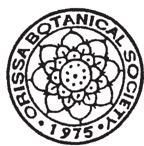

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Evaluation of cytotoxic potential of methanolic extract of Guduchi [*Tinosporia cordifolia* (Willd.) Hook. f. & Thoms.] on root meristematic tissues of *Allium cepa* L.

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ABSTRACT

Tinospora cordifolia (Menispermaceae), commonly known as 'Guduchi', is a robust climber with greenish yellow flowers and having a number of bioactive compounds such as berberine, palmatine, magnoflorine, syringin, furanolactone, jatrorrhizine with high medicinal activity. Extracts of *T. cordifolia* reported to have anti-oxidant, anti-tumour and anti-inflammatory activities. Present study deals with an investigation on isolation of secondary metabolites from bark in methanolic extracts and evaluation of its cytotoxic potential on *Allium cepa* root meristems. Three concentrations (20µg, 40µg and 100µg) of crude extracts of *T. cordifolia* were studied under 6h and 24h of treatment on root meristematic tissues. The cytotoxic compounds present in the bark extracts brought about significant reduction of mitotic index in 24h of treatment at 20 µg ml^{-1}, 40µg ml^{-1} and 100 µg ml^{-1} concentrations as compared to control. Different cytological abnormalities like clumping of chromosomes, DNA fragmentation, spindle arrest with scattered chromosomes, chromatin condensation, diplochromatin chromosome erosion, denucleation and chromosome break were observed. The preliminary investigation showed that this plant-derived bioactive compounds can destroy the cells at micromolar concentration and hence may be a potential drug for treatment of cancer.

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1. Introduction

Since the beginning of human civilisation, plants played an important role in the protection of human and animal health against chronic degenerative diseases. Hence an impressive number of phytochemicals (bioactive compounds) have been isolated from nature. As per an estimate of WHO, about 80% of total inhabitants on earth rely on traditional medicines for their primary health care needs (Anonymous, 1948). About 8,000 species of medicinal plants belonging to 450 genera are reported to occur wild in India, which is about 50% of the known flowering plants of the country (Owolabi *et al.,* 2007). *Tinospora cordifolia,* commonly known as 'Guduchi' is a large, glabrous, deciduous climber belonging to the family Menispermaceae. It is distributed throughout the tropical parts of Indian subcontinent including Pakistan, Sri Lanka, Bangladesh and China, ascending to an altitude of 300 m (Garg *et al.,* 2007). In Hindi, the plant is commonly known as 'Giloi', which is a Hindu mythological term, refers to the heavenly elixir that has saved celestial beings from old age and kept them eternally young (Bhandari, 2006). In India, the species is distributed from Kumaon to Assam in north extending through West Bengal, Odisha, Bihar, Deccan, Konkan, Karnataka and Kerala (Wani *et al.,* 2011). A large number of compounds have been isolated from the aerial parts and roots of *T. cordifolia* which includes berberin, tinosporacide, tinosporin, tinocordifolioside, cordifolioside A, cordifolioside B, isocolumbin, magnoflorine (Mehra *et al.,* 2016). The presence of several terpenoids, alkaloids, lignin, carbohydrates, bitters, steroids and glycosides has also been reported in the species. One of the most important constituents present in the stems of *T. cordifolia* is berberine, an isoqunoline alkaloid, having various pharmacological

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actions which enhance the therapeutic values of the plant (Andola, 2000). In modern medicine, it is used for the treatment of general weakness, fever, dyspepsia, dysentery, gonorrhoea, urinary diseases, viral hepatitis and anaemia. Recently, it is also being used as an immunomodulatory, antioxidant, anti-neoplastic, anti-stress, anti-hyperglycemia, anti-diabetic agent (Joshi and Joshi, 2013). There are few reports on the cytotoxic properties of different extracts of *T. cordifolia* (Uddin *et al.,* 2011). The bark of *T. cordifolia* is used as an astringent and for treatment of fever and tumours, while the leaves are used to treat ulcers, sore throat, inflammatory conditions in traditional medicine (Adhvaryu *et al.,* 2008). Although chromosome number of *T. cordifolia* has been determined as 2n =22, no detailed study on genetic variability in terms of ploidy level and phytochemical constituents has been done till date (Rana *et al.,* 2012). In consideration of the facts that very scanty information on the cytotoxic and anti-inflammatory properties of the tinosporin derivatives are available in different parts, we report here the cytotoxic activity of bark extracts of *T. cordifolia* on *Allium cepa* root meristems.

2. Materials and methods

2.1. Plant materials

The plant material consists of dried powdered bark of *Tinospora cordifolia*, collected from Dhansole village of Baripada block, Mayurbhanj district, Odisha and identified with the help of Flora of Orissa (Saxena and Brahmam, 1995). The herbaria specimens and dried bark samples were deposited in the herbarium of the Department of Botany, Utkal University, Bhubaneswar, Odisha.

The test plant was onion *i.e*. *Allium cepa* L. (Liliaceae), which has 16 long chromosomes. This species is an excellent plant material to be used as a biomarker for environmental monitoring with many advantages, such as low cost, profuse rooting from the bulb, ease of storage and handling, short duration to conduct a test, large cells with easily visible long chromosome and the ease at which the abnormalities in chromosomes during mitosis can be observed (Banerjee and Giri, 2014).

2.2. Extraction of crude bark extract

The fresh bark of *T. cordifolia* was collected and dried under room temperature and powdered using a grinder. The powdered bark sample was kept in air tight containers until the time of use. The powdered bark (50 g) was exhaustively extracted with 200 ml 99.8% methanol (Merck) using Soxhlet apparatus at 40°C. The methanolic extract was filtered and the filtrate was condensed under reduced pressure and concentrated to dryness under controlled temperature (40°C) with the help of IKA RV10 Rotary Evaporator (Germany) fitted with IKA HB10 digital temperature controller, vacuum pump and water chiller (Cole-Parmer).

2.3. Cytotoxic study

Allium cepa var. Deshi was grown in sand in the green house of Botany Department, Utkal University, Vani Vihar, Bhubaneswar, Odisha and was used as experimental material for cytotoxicity study. After 5-6 days, bulbs with 3-4 cm long roots were washed in running tap water and subjected to treatment of 0µg ml^{-1} (Control), 20µg ml^{-1}, 40 µg ml^{-1} and 100µg ml^{-1} concentration of bark extract dissolved in dimethyl sulfoxide (DMSO) followed by double distilled water and kept for 6 h and 24 h at room temperature. A control experiment was conducted without any bark extract.

2.4. Mitotic index and chromosome study

Root tips from each treatment were collected and fixed in 1:3:: acetic acid: ethanol overnight at room temperature. Fixed root tips were treated with 45% glacial acetic acid for 15 min and were stained in 2% aceto-orecin: 1N HCl (9:1) for 4-5 h. Stained root tips were squashed in 45% acetic acid on a clean glass slide. For each treatment ~100 cells from root tips were scored at random from each slide and the data were pooled for each treatment. The mean data were taken from each treatment and each experiment was replicated thrice. Cells from each root tip were scored at different stages of chromosome under Olympus BX56 microscope (Japan) attached with a digital camera. All the observations were recorded for abnormalities during the cell and chromosome division under different concentrations of crude bark extract.

2.5. Cell death measurement

The cytotoxicity levels were measured for both treated and control roots by staining them in 0.25% Evan's Blue (w/v) for 30 min (Baker and Mock, 1994). Stained root tips were transferred to 1 ml of N, N – Dimethylformamide for 1 h at 37°C. The absorbance of the dissolved Evan's Blue solution was measured at 600 nm in a UV Visible Spectrophotometer and plotted in a graph and calculated statistically.

3. Results and discussion

3.1. Mitotic index and chromosomal anomalies

The treated roots became brown in colour and growth was restricted in 24 h of direct treatment in 100 µg ml^{-1} as compared to control or 6 h of direct treatment. The concentration of the bark extract and the time of exposure played an important role in the reduction of mitotic index

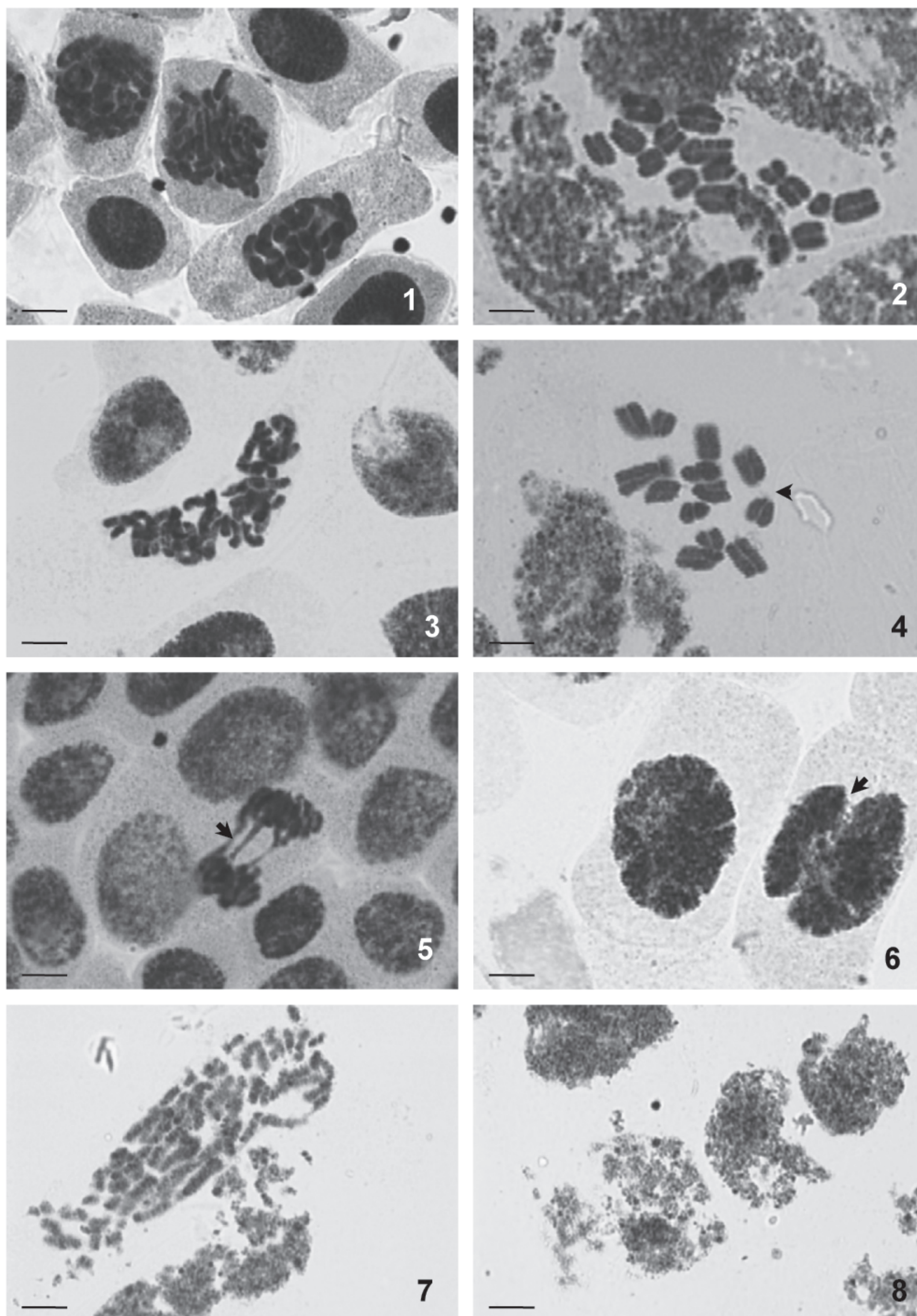
in a dose-dependent manner. Mitotic Index (MI) decreased progressively with increase in concentration as well as the duration of the treatment (Table 1). The MI was 41.20 in 6h and 29.48 in 24 h of treatment in 20 µg ml^{-1}, which was slightly higher than 40 and 100 µg ml^{-1} concentration. The mitotic index dropped significantly by about ~1.90 to ~2.78 fold in 24 h of treatment at 20 µg ml^{-1} and 100 µg ml^{-1} concentrations respectively as compared to respective control. Control root tip cells showed normal mitosis at all stages of divisions. However, C-mitosis, chromosome bridges, chromosome fragments, chromosomal clumping, chromosome break and chromosome erosions and spindle abnormalities with spread metaphase, denucleation and chromosome condensation were recorded (Figs. 1-8). Spindle fibre abnormality (SFA) included C-mitosis, chromosome stickiness, chromosomal clumping (Fig. 1) and metaphase chromosomal abnormality (Fig. 3) including chromosomal breaks (Fig. 4) and chromosomal bridges (Fig. 5). The frequency of chromosome break and chromosome erosions (Fig. 7) increased significantly with increasing concentration of bark extract and increase of treatment time. Very condensed chromosomes with mitotic effects in the cell as well as chromosome fragments were noticed in 100 µg ml^{-1} (Fig 2. 2 and 4). Sticky chromosome bridges were found in low doses *i.e*., 20 µg ml^{-1} of extract. Chromosomal stickiness as well as break and erosion (Figs. 4 and 7) are usually irreversible leading to denucleation of chromatin materials (Fig. 8) and could be due to the toxic effects of plant extracts leading to cell death. Moreover, high mitotic index with more percentage of cells in metaphase stage was noticed at lower concentrations compared to other concentrations. Likewise, the percentage of cells in anaphase stage also varied in different concentrations. The oxidative damage by bark extract might have induced various chromosomal abnormalities, which was found to be dose-dependent (Table 1). The highest percentage of abnormalities was noted with treatment of 100µg ml^{-1} of extract as compared to treatment with 20 µg ml^{-1}. A comparative analysis of all abnormalities with different hours of treatment showed that a comparatively high percentage of metaphase cells were damaged as compared to anaphase. Metaphase and anaphase displayed various types of abnormalities such as spindle fibre anomalies leading to pre-treatment effect, chromosome break with lagging chromosomes, chromosome erosion, chromosome clumping, sticky chromosomal bridge formation and C-mitosis formation. The maximum number of chromosome breaks and erosion was found with treatment of 100 µg ml^{-1} extract for 24 h. At 20 µg ml^{-1} treatment, significant percentage of abnormalities was also registered. The increase in percentage of chromosomal abnormality was dependent on dose- and time of treatment. The treated root tips showed abnormal spindle formation in low dose leading to metaphase arrest and separation due to direct bark extract treatment. Chromosomal break was observed in 40 µg ml^{-1} treatment, while 20 µg ml^{-1} treatments produce early separation of chromosomes in anaphase. Chromosomal damages were prominent resulting in erosion and intense break of chromosome at 100 µg ml^{-1} treatment. This clearly indicates that methanolic extracts of *Tinospora cordifolia* have potential carcinogenic chemicals, which can kill the cells. Hence, isolation and extraction of individual chemicals; separation and testing of their efficacy at cellular level are very important considerations for discovery of cytotoxic molecules. Since, this extract has serious impact on cell cycle, the extract in crude form may have potential anti-cancer activity.

Root is the most sensitive and accessible part of *Allium cepa*. The inhibition of root growth upon exposure to bark extract clearly showed cytotoxic effect of plant extract and consequent chromosomal aberrations similar to heavy metal stress (Zhang *et al.,* 2009). Chromosome stickiness is a lethal type of aberration besides chromosome fragments and bridges, which was also observed in the present study. Increased frequency of chromosome bridge and presence of more chromosome fragments in the cells might be due to chromosome replication and protein synthesis in roots induced under plant extract stress. The active molecules of bark extracts might be interfering with calmodolin, a calcium modulating protein, located in the mitotic spindle by influencing the uptake of Ca^{++} causing abnormal processes of chromosome movements leading to mitotic abnormalities (Liu *et al.* 1995).

There are not many reports on toxicity of plant extracts causing chromosomal abnormalities such as chromosome break, lagging, erosion and effect on cell division. Various types of abnormalities were noticed by treating the *Allium cepa* cells with methanolic bark extract of *Tinospora cordifolia* at the cell and tissue levels, affecting the elongation zones of root apex. The effect of low doses of methanolic bark extract was found to have significant effect on oxidative damage of chromosome structure. Chromosomal damage includes gross structural changes, which are initiated by chromosome breaks and erosion.

3.2. Cell death and cytotoxicity

Increased Evan's blue uptake of 10.25% was found with treatment of 20 µg ml^{-1} and 18.65% in 100 µg ml^{-1} at 24 h treatment as compared to control. However, in 6 h treatment with both the concentrations, no significant cell death was observed. Pronounced cytotoxic effect of bark extract on roots of *A. cepa* in both the concentrations was



Figs. 1 - 8 : Cytotoxic effects of crude extract of *T. cordifolia* on *Allium cepa* root tip cells; Fig. 1: Clumping of chromosomes at 100 µg ml^{-1} concentration at 6 h direct treatment; Fig. 2 & 3: Spindle abnormalities with metaphase chromosome effect and abnornal metaphse at 20µg ml^{-1} concentration at 24hr; Fig. 4 & 5: Condensation diplochromatin with chromosome break (arrow head) and sticky chromosome bridge at anaphase (arrow head) at 40µg ml^{-1} concentrationat 24 hr treatment; Fig. 6: Unequal binucleate nuclei without cell plate formation (arrow head); Fig. 7 & 8: Chromosome errosion and chromatin denucleation at 100µg ml^{-1} concentration at 24 hr treatment. Bar= 10µ.

Table 1

Effect of crude bark extract of *Tinospora cordifolia* on mitotic index of *Allium cepa* root apical meristems

	Concentrations					
	20µg ml^{-1}		40µg ml^{-1}		100µg ml^{-1}	
Treatment	Mitotic index (±S.D.)	Cell aberration percentage (±S.D.)	Mitotic index (±S.D.)	Cell aberration percentage (±S.D.)	Mitotic index (±S.D.)	Cell aberration percentage (±S.D.)
Control	49.16 ±1.25	1.23 ±0.54	47.26 ±0.96	1.03 ±0.61	46.24 ±0.94	1.01 ±0.66
6h	41.20 ±2.15	55.26 ±2.17	37.46 ±2.44	62.45 ±1.27	35.38 ±2.56	67.36 ±1.29
24h	29.48 ±1.53	69.23 ±1.58	24.31 ±2.10	74.13 ±1.58	22.30 ±2.08	88.73 ±1.78

found to vary with duration of treatment. The uptake of Evan's blue stain by the samples with longer period of exposure was significantly more at higher concentrations in comparison to those exposed to low concentrations for short duration. The increase in Evan's blue colour uptake in the roots of *A. cepa* at different concentrations of bark extracts indicates its cytotoxicity effect even at micromolar concentrations, which may be due to mitotic arrest leading to cell death (Arya and Mokherjee, 2014). Our observation on DNA and chromosomal damage in *A. cepa* caused by the crude bark extract of *T. codiforlia* in the present investigation is comparable with the findings of Figueiro *et al.* (2016) using *Glandularia selloi* leaf extract. Root extract of *Coccinia grandis* also showed cytotoxic and pesticidal effect (Hasan and Sikdar, 2016). *Rhaphidophora korthalsii* - a root climber used in Chinese traditional medicine for cancer and skin diseases has been reported to have cytotoxic effect on NK cells against the NK sensitive target K562 cell line (Yeap *et al.,* 2013). The higher percentage of cell death might be due to higher lipid peroxidation activity that might be leading to membrane instability.

4. Conclusion

The findings of our study established the cytotoxic effect of the crude bark extract of *T. codifolia* at very low dose on root tip cells of onion (*Allium cepa*). It can be concluded that onion is sensitive to plant alkaloids similar to animal cells at very low concentrations and therefore, can be used as an indicator for cytotoxicity. The active principles of the methanol fraction of bark extract have high cytotoxic effect, which necessitates detailed study to elucidate the molecular mechanism of cell death.

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