

EVALUATION OF GENOTOXICITY AND CYTOTOXICITY OF *TINOSPORA CORDIFOLIA* (THUNB.)

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ABSTRACT

The communication deals with the genotoxic and cytotoxic effects of commonly used medicinal plant, *Tinospora cordifolia* (Thunb.). Genotoxicity was studied using the *Allium cepa* L. root tip model using three dose concentrations (10, 20 and 30 mg/mL) for 24, 48 and 72 h. T-test was performed to validate the significance of data. Data revealed dose dependent alterations in the Mitotic Index (M.I.). The M.I. first showed a significant increase and then reduced as the concentration of extract was raised. The extract increased the rate of growth in the *A. cepa* root meristem cells. Moreover, no spindle disturbances, or micronuclei formation was observed indicating that the stem extract did not exhibit prominent genotoxic activity. Cytotoxicity of *T. cordifolia* extract was evaluated using RBCs. The extract showed significant dose dependent cytotoxic activity ($p=2.98$). Considering the above results, the use of aqueous stem extract of *Tinospora cordifolia* can be considered safe at low dose.

INTRODUCTION

Use of plants and products thereof as remedies to ease pain and cure diseases has a long cultural history (Rojas *et al.*, 2003), which was trounced by the formulation of synthetic drugs during the twentieth century (Russell, 2002). In modern times however, these drugs have proved to be ineffective to control many of the diseases against which they are administered (Rojas *et al.*, 2003; Benkeblia, 2004). Dreaded of the side-effects and cynical due to the failure of these chemotherapeutics, people are once again turning their interests to the time-tested herbal remedies (Ghani, 2003; Mahesh and Satish, 2008; Thenmozhi and Rajeshwari, 2010), which have been proven safe and effective (Parekh and Chandra, 2007). A number of studies have been concluded proving the efficacy of several plant extracts for containment of a wide range of diseases, however the reports pertaining to the precise dosage of these extracts are lacking (Adegbite and Sanyaolu, 2009). It is an established fact that plants are the primary source of natural toxins, which induce cytotoxic and genotoxic effects (Plewa and Wagner, 1993). Hence, an assessment of the cytotoxic and genotoxic potentiality of a plant product is desirable to ensure safety, such that ill-consequences of overdose if any could be avoided (Fateme and Khosro, 2012).

Tinospora cordifolia (Menispermaceae) is an important medicinal plant native to tropical Indian-subcontinent (Srivastava, 2011). Widely used in traditional Ayurvedic and folk medicine systems, *T. cordifolia* has been screened for its anti-pathogenic, anti-oxidative, immunomodulatory, anti-allergic rhinitis, anti-ulcer, anti-hyperglycemic,

cardioprotective, chemopreventive, hepatoprotective, hypolipidaemic, neuroprotective and radioprotective actions and against obstructive jaundice (Thatte *et al.*, 1992; Dhuley, 1997; Grover *et al.*, 2000; Stanley *et al.*, 2000; Premanath and Lakshmidevi, 2010; Megraj *et al.*, 2011; Dandapat *et al.*, 2013; Kullu *et al.*, 2013; Kumar *et al.*, 2013a,b; Tabassum *et al.*, 2013). The genotoxic and cytotoxic activities of *T. cordifolia* however have not been studied. Hence it is imperative to screen *T. cordifolia* for its genotoxic and cytotoxic activities for acceptance of the health claims in terms of safety.

Among the various possible tests that would serve the purpose, *Allium cepa* root model and erythrocyte model are the most feasible ones for genotoxic and cytotoxic bioassays respectively (Sousa *et al.*, 2009; Rajeshwari *et al.*, 2012). With this pre-context, the present study was undertaken to assess the genotoxic and cytotoxic activities of *Tinospora cordifolia* stem.

MATERIALS AND METHODS

Collection of plant material

The fresh mature parts of stem were collected, chopped, dried in a shade under room temperature for six to seven days and then crushed into coarse powder using electric grinder. The powder was sieved to get fine powder using fine plastic sieve which was stored in air tight bottle in the laboratory until required.

Preparation of the extract

50g of the powder was subjected to extraction by soxhlet

using distilled water. The extract obtained was filtered, concentrated, dried and was stored in air tight containers at room temperature for further studies.

Test for genotoxicity

The genotoxicity of *T. cordifolia* was determined using *Allium cepa* root model following Sousa *et al.* (2009). Onions weighing 35 ± 5 g were bought, the dry roots were removed and the bulbs were placed in tap water for 48h. at room temperature for germination of roots. Three concentrations, 10 g/L, 20g/L and 30 g/L of the extract were administered and the root tips were exposed to the treatment for 24, 48 and 72 h. At end of each period, the root tips were collected and squash prepared in acetocarmine. The squash was examined under a microscope for micronuclei formation, while the cells at different stages of division were counted. Mitotic index (M.I.) was calculated using the formula:

$$M. I. = \frac{\text{Number of dividing cells}}{\text{Total number of cells studies}} \times 100$$

Test for cytotoxicity

The cytotoxic effect of *T. cordifolia* stem was evaluated by determining the hemolytic activity of the aqueous stem extract using goat blood. An erythrocyte suspension was prepared by adding 5% (by volume) of sodium citrate (36.5 g/L) to fresh blood and centrifuged at 3000 rpm for 5 min to separate the erythrocytes. 2% erythrocyte suspension was prepared by adding 49mL phosphate buffer (pH 7.4) to 1mL packed erythrocytes. Serial dilution of plant extract was prepared using phosphate buffer. 1mL of citrated blood was mixed with equal volume of diluted plant extracts and the volume was adjusted to 5mL by phosphate buffer. The mixture was allowed to stand for 6h. at room temperature. Hemolysis was monitored spectrophotometrically at 540 nm, depicted by an increase in the optical density of the solution due to the release of hemoglobin through time (Noudeh *et al.*, 2011).

Statistical analysis

T-test and two-way anova were performed to test the influence of dose and duration on significance on genotoxic and cytotoxic activities respectively.

RESULTS AND DISCUSSION

The chemical constituents of the extract may alter the cell cycle. The interaction of chemical constituents of the plant with DNA, in one hand may stimulate the cell growth (Trivedi and Ahmad, 2013), while it may induce mutation and prove deleterious to the cell on the other hand (Celik, 2012). Genotoxicity of the aqueous stem extract of *T. cordifolia* was determined using the *Allium cepa* root model (Fig. 1).

In the present study, at an average, 1500 cells were observed for each concentration. In general, the mitotic index increased with low dose of the extract and then reduced as the dose concentration was increased. T-test was performed to test the significance of the changes in M.I. ($p=0.05$). The results show that after 24 h. of incubation, the M.I. of control was 17.97, which increased significantly to 18.18 ($p=0.350$) when treated with 10 mg/mL of the stem extract. Further with 20 mg/mL of extract, the M.I. reduced significantly to 18.44 ($p=0.332$),

while with 30 mg/mL extract the M.I. was found 18.70 ($p=0.238$). After 48h. of incubation, the M.I. of control was 17.94, which increased significantly to 18.00 ($p=0.460$) when treated with 10 mg/mL of extract. When treated with 20 and 30 mg/mL of stem extract, the M.I. increased to 18.25 and 18.69 respectively. After 72 h. of incubation, the M.I. of control was found to be 17.75, which increased to 17.84 when treated with 10 mg/mL of extract. On treatment with 20 mg/mL of the stem extract, a significant increase in M.I. was observed, which was calculated as 18.22 ($p=0.277$). The M.I. further increased to 18.567 when treated with 30 mg/mL of the extract. This increase in M.I. is a clear indicative that the *T. cordifolia* stem extract enhanced the rate of growth in root meristem cells of *A. cepa* which is obvious in different stages of the cell cycle.

A time and concentration dependant increase in the percentage of cells in prophase was observed (Fig. 2). This may be an indicative of impaired development of prophase bands due to inhibition of RNA synthesis, since RNA species are required for proper microtubular orientation in the root meristem cells (Utrilla and Torre, 1991). At 24 h. of incubation, 3.12 % cells were found in metaphase stage in the control, which reduced to 2.73 % after treatment with 10 mg/mL of the extract. The % of cells in metaphase was found to decrease to 2.05 and 1.51 % respectively when treated with 20 and 30 mg/mL of extract. The cells in metaphase were observed to be 3.43 % in the control after 48 h. of incubation, which reduced to 2.38 and 1.96 % when the treated with 10 and 20 mg/mL of the extract respectively, and decreased to 1.36 % when treated with 30 mg/mL of extract. After 72 h. of incubation, 3.8 % of cells were found to be in metaphase stage in control. When treated with 10 mg/mL of the stem extract, the number of cells in metaphase was found to reduce by 1.54 %, while after treating with 20 mg/mL of extract, only 1.73 % of cells were found in metaphase state. When treated with 30 mg/mL of extract, 1.06 % of cells were found to be in metaphase stage (Fig. 3). After 24 h. of incubation, the percentage of cells in anaphase decreased from 1.53 % in control to 1.06 % on treatment with 10 mg/mL of extract. When treated with 20 mg/mL of stem extract, the cells in anaphase showed decrease of 0.13 % as compared to control, while on treatment with 30 mg/mL of extract, 0.53 % cells were found in anaphase stage. 2.06 % cells were found in anaphase stage in the control at 48 h. of incubation, which was found to reduce by as much as 1.6 % when treated with 10 mg/mL of extract. A further decrease of 0.13 % was observed on treatment with 20 mg/mL of extract. Cells in anaphase stage were found to be 0.46 % on treatment with 30 mg/mL of extract at 48 h. of incubation. At 72 h. of incubation, 2.26 % cells were found to be in anaphase stage in the control, while on treatment with 10 mg/mL of extract, only 0.2 % cells were in anaphase. On treating with 20 mg/mL extract, cells count in anaphase decreased by 0.2 %. A further increase of 0.14 % was observed on treatment with 30 mg/mL of the extract at 72h. of incubation (Fig. 4).

A direct correlation exists between the amount of DNA and the duration of cell cycle. However, when treated with the pesticides and other genotoxins, the duration of cell cycle has been reported to increase due to induction of chromosome stickiness as a result of mutation in non-histone proteins required for chromosome segregation (Kowalska and

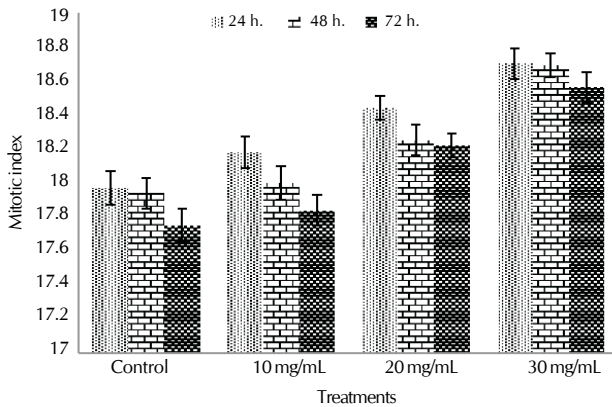


Figure 1: Effect of aqueous stem extract of *T. cordifolia* on Mitotic Index in *A. cepa* root meristems (values in $M \pm SD$; $n=3$)

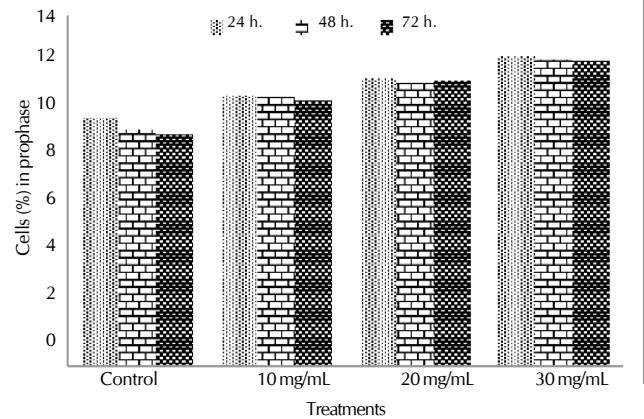


Figure 2: Effect of aqueous stem extract of *T. cordifolia* on prophase stage of cell cycle

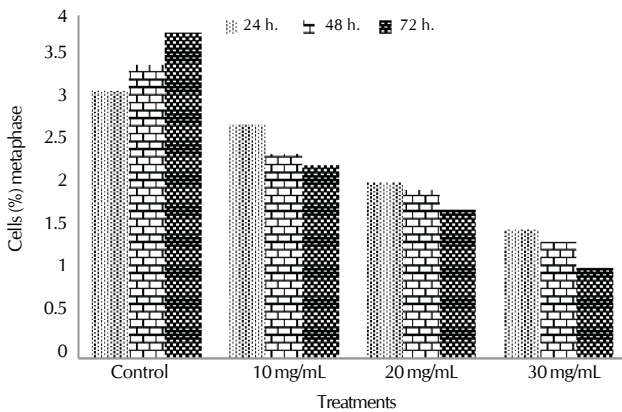


Figure 3: Effect of aqueous stem extract of *T. cordifolia* on metaphase stage of cell cycle

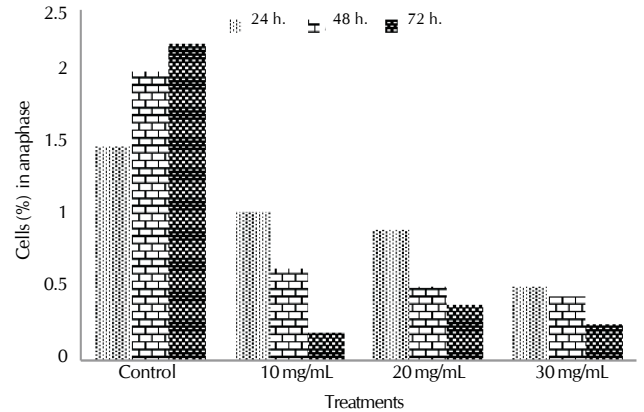


Figure 4: Effect of aqueous stem extract of *T. cordifolia* on anaphase stage of cell cycle

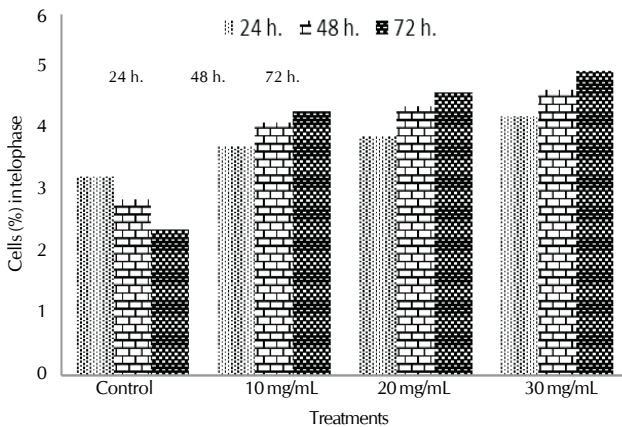


Figure 5: Effect of aqueous stem extract of *T. cordifolia* on telophase stage of cell cycle

Oscilowicz, 2002). This results into mitotic arrest and hence number of cells in metaphase and anaphase increases (Trivedi and Ahmad, 2013). In the present study, for a contrast, significant reduction in number of cells in metaphase and anaphase stages has been observed as compared to the control. This indicates that the *T. cordifolia* stem extract did not induce any chromosome stickiness or mutation, rather

the extract shortened the duration of these stages, hence increasing the rate of growth in *Allium cepa* roots.

A time and concentration dependant increase in telophase cell-count was observed, which may be an outcome of shortening of the metaphase and anaphase stages of the cell cycle. In control, at 24 h. of incubation, 3.3 % of the cells were in telophase. After treating with 10 mg/mL of extract, the count increased to 3.8 %. A further increase of 0.16 % in cell count was observed on treatment with 20 mg/mL of extract, while after treatment with 30 mg/mL of extract, cell count was 4.3 %. At 48h. of incubation, the percentage of cells in telophase showed marked increase with concentration of the extract. The cell count at 48 h. of incubation was 2.93 % in control, which increased to 4.2, 4.46 and 4.74 % after treatment with 10, 20 and 30 mg/mL of extract respectively. At 72 h. of incubation, 2.43 % of cells were found in telophase in control, while after treatment with 10 mg/mL of extract, the cell count increased by 1.95 %. When treated with 20 mg/mL of extract, the cell count however increased only by 0.31 %, while after treating with 30 mg/mL, 5.06 % cells were found in telophase (Fig. 5).

The genotoxicity of some other plant extracts have been worked out. The mitotic index (M.I.) was reported to decrease from 32.37 to 22.52 after treating with 100 g/L of aqueous leaf extract of *Vernonia amygdalina*, while the M.I. dropped to

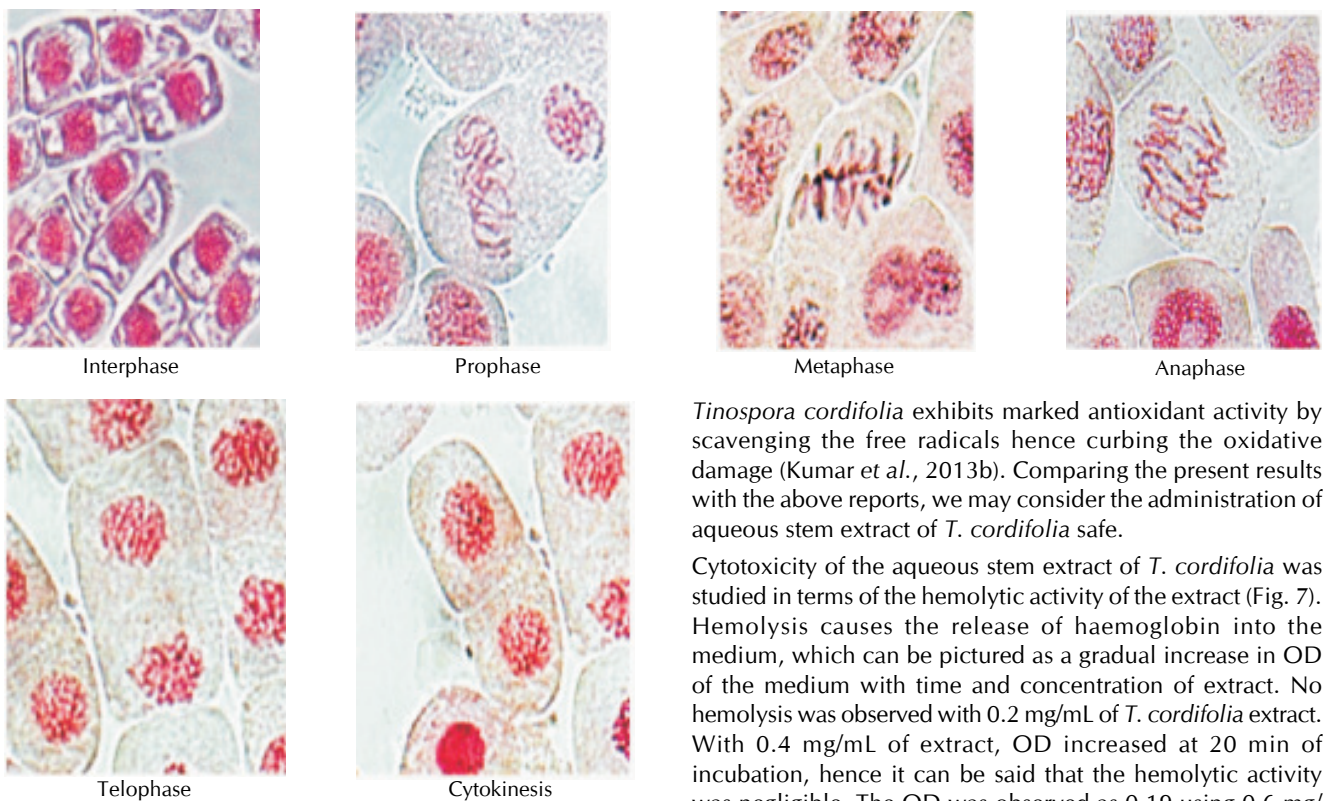


Figure 6: Different stages of cell cycle after treatment with aqueous stem extract of *T. cordifolia* (30 mg/mL) for 72 h. (1000X)

12.85 when treated with 500 g/L of the same extract. The extract also induced chromosomal aberrations with 200, 400 and 500 g/L of the extract (Adegbite and Sanyaolu, 2009). Significant dropdown in the M.I. of *A. cepa* was reported on treatment with aqueous and petroleum ether leaf extracts of *Mimosa pudica*, from 8.43 in control to 3.19 with 2 mg/mL of extract. The *M. pudica* extracts also induced cellular abnormalities even at low concentration of 2 mg (Malode *et al.*, 2012). Similar prominent decrease in the mitotic index and induction of cellular anomalies in *A. cepa* root tips was demonstrated by Fatemeh and Khosro (2012) using aqueous root extracts of *Arctium lappa*. The mitotic index of control was reported as 7.26, while that treated with 12.5 mg/mL of root extract dropped to 3.4 i.e. by 3.86 %. On treating the *A. cepa* roots with even high dose of 30 mg/mL of *T. cordifolia*, the decrease in M.I. was not as much. Further the mitotic index dropped to 1.05 and 0.583 on application of 62.5 and 125mg/mL of *A. lappa* root extract.

Micronucleus assay is quite easy but sensitive approach for detection of genotoxins, which has been considered at par with the chromosome aberration assay (Adhvaryu *et al.*, 1991). Micronuclei are formed due to disturbances in chromosome apparatus, like chromosome breakage and are also associated with detection of cancer (Trivedi and Ahmad, 2013). In the present study since no micronuclei were observed, hence it is confirmed that *T. cordifolia* does not induce genotoxicity. Furthermore, it is established that the common effect of toxicants is that they generate free radicals, which enact deleterious effects on protein, lipid and DNA, and hence induce cellular damage (Foyer *et al.*, 1997). On the contrary,

Tinospora cordifolia exhibits marked antioxidant activity by scavenging the free radicals hence curbing the oxidative damage (Kumar *et al.*, 2013b). Comparing the present results with the above reports, we may consider the administration of aqueous stem extract of *T. cordifolia* safe.

Cytotoxicity of the aqueous stem extract of *T. cordifolia* was studied in terms of the hemolytic activity of the extract (Fig. 7). Hemolysis causes the release of haemoglobin into the medium, which can be pictured as a gradual increase in OD of the medium with time and concentration of extract. No hemolysis was observed with 0.2 mg/mL of *T. cordifolia* extract. With 0.4 mg/mL of extract, OD increased at 20 min of incubation, hence it can be said that the hemolytic activity was negligible. The OD was observed as 0.19 using 0.6 mg/mL of extract, which increased to 0.2 at 15 min of incubation and no further increase was observed. Similarly, with 0.8 mg/mL of extract, the OD was 0.21 which saturated at 0.22 at 15 min of incubation; while with 1 mg/mL of extract, OD was 0.27, which increased to 0.28 at 5 min of incubation, with no further increase within the experimental time frame. Two-way anova was performed at 4 d.f. and $p=0.05$. The data revealed that the dose-dependence of the plant extract was significant ($p=2.98$), while the duration dependence of cytotoxicity was found insignificant ($p=0.010665$).

The chemical constituents of the plants, particularly saponins

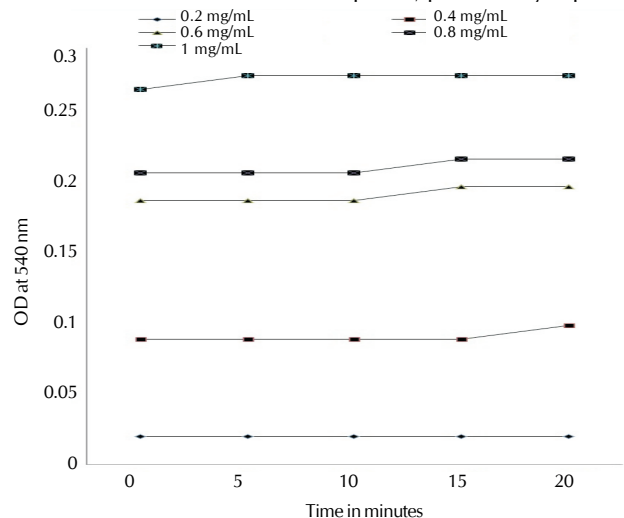


Figure 7: Cytotoxic activity of aqueous stem extract of *Tinospora cordifolia* at different concentrations

are known to increase the membrane permeability ((Urbanska et al., 2009; Noudeh et al., 2011) and hence are considered as potential adjuvants. However, because of the same property, they cause hemolysis (Urbanska et al., 2009) and results in cell-death. The present results show concentration-dependent increase in the hemolytic activity and a slight increase in the same with time of incubation. Aqueous leaf extract of *Piper betel* has been reported to induce 10.06 % hemolysis while the methanolic extract of the same induced 8.25 % hemolysis (Chakraborty and Shah, 2011). The hemolytic activity of *Ulva fasciata* has been reported in terms of hemolytic units (HU); for chicken blood, the extract showed a maximum of 64 HU/mg, while for goat blood it was 32 HU/mg (Priyadarshini et al., 2012). *Tribulus terrestris*, *Trigonella foenum-graceum* and *Echium amoenum* were reported to cause 11.7, 2.9 and 4.7 % hemolysis respectively (Noudeh et al., 2011). From the present results, it is evident that since OD value is quite low than unity, we may say that the hemolytic activity, and hence cytotoxicity of *T. cordifolia* is not very pronounced and hence the use of crude stem extract is quite safe.

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