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CYTOTOXICITY AND IMMUNOMODULATION ACTIVITY OF DIFFERENT METHANOLIC STEM EXTRACTS OF *Tinospora cordifolia* (WILD.) MIERS EX HOOK. F. & THOMS.

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ABSTRACT

The stem, leaves and root parts of *Tinospora cordifolia* (Wild.) Miers ex Hook. F. & Thoms, an important medicinal plant, have been used as treatment for various ailments in traditional and modern medicine. The present study explored the cytotoxicity and immunomodulatory activity of methanolic extracts of *T. cordifolia* grown on *Azadirachta indica* and *Mangifera indica,* wild grown and *in vitro* callus. The extracts of *T. cordifolia* revealed substantial cell viability for these samples and showed remarkable survival of peripheral blood mononuclear cells (PBMC) at 50-10 µg mL^{-1} concentration of extract. The comparison of immunomodulatory activity of four extracts of *T. cordifolia* by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] and ELISA kit assay revealed that maximum response for cell proliferation and expression of human interleukin IL-2 and IL-6 was in *T. cordifolia* grown on *A. indica,* followed by *T. cordifolia* grown on *M. indica,* grown in wild and callus extracts. The study illustrates that by using modern scientific approaches the properties of this medicinal plant can be exploited for human welfare on the basis of their ethnobotanical knowledge.

Key words: Cytotoxicity, ELISA assay, immunomodulatory activity, medicinal plant, *Tinospora cordifolia*

INTRODUCTION

The ethnobotanical knowledge of medicinal plants has a significant role in medicinal industry world-wide. Their role in defense of various diseases has been validated by modern scientific methodologies and these medicinal plants still serve as important source in the discovery of new drugs. Immunomodulators are the substances that affect immune system response. Immune system protects human body against invading toxins, foreign cells and microorganisms (Roshan and Savitri, 2013). Herbal plants reportedly have immunomodulatory properties and generally act by stimulating both humoral and cell-mediated immunity and some shows activation of cellular components of the immune system (Sonkar and Mishra, 2011). Some medicinal plants possess *rasayana* property which means that the use of these plants imparts human body resistance against infections and improves immune system. The plants listed as *rasayana* have been claimed to have immunomodulatory activity (Dahanukar *et al.*, 1999).

Immunomodulation is the process that involves alteration of immune response to the desired level. There are some reports describing the number of analysis tests performed *in vitro* and *in vivo*

to screen a drug for its immunomodulation activity like lymphocyte proliferation assay, delayed type hypersensitivity assay (DTH), plaque forming cell assay (PFC), carbon clearance test, neutrophil adhesion test, etc. (Aher and Wahi, 2010; Roshan and Savitri, 2013). The immune-modulators may modulate immune mechanism by either suppressing or by stimulating one or more functions such as antigen recognition and phagocytosis, lymphocyte proliferation/differentiation, release of mediators due to immune response and antigen-antibody interaction (Roshan and Savitri, 2013).

Cytotoxicity assay is one of the preliminary analysis in standardization of drug dosage so as to check the survival rate of cells and toxicity effect of drug on cells. The tetrazolium salt assays, like MTT, are used for the study of cytotoxicity of cells which detects living cells and is dependent on the degree of cell activation. Hence, MTT method can be used for analysis of cytotoxicity, proliferation and activation studies (Mosmann, 1983). The dichloromethane extract of *Tinospora cordifolia* (Wild.) Miers ex Hook. F. & Thoms reportedly affects HeLa cells (Jagetia and Rao, 2006) and the cytotoxic effect of dichloromethane extract may be due to lipid peroxidation, release of LDH and decline in glutathione-s-transferase with dose-dependent decline in clonogenicity.

T. cordifolia has been mentioned in traditional medicine for its immunomodulatory activity. In Ayurveda it is considered as a *rasayana* drug. The *rasayana* agents inflict preventive action against various diseases when taken by healthy individuals; as well as strengthen the defense system and body function (Upadhyaya, 2000). The ability of *T. cordifolia* as a potent immuno-modulator has been demonstrated by researchers (Patel *et al.*, 2013). Aher and Wahi (2010) showed the activity of *T. cordifolia* as immunomodulator by assessing the delayed-type hypersensitivity and effect on bone marrow cellularity and α-esterase cells in rat for alcoholic stem extracts. The study revealed enhancement in bone marrow cellularity and α-esterase activity in male Wister rats. The *in vivo* administration of *T. cordifolia* extracts in Swiss Albino mice illustrated immune-protective activity in CCl_4-induced pathological manifestation of immunotoxicity with the help of phagocytosis assay, *in vitro* cell adhesion assay and myeloperoxidase assay (Chakraborty *et al.*, 2009). The polysaccharide from *T. cordifolia* showed significant antioxidant properties against peroxy-nitrite and photosensitization induced damage (Desai *et al*., 2002). *T. cordifolia* extract treatment on HIV positive patients caused significant reduction in eosinophil and haemoglobin as validated by clinical evaluation. However, the report concluded that further studies need to be undertaken for the elucidation of the information generated (Kalikar *et al*., 2008). The potential of *T. cordifolia* as immunomodulator in glioblastoma therapy has been illustrated which revealed that *T. cordifolia* strong inhibited proliferation and migration of glioma cells and programmed cell death (Mishra and Kaur, 2013). As per the traditional Ayurveda system, *T. cordifolia* grown on either *Azadirachta indica* or *Mangifera indica* tree has more potential medicinal properties (Panchabhai *et al*., 2008). The present study evaluated the therapeutic properties of four different methanolic extracts of *T. cordifolia* for their cytotoxicity and immunomodulation activity with the help of MTT and ELISA assays. The extracts used were *T. cordifolia* grown on *A. indica* and *M. indica*, wild grown and *in vitro* callus samples.

MATERIALS AND METHODS

Plant materials

The stems of *T. cordifolia* were collected from Tarapur, Maharashtra (India) in December 2012. The samples were then grown on *A. indica* or *M. indica*, wild and i*n vitro* callus. The plant samples were got identified by St. Xavier's Blatter Herbarium, Mumbai (Specimen No: 21641). The stem samples were sun-dried and ground to powder form, sieved and stored in air-tight containers.

Callus culture

For callus culture, the young and tender internodes were used as explant material. Callus culture was established on Murashige & Skoog medium fortified with 6-benzylaminopurine (BAP) (2.5 mg L^{-1}) and 1-naphthalene acetic acid (NAA) (1.0 mg L^{-1}). The growth conditions maintained were: illumination 1,000 lux, photoperiod 16 h light/8 h dark, and culture room temperature 24±2°C.

Extract preparation

Thirty grams of weighed powder of *T. cordifolia* was defatted with petroleum ether for 24 h and then subjected to Soxhlet extraction with methanol till exhaustion of the material. Subsequently, the crude methanol extract was evaporated and dried under vacuum pressure at 60ºC in a rotary vacuum evaporator (Superfit Rotavap PBU-6). The concentrated crude extract was collected and used for further analysis. This procedure was repeated with each sample used.

Extract concentration for assay

Different extracts used in assay were labelled as Tc-N (*T. cordifolia* grown on *A. indica*), Tc-M (*T. cordifolia* grown on *M. indica*), Tc-W (*T. cordifolia* grown wild i.e. without the support of any plant) and Tc-C (*T. cordifolia* grown under *in vitro* conditions of callus culture). The range for the extract dosage was selected between 50 and 10 µg mL^{-1}, dissolved in 0.5% DMSO (dimethyl sulfoxide) in sterile PBS (phosphate buffer saline). The colour impurities and particles were observed in the extract concentrations above 50 µg mL^{-1}. The positive control used in the assay was phytohemagglutinin (PHA - Sigma) (10 µg mL^{-1}) while solvent control was 0.5% DMSO dissolved in sterile PBS and negative control 1% SDS (sodium dodecyl sulfate) dissolved in sterile PBS.

Collection of blood samples

Fresh heparinized human blood samples collected from > 9 healthy adult donors with the approval from the Kelkar Educational Trust, Mumbai's Institutional Scientific & Ethical Committee (vide their communication dated 21 October, 2014). The donors with no chronic disease history were selected and 4 mL blood sample was withdrawn from each donor. These blood samples were used to culture the peripheral blood mononuclear cells.

PBMCs culture

The peripheral blood mononuclear cells (PBMC) were isolated using Ficoll Hypaque (Sigma) density gradient centrifugation (Boyum, 1968, Hokland and Heron, 1980). The mononuclear cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640 supplemented with glutamine, HiMedia) with 10% fetal bovine serum (FBS, Sigma). The PBMC culture was maintained aseptically in T-flasks in CO_2 incubator maintained at 37ºC with 5% (v/v) CO_2 supply. After sufficient confluent, the cell growth was attained and the cells were counted using trypan blue dye exclusion method (Strober, 2001). A cell density of 1.5×10^6 was used for cytotoxicity and immunomodulation assay.

Cytotoxicity assay and cell proliferation-immunomodulation assay (MTT assay)

The cells were counted with the help of a compound microscope (Labomed TMC 400) using trypan blue dye exclusion method (Strober, 2001) and the desired concentration of cell suspension was adjusted in separate T-flask with RPMI 1640 medium with 10% FBS. The 96-well microplate was incubated for 24 h in a CO_2 (5% v/v) incubator. Next day, the desired set of dosage of samples were added in triplicate and incubated for 24 h in CO_2 incubator. Then 10 µL sterile MTT solution (5 mg L^{-1} in PBS stored in dark condition) was added in each well, followed by incubation at 37ºC for 5 h. For non-adherent cell suspension, the cells were centrifuged at 1500 rpm for 5 min. The supernatant was collected in another 96-well microplate. Then 100 µL DMSO was added to each well and absorbance read at 540 nm with ELISA plate reader. The data generated was carefully recorded.

$$\%\text{Cell viability} = \{(At-Ab)/(Ac-Ab)\} \times 100$$

$$\%\text{Proliferation} = 100 - \{(At-Ab)/(Ac-Ab)\} \times 100$$

Where, At: Absorbance of test sample, Ab: Absorbance of blank; and Ac: Absorbance of control

Quantification of human IL-2 and IL-6 with ELISA kit

The quantification of cytokines - interleukin IL-2 and IL-6 were done by using ready-to-use Corning Costar 9018 ELISA kits (Invitrogen). The protocol for human IL-2 and IL-6 ELISA was carried out as per the instruction manual of manufacturer's kit. The plate was read at 450 nm and data recorded. For the quantification of cytokine expression of the samples, the standard curve points were plotted and cytokines quantification extrapolated from the curve.

Data analysis

The cytotoxicity and immunomodulation activities were assessed using blood sample of 7 volunteers in triplicates. The ELISA kit method was used according to the standardized protocol in triplicate readings and data extrapolated from standard curve. The MTT assay data was statistically analyzed using statistical analysis tool, standard deviation and analysis of variance (ANOVA) at the significant level of <0.05 with the help of Microsoft excel. The ANOVA (two way) without replication test was used in the study where two or more samples were analyzed to check the degree of differences between the means of samples. In this study the comparison of four methanolic extracts of *T. cordifolia* in five different concentrations range 50-10 μg mL^{-1} were made.

RESULTS AND DISCUSSION

In PBMC culture, established after Ficoll Hypaque density gradient centrifugation, the appropriate extract dosage range while examining the cytotoxicity and immunomodulation activity of extracts was found between 50 - 10 μg mL^{-1}. The absorbance of four extract concentrations was recorded along with absorbance for control data.

Cytotoxicity of methanolic extracts of T. cordifolia

The MTT assay was successfully performed for cytotoxicity analysis of *T. cordifolia* for four different extracts. The assay was carried out on seven individuals in triplicates and statistical significance worked out ($p<0.05$). The percentage viability was tested for peripheral blood mononuclear cell culture by exposure to *T. cordifolia* extracts at 50 to 10 μg mL^{-1} range. The percentage viability values ranged from 87.95±10.62 to 74.33±10.24% for *T. cordifolia* grown on *A. indica*, 90.18±10.22 to 80.15±10.46% for *T. cordifolia* grown on *M. indica*, 89.54±13.61 to 81.06±11.23% for *T. cordifolia* grown under wild conditions and 92.42±12.26 to 84.75±11.52% for *T. cordifolia* grown under *in vitro* callus culture conditions (Table 1). The highest viability values were obtained at lower dose concentration (10 μg mL^{-1}) for samples. As the concentration of extract increased the survival rate was observed upto 80%. The lowest per cent proliferation value was observed at 50 μg mL^{-1} concentration. The survival rate for the extracts of four samples ranged from 74 to 92%. The *T. cordifolia in vitro* grown-callus extracts illustrated higher survival rate as compared to the other extracts. All the extracts at various concentration doses showed negligible

Table 1: PBMC cell viability (%) of four *T. cordifolia* extracts

Concentration (μg mL^{-1})	Per cent cell viability (±SD)			
	Tc-N*	Tc-M*	Tc-W*	Tc-C*
50	74.33±10.24	80.15±10.46	81.06±11.23	84.75±11.52
40	79.42±11.73	81.51±11.17	83.27±11.93	86.38±11.82
30	80.68±11.95	83.96±9.83	86.09±11.60	88.31±11.85
20	82.12±11.75	90.18±10.22	87.68±12.77	91.35±11.99
10	87.95±10.62	87.38±10.04	89.54±13.61	92.42±12.26

*Tc-N = *T. cordifolia* grown on *A. indica*, Tc-M = *T. cordifolia* grown on *M. indica*, Tc-W = *T. cordifolia* grown wild (without support of any plant) and Tc-C = *T. cordifolia* grown under *in vitro* condition callus culture

Table 2: Percentage of PBMC cell proliferation of four *T. cordifolia* extracts

Concentration (µg mL^{-1})	%Cell Proliferation (±SD)			
	Tc-N	Tc-M	Tc-W	Tc-C
50	29.77±5.52	22.94±2.04	22.23±3.28	19.47±2.64
40	23.73±4.80	21.00±3.02	20.19±3.02	17.06±2.49
30	22.38±6.46	18.55±2.41	17.71±3.36	15.00±1.97
20	20.55±2.42	15.29±1.73	14.69±3.32	11.41±1.51
10	15.89±5.50	13.23±2.23	12.28±3.28	9.98±1.92

*Tc-N = *T. cordifolia* grown on *A. indica*, Tc-M = *T. cordifolia* grown on *M. indica*, Tc-W = *T. cordifolia* grown wild (without plant support) and Tc-C = *T. cordifolia* grown under *in vitro* condition callus culture

toxicity effect and higher survival rate. Hence, the present study reveals the safety of *T. cordifolia* extracts in *in vitro* experiments which need to be further evaluated under *in-vivo* conditions.

Immunomodulation activity: Cell proliferation assay

The MTT assay was successfully performed to analyze immunomodulation activity of *T. cordifolia* by quantification of PBMC cell proliferation. The assay was carried out between data from seven individuals performed in triplicates and statistical significance analyzed by ANOVA ($p<0.05$). The percentage cell proliferation of four different extracts of *T. cordifolia* observed after MTT assay at 24 h incubation period ranged from 29.77±5.53 to 15.89±5.50% for *T. cordifolia* grown on *A. indica*, 22.94±2.04 to 13.23±2.24% for *T. cordifolia* grown on *M. indica*, 22.23±3.28 to 12.28±3.29% for *T. cordifolia* grown in wild conditions and 19.47±2.65 to 9.98±1.93% for *T. cordifolia in vitro* grown callus sample (Table 2). The highest value was observed at 50 µg mL^{-1} for *T. cordifolia* grown on *A. indica* sample. At higher concentration (50 µg mL^{-1}), the extracts showed higher percentage proliferation and the activity showed decreasing trend for lower concentrations with lowest activity at 10 µg mL^{-1}. The results revealed that *T. cordifolia* grown on *A. indica* extracts induced maximum per cent proliferation response, followed by *T. cordifolia* grown on *M. indica* > *T. cordifolia* grown in wild conditions > *T. cordifolia in vitro* grown - callus values at each concentration of test dose. *T. cordifolia* extract dosage developed in present study could be used as a reference for the studies considering the positive survival rate of used concentration and the proliferation activity observed in this data.

T. cordifolia in vitro grown - callus extracts when compared with other three extracts displayed notable results for lymphocyte proliferation response, since other *T. cordifolia* samples were collected from the well grown *T. cordifolia* plant raised under natural conditions. *T. cordifolia in vitro* grown-callus being the extract from young plant material than other samples; its proliferation activity confirms the immunomodulation activity as a characteristic for *T. cordifolia* plant. *T. cordifolia in vitro* grown callus extract with significant results for viability indicated its safety for medicinal use.

The F-value for viability and proliferation activity were higher than F-critical values for different *T. cordifolia* samples as well as for the concentrations used (Table 3 and 4). The means of samples differed significantly from each other. Hence, as per test we consider that the observations are significantly different from each other.

Table 3: ANOVA of PBMC cell viability for four *T. cordifolia* extracts

Source of variation	SS	Df	MS	F	P-value	F crit
Rows	226.23	4.00	56.56	25.09	9.42 x 10^{-6}	3.26
Columns	152.32	3.00	50.77	22.53	3.22 x 10^{-5}	3.49
Error	27.05	12.00	2.25	-	-	-
Total	405.59	19.00	-	-	-	-

Note: SS - sum of squares, df - degrees of freedom, MS - mean square, F- statistic value

Table 4: ANOVA of PBMC cell proliferation for four *T. cordifolia* extract

Source of variation	SS	df	MS	F	P-value	F crit
Rows	282.16	4.00	70.54	88.91	8.23×10^{-9}	3.26
Columns	159.31	3.00	53.10	66.93	9.20×10^{-8}	3.49
Error	9.52	12.00	0.79	-	-	-
Total	450.99	19.00	-	-	-	-

Note: SS - sum of squares, df - degrees of freedom, MS - mean square, F - statistic value

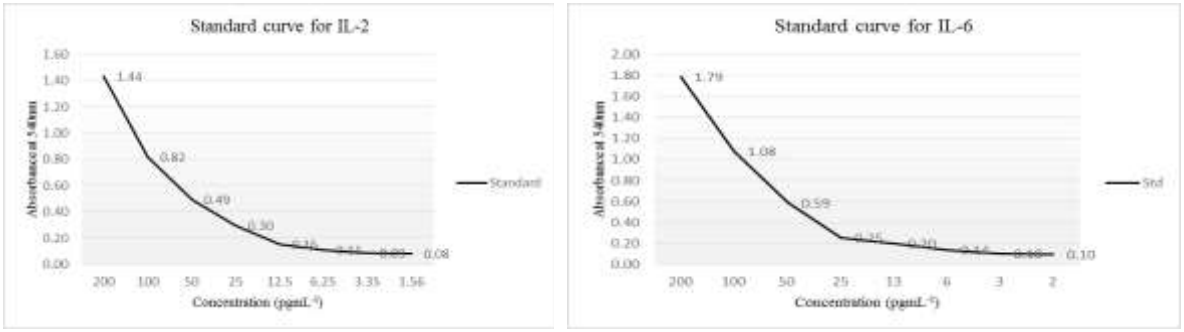


Fig. 1: Standard curve for IL-2 (left side) and IL-6 (right side)

Table 5: IL-2 quantification for four *T. cordifolia* extracts (pgmL^{-1})

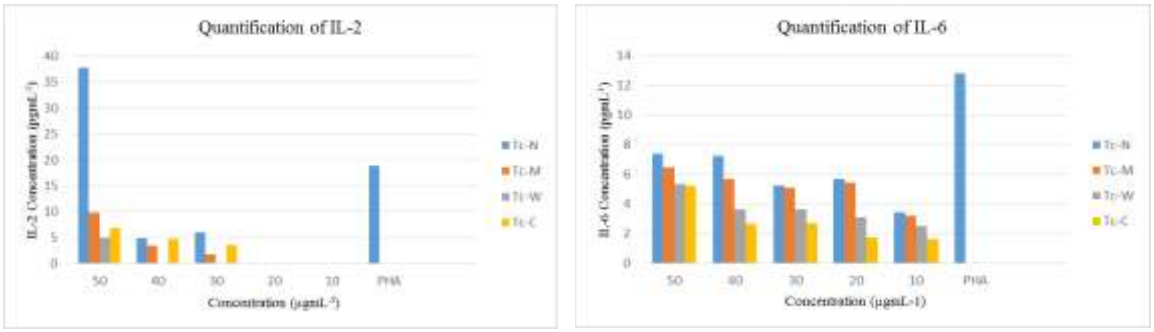
Conc. of extract (µg mL^{-1})	IL-2 quantification (pg mL^{-1})			
	Tc-N	Tc-M	Tc-W	Tc-C
50	37.78	9.87	4.97	6.84
40	4.83	3.53	-	4.69
30	5.98	1.81	-	3.68
20	0.08	-	-	-
10	-	-	-	-
PHA (10 µg mL^{-1})	18.93	-	-	-

Table 5: IL-6 quantification for four *T. cordifolia* extracts (pg mL^{-1})

Conc. of extract (µg mL^{-1})	IL-6 quantification (pg mL^{-1})			
	Tc-N	Tc-M	Tc-W	Tc-C
50	7.37	6.47	5.34	5.22
40	7.26	5.68	3.64	2.63
30	5.22	5.11	3.64	2.71
20	5.68	5.45	3.08	1.72
10	3.42	3.19	2.51	1.61
PHA (10 µg mL^{-1})	12.79	-	-	-

Immunomodulation activity: Quantification of human IL-2 and IL-6

The standard curves for IL-2 and IL-6 are depicted in Fig. 1 and 2, respectively. The standard curve absorbance values for IL-2 quantification were found in the range of 1.44 to 0.08 at A_{450} (Fig. 1). Table 5 and Fig. 3A depicts the IL-2 quantification (pg mL^{-1}) for the extract range of 50-10 µg mL^{-1} was depicted. The maximum expression of IL-2 was seen in *T. cordifolia* grown on *A. indica* sample at 37.78 for 50 µg mL^{-1}, while it showed minimum expression at 0.08 for 20 µg mL^{-1}. The dosage of 10 µg mL^{-1} showed negligible IL-2 expression for all the four samples. *T. cordifolia* grown under wild conditions showed IL-2 expression according to the standard curve for only 50 µg mL^{-1} dosage. *T. cordifolia* grown on *M. indica* and *T. cordifolia* grown *in vitro* callus showed



A **B**

Fig. 3: Comparison of four *T. cordifolia* methanolic extracts for quantification of IL-2 (left side) and IL-6 (right side)

significant quantification of IL-2 for 50, 30 and 20 μg mL^{-1} concentration. The phytohemagglutinin (PHA) used as positive control showed 18.93 pg mL^{-1} IL-2 quantification at 10 μL stock solution in sterile PBS. The comparison between four samples illustrated that *T. cordifolia* grown on *A. indica* showed maximum IL-2 expression than other three extracts.

The IL-6 quantification (pg mL^{-1}) for the extract range 50-10 μg mL^{-1} has been illustrated in Table 6 and Fig. 3B. The standard curve absorbance values for IL-6 quantification were found in the range of 1.79 to 0.10 at A_{450} (Fig. 2). Maximum expression of IL-6 was seen in *T. cordifolia* grown on *A. indica* sample at 7.37 at 50 μg mL^{-1} while minimum expression was observed at 5.22 for *T. cordifolia* grown *in vitro* callus at 50 μg mL^{-1}. *T. cordifolia* grown under wild conditions and *in vitro* callus extracts displayed closely related values of 5.33 and 5.22, respectively. The phyto-hemagglutinin (PHA) used as positive control showed 12.79 pg mL^{-1} IL-6 quantification at 10 μL stock solution in sterile PBS. The comparison between four samples illustrated that *T. cordifolia* grown on *A. indica* have maximum IL-6 expression than other three extracts.

The cytokines quantification with the help of ELISA kit demonstrated comparable results for the activity *T. cordifolia* on *A. indica* being maximum, followed by *T. cordifolia* on *M. indica*, *T. cordifolia* grown wild and *T. cordifolia* grown *in vitro* callus which also supports the data obtained for lymphocyte proliferation assay. The present study confirms that *T. cordifolia* when grown on *A. indica* or *M. indica* plant enhances medicinal value as mentioned in Ayurveda (Panchabhai *et al*., 2008). The present study showed enhanced outcome of results for *T. cordifolia* grown on *A. indica* plant which is supported by Narkhede *et al*. (2014). The immunomodulation assay with the help of human polymorphonuclear lymphocytes cells (PMN) was assayed along with *Candida* culture as an antigen and *T. cordifolia* extracts showed stimulation of PMN cells for phagocytosis of *Candida* culture (Salskar *et al*., 2014). *T. cordifolia* has also been studied as immunomodulator for activation of macrophages and established enhanced inhibitory effects of extracts (direct effect and indirect effect) on the *E. coli* (More and Pai, 2011). The importance of cytokines has been described in the reports as an efficient therapy for various diseases. The study with sixteen patients with metastatic non-small cell lung cancer has shown significant results when treated with a combination of low-dose IL-2 and TNF-α. This study was a part of phase I dose escalation trail to assess the immunomodulatory effects and anti-tumor efficacy of cytokines (Yang *et al*., 1991). A report by Kolodziej (2011) describes the immunomodulatory activity of *Pelargonium sidoides* in the context of its health promotion with the help of cytokines- IL-1, IL-2. IL-18, TNF-α.

Kapil and Sharma (1997) have reported that immunopotenting compounds (syringin, cordioside, cordifolioside A and cordiol- active phytochemicals) from *T. cordifolia* with the help of inhibition of C3-convertase of the classical complement pathway and macrophage activation protocol. Seven compounds from different class of phytochemicals were isolated and analyzed for their immunomodulatory activity using PMN phagocytic function studies from *T.cordifolia*. The compounds isolated were 11-hydroxymustakone, N-methyl-2-pyrrolidone, N-formylannonain, cordifolioside A, agnoflorine, tinocordiside, syringing (Sharma *et al*., 2012). Aher and Wahi (2012) also reported the immunomodulatory properties of *T. cordifolia* grown on support of *M. indica* plant. They performed the study by spleen lymphocyte proliferation assay using cytokines IL-2, IL-10 and TNF-α ELISA reader quantification and also determined their expression by Real Time PCR. Through results it was concluded that *T. cordifolia* stem (*M. indica* plant climber) showed potent immunomodulation activity.

Conclusions: *T. cordifolia* samples when treated with peripheral blood mononuclear cells resulted in good survival rate at 50-10 μg mL^{-1} concentration of extract. This reveals the safety of *T. cordifolia* for pharmaceutical use. The comparison of extracts for immunomodulation activity depicted significantly higher response for cell proliferation for *T. cordifolia* grown on *A. indica* than other extracts for MTT assay. The expression of IL-2 and IL-6 cytokines signified the medicinal value of *T. cordifolia*. *T. cordifolia* grown on *A. indica* or *M. indica* seems potent drug as compared to other *T. cordifolia* samples. The callus extract of *T. cordifolia* depicted noteworthy results as

compared to other extracts of *T. cordifolia* for the presence of immunomodulation activity. This illustrated that biotechnological tools like *in vitro* culture of medicinal plants can be helpful in enhancing medicinal value of plants, as well as for their bioprospectation and conservation.

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