Immunomodulatory activity of ethanolic extract of *Pueraria Tuberosa* D.C.

Jignesh Patel*, Nitin Doshi*, Abheejit Bhalerao#, Rajesh Bonagiri

Abstract— *Pueraria tuberosa D.C*. has been used as a 'Rasayana' in the ayurvedic medicine as well as in swelling of joints and treatment of rheumatism. In current study; Immunomodulatory potential of the ethanolic extract of tubers of *Pueraria tuberosa D.C*. has been evaluated *in vivo* using animal models like Delayed type of hypersensitivity, Haemagglutination antibody titre assay, carbon clearance assay and cyclophosphamide-induced myelosuppression in mice. Results demonstrated that the Ethanolic extract of *Pueraria tuberosa* caused a significant inhibition of delayed-type hypersensitivity (DTH) reaction and Humoral antibody titre suggesting a potential role in inhibition of adaptive immune response or cells. Ethanolic extract of *Pueraria tuberosa* also potentiated function of the mononuclear phagocytic system by increase in phagocytosis and proection from myelosuppression in mice treated with cyclophosphamide.

Our preliminary study demonstrate the immunomodulatory potential of *Pueraria tuberosa* which should be further evaluated in various autoimmune disease models for efficacy in different pathological conditions.

Key words: Immunomodulation, Delayed type of hypersensitivity, Humoral antibody titre, Phagocytic response

1 INTRODUCTION

Modulation of the immune system denotes to any change in the immune response that can involve induction, expression, amplification, or inhibition of any part or phases of immune system. While several types of immunomodulatory agents are available, undesirable side effects often limit their use. Complementary or alternative medicines have become popular for treating different immune disorders. Increasingly among these are extracts from medicinal plants[1]. Hence, mechanistic studies and the identification of active compounds of these medicines could lead to new discoveries in the biological and biomedical sciences. Modern translational research on herbal medicines beyond basic science and clinical perspectives could contribute to the development of new therapeutics[2].

Pueraria tuberosa D.C. (family: Leguminoseae), commonly known as Vidarikand in india, is a large perennial climber or a twiner with shrubby stem and tuberous roots found throughout the warm and moist regions of India up to 4,000 ft[3]. It has been used as a 'Rasayana' in the ayurvedic system of medicine[4]. In traditional medicine, the tubers of *Pueraria tuberosa* is used in swelling of joints and treatment of rheumatism whose etiology is known due to alteration in function of immune system[3]. The tubers have also been reported to possess adaptogenic activity[5]. Although, there are several reports on the medicinal properties of flavonoids isolated from plant, there are no reports regarding characterization of Immunomodulatory activity from Pueraria Tuberosa Linn. Hence, The present study was undertaken to explore the immunomodulatory activity of *Pueraria tuberosa D.C* on cellular and humoral immune responses.

* *Corresponding Author: Mr. Jignesh Patel, Department of Pharmacology, Bombay college of pharmacy, Kalina, santacruz (East), Munbai-400098 Maharashtra,India. Mob. +91 9867962824 E mail: jiggipatel.007@gmail.com*

Mr. Abhijeet Bhalerao. Department of Pharmacology, K. M Kundnani college of pharmacy, Cuffe Parade road, Mumbai-400005 Maharashtra,India bhalerao.abhi@gmail.com

2. MATERIALS AND METHODS

2.1. Reagents

The tubers of *Pueraria tuberosa* were collected, identified and authenticated by Dr. Ashish Phadke, Dept. of Dravyaguna (Herbal Pharmacology), Y.M.T. Ayurvedic Medical College, Mumbai. Cyclophosphamide was obtained as gift sample from Khandelwal laboratory, Thane. Sodium carboxy methyl cellulose was purchased from Sigma. Fresh Sheep red blood cells (SRBCs) was collected in alseiver's solution from a healthy sheep from the Bombay veterinary hospital, Mumbai.

2.2. Animals

Male Swiss albino mice weighing 25 to 30 g were used for all studies. Mice were housed five to six per cage in a temperature and humidity-controlled environment with food and water available ad libitum. They were maintained on a 12-h light/dark cycle (lights on 7:00 AM, lights off 7:00 PM) and all

studies were conducted between 9:00 AM and 5:00 PM. These procedures involving the use of animals and their care were conducted in conformity with CPCSEA Guidelines. All studies involving animals were approved by Institutional animal ethical committee of Bombay College of pharmacy, Mumbai.

2.3 Preparation of Ethanolic extract of tubers of *Pueraria tuberosa*

Two hundred gram Air-dried fine powder of the dried tubers of *P. tuberosa* were defatted by soxhlet extraction using petroleum ether for 24 hr and then defatted mark was subjected to ethanolic extraction using 400 ml 95% ethanol. The extract was concentrated by evaporating the solvent on a water bath at 60°C. Suspensions of ethanolic extract of *P. tuberosa* (EPT) were prepared by constant trituration in 0.5% of sodium carboxy methyl cellulose (w/v) and used for dosing animals.

2.4 In vivo Delayed type of hypersensitivity and Humoral antibody titre response[6,7,8]

Mice were divided into five groups, each consisting of six animals. Group I was administered with vehicle (0.5% w/v sodium carboxy methyl cellulose in water, PO, qd) and Group III-V were administered with different dose of EPT (100, 200, 400mg/kg, p.o,qd.), daily starting 8 day prior to sensitization till the challenge. Group II was administered with cyclophosphamide 200mg/kg, i.p, one day before immunization. The animals were immunized by injecting 2 X 10^8 Sheep red blood cells, intraperitoneally, on day 0 and challenged on day 5 by injecting 2x10^8 SRBC in 0.05ml of saline in the right hind paw. The contralateral paw received same volume of saline. The thickness of the paw was measured at 24 hr after challenge using Dial caliper (Mitutoyo, Japan). The difference in the thickness of the left hind paw and right hind paw was used to calculate the % change in DTH reaction different groups were statistically compared with vehicle treated group..

On day 6 post immunization, Blood from the individual animal was withdrawn from retro orbital plexus and serum was separated. Twenty five µl of two fold-diluted sera was challenged with 25 µl of 0.1% v/v of SRBC suspension in U-bottom microtitre plates. The plates were incubated at 37°C for 90 minute and then observed for haemagglutination. The highest dilution showing haemagglutination was taken as antibody titre. The antibody titres were expressed in a graded manner, the minimum dilution to be taken as (1/2) being ranked as 1. The mean ranks of different groups were statistically compared with vehicle treated group.

2.5 In vivo Carbon clearance assay[6,9]

Mice were divided into four groups, each consisting of five animals. Group I was administered with vehicle (0.5% w/v sodium carboxy methyl cellulose in water, PO, qd) and Group II-IV were administered different dose of EPT (100, 200, 400mg/kg, p.o, qd.) for 14 days. After the last dose of extract, all mice were injected intravenously with 0.1 ml/10gm b.w of carbon suspension (Rotring ink, Germany). Blood sample were withdrawn from retroorbital plexus immediately before and at 3, 6, 9, 12 and 15 minute after the injection of carbon suspension. Aliquot of 25 µl of blood sample was lysed in 2 ml of 0.1% sodium carbonate solution and absorbance was measured spectrophotometrically at 675 nm. Absorbance was plotted against time was plotted and phagocytic index as the rate of carbon clearance was calculated from the regression line. The stimulation of phagocytic activity, known as jurcic indices was obtained as the ratio of the mean regression coefficient of the substance to the mean regression coefficient of the control.

2.6 Cyclophosphamide induced Myelosuppression[6]

Mice were divided into 5 groups, each consisting of 6 animals. Group I (Control) was administered vehicle (0.5% w/v sodium carboxy methyl cellulose in water, PO, qd) and Group III-V were administered different dose of EPT (100, 200, 400mg/kg, p.o., qd respectively) for 14 days. On last day of treatment, Groups II to V was administered with a single dose of cyclophosphamide (200 mg/kg, i.p.). Next day, blood was collected from retroorbital plexuses of individual animal and white blood cell count and % neutrophils were determined. White blood cells were determined using Erma PC-607 cell counter (Erma Inc., Japan). Dry smear of the blood was prepared and stained with Field 'A' and Field 'B' stain for differential leucocytes counts. % neutrophils of total leucocytes were counted for the various group using compound microscope. WBC and neutrophil counts of the treatment groups were compared with the cyclophosphamide treated group.

2.7 Acute Oral toxicity Study

Acute oral toxicity study of crude ethanolic extract of *Pueraria tuberosa* was carried out in mice according to the OECD 425 guideline, an Up and Down procedure, using software "aot 425" (aot 425statpgm, version 1.0, westat, USA).

2.7 Statistical analysis

Results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnett's test was applied to calculate the statistical significance between various groups using GraphPad software. A value of $p < 0.05$ was considered to be statistically significant.

3. RESULTS

3.1 Effect of EPT on Delated type hypersensitivity and Humoral antibody titre assay.

Administration of ethanolic extract of *P. tuberosa* caused significant inhibition in paw edema induced by SRBC at 24 hr. The percentage inhibition of paw edema at doses of 100, 200

and 400 mg/kg was found to be 49.89, 64.08 and 64.86% respectively as compared with Vehicle control at 24 hrs. However, the inhibition was maximum at 200mg/kg and the effect was not found to be dose dependent manner. Cyclophosphamide (200mg/kg) was found to increase the DTH reaction to the extent of 58.94% as compared to Vehicle control (Figure-1).

A dose-dependant decrease in antibody production by B cells was evidenced by decrease in antibody titre in mice treated with ethanolic extracts of *P. tuberosa* compared to Vehicle control group. The percentage inhibition of humoral antibody titer at doses of 100, 200 and 400 mg/kg was found to be 21.27, 51.06 and 57.44% as compared with control respectively (Figure-2). Cyclophosphamide inhibited the humoral antibody titre to the extent of 93.61% as compared with Vehicle control (Figure-2).

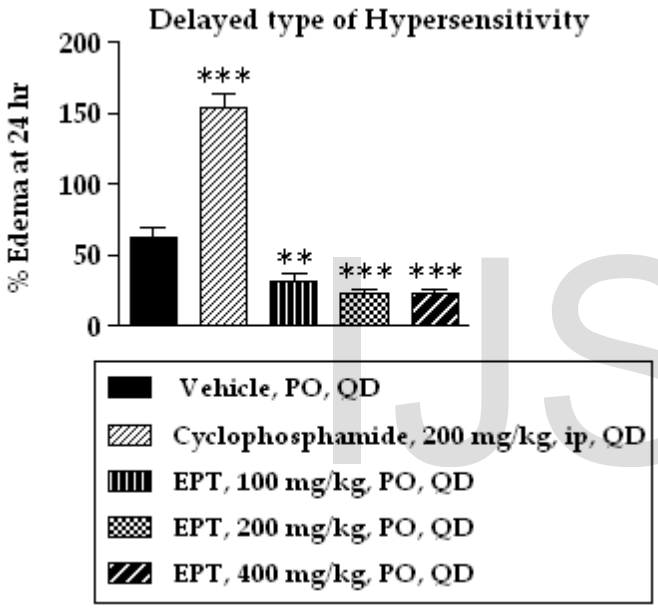

Fig. 1. Treatment with ethanolic Extract of *P. tuberose* significantly inhibited the paw edema and swelling at all tested doses as compared to vehicle group post 24 hr of SRBC challenge. The data represent as Means ± SEM. (n = 6) **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Vehicle

3.2 Effect of EPT on Phagocytosis and Carbon clearance assay

Treatment with ethanolic extract of *P. tuberosa* produced a significant increase in phagocytic index as a function of decrease carbon particles in blood. The phagocytic index at doses of 100, 200 and 400 mg/kg was found to be 0.0334, 0.0438 and 0.0392 respectively. However, the increase in phagocytic index was maximum at 200mg/kg and the effect was not found to be dose dependent manner (Figure-3). Phagocytic rate (Jurcic Indices) calculated from mean regression coefficient also shown to be increased for 100 and 200 mg/kg dose of ethanolic extract of *Pueraria tuberose* which was found to be 1.090 and 1.749 respectively (Table-1).

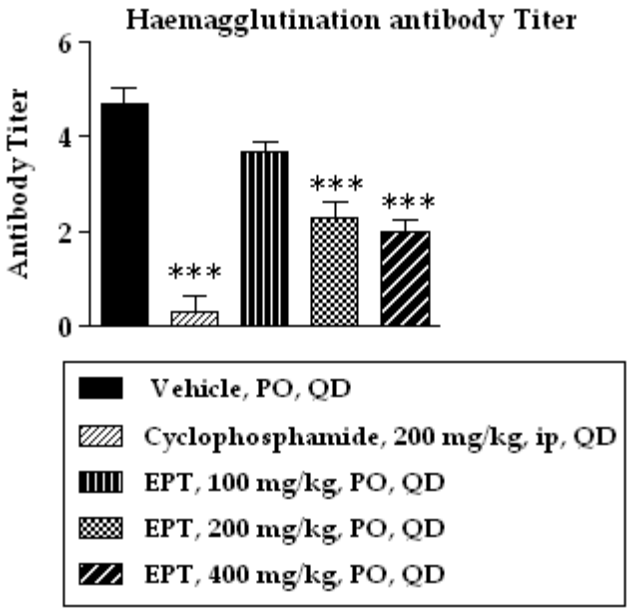

Fig. 2. Treatment with ethanolic Extract of *P. tuberose* significantly inhibited Antibody titer at 200 and 400 mg/kg as compared to vehicle group. The data represent as Means ± SEM. (n = 6) **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Vehicle

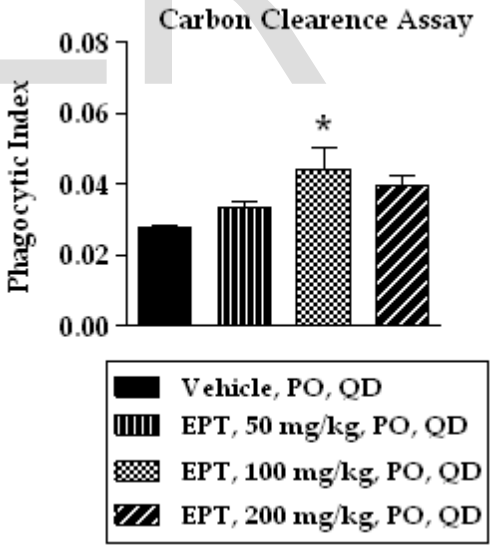

Fig. 3. Treatment with ethanolic Extract of *P. tuberose* significantly increase clearance of carbon particles from blood as function of stimulation in Phagocytic system as compared to vehicle group. The data represent as Means ± SEM. (n = 5) **P* < 0.05 vs. Vehicle

Table- 1

Effect of ethanolic extract of *Pueraria tuberosa* (EPT) on Jur-

cic indices.

Treatment groups	Mean Regression Coefficient	Jurcic Indices (Phagocytic rate)
Vehicle	0.0384 ± 0.02	-
EPT, 50 mg/kg	0.0418 ± 0.01	1.090
EPT, 100 mg/kg	0.0672 ± 0.04	1.749
EPT, 200 mg/kg	0.0315 ± 0.01	0.821

The data represent as Means ± SEM. (n = 5)

3.3 Effect of EPT on Cyclophosphamide induced Myelosuppression

Cyclophosphamide alone at the dose of 200 mg/kg, i.p. caused a significant reduction in the WBC and % neutrophils count as compared to Vehicle control. The WBC count in Cyclophosphamide treated group was found to be 1910 cells and %neutrophil count was found to be 14.83% (Table 2). The WBC count of ethanolic extract of *P. tuberosa* at doses 100, 200 and 400 mg/kg when given with cyclophosphamide was found to be 5010, 4500 and 5260 cells respectively and the % neutrophil count was 31.83, 27.33 and 28.33 % respectively (Table 2). Combined treatment of cyclophosphamide and ethanolic extract of *P. tuberosa* resulted in a restoration of WBC and neutrophils count in blood and bone marrow activity as compared with cyclophosphamide treatment alone (Table-2). However, the effect was not found to be dose dependant.

Table- 2

Effect of ethanolic extract of *Pueraria tuberosa* (EPT) on cyclophosphamide induced myelosuppression.

Treatment groups	WBC count (×1000 cells)	% Neutrophil count
Vehicle	7.13 ± 0.988***	24 ± 3.044
Cyclophosphamide, 200 mg/kg	1.91± 0.185	14.83 ± 1.249
EPT 100 mg/kg + Cyclophosphamide, 200 mg/kg	5.01± 0.510**	31.83 ± 4.4**
EPT 200 mg/kg + Cyclophosphamide, 200 mg/kg	4.5 ± 0.543*	27.33 ± 1.820*
EPT 400 mg/kg + Cyclophosphamide, 200 mg/kg	5.26 ± 0.418**	28.33 ± 2.603**

The data represent as Means ± SEM. (n = 6) *P < 0.05, **P < 0.01, ***P < 0.001 vs. Cyclophosphamide (200 mg/kg).

3.4 Acute Oral toxicity of EPT

The LD_{50} of ethanolic extract of *Pueraria tuberosa* was estimated to be 1502 mg/kg (p.o) in mice. During observation, animal did not reveal any changes in behavioral pattern, Body weight or change in gross pathophysiology of any organs.

4. DISCUSSION

The prime objective of the study was to investigate the immunomodulatory effect of *Pueraria tuberosa D. C.* The findings outlined above have demonstrated that ethanolic extract of *Pueraria tuberosa* possesses potent immunomodulatory activity.

The cell-mediated immune response (CMI) involve activation and differntiation of mainly CD4+ T cells in effector and memory T cells and also Cytotoxic CD8+ which further involve other polymorphonuclear cells and macrophages. The Cell-mediated immune response was assessed by Delayed type of hypersensitivity (DTH) reaction. DTH is a part of the process of transplantation immunity, cancer immunology, defense against intracellular parasites and autoimmune inflammatory diseases[10]. DTH requires activation of sensitized Th1 cells by specific antigen results in the secretion of various cytokines which increase vascular permeability, increase vasodilation, recruitment of leukocytes and macrophages to the site of inflammation and activate them for phagocytic activity and more effective killing[11]. Ethanolic extract of *P.tuberosa* significantly inhibited the delayed type of hypersensitivity reaction suggesting decrease infiltration of immune cells to inflammatory site. Inhibition of DTH reaction indicate inhibitory effect of ethanolic extract of *P. tuberosa* on T lymphocytes proliferation and/or cytokines secretion and accessory cell types required for the expression of reaction.

Haemagglutination antibody titre was determined to establish the humoral response against antigen. Humoral immunity involves interaction of B lymphocytes with antigen which leads its proliferation and differentiation into plasma cells which secrete antigen specific antibodies. Antibodies secreted against toxic products of microorganisms neutralizes the toxin and facilitates its elimination, activate the complement system for cytolytic action and assist in phagocytosis by opsonization[10, 12]. Also, now a day growing scientific evidences proves that Pathogenic antibodies are known to participate in many Autoimmune diseases mechanism like Rhematoid Arthritis, Multiple sclerosis and Lupus and are good targeted approach to find new cure to autoimmune diseases. Ethanolic extract of *P. tuberosa* significantly inhibited the humoral immunity to SRBC as evidenced by decrease in antibody titre. Inhibition of humoral immune response by treatment of ethanolic extract of *P. tuberosa* may be due to on B lymphocytes proliferation, antibody synthesis or interaction of accessory cell types required for the antibodies production.

Phagocytosis plays an important defense mechanism against various foreign bodies including pathogenic microor-

ganisms and also in processing and presentation of antigens to B cell and T cells [6, 13]. A key challenge is to develop an agent that will limit inflammation without having a deleterious effect on innate immunity, as the inflammatory response is a prerequisite for an effective host defence response to infection[14]. Carbon clearance assay is used to assess the rate of removal of injected colloidal carbon particles by macrophages which correlates the reticuloendothelial cell phagocytic activity[15]. In present study, *P. tuberosa* was found to stimulate the phagocytic activity of macrophages as evidenced by increased phagocytic index. Increase in the phagocytic capability by macrophages was also confirmed by phagocytic rate (jurcic indices). Indices of 0 were considered to represent an absence of activity, values between 1.0 and 1.5 represent a good stimulating activity and values > 1.5 represent a very good stimulating activity[9]. *P. tuberosa* extract showed to stimulate the phagocytic rate at 100 and 200mg/kg which indicates that ethanolic extract of *P. tuberosa* has stimulatory effect on innate immune system.

Now a day, many synthetic immunosuppressive and chemotherapeutic agents are used for treatment of various Autoimmune diseases and cancers like cyclophosphamide, Dexamethasone which are known to have myelosuppressive and cytotoxic effects, resulting in leucopenia and cytopenia. However, such therapies require chronic use and non-specifically suppress the entire immune responses and bone marrow, exposing patients to considerably higher risks of infections[16, 17]. It is important to avoid untoward effects of immunosuppresive drugs in autoimmune diseases without compromising antiinflammatory activity[17]. Cyclophosphamide alone at single dose of 200 mg/kg significantly reduced both the total leucocyte count as well as %neutrophil count with compared to the control. Combined treatment of cyclophosphamide and ethanolic extract of *P. tuberosa* resulted in restoration of bone marrow activity and WBC count in blood as compared to cyclophosphamide alone. Hence *P. tuberosa* has prevented myelosuppressive activity of cyclophosphamide and it could be used as an adjuvant or supportive therapy to chemotherapeutic agents.

We conclude that; *Pueraria tuberosa* has immunomodulatory potential. The ethanolic extract of *P. tuberosa* was found to increase phagocytic capability of macrophages. The extract protected myelosuppressive effect of cyclophosphamide. The extract has inhibited both Cell mediated immunity and Humoral immunity. Hence, it can be concluded that, the plant extract has suppressive effect on adaptive immunity without affecting innate immune system and bone marrow cells proliferation. Further fractionation and purification of extract may yield potent immunomodulatory compounds.

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