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Cytotoxic potential of bark extract of *Hymenodictyon orixense* (Roxb.) Mabb. - a medicinally important tree, on root meristematic tissues of *Allium cepa* L.

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ABSTRACT

There exist a large number of bioactive compounds in plants, out of which a few have been examined and these continue to be important sources of cytotoxic agents. Now-a-days, worldwide effort has been made to find out new cytotoxic compounds from plants. The present study deals with cytotoxic effect of anthroquinone isolated from *Hymenodictyon orixense* bark extract on *Allium cepa* root meristems. Two concentrations (20µg and 50 µg) of bark crude extract of *H. orixense* were studied under 6 h and 24 h of treatment on root meristematic tissues. The direct effect of cytotoxic chemicals of bark methanolic extract revealed significant reduction of mitotic index. The mitotic index reduced significantly (~1.7 to ~2.5 fold) in 24 h of treatment at 20 ìg/ml and 50 ìg/ml concentration respectively as compared to control. Different cytological abnormalities like clumping of chromosomes, DNA fragmentation, spindle arrest, diplochromatin, chromosome erosion and chromosome break were observed. The preliminary investigation showed that this plant-derived anthraquinone can destroy the cells in micromolar concentration and hence may be a potential anticancer drug.

1. Introduction

The use of medicinal plants by human is very ancient and WHO estimated that about 80% of the world population needs herbal medicines to cure diseases. *Hymenodictyon orixense* (Roxb.) Mabb. [Syn: *H. excelsum* (Roxb.) Wall.], belonging to the family Rubiaceae, is a deciduous tree up to 20 m tall having dark grey bark, simple and opposite leaves, terminal racemose inflorescence. It is found mostly in semi-evergreen forests throughout the Western Ghats up to 500 meters, but also occur in moist and dry deciduous forest patches of Mayurbhanj, Kalahandi, Keonjhar, Nayagarh, Phulbani, Sonepur, Gajapati, Sambalpur, Balangir and Koraput districts of Odisha.

The stem bark contains tannin, toxic alkaloids, hymenodictine, aesculin, an apioglucoside of scopoletin and hymexelsin (Rao *et al*., 1988). Anthraquinones, rubiadin and its methyl ether, lucidin, 2-benzylxanthopurpurine, anthragallol, soranjidiol and morindone have also been known from the roots of this plant (Rastogi & Mehrotra, 1996). Leaves contain acetylene fatty acids, triglycerides and triterpenes (Anonymous, 1948). Accumulation of scopoletin has been reported from this plant in addition to other related coumarins and coumarin glycosides such as scopolin, esculetin and esculin (Swe, 2008). A variety of biological activities such as anti-inflammatory, anti-allergic and anti-angiogenesis have been reported from this medicinal plant.

There are only a few reports on the cytotoxic properties of different extracts of *Hymenodictyon orixense* (Khairunnisa and Karthik, 2014). Molecular docking studies revealed that anthraquinones and scopoletin obtained from the plant have anticancer property mainly for prostate cancer, which needs to be validated (Rahman, 2015). The bark of *H. excelsum* is used as an astringent and febrifuge and for treatment of fever and tumors, while the leaves are used to treat ulcers, sialitis, sore throat, tonsillitis and inflammatory conditions in traditional medicine system (Nareeboon *et al*., 2009).

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Although chromosome number of *Hymenodictyon orixense* has been determined as n=33 indicating hexaploidy (Bedi, 1991), no detailed study on genetic variability in terms of ploidy level and phytochemical constituents has been done till date. Keeping in view that very scanty reports on cytotoxic and anti-inflammatory properties of the coumarin derivatives are available in different parts, we report here the cytotoxic activity of bark extract of *Hymenodictyon orixense* on *Allium cepa* root meristems.

2. Materials and methods

2.1 Plant material

The plant material consists of dried powdered bark of *Hymenodictyon orixense,* collected from Hatiasila village of Nuagaon block, Nayagarh District, Odisha State and identified with the help of Flora of Orissa (Saxena & 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 or *Allium cepa* (Liliaceae), which has 16 long chromosomes. This species is an excellent plant material and a useful biomarker for environmental monitoring with many advantages, such as large number of roots from a bulb, low costing plant material, short duration to conduct a test, easy storage and handling, large cells with easily visible long chromosome and ease of observing abnormal phenomena of chromosome during mitosis (Banerjee and Giri 2014).

2.2 Extraction of crude bark extract

The fresh bark of *H. orixense* was collected and dried under room temperature and powered mechanically. The powered bark sample was kept in air tight container until the time of use. The powered bark (50 gm) was exhaustively extracted with 99.8% methanol (200 ml) using Soxhlet apparatus at 40^{o}C. The methanolic extract was filtered and the filtrate condensed under reduced pressure and was concentrated to dryness under controlled temperature (40^{o}C) with the help of IKA RV10 Rotary Evaporator (Germany) fitted with IKA HB10 digital temperature controller, vacuum pump and water chillier (Cole-Parmer).

2.3. Cytotoxic study

Allium cepa var. Deshi was grown in sand in the net-house of the Department of Botany, Utkal University, Vani Vihar, Bhubaneswar and was used as experimental material for cytotoxicity test. After 4-5 days, bulbs with 3- 4 cm long roots were washed in running tap water and subjected to treatment of 0µg/ ml (control), 20µg/ ml and 50 µg/ ml 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% acetic acid for 15 min and were stained in 2% aceto-orcin: 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 the provided stress conditions.

2.4 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^{o}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 50 µg ml^{-1} as compared to control or 6 h of treatment. The concentration of the bark extract and the time of exposure played an important role in 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 40.21 and 28.49 respectively in 6 h and 24 h treatment in 20 ìg/ml, which was slightly higher that 50 ìM ìg/ml concentration. The mitotic index dropped significantly by about ~1.7 to ~2.5 fold in 24 h of treatment at 20 ìg/ml and 50 ìg/ml concentration respectively when compared to that of control. Control root tip cells showed normal mitosis. However, C-mitoses, chromosome bridges, chromosome fragments, chromosomal clumping, chromosome stickiness, chromosome break and chromosome erosions were recorded (Fig. 1 a-h). Spindle fiber abnormality (SFA) included C-

mitosis, chromosome stickiness, chromosomal clumping; and chromosomal abnormality (CA) included chromosomal breaks and chromosomal bridges (Fig. 1). Laggard chromosomes and distorted chromatin (Fig. 1e) were observed in direct treatment. The frequency of chromosome break and chromosome erosions increased significantly with increasing concentration of bark extract and prolongation of treatment time. Very condensed chromosomes with mitotic effects in the cell as well as chromosome fragments were noticed in 50 ìg/ml (Figs. 1 b & c). Sticky chromosome bridges were found in low doses *i.e.* 20 ìg/ml of extract. Chromosomal stickiness as well as break and erosion are usually irreversible (Fig. 1 g & h) and could be due to the toxic effects of plant extracts leading to cell death. Like mitotic index, more metaphase percentage was noticed at lower concentration compared to other concentrations. Likewise, the percentage of anaphase also varied in different concentrations. The oxidative damage by bark extract might have induced various chromosomal abnormalities, which are dose-dependent (Table 1). The highest percentage of abnormalities was noted with 50 *ì*g/ml treatment of extract as compared to treatment with 20 *ì*g/ml.

A comparative analysis of all abnormalities percentage of 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 chromosome abnormalities like spindle fiber anomalies leading to pretreatment effect, chromosome break, chromosome break with lagging chromosomes, chromosome erosion, chromosome clumping, sticky chromosomal bridge formation and C-mitosis formation. The maximum number of chromosome breaks and erosions was found in the 50 *ì*g/ml treatment for 24 h. At 20 *ì*g/ml, a number of abnormalities were also found with significant percentages. The chromosomal abnormality percentage showed dose- and time-dependent increase. The treated root tips showed abnormalities of spindle formation in low dose that leads to metaphase arrest and separation due to direct bark extract treatment. Chromosomal break was observed in 50 *ì*g/ml treatment, while 20 *ì*g/ml treatment produced early separation of chromosomes in anaphase. Chromosomal damages were prominent resulting in chromosomal erosion and intense break of chromosome at 50 *ì*g/ml treatment. That clearly indicated that methanolic extracts have potential carcinogenic

Table 1

Effect of crude bark extract of *Hymenodictyon orixense* on mitotic index of *Allium cepa* root apical meristems

Treatment	20 µg/ml		50 µg/ml	
	Mitotic index Mean (±S.D.)	% Cell aberrations Mean (±S.D.)	Mitotic index Mean (±S.D.)	% cell aberrations Mean (±S.D.)
Control	48.17±1.23	1.21±0.56	46.25±0.98	1.02±0.67
6 h	40.21±2.14	58.27±2.13	35.49±2.56	64.70±1.29
24 h	28.49±1.56	72.21±1.59	22.31±2.09	83.14±1.78

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 anticancer 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 bridges and presence of more chromosome fragments in the cells might be due to chromosome replication and enhanced protein synthesis in roots induced under plant extract stress. The active molecules of bark extract might be interfering with calmodulin, a calcium modulating protein, located in the mitotic spindle by influencing the uptake of Ca^{++} and causing abnormal processes of chromosome movements leading to mitotic abnormalities (Liu *et al*., 1995).

There are not many reports on biotoxicity in plants 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 *Hymenodictyon orixense* at the cell and tissue levels, affecting the elongation

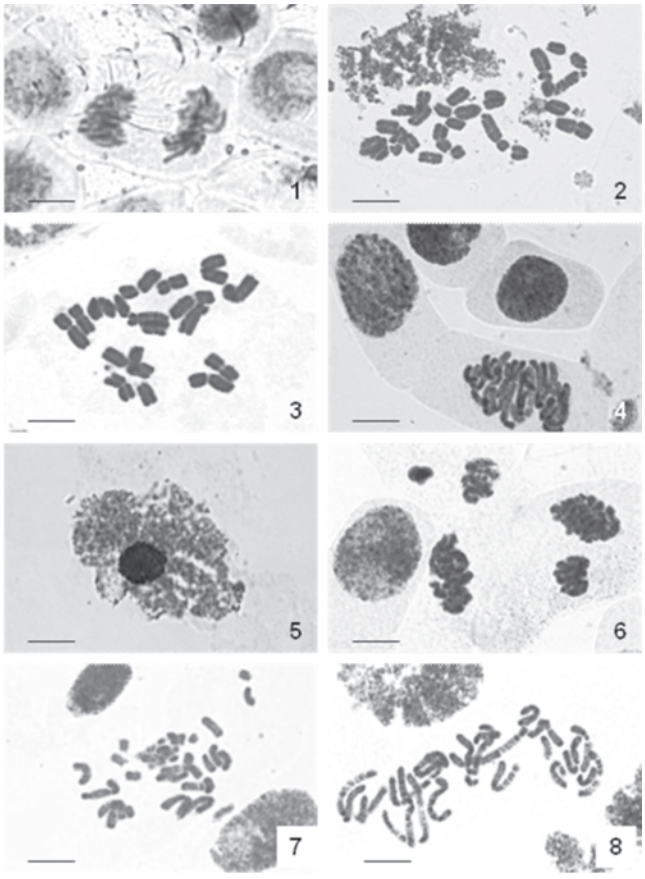

Fig. 1: Cytotoxic effects of crude extract of *Hymenodictyon orixense* on *Allium cepa* root tip cells- (a): Sticky chromosome bridge at 20 Bar = 20µg/ml concentration at 6 h direct treatment. (b-c): Chromosome condensation with spindle arrest with metaphase effect at 20 µg/ml concentration at 24 h and chromosome break at 50µg/ml concentration at 24 h treatment. (d-e): Nuclear clumping and nuclear chromatin decondensation and fragment at 50µg/ml concentration at 24 h treatment. (f-h): Chromosome break and erosion at 50µg/ml concentration at 24 h treatment.

zones of root apex. The effect of low doses of methanolic bark extract was found to be significant on oxidative damage of chromosome structure. Chromosomal damage includes gross structural changes that are initiated by chromosome breaks and erosion.

3.2 Cell death and cytotoxicity

Increased Evans Blue uptake of 10.25 % was found with treatment of 20 µg/ml and 18.65 % in 50 µg/ml 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 found to vary with duration of treatment. The uptake of Evans 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 of treatment. The increase in Evan's Blue uptake in the roots of *A. cepa* at different concentrations of bark extract 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 *H. orixense* in the present investigation is comparable with the findings of Figueiró *et al.* (2016) using *Glandularia selloi* leaf extract. Root extracts of *Coccinia grandis* also showed cytotoxic and pesticidal effect (Hasan and Sikdar, 2016). *Rhaphidophora korthalsii*- a root-climber plant used in Chinese traditional medicine for cancer and skin disease, is also reported to have cytotoxic effect on NK cell 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 establish the cytotoxic and genotoxic effects of crude bark extract of *Hymenodictyon orixense* 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 cytotoxic study. The active principles of the methanol fraction of bark extract have high cytotoxic effect, which necessitates detailed study to elucidate the molecular mechanisms of cell death.

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References

Anonymous (1948). The Wealth of India: A dictionary of Indian raw materials and industrial products. Volume 4, National Institute of Science Communication and Information Resources (CSIR), New Delhi.

Arya, S. K. and Mokherjee, A. (2014). Sensitivity of *Allium cepa* and *Vicia faba* towards cadmium toxicity. J. Soil Sci. Plant Nutr. 14:447–458.

Baker, C. J. and Mock, N. M. (1994). An improved method for monitoring cell death in cell suspension and leaf disc assays using Evan's Blue. Plant Cell Tissue Organ Cult. 39: 7–12.

Banerjee, N. and Giri, A. K. (2014). Arsenic induced health effects, genetic damage and genetic variants in the population exposed to arsenic through drinking water in West Bengal. Proc. Indian Natl. Sci. Acad. 80: 565–581.

Bedi, Y. S. (1991). Chromosomal conspectus and evolutionary status of Indian commercial timbers (Hardwoods). Cytologia 56: 665-672.

Figueiró, L. R., Comerlato, L. C., Da Silva, M. V., Zuanazzi, J. A. S., Von Poser, G. L., Ziulkoski, A. L. (2016). Toxicity of *Glandularia selloi* (Spreng.) Tronc. leave extract by MTT and neutral red assays: influence of the test medium procedure. Interdiscip. Toxicol. 9: 25–29.

Hasan, F. M. and Sikdar, B. (2016). Screening of antimicrobial, cytotoxic and pesticidal activities of *Coccinia grandis* (L.) Voigt. J. Microbiol. Biotech. Food Sci. 5:584-588

Khairunnisa, K. and Karthik, D. (2014). Evaluation of *in vitro* apoptosis induction, cytotoxicity activity of *Hymenodictyon excelsum* (Roxb) Wall. in Dalton's lymphoma ascites (DLA) and lung fibroblast - Mouse L929 cell lines. J. App. Pharmaceut. Sci. 4(8):11-17.

Liu, D. H., Jiang, W. S., Wang, W. and Zhai, L. (1995). Evaluation of metal ion toxicity on root tip cells by the *Allium* test. Israel J. Plant Sci. 43: 125–133.

Nareeboon, P., Komkhunthot, W., Lekcharoen, D., Wetprasit, N., Piri-yapolsart, C., Sutthivaiyakit, S. (2009). Acetylenic fatty acids, triglyceride and triterpenes from the leaves of *Hymenodictyon excelsum*. Chem. Pharm. Bull. (Tokyo) 57(8): 860–862.

Rahman, M. M. (2015). Evaluation of *Hymenodictyon excelsum* phytochemical's therapeutic value against prostate cancer by molecular docking study. Jundishapur J. Nat. Pharm. Prod. 10(1):e18216.

Rao, P. S., Asheervadam, Y., Khaleelullah, M., Rao, N. S., Murray, R. D. (1988). Hymexelsin, an apiose-containing scopoletin glycoside from the stem bark of *Hymenodictyon excelsum*. J. Nat. Prod. 51(5):959–961.

Rastogi, R. P. & Mehrotra, B. N. (1996). A compendium of Indian medicinal plants, Vol. 2, Central Drug Research Institute, Lucknow.

Saxena, H. O. & Brahmam, M. (1995). The flora of Orissa, Vol. 2, RRL and Orissa Forest Development Corporation Ltd., Bhubaneswar.

Swe, N. N. (2008). Scopoletin from *Hymenodictyon orixense* (Roxb.) Mabb. Jour. Myan. Acad. Arts & Sci. 4:193-203.

Yeap, S. K., Omar, A. R., Ho, W. Y., Beh, B. K., Ali, A. M., Alitheen, N. B. (2013). *Rhaphidophora korthalsii* modulates peripheral blood natural killer cell proliferation, cytokine secretion and cytotoxicity. BMC Complementary and Alternative Medicine 13:145.

Zhang, S. S., Zhang, H. M., Qin, R., Jiang, W. S. and Liu, D. H. (2009). Cadmium induction of lipid peroxidation and effects on root tip cells and antioxidant enzyme activities in *Vicia faba* L. Ecotoxicology 18: 814–823.