Phytochemical Analysis and Cytotoxicity studies of *Tinosporacordifolia* leaves in BHK-21 Cells

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**Abstract**

*Tinosporacordifolia* is a common medicinal plant used in traditional medicine of India. Traditionally, the plant is being used for the treatment of various diseases. Phytochemical analysis of plant revealed the presence of alkaloids, flavonoids, steroids, saponins, total phenols, cardiac glycosides and reducing sugars. Therefore, it had been planned to study its anticancer properties in BHK-21 cells. Methanolic, ethanolic and aqueous extracts of the leaves of *Tinosporacordifolia* were screened for their anticancer properties. The cells were seeded with all the extracts and then allowed to grow for 24hrs; the cell growth was inhibited within 24hrs. The cytopathology included rounding and clumping of cells, detachment of cells, flagging of cells and apoptosis. Methanolic and ethanolic extracts showed better response than that of its methanolic extract. The concentration of 10 mg/ml of methanolic extract inhibited the cell growth with high affinity.

**Keywords:** BHK-21 cells, *Tinosporacordifolia*, Phytochemical, Cytotoxicity.

**Introduction**

*Tinosporacordifolia*, which is known by the common name Guduchi, is an herbaceous vine of the family Menispermaceae. The plant is a glabrous climbing shrub found throughout India, typically growing in deciduous and dry forests. The leaves are heart shaped. A standardized extract from *Tinospora* known as Tinofend has been studied clinically. One study in 75 patients with allergic rhinitis (hay fever) showed statistically significant reduction of symptoms compared to placebo (Singh et al., 2003). *Tinosporacordifolia* and related species such as *Tinosporacrispa* and *TinosporarumphiiBoerlare* used in Ayurvedic and Jamu herbal medicine. Recent research has demonstrated that a combination of *T. cordifolia* and turmeric extracts are effective in reducing the hepatotoxicity which is induced by the combination of isoniazid, rifampicin, pyrazinamide and ethambutol for treating tuberculosis. Alcoholic extract of the stem shows activity against *Escherichia coli.*
The decoction of the leaves is used for treatment of gout. Its fruit is used in the treatment of jaundice and rheumatism. According to the 1918 United States Dispensatory, the plant has a long history of use in India as a medicine and in the preparation of a starch known as Giloe-ka-sat or as Palo (Badar et al., 2005). Traditionally, the plant has been in use as an anti-spasmodic, anti-inflammatory, jaundice, diabetes, seminal weakness, urinary tract infections, fever, general debility, skin diseases, expectorant, carminative, digestive, anti-stress and aphrodisiac. Piles problem can be controlled by eating this plant mixed with milk or water and thus, preventing the bleeding and constipation. A variety of chemical constituents have been isolated from this plant and their structures have been established. The active ingredients include alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoids, phenolics, aliphatic compounds and polysaccharides (Farooq et al., 2001). In the present study, an effort has been made to establish the anticancerous as well as phytochemical study of _Tinospora cordifolia_ leaves grown in polyhouse of the institute of Biotechnology.

**Materials and Methods**

Plant has been selected from high altitude area (1600m from sea level) from the polyhouse of Institute of Biotechnology, Patwadangar (Nainital), Uttarakhand and leaves of _Tinospora cordifolia_ were collected and were washed with tap water thrice. Washing was again repeated five times by using distilled water. Then the leaf samples were air dried and there after kept in incubator at 37°C for 24 hrs. The dried leaf material was then crushed in mechanical grinder in order to make fine powder which was stored at room temperature for further use.

**Extract Preparation**

**Aqueous Extract**

The aqueous extract was prepared according to the standard method with slight modifications. 5 g of leaf powder was mixed in 120 ml of water and was kept in incubator shaker at 36°C and 100 rpm. The extract so obtained was evaporated to drying through heating in a china dish. Dry extract was then scrapped off, weighed and reconstituted in normal saline (Marks et al., 2010).

**Solvent Extraction**

Ethanol and methanol extracts were prepared in Soxhlet’s apparatus at room temperature. Dried Leaves of _Tinospora cordifolia_ weighed accurately 5gm and taken in thimble and subjected to extraction in a Soxhlet’s apparatus at room temperature using ethanol (150ml) and methanol (165ml) (Govindachari et al., 1999). The extract obtained was first filtered using Whatman No. 1 filter paper and solvent was then removed under reduced pressure in a vacuumed rotary evaporator and dried. Cell culture for the present study, BHK-21 cells were cultured in 24 well sterile polystyrene plates using GMEM media supplemented with 5% fetal bovine serum as per standard procedure. The cells were seeded into 24 well sterile polystyrene plates and were incubated for 24 hours at 37°C. Thereafter, the medium was removed and 0.5ml of each dilution (10mg, 1mg, 10µg/ml) of each extracts added to the assigned wells. Control well were also kept (medium without test sample) and triplicate sets of each dilution were maintained. Finally the cells were incubated for 24 hours at 37°C and thereafter, examined under inverted microscope for their morphological studies.

**MTT Assay**

The MTT Assay is a sensitive, quantitative and reliable colorimetric assay that measure viability, proliferation and activation of cells. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) into a dark formazan product that is insoluble in water. The amount of formazan produced is directly proportional to the cell number in a range of cell lines. It was performed as, prepared an MTT stock solution of 5 mg ml-1 in phosphate-buffered saline (PBS), pH 7.5, and filter sterilized and the small amount of insoluble residue was removed. Add 10 µl of MTT (5mg ml-1), after 24 h of incubation and the cells were further incubated in incubator at 37°C for 3 h. Then 100 µl 0.04 M HCl in propan-2-ol to each well were added and mixed thoroughly to dissolve insoluble blue formazan crystals. The Plates were read on a micro-ELISA reader using a test wavelength of 570nm (Mosmann, 1983).

**Neutral Red Assay**

Neutral red (3-amino-7-dimethyl-2- methylphenazine hydrochloride) is a water soluble, weakly basic, supravital dye that accumulates in lysosomes of viable cells. The neutral red (NR) assay is an invitro cell viability test that was developed and extensively studied for in vitro cytotoxicity determination. After incubation of cells with extracts, 0.33% of NR (NR in PBS) was added in each well and incubated for 1 h at37°C. Dye-containing medium was removed and the well was washed twice with 150µl/well warmed PBS. The cells were then lysed with 125 µl of 50% of v/v mixture ofethanol and 0.1M monobasic sodium phosphate to solubilise the neutral red. The plate was then incubated for 15 min and take O.D at 550 nm (Flick and Gifford, 1984).
Cytotoxicity % = \frac{A - B}{A} \times 100
A = O.D of untreated well;
B = O.D of wells treated with plant extract.

**Phytochemical analysis**

The qualitative tests performed to analyze the presence of various phytochemicals such as alkaloids, tannins, flavonoids etc. in plant (Wagner and Bladt, 1996)(Govindachari et al., 1999).

**Results and Discussion**

The ethanolic and aqueous extracts of *Tinosporacordifolia* were subjected to qualitative phytochemical screening for the detection of phytoconstituents like carbohydrates, alkaloids, proteins, amino acids, tannins, phenolics, saponins, flavonoids, triterpenoids, steroids, glycosides, fixed oils, gums and mucilages. The results revealed the presence of alkaloids, steroids, carbohydrates, glycosides, proteins, saponins, gums and mucilages. Methanolic extract of *T. cordifolia* tested positive for all tested metabolites (reducing sugar, alkaloids, saponins, cardiacglycosides, steroids and flavonoids) except tannins. The results showed that the chemicalconstituents reported from the plant *Tinospora* belonged to different classes such as alkaloids,flavonoids, glycosides, steroids, terpenoids,phenolics and saponins (Kavitha et al., 2011). The presence ofthese phytochemicals is an indicator that theplant can be a potential source of precursors inthe development of natural drugs. Plant steroids are known to be important fortheir cardiotoxic activities; they possessinsecticidal and antimicrobial properties. Theyare also used in nutrition, herbal medicine andcosmetics; they are routinely used in medicinebecause of their profound biological activities (Ayoola et al., 2008).

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Extracts</th>
<th>10mg/ml</th>
<th>1mg/ml</th>
<th>10µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aqueous</td>
<td>65.21±3.29*</td>
<td>63.36±3.25</td>
<td>60.42±3.21*</td>
</tr>
<tr>
<td>2.</td>
<td>EtOH extract</td>
<td>79.62±3.41</td>
<td>76.59±3.31*</td>
<td>73.72±3.36*</td>
</tr>
<tr>
<td>3.</td>
<td>MeOH extract</td>
<td>84.42±2.91*</td>
<td>82.21±2.47</td>
<td>80.17±1.42</td>
</tr>
</tbody>
</table>

**Table 2:** Percent cell cytotoxicity due to the extract of *Tinosporacordifolia* leaves measured by NR Assay (mean±SE)

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Extracts</th>
<th>10mg/ml</th>
<th>1mg/ml</th>
<th>10µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Aqueous</td>
<td>59.63±2.91*</td>
<td>56.42±2.67</td>
<td>51.39±2.05*</td>
</tr>
<tr>
<td>2.</td>
<td>MeOH extract</td>
<td>86.94±2.07</td>
<td>84.31±2.19*</td>
<td>81.56±1.49</td>
</tr>
<tr>
<td>3.</td>
<td>EtOH extract</td>
<td>81.72±1.89</td>
<td>78.67±1.76*</td>
<td>75.49±1.35</td>
</tr>
</tbody>
</table>

The anti-tumor activity and chemopreventive potential of four Ayurvedic herbs viz. *Curcuma longa L.*, *Ocimum sanctum L.*, *Tinosporacordifolia* were evaluated using Dalton Lymphoma ascites (DLA) tumor model in Swiss Albino mice. The outcome was assessed using survival time, peritoneal ascitic fluid (Tumor volume) and
hematological indices as parameters. Animals were divided into five groups (n = 6) viz. one DLA control and four Herb + DLA treated groups. All the four herb + DLA

Fig: 1. Tinosporacordifolia

Fig.2. Normal BHK-21 cells

Fig.3. Cells treated with extract of Tinosporacordifolia leaves
groups were pre-treated with respective herbs for 7 days and hematological indices were measured for entire five groups. On day-8 animals were inoculated with 1x10^6 DLA cells i.p., and Herb + DLA groups were continued with oral herbal treatment for 21-days. Hematological parameters and tumor volume were assessed to find the effects of herbs. Short term in vitro cytotoxicity was determined by Trypan Blue exclusion method and LDH leakage assay using different concentrations of herbal extracts and 5-FU as a positive control and IC_{50} for each herbal extract and 5-FU were determined. All the four herbs showed in vitro cytotoxic activity against DLA cell-line. Better anti-tumor activity showed by T. cordifolia (Meghna et al., 2008). A formulation containing Tinosporacordifolia, Asparagus racemosus, Withaniasomnifera and Picrorrhizakurrooa markedly inhibited the suppression of chemotactic activity and production of interleukin-1 and tumour necrosis factor induced by the carcinogen ochratoxin in mouse macrophages(Methewsand Kuttan, 1997). Aqueous, methanolic and dichloromethane extracts of Tinosporacordifolia showed dose-dependent increases in lethality to HeLa cells in vitro. The most potent activity was found in the dichloromethane extract (Maryamma et al., 1990).

T. cordifolia plant extracts made with water, ethanol/methanol, or methylene chloride extract have been evaluated for antineoplastic effects in various animal experiments. Tumor mass reduction and increased survival time have been observed with administration of the extract in several experiments in mice with induced carcinomas (Leyon and Kuttan, 2004) (Jagetia; SK Rao, 2006). At low doses, an ethanol extract of T. cordifolia increased bone marrow cell counts, while higher doses resulted in decreased counts in mice with induced lymphoma (Singhet al., 2006). Thus, on the basis of observed encouraging cytotoxic effects by the in-vitro bioassays in present investigation, it can be revealed that methanolic and ethanolic extracts of T. cordifolia leaves had promising anticancer bioefficacy than its aqueous extract and must have some phytochemical moiety in the leaves of this plant which might be responsible for observed beneficial effects. It is suggested that the detailed in-vivo studies should be carried out in an animal experimental model of cancer to further prove the anticancerous activity of T. cordifolia.

**Conclusion**

The active phytochemicals present in T. cordifolia leaves had cytotoxic potential in BHK-21 cells. The aqueous, methanolic and ethanolic extracts, showed cytotoxic effects on the cancer cells. It therefore, provides an important lead for development of anti-cancer therapeutics for management of cancer, which needs further research in animal models.

**Acknowledgement**

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**References**