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Research Article

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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 presenceof alkaloids, flavonoids, steroids, saponins, total phenols, cardiac glycosides andreducing 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 *TinosporarumphiiBoerl*are used in Ayurvedic and Jamu herbal medicine. Recent research has demonstrated that a combination of *T. cordifolia* and turmeric extractsare 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, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides (Farooqi*et al.*, 2001).In the present study, an effort has been made to establish the anticancereous as well as phytochemical study of *Tinosporacordifolia* 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 *Tinosporacordifolia*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 37^0C for 24 hrs. The dried plant 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 *Tinosporacordifolia*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 polysterene plates using GMEM media supplemented with 5% fetal bovine serum as per standard procedure. The cells were seeded into 24 well sterile polysterene plates and were incubated for 24 hours at 37ºC. Thereafter, the medium was removed and 0.5ml of each dilution (10mg, 1mg,10µgm) 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 37ºC 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 through a 0.22-µ filter to sterilize 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 at37ºC. 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. Theplate was then incubated for 15 min and take O.D at 550 nm (Flick and Gifford, 1984).

Cytotoxicity % = A-B/A X 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 fortannins. The results showed that the chemicalconstituents reported from the plant *Tinospora*belonged to different classes such as alkaloids,flavanoids, 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 cardiotonic 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 1: Percent cell cytotoxicity due to the extract of *Tinosporacordifolia*leaves measured by MTT Assay (mean±SE).

Sl.No.	Extracts	10mg/ml	1mg/ml	10µgm/ml
1.	Aqueous extract	65.21±3.29*	63.36±3.25	60.42±3.21*
2.	EtOH extract	79.62±3.41	76.59±3.31*	73.72±3.36*
3.	MeOH extract	84.42±2.91*	82.21±2.47	80.17±1.42

Significant difference (*p≤0.5)

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

Sl.No.	Extracts	10mg/ml	1mg/ml	10µgm/ml
1.	Aqueous extract	59.63±2.91*	56.42±2.67	51.39±2.05*
2.	MeOH extract	86.94±2.07	84.31±2.19*	81.56±1.49
3.	EtOH extract	81.72±1.89	78.67±1.76*	75.49±1.35

Significant difference (*p≤0.5)

The effect of ethanolic and methanolic extracts was much better than that of aqueous extract. Rounding of cells were found in methanolic as well as ethanolic extracts of *Tinosporacordifolia*. There was clumping of cells more in the methanolic extract of *Tinosporacordifolia* then in aqueous extract of *Tinosporacordifolia*. The cytotoxity percentage was also higher in methanolic extract then aqueous extract of *Tinosporacordifolia*. The cytotoxicity percentage in MTT was 84% at 10 mg/ml, in ethanolic extract the cytotoxicity was 79% which was 65% in aqueous extract of *Tinosporacordifolia*. The cytotoxicity at other concentration of methanolic and ethanolic extractswere also higher than that of aqueous extract of *Tinosporacordifolia*. Such as the concentration at 1 mg/ml and 10 µgm/ml of methanolic extract of *Tinosporacordifolia*the cytotoxicity was 82% and 80%, respectively and at the same concentration the cytotoxicity of ethanolic extract was found 76% and 73%, respectively. The concentration of aqueous extract at 1 mg/ml and 10 µgm/ml were 63% and 60%, respectively. Thus, it was clear that the methanolic extract showed much better cytotoxicity than that of its aqueous extract. In case of NR assay, the cytotoxicity of methanolic extract was 86% at 10mg/ml, at 1mg/ml and 10µgm/ml the cytotoxicity was found 84% and 81%, respectively. Cytotoxicity percentage of ethanolic extract at 10mg/ml was 81% and it was 78% and 75% at 1mg/ml and 10µgm/ml, respectively. Aqueous extract at 10mg/ml showed 59% cytotoxicity, which was 56% and 51% at 1mg/ml and 10µmg/ml, respectively.

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*

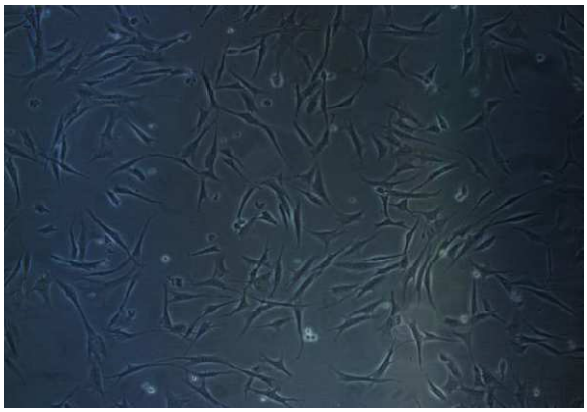

Fig.2. Normal BHK-21 cells

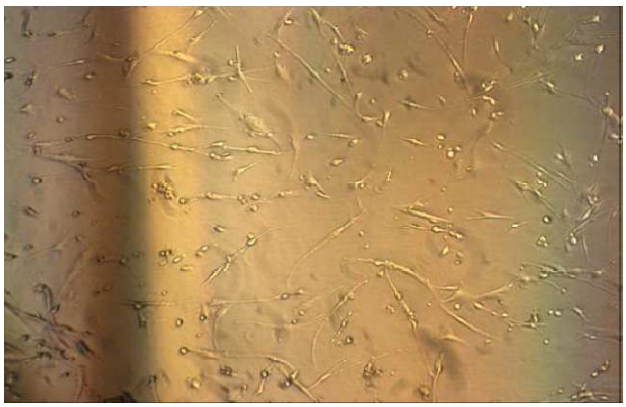

Fig.3. Cells treated with extract of *Tinosporacordifolia* leaves

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* markedlyinhibited the suppression of chemotacticactivity and production of interleukin-1 andtumour necrosis factor induced by thecarcinogen ochratoxin in mousemacrophages(Methewsand Kuttan, 1997) .Aqueous, methanolic and dichloromethaneextracts of *Tinosporacordifolia* showeddose-dependent increases in lethality toHeLa cells in vitro. The most potent activitywas found in the dichloromethane extract (Maryamma*et al.,* 1990).

T.cordifolia plant extracts made with water, ethanol/methanol, or methylene chloride extractshave been evaluated for antineoplastic effects in various animal experiments. Tumor massreduction and increased survival time have been observed with administration of the extract inseveral experiments in mice with induced carcinomas (Leyon and Kuttan, 2004)(Jagetia; SK Rao, 2006). At low doses, an ethanol extractof *T. cordifolia* increased bone marrow cell counts, while higher doses resulted in decreasedcounts in mice with induced lymphoma (Singh*et 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 animal experimental model of cancer to further prove the anticancerous activity of *T. cordifolia*.

Conclusion

The active phytochemical constituents 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.

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