

Assessment of the Multifaceted Immunomodulatory Potential of the Aqueous Extract of *Tinospora Cordifolia*

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Abstract

Immunomodulation relates to potentiation or suppression of the immune responses of the host, depending on the requirement of the situation. *Tinospora cordifolia* (T.C.), an indispensable medicinal plant, has been used for the treatment of various diseases and has been recommended for improving the immune system. In the current study, we have evaluated the cytotoxicity and immunomodulatory activity of the aqueous extract of T.C. using *in vitro* and *ex vivo* models.

Results show that there was dose-dependant cytotoxicity in B16F10 mouse melanoma cells. ~90% cytotoxicity was observed 72 hours post-treatment with 100µg/ml of T.C. extract. Interestingly, a low dose of 1µg/ml also showed ~60% cytotoxicity. Further, the aqueous extract of T.C. exhibited boosting of phagocytic ability of macrophages, remarkable enhancement in nitric oxide production by stimulation of splenocytes and macrophages at 1mg/kg dosage of the extract. Immunomodulatory ability of a compound can be determined by its capability to influence the cytokine production, mitogenicity, stimulation and activation of immune effector cells. The extracts have been investigated for their effect on the cytokine profile (IL-6) by ELISA. Splenocytes cultured in low concentration of T.C, as low as 1.56 µg/ml, produced significantly higher levels of IL-6 as compared to un-stimulated cells. The present study reveals the multifaceted immunomodulatory potential of T.C..

Keywords: *Tinospora cordifolia*, cytokines-IL-6, cytotoxicity, Immuno-modulation.

Introduction

Alternative medicine is now recognized as an invaluable resource even by the most intransigent clinicians of advanced countries. Plant extracts have been widely investigated for their possible immunomodulatory properties¹⁻⁴. Natural products are generally known to be efficacious with minimal side effects⁵, but some natural compounds have been reported to act as mutagens and/or carcinogens⁶⁻⁸. These genotoxic insults often lead to cancer⁹, Alzheimer's disease¹⁰ and other chronic degenerative diseases, such as atherosclerosis and heart diseases, which are the leading cause of death in human and animal populations¹¹.

There is great interest in development of new drugs from traditionally used medicinal plants like *Tinospora cordifolia* [TC]. Ayurveda refers to *Tinospora cordifolia* as 'Amrita' or the 'Nectar of Immortality'. The term 'Amrita' is attributed to this drug in recognition of its ability to impart youthfulness, vitality and longevity. Immunomodulation can be determined by the capacity of the compounds to influence the cytokine production, mitogenicity, stimulation and activation of immune effector cells¹². *Tinospora cordifolia*, a *rasayana* drug is widely used in the Ayurvedic system of medicine as an anti-inflammatory, anti-arthritic, anti-allergic, anti-malarial and as an anti-diabetic¹³ drug. The extract from the stem of *T. cordifolia* is often used in dyspepsia, fever and urinary infections¹⁴. The anti-tumor promoting activity of *Tinospora* extract in a two-stage skin carcinogenesis model has already been explored¹⁵.

Owing to its ability to impact a plethora of diseases, the present study was undertaken to understand the effect of TC on the production of IL-6 in mice. IL-6 - being a pleiotropic cytokine involved in a variety of normal processes including acute reactions to injury, inflammation, activation of cytotoxic T-cells, and differentiation of B-cells - has generated interest as a new drug molecule against a variety of diseases. IL-6 may be of use in activating antitumor responses since increased levels of circulating IL-6 have been observed following systemic treatment of patients or animals with endotoxin¹⁷. Many other factors such as adhesion molecules affect metastasis, and it is possible that IL-6 interacts with them¹⁸. Hence, we went ahead to investigate molecular drugs from medicinal plants that could modulate the immune response. The therapeutic potential of *Tinospora cordifolia*, commonly known as 'Guduchi', was studied to assess the immunomodulatory activity using *in-vitro* as well as *ex-vivo* models.

Material and Methods

Sodium dodecyl sulphate, N,N,N',N' tetramethylethylenediamine, acrylamide, bisacrylamide, bromophenol blue, β mercaptoethanol, ethylenediaminetetra acetic acid, trypan blue and MTT were procured from Sigma St Louis. 3,5-Dinitrosalicylic acid, potassium sodium tartarate, sodium hydroxide, dinitrosalicylic acid, sulphanilamide, N-1 naphthylene diamine, Coomassie brilliant blue R-250, ammonium per sulphate, glycine, glycerol, acetic acid and methanol were purchased from MERCK. RPMI-1640

medium and foetal calf serum were purchased from Gibco, BRL. Lipopolysaccharide LPS, *E coli* 055-B5 and Sodium thioglycollate were procured from Difco and Micro BCA protein assay reagent kit was purchased from Pierce.

Preparation of Aqueous Extract of *T. cordifolia* (TC):

Dried 60gm fine powder from stem of *Tinospora cordifolia* was soaked in 600ml of water. It was kept at room temperature (RT) for 48 hours with intermittent mixing. Aqueous extract of *Tinospora cordifolia* obtained after 48 hours of soaking was filtered using Whatman paper. Filtrate was stored at 4°C. 90% Ammonium sulphate saturation was performed for protein precipitation. Precipitate of 90% ammonium sulphate saturation, stored at 4°C, was centrifuged at 10000 rpm for 30 minutes. Supernatant was decanted off and pellet was resuspended in PBS¹⁹.

Protein Purification and Quantitation: The protein content of the dialyzed sample was estimated by BCA Method²⁰. The BCA protein assay combines the well-known reduction of Cu²⁺ to Cu¹⁺ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu¹⁺) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium potassium tartarate. In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is a water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. The BCA reagent is approximately 100 times more sensitive (lower limit of detection) than the pale blue color of the first reaction.

The molecular weight of the protein obtained from the aqueous extract of *Tinospora cordifolia* was determined by Sodium dodecyl sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE)²¹.

HPLC-LC/MS: The HPLC of TC extract was run in Agilent 1200 series XBD C-18 column (4.6x150mm) (USA). The injection volume was 5µl. The sample was run in gradient solvent (90% water 10% ACN & 90% ACN 10% water) at the UV 254nm. For LC/MS, the sample was run in Agilent technologies 6310 Ion trap LCMS.

Fourier transform infrared spectroscopy [FTIR]: The transmission FTIR spectrum of the lyophilized powder of TC extract was done in KBr pellet by Perkin Elmer, UK RX1 model (Resolution 4cm⁻¹; KBr Beamsplitter; Source MIR; Detector LiTaO₃).

¹H NMR: For the ¹H NMR experiments, lyophilized samples were dissolved in D₂O and the experiments were carried out at 25±0.05°C, using a Bruker Spectrospin, operating at 300MHz and 7.05T with an inverse 5mm probe using PULSPROG. Chemical shifts were measured in parts per million (ppm).

In-vitro Cytotoxicity Assay: MTT Assay was carried out in accordance with previously published protocol²². Briefly, B16F10 melanoma cells (5x10³ cells/well) were cultured on a flat-bottomed 96 well plate. After 48 hours incubation, 20µl of MTT stock solution was added to each well of the assay plate, which was then incubated for 2 hours at 37°C. After incubation, the formazan crystals formed by the reduction of tetrazolium salt by the mitochondria of living cells were dissolved in DMSO. The plates were read in ELISA plate reader at wavelength of 540nm.

Nitric Oxide [NO] Assay: The peritoneal exudates from mice were collected after 72 hours of an i.p. injection of 4% sodium thioglycolate solution. The exudate was centrifuged at 1100 rpm for 10 minutes at 4°C. The pellet was resuspended in RPMI. The cells [~0.5 million/well] were incubated for 3 hours at 37°C in the presence of 5% CO₂ and then washed with RPMI. Cells with and without LPS (5mg/ml) in RPMI were incubated for 48 hours for production and estimation of NO, since nitric oxide is highly reactive and converted into sodium nitrite to form a stable end product of the reaction. Equal volumes of supernatant collected after incubation of macrophage culture was incubated with freshly prepared Griess reagent at room temperature for 5 minutes. Absorbance at 550 nm was measured by using ELISA reader. Nitrite was quantified by comparison with NaNO₂ as a standard.

Immunization of Mice: Equal volumes of collagen solution (in acetic acid) and complete Freund's adjuvant (Chondrax) were taken in separate glass syringes. Emulsion was prepared by continuous passage between two syringes connected by a tube. The process was carried out in cold under ice. Emulsion formation was confirmed by pouring a drop on water surface. Prepared emulsion was dispensed in 1ml syringe and used for immunization of mice. Mice (Balb/c) were immunized by subcutaneous injection of 100 µl emulsion using 26g needle. Simultaneously, the mice were given i.p. injection with 180 µl of crude protein (0.6mg/ mice).

Results and Discussion

The aqueous extract of *Tinospora cordifolia* was precipitated with 90% ammonium sulfate, dialyzed against water and dried in vacuum to obtain crude *T.C.* (table-1). This crude *TC* was subjected to Q-Sepharose ion-exchange chromatography, resulting in the isolation of *TC* extract (figure-1). Its protein concentration was determined by BCA method (figure-1), and molecular weight was determined by SDS-PAGE (figure-2).

Flow chart for purifying protein from aqueous *Tinospora cordifolia*

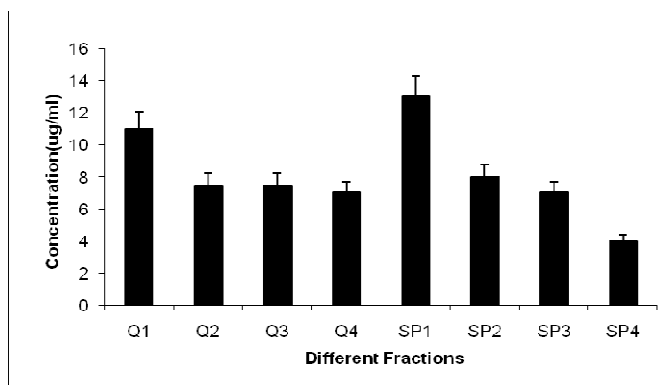
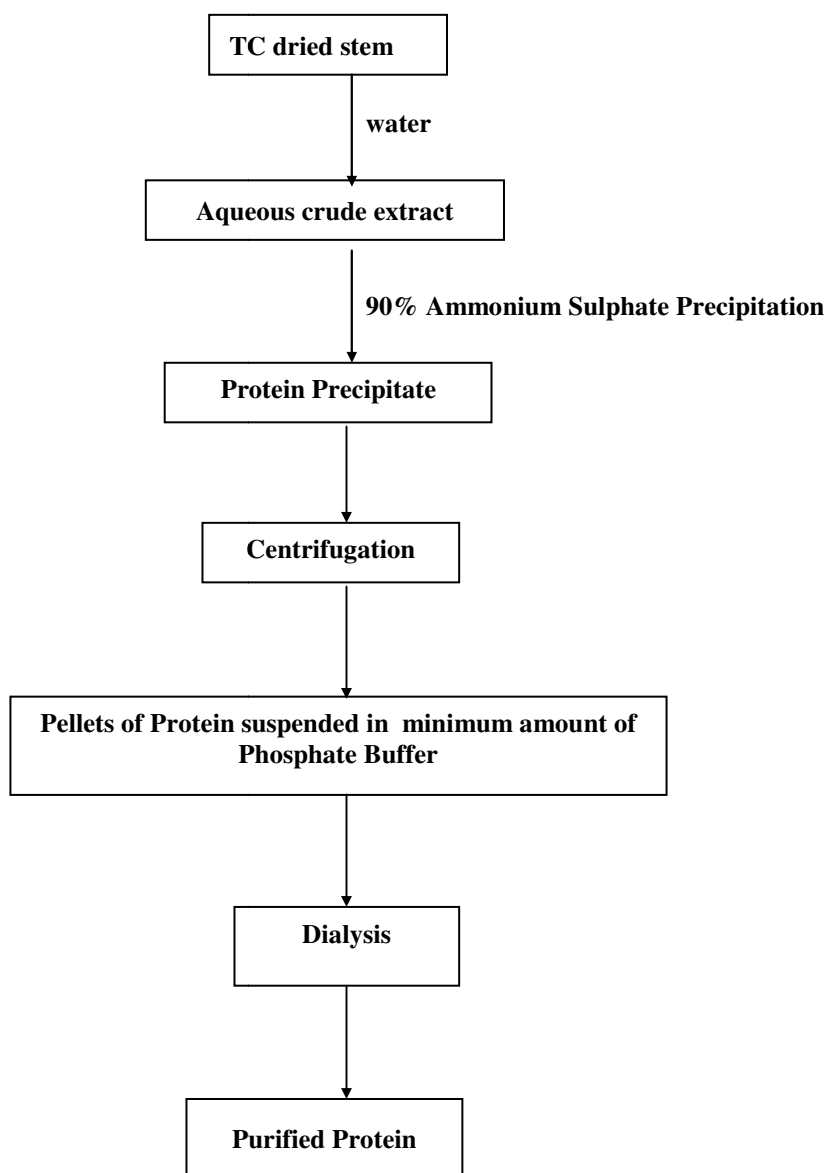


Figure-1
Q-Sepharose anion exchange chromatographic separation of different fractions from aqueous extract of *Tinospora cordifolia*

MW: KDa

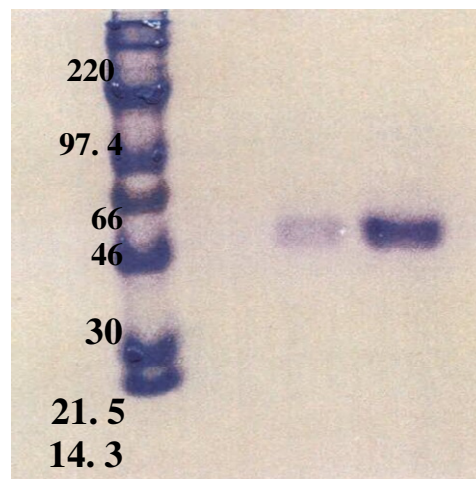
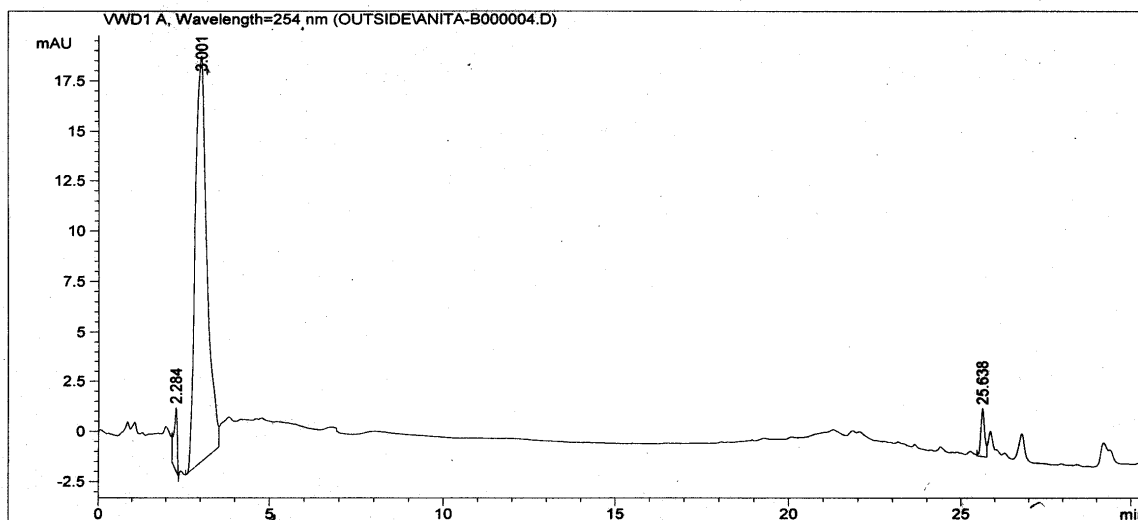


Figure-2
SDS PAGE indicating the molecular weight of the unknown protein



HPLC-LC/MS

Figure-3
HPLC of the aqueous extract of TC

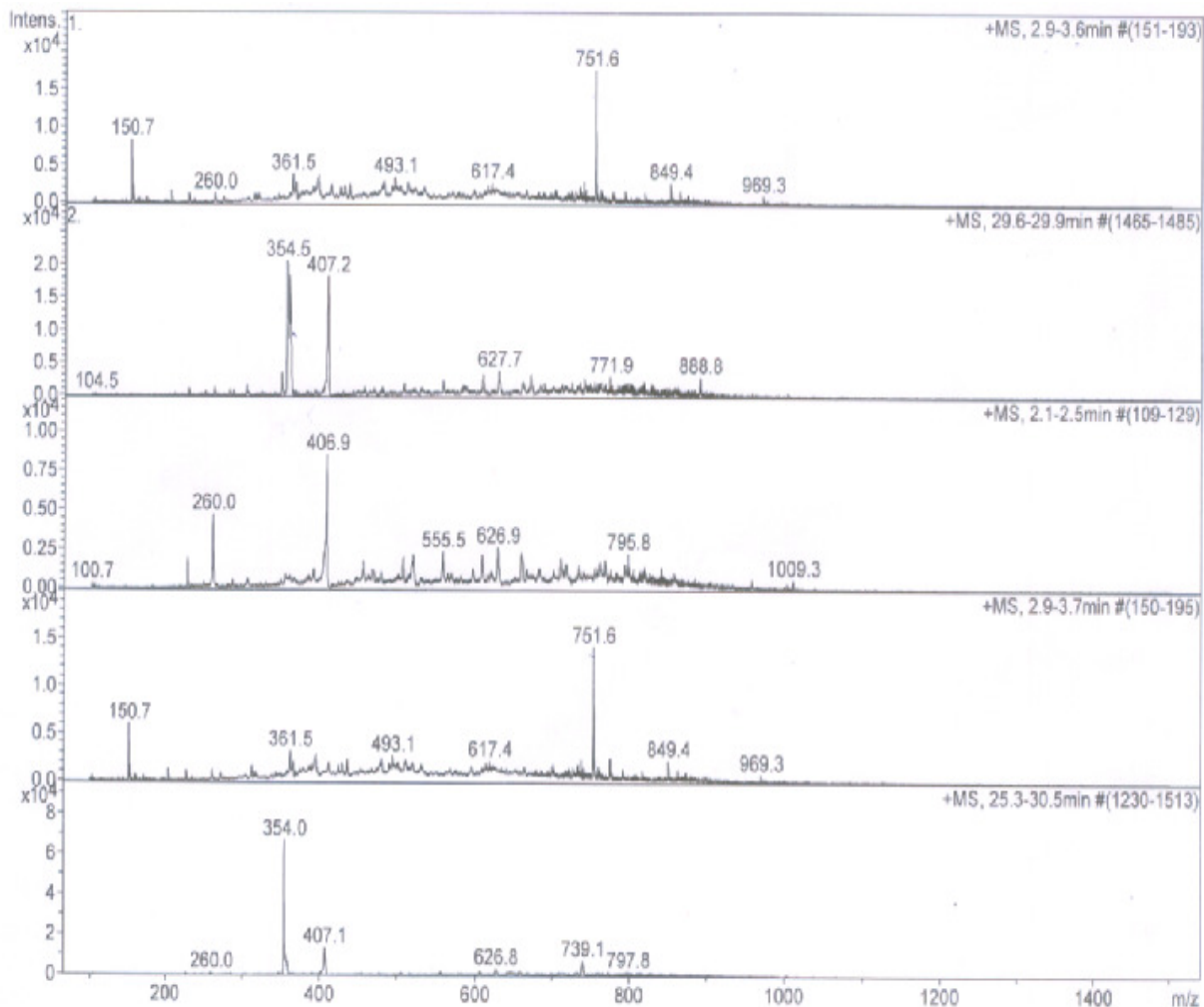
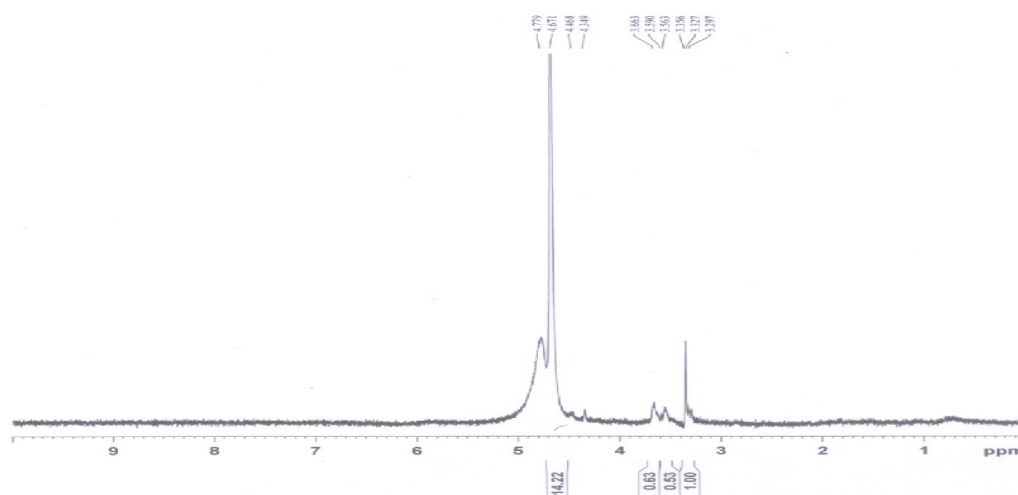


Figure- 4
LC/MS Scan of the aqueous extract of TC



NMR

Figure- 5
 The Proton NMR Chemical Shifts of the aqueous extract of TC

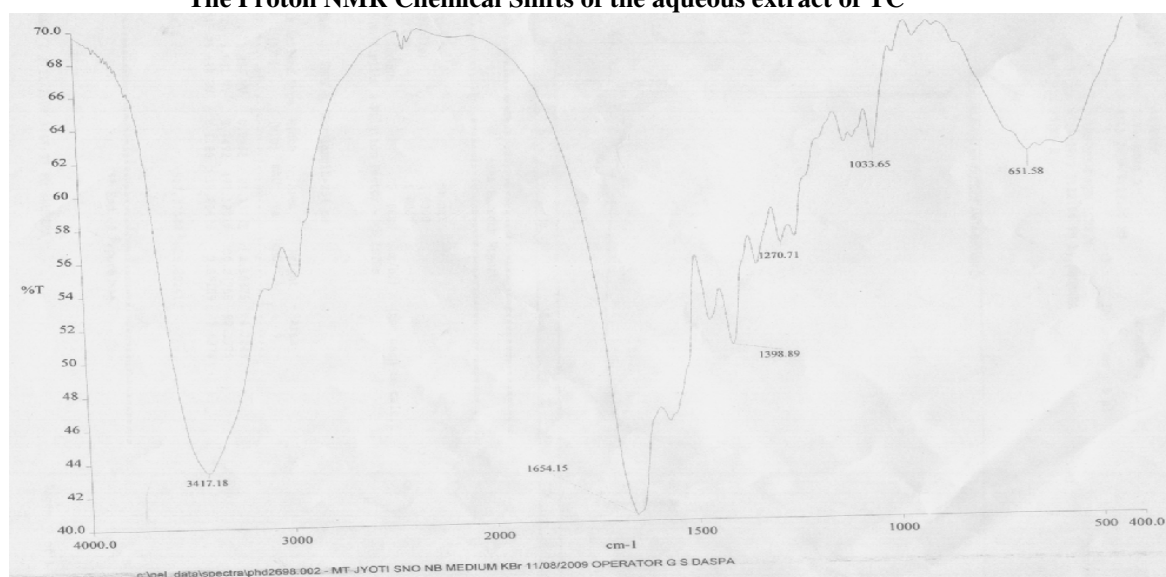


Figure- 6

Fourier Transform Infra Red Spectroscopy of the aqueous extract of TC

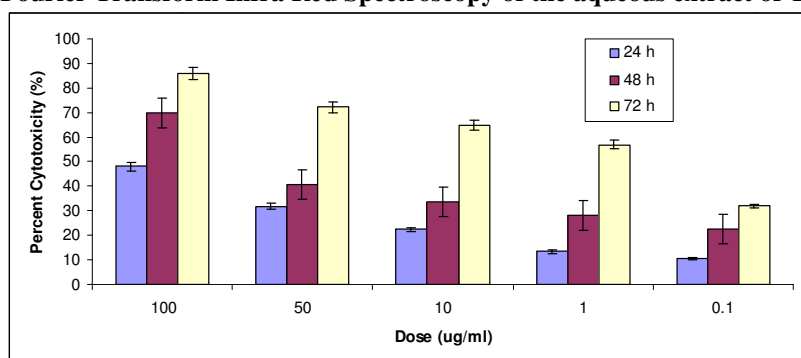


Figure-7

Dose Dependent Cytotoxicity of aqueous extract of Tinospora in B-16 mouse melanoma cell line

Satisfactory retention times and good resolution of the TC extract was achieved using reverse phase C-18 column eluted with acetonitrile-water (10:90 v/v) at a flow rate of

0.6 ml/min. A sharp and symmetric peak was obtained, with good baseline resolution and minimal tailing, thus facilitating the accurate measurement of peak area. Two

peaks were found, one at 3.001 minute along with another one minor peak at 25.638 minute. In LC/MS Scan also two peaks were found among which one coincided with HPLC peak and the second peak after 25 minute was prominent in LC/MS. The sample clearly has more than one compound with expecting more ionic compound elution after 25 minute figure-3. In LC/MS the four different mass of 751.6, 354.5, 406.2 260 were found in between 2.1 to 3.6 minute & 25.3 to 30.5 minute. It conclude that the sample have the mixture of at least 4 different compound figure- 4.

The Proton NMR chemical shifts of the TC extract was very sharp in between 3.2ppm to 3.6ppm and 4.7ppm to 4.3ppm, which represents the amide containing aliphatic compound in the samples (figure- 5).

In FTIR, the prominent transmission peak was found at 3417.18cm^{-1} , 1654.15cm^{-1} 1398.89cm^{-1} . The peak value corresponds to the presence of amide N-H and carboxylic stretch containing compound (figure- 6).

The aqueous extract of *T. cordifolia* mainly contained polysaccharides, phenolic compounds and bitter principles^{23, 24}. The cytotoxic effect of TC increased in a dose-dependent manner in B-16F10 melanoma cell line in a 72

hours assay. 90% cytotoxicity was observed at $100\mu\text{g/ml}$ dose after 72 hours. The cytotoxic action of '*guduchi*' could be attributed to the presence of alkaloids, di-terpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds or polysaccharides. Interestingly, ~60% cytotoxicity was observed at a low dose of $1\mu\text{g/ml}$ of the purified extract (figure-7).

Results show that there is increase in nitric oxide [NO] production by the macrophages obtained from the aqueous extract of TC treated mice when stimulated with LPS (figure-8). 1mg/kg of extract shows remarkable enhancement in NO production. NO has been considered to be a key molecule in the regulation of the immune response to tumors. We measured the production of NO from the peritoneal murine macrophages and observed that when macrophages were treated with the aqueous extract of TC (1mg/kg), there was dose-dependent enhancement in NO production. This increase in the NO production is supposed to have been due to the cascade of the signaling pathway. These data further strengthens the role of macrophage in producing NO by direct lysis of B16F10 melanoma cells *in vitro*.

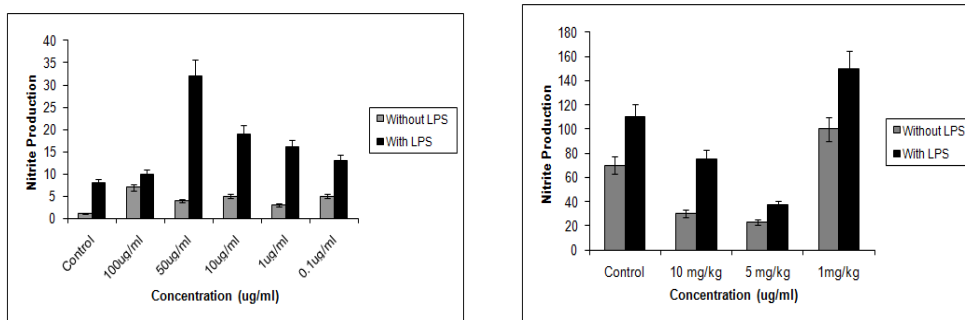


Figure-8a and 8b
In vitro effect of varying concentrations of aqueous extract of *Tinospora* on NO production by peritoneal macrophages [a] and splenocytes [b]

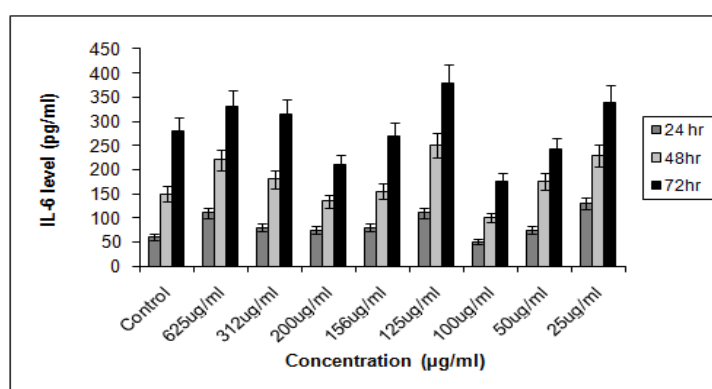


Figure-9
Dose dependent IL-6 production by Aq extract of TC. The values are means of triplicates at each point and the error bars represent SD. Data are representative of five different experiments

The data reveals immune modulation and cytotoxicity against tumor cells. NO has been recognized as the major effector molecule concerned in the demolition of tumor cells by activated macrophages and our data confirms the possibility that the extract of TC might be an

immunomodulator and the possibility that the induction of nitric oxide may contribute to tumoricidal activities.

Tinospora cordifolia has also been found to contain an immunomodulator arabinogalactan in polar fraction²⁵.

Although scientific studies have been done on a large number of Indian medicinal plants, a considerably smaller number of marketable drugs or phyto-chemical entities have entered the evidence-based therapeutics²⁶.

It has been reported that aqueous extract of TC has potent immunomodulatory properties. However, the mechanism of immunomodulation remains ambiguous. Since plant extracts have been reported to reveal various immunomodulating activities, we focused on the activation of splenocytes and lymphocytes by the TC extract, to augment the production of cytokines. The induction of cytokine is one of the methods to measure the augmentation activity of the immune system that plays a fundamental role in controlling the homeostasis of the whole organism. IL-6 is vital for B-cell stimulation and regulation of acute-phase responses and inflammation²⁷. In this study, we have shown that TC is a potent regulator of IL-6 production and plays a key role in the cytokine network. The finding that IL-6 was up-regulated might provide a decisive key to the understanding of the mechanism of action for clinical relevance and the rationale for TC treatment. IL-6 deficiency is associated with enhanced susceptibility to, and diminished clearance of, a wide range of pathogens including extracellular and intracellular bacteria²⁸⁻³¹, parasites³²⁻³⁵ and fungi³⁶. IL-6-deficient mice also exhibit impaired wound healing and tissue regeneration subsequent to injury³⁷⁻⁴⁰, altered nociceptive responses and higher tumor progression⁴¹. Recently, it was shown that IL-6 induces hepcidin in hepatocytes which decreases the availability of iron by inhibition of macrophage iron release and intestinal iron absorption⁴². These results represent an approach to determine immunomodulatory effects and can be used for specific and differential immunomodulation. The key finding of our experiments was the impact of TC extract on IL-6 release (figure-9). It has opened a new window for investigation of the signaling pathway. The era is of regenerative and alternative medicine for treatment of various diseases. But, there are a lot of gaps to be filled by new investigations. There is a great interest in development of new drugs from traditionally used medicinal plants; *Tinospora cordifolia* may provide new lead molecules for the development of alternative drugs.

Conclusion

The results of this study confirm the earlier claims that *Tinospora cordifolia* has immunomodulatory potential and further demonstrates that this activity is mainly due to the aqueous extract of TC, a glycoprotein isolated from the stem of *Tinospora cordifolia*. This aqueous extract primarily acts on the macrophages and stimulates the production of effector molecules like nitric oxide and cytokine mediators and also augments the antigen specific recall response indicating its ability to enhance antigen presenting ability of macrophages.

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