ORIGINAL ARTICLE

An experimental evaluation of *in vitro* immunomodulatory activity of isolated compound of *Ricinus communis* on human neutrophils

Arvind Kumar, Vaishali Singh, Sourav Ghosh
Department of Pharmacology, S. D. College of Pharmacy and Vocational Studies, Muzaffarnagar, Uttar Pradesh, India

In the present study, the *in vitro* immunomodulatory activity of *Ricinus communis* Linn (Euphorbiaceae) was determined on human neutrophils. The isolated compound (tannin) of *R. communis* leaves was screened for its possible immunomodulatory activity by carrying out nitroblue tetrazolium test, phagocytosis of killed *Candida albicans*, neutrophil locomotion and chemotaxis. The isolated compound was tested at concentrations, viz. 10 µg/ml, 20 µg/ml, 40 µg/ml, 100 µg/ml and 1000 µg/ml. The isolated compound of *R. communis* showed predominantly significant activity on human neutrophils in all the parameters tested, which was comparable to the standard and control at different concentrations, indicating the possible immunostimulating effect.

Key words: Human neutrophils, immunomodulatory, *Ricinus communis*, tannin

INTRODUCTION

The immune system is designed to protect the host from invading pathogens and to eliminate disease.[1] The primary objective in the past had been to suppress the immune system to permit all organ transplantation. Activation of immune system by "non-self" antigen (alloantigen) or "self" antigen (auto antigen) is generally believed to require processing of the antigen by phagocytic cells such as macrophages, monocytes, or related cells.[2]

Immune system dysfunction is responsible for various diseases like arthritis, ulcerative colitis, asthma, allergy, parasitic diseases, cancer and infectious diseases.[3] The degree to which the patient becomes abnormally susceptible to infections by this microbial environment depends on the extent of immunosuppression. The suppression of the immune system is characterised by reduction in the number and phagocytic function of the neutrophils and macrophages, as well as an impairment of the intracellular bactericidal capacity of these cells. This immunosuppression allows opportunistic pathogens to overwhelm the host to cause secondary infections.[4] This problem can be overcome by boosting the immune system by the use of immunomodulatory drugs.[5]

Chemotherapeutic agents available today have mainly immunosuppressive activity. Most of them are cytotoxic and have various side effects. This has led to the search for investigating natural resources showing immunomodulatory activity. Many medicinal plants are known to have immunomodulatory properties. Medicinal plants, which are used for their immunomodulatory effect, provide alternative potential to conventional chemotherapy for a variety of diseases, especially in relation to host defence mechanism. Plant products like polysaccharides, lectins, peptides, flavonoids and tannins have been used for the immune response or immune system in various *in vitro* models.[6]

In the present study, we have evaluated the immunomodulatory potency of *Ricinus communis* Linn (Euphorbiaceae) on human neutrophils.

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MATERIALS AND METHODS

Plant Material

The leaves of *R. communis* Linn were collected from a local area of Muzaffarnagar, Uttar Pradesh, India, in the month of February 2011 and authenticated by the Botanical Survey of India (vide no. CNH/I-I/(292)/2011/Tech.II/333).

Address for correspondence: Dr. Arvind Kumar, Department of Pharmaceutical Chemistry, S. D. College of Pharmacy and Vocational Studies, Muzaffarnagar, Uttar Pradesh, India. E-mail: karvind77@gmail.com
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Preparation of Extracts

Leaves were shade dried at room temperature and powdered to #40 mesh particle size. The powder (250 g) of crude plant was defatted with petroleum ether and subjected to extraction with methanol using Soxhlet apparatus. The extract was filtered and evaporated at 40°C under vacuum and the residue was freeze-dried.

Phytochemical Tests

R. communis leaf extract was tested for the presence of tannins, alkaloids, carbohydrates, steroids, and flavonoids separately.[7]

Test for alkaloids

1. Mayer's test: 2-3 ml of solution of extract was added to a few drops of Mayer's reagent.
2. Dragendroff's test: 2-3 ml of solution of extract was added to a few drops of Dragendroff's reagent.
3. Hagger's test: 2-3 ml of solution of extract was added to a few drops of Hagger's reagent.

Test for tannins

2-3 ml of solution of extract was added to a few drops of 5% $FeCl_3$ solution.

Test for flavonoids

1. 2-3 ml of solution of extract was added to 95% ethanol and neutral $FeCl_3$ solution.
2. 2-3 ml of solution of extract was added to 95% ethanol and lead acetate solution.

Test for carbohydrate

Molish test: 2-3 ml of solution of extract was added to a few drops of alpha napthol solution in alcohol, shaken well and conc. H_2SO_4 was added from the sides of the test tube.

Test for steroid

Salkowski reaction: 2-3 ml of solution of extract was added to 2 ml of chloroform and 2 ml of conc. H_2SO_4.

Isolation of Tannins

About 5 g of dried methanolic extract of leaf of *R. communis* was digested with boiling water for 30 min. To this saturated solution, lead acetate was added to precipitate out tannins. The solution was filtered and the residue containing tannins was collected and the filtrate discarded. Residue was collected in water and hydrogen sulphide (H_2S) gas was passed to remove the excess lead acetate as lead sulphide. The solution was filtered, and the residue which contained excess of lead acetate was discarded. The filtrate was concentrated to get tannins. The isolated tannin was confirmed by chemical tests and using benzene: glacial acetic acid (1:1) as the solvent system for thin layer chromatography (TLC).

Preparation of Test Sample

Sample solutions for *in vitro* studies were prepared by dissolving 5 mg of the isolated compound of *R. communis* leaf in 0.25 ml water and phosphate buffer salt solution to prepare solutions with concentrations of 10 µg/ml, 20 µg/ml, 40 µg/ml, 100 µg/ml and undiluted (1000 µg/ml) extract.

Study of Immunomodulatory Activity

Qualitative nitroblue terazolium test

A suspension of leucocytes (5×10^6/ml) was prepared in 0.5 ml of phosphate buffered saline (PBS) solution in different tubes. 0.1 ml of PBS solution (control) and 0.1 ml of endotoxin-activated plasma (standard) were added to 1st and 2nd tubes, respectively, and to the other tubes were added 0.1 ml of different concentrations (10 µg/ml, 20 µg/ml, 40 µg/ml, 100 µg/ml and 1000 µg/ml) of the test samples. 0.2 ml of freshly prepared 0.15% nitroblue tetrazolium (NBT) solution was added to each tube and incubated at 37°C for 20 min. The tubes were centrifuged at 400 *g* for 3-4 min to discard the supernatant.

The cells were resuspended in a small volume of PBS solution. A thin film was made with the drop on the slide, dried, fixed by heating, counterstained with dilute carbol-fuchsin for 15 sec. The slide was washed under tap water, dried and focussed under 100× oil immersion objective; 200 neutrophils were counted for the % of NBT positive cells containing blue granules/lumps.[8]

Phagocytosis of Killed *Candida albicans*

Preparation of Candida albicans suspension

Candida albicans culture was incubated in Sabouraud broth overnight and then centrifuged to form a cell button and the supernatant was discarded. The cell button was washed with sterile Hank's Balanced Salt Solution (HBSS) and centrifuged again. This was done 3-4 times. The final cell button was mixed with a mixture of sterile HBSS and human serum in a proportion of 4:1. The final cell suspension of concentration 1×10^8 was used for the experiment.[9]

Slide preparation

Human blood (0.2 ml) was obtained by finger prick method on a sterile glass slide and incubated at 37°C for 25 min to allow clotting. The blood clot was removed very gently and the slide was drained slowly with sterile normal saline, taking care not to wash the adhered neutrophils (invisible). The slide consisting of polymorphonuclear neutrophils (PMNs) was flooded with a concentration of test sample and incubated at 37°C for 15 min. The PMNs were covered with *C. albicans* suspension and incubated at 37°C for 1 hour. The slide was drained, fixed with methanol and stained with Giemsa stain.

Phagocytosis evaluation

The mean number of *Candida* cells phagocytosed by PMNs on the slide was determined microscopically for

100 granulocytes using morphological criteria. This number was taken as Phagocytic Index (PI) and was compared with basal PI of control. This procedure was repeated for different concentrations (10 µg/ml, 20 µg/ml, 40 µg/ml, 100 µg/ml and 1000 µg/ml) of the isolated compound. Immunostimulation in % was calculated by using the following equation:

Stimulation (%) = PI (test) − PI (control) × 100/PI (control)

Neutrophil Locomotion and Chemotaxis Test

Neutrophil cell suspension was prepared in PBS solution at about 10^6 cells/ml. The lower compartment of chemotactic chamber was filled with appropriate chemotactic reagents preadjusted to a pH of 7.2 [e.g. chamber 1 had PBS solution (control), chamber 2 had casein 1 mg/l (standard) and chambers 3, 4, 5, 6, 7 had different concentrations (10, 20, 40, 100 and 1000 µg/ml) of the isolated sample]. The upper compartment (1 ml syringe) was filled with neutrophil cell suspension and the wet filter (Millipore) of 3 mm pore size was fixed at the bottom of the upper compartment. The upper compartment was placed into the lower compartment and incubated at 37°C for 180 min.

The upper compartment was removed and inverted to empty the fluid. The lower surface of the filter was fixed with 70% ethanol for 2 min and then stained with haematoxylin dye for 5 min. The fixed filters were observed under microscope using 100× lens and the number of neutrophil cells that reached the lower surface was counted.[8]

Statistical Analysis

The values were expressed as mean±standard error of mean (SEM) (n=4). The results were analysed by using one-way analysis of variance (ANOVA) followed by Dunnet's "t" test determine the statistical significance.[10]

RESULTS

Phytochemical Screening

The preliminary phytochemical investigation revealed the presence of tannins. The isolated compound had shown significant activity in all the parameters only at higher concentrations.

Nitroblue Tetrazolium Test

The isolated compound (tannin) stimulated the neutrophils to phagocytic activity to the extent of 64.95% and 60.67% at concentrations of 1000 µg/ml and 100 µg/ml, respectively, when compared to normal control (22.17% and 20.12%) and standard, i.e. endotoxin-activated plasma (67.15% and 57.97%) at the same concentrations. However, at low concentrations of 40 µg/ml, 20 µg/ml and 10 µg/ml, the stimulation of neutrophil was comparatively lower. Significant stimulation of neutrophil was observed. The results are shown in Table 1.

Phagocytosis of Killed *C. albicans* Assay

The isolated compound (tannin) stimulated the phagocytosis

Table 1: Percentage of reduced neutrophils after treatment with the isolated compound of *R. communis* leaf extract, determined by nitroblue tetrazolium test

Samples	Concentration (µg/ml)				
	1000	100	40	20	10
Phosphate buffered saline	22.17±0.31	20.12±0.06	19.34±0.04	19.20±0.03	19.16±0.03
Endotoxin-activated plasma	67.15±0.09	57.97±0.95	51.49±0.25	50.37±0.11	36.69±0.07
Isolated compound	64.95±0.67*	60.67±0.15*	44.81±0.18*	31.46±0.06*	25.19±0.04

The data are expressed as mean percentage reduced neutrophils±standard error of mean. Significant difference found from positive control (standard) by one-way ANOVA followed by Dunnet's "t" test (n = 4). *P<0.01. Phosphate buffered saline (normal control), endotoxin-activated plasma (positive control)

Table 2: Mean particle number in phagocytosis of killed *Candida albicans* after treatment with isolated compound of *R. communis*

Samples (extracts)	Concentration (µg/ml)				
	1000	100	40	20	10
Hank solution	0-1	0	0	0	0
Pooled serum	6	4-5	4	3-4	3
Isolated compound	5-6	4	4	3	2

The data are expressed as mean particle number phagocytosed; Hank solution (normal control), pooled serum (positive control)

Table 3: Number of neutrophils per field after treatment with the isolated compound of *R. communis* leaf extract, determined by neutrophil locomotion and chemotactic activity

Samples (extracts)	Concentration (µg/ml)				
	1000	100	40	20	10
Phosphate buffered saline	16.25±0.29	14.25±0.25	13.75±0.49	12.50±0.29	12.25±0.29
Casein	149.8±0.25	135.0±0.41	132.0±0.41	129.5±0.29	120.3±0.48
Isolated compound	156.5±0.29*	150.5±0.29*	138.5±0.29*	138.5±0.29*	120.3±0.25

Mean number of neutrophils per field±standard error of mean; significant difference from positive control (std.) by one-way ANOVA followed by Dunnet's "t" test (n=4). *P<0.01. Phosphate buffered saline (normal control), casein (positive control)

of killed *Candida albicans*. The mean particle numbers (MPN) were found to be 5-6, 4 and 4 for the isolated compound at concentrations of 1000 µg/ml, 100 µg/ml and 40 µg/ml, respectively, when compared to positive control (standard), i.e. pooled serum (6, 4-5 and 4) at the same concentrations. However, at low concentrations of 20 µg/ml and 10 µg/ml, the stimulation of phagocytic activity was comparatively lower. The results are shown in Table 2.

Neutrophil Locomotion and Chemotaxis

The isolated compound of leaves of *R. communis* showed very significant chemotactic activity at all concentrations. The mean number of neutrophils per field for the isolated compound (tannin) was found to be 156.5, 150.5 and 138.5 at concentrations of 1000 µg/ml, 100 µg/ml and 40 µg/ml, respectively, when compared to the standard, i.e. casein (149.8, 135.0 and 132.0) and normal control (16.25, 14.25 and 13.75) at the same concentrations. However, at low concentration of 20 µg/ml, significant stimulation of chemotactic activity was observed. The results are shown in Table 3.

DISCUSSION

Immunomodulatory agents of plant and animal origin increase the immune responsiveness of the body against pathogens by activating the non-specific immune system. However, there is a need to subject such medicinal plants to systemic studies to substantiate the therapeutic claims made with regard to their clinical utility.[11]

In the present study, the isolated compound of *R. communis* leaves significantly increased the phagocytic function of human neutrophils when compared to control, indicating the possible immunostimulating effect.

The engulfment of microorganism by leucocytes called phagocytosis is one of the main defence mechanisms of an organism.[12] The isolated compound from *R. communis* leaf extract significantly increased the neutrophil chemotactic movement as indicated by the increase in number of cells reaching the lower surface of filter; therefore, the extract acts as a chemo attractant.

The final step of phagocytosis is the intracellular killing of microorganisms by the neutrophils, which is dependent on metabolic thrust generated through the hexose monophosphate shunt activation, and activation which is also necessary for the normal microbicidal activity.[13]

The isolated compound significantly increased the intracellular reduction of NBT dye to formazan (deep blue compound) by the neutrophils, confirming the intracellular killing property and overall metabolic integrity of phagocytosing neutrophils.

Tannins obtained from the leaves were found to possess anti-inflammatory and immunomodulatory properties. Thus, it can be also concluded that immunomodulatory activity may be due to the presence of tannins in the *R. communis*.

REFERENCES

1. Singh V, Sharma P, Dudhe R, Kumar N. Immunomodulatory effects of some traditional medicinal plants. J Chem Pharm Res 2011;3:675-84.
2. Sainis KB, Sumariwalla PF, Goel A, Chintalwar GJ, Sipahimalani AT, Banerji A. Immunomodulatory properties of stem extracts of Tinospora cordifolia. Cell Targets and Active Principles. New Delhi, India: Narosa Publishing House; 1997. p. 95.
3. Patwardhan B, Kalbag D, Patki PS, Nagsampagi BA. Search of immunomodulatory agents. Indian Drugs 1990;28:56-63.
4. Rao CS, Raju C, Gopumadhavan S, Chauhan BL, Kulkarni RD, Mitra SK. Immunotherapeutic modification by an ayurvedic formulation Septilin. Indian J Exp Biol 1994;32:553-8.
5. Fulzele SV, Satturwar PM, Joshi SB, Dorle AK. Study of immunomodulatory acticity of Haridradi Ghrita in rats. Indian J Pharmacol 2003;35:51-4.
6. British Pharmacopoeia, Department of Health. British Pharmacopoeia Commission. London: The Stationary Office; 1999.
7. Khandelwal KR. Techniques and Experiments. 9th ed. Practical Pharmacognosy. Pune: Nirali Prakashan; 2002. p. 149-59.
8. Wilkinson PC. Neutrophil leucocytes function test. In: Thomas RA, editor. Techniques in clinical immunology, 2nd edition: London: Blackwell Scientific Publication; 1981. p. 278-539.
9. Ponkshe CA, Indap MM. *In vivo* and *in vitro* evaluation for immunomodulatory activity of three marine animal extracts with reference to phagocytosis. Indian J Exp Biol 2002;40:1399-402.
10. Kulkarni SK. Biostatics in experimental pharmacology. Handb Exp. Pharmacol. Delhi: Vallabh Prakashan; 1999. p. 179-80.
11. Fulzele SV, Satturwar PM, Joshi SB, Dorle AK. Study of immunomodulatory activity of Haridradi Gharita in rats. Indian J Pharmacol 2003;35:51-4.
12. König P, Hordvik NL, Kreutz C. The preventive effect and duration of action of nedocromil sodium and cromolyn sodium on exercise-induced asthma (EIA) in adults. J Allergy Clin Immunol 1987;79:64-8.
13. Daniel PS. In: Daniel PS, Abba IT, Tristram GP (Eds): Basic and Clin Immunology, 8th edition, USA. Appleton and Lange; 1994. p. 195-2.

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