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# *In vitro* cytotoxicity of *Tinospora cordifolia* stem extract in Daltons Lymphoma Ascites cell lines#

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**Abstract**

*Tinospora cordifolia (TC) is one of the most versatile medicinal plants in Ayurvedic medicine used for the treatment of various ailments. The present study was carried out to investigate the in vitro cytotoxicity of the aqueous extract of TC stems on Dalton's lymphoma ascites (DLA) cell lines in comparison with a reference drug, cisplatin through 3-(4, 5-dimethylthiazol-2-yl)-2, 5-phenyl tetrazolium bromide assay (MTT) and Trypan blue dye exclusion methods. Phytochemical analysis of aqueous extract of TC revealed the presence of alkaloids,* flavonoids, steroids, glycosides, phenols, anthraquinones, quinones, saponin, carbohydrates and proteins. The *MTT assay revealed that the mean per cent inhibition of cells at the test concentrations of 5, 10, 20, 40, 80, 160, 320, 640 µg/mL ranged from 6.848 ±0.01 to 59.686 ±0.13 for TC extract and from 29.129 ±0.13 to 80.902 ±0.05 for cisplatin, respectively. The $IC_{50}$ values of TC and cisplatin in DLA cells were estimated to be 72.05* µg/mL and 65.44µg/mL *respectively. Trypan blue dye exclusion revealed the mean per cent cell viability to be 59.40 ±0.04 and 55.17 ±0.01 for TC stem extract and cisplatin respectively. The mean percent cell viability and inhibition differed between TC extract and cisplatin at $IC_{50}$ as well as at any given concentration significantly ($p<0.01$). The tests revealed that the cytotoxic*

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*effect of TC extract on DLA cells was moderate and to a comparable extent with cisplatin. The results pointed out that TC induced cytotoxicity in DLA cells indicative of its probable apoptotic potential and therapeutic merit in the treatment of lymphomas.*

**Keywords**: *Tinospora cordifolia, cytotoxicity, MTT assay, Trypan blue dye*

Traditional herbal medicines are gaining importance worldwide. The genus *Tinospora* under the family *Menispermaceae* is known to contain phytochemicals with marked therapeutic activity. *Tinospora cordifolia* (TC) is one of the most versatile medicinal plants in Ayurvedic medicine known for medicinal properties like anti-diabetic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-leprotic, anti-malarial, hepato-protective, immune-modulatory and anti-neoplastic actions (Tambekar *et al.,* 2009; Parthipan *et al*., 2011; Saha and Ghosh*,* 2012). It is also used as a *rasayana* to improve the immune system and body resistance against infections. The leaves, stem and bark are reported in the treatment of fever, chronic diarrhea, dysentery, jaundice, cancer, bone fracture, general debility, cough, pain in the ear, leucorrhea, asthma, skin disease, insect and snake bites and eye disorders. *Tinospora cordifolia* is also termed a 'divine nectar' or 'heavenly elixir' in Ayurveda due to its high medicinal value and rejuvenating potential. Its pharmacological action is categorised according to taste, property (*guna*), digestion and metabolism, potency and mechanism of action. The stem of the plant is approved for use by the Ayurvedic Pharmacopoeia of India (Anonymous, 2001). The anti-cancer activity of various alkaloids present in the plant have been reported in Ehrlich ascites carcinoma in mice (Jagetia and Rao, 2006), lung adenocarcinoma cell lines (Pandey *et al*., 2008), DMBA induced skin carcinoma in mice (Ali and Dixit, 2013), C6 glioma cell lines (Mishra and Kaur, 2013) and HeLa cells (Jagetia and Rao, 2015). In this context, the present study was carried out to screen the aqueous extract of *T. cordifolia* stem (TC) for the presence of different phytochemicals and assess its *in vitro* cytotoxicity in DLA cell lines through MTT assay and Trypan blue dye exclusion methods. The study is a pioneer attempt to verify the above property of the traditional Ayurvedic form and formulation of *Tinospora cordifolia* used widely in human clinical practice.

## Materials and methods

### *Preparation of aqueous extract of T. cordifolia stem*

The fresh stems of *Tinospora cordifolia* (TC) were collected from Chelannur, Calicut. The specimens were authenticated at the Centre for Medicinal Plants Research, Arya Vaidyasala, Kottakkal, Kerala. The aqueous crude extract commonly used in human clinical practice (called "*ghana*" in Ayurveda) was prepared in the traditional way as per Siddha Yoga Samgraha (Acharya, 2006) from the stems which were cleaned, dried under shade, cut into small pieces, soaked four times in water and heated to get a decoction. The decoction was reheated until it became semisolid and dried in the oven at 55°C. The aqueous extract was then stored under refrigeration (4ºC) in an air-tight container. The fresh stems yielded 9.54 per cent aqueous extract with reference to the initial material obtained after cutting the stem.

### *Qualitative phytochemical analysis of TC stem extract*

Phytochemical analysis of the aqueous stem extract of TC was carried out to detect the presence various bioactive compounds. The different tests adopted were Mayer's test (Ugochukwu *et al.*, 2013) for the detection of alkaloids, Molish's test for the presence of carbohydrates (Foulger, 1931), Biuret test for the presence of proteins (Wokes and Still,

1942), foam test for the presence of saponins (Kareru *et al*., 2008), alkaline reagent test for the presence of flavonoids (Ugochukwu *et al*., 2013), Ferric Chloride Test for the presence of phenols (Soloway and Wilen, 1952), Salkowski test for the presence of terpenoids (Soni and Sosa, 2013), Borntrager's test for the presence of Anthraquinones, Libermann – Buchard test for the presence of steroids (Nath *et al*., 1946) and Keller-Killiani test for the presence of glycosides (Ugochukwu *et al*., 2013). The qualitative assessment was based on colour development classified as present or absent for alkaloid. A 10 per cent stock solution was used for carrying out the phytochemical analysis.

***Daltons lymphoma ascites (DLA) cell lines and the experimental animals***

Ten adult Swiss albino mice, six to eight week old, weighing 25-30 g each bought from the Small Animal Breeding Station of College of Veterinary and Animal sciences, Mannuthy (KVASU) were used to maintain the Dalton's lymphoma ascites cells intra-peritoneally. The DLA cells authenticated by NCCS, Pune and propagated at Amala Cancer Research Centre, Thrissur for nearly 20 years were used for the study. The animals were housed in polypropylene cages under standard management, feeding and optimal environmental conditions of air and illumination and acclimatised for a period of one week before the start of the experiment. Routine clinical examinations of all the animals were performed throughout the period of the experiment. The DLA cells were maintained continuously as ascitic fluid in the Swiss albino mice by intra-peritoneal injections @ $5x10^5$ cells / mouse counted using a cell counter as 2.5 million cells/ mouse as per the protocol followed by Thummar *et al*. (2016). The DLA cells thus maintained *in vivo* in the mice were used for the subsequent *in vitro* studies. The animal experimentation procedures were approved by the Institutional Animal Ethics Committee of the College of Veterinary and Animal Sciences, Mannuthy as per proposal No. IAEC/22/01 dt. 06/04/2022.

***MTT assay***

Cytotoxicity as indicated by the reduction in cell viability or the percent cell inhibition of TC extract and cisplatin (Batch No. HHBL 2109CI, Hetero Health Care Limited, Hyderabad) was monitored in DLA cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl tetrazolium bromide assay (MTT) performed in triplicate (Mosmann, 1983; Riss *et al.*, 2016). Viable cells were detected by the presence of purple formazan crystals formed through the reduction reaction of MTT by succinate dehydrogenase enzyme. The aqueous extract of TC was suspended in distilled water at a concentration of 1.3 mg/mL to get a stock solution, further diluted with RPMI medium to the desired concentrations. The DLA cells aspirated from the peritoneal cavity of tumour-bearing mice were washed thrice with PBS and seeded in 96-well plates at a density of $5x10^3$ cells per well in 100 µL RPMI medium. Cells were then exposed to different concentrations (5 µg/mL, 10 µg/mL, 20 µg/mL, 40 µg/mL, 80 µg/mL, 160 µg/mL, 320 µg/mL and 640 µg/mL) of TC stem extract (test) and cisplatin for a period of 24 hours. Six wells seeded with the DLA cells and left untreated with test extracts or drug were used as the experimental control. After 24 h, 10 µL of MTT (5 mg/mL) was added and the cells incubated at 37º for four hours. Viable cells developed dark purple formazan crystals. The crystals were solubilised with an organic solvent (200 µL of DMSO) and the absorbance or optical density (OD) at 595 nm was measured in an ELISA plate reader (Varioskan Flash, Thermo Fisher Scientific, Finland). Per cent cell inhibition in cells treated with test and control was calculated by the formula:

$$\text{Per cent cell inhibition} = \frac{(\text{Mean OD of Control} - \text{Mean OD of treated cells})}{\text{Mean OD of Control}} \times 100$$

The cytotoxicity of TC extract and cisplatin were expressed as the concentration of the extract/ drug inhibiting cell growth in DLA cells by 50 per cent ($IC_{50}$ values) and these were estimated from the per cent cell inhibition under various concentrations using the AAT Bioquest software (https://www.aatbio.com ).

***Trypan blue dye exclusion method***

The DLA cells aspirated from the peritoneal cavity of tumour-bearing mice were washed thrice with PBS. The cells ($5\times10^5$ cells/ mL) were added to tubes containing $IC_{50}$ levels of the TC extract and cisplatin and the volume made up to one millilitre using PBS. After the incubation of tubes for three hours at 37ºC, the cell viability was assessed by staining one part of the cell suspension (10 µL) with one part of Trypan blue dye (10 µL). Using an automated cell counter (Invitrogen Life Technologies USA), the viable and non-viable cells were counted. The test was performed in triplicate as per Strober (2015). The cell viability per cent was calculated as:

$$\text{Per cent viable cells} = \frac{\text{Total number of viable cells}}{\text{Total number of cells}} \times 100$$

**Statistical analysis**

The means and standard errors were estimated and tested for significance at one per cent level ($p<0.01$) using one-way ANOVA with equal replications (SPSS V. 22).

**Results and discussion**

***Qualitative phytochemical analysis of TC stem extract***

Phytochemical screening of the aqueous extract of TC stem (Fig 1) revealed the presence of nine different bioactive anti-oxidant and anti-proliferative compounds such as alkaloids, flavonoids, glycosides, phenols, saponins, anthraquinones, quinones, proteins and carbohydrates which are in agreement with the earlier reports (Nazir and Chauhan, 2018; Modi *et al*., 2021). Alkaloids in TC have been reported to exhibit potent cytotoxicity towards H1299 lung adeno carcinoma cell lines and further proved to prevent the cell proliferation by inducing apoptosis (Pandey *et al*., 2008).

**Fig. 1** *Tinospora cordifolia*

***MTT assay***

The MTT reduction assay (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) was performed to determine the per cent cell viability of DLA cells on treatment with the TC and cisplatin. After 24 hours of treatment, the mean per cent inhibition of cells for TC stem extract and cisplatin at the concentrations of 5, 10, 20, 40, 80, 160, 320, 640 µg/mL increased from 6.848 ±0.01 to 59.686 ±0.13 for TC extract and from 29.129 ±0.13 to 80.902 ±0.05 for cisplatin, respectively. Accordingly, at these concentrations, the mean per cent viability of cells was found to be decreasing from 93.151±0.01 to 40.314±0.13 for TC extract and from 70.870±0.13 to 19.097 ±0.05 for cisplatin, respectively. The mean per cent cell viability and per cent cell inhibition (Table 1) differed between TC and cisplatin at any given concentration significantly ($p<0.01$). The viability of the cells was found to be significantly higher with TC when compared to

cisplatin at any given concentration. The number of viable cells reduced on account of increased cell inhibition as the concentrations of TC extract and cisplatin increased. The decline in cell density indicated that the aqueous extract of TC produced a cytotoxic effect comparable to cisplatin in a similar concentration-dependent manner. The per cent cell viability and inhibition for the DLA cells under different concentrations of the TC extract and cisplatin are presented as dose-response curves (Fig.2 and 3).

The $IC_{50}$ values estimated in DLA cell lines under study were 72. 05 µg/mL and 65.44 µg/mL for TC stem extract and cisplatin respectively. The $IC_{50}$ values of TC extract and cisplatin were in close range of 72. 05 µg/mL and 65.44 µg/mL and this indicated both the compounds to cause 50 per cent of cell inhibition in DLA tumour cells in a close range of their concentrations. Based on the $IC_{50}$ value and the concentrations used in the study, the optimum cytotoxic level for TC stem extract in DLA cells was deduced as 80 µg/mL (Table 1). This finding is contradictory to the reports in TC-treated macrophage J774A.1 cell line where there was no decline in cell viability at 80 µg/mL (More and Pai, 2011). However, the result is indicative of the possibility of a selective cell inhibition of TC stem extract at the optimal cytotoxic concentration depending on the status of the cell lines as normal or

**Table 1.** The mean ± SE of per cent cell inhibition and viability of DLA cells with various concentrations of TC stem extract and Cisplatin in MTT assay

| Concentration (µg/ml ) | Cell viability % | | Cell inhibition % | |
|---|---|---|---|---|
| | TC stem extract | Cisplatin | TC stem extract | Cisplatin |
| 5 | 93.151 ± 0.01[a] | 70.870 ± 0.13[a] | 6.848 ± 0.01[a] | 29.130 ± 0.13[a] |
| 10 | 89.750 ± 0.04[b] | 64.844 ± 0.05[b] | 10.250 ± 0.42[b] | 35.155 ± 0.05[b] |
| 20 | 85.280 ± 0.10[c] | 59.423 ± 0.13[c] | 14.547 ± 0.10[c] | 40.577 ± 0.13[c] |
| 40 | 80.720 ± 0.07[d] | 52.035 ± 0.03[d] | 19.280 ± 0.07[d] | 47.964 ± 0.03[d] |
| 80 | 61.989 ± 0.42[e] | 40.654 ± 0.04[e] | 38.011 ± 0.20[e] | 59.345 ± 0.04[e] |
| 160 | 51.693 ± 0.05[f] | 31.643 ± 0.31[f] | 48.307 ± 0.05[f] | 68.357 ± 0.03[f] |
| 320 | 45.546 ± 0.02[g] | 26.923 ± 0.03[g] | 54.453 ± 0.20[g] | 73.077 ± 0.03[g] |
| 640 | 40.310 ± 0.13[h] | 19.097 ± 0.05[h] | 59.686 ± 0.13[h] | 80.902 ± 0.05[h] |

Means with different superscripts within each column differ significantly ($p<0.01$)

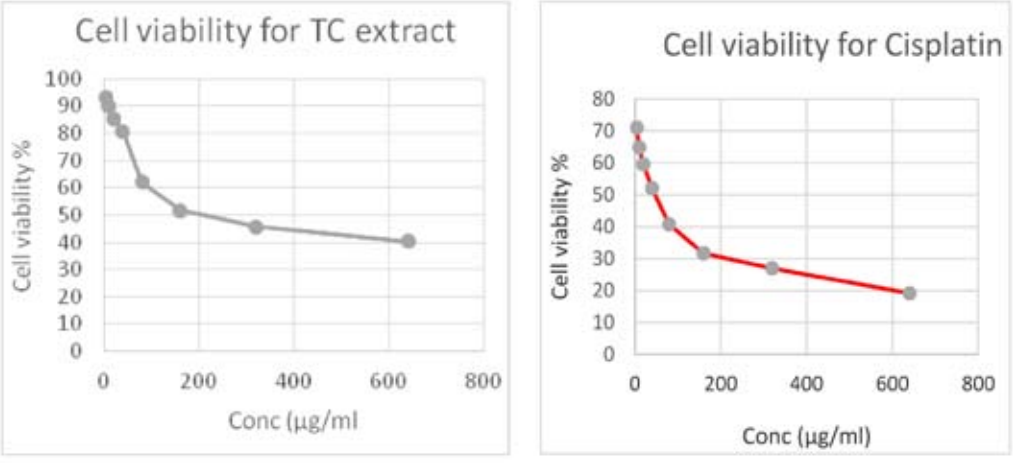


**Fig 2**. Cell viability of TC stem extract and Cisplatin in DLA cell lines under MTT assay

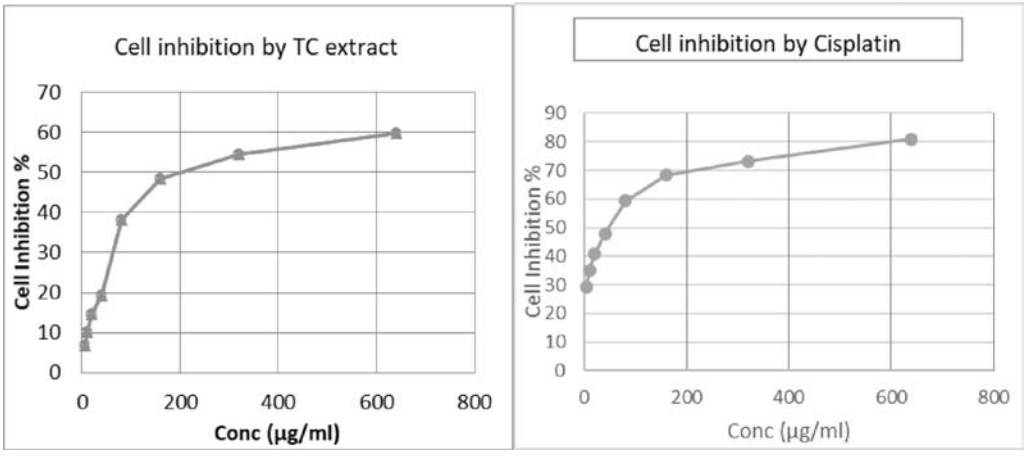


**Fig 3**. Cell inhibition by TC stem extract and Cisplatin in DLA cell lines under MTT assay

**Table 2.** The mean ± SE of viable cells and percent cell viability in DLA cell line on treatment with TC stem extract and cisplatin at their $IC_{50}$ in Trypan blue dye exclusion assay

| Treatment compounds | Number of viable cells (×$10^5$) | % Cell viability |
|---|---|---|
| Control | 6.133 ± 0.03[a] | 92.00 ± 0.00[a] |
| TC stem extract @ $IC_{50}$ | 3.733 ± 0.03[b] | 59.40 ± 2.15[b] |
| Cisplatin @ $IC_{50}$ | 3.467 ± 0.03[c] | 55.16 ± 2.23[c] |

Means with different superscripts within each column differ significantly (p<0.01)

abnormal. The results of MTT assay revealed that the TC stem extract exhibited cytotoxic and cell inhibitory action as that of cisplatin on the DLA cells.

***Trypan blue dye exclusion method***

Trypan blue dye exclusion revealed the number of viable cells (×$10^5$) on treatment with $IC_{50}$ concentrations of TC extract and cisplatin in DLA cell line to be 3.733 ± 0.03 and 3.467 ± 0.03 respectively. The viable cell count was found to be significantly higher with the TC extract when compared to cisplatin and they differed significantly between the groups and with the control (p<0.01). The per cent cell viability on an average as against the control (92%) following three hours of incubation of DLA cells with their $IC_{50}$ concentrations was found to be 59.40 ± 0.04 and 55.17 ± 0.01 for aqueous TC stem extract and cisplatin respectively (Table 2). The mean per cent cell viability also varied between TC and cisplatin significantly (p<0.01). The results indicated that TC stem extract has potent cytotoxic property. This result was in accordance with the findings of Bansal and Das (2010) who reported the *in vitro* cytotoxicity of TC in oral squamous cell carcinoma cell line in a dose-dependent (25-75µg/ml) and time- dependent (24-120 hours) manner observing the mean cell viability to range from 37.24 to 44.69 per cent. In the same study, under identical conditions however, the viability of another cell line of peripheral blood mononuclear cells was found to be unaffected by the treatment with TC extract.

**Conclusion**

The present study revealed that TC stem extract exhibited a potent cytotoxic effect on Dalton's lymphoma ascites cells and can emerge as a good anti-neoplastic herbal option against lymphomas due to its cell inhibition properties as evident from the MTT assay and Trypan blue dye exclusion methods. The

cytotoxicity exhibited by *Tinospora cordifolia* stem extract in the present study may be attributed to the presence of the various naturally occurring phytochemicals that are reported to be of ant-oxidative, anti-proliferative and cytotoxic nature. The specific components present in each of the bioactive compound found in the extract may be responsible for cytotoxicity and hence they need to be investigated and isolated in the future studies. *Tinospora cordifolia* stem extract is already in human clinical practice for the treatment of tumours and the present study throws light on its efficacy through validation techniques.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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