ABSTRACT
1. INTRODUCTION

Vaccine formulations generally contain adjuvants such as alum to elicit or potentiate antigen specific immune responses. Currently, aluminum compounds (alum) are the only vaccine adjuvants licensed by US Food and drug Administration (FDA) for human use. The limitations of alum especially in inducing cellular immunity are well reported. This has resulted in interest and need for newer adjuvants towards development of safer and improved antigen sparing vaccine formulations. Combined use of vaccines and immunomodulators is emerging as one of such strategies to elicit efficient protective immune responses. Such approaches have resulted in development of immunoadjuvants such as QS-21, CpG oligonucleotides, MPL, cytokines which have shown encouraging results when used in various vaccine formulations. T helper cells (Th) have two subsets known as Th1 and Th2, and the cytokines produced are known as Th1-type and Th2-type cytokines. Th1-type cytokines (IFN-gamma, IL-2) promote cell-mediated immunity responsible for killing intracellular parasites while Th2-type cytokines (IL-4, IL-10) are associated with humoral immunity leading to increased IgE and eosinophilic responses. Modulation of Th1 and Th2 immunity has remained as central target for immunomodulators. Immunomodulators generally are classified as Th1, Th2 or mixed Th1/Th2 agents characterized by differences in their modulatory effects on T-helper cell (Th) cytokines. Several immunomodulators that selectively boost either Th1 or Th2 responses are currently being pursued with great hope as possible adjuvants in vaccine formulations.

Traditional medicine driven bioprospecting remains as important approach towards discovery of newer and safer drugs. Indian Traditional System of Medicine, Ayurveda mentions a special class of immunomodulatory botanicals known as Rasayana. Several immunomodulatory botanicals from this class have been reported to have significant effect on Th1/Th2 immunity. This study is an attempt to establish immunopharmacological profile of selected rasayana botanical with reference to Th1/Th2 immunity and further explore its possible potential as an immunoadjuvant for vaccine formulations.

_Tinospora cordifolia_ (Willd.) Miers (Family: Menispermaceae) is commonly known as Guduchi in India and widely distributed throughout the tropical Indian subcontinent and China. The monograph of this herb is mentioned in Indian Pharmacopoeia.  _Tinospora cordifolia_ extract (TCE) and its active principles have
reportedly been linked to exhibit various medicinal properties. Further, several extensive studies to demonstrate the immunomodulatory potential of TCE and its active principles with antigens have been carried out\(^4\). However, systematic study on the modulation of Th1/Th2 immunity to confer T cell response has not been reported. In the present study, we have proven the immunomodulatory (Th1/Th2 immunity) and immuno-adjuvant potential of TC aqueous extract.

The research work described in present synopsis is aimed at development of a Rasayana botanical as an immunoadjuvant for use in vaccine industry. The work involves studies on characterizing modulatory effects of TCE on Th1/Th2 immunity using flowcytometry and further evaluates its immunoadjuvant potential with DPT vaccine with special reference to protective immunity against diphtheria. Levamisole, Quillaja saponin (QS) from the bark of American soaptree *Quillaja saponaria* were used as reference.

**AIM OF THE STUDY**

To establish immunopharmacological profile of a shortlisted Ayurveda based botanical extract with special reference to modulation of Th1/Th2 immunity and further explore its applications as an immuno-adjuvant for use in vaccine industry.

**STUDY OBJECTIVE**

- To determine the quality of the test material and its chemical characterization with respect to marker content analysis.
- To carry out immuno-pharmacological evaluation of extract with reference to Th1-Th2 immunity using flowcytometry
- To study the comparative immuno-adjuvant potential of test extract and Quillaja saponin (QS) for their adjuvant potential in DPT vaccine with special reference to protective immunity against diphtheria.

**2. LITERATURE REVIEW**

Scientific literature on following aspects was reviewed in detail:

- Vaccine adjuvants currently in research and development including CpG oligonucleotides, QS-21, Cytokines, MPL, MF-59 and Alum salts.
• Scope and potential of natural products in adjuvant discovery.
• Traditional Medicine and its role in bioprospecting of immunoadjuvan	special reference to Ayurveda based Rasayana drugs.
• Pharmacology of *Tinospora cordifolia*.

A brief overview reference drugs and selected test material is provided below:

**Reference Drugs:**

**Levamisole:** Levamisole is reported as non-specific stimulator of lymphocyte. It is known to enhance humoral and cellular immunity towards T cell dependent antigens (at lower doses). Levamisole at a concentration of 2.5 mg/kg dose was used during the studies.

**Alum:** alum is a licensed vaccine adjuvant for human use. It is efficient in inducing a strong antibody response and associated with humoral immunity (Th2 response).

**Cyclophosphamide:** Cyclophosphamide induces immunosuppression especially targeting B cells leading to inhibition of antibody production. It was used at 250 mg/kg/b.w/p.o administered 48 hrs prior to immunization.

**Cyclosporin:** Cyclosporin A (Cys) is used as a clinical immunosuppressive agent. It affects calcium-ion uptake in a specific white blood cell (lymphocytes), rendering that cell ineffective. Cys was used at 5 mg / kg / b.w /p.o administered 48 hrs prior to immunization.

**Quillaja saponin (QS):** Saponins are plant glycosides which are widely distributed in plants. Saponins and its purified fractions from *Quillaja saponaria*, have been shown to have adjuvant activity. Quillaja saponin is obtained from the bark of the South American soaptree, *Quillaja saponaria* Molina (Family: Rosaceae). Partially purified Quillaja saponins were reported to associate with hydrophobic or amphipathic proteins and lipids to form detergent/lipid/saponin complexes termed as ‘ISCOMs’ (immunostimulating complexes). ISCOMs are highly immunogenic by both oral and parenteral routes, inducing a wide range of immune responses, including delayed-type hypersensitivity (DTH) and antibody responses. In addition, mucosal immunisation with ISCOMs stimulates production of local IgA antibodies and cell mediated immunity. Further, orally fed Quillaja saponin was reported to enhance the immunopotentiating ability of an intraperitoneally administered inactivated rabies vaccine in mice. Purified saponin from crude *Quillaja saponaria* amplified antigen-
specific immune responses to an experimental HIV-1 vaccine and potentiated an immune response elicited by albumin and venom both in mice.

*Tinospora cordifolia* (TC): (part to be used: Dried stems)

TC (commonly known as Guduchi/Gulvel) is reported in Ayurvedic and other ancient literature for its immunomodulatory and cytoprotective activities. TC has been shown to exhibit creditable medicinal properties like general tonic, antipyretic, anti-inflammatory and prevention of complement disorders and protection against tumors. It has also been shown to protect against abdominal infections by enhancing the phagocytic efficiency and intracellular bactericidal activity of macrophages and neutrophils in *E. coli* induced peritonitis. Its chemical constituent can be broadly divided into alkaloids, diterpenoids, steroids, flavanoids and lignans. Aqueous extract obtained from stems (TCE) is well researched for its immunomodulatory activities. TCE is reported to have modulatory effects on phagocytosis and B cell mediated immunity. Further in another report, treatment with TCE resulted in reversal of cyclophosphamide induced immune suppression.

3. MATERIALS AND METHODS

The study was carried out as per standard protocols based on international guidance and regulatory provisions for quality, safety and efficacy.

3.1. Quality and safety

The study test material (dried stems and aqueous extract of *Tinospora cordifolia*; TCE) was procured from Natural Remedies, Bangalore and were authenticated from National Institute of Science Communication (NISCOM), New Delhi. Test material was ensured to be within limit of W.H.O. for contaminants such as Aflatoxins, Pesticidal residue, Heavy metal content and Microbiological tests. Markers compounds in TC were identified and quantified (Berberin and palmatine) towards chemoprofiling and quality control. Extract was further subjected to safety studies as per OECD guidelines.
3.2. Efficacy

The study protocols were approved by Institutional Animal Ethics Committee (IAEC).

3.2.1. Effect of TC extract on Th1-Th2 immunity

3.2.1.1. Animals, antigenic stimulus and route of administration: Balb/c mice of either sex weighing 12-16 gm. were sensitized and challenged using sheep red blood cells (SRBC) in the concentration of $5 \times 10^6$ cells/ml as per study protocol.

3.2.1.2. Parameters: Following parameters were studied using flowcytometry and immunopharmacological models.

- Lymphocyte percentages: *in vivo* (Flowcytometry, CD3 and CD19 percentages) methods.
- Lymphocyte phenotyping: CD4$^+$ and CD8$^+$ T cell percentages. (Flowcytometry)
- Cytokine assays: Th1 (IFN-gamma, IL-2) and Th2 (IL-4) *in vivo* cytokine modulation in SRBC sensitized animals. (Flowcytometry)
- *In vivo* humoral and cellular immune response (CMI) modulation: as per method described by Doherty$^{19}$ and Nelson model$^{20}$ respectively.

3.2.2. Immunoadjuvant potential of TCE and QS using DTP vaccine as a test system:

3.2.2.1. Animal and antigenic stimulus: Pathogen free, guinea pigs of either sex weighing 200-250 gm. were used for immunoadjuvant study. Animals were immunized with Alum adsorbed DTP vaccine. Test extract was evaluated on optimized doses derived from studies on Th1-Th2 immunity.

The potency of diphtheria component in DPT vaccine is determined in guinea pigs using a well established pharmacopoeial procedure$^{21}$. The test was modified with respect to dilutions of DPT vaccine, treatment schedule, diphtheria toxin and post challenge observation period. As a result, 1:160 dilution of DPT vaccine, Day 0-14 treatment, 10 LD$_{50}$ challenge and observations up to 42 day after challenge procedure was selected for the study. Further, scoring system for diphtheria symptoms was developed as per reported procedures$^{22}$. 
The brief details of the experiment is given below:

**Day 0:** Immunization with DPT vaccine

**Day 0 to 14:** Oral treatment of TCE or QS at respective doses in specified groups.

**Day 26:** Estimation of neutralizing antitoxin level by vero cell neutralization assay (VCA) in predetermined control and treatment groups.

**Day 28:** Challenge with diphtheria toxin at 10 LD$_{50}$ dose in control and treatment groups.

**Day 28 to 42:** Observation for mortality, morbidity, scoring of challenged animals using scoring pattern and adrenal gland toxicity.

**Day 42:** Estimation of neutralizing antitoxin level in control and treatment groups.

---

**3.2.2.2. Parameters:**

The antibody response to diphtheria component of DPT vaccine was evaluated by Vero cell assay (VCA).

**Immunoprotection:** The treated and untreated vaccinated animals were challenged with diphtheria toxin and resulting morbidity and mortality was observed and graded in form of scores. Further, toxic effects of diphtheria toxin on adrenal glands is known and established in guinea pig models. Sera cortisol levels and histopathology was carried out to grade adrenal hemorrhages in control and treated animals.

**3.2.3. Haemolytic Activity:** Rupture of erythrocyte membranes (haemolysis) by saponins is one of the most spectacular properties of saponin based molecules. In this
study, we compared the percentage hemolytic activities of test materials QS and *Tinospora cordifolia* at different dose levels.

### 3.3 Analysis

Data are expressed as mean ± standard deviation. Statistical significance of differences was assessed by Post ANOVA (Bonferroni test for multiple comparisons) $P<0.05$ was set as the level of significance. For immunoadjuvant studies, Mann Whitney test was used to assess the value of significance in Vero cell assay and protection scores.

### 4. RESULTS AND DISCUSSION

Vaccines based on toxoid, synthetic peptides, and plasmid DNA are often less immunogenic. Such vaccines need adjuvants for increasing the potency or stimulating the appropriate immune response. Several different adjuvants have been proposed over the last few decades, however has limitations including efficacy, cost and safety concerns. There is a major unmet need for a safer and efficacious adjuvants capable of triggering appropriate protective immune responses. Immunomodulators obtained from different sources like bacterial, viral, synthetic have been used for enhancement of immune response to vaccines. Traditional systems of medicines are becoming important bioprospecting tools for discovery of immunoadjuvants for use in several immune disorders including vaccines. With this viewpoint, the present study is an attempt to explore the potential of Ayurveda based Rasayana drug, *Tinospora cordifolia*, for possible use as an immunoadjuvant in vaccine industry.

As a first step, test extract was prepared as per Ayurveda guidance and standardized as per international guidance on quality control and standardization. HPLC-DAD method was developed for chemoprofiling of TCE with the help of commercially available marker compounds for standardization purpose only. Palmatine ($0.023 \pm 0.005$ mg g$^{-1}$) and Berberine ($0.031 \pm 0.009$ mg g$^{-1}$) were identified and quantified in crude (stem) and stem aqueous extract (TCE) for quality control. Several *in vitro* methods involving cell populations or cell lines are reported for examining effects on Th1 and Th2 immunity. However, these methods have reported limitations with complex mixtures such as herbal extracts. In this study, we have used flow cytometry to characterize *in vivo* effects of TCE on Th1/Th2 immunity. The results suggest the following:
4.1. Effect of TCE on lymphocyte percentages (T and B cell):

The study was carried out in presence of antigenic stimulus (SRBC). Treatment of levamisole (2.5 mg/kg/b.w./7 days), and TCE at 100 mg/kg/b.w/7 days showed a significant stimulatory effect on CD3^ and CD19^ percentages (P<0.001) in peripheral blood of normal and SRBC sensitized animals whereas alum (500 ug/mouse) showed significant increase in CD19^ percentages (P<0.001) as compared to SRBC alone which are consistent with previous reports. Agents having such stimulatory effect on T and B cell populations have applications in adjuvant therapy.

4.2. Effect of TCE on CD4^ and CD8^ percentages:

As a next step, effect of TCE, levamisole and alum was studied on CD4^ (T-helper) and CD8^ (T-cytotoxic) percentages in peripheral blood of SRBC sensitized animals. Interestingly, TCE (P<0.01, P<0.001) and levamisole (P<0.001, P<0.001) showed a significant stimulatory effect on CD4^ and CD8^ percentages. Levamisole showed a higher percent immunomodulatory activity for CD4^ and CD8^ as compared to TCE and alum treatment. While, modulatory effect of TCE was higher on CD8^ percentages. In similar conditions, percent immunomodulatory activity of alum was lower as compared to TCE and/or levamisole. This is further consistent with the previous reports on alum which suggests direct activation of B cells as major mechanism of its adjuvanticity. Agents having up-regulating or restoring effect on CD4/CD8 percentages have important applications as immuno-adjuvants in the area of cancer, HIV, etc.

4.3. Effect of TCE treatment on CD4^ (IL-2, IFN-gamma) and (IL-4) cytokine percentages:

TCE treatment at optimal dose 100 mg/kg enhanced both Th1: IL-2 (P<0.001), IFN-gamma (P<0.001) and Th2: IL-4 (P<0.01) percentages in peripheral blood in SRBC immunized animals as compared to control, suggesting mixed modulatory effect on Th1/Th2 immunity. Levamisole also showed significant upregulation of IL-2 and IFN-gamma expression (P<0.001). However, IL-4 expression in levamisole treated animals was significantly low, indicating preferential effects on Th1 immunity. While, alum showed significant enhanced IL-4 expression (P<0.001), indicating
Abstract

effects on Th2 immunity. Cyclosporin treated group showed significant reduction in Th1 and Th2 cell percentages indicating immunosuppressive effect (P<0.001).

4.4. Effect of TCE on SRBC specific humoral and cellular responses:

Oral administration of TCE (25-200 mg/kg/p.o) showed a dose dependent increase in anti SRBC antibody level and DTH (foot pad thickness) responses suggesting significant effects on humoral and cell mediated immunity respectively. Levamisole, alum (positive control) and cyclosporin, cyclophosphamide (negative controls) showed significant up-regulation and down regulation of immune responses respectively which is consistent with their previous reports.

Overall the study suggests dual Th1/Th2 phenotype of TCE which was evident from significantly higher CD4/CD8, CD3/CD19 and respective CD4 positive Th1/Th2 cytokine percentages as compared to control. TCE also showed significant upregulation of SRBC specific humoral and cellular immune responses which further supports mixed Th1/Th2 phenotype of TCE. The study also establish that TCE shares a similar immunopharmacological profile with levamisole with respect to modulation of Th1/Th2 immunity. Immunomodulators with such activity are currently desired as adjuvants in vaccine formulations. This further prompted us to explore immunoadjuvant potential of TCE with specific reference to protective immunity against diphtheria in DPT immunized animals.

4.5. Effect of TCE on protective efficacy of DPT vaccine: Applicability studies

Immunoadjuvant potential of TCE was studied as compared to QS. Oral administration of TCE and QS resulted in significantly higher diphtheria antitoxin levels even at higher dilutions of DPT vaccine suggesting antigen sparing effect (P<0.001, P<0.01 respectively). However, TCE showed higher adjuvant effect on antitoxin levels as compared to QS as evident by percent immunomodulatory activity (TCE=616.66 and QS=133.33). In order to further assess the immunoadjuvant effect of TCE and QS on in vivo protective immunity, DPT immunized animals in vaccine alone and treated groups were challenged with 10 LD50 dose of diphtheria toxin. Antitoxin levels, survival rate and challenge associated morbidity (severity scores, weight loss) was measured in vaccine alone and treatment groups. The challenge procedure resulted in significant increase in antitoxin levels in control and treated
animals. This phenomenon is reported for various lethal challenges such as in pertussis\(^4\). Interestingly, a significant and higher antitoxin response was observed in TCE (P<0.01) treated animals. Whereas, antitoxin levels in QS treated animals were not significant as compared to control. These observations were found consistent with survival where survival rates in TCE and QS were 100 and 87 % respectively as compared to 37 % with vaccine alone. This was accompanied by significant reduction in challenge associated morbidity in TCE or QS groups as evident by mean severity scores (TCE = 6.00 ± 2.07, QS = 5.00 ± 2.94). This suggest higher modulatory potential of TCE as compared to QS. Further, diphtheria toxin is known to have toxic effect (diphtheria toxaemia) on adrenal glands. Sera cortisol values and histopathology was assessed as markers of challenge associated adrenal toxicity. Treatment with TCE showed higher modulation as evident by higher reduction of cortisol levels as compared to QS (TCE = 9.08 ± 2.01, QS = 27.70 ± 3.96) which is further consistent with diphtheria antitoxin, survival rate and severity score observations. Furthermore, enhanced cytokine level, IFN-gamma and IL-4, in TCE treated group suggests its dual effect on Th1/Th2 immunity whereas QS treatment results in enhanced Th1 response (increased IFN-gamma level).

5. CONCLUSION
The study demonstrates immuno-adjuvant potential of TCE treatment and its modulatory effects on Th1/Th2 immunity. The investigations covered effect of TCE on T cell percentages, intracellular cytokines and antigen specific responses which will be useful to better understand the immunopharmacological properties of TCE. Such agents can be a alternative and safer source for development of newer vaccine adjuvants. TCE showed modulatory effects on Th1/Th2 immunity, resulting in Th-1 biased responses, which will further improve efficacy of DPT vaccine formulations especially in immunocompromised conditions such as in elderly and patients on cancer therapy. Overall, the study establishes in vivo Th1/Th2 (mixed) and Th1 up-regulating activity of TCE and QS respectively and suggests its newer applications in vaccine industry as immuno-adjuvants. These findings also contribute to better understanding of traditional uses of TCE, which will allow more rational use of this botanical in integrative medicine.
6. PUBLICATIONS

- Dada Patil, Sanjay Mishra, Manish Gautam, PrajAKta Kulkarni, K. Suresh, Sunil Gairola, Suresh Jadhav and Bhushan Patwardhan; (2010); Estimation of protoberberine alkaloids in *Tinospora cordifolia* stem crude powder, processed extracts and marketed formulations using HPLC-DAD method; *Chromatographia*; Volume 71; Number 3-4; 341-345.

- Dada Patil, Manish Gautam, Sanjay Mishra, Umesh Jadhav, K. Suresh, S. Gairola, S. S. Jadhav, Bhushan Patwardhan; (2010); Physicochemical stability and biological activity of *Withania somnifera* extract on real time and accelerated storage conditions; *Planta Medica*; 76 (5); 481-8.


- Sanjay Mishra, Manish Gautam, Dada Patil, S. Gairola, Y. Shinde, K. Suresh, S. S. Jadhav, Bhushan Patwardhan; Immuno-adjuvant potential of *Tinospora cordifolia* with DPT vaccine in an experimental system using diphtheria as a test system.
  (To be communicated).

- Sanjay Mishra, Manish Gautam, Sarang Bani, S. S Jadhav, Bhushan Patwardhan; Modulation of Th1/Th2 immunity by *Tinospora cordifolia* and Vaccine Efficacy: Exploratory study with DPT vaccine using pertussis as a test system.
  (Manuscript).

7. PRESENTATION:

- Mishra S K, Goyal C, Patwardhan B, Gairola S; (2008); Immunomodulators and Protective Efficacy of Vaccines: Co-adjuvant potential of QS on protective efficacy of DPT Vaccine; 60th Indian Pharmaceutical Congress (IPC); New Delhi.
REFERENCES:

1. Nikolai Petrovsky; (2008); Freeing vaccine adjuvants from dangerous immunological dogma; Expert Rev. Vaccines; 7(1); 7–10.
2. Bhushan Patwardhan; (2005); Ethnopharmacology and drug discovery; JEP; 50–52.
5. Indian Pharmacopoeia; (2007); Indian Pharmacopoeia commission; Ghaziabad; 2037-38.
6. P. K. Raveendran Nair et al; (2005); Mechanism of macrophage activation by (1,4)- α-D-glucan isolated from Tinospora cordifolia; Int. Immuno; 6 ; 1815–1824.
10. A. Aguila, A. M. Donachie, M. Peyre, C P. McSharry, D. Sesardic, A. M. Mowat; (2006); Induction of protective and mucosal immunity against diphtheria by ISCOMS based vaccine; Vaccine; 24, 5201–5210.
14. M. Ahmed et al; (2006), Quantitative Determination of four Constituents Tinospora sps. by a Reversed-Phase HPLC–UV–DAD Method; Broad-Based Studies Revealing...
Abstract

Variation in Content of Four Secondary Metabolites in the Plant from Different Eco-Geographical Regions of India; JCS; Volume 44; Number 8; 504-509.

15. M. S. Ranjith et al.; (2008); Enhanced Phagocytosis and Antibody Production by Tinospora cordifolia - A new dimension in Immunomodulation; AJB; 7 (2); 081-085.

16. Veena R. Desai et al; (2005); G1-4A, an immunomodulatory polysaccharide from Tinospora cordifolia modulates macrophage responses and protects mice against lipopolysaccharide induced endotoxic shock; Int. Immunoph; 7; 1375–86.

17. P N Manjrekar et al.; (2000); Comparative studies of the immunomodulatory activity of Tinospora cordifolia and Tinospora sinensis; Fitoterapia; 71; 254 -257.


Fixed dose procedure – Acute oral toxicity; Limit test; Page No. 4.


21. Indian Pharmacopoeia; (2007); Indian Pharmacopoeia commission; Ghaziabad, 1928-29.

22. Tadashi et al.,(2004); Observation period of animals in toxoid based potency tests; Jpn. Journal of Infectious Diseases; 57; 257-59.