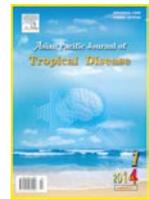




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Immunostimulant activity of *Phyllanthus reticulatus* Poir: a useful plant for infectious tropical diseases

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PEER REVIEW

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Comments

This is a good quality work in which the authors have evaluated immunostimulant effect of *P. reticulatus* in albino mice. The results are interesting and well presented.

Details on Page S494

ABSTRACT

Objective: To confirm the ethnomedicinal claim of *Phyllanthus reticulatus* Poir as immune enhancer.

Methods: The hydroalcoholic extract of fruits and leaves of the plant were evaluated for immunostimulant activity on albino mice at the dose levels of 100 and 200 mg/kg orally. The assessment of immunostimulant activity on specific and non-specific immunity was studied by neutrophil adhesion test, carbon clearance test and cyclophosphamide induced myelosuppression. Cyclophosphamide (30 mg/kg) was used to induce immunosuppression in mice and levamisole (50 mg/kg) was used as immunostimulating agent.

Results: Oral administration of both the doses of fruit and leaf extracts showed significant increase in phagocytic activity (** $P < 0.001$) and the percentage of neutrophil adhesion ($P < 0.01$). Significant increase in white blood cell ($P < 0.05$, ** $P < 0.01$) count was seen on administration of both the doses of fruits and leaves extracts.

Conclusions: The study demonstrates that both the plant parts triggers specific and non-specific responses and thus reveals a promise to hold immunostimulant activity.

KEYWORDS

Carbon clearance test, Immunostimulant, Neutrophil adhesion, *Phyllanthus reticulatus*

1. Introduction

The plants labeled as 'rasayana' have been endowed with properties like strengthening the phycho-neuro-immune axis[1]. Dysfunction of immune system is responsible for various diseases like cancer, allergy, arthritis, ulcerative colitis, asthma, parasitic diseases and infectious diseases[2]. Plants are the invaluable, incredible and traditional sources for the curability of various diseases in the form of medicines. The first thing done in the field of

immunomodulation was the search of immunomodulatory agent for the treatment of residual cancer[3].

Phyllanthus reticulatus Poir (*P. reticulatus*) (Family: Euphorbiaceae) is a large, often scandent, shrub which grows throughout tropical areas of India, Bangladesh, China and the Malay Islands[4]. The leaves of the plant are diuretic and also used for diarrhoea in infants[5]. The leaf juice is a remedy for spongy and bleeding gums[6]. The biological work performed so far on this plant showed anti-diabetic[7], anti-plasmodial[8], hypocholesterolemic[9], antimicrobial

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and cytotoxic^[10], hepatoprotective^[11], antinociceptive and antihyperglycemic^[12], analgesic and anti-inflammatory^[13], and antibacterial^[14] activities.

Three compounds (lupeol, lupeol acetate and stigmasterol) were isolated and identified by phytochemical study conducted on the leaves of *P. reticulatus*^[15]. Other isolated compounds were tannic acid, friedelin, epifriedelinol, betulin, taraxerone, β -sitosterol, glochidonol, octacosanol, taraxeryl acetate and 21- α -hydroxyfriedelan-3-one, betulinic acid^[10]. Eight compounds (β -sitosterol-3-O- β -glucoside, stigmasterol-3-O- β -glucoside, methyl gallate, ellagic acid, corilagin, methyl brevifolin carboxylate, kaempferol, astragalgin), including two flavonoid glycoside rutin (quercetin-3-rutinoside) and quercetin-3-O- β -D-glucopyranoside (isoquercitrin) were isolated from the butanol soluble fraction of the methanolic extract of the leaves of *P. reticulatus* by conventional methods^[16]. Ellagic acid was found as the chemical constituent for the inhibition of rheumatoid arthritis^[17].

2. Materials and methods

2.1. Plant material

Leaves and fruits of *P. reticulatus* were collected from the campus of Kurukshetra University, Kurukshetra in the month of July–August. The plant was identified by Dr. HB Singh, Head, Raw Material Herbarium and Museum Division, NISCAIR, New Delhi. (Ref–NISCAIR/ RHMD/ consult/2010–11/1472/70).

2.2. Extract preparation

The plant material was coarsely powdered using dry grinder and extracted with pet ether (60–80 °C) in Soxhlet apparatus at a temperature not exceeding 60 °C. The defatted plant material was then extracted with hydroalcohol. The extracts were concentrated under reduced pressure in rotary evaporator to yield a crude semi-solid mass. Semisolid blackish extract was obtained from dried ripe fruits and brownish black extract was obtained from dried leaves of the plant.

2.3. Carbon clearance test

Mice were divided into six groups of five animals each. The control group I received vehicle (normal saline), while group II received levamisole (50 mg/kg) for 14 d. The animals of treatment group III and IV were given leaf extracts (100 mg/kg and 200 mg/kg respectively) daily for 14. The groups

V and VI were given fruit extracts (100 mg/kg and 200 mg/kg respectively) daily for 14. On the 14th day of treatment, three hour after the last dose, mice were injected with 0.1 mL of carbon suspension (Pelican ink) intravenously through tail vein. Blood samples (25 μ L) were collected from retro-orbital plexus just at 0 and 15 min after injection. Blood samples were mixed with 2 mL of 0.1% w/v Na₂CO₃. The carbon clearance *i.e.*, rate of elimination of carbon from blood was determined by turbidometric spectroscopy at 650 nm using UV spectrophotometer. The phagocytic index (K) was calculated using the formula:

$$K = (\ln OD_1 - \ln OD_2) / t_1 - t_2$$

Where OD₁ and OD₂ are the optical densities at time t₁ and t₂^[18].

2.4. Cyclophosphamide-induced myelosuppression

In cyclophosphamide induced myelosuppression, mice were divided into seven groups of five animals each. Group I (control group) and group II (cyclophosphamide group) received the vehicle for a period of 13 d. Group III received levamisole (50 mg/kg) for 13 d. The animals of treatment group IV and V were given leaf extracts (100 mg/kg and 200 mg/kg respectively) daily for 13 d. The groups VI and VII were given fruit extracts (100 mg/kg and 200 mg/kg respectively) daily for 13 d. The animals of groups II to VII were injected with cyclophosphamide (30 mg/kg, *i.p.*) on the 11th, 12th and 13th day, 1 h after the administration of the respective treatment. Blood samples were collected on the 14th day of the experiment and the total white blood cell (WBC) count was determined by routine hematological method using Neubaure chamber with haemocytometer^[19].

2.5. Neutrophil adhesion test

In neutrophil adhesion test, the control group I received vehicle, while group II received levamisole (50 mg/kg) for 14 d. The animals of treatment group III and IV were given leaf extracts (100 mg/kg and 200 mg/kg respectively) daily for 14 d. The groups V and VI were given fruit extracts (100 mg/kg and 200 mg/kg respectively) daily for 14 d. On the 14th day of the treatment, blood samples from all the groups were collected by puncturing retro-orbital plexus under mild ether anesthesia. Blood was collected in vials pre-treated by disodium EDTA and analysed for total leukocyte count (TLC) and differential leukocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with nylon fiber (80 mg/mL of blood sample) for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and percent neutrophil gives neutrophil index of

blood sample. Percent neutrophil adhesion was calculated as follows:

$$\text{Neutrophil adhesion} = \frac{NI_u - NI_i}{NI_u} \times 100$$

Where NI_u is neutrophil index before incubation with nylon fiber; NI_i is neutrophil index after incubation with nylon fiber[18].

2.6. Statistical analysis

The data was analysed using one way analysis of variance (ANOVA) followed by Dunnett's test. All the values were expressed as mean±SEM.

3. Results

3.1. Effect of *P. reticulatus* on carbon clearance test

Effect of hydroalcoholic extract of fruit and leaf on the phagocytic activity by the carbon clearance test is shown in Table 1. The phagocytic activity of the reticuloendothelial system is generally measured by the rate of removal of carbon particles from the blood stream. Both the doses of fruit and leaf extracts showed significant ($**P<0.001$) increase in the phagocytic index when compared to control, indicating that there was increase in the clearance of colloidal carbon from the blood after administration of these extracts.

Table 1

Effect of extracts on rate of carbon clearance and neutrophil activation.

Groups	Treatment	Phagocytic Index	Neutrophil adhesion (%)
Group I	Normal saline	0.0189±0.0010	9.537±2.188
Group II	Levamisole (50 mg/kg)	0.0683±0.0005**	73.24±0.06**
Group III	leaf extract (100 mg/kg)	0.0469±0.0020**	27.537±3.496**
Group IV	leaf extract (200 mg/kg)	0.0592±0.0016**	46.350±5.096**
Group V	fruit extract (100 mg/kg)	0.0498±0.0030**	43.037±7.555**
Group VI	fruit extract (200 mg/kg)	0.0618±0.0002**	52.113±4.652**

Results are expressed as Mean ± SEM, $N=5$, $**P<0.001$ when groups III to VI were compared with group II.

3.2. Effect of *P. reticulatus* on cyclophosphamide-induced myelosuppression

Effect of fruit extract and leaf extract on total leukocyte count by cyclophosphamide induced neutropenia test is shown in Table 2. Administration of cyclophosphamide produced significant decrease in the total leukocyte count. A significant reduction in total WBC count was observed in mice treated with cyclophosphamide alone (Group II) compared to control group (Group I). Both the doses (100 and

200 mg/kg, *p.o*) with cyclophosphamide showed significant ($*P<0.05$, $**P<0.001$) increase in the levels of total WBC count compared to cyclophosphamide treated group. The rise in the total WBC count lowered by cyclophosphamide was observed at 100 mg/kg and 200 mg/kg of both extracts but the results were not significant with 100 mg/kg of leaf extract.

Table 2

Effect of extracts on cyclophosphamide induced immunosuppression.

Groups	Treatment	WBC ($1 \times 10^9/L$)
Group I	Normal saline	4.3750±0.1377
Group II	Cyclophosphamide (30mg/kg)	3.2750±0.1109**
Group III	Levamisole (50 mg/kg)	6.2500±0.0645**
Group IV	Leaf extract (100 mg/kg)	4.8750±0.1315*
Group V	Leaf extract (200 mg/kg)	5.9000±0.0912**
Group VI	Fruit extract (100 mg/kg)	4.7000±0.1225
Group VII	Fruit extract (200 mg/kg)	5.7500±0.1936**

Results are expressed as Mean±SEM, $N=5$, $**P<0.001$ when groups III to VII were compared with group II.

3.3. Effect of *P. reticulatus* on neutrophil adhesion test

Effect of fruit extract and leaf extract on neutrophil activation by the neutrophil adhesion test is shown in Table 1. Cytokines are secreted by activated immune cells for margination and extravasation of the phagocytes, mainly polymorphonuclear neutrophils[18]. Incubation of neutrophils with nylon fibres produced a decrease in the neutrophil counts due to adhesion of neutrophils to the fibres. The percentage of neutrophil adhesion was significantly ($**P<0.001$) increased by both the doses of fruit and leaf extract when compared with the control group, showing possible immunostimulant effect.

4. Discussion

In our previous work, we have studied many medicinal plants with immunomodulatory activity for treatment and management of various diseases[20]. In the present work, immunostimulant activity of plant *P. reticulatus* was carried out which is used as folkmedicine for the management of HIV/AIDS related conditions in Bukoba rural district of Tanzania[21]. To prove its claim, immunostimulant activity of the plant was carried out by different *in vivo* models.

The carbon clearance test was done to evaluate the effect of extracts on the reticuloendothelial system. The reticuloendothelial system is a diffuse system consisting of phagocytic cells. Cells of reticuloendothelial system play a vital role in the clearance of foreign particles from the bloodstream[22]. The function of phagocytosis is the removal of microorganism, foreign bodies, dead and injured cells.

The increase in the carbon clearance index of both extracts reflects the enhancement of the phagocytic function of mononuclear macrophage and nonspecific immunity^[18]. When colloidal carbon particles in the form of black ink are injected directly into the systemic circulation, the rate of clearance of particles from the blood by macrophage is governed by an exponential equation^[22]:

$$K = (\log_e OD_1 - \log_e OD_2) / 15$$

Where OD_1 and OD_2 are the optical densities at 0 and 15 min respectively.

Both the doses of fruit extract as well as leaf extract showed remarkable increase in the phagocytic index due to their ability to increase the activity of the reticuloendothelial system.

Neutrophils are the main components of immune protection system from infections by migration towards the challenge^[23]. The neutrophil adhesion to nylon fibres describes the margination of polymorphonuclear lymphocyte in the blood vessels and the number of macrophages reaching to the site of inflammation^[22]. Both the doses (100 mg/kg and 200 mg/kg) of fruit and leaf extract showed a substantial rise in the neutrophil adhesion to nylon fibres. This might be due to the upregulation of the $\beta 2$ integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres. Hence it was inferred that both extract causes stimulation of neutrophils towards the site of inflammation.

The cyclophosphamide induced neutropenia model concentrates on the protective effects against cyclophosphamide induced myelosuppression in experimental animals. A high degree of cell propagation provides the bone marrow a sensitive target particularly to cytotoxic drugs. In fact, bone marrow is the organ which is most affected during any immunosuppression therapy. Loss of stem cells and inability of the bone marrow to regenerate new blood cells results in thrombocytopenia, and cyclophosphamide at the dose of 30 mg/kg caused a significant reduction in total WBC count in mice when compared to control group^[24].

Cyclophosphamide belongs to nitrogen mustard subclass of alkylating agents and acts as an immunosuppressive agent by causing alkylation of DNA, in turn by interfering in DNA synthesis and function. *P. reticulatus* caused reduction in the cyclophosphamide induced neutropenia, suggesting that it may have an effect on the haemopoetic system. The prevention of neutropenia induced by cyclophosphamide may be through activation of macrophages, which secrete a large number of substances including colony stimulating factor and interleukin-1^[25]. Both the doses of leaf extract and fruit extract of *P. reticulatus* attenuates the effect of cyclophosphamide on the haemopoetic system.

The present study reports the scientific basis for the use of *P. reticulatus* as immunostimulant for the first time. From the above study it could be speculated that the activity of *P. reticulatus* might be related to the presence of tannins and phenolic compounds. The study revealed that leaf and fruit of *P. reticulatus* could be added in the herbal formulations beneficial for the enhancement of immunity. Further studies can be undertaken at the cellular and molecular level, which may further elucidate its mechanism in detail. The present investigation has also opened an avenue for further research especially with reference to the development of potent formulation for enhancement of immunity from *P. reticulatus*.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Dysfunction of immune system is responsible for various diseases like cancer, allergy, arthritis, ulcerative colitis, asthma, parasitic diseases and infectious diseases. The plants have been used to enhance immunity from the ancient time. The authors experimentally confirmed that *P. reticulatus* has immunostimulant properties. So, the plant is beneficial for immune related disorders.

Research frontiers

The immunostimulant activity was performed by well established methods: carbon clearance test, cyclophosphamide induced myelosuppression and neutrophil adhesion test in albino mice. The present study reports the scientific basis for the use of *P. reticulatus* as immunostimulant for the first time.

Related reports

The different species of same genus have also immunostimulant and immunomodulator activities e.g. *Phyllanthus niruri*, *Phyllanthus embelica* etc. as previously

reported by some other authors.

Innovations & breakthroughs

The paper has innovations as the study reports scientific basis for the use of *P. reticulatus* as immunostimulant for the first time in different *in vivo* methods.

Applications

The plant *P. reticulatus* will be highly useful for treatment of various diseases related to immune system.

Peer review

This is a good quality work in which the authors have evaluated immunostimulant effect of *P. reticulatus* in albino mice. The results are interesting and well presented.

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