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Evaluation of Immunomodulatory Activity of Protocatechuic Acid

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Abstract

Several drugs such as anti-cancer drugs and important antibiotics such as linezolid pose the serious disadvantage of causing myelosuppression. Cyclophosphamide, a known anti neoplastic agent of the alkylation class suppresses T-lymphocyte activity, leading to a suppressed immune function. It is used experimentally to mimic an immunodeficient condition. Protocatechuic acid is water soluble monomeric phenolic acid with strong free radical scavenging effects. The immunity system play main role in protection of body from infection. The system is divided into two major types.

- Innate immune system
- Adaptive immune system

Rational behind this study was to investigate Protocatechuic Acid for its Immunomodulatory activity in laboratory animals using different preclinical screening models and Antibody molecules secreted by plasma cells mediate humoral response, increase in Hemagulation indicates immunostimulant activity. Different groups of animals receiving PCA at 10 mg/kg, 30 mg/kg, and 100 mg/kg, and of the normal control group that did not receive cyclophosphamide indicating that PCA restored the TLC back to the baseline values. The evaluation parameters are Haemagglutinating Antibody (HA) titre value. Neutrophil adhesion test Delayed Hypersensitivity reaction (DTH). The estimation of study parameter here we can conclude that the highest dosed animals i.e. 100 mg/kg shown significant effect.

Keywords: Immunomodulatory; Protocatechuic acid; Cyclophosphamide

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Introduction

Protocatechuic acid is a polyphenolic compound found in several plants such as onion (Allium cepa L) especially in the scales [1], plums (*Prunus domestica* L) [2]; grapes (*Vitis vinifera*) [3]; kernels of *Alpinia* oxyphylla [4] and nuts, such as almonds ordinary almonds (*Prunus Amygdalus*) etc [5]. Several studies have demonstrated that PCA possesses various activities such as anti-tumor activity anti-carcinogenesis [6,7]; Anti-inflammatory effect [8]; promotes cell proliferation and reduces basal apoptosis [9,10]; anti-oxidant [11] and radical scavenging activity [12]; hepatoprotective [13]; Antibacterial activity [14]; Neuroprotective [15].

However, its immunomodulatory potential has not been scientifically explored. The immune system plays a vital and complex role in the host defense system. Disturbances in this system are associated with several disease conditions. Traditionally, consumption of plants/fruits rich in polyphenolic compounds is associated with improved immune function and resistance to disease (*Holodiscus discolor* [16]; *Hippophae rhamnoides* [17], *Hibiscus sabdariffa L* [18]). Thus, the present investigation of the immunomodulatory effects of PCA is justified.

An immunomodulatory agent can act via its effect on the humoral immune system; cell mediated immune system, both or through non-specific interaction with various components of immune functions. Thus, there are several preclinical studies that can be employed for the evaluation of the immunomodulatory potential of a compound. Therefore, in present study, an attempt has been made to evaluate Protocatechuic acid for its immunomodulatory activity.

Table 1: Rank of the highest dilution of serum showing hemagglutination *P<0.05, **P<0.01, ANOVA followed by Dunnetts test, all groups compared to control group. Effect of PCA on humoral and delayed–type hypersensitivity response in rat.

Group no.	Treatment (For 10 days period)	Hemagglutination Titer
I (Control)	0.9% saline	4.83 ± 0.31
II	Std. Drug Levamisole 50 mg/kg/bw) p.o.	7.16 ± 0.40**
III	PCA (10mg/kg) p.o.	6.33 ± 0.21*
IV	PCA (30mg/kg) p.o.	9.16 ± 0.40**
V	PCA (100mg/kg) p.o.	7.166 ± 0.31**

Table 2: Rank of the highest dilution of serum showing hemagglutination *P<0.05, **P<0.01, ANOVA followed by Dunnetts test, all groups compared to control group. Effect of PCA on humoral and delayed–type hypersensitivity response in rat.

Group no.	Treatment (For 10 days period)	DTH Respose (% increase in paw volume)
I (Control)	0.9% saline	57.101 ± 3.702
II	Std. Drug Levamisole 50 mg/kg/ bw) p.o.	27.407 ± 2.480**
III	PCA (10mg/kg) p.o.	38.970 ± 5.988**
IV	PCA (30mg/kg) p.o.	21.109 ± 1.190**
V	PCA (100mg/kg) p.o.	36.602 ± 1.767**

Materials and Methods

Drug material

Protocatechuic acid was procured from Sigma-aldrich, USA.

Chemicals and drugs

Sodium nitrate, dextrose, sodium citrate were obtained from Rajesh chemicals Mumbai. Levamisole (Dicaris) an immunomodulator used as a standard drug, Bovine Serum Albumin (BSA) Hi Media Lab Pvt Ltd., Mumbai, cyclophosphamide (Endox) used as immunosuppressant drug. Sheep Red Blood Cells (SRBC's) were used as an antigen, obtained from local slaughter house.

Animals

The study was approved by Institute's animal ethical committee and confirmed to national guidelines on the care and use of laboratory animals (CPCSEA/IAEC/PC-10/07-2K8). Male albino rats (Wistar Strain), 10 to 12 weeks old, weighing 125 gms to 150 gms were obtained from Yash farms, Pune used for the study. The animals were maintained at 25 ± 2°C in the Institute's animal house with food (nutriwet, Pune, India) and water ad *libitum*.

Antigen

Fresh sheep blood was collected from local slaughter house in sterile Alsever's solution in 1:1 proportion of Alsever's solution (freshly prepared). Sheep erythrocytes (SRBC) were separated from the blood by centrifugation and washed three times with large volume of pyrogen free sterile saline. Their count was adjusted to 5×10^9 cells/mL in saline using a hemocytometer.

Selection of dose

PCA was weighed accurately and prepared appropriate stock solution (10 mg/kg, 30 mg/kg, and 100 mg/kg) using double distilled water as a vehicle. The drug solutions were prepared daily freshly.

Immunomodulatory activity

Determination of humoral immune response [19]**:** To study humoral antibody response sheep erythrocyte agglutination test was performed. Animals were divided into five groups, each group having six wistar rats. Group I was designated as control and received vehicle only. Group II received the standard drug Levamisole, while groups III, IV and V received various dose levels of PCA orally daily for ten days.

The rats were primed on the third day by an intraperitoneal injection of 100 µL of sheep SRBC (5 × 109 mL-1). Blood samples were collected from the anesthetized rats on the 10^{th} day by retro-orbital puncture. The anti-sheep anti-SRBC titer of rat serum was estimated using the hemagglutination technique. Serial two-fold dilutions of serum samples were made in 100 µL of normal saline containing 0.1% w/v Bovine Serum Albumin (BSA) in microtiter wells. Next, 100 µL of 0.1% SRBC suspension in phosphate buffered saline (count adjusted to 5 × 109 mL-1) was added to each well. The microtiter plates were incubated for 4 hr at 37°C and observed for hemagglutination at the end of the incubation period. Minimum serum dilution (1:2) was ranked as 1 and subsequent dilutions were expressed in a graded manner (1-15). The value of the highest serum dilution showing hemagglutination was taken as the antibody titer of that sample. The mean ranks of the different treatment groups were statistically compared with the mean ranks of the vehicle control groups I and II.

Delayed type hypersensitivity response (DTH response) [20]**:** Six animals per group (control and treated) were immunized on day 0 by i.p. administration of 0.5×10^9 SRBC/rat and challenged by subcutaneous administration of 0.025×10^9 SRBC/ml in to right hind foot pad on day +14. The PCA was administered orally from day -14 until day +13. DTH responses were measured at 24 hr after SRBC challenged on day +14 and expressed as mean percent increase in paw volume by using a calibrated plethysmometer- LE7500 (Panlab).

Neutrophil adhesion test [21]**:** This method is used to evaluate the effect of PCA on neutrophil adhesion. In this test five groups of animals were used, each group containing six rats. Group I was kept as a control and received vehicle only. Standard control group II with levamisole (50 mg/kg), group III PCA (10 mg/kg) group IV PCA (30 mg/kg), group V PCA (100 mg/kg) were administered orally daily for 14 days. After 14 days of treatment of all five groups, blood samples were collected by retro-orbital puncture, anticoagulated and subjected to total (TLC) as well as Differential Leukocyte Count (DLC). After initial counts the blood sample were incubated with 80 mg/ml of nylon fibers at 37°C for 15 minutes. The incubated samples were again analyzed for DLC and TLC.

The product of TLC and % neutrophil known as neutrophil index was determined for each of the respective group.

% Neutrophil Adhesion = (*Difference of neutrophil count of untreated and fiber treated group*) / (*neutrophil count of untreated blood*) × 100

Cyclophosphamide induced myelosuppression model [22]**:** The rats were divided into five groups, each group containing six rats. Group I received 0.9% normal saline, whereas group II was administered only cyclophosphamide at the dose of 50 mg/kg, i.p. Group III was treated with cyclophosphamide along with standard (levamisol 50 mg/kg) Group IV, Group V and Group VI received cyclophosphamide with varied concentrations of PCA (10 mg/kg, 30 mg/kg & 100 mg/kg respectively) for 10 days.

On day 11, blood samples were collected from the retro-orbital plexus of individual animals and analyzed for hematological

Table 3: P<0.05, **P<0.01, ANOVA followed by Dunnetts test, all groups compared to control group. Effect of PCA on neutrophil in rat using % neutrophil adhesion test.

Animal group	TLC (10^3 MM^{-3}) (X)		% Neutrophils(Y)		Neutrophils Index(XY)		% Neutrophil Adhesion
	UnTB	FTB	UnTB	FTB	UnTB	FTB	
Group I	13450 ± 106.77	12850 ± 128.07	79.33 ± 0.67	74.50 ± 0.73	1019516.67 ± 13397.02	1001900 ± 10808.58	1.71 ± 0.43
Group II	14200 ± 183.31	13800 ± 207.85	76.17 ± 1.25	73 ± 1.26	1081716.67 ± 22860.96	1007350 ± 22860.96	6.86 ± 0.89**
Group III	15400 ± 113.14	15100 ± 116.62	81 ± 1.06	78.50 ± 0.88	1247650 ± 22610	1185316.67 ± 15710.36	4.95 ± 1.00**
Group IV	14300 ± 141.43	14100 ± 152.32	80 ± 1.6	76 ± 1.35	110583 ± 33822.87	1079450 ± 29980.13	4.48 ± 1.16
Group V	14050 ± 43.53	13500 ± 120	7.17 ± 0.969	74 ± 1.17	1084350 ± 19714.37	998850 ± 16020.32	7.86 ± 0.81**

parameters.

Statistical analysis

The values were calculated as mean ± SEM. The significance of the difference of the mean value with respect to control group was analyzed by one way ANOVA followed by Dunnett's test using software Graphpad Prism 6.0. P<0.01 or above was considered to be significant.

Results

Hemagglutinating antibody (HA) titer

On tenth day of the study Hemagglutinating Antibody (HA) titer was represented by the rank of the highest dilution of serum showing hemagglutination [19]. A representative 96 well plates has been shown to provide a visual reference. The mean H.A was determined for each group and compared with the mean H.A of the control (Table 1).

Delayed type hypersensitivity (DTH) reactions

In the present study the effect of PCA on cell mediated immune response was studied by Delayed Type of Hypersensitivity (DTH) to SRBC's. The result shown in Figure 1 indicates that there was significant decrease in mean difference, in the foot paw thickness at doses of 10 mg/kg, 30 mg/kg and 100 mg/kg of PCA administered group when compared with normal control (Table 1).

Group IV treated with PCA 30 mg/kg decrease DTH response in terms of mean difference, in the foot paw thickness, significantly (**P<0.01) when compared with control and standard, wherein group III (PCA 10 mg/kg) and group V (PCA 100 mg/kg) decrease DTH response in terms of mean difference, in the foot paw thickness, significantly (**p<0.01) when compared with control. The drug influences cell mediated immune response in dose dependent manner (Table 2).

Neutrophil adhesion test

The % neutrophil adhesion rate in control group animals was noted to be 1.71 ± 0.43; whereas, in PCA-treated groups it was found with increased pattern as compared to their respective control. However, the dose concentrations ranging from 10 mg/kg to 100 mg/kg revealed significant increase in neutrophil adhesion same like that of standard (levamisole) as compared to control, suggesting possible immunostimulant action of the PCA (Table 3).

Cyclophosphamide-induced myelosuppression

A significant (P<0.01) reduction in WBC, RBC and platelets cell count was observed in rats treated with cyclophosphamide alone (Group II) as compared to control group (Group I). This indicates that the cyclophosphamide induced myelosuppression model was successful (Figure 1). Analysis of the groups which received various treatments in addition to cyclophosphamide reveals a marked

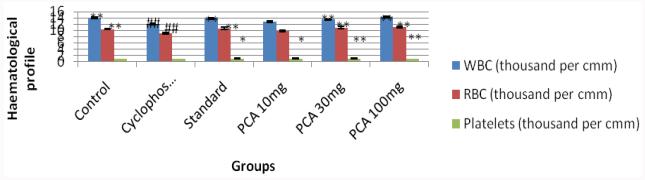

Figure 1: Effect of PCA on WBC count in rat using cyclophosphamide induced myelosuppression model.

improvement in the various hematological parameters. Group III, Group IV and Group V treated with PCA 10 mg/kg, 30 mg/kg and 100 mg/kg respectively increased the levels of total WBC, RBC and Platelets count as compared to cyclophosphamide treated group.

Discussion

To assess the effect of PCA on humoral immune response the SRBC agglutination test was employed. Protocatechuic acid at 30 mg/kg and 100 mg/kg demonstrated a significantly (P<0.01) higher hemagglutination titre compared to the untreated control group. The hemagglutination titre is a measure of the antibodies generated against the SRBC antigen.

Antibody molecules, a product of B lymphocytes and plasma cells, are central to humoral immune responses. IgG and IgM are the major immunoglobulins which are involved in the complement activation, opsonization, neutralization of toxins, etc [23]. Humoral response involves interaction of B-cells and antigen and their proliferation plasma cells. Antibody molecules secreted by plasma cells mediate humoral response. Antibody production to T-dependent antigen SRBC requires co-operation of T- and B-lymphocytes and macrophages i.e. T and B lymphocytes involved in antibody synthesis [24]. The high values of haemagglutinating antibody titre obtained in case of treatment with PCA indicate its potential to interact with B cell towards activation of antibodies.

The effect of PCA on Cell-mediated immune response was studied by Delayed Type Hypersensitivity (DTH) reaction. DTH responses have been well characterized. The reaction is antigen specific and causes erythema and indurations at the site of antigen injection in immunized animals or humans. In general characteristics are an influx of immune cells at the site of injection and indurations which becomes apparent within 24 hr to 72 hrs. Even though they make up only a small percentage (10% to 20%) of the total inflammatory infiltrate at 48 hrs, T cells (either CD4 or CD8 depending on the antigen) are required to initiate the reaction. DTH reaction is antigen specific and causes erythema and indurations at the site of antigen injection in immunized animals when encountered with activated Th1 cells by certain antigens, viz SRBC's DTH comprises of two phases, an initial sensitization phase and effectors phase.

The result obtained in delayed type hypersensitivity reaction indicated that there was significant decrease in % increase in paw volume i.e. foot paw edema of rats treated with PCA at all dose levels when compared against Inducer control. Suppression of immunological edema suggests immunosuppressant activity. Similar results were also observed with the standard levamisole, which is contrary to the reported findings of immunostimulant effect [25,26]. Hence, the results of this study were considered inconclusive, as further investigation of Immunosuppressant activity of the PCA as well as standard (levamisole) should be done before drawing any conclusion of this model.

The neutrophile adhesion is an indication of the migration of phagocytic cells in the blood vessels, i.e. an indication of immunostimulation. Increase in % neutrophil adhesion is attributed in defensive response under normal circumstances. Neutrophils circulate in the vasculature in passive state and become more adhesive upon stimulation at site of inflammation, while it was migrated to the vessel wall, subsequently by transmigration and phagocytosis [27].

The neutrophil adhesion to nylon fibres describes the migration of polymorphonuclear lymphocyte in the blood vessels and the number of macrophages reaching the site of infection. Both low and high doses of PCA (10 mg/kg, 30 mg/kg and 100 mg/kg, p.o) showed a substantial rise in the neutrophil adhesion to nylon fibres. This might be due to the upregulation of the b2 integrins, present on the surface of the neutrophils through which they adhere firmly to the nylon fibres [28]. Hence, it was inferred that PCA causes stimulation of neutrophils towards the site of infection/inflammation and may potentially help in increasing immunity of the body against microbial infection.

Besides countering infection, immunomodulators are also needed to mitigate drug induced myelosuppression.

Conclusion

The present study demonstrated that, Protocatechuic Acid (PCA) treatment at a dose of 100 mg/kg exerted a strong immunomodulatory activity in laboratory animals. It showed a significant immunostimulant effect on specific arms of immune system. Wherein PCA exhibited decrease in cell mediated immunity in DTH revealed its reported anti-inflammatory activity.

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