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Antioxidant and Immunosuppressant Activity of *Pueraria tuberosa*

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ABSTRACT

Pueraria tuberosa has been traditionally used in the treatment of various immune-related disorders but, no scientific data has been published supporting the claimed ethnomedical uses. The study was designed to investigate the antioxidant and immunosuppressant activities of *Pueraria tuberosa*. The ethanolic extract of *Pueraria tuberose* possess strong antioxidant activity which was revealed by its prominent reducing power and ability to scavenge the stable free radical H_2O_2. The extract also exhibited significant dose-dependent immunosuppressant activity evident by decrease in antibody titer method and also reduction in hematological parameters like total WBC, RBC and Hb% in drug-induced myelosuppression model. The presence of flavonoids and steroids in the extract might be responsible for the marked antioxidant and immunosuppressant activity of the plant.

INTRODUCTION

Immunomodulation using medicinal plants, especially 'Rasayana' drugs, can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders. This concept of using rasayanas for health, gained little more credibility when it was realized that herbal antioxidants concurrently exhibit significant immunomodulatory activities, like Shilajit and Chyavanprash Awaleha[1]. Further, Indian medicinal plants are a rich source of substances which are claimed to induce innate immunity, the non-specific immunomodulation of essential granulocytes, macrophages, natural killer cells and complement fractions[2].

About 34 plants are identified as rasayanas in Indian Ayurvedic System of medicine having various pharmacological properties such as immunostimulant, tonic, neurostimulator, antiaging, antibacterial, antiviral, antirheumatic, anticancer, adaptogenic, antistress, antioxidant etc. Many plants with potential immunomodulatory and antioxidant activities are reported, some of these have already been undertaken for evaluation of their activities in animals, and also to some extent in humans. Some glaring examples with promising activity are *Asparagus racemosus*, *Azadirachta indica*, *Curcuma longa*, *Ocimum sanctum*, *Panax ginseng*, *Picrorhiza kurroa*, *Tinospora cordifolia*, *Withania somnifera* etc. A lot more are still to be explored and offer scope for further investigation[3].

Pueraria tuberosa DC. Family: Fabaceae, is a perennial herb, commonly known as "vidarikand" distributed throughout the tropical parts of India[4]. 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[5]. Traditionally, the tubers are also used against sexual debility, pain, inflammations, burning sensations and skin problems[5]. Phytochemical investigations reported that plant tubers contain puerarin, daidzein, genistein, puerarone, coumarin, anthocyanin, lupinoside, tuberosin, pterocarpintuberosin, puetuberosanol, hydroxytuberosone and β-sitosterol[6,7]. Among the phytochemicals listed above, the isoflavones and sterols are most important in terms of their immunomodulatory functions[7]. Based on the above context, the present study was designed to explore the

antioxidant and immunomodulatory potential of ethanolic extract of *Pueraria tuberosa* in rats.

MATERIALS AND METHODS

Drugs and chemicals:

Chemicals used in the study were of analytical grade and were procured from SD Fine Chem. Limited, Mumbai, India. Azathioprine was purchased from Roxane Laboratories, Columbus, USA. Sheep red blood cells (SRBCs) were washed thrice with normal saline and adjusted to the required concentration for immunization and for challenge as an antigen.

Preparation of herbal extract:

250 gm of powdered tubers of *Pueraria tuberosa* was weighed and taken in a 500 mL round bottomed flask. To this 250 mL of ethanol was added then the round bottomed flask was attached to a condenser and distilled at temperature of 30ºC for 48 hours. The extract obtained was air dried in an evaporating dish till constant weight was obtained.

Phytochemical analysis:

The phytochemical screening of ethanolic extract of *Pueraria tuberose* was performed as per the standard procedure described in Khandelwal[8].

Animals:

Wistar albino rats (150-200g) were procured from Gentox Bioservices, Hyderabad and acclimatized to laboratory condition for 10 days after their arrival. They were housed in groups under standard light/dark cycle with food and water provided ad libitum. All the experimental procedures and protocols used in the study were approved by IAEC.

Antioxidant activity:

Reducing Power Assay:

This method is based on the principle of increase in the absorbance of the reaction mixtures. Increase in the absorbance indicates an increase in the antioxidant activity. 2.5 mL of potassium ferricyanide (1% w/v) and 2.5 mL of phosphate buffer pH 6.6 was added to 1 mL of test and standard compounds and incubated at 50°C for 30 min.2.5 mL of distilled water

and 0.5 mL of $FeCl_3$ solution (0.1% w/v) were added to 2.5 mL of above supernatant liquid. The absorbance of ferric ferrous complex was measured using phosphate buffer pH 6.6 as control at 700 nm using UV-Visible spectrophotometer and increase in absorbance was estimated[9].

The percent increase in reducing power was calculated using the following equation,

$$\text{Percentage increase in reducing power (\%)} = \frac{A_{test} - A_{blank}}{A_{blank}} \times 100$$

Where 'A_{test}' is absorbance of test solution; 'A_{blank}' is absorbance of blank.

H_2O_2 radical scavenging assay:

A solution of hydrogen peroxide (2mmol/L) was prepared in phosphate buffer (pH7.4). Test compounds (10–100 µg/M) were added to hydrogen peroxide solution (0.6 mL). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without hydrogen peroxide and compared with ascorbic acid, the reference compound[10].

$$\% \ H_2O_2 \text{ activity} = \frac{(\text{Abs control}) - (\text{Abs sample})}{(\text{Abs control})} \times 100$$

Where, Abs (control): Absorbance of the control

Abs (sample): Absorbance of the extract/standard.

Methods for immunosuppressant activity:

Antibody (HA) titer response to SRBC:

Antigen Challenge: On 0^{th} day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, *i.p.*

Experimental design:

Animals were divided into different groups each containing 6 animals.

Group I: Normal control, received normal saline.
Group II: received 250 mg/kg bd. wt. of EEPT *p.o.* (1^{st} to 7^{th}day).
Group III: received 500 mg/kg bd. wt. of EEPT *p.o.* (1^{st} to 7^{th}day)

Group IV: received 2 mg/kg bd.wt. of standard, Azathioprine, *i.p.* (1^{st} to 7^{th}day)

Method:

On 7^{th} day before challenge, blood was withdrawn from retro-orbital plexus of each animal. Blood was centrifuged, and serum was separated. Serial two-fold dilutions were made i.e. 50 µL of serum was added to 1^{st} well of 96-well microtitre plate containing 50 µL normal saline. To this 1% SRBC (50 µL) dissolved in normal saline was mixed. From 1^{st} well 50 µL of diluted serum was added to 2^{nd} well containing 50 µL normal saline and 50 µL 1% SRBC. Such dilutions were done till 24^{th} well. Plates were incubated at 37ºC for 1h. Highest dilution that has shown visible agglutination was considered as haemagglutination antibody titre[11].

Drug-induced myelosuppression:

Antigen Challenge: On 0^{th} day, all groups were sensitized with 0.1 mL of SRBC containing 1×10^8 cells, *i.p.*

Experimental design:

Animals were divided into different groups each containing 6 animals.

Group I: Normal control, received normal saline
Group II: Disease control, received only antigen.
Group III: received 250 mg/kg bd. wt. of EEPT *p.o.* (1^{st} to 13^{th}day)
Group IV: received 500 mg/kg bd. wt. of EEPT *p.o.* (1^{st} to 13^{th}day)
Group V: received 2 mg/kg bd. wt. of standard, Azathioprine, *i.p.* (11^{th}, 12^{th} and 13^{th} day)

Method:

On 0^{th} day, blood was withdrawn from retro-orbital plexus of animals of each group and subjected to hematological parameter determination. Drugs were fed as per the schedule from 1^{st} to 13^{th}day. Azathioprine (2 mg/kg, bd. wt.) is given to all animals on 11^{th}, 12^{th} and 13^{th}day, 1 h after extracts administration except control and standard group. On 14^{th} day, blood was again withdrawn from retroorbital plexus of animals of each group and subjected to hematological parameters determination and restoration of parameters was observed[12].

Statistical analysis:

The data were expressed as mean ± SEM. The results were analyzed statistically by software (Graph pad prism 3) using ANOVA followed by Dunnet's 't' test. Significance was observed at *P<0.05, **P<0.01 & ***P<0.001.

RESULTS

Preliminary phytochemical investigation of ethanolic extract of *Pueraria tuberosa* revealed the presence of flavonoids, steroids, carbohydrates and proteins.

Antioxidant activity:

Reducing power assay:

Table No. 1 shows the reducing power of the ethanolic extract of *Pueraria tuberosa* as a function of its concentration. In this assay, the yellow color of the test solution changes to various shades of green and blue, depending on the reducing power of the extract. The reducing power of the extract increased with concentration.

The antioxidant activity of the ethanolic extract of *Pueraria tuberose* was carried out by using reducing power assay. EEPT had shown dose-dependent inhibition of free radicals and its IC_{50} value was found to be 41 µg/mL. The potential of the extract was comparable to that of standard ascorbic acid (IC_{50} = 36 µg/mL).

Hydrogen peroxide radical scavenging assay:

The antioxidant activity of the extract was further confirmed by hydrogen peroxide radical scavenging assay. The standard ascorbic acid showed prominent dose-dependent inhibition of hydrogen peroxide radicals and its IC_{50} value was found to be 32 µg/mL. Similarly, the test extract exhibited the dose-dependent inhibition of free radicals and its IC_{50} value (38 µg/ mL) was comparable to that of standard.

The IC_{50}values of standard ascorbic acid and the test extract were found to be 36, 32 respectively (Table No. 2).

Immunosuppressant activity:

Haemagglutination antibody titer:

The immunosuppressant activity of the extract was carried out by antibody titer method using microtitre plate. The standard azathioprine (2 mg/kg bd.wt.) showed significant reduction in antibody titre value (0.50 ± 0.42) compared to control (1.33 ± 0.21). The extract produced significant dose-dependent reduction of antibody titer. Antibody titer value of 500 mg/kg bd. wt. of ethanolic extract (0.70 ± 0.36) was better than that of 250 mg/kg bd. wt. (1.06 ± 0.60) and almost comparable to that of standard (azathioprine) (Figure No. 1).

Drug-induced myelosuppression:

The immunosuppressant activity of the ethanolic extract was also performed by drug-induced myelosuppression. Standard azathioprine at a dose of2 mg/kg bd. wt. significantly reduced RBC count (4.78 ± 0.39), total WBC count (6320 ± 0.45) and Hb% (12.8 ± 0.53) compared to control group. Ethanolic extract of *Pueraria tuberose* exhibited significant dose-dependent reduction in all the parameters mentioned above. 500 mg/kg bd. wt. of the extract showed better diminution of RBC count (4.91 ± 0.39), total WBC count (6820 ± 0.53) and Hb % (13.1 ± 0.73) than 250 mg/kg bd. wt. dose and its activity were comparable to that of standard azathioprine (Figure No. 2).

DISCUSSION

The immune system is composed of many interdependent cells collectively protect the body from bacterial, parasitic, fungal, viral infections and from the growth of tumor cells. Many of these cell types have specialized functions. The cells of the immune system can engulf bacteria, kill parasites or tumor cells, or kill viral-infected cells. Often, these cells depend on the T helper subset for activation signals in the form of secretions formally known as cytokines, lymphokines, or more specifically interleukins.

The immune system is involved in the etiology as well as pathophysiologic mechanisms of many diseases. Modulation of the immune response through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide a supportive therapy to conventional chemotherapy[13]. Immunostimulation in, a drug–induced immunosuppression

and immunosuppression in an experimental hyperreactivity model by the same preparation can be said to be true immunomodulation[14]. The presence of immunostimulant compounds in higher plants has been extensively reviewed but only a limited amount of immunosuppressive products of plant origin have been reported. Such products, if well tolerated by the patient, may be developed into alternative co-adjuvants in the treatment of disorders caused by an exaggerated or unwanted immune response, such as in autoimmune diseases, allergies, glomerulonephritis, chronic hepatitis, etc[15,16]. In the present investigation, the immunosuppressant activity of ethanolic extract of *Pueraria tuberose* was evaluated by haemagglutination antibody titer and drug-induced myelosuppression models.

Antioxidant activity was evaluated by two methods. The standard ascorbic acid showed significant dose-dependent increase in absorbance value. Similar pattern of activity was shown by EEPT. The IC_{50} of EEPT was found to be significant compared to that of standard ascorbic acid.

The haemagglutination antibody titer was used to assess humoral immune response. Azathioprine (2 mg/kg, bd.wt. *i.p*) showed significant inhibition in antibody titer response. Similarly, the EEPT showed significant dose-dependent suppression of humoral antibody immune response as evidenced by decreased antibody titer in albino rats challenged with SRBC. The activity of the 500 mg/kg bd. wt of the extract was comparable to that of standard azathioprine. This suppression of the humoral response to SRBC antigen by decrease in haemagglutination antibody titer indicated the reduced responsiveness of macrophages and T and B lymphocyte subsets involved in antibody synthesis.

The activity of the extract was also evaluated by drug-induced myelosuppression method to further confirm the results. Azathioprine at the dose of 2 mg/kg bd. wt., *i.p* caused a significant reduction in RBC count, total WBC count and Hb% as compared to control group. Ethanolic extract of the plant showed significant dose-dependent decrease in RBC count, total WBC counts and Hb% when compared with azathioprine group. Bone marrow is a site of continued proliferation and turnover of blood cells and is also a source of cells involved in immune activity. A high degree of cell proliferation renders bone marrow a sensitive agent, particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immunosuppression therapy with this class of drugs[17,18]. The extract might have shown immunosuppression by destroying stem cells and rendering the bone marrow unable to regenerate them.

CONCLUSION

The results clearly indicate that the tubers of *Pueraria tuberosa* possess immunosuppressant property and suggest usefulness in the disorder of immunological origin where the antioxidant system is adversely affected. Further study regarding the isolation of active chemical constituents responsible for immunomodulatory activity and its mode of action need to be determined.

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Tables:

Table No. 1: Antioxidant activity of *Pueraria tuberosa* by using reducing power assay:

Standard			
Concentration	**Absorbance**	**% inhibition**	**IC_{50} (µg/mL)**
10	0.049	23.36	36
20	0.243	36.12	
30	0.287	42.28	
40	0.373	56.32	
50	0.585	69.73	
Test			
Concentration	**Absorbance**	**% Inhibition**	**IC_{50} (µg/mL)**
10	0.040	18.36	41
20	0.171	29.36	
30	0.180	37.28	
40	0.189	49.32	
50	0.218	62.73	

Values are expressed as mean ± SEM

Table No. 2: Effect of *Pueraria tuberosa* on antioxidant activity by using hydrogen peroxide radical scavenging assay.

Standard			
Concentration	**Absorbance**	**% Inhibition**	**IC_{50} (µg/mL)**
10	0.065	22.39	32
20	0.166	31.13	
30	0.476	51.16	
40	0.675	65.22	
50	0.999	78.07	
Test			
Concentration	**Absorbance**	**% Inhibition**	**IC_{50} (µg/mL)**
10	0.050	20.15	38
20	0.114	27.30	
30	0.261	45.16	
40	0.189	56.22	
50	0.289	70.07	

Values are expressed as mean ± SEM

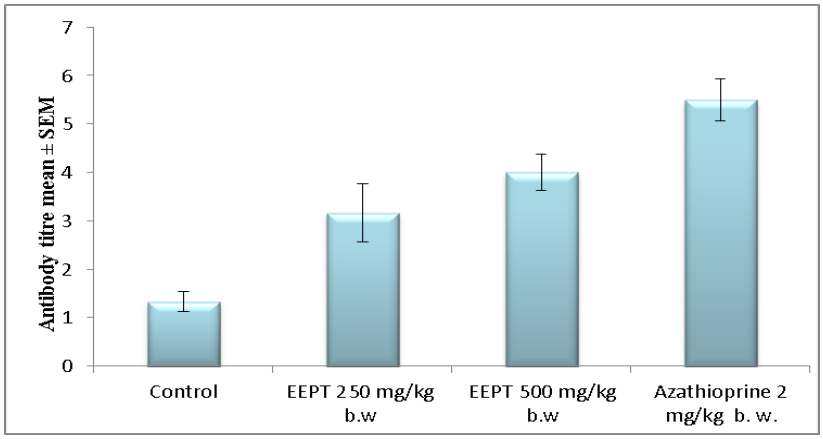

Figure No. 1: Immunomodulatory activity of ethanolic extract of *Pueraria tuberosa* by using antibody titer method

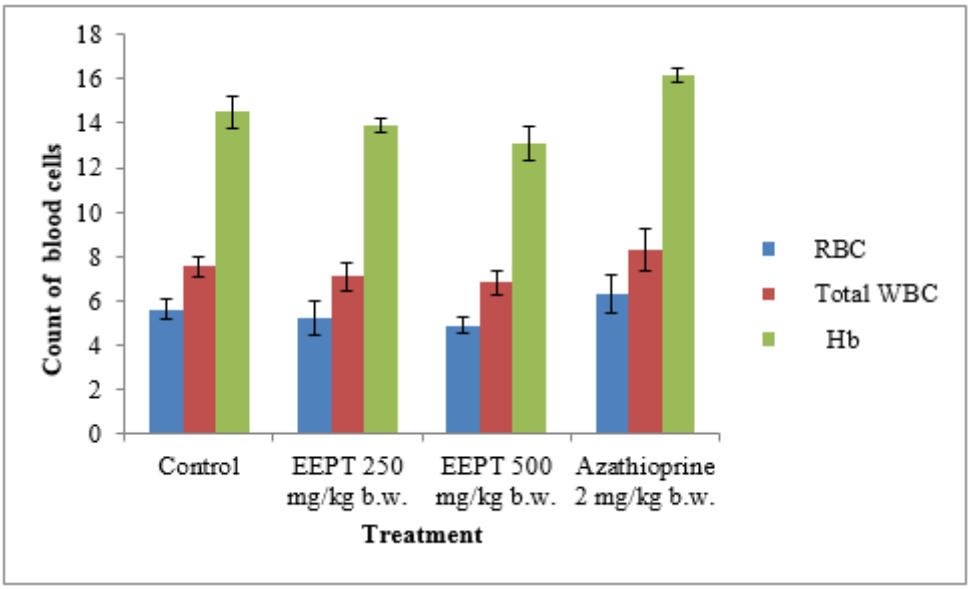

Figure No. 2: Hematological parameters of ethanolic extract of *Pueraria tuberosa* in drug-induced myelosuppression