{2,3,4}$±0.05	0.01^{2}±0.01	$0.02^{2,3}$±0.02	$0.27^{1,2,3}$±0.12	0.38^{2}±0.05	$1.41^{2,3}$±0.40	3.86^{4}±0.29
5000	0	0	0	0	0	0.03^{4}±0.02	0.03^{5}±0.02	0.49^{5}±0.12
Sodium azide (1 µM)	0.82^{2}±0.13	$0.14^{2,3}$±0.03	0.28^{1}±0.08	0.123^{1}±0.03	$0.14^{2,3,4}$±0.04	$0.31^{2,3}$±0.05	1.82^{2}±0.34	5.57^{3}±0.25
Bleomycin (50 µM)	0.21^{4}±0.03	0.42^{1}±0.04	0.31^{1}±0.03	$0.06^{1,2,3}$±0.03	$0.13^{2,3,4}$±0.06	$0.32^{2,3}$±0.06	$1.46^{2,3}$±0.13	5.02^{3}±0.54
Vinblastine (15 µM)	1.74^{1}±0.22	0.17^{2}±0.04	0.07^{2}±0.04	$0.11^{1,2}$±0.05	$0.28^{1,2}$±0 .02	$0.36^{2,3}$±0.01	2.72^{1}±0.19	9.74^{1}±0.24
Control	0.08^{4}±0.04	$0.02^{4,5}$±0.01	0	0	0.06^{4}±0.01	0.04^{4}±0.03	$0.20^{4,5}$±0.07	7.23^{2}±0.32

Since, control and vehicle control (2% DMSO) results were similar therefore the data was pooled
Total number of cells observed = 9000 in control and for each treatment
Each value represents percent mean ± SEM of mitotic index and different chromosomal aberrations
The dissimilar numerical superscripts (1- 5) in columns are significantly ($P < 0.05$) different from each other, whereas, homogenous means are represented by similar numerical superscripts



Control (a-d): (a) prophase (b) metaphase (c) anaphase (d) telophase.
Test agents showing various chromosomal aberrations which are represented by arrows: MECR (100 µg/ml): (e) aberrant metaphase and (f) spindle disturbance.
Bleomycin (50 µM): (g) abnormal anaphase with bridge and (h) abnormal telophase with fragment and laggard.

Fig. 3: Mitotic stages in meristematic cells of *A. cepa* in the absence and presence of test agents.

azide, bleomycin and vinblastine were used as their corresponding MI were more than 50% of control value (Rank and Nielsen 1994). fig. 3 (e-h) demonstrates various types of CAs in the root cells of the *A. cepa* induced by MECR, bleomycin and vinblastine. The frequency of CAs (table 3) was significantly increased from ~0.20% (control) to 2% and 1.4% in MECR treated group at 100 and 1000µg/ml, respectively. The most frequent CAs were metaphase aberrant, sticky chromosomes and spindle abnormalities in both the *Allium* species. Sodium azide and bleomycin treatment also significantly increased the frequency of CAs to ~1.6%, with predominantly metaphase aberrant and bridges, respectively. Vinblastine caused maximum CAs (2.72%) predominantly spindle abnormalities.

In *A. sativum*, pure compounds odoroside H and neitaloside as well as sodium azide induced similar types of CAs which were increased (~6x – 11x) as compared control and most notable effects were metaphase aberrant, sticky chromosome and spindle abnormality. At 1µM, both odoroside H and sodium azide induced maximum CAs (~9x) whereas, strospeside was ineffective.

DISCUSSION

In the present study, three important parameters such as i) root length, ii) MI and iii) CAs were taken into account for the assessment of genotoxic effects of MECR and pure compounds derived from it. The root tip cells of *A. cepa* with manageable chromosome number (2n = 16) and large size provides an excellent system for cytological tests that allows an assessment of different genetic endpoints, i.e MI and CAs. It is one of the most popular and sensitive methods for genotoxic evaluation and identification of mutagenic substances (Sehgal *et al.,* 2006; Shaymurat *et al.,* 2011; Akintonwa *et al.,* 2009). It has also been recommended by the World Health Organization (WHO), for evaluation of chemical safety in the international testing program. Another related member, *A. sativum* has also been successfully used to assess the cytotoxic and genotoxic potential of substances such as cadmium (Rank and Nielsen, 1994) and zinc oxide nanoparticle on reduction of root growth and MI with an increase in CAs (Herrero *et al.,* 2012).

Usually for toxicity studies mean root lengths at each concentration of test agent are calculated as percentages of the control (Sehgal *et al.,* 2006). However, in the present study with *A. cepa* root the IC_{50} for *C. reflexa* extract (MECR) could not be determined (data not shown). This could be due to the variations in the root lengths (~2-3 cm) at the start of the experiment (0 h) in the control and treated groups. Therefore, measurements of root lengths at 0 h were subtracted from their respective values at 24h or 48h, thus, providing a more reliable estimate of the increase in root length at 24 h and 48 h of incubation. After this modification using the same data, IC_{50} values were obtained that correlate with the visual observations as well.

MECR induced a dose dependent and time dependent reduction in root length clearly indicating its cytotoxic effects. In parallel, reduction in MI (~1.4x-16x) by MECR (100-5000µg/ml) as compared to control, followed by complete inhibition of root length at 10000 µg/ml with cells predominantly in interphase stage, suggesting its turbagenic effect at high concentration. Similarly concentration dependent decline in both root length and MI have also been observed in aqueous extracts of *Alstonia boonei, Newbouldia laevis, Terapluera tetraptera* and *Rauvolfia vomitoria* (Soliman, 2001) which has been linked to their toxic effects. In the present study, initially MECR at low concentrations (1 and 10µg/ml) caused a significant increase in onion root length at 24h with further increment by 2x at 48h without effecting mitotic index supporting that possibly cell elongation is responsible for it. As, it is well established that a reduction in root length can occur either due to decrease in cell division or by impairment in cell elongation (Kong and Ma, 1999).

The genotoxic potential of MECR was further supported by the induction of various types of CAs, such as aberrant metaphase, stickiness, bridges and fragments in the root tips of *A. cepa*. The most prevalent being aberrant metaphase and anaphase, possibly due to disturbances in spindle apparatus leading to impairments in cell division. The sticky chromosomes forms chromosome agglomeration (Singh, 2003) which is an irreversible process inducing cross-linkages of chromo-proteins (Nakamura *et al.,* 2000) leading to incomplete separation of daughter chromosomes at the anaphase stage and finally cell death. Additionally, sticky chromosome also leads to chromosomal bridges causing impairment in their segregation to the two poles. In the presence of MECR, the frequency of chromosomal fragments was low but is also an indicative of chromosomal breaks, and may be a consequence of anaphase/telophase bridges (Rashan *et al.,* 2011).

Since, odoroside H, neritaloside and strospeside were not available in sufficient amounts to perform genotoxicity test in onion system, therefore, *A. sativum* was preferred. Consistently, in *A. sativum*, MECR (100µg/ml) also caused inhibition (87.13±1.93%) in root length and reduction (6.4x) in MI as compared to control and was ~8x more sensitive than *A. cepa* assay. It is also reported for the first time that both odoroside H (1) and neritaloside (3) at ~10 µM reduced the root length (~37%) and MI (~3x) with a dramatic reduction in the number of cells which were arrested in the interphase stage.

MECR induced similar types of CAs in both *Allium* systems, most frequent being aberrant metaphase and spindle abnormalities (aneugenic effects) which were enhanced (~5x) in the presence of odoroside H (1) and neritaloside (3) as compared to control, whereas, strospeside (4) was ineffective indicating that former two compounds are genotoxic. Both 3 and 4 compounds are derivatives of odoroside H having acetyloxy and hydroxyl groups at their 16-position, respectively. The weak activity of compound 4 as compared to the parent cardenolide 1 may be due to the presence of hydroxyl group at C-16 in 4; while in 3 hydroxyl is in the acetate form elicited genotoxic effect. Recently, GLI transcriptional inhibition (IC_{50} in µM) has been reported for cardenolides, in which the importance and the influence of substitution at C-16 of the steroidal skeleton (Arai *et al.,* 2011) have also been recognized.

In conclusion, MECR is non-genotoxic at low concentrations (1 and 10 µg/ml). Whereas, at higher concentrations (100-5000µg/ml) exhibited a dose-dependent reduction in root length, MI accompanied by an increase in CAs, predominantly metaphase aberrant, sticky chromosome and spindle disturbances. Therefore, MECR showed a stronger aneugenic effect as well as a weaker clastogenic effect in both *A. cepa* and *A. sativum*. This genotoxic effect is more likely due to the presence of equipotent odoroside H (1) and neritaloside (3). Therefore, it is important to use *C. reflexa* with great caution at safe dose for medicinal purposes. In future, additional *in vivo* and *in vitro* genotoxic assays should be conducted to evaluate its mechanism of genotoxicity.

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