

EVALUATION OF IMMUNOMODULATORY ACTIVITY OF DIOSGENIN IN RATS

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ABSTRACT

Background: The immune system is intrinsic to health. Modulation of the immune responses to alleviate the diseases by using herbal plants has been of interest for many years. Diosgenin, a naturally occurring steroid saponin mainly present in the seeds of fenugreek (*Trigonella foenum graecum*) and in the root tubers of wild yams (*Dioscorea villosa*). Activation of specific and nonspecific immunity results in stimulation of immune response. Diosgenin has the positive effects on both specific and nonspecific immunity. **Aim:** To study the immunomodulatory activity of Diosgenin in rats. **Method:** The suspension of Diosgenin was given orally at the dosage level of 50, 100 and 150 mg/kg for 21 days in a rat. The immunomodulatory activity on specific and non-specific immunity was studied by haemagglutination antibody (HA) titer, delayed type hypersensitivity (DTH) response and carbon clearance test. Immunosuppression in a rat was induced by using Cyclophosphamide (100 mg/kg, p.o.). Sheep red blood cells (SRBCs) were used as antigen (0.1ml 20% SRBCs) in haemagglutinating antibody titer and delayed type hypersensitivity response methods. **Result:** Diosgenin exhibited significant increase in the production of antibody titer in response to SRBC antigen. A significant increase in both primary and secondary HA titer was observed in immunosuppressed group treated with Diosgenin when compared with negative control. A significant increase in the DTH response was observed in immunosuppressed animals treated with Diosgenin, pre-sensitized with SRBCs antigen. Diosgenin exhibited significant increase in phagocytic index against control group, indicating the stimulation of the reticuloendothelial system. **Conclusion:** The study indicates that Diosgenin triggers stimulatory effect on specific and nonspecific immune response. The immunostimulant effect of Diosgenin could be attributed due to its saponin glycoside.

KEYWORDS: Diosgenin; Immunomodulation; Antibody titer; Delayed type hypersensitivity; Phagocytic index.

INTRODUCTION

Immune system is a host defense system comprising many biological structure and processes within an organism that protects the body against diseases, while simultaneously maintaining self-tolerance. The basic function of immune system is to protect against foreign pathogens and infectious agents. This is achieved either through innate or natural immunological mechanisms which essentially serve as a short term first line defense or through adaptive mechanisms which are highly specific, complex, and marked by diversity and memory [1]. Modulation of immune responses to alleviate the diseases has been of more interest for many years [2]. The term immunomodulators means regulation of the immune system by suppression and stimulation of the cells and organs of the immune system [3].

Immunostimulation implies stimulation of nonspecific system i.e. stimulation of function and efficacy of macrophages, granulocytes, T-lymphocytes and complement natural killer cells. There is need to stimulate immune system in conditions of immune deficiency and variety of disease conditions (e.g., the treatment of AIDS, bacterial infections) or suppress it in case of normal or excessive immune function (e.g., the treatment of graft rejection, hypersensitivity reactions, autoimmune disease) [4]. Immunostimulation and immunosuppression both need to be tackled in order to regulate the normal immunological functioning. An immunomodulatory therapy is now being recognized as an alternative to conventional chemotherapy for a variety of disease conditions, involving impaired immune response of the host [5]. Due to wide range of effector mechanism possessed by various groups of immune cells and its ability to exert effects with exquisite specificity, immune system provide a good target in cancer therapy. Currently, there is a growth in diseases especially infectious diseases that requires efficient body defense mechanisms to control them through the process of immunomodulation [6].



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Presently, focus on plant research has been intensified all over the world and a large amount of evidence has been collected to show immense potential of medicinal plants used in various traditional systems. In Indian system of medicine, many herbal drugs have been advocated for various types of diseases. A large number of plants and their isolated constituents have been known to possess immunomodulatory activity [7]. Diosgenin (C₂₇H₄₂O₃) is a steroid saponin mainly present in plants like Fenugreek (*Trigonella foenum-graecum*) and other species of Dioscorea. It is also considered as the active constituent in the plant extract. Some of the therapeutic uses of Diosgenin include as an antioxidant, anticancer, anti-inflammatory, antihyperlipidemic etc. [8]. The beneficial effect of Diosgenin in immunomodulatory activity is attributed due to saponin glycosides. Therefore, the aim of present study was to assess immunomodulatory effect of Diosgenin.

MATERIALS AND METHODS

Study design: An experimental animal based study

Ethical approval: The experimental protocol was approved by the Institutional Animal Ethics Committee (COPH/IAEC/PG/02/2018).

Study location: Dr.V.V.P. Foundation's College of Pharmacy, Ahmednagar.

Study duration: 21 days

Animals: The healthy albino wistar rats of either sex, weighing between 150-250 g were taken for the study. They were housed under standard environmental conditions of temperature (23±2°C) humidity (55±5%) and 12h light and 12h dark cycles. The animals were fed with standard pellet diet and water ad libitum.

Sample size: Forty eight rats were used (in each group n=6)

Drug and chemicals: Diosgenin was procured from Yarrow Chem Products, Mumbai. Cyclophosphamide was procured as a marketed product "Cyclocel" of Celon Laboratories Ltd. Hyderabad. Colloidal carbon (Pelikan fount Indian ink, Hannover, Germany) all other chemicals were of analytical grade and purchased commercially. Water used was double distilled throughout the study.

Experimental protocol

Dose selection of drug: Studies assessing steroidal saponins for toxicity have shown that they did not show any sign of toxicity up to oral dose of 562.5 mg/kg. Therefore in the present study the dose of Diosgenin 50, 100 and 150 mg/kg has been fixed. They were given by oral route using oral gavage [9].

Preparation of drug suspension: Diosgenin suspension was prepared in 0.5% w/v carboxymethylcellulose

(CMC) in distilled water prior to oral administration to animals [12].

Preparation of SRBC suspension

The blood was collected from a healthy sheep from the local slaughter house in sterile Alsever's solution in 1:1 proportion of Alsever's solution (freshly prepared). It was preserved at a temperature of 2-8°C. On the day of immunization, the blood sample was centrifuged at 2000 rpm for 10 min and then washed three times with 0.9% sodium chloride solution. The SRBC (20% v/v) suspension was then prepared in 0.9% sodium chloride solution [10].

Preparation of Alsever's solution: Formula of Alsever's Solution is Citric acid 0.055g, Sodium citrate 0.8g, Glucose 2.05g, Sodium chloride 0.42g, Distilled water to make volume up to 100 ml. The solution was stored in refrigerator [11].

Preparation of Carbon ink suspension: Pelikan fount, Germany, ink was diluted eight times with saline and used for carbon clearance test in a volume of 1ml/100 g [12].

Methods

Immunomodulatory activity was evaluated using following models of specific (humoral, cellular) and non-specific immunity.

Assessment of humoral immune response to SRBC

Haemagglutination Antibody (HA) titer [12] : The animals were divided into eight groups consisting of six animals each.

Group I (Normal control) received distilled water (1ml/kg, p.o.) for 21 days.

Group II (Negative control) received Cyclophosphamide (100 mg/kg, p.o.) on 9th and 16th day as a single dose.

Group III, IV and V received Diosgenin (50, 100, 150 mg/kg, p.o.) respectively for 21 days. Immunosuppressed animals in Group VI, VII, VIII received Diosgenin (50, 100, 150 mg/kg, p.o.) for 21 days plus Cyclophosphamide (100 mg/kg, p.o.) on 9th and 16th day as a single dose.

On 7th and 14th day, all the groups (i.e. Group I to VIII) were immunized and challenged respectively, with SRBCs in normal saline (0.1ml of 20% SRBCs, i.p.) blood was withdrawn on 14th and 21st day from retro-orbital plexus under mild ether anaesthesia from all antigenically sensitised and challenged rat respectively. Blood was centrifuged to obtain serum, normal saline was used as a diluent and SRBCs count was adjusted to (0.1% of SRBCs). Each well of a microtiter plate was filled initially with 20 µl of saline and 20 µl of serum was mixed in the first well of microtiter plate. Subsequently the 20 µl diluted serum was removed from first

well and added to the next well to get twofold dilutions of the antibodies present in the serum. Further twofold dilutions of this diluted serum were similarly carried out till the last well of the second row (24th well), so that the antibody concentration of any of the dilutions is half of the previous dilution. 20 μ l SRBC (0.1% of SRBCs) were added to each of these dilutions and the plates were incubated at 37°C for one hour and then observed for haemagglutination. The highest dilution giving haemagglutination was taken as the antibody titer. The antibody titer were expressed in the graded manner, the minimum dilution (1/2) being ranked as 1, and mean ranks of different groups were compared for statistical significance. Antibody titer obtained on 14th day after immunization (on 7th day) and on 21st day after challenge (on 14th day) with SRBCs was measured as primary and secondary humoral immune response respectively.

Assessment of cellular immune response induced by SRBC

Delayed type hypersensitivity (DTH) response [13]

The drug treatment was exactly the same as described for HA titer. On 14th day of the study, all the groups I to VIII were immunized with SRBCs (0.1ml of 20% SRBC, i.p.) in normal saline. On day 21st all animals from all the groups were challenged with 0.03 ml of 20% SRBCs in subplantar region of right hind paw and normal saline in left hind paw in same volume. Foot pad oedema in rat was used for detection of cellular immune response. Foot pad reaction was assessed after 24 hr. on 22nd day, in terms of increase in the thickness of footpad as a result of hypersensitivity reaction due to oedema, the paw volume of the right hind footpad was measured by digital plethysmometer. The footpad reaction was expressed as the difference in the volume (ml) between the right foot pad injected with SRBC and the left footpad injected with normal saline.

Assessment of nonspecific immune response: Carbon clearance test [12, 14]

The animals were divided into four groups consisting of six animals each.

Group I (Normal control) received distilled water (1ml/kg, p.o.) for 14 days.

Group II, III, and IV received Diosgenin (50, 100, 150 mg/kg, p.o.) respectively for 14 days. On 14th day, 3 hours after the last dose all the animals of each group were given colloidal carbon intravenously in a volume of 1 ml/100 g. Blood samples were then collected (25 μ l) from retro orbital plexus at 0 and 15 minutes after injection of colloidal carbon ink and mixed in 0.1% sodium carbonate solution (3ml). The optical density was measured spectrophotometrically at 650 nm. The phagocytic index (K) was calculated using the formula:

$$K = \frac{(\ln OD1 - \ln OD2)}{(t2 - t1)}$$

Where, OD1 and OD2 are the optical densities at time t1 and t2 respectively.

Statistical Analysis: All the results were expressed as Mean \pm Standard Error (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test. Value of P less than 5% ($P < 0.05$) was considered statistically significant

RESULTS

1. Effect of Diosgenin on Haemagglutination antibody (HA) titer

Effect of Diosgenin on primary and secondary antibody titer is shown in [Table 1]. Primary antibody titer on 14th day negative control (Cyclophosphamide treated, 100 mg/kg, p.o.) group had shown significant decrease (# # $p < 0.01$) in primary antibody titer when compared with normal control. In Diosgenin-treated (50, 100 and 150 mg/kg, p.o.) groups with normal immune status, showed significant rise (# # $p < 0.01$) in primary antibody titer when compared with normal control. In immunosuppressed groups, where immunity was suppressed by administration of Cyclophosphamide on day nine, Diosgenin (50, 100 and 150 mg/kg, p.o.) administration produced a significant rise (** $p < 0.01$) in primary antibody titer when compared with negative control.

Secondary antibody titer on 21st day negative control (Cyclophosphamide treated, 100 mg/kg, p.o.) group had shown significant decrease (### $p < 0.01$) in secondary antibody titer when compared with normal control. In Diosgenin-treated (50, 100 and 150 mg/kg, p.o.) groups with normal immune status, showed significant rise (### $p < 0.01$) in secondary antibody titer when compared with normal control. In immunosuppressed groups, where immunity was suppressed by administration of Cyclophosphamide on day sixteenth, Diosgenin (50, 100 and 150 mg/kg, p.o.) administration produced a significant rise (** $p < 0.01$) in secondary antibody titer when compared with negative control.

2. Effect of Diosgenin on delayed type hypersensitivity response

The Effect of Diosgenin on SRBC induced DTH response is shown in [Table 2]. On twenty-first day negative control (Cyclophosphamide treated, 100 mg/kg, p.o.) group showed significant decrease (### $p < 0.01$) in the mean difference of paw volume when compared with normal control. In the all groups of rat with normal immune status, treated with Diosgenin (50, 100, 150 mg/kg, p.o.) showed significant potentiated (### $p < 0.01$) DTH response in terms of increase in the mean difference of paw volume when compared with normal control. In the all groups of rat treated with Cyclophosphamide i.e. an immunosuppressed groups, with Diosgenin (50,100,150 mg/kg, p.o.) showed significant potentiated (** $p < 0.01$) DTH response in terms of increase in the mean difference of paw volume when compared

Table 1. Effect of Diosgenin treatment on primary and secondary antibody titer

zSr. No.	Treatment	primary antibody titer (unit/ml)	secondary antibody titer (unit/ml)
1	Normal control	4.08 ± 0.060	5.65 ± 0.022
2	Negative control	3.48 ± 0.135 ^{##}	4.65 ± 0.030 ^{##}
3	Diosgenin (T-50)	5.18 ± 0.060 ^{##}	8.11 ± 0.096 ^{##}
4	Diosgenin (T-100)	6.16 ± 0.066 ^{##}	8.22 ± 0.022 ^{##}
5	Diosgenin (T-150)	7.18 ± 0.060 ^{##}	8.65 ± 0.025 ^{##}
6	Diosgenin (T-50) + Cyp.	5.26 ± 0.053 ^{**}	7.19 ± 0.092 ^{**}
7	Diosgenin (T-100) + Cyp.	6.11 ± 0.099 ^{**}	7.31 ± 0.023 ^{**}
8	Diosgenin (T-150) + Cyp.	6.82 ± 0.018 ^{**}	7.63 ± 0.030 ^{**}

The values are expressed as (Mean ± S.E.M.), n=6, comparison were made as follows,

^{##}p<0.01, when negative control, normal immune status

(i.e. Diosgenin-50, 100, 150 mg/kg) treated groups were compared with normal control.

^{**}p<0.01, when immunosuppressed drug (Diosgenin-50, 100, 150 mg/kg + Cyp.)

treated groups were compared with negative control.

Statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparison test.

T-50 = Diosgenin treated group (50 mg/kg)

T-100 = Diosgenin treated group (100 mg/kg)

T-150 = Diosgenin treated group (150 mg/kg)

Table 2. Effect of Diosgenin treatment on SRBC induced delayed type hypersensitivity response

Sr. No.	Treatment	Mean difference of paw volume in ml
1	Normal control	0.22 ± 0.0106
2	Negative control	0.301 ± 0.0101 ^{##}
3	Diosgenin (T-50)	0.821 ± 0.0107 ^{##}
4	Diosgenin (T-100)	0.905 ± 0.0117 ^{##}
5	Diosgenin (T-150)	0.921 ± 0.008 ^{##}
6	Diosgenin (T-50) + Cyp.	0.508 ± 0.009 ^{**}
7	Diosgenin (T-100) + Cyp.	0.643 ± 0.013 ^{**}
8	Diosgenin (T-150) + Cyp.	0.721 ± 0.011 ^{**}

The values are expressed as (Mean ± S.E.M.), n=6, comparison were made as follows,

[#]p<0.01, when negative control, normal immune status (i.e. Diosgenin-50, 100 and 150 mg/kg) treated groups were

compared with normal control. ^{**}p<0.01, when immunosuppressed drug (Diosgenin-50, 100, 150 mg/kg + Cyp.)

treated groups were compared with negative control. Statistically analyzed by one-way analysis of variance (ANOVA)

followed by Tukey-Kramer multiple comparison test.

Table 3. Effect of Diosgenin treatment on reticuloendothelial system by carbon clearance test

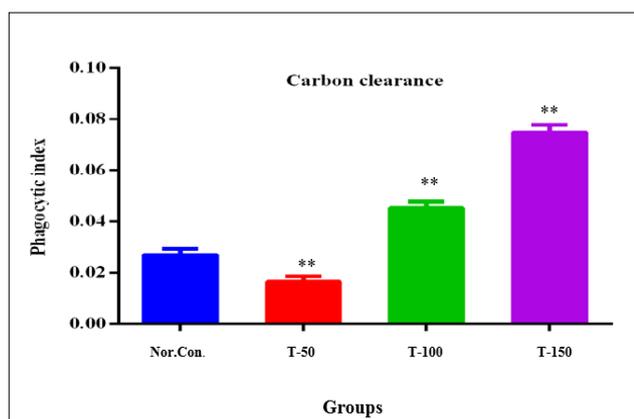
Sr. No.	Treatment	Phagocytic Index
1	Normal Control	0.027 ± 0.00096
2	Diosgenin (T-50)	0.0165 ± 0.00088 ^{**}
3	Diosgenin (T-100)	0.0453 ± 0.00098 ^{**}
4	Diosgenin (T-150)	0.0748 ± 0.0012 ^{**}

The values are expressed as (Mean ± S.E.M), n=6, comparison were made as follows,

^{**} p<0.01, when all test drug (Diosgenin-50, 100 and 150 mg/kg) treated groups were compared with normal con-

trol. Statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple compari-

son test.



Graph 1. Effect of Diosgenin treatment on reticulo-endothelial system by carbon clearance test

with negative control. Highlighted delayed type hypersensitivity reaction suggests the activation of cellular immune system.

Diosgenin treatment on the reticuloendothelial system by carbon clearance test is shown in [Table 3]. The phagocytic activity of the reticuloendothelial system is generally measured by the rate of removal of carbon particles from the blood stream. In carbon clearance test, the phagocytic index of Diosgenin-treated (50, 100 and 150 mg/kg, p.o.) groups showed significant increased (** $p < 0.01$) in phagocytic index when compared with normal control. This indicates stimulation of the reticuloendothelial system.

DISCUSSION

Immunomodulation is a procedure which can regulate the immune system of an organism by interfering with its function. Modulation of the immune system by stimulation or suppression helps to maintain a disease-free state within an individual. Therefore, Immunomodulators have been used worldwide to control disease conditions. The study explored the immunomodulatory activity of Diosgenin by evaluating its effect on Haemagglutination antibody (HA) titer, DTH reactions, and on Phagocytic capacity of the reticuloendothelial system.

A haemagglutination test was performed to determine the effect of Diosgenin on humoral immunity. The humoral immunity is mediated by B lymphocytes which involves interaction of B cells with the antigen and their subsequent proliferation and differentiation into plasma cells which synthesize and secrete specific proteins called antibodies. Antibody functions as the effector of the humoral response by binding to antigen and neutralizing it or facilitating its elimination [15]. To evaluate the effect of Diosgenin on humoral response, its influence was tested by using SRBC's as antigen in HA titer. Cyclophosphamide showed significant inhibition in antibody titer response as it suppresses B lymphocytes proliferation while, Diosgenin counteract the sup-

pression of both primary and secondary humoral responses induced by Cyclophosphamide. The results from this study demonstrated that Diosgenin stimulate the production of antibodies in an immunocompromised animal.

The DTH reaction is type IV delayed or Cell mediated hypersensitivity reactions, results from the interactions of sensitized T cells with a specific antigen [16]. The resulting cell mediated immune response is mediated by either direct cytotoxicity or by the release of lymphokines which causes activation and accumulation of macrophages, increases vascular permeability, induces vasodilation and produces inflammation [4]. This results in the net increase in the thickness of the foot pad in previously immunized animals. In this study, the increase in footpad thickness of the rat that was subjected to Diosgenin treatment could be attributed to the ability of the Diosgenin to activate lymphocytes and enhancement in the production of antibodies in the previously immunosuppressed animal, responsible for increasing cell-mediated immunity.

Diosgenin showed stimulatory effect on the phagocytic capacity by exhibiting a clearance rate of carbon by the cells of the reticuloendothelial system (RES). The role of phagocytosis is the removal of microorganisms, foreign bodies, dead or injured cells. The increase in the carbon clearance rate reflects the enhancement of the phagocytic function of mononuclear macrophage and non-specific immunity [17].

CONCLUSION

Based on the findings from the study, Diosgenin showed immunostimulant effect on specific arms of immune system, the humoral and cell mediated immunity and nonspecifically activated the immune system by the activation of reticuloendothelial system because of saponin glycoside. Thus, it can be concluded that Diosgenin has potential therapeutic value in immunosuppression clinical conditions and useful to alleviate disease conditions.

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