Research Article

IN-VIVO IMMUNOMODULATORY POTENTIAL OF STANDARDIZED PUERARIA TUBEROSA EXTRACT AND ITS ISOFLAVONOIDS

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ABSTRACT

Objective: The present study was designed to investigate the immunomodulatory potential of *Pueraria tuberosa* tuber extract and isoflavones puerarin, daidzein and genistein in rats.

Methods: The immunomodulatory potential of standardized *P. tuberosa* tuber extract (100-200 mg/kg; p.o.) and isoflavones puerarin (25-50 mg/kg; p.o.), daidzein (25-50 mg/kg; p.o.) and genistein (25-50 mg/kg; p.o.) was evaluated in swiss albino rats. Immunomodulatory potential was evaluated against sheep red blood cells (SRBCs) antigenic challenge using the models include total and differential leukocyte cell counts, nitroblue-tetrazolium reduction test, haemagglutinin titre assay, neutrophil adhesion test, carbon clearance test and delayed type hypersensitivity reaction. The study was also constituted the active marker based RP-HPLC standardization of PTTE.

Results: RP-HPLC analysis revealed those *P. tuberosa* tubers extract contains (w/w) puerarin (8.31% ± 0.06), daidzein (1.70% ± 0.04) and genistein (1.37% ± 0.02). Oral administration of the *P. tuberosa* extract, puerarin and daidzein dose dependently increased total leukocyte cell count, monocyte and lymphocyte counts, nitroblue-tetrazolium reduction, haemagglutinin titre and phagocytic index whereas suppressed delayed type hypersensitivity response in their specified concentration range in SRBC immunized rats. The *P. tuberosa* extract was also enhanced the neutrophil adhesion, while puerarin and daidzein had no effect on the neutrophil adhesion. Genistein dose dependently suppressed all the above parameters. Results suggest that the *P. tuberosa* extract augmented some innate as well as humoral immune responses against SRBC challenged in rats.

Conclusion: *P. tuberosa* holds a promising therapeutic potential as an immunomodulator and the effects mostly due to the effects of isoflavones puerarin, daidzein and genistein.

Keywords: *P. tuberosa*, RP-HPLC, puerarin, daidzein, genistein, immunomodulatory activity

INTRODUCTION

The Indian traditional system of medicines, especially Ayurveda, plays a significant role in disease prevention and promotion of health towards longevity with better quality of life[1]. The concept of "rasayana" drugs of Ayurveda complemented by various research findings suggested that the "rasayana" drugs may influence the host immunity and resistance against infections[2]. Many medicinal plants enlisted as "Rasayana" drugs in Ayurveda are believed to improve defense mechanisms of the body, promote physical and mental health and enhance longevity[3]. Indian medicinal plants are a potential source of bioactive compounds which are reported to potentiate immune responses by modulating the functions of lymphocytes, granulocytes, macrophages, natural killer cells, complements etc[4]. There is a growing concern in herbal drug research about the actions of bioactive constituents on immunomodulation. Since the active components cannot always be reliably quantified in a complex plant extract, the interpretation of assay data must be critically addressed using multiple strategies. Therefore, the standardization of a plant extract is essential to maintain the quality as well as to get the most favourable concentrations of known active constituents during biological and toxicological studies[5].

Pueraria tuberosa DC. Family: Fabaceae, is a perennial herb, commonly known as "Vidarikand" distributed throughout the tropical parts of India[6]. It is a reputed herb in "Rasayana" drugs in Ayurveda for its aphrodisiac, longevity and rejuvenation properties. In Ayurveda and folk medicines, plant tubers are used as restorative tonic, antiaging, energizer, vital energy booster, galactagogue, spermatogenic and immune booster[2]. Traditionally, the tubers are also used against sexual debility, pain, inflammations, burning sensations and skin problems[2]. Phytochemical investigations reported that plant's tubers contain puerarin, daidzein, genistein, genistein, puerarone, coumarin, anthocyanin, lupinoside, tuberosin, pterocarpintuberosin, puetuberosanol and hydroxytuberosone[7,8]. Among the phytochemicals listed above, the isoflavones puerarin, daidzein and genistein are most important in term of their immunomodulatory functions[8]. Recently, *in-vivo* antibody response and *in-vitro* phagocytic effect of the *P. tuberosa* tuber extract (PTTE) has been evaluated[8]. However, the effects of PTTE and its active isoflavones (puerarin, daidzein and genistein) on haematopoietic system and on the functions of T cells and neutrophils have not been studied extensively which can correlate the immunomodulatory functions of PTTE. Based on the above context, the present study was designed to explore the immunomodulatory potential of standardized PTTE and its three isoflavones, puerarin, daidzein and genistein in rats.

MATERIALS AND METHODS

Chemicals and Reagents

Reference standards (purity ≥ 98%), puerarin (PR), daidzein (DZ) and genistein (GS) were purchased from Sigma-aldrich, St. Louis, MO, USA. The standard drug, levamisole (LEV) was purchased from local medical store. The nitroblue tetrazolium (NBT) was purchased from HiMedia laboratories Pvt. Ltd., Mumbai. All other analytical grade solvents were purchased from Merck Ltd, Mumbai, India.

Collection and extraction of plant material

Tubers of *P. tuberosa* were procured from local vendor and the sample was authenticated from the Department of Botany and Forestry, Vidyasagar University, India. A voucher specimen (VU/BOT/DB/17/12) has been deposited in the Department of Botany and Forestry, Vidyasagar University, India, for future reference.

The air dried (20-25°C) plant material (500 g) was powdered in a grinder and extracted with ethanol (70%) by cold maceration (25°C ± 2) process for 15 days. The extract was filtered and dried using rotary evaporation followed by lyophilization, yielding 11.52% (w/w) crude extract. The extract was then analyzed to determine the contents of PR, DZ and GS using RP-HPLC technique.

Instrumentation and HPLC standardization

The hydro-ethanolic PTTE was standardized against PR, DZ and GS as reference standards using a RP-HPLC system (Waters

Corporation, Milford, MA, USA) consisted of Luna C18 (2) 100 Å, 250 × 4.6 mm (Phenomenex, Torrance, CA, USA) column, a 600 controller pump, a multiple-wavelength ultraviolet-visible detector, an in-line AF 2489 series degasser, a rheodyne 7725i injector with a 20 μl sample loop. The HPLC analysis was performed according to the method described by Vaishnav et al[9]. Stock solutions of reference standards, PR (1 mg/ml), DZ (1 mg/ml) and GS (1 mg/ml) were prepared in methanol. The appropriate amount of each standard was mixed separately to prepare working standard solutions containing six different concentrations (100, 200, 400, 600, 800 and 1000 μg/ml) of each standard. The aliquot was analyzed to construct calibration curves by plotting peak areas against concentrations injected by means of linear regression. The quantification of PR, DZ and GS in the extract was determined from the corresponding calibration curves.

Experimental animal

Swiss albino rats (70-80 g) of both sexes were used in these experiments. Animals were housed under standard conditions of temperature (25°C ± 2), relative humidity (55.6% ± 10) and 12 h light/dark cycle with standard food and water ad libitum. The experimental protocols were approved by the Institutional Animal Ethics Committee of Vidyasagar University, Midnapore, India.

Selection of dose

Animals were randomly distributed in ten different groups and each group consisted of six animals (n = 6). Effective experimental doses were selected on the basis of previous studies i.e. PTTE 100 and 200 mg/kg[10]; PR 25 and 50 mg/kg; DZ 25 and 50 mg/kg[11] and GS 25 and 50 mg/kg[12]. Animals were grouped and orally treated in the fashion as: Group I: control, normal saline; Group II: PTTE, 100 mg/kg; Group III: PTTE, 200 mg/kg; Group IV: PR, 25 mg/kg; Group V: PR, 50 mg/kg; Group VI: DZ, 25 mg/kg; Group VII: DZ, 50 mg/kg; Group VIII: GS, 25 mg/kg; Group IX: GS, 50 mg/kg; and Group X: LEV, 50 mg/kg.

Antigen (immunization)

Fresh sheep red blood cells (SRBCs) collected from the local slaughterhouse were used to immunize the animals. SRBCs were stored in a sterile Alsever's solution and washed thrice with pyrogen free normal saline and cell numbers were adjusted to 0.5×10^9 cells/ml. Animals were immunized by intraperitoneal injection of 1 ml of 20% SRBCs suspension. The day of immunization was considered as day '0'. On the 5th day, blood samples were collected from each animal through retro-orbital puncture to perform total and differential leukocyte counts and NBT reduction test.

Total and differential leukocyte cell (TLC and DLC) counts

Collected blood samples were diluted with Turk's fluid using WBC pipette to lyses the red blood cells. The diluted blood samples were subjected for total leukocyte counts using a neubauer's chamber. In DLC count, smears of each blood sample were made on glass slides and stained with Leishman's stain. The slides were examined under oil immersion microscopy and the DLC counts were performed.

Nitroblue tetrazolium (NBT) reduction test

Smears of blood samples made on glass slides were treated with 0.4 ml of NBT medium and incubated at 37°C for 30 min. After washing with cold saline water, the slides were stained with safranin solution. The slides were examined under microscope and the percentages of cells with reduced NBT dye were determined[13].

Haemagglutinating antibody (HA) titre

On day 7, the blood samples were collected from each animal through the retro-orbital puncture. The blood samples were centrifuged to collect serum samples. Antibody titres were determined using the haemagglutination technique. Briefly, twofold serial dilutions of serum samples were made in 25 μl of normal saline in microtitration plates and then 25 μl of 1% SRBCs (0.025×10^9 cells) suspension made with normal saline was added. The plates were incubated (37°C) for 1 h and examined under the microscope for haemagglutination. Reciprocal of the highest dilution of test serum which gave positive haemagglutination reaction was considered as the antibody titre[3].

Delayed-type hypersensitivity (DTH) response

DTH response was performed according to the method described by Maji et al[5]. On day 7 of drug treatment, animals were challenged by the injection of 20 μl of 0.5×10^9 SRBCs suspension in the right hind foot pad. On day 14, second challenged was made with the same antigen in the left foot pad. Foot thickness was measured after 24, 48 and 72 hrs of second challenge. The difference between the pre and post challenge foot thickness (mm) was taken as a measure of DTH.

Neutrophil adhesion test

On 14th day of drug treatment, blood samples of each animal were collected by retro-orbital puncture and blood samples were analyzed for TLC and DLC counts. After initial counts, blood samples were incubated (37°C) with nylon fibers for 15 min. The incubated blood samples were again analyzed for TLC and DLC counts. The product of TLC and percent neutrophil gives the neutrophil index of blood sample[5]. The percent neutrophil adhesion was calculated.

Phagocytic activity by carbon clearance test

The method was described by Maji et al[5]. On the 15th day of drug treatment, all animals were received an intravenous injection (0.1 ml) of carbon suspension (Indian ink, Camel) through the tail vein. Blood samples (50 μl) were collected from each animal from the tip of the tail vein at an interval of 2 and 10 min after the injection of carbon suspension. Blood samples were added to 2 ml of 1% acetic acid solution to lyse the erythrocytes. Absorbencies of the samples were measured at 675 nm using a spectrophotometer. The rate of carbon clearance (phagocytes index, K) was calculated.

Statistical analysis

All data are presented as mean ± SEM. The statistical analysis of the experimental results was performed by one-way ANOVA followed by Dunnett's test or Bonferroni's multiple comparison tests wherever applicable using Graph Pad Prism 5.0 statistical software.

RESULTS

HPLC standardization

The HPLC standardization of PTTE was performed prior to the evaluation of its immunomodulatory potential. The HPLC chromatogram of PTTE (Fig. 1) showed sharp peaks for PR, DZ and GS at retention time of 13.08 ± 0.12, 20.46 ± 0.16 and 23.16 ± 0.28 min, respectively. From the calibration curves, the contents (w/w, n = 6) of PR, DZ and GS in hydro-ethanolic extract were found to be 8.31% ± 0.06, 1.70% ± 0.04 and 1.37% ± 0.02, respectively.

TLC and DLC count

The results of TLC and DLC counts are summarized in Table 1. Administration of hydro-ethanolic PTTE (100 - 200 mg/kg), PR (25 - 50 mg/kg) and DZ (25 - 50 mg/kg) dose dependently increased TLC count, while GS (25 - 50 mg/kg) decreased the TLC count in rats when compared with the control group. In DLC count, DZ and GS suppressed the neutrophil population, while PTTE and PR had no effects on the neutrophil population. On the other hand, PTTE, PR and DZ increased the populations of monocyte and lymphocyte while GS suppressed the monocyte population without affecting the lymphocyte population. The standard drug, LEV (50 mg/kg) was significantly (P < 0.001) increased the neutrophil population with a decreased pattern of eosinophil, basophil, monocyte and lymphocyte population.
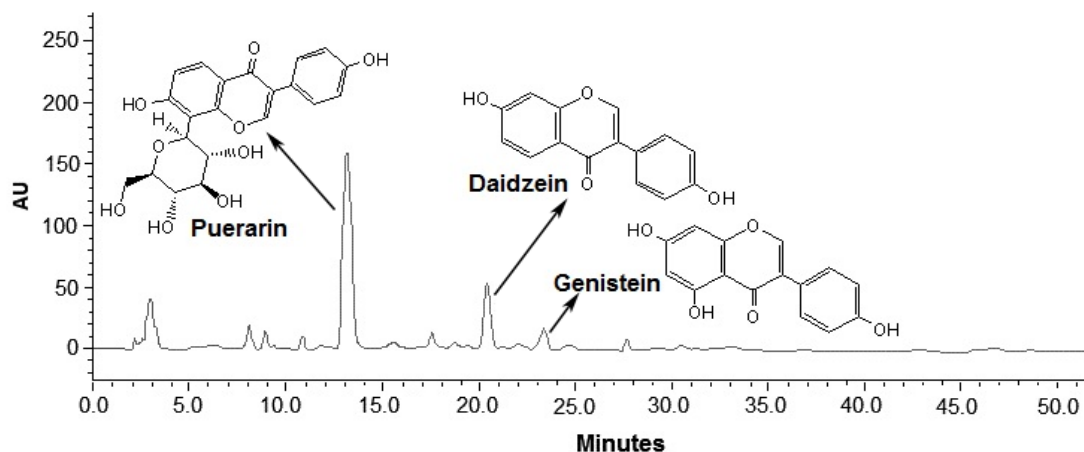
Fig. 1: RP-HPLC chromatogram of hydro-ethanolic *P. tuberosa* extract

Table 1: Total and differential leukocyte counts in PTTE, PR, DZ and GS treated rats (n = 6).

Treatment (mg/kg)	TLC count (10^3/mm³)	DLC count (%)					Phagocytic index (K)
		Neutrophil	Eosinophil	Basophil	Monocyte	Lymphocyte	
Control	6.89 ± 0.16	39.01 ± 1.67	2.02 ± 0.09	1.47 ± 0.05	1.19 ± 0.06	54.15 ± 1.14	0.019 ± 0.001
PTTE 100	7.37 ± 0.13	38.87 ± 1.42**	1.27 ± 0.07***	1.21 ± 0.08*	1.56 ± 0.04**	57.55 ± 1.54	0.026 ± 0.002
PTTE 200	7.85 ± 0.12**	38.66 ± 1.29***	0.89 ± 0.05***	1.05 ± 0.09***	2.16 ± 0.08***	59.94 ± 1.29*	0.034 ± 0.003***
PR 25	7.30 ± 0.15	38.30 ± 1.38	0.80 ± 0.04***	0.90 ± 0.07***	1.11 ± 0.05	58.40 ± 1.20	0.032 ± 0.001***
PR 50	7.61 ± 0.14*	38.74 ± 1.30	0.62 ± 0.09***	0.75 ± 0.06***	1.20 ± 0.07	60.86 ± 1.35*	0.041 ± 0.002***
DZ 25	7.56 ± 0.17	35.91 ± 1.33	2.12 ± 0.07	1.12 ± 0.09**	1.87 ± 0.06***	59.15 ± 1.46	0.030 ± 0.002**
DZ 50	8.27 ± 0.19***	32.72 ± 1.46*	2.18 ± 0.04	0.83 ± 0.03***	2.34 ± 0.05***	62.39 ± 1.54**	0.037 ± 0.003***
GS 25	6.66 ± 0.15	34.85 ± 1.23	1.47 ± 0.07***	0.98 ± 0.05***	1.10 ± 0.09	54.58 ± 1.87	0.015 ± 0.002
GS 50	5.68 ± 0.20***	31.70 ± 1.53**	1.18 ± 0.05***	0.71 ± 0.02***	0.72 ± 0.04***	54.77 ± 1.51	0.011 ± 0.001
LEV 50	11.10 ± 0.24***	55.10 ± 1.39***	0.35 ± 0.08***	0.32 ± 0.04***	0.62 ± 0.07***	45.13 ± 1.31***	0.081 ± 0.003***

Values are tabulated as mean ± SEM; one-way analysis of variance (ANOVA) followed Dunnett's t-test, values are compared with control animals, ***P < 0.001; **P < 0.01 and *P < 0.05.

Nitroblue tetrazolium (NBT) reduction test

The dose dependent effects of PTTE (100 - 200 mg/kg), PR (25 - 50 mg/kg) and DZ (25 - 50 mg/kg) on the intracellular reduction of NBT dye were illustrated in Fig. 2. In comparison to the control group, the NBT dye reduction was increased in PTTE, PR and DZ treated groups as like standard drug LEV (50 mg/kg;) treated group (P < 0.001), whereas NBT reduction was decreased in GS treated group.
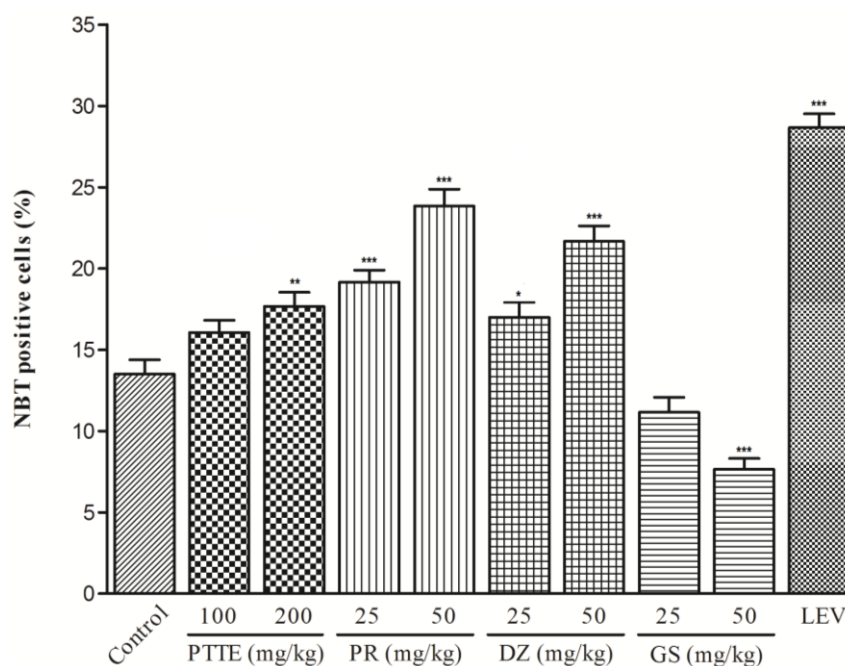

Fig. 2: NBT reduction in PTTE, PR, DZ and GS treated rats (n = 6). The bars of the graph represent mean ± SEM of six observations. Statistical analysis was done through One-way ANOVA followed by Dunnett's multiple comparison tests. Statistically significant differences compared as: Control Vs PTTE, PR, DZ, GS and LEV treated groups (*P < 0.001; **P < 0.01 and *P < 0.001)**

Haemagglutinating antibody (HA) titre

HA titre was determined to assess the effect of PTTE, PR, DZ and GS on humoral immune response against the immunization with SRBCs. In response to SRBCs, the serum HA titre was markedly augmented in PTTE (100 - 200 mg/kg), PR (25 - 50 mg/kg), DZ (25 - 50 mg/kg) and LEV (50 mg/kg) treated groups in comparison to control group (Fig. 3). The HA titre was dose dependently suppressed by GS (25 - 50 mg/kg) but the results were non-significant.
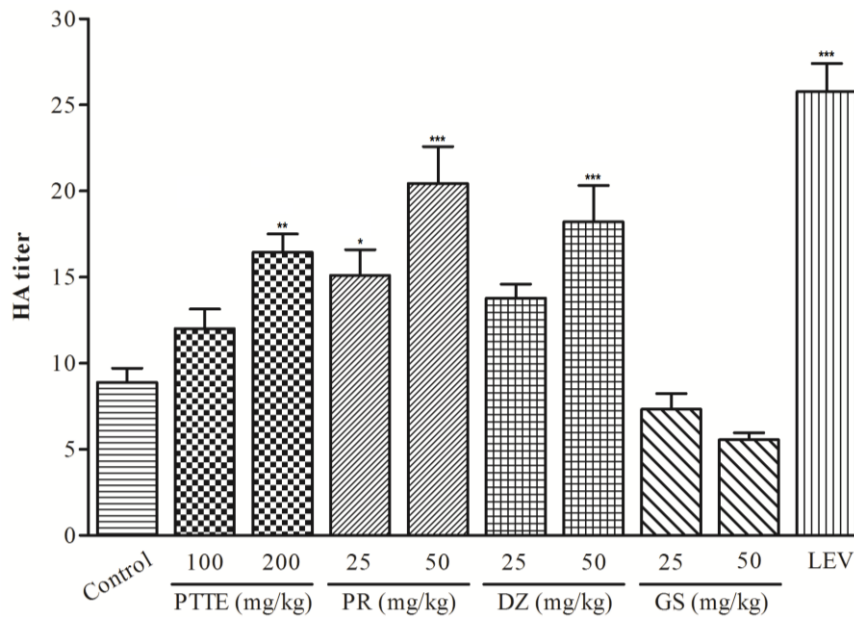

Fig. 3: HA titres against SRBCs-challenged in PTTE, PR, DZ and GS treated rats (n = 6). HA titre was dose dependently increased in PTTE, PR and DZ treated groups and decreased in GS treated group when compared to the control group. The bars of the graph represent mean ± SEM of six observations. Statistical analysis was done through one-way ANOVA followed Dunnett's t-tests (*P < 0.001; **P < 0.01 and *P < 0.05)**

Delayed-type hypersensitivity (DTH) response

The cell-mediated immune response of PTTE, PR, DZ and GS was assessed by DTH reaction, i.e. foot pad reaction. A dose dependent suppression of SRBC-induced DTH response at 24, 48 and 72 hrs was observed in PTTE, PR, DZ and GS treated groups. A stimulatory effect on DTH reaction was observed in LEV (50 mg/kg) treated group (Fig. 4).
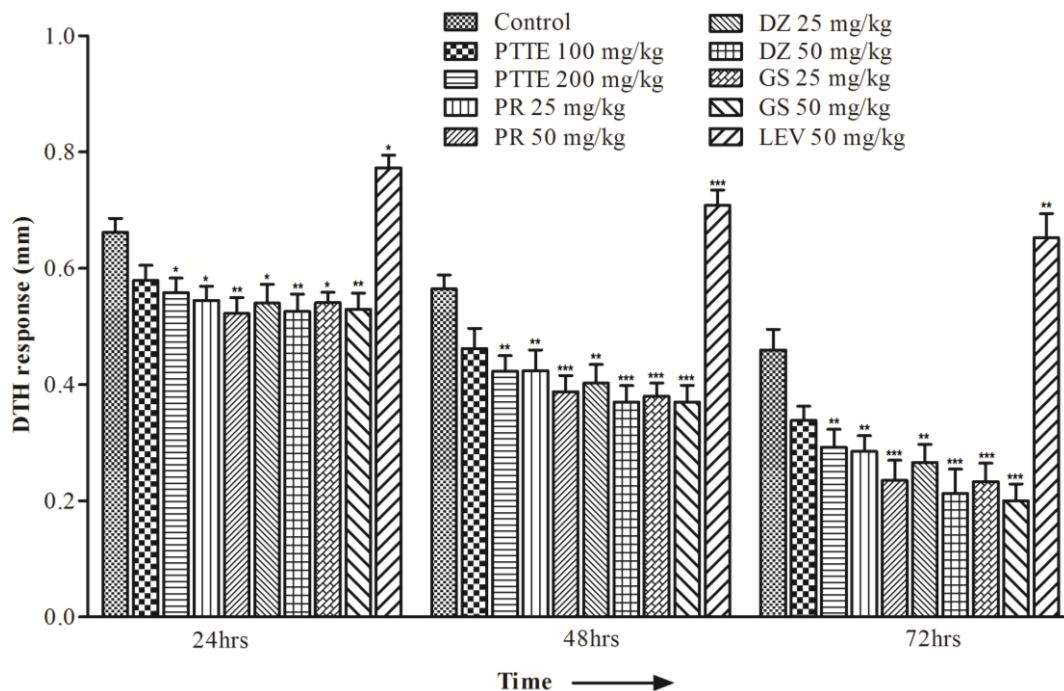

Fig. 4: DTH response in PTTE, PR, DZ and GS treated rats (n = 6). DTH response was dose dependently suppressed by PTTE, PR, DZ and GS in compared with the control group. The bars of the graph represent mean ± SEM of six observations. Statistical analysis was done through one-way ANOVA followed Dunnett's t-tests (*P < 0.001; **P < 0.01 and *P < 0.05)**

Neutrophil adhesion test

Neutrophil adhesion to nylon fibers was enhanced by PTTE (100 - 200 mg/kg) and LEV (50 mg/kg), while the adhesion of neutrophils was suppressed by GS (25 - 50 mg/kg) in compared to the control group. The effects of PR (25 - 50 mg/kg) and DZ (25 - 50 mg/kg) on neutrophil adhesion were ineffective at specified concentrations (Table 2).

Table 2: Effect of PTTE, PR, DZ and GS on neutrophils adhesion (n = 6)

Treatment (mg/kg)	TLC count (10^3/mm^3) [A]		Neutrophils, % [B]		Neutrophil index [A × B]		Neutrophil adhesion (%)
	UB	FTB	UB	FTB	UB	FTB	
Control	6.73 ± 0.22	6.31 ± 0.26	38.40 ± 1.10	36.55 ± 1.25	259.19 ± 14.93	231.72 ± 15.84	10.88 ± 1.10
PTTE 100	7.42 ± 0.14	6.94 ± 0.23	38.23 ± 1.15	32.73 ± 1.24	284.47 ± 13.78	228.28 ± 15.56	20.14 ± 1.83***
PTTE 200	7.93 ± 0.15**	7.38 ± 0.18**	38.34 ± 1.25	31.68 ± 1.45	304.99 ± 15.80	234.57 ± 15.24	23.34 ± 1.32***
PR 25	7.32 ± 0.18	6.75 ± 0.20	38.17 ± 1.15	37.01 ± 1.26	280.38 ± 15.40	250.62 ± 15.11	10.73 ± 1.16
PR 50	7.71 ± 0.20*	7.05 ± 0.21	38.37 ± 1.38	37.47 ± 1.57	296.79 ± 17.57	264.82 ± 16.53	10.82 ± 1.23
DZ 25	7.63 ± 0.29*	7.04 ± 0.26	34.87 ± 1.25	33.64 ± 1.47	267.50 ± 19.23	237.98 ± 17.63	11.06 ± 1.77
DZ 50	8.34 ± 0.27***	7.60 ± 0.23	32.28 ± 1.32*	31.52 ± 1.31	270.59 ± 19.10	240.75 ± 16.51	10.95 ± 1.49
GS 25	6.48 ± 0.22	6.15 ± 0.15	34.04 ± 1.43	32.81 ± 1.68	221.60 ± 15.29	202.24 ± 13.07	8.55 ± 1.69
GS 50	5.58 ± 0.21**	5.31 ± 0.10**	30.25 ± 1.21***	30.14 ± 1.54*	170.02 ± 12.95**	160.42 ± 10.54*	5.25 ± 1.30*
LEV 50	11.23 ± 0.25***	10.06 ± 0.16***	50.31 ± 2.01***	37.69 ± 1.58	566.29 ± 32.11***	380.07 ± 21.34***	32.83 ± 1.36***

Values are tabulated as mean ± SEM; one-way analysis of variance (ANOVA) followed Dunnett's t-test, values are compared with control animals, ***P < 0.001; **P < 0.01; *P < 0.05; UB = untreated blood and FTB = fibre treated blood.

Phagocytic activity by carbon clearance test

The effects of PTTE, PR, DZ and GS on the clearance of carbon particles, expressed as phagocytic index (K) are shown in Table 1. In comparison to the control group, PTTE, PR and DZ augmented the rate of carbon clearance at their specified concentration range in rats. The phagocytic index was also increased by LEV (P > 0.001). GS showed a dose dependent suppressive effect on the clearance of carbon particles from the circulatory system of rats.

DISCUSSION

Modulation of the immune functions either by stimulation or suppression may help to maintain a disease-free state[3]. Medicinal plants used in traditional medicines are demonstrated to modulate either cellular or humoral or both arms of the immune responses of the body[14]. Plants contain several chemical constituents, of which some constituents may have immunosuppressive activity, whereas others possess immunostimulatory action[15]. The biological activity of a plant extract is greatly dependent on its chemical nature, composition and structure of the major active metabolites[16]. Therefore, the quantitative determination of specific known bioactive compounds in herbal medicines is essential for quality control and dose determination in the toxicological and biological activity studies. Therefore, PTTE was standardized to quantify the major active constituents present, prior to evaluation of its immunomodulatory potential. The isoflavones, PR, DZ and GS are the most important bioactive constituents of *P. tuberosa* tubers used as chemical markers for standardization. Results of HPLC analysis revealed the abundant presence (%, w/w; n = 3) of PR (8.31 ± 0.06), DZ (1.70 ± 0.04) and GS (1.37 ± 0.02) in hydro-ethanolic PTTE.

The leukocytes including neutrophils, lymphocytes, monocytes, eosinophils and basophils are involved in the development of an immune response. Of these cells, lymphocytes are the hallmark of an immune response because of its diversity, specificity, memory and self/non-self recognition characteristics. All the other cells play accessory roles like activation lymphocytes, stimulation of antigen clearance by phagocytosis, secretion of various immune effector molecules[15]. The haematopoietic action of a plant extract is demonstrated to be a direct action on the hematopoietic stem cells[17]. In TLC and DLC counts, PTTE, PR and DZ showed a dose dependent increase in TLC count and populations of monocyte and lymphocyte in rats (Table 1). However, PTTE and PR had no effect on neutrophil population, while DZ and GS decreased neutrophil population. The possible immunostimulatory effect of PTTE was evidenced by an increase in the TLC count with the increase in the population of monocytes and lymphocytes. These effects can be explained by the effects of PR and DZ. Interestingly, the suppressive effect of DZ and GS on neutrophil population was not reflected in effect of PTTE which indicated that some other molecules may be present in PTTE which may balance the suppressive effects of DZ and GS on neutrophil population, either by competitive inhibition or by transient chemical interaction.

The NBT reduction test gives the information about the phagocytic and intracellular killing potential of phagocytes. In this test, the NBT dye is readily ingested by phagocytes and consequently reduced by intracellular superoxide anion radicals (O2−.) to form formazone crystals. Higher amount of NBT reduction represented higher activity of oxidase enzyme reflecting the stimulation of phagocytes in proportion to intracellular killing[16]. The stimulation of the intracellular killing potential of phagocytes by PTTE and its isoflavones, PR and DZ was evidenced by an increase in the intracellular reduction of the NBT dye (formazone crystal) (Fig. 2). GS exhibited dose dependent suppressive effect on NBT reduction. The resultant effect of PTTE in intracellular killing activity of phagocytes may be predominantly attributed to the presence of PR and DZ.

Antibody molecules, produced from B lymphocytes and plasma cells play a central role in humoral immune response. In humoral immunity, IgG and IgM are the major immunoglobulins involved in the complement activation, opsonization, neutralization of toxins, etc[3]. Previous study reported that, PTTE enhanced the serum IgG and intestinal IgA levels[8]. The enhancement of humoral response to antigenic stimuli upon the drug treatment is evidenced by the increase in serum HA titre. However, the correlation between the effects of PTTE and its isoflavones on humoral response has not been evaluated previously. In this study, the serum HA titres were markedly augmented by PTTE, PR and DZ, while the titres were decreased by GS in response to SRBCs challenge in rats (Fig. 3). Thus, the results of this study re-established the stimulatory effect of PTTE on humoral immune response and this resultant effect may be due to the stimulatory effect of isoflavones PR and DZ. Thus, the elevation of serum HA titres indicated the stimulation of the responsiveness of macrophages and lymphocytes which are responsible for the antibody synthesis.

In DTH test, the PTTE and its isoflavones PR, DZ and GS produced dose dependent reduction in DTH responses i.e. foot paw edema in rats (Fig. 4). The inhibitory effects of PTTE and isoflavones on DTH response were demonstrated to be more significant with the increase of doses. The DTH test is performed to assess the antibody independent cell mediated immune (CMI) responsiveness where macrophages, T helper 1 (Th1) and cytotoxic T (Tc) cells play important roles[17]. In CMI response, T lymphocytes are activated and differentiated into effector T cells including Tc cells, Th1 and

Th2 subsets which have subsequently released various cytokines of which Th1 cytokines (e.g. IL-2, IFN-γ, TNF-β etc.) activate macrophages, Tc cells, NK cells and mediate DTH responses while Th2 cytokines (e.g. IL-2, IL-4, IL-10 etc.) stimulate the activation and proliferation of B cells[18]. These in turn increase vascular permeability, induce vasodilation, macrophage accumulation and activation to promote phagocytic activity *vis-a-vis* increase the concentration of lytic enzymes for more effective killing[19]. Thus, the reduction of DTH response by PTTE evidenced the suppressive effect on T cells and it may as well possess anti-inflammatory activity as already reported[20]. Taken the effects of isoflavones (PR, DZ and GS) together, the suppressive effect of PTTE on DTH response is mainly because of the influences of the isoflavones, each in different aspects of the immunomodulatory system in rats.

Neutrophil is one of the most important effector cells of the immune system involved in chemotaxis, phagocytosis, exocytosis and both intracellular and extracellular killing of invading pathogens[16]. At the onset of an inflammation, neutrophils rapidly migrate and recruit at the site of inflammation and firmly adhere to the vascular endothelial surface through the interactions with β-integrin molecules present on the neutrophils[13, 5]. Results suggest that oral administration of PR and DZ had no effect on the adhesion of neutrophils, while GS dose dependently suppressed adhesion of neutrophils to the nylon fibers (Table 2). Since, PTTE increased the adhesion of neutrophils and this effect may be due to the up-regulation of the expression of β-integrin molecules. This stimulatory effect of PTTE on integrin-mediated adhesion of neutrophils is not correlated with the effects of the isoflavones (PR, DZ and GS) and this effect may be mediated by other constituents present in the PTTE.

Phagocytosis provides the body's first-line defense mechanism by eliminating microorganisms, inhaled carbon particles, malignant cells, tissue debris and other non-biological foreign materials[21,22]. The process mainly involves specialized immune cells including neutrophils, macrophages and dendritic cells collectively called phagocytes[23]. The phagocytic activity of the reticuloendothelial system (RES) has been correlated with the rate of the removal of carbon particles[3]. An increase in the rate of carbon clearance indicated the augmentation of the phagocytic activity and competency of the granulopoetic system in the removal of foreign particles[24]. The result of the study showed that PTTE (100 - 200 mg/kg) dose dependently increased the rate of clearance of carbon particles from the blood circulation of rats, expressed as phagocytic index (K) (Table 1). PTTE has a stimulatory action on the cells of mononuclear phagocytic system and this is mainly because of the presence of PR and DZ which may synergistically overcome the suppressive effect of GS on phagocytic activity.

CONCLUSION

The present study, suggests that PTTE potentiate some innate immune functions as well as humoral immunity in albino rats. Thus, the extract augmented the host defense mechanisms by influencing some immune cells' functions and therefore it holds a promising therapeutic potential as to modulate the host immune responses of humans. In conclusion, the results of the present study preliminarily validate the immunomodulatory function of *P. tuberosa* in animals however clinical studies would mandate the validation of its utilization in the herbal formulations for humans. Specific observations lead to the conclusion that PR and DZ increase the TLC, monocytes and lymphocyte populations, HA titre, phagocytic index, NBT reduction and suppress the DTH response, while GS possess suppressive effect on all the above parameters including neutrophil adhesion. The net effect of PTTE is therefore immunostimulatory, while it stimulates TLC, monocytes and lymphocyte populations, HA titre, phagocytic index, NBT reduction, neutrophil adhesion and also suppresses DTH response, the overall impact dose dependently dose dependently stimulates the immune system. However, further studies need to be designed and conducted with PR, DZ and GS in various combinations taken two at a time and all three combined as against PTTE with control groups to determine the specific mechanism by which the GS's immunosuppressive action is

overcome. Additionally as discussed, the stimulatory effect of PTTE on neutrophil adhesion was not evidenced to be caused by any of the known isoflavones (PR, DZ AND GS), there is obviously some other compounds present in PTTE which has caused this activity and therefore is an important constituent that requires to be identified, isolated and studied in the same context to fully understand the mechanism of the complex immunomodulatory action of PTTE.

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