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Research Paper

Evaluation of Immunomodulatory Activity of Diadzein in Balb/C Mice Using A Panel of *In- Vivo* Assays

Dhiman S., Neelu Sharma, Sisodia S.S. and Rajora M.

B. N. College of Pharmacy, Udaipur (Raj.) 313002 India

In order to evaluate the role of Diadzein purchased from Sigma Aldrich in the modulation of immune responses, detailed studies were carried out using a panel of *in vivo* assays. Oral administration of daidzein (12.5-100mg/kg) stimulated the IgM and IgG titer expressed in the form of hemagglutination antibody (HA) titer. Further it elicited a dose related increase in the delayed type hypersensitivity reaction (DTH) after 24, 48, and 72 h in BALB/c mice. The results in these studies demonstrated the immunostimulatory effect of Diadzein.

Keywords: Diadzein, Hemagglutination antibody titer, Delayed type hypersensitivity reaction, BALB/c mice, Alsever's solution.

INTRODUCTION

Treatment of human infections is challenging from time immemorial to present day, as many new infections have been taken birth and some old infections are resurfacing. Development of resistance to antibiotics and chemotherapeutic agents is observed in microorganisms. Due to this reasons there is need for continuous development of antibacterial agents. Synthetic compounds of derivatives quinazolinone have shown antibacterial activities, antifungal activities[1-6] antitumor activities[7]. Literature study reveals the antitumor[8-13], anti inflammatory[14-19] activity. The methods of synthesis of quinazolinone derivatives with different techniques have been reported.

***Address for Correspondence**
anilneelusharma@gmail.com

The immune system is organisation of cells and molecules with specialized roles in defending against infection.[1] Immunity is divided into two parts determined by the speed and specificity of the reaction. These are named the innate (nonspecific) and adaptive (specific) responses.[2] Defects or malfunction in either the innate or adaptive immune response can provoke illness or disease.[3] In recent days, lot of medicines, chemicals, as well as natural products have been introduced in order to stimulate the non-specific defense mechanism as well as specific immune response, these are termed as immunostimulants. The principal nutrients thought to provide the protection afforded by fruits and vegetables are antioxidants such as vitamin C, vitamin E, ß-carotene, and flavonoids

(including flavone, isoflavones, and anthocyanins). Citrus fruits are considered as good sources of both flavonoids and phenolic acids like anthocyanins, etc.[6,7] isoflavones (Daidzein) also possess characteristics such as antioxidant, antiproliferative, anti-inflammatory and differentiation-inducing abilities that may modulate immunity[8]. In an *in vitro* study conducted by Wang et al.[9] daidzein at concentrations of 0.01-10 µM significantly increased lymphocyte proliferation as well as secretion of the cytokines IL-2 and IL-3 by murine splenocytes in response to Con A and lipopolysaccharide (LPS) stimulation. This in vitro study showed the immunomodulatory activity of diadzein. In 1997, a study conducted by Zhang and colleagues was one of the first to generate *in vivo* evidence for daidzein-induced nonspecific immune function. Swiss mice fed either 20 or 40 mg/kg daidzein for 7 d showed significant increases in thymus weights and phagocytic activity of peritoneal macrophages. Lower daidzein feedings of 10 mg/kg did not seem to be effective.[10] The present aim of our study is to evaluate immunomodulatory activity of daidzein using different models in BALB/c mice.

Bioactivity studies of Diadzein

There is considerable epidemiological evidence, including a review of 21 studies on 26 different cancer sites that daidzein and genistein might provide protection from several types of cancer.[10] There is also epidemiological, animal, and *in vitro* evidence of daidzein and genistein effectiveness in the prevention of prostate cancer.[11] Daidzein inhibit atherosclerotic plaque formation by intervening at several steps in thrombus formation.[12] The pathogenesis of atherosclerotic plaque formation also involves, in addition to lipid accumulation, the infiltration of monocytes and T-lymphocytes into the artery wall, contributing to the thickening of the wall and occlusion of the vessel. Monocytes and lymphocytes adhere to the endothelial cell surfaces via the expression of certain "adhesion molecules." Infiltration and proliferation appear to be controlled by peptide growth factors. Increased levels of isoflavones, Diadzein particular, appear to alter growth factor activity, and inhibit cell adhesion and proliferation, all activities necessary for lesion formation in the intima of blood vessels. Animal studies demonstrated important lipid lowering effects of daidzein and genistein.[13] Animal studies have found soy protein isolates seem to enhance bone density, and epidemiological evidence points to diets high in soy as a possible protection against osteoporosis. Daidzein's weak estrogenic effect may also be involved in its possible

anti-osteoporotic activity. Daidzein has been found to have an anabolic effect in an osteoblastic cell line in culture, suggesting that it may be able to stimulate osteoblastic bone formation.[14] Daidzein has been found to have both weak estrogenic and weak anti estrogenic effects.[15] In vivo, Daidzein's estrogenic activity is one-fourth that of genistein .[16] Daidzein is also an antioxidant. [17]

MATERIALS AND METHODS

Reagents

96 V well microtitration plates and epindroff tubes from Tarson, trypan blue (Microlabs, Bombay), gum acacia, Alsever's solution, from Sigma were used.

Experimental animals

The study was conducted on male BALB/c mice (18–22 g). The ethical committee of the Bhupal Nobles College of Pharmacy Udaipur (Rajasthan) instituted for animal handling approved all the protocols. The animals were bred and maintained under standard laboratory conditions: temperature (25±2 °C) and a photoperiod of 12 h. Commercial pellet diet and water were given ad libitum.

Immunization schedule

Sheep red blood cells (SRBC) were used as a source of T-dependent antigen. For this purpose the blood was withdrawn from a healthy sheep in Alsever's solution.[18] SRBC used for immunization were prepared in pyrogen free normal saline. Mice were divided into eight groups, each consisting of six animals. Diadzin at 12.5 mg, 25 mg, 50 mg and 100 mg/kg (in 200 µL of normal saline) was administered orally by gavage for 15 days, daily. The dose volume was 0.2 ml, Control group received normal saline. Levamisole, a known immunostimulatory reported to augment the antibody response [19] was given orally as positive control, at a dose of 2.5 mg/kg body weight. All groups were immunized with 0.2 ml of SRBC (5×10^9) per mouse intraperitoneally (i.p.) on day 0 of drug treatment. Additional three immunized groups, challenged on day 7 with SRBC were used for DTH and different immunoglobulin assays.

Hemagglutination antibody (HA) titre

The animals were immunized by injecting 0.2 mL of 10% of fresh SRBC suspension intraperitoneally on day 0. Blood samples were collected in micro-centrifuge tubes from individual animals by retro-orbital plexus on day 7 for primary antibody titre and day 14 for secondary antibody titre. Serum was separated and antibody levels were determined by the hemagglutination technique.[20] Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25 µL volumes of normal saline in a micro-

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Table 1: Effect of Diadzin on antibody (IgG and IgM) titer

Samples	Dose (mg/kg)	Primary Antibody (IgM) Titre (After 7days)		Secondary Antibody (IgG) Titre (After14days)	
		Mean±S.E.M	%stimulation	Mean±S.E.M	% stimulation
Control		7.4 ± 0.37		7.6±0.26	
Levamisole	2.5mg/kg	9.2±0.29**	24.32 %	9.6±0.34**	26.31 %
Diadzein	11 12.5mg/kg	7.6±0.36	2.70%	7.9±0.37	3.94%
	25 25.0mg/kg	7.8 ±0.33	5.40 %	8.2 ± 0.18	7.89 %
	50.0mg/kg	11.6±0.26***	56.75 %	12± 0.41***	57.89 %
	100 mg/kg	9.6±0.21**	29.72 %	9.9 ± 0.28**	30.26 %

Antibody titer (IgM and IgG) in mice sera were measured on 7 and 14 days after immunization. Data are mean± S.E.M of five animals. **P<0.01 and ***P<0.001compared with control group determined by one way ANOVA (Bonferroni correction multiple comparison test)

titration plate to which were added 25 µL of 1% suspension of SRBC in saline. After mixing, the plates were incubated at room temperature for 1 h and examined for Hemagglutination under the microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

Delayed type hypersensitivity (DTH)

Daidzin (12.5, 25, 50 and 100 mg/kg) was administered 2 h after SRBC injection and once daily on consecutive days. Six days later, the thickness of the left hind footpad was measured with a spheromicrometer (pitch, 0.01 mm) and was considered as a control. The mice were then challenged by injecting 20 µL of 5×10^9 SRBC/mL intradermally into the left hind footpad. The footpad thickness was measured again after 24, 48, and 72 h. [20]

Statistical analysis

Data are expressed as Mean ± Standard error means (S.E.M). and statistical analysis was carried out using one-way ANOVA (Bonferroni correction multiple comparison test). Dunnett's test was used to analyze the different variables in the same subject and P values less than 0.05 were being taken as statistically significant.

RESULTS

Effect of Daidzein on anti-SRBC antibody titre

Anti-SRBC antibody (IgM and IgG) titres were measured in mice sera of different groups, collected retro-orbitally on 7 and 14 days after immunization and treatment. Anti-SRBC antibody titres increased in mice treated with three doses of Daidzein (12.5, 25, 50 and 100 mg/kg) after seven days when compared with control. A similar profile was obtained after 14 days, with IgG predominating over IgM (Table1).

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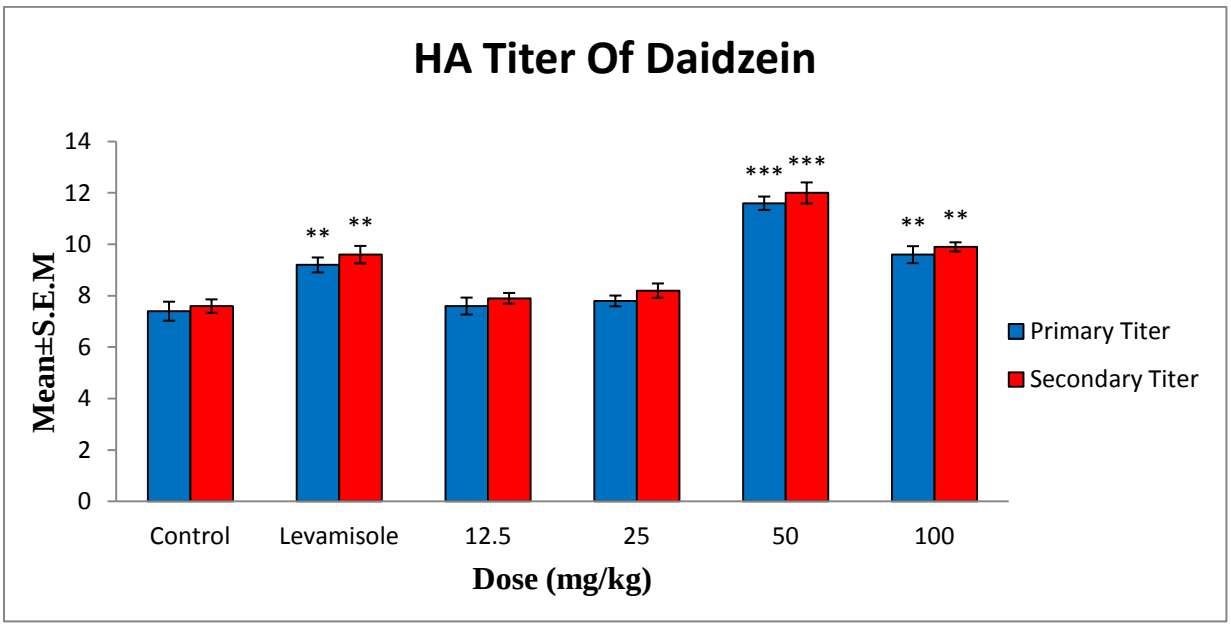

Figure 1: Effect of Daidzein on antibody (IgG and IgM) titer.

The maximum effect was observed at 50mg/kg in both primary and secondary antibody titre ($P < 0.01$). Further increase in dose (100 mg/kg) showed a decreased response. Administration of levamisole (2.5 mg/kg, p.o.), used as a positive control resulted in a significant increase in the humoral antibody titre compared with the control animals.

Effect of Daidzein on delayed type hypersensitivity (DTH)

In order to assess the cell-mediated immune response, DTH reaction to SRBC was measured as given in Table 2, in which data are expressed in terms of the swelling of the footpad. After administration of the Diadzin (12.5–100 mg/kg, p.o.), a significant increase

Table 2: Effect of Daidzein on delayed type hypersensitivity reaction (DTH) to T-dependent antigen SRBC

Sample	Dose(mg/kg)	Foot pad thickness(mm)		
		Mean±S.E.M (24hr)	Mean±S.E.M (48hr)	Mean±S.E.M (72hr)
Control		1±0.048	2±0.029	1.33±0.034
Levamisole	2.5mg/kg	4±0.36**	8±0.037**	1.59±0.028**
Daidzein	12.5mg/kg	5±0.041	9±0.035	1.41±0.037
	25mg/kg	8±0.028	3±0.022	1.47±0.032
	50mg/kg	2±0.031***	9±0.044***	1.78±0.027***
	100mg/kg	7±0.029*	1±0.034*	1.53±0.045*

DTH response was determined in SRBC immunized, Daidzein treated mice at 24, 48, and 72 h after antigen challenge. Data are mean± S.E.M of five animals.*P<0.05, **P<0.01 and ***P<0.001compared with control group determined by one way ANOVA (Bonferroni correction multiple comparison test)

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(P<0.01) in footpad thickness was found at 24 , 48 and 72 h as compared with the control group: maximum increase being observed at 50 mg/kg. Further increase in dose (100 mg/kg) showed a decreased response.

DISCUSSION & CONCLUSION

There are a number of diseases where immunostimulant drugs are required to overcome the immunosuppression induced by drugs or environmental factors and Immunosuppressants are required where there is undesired immunopotentiation.

There is strong requirement of the drugs which can boost immune system to combat the immuno-suppressive consequences caused by stress, chronic diseases like tuberculosis, conditions of impaired immune responsiveness (e.g. research studies show that citrus fruits are one of the few known food sources that are a rich source of AIDS),etc.[22] Convincing phytochemical powerful agents especially anthocyanins and flavonoids. Anthocyanins convey marked antioxidant activity via the donation of electrons or hydrogen atoms from hydroxyl moieties to free radicals.[23, 24] Flavonoids play some important pharmacological roles against diseases, such as cardiovascular disease, cancer, inflammation and allergy.[25,26] Isoflavone daidzein found in sour cherries could stimulate murinenon-specific immunity, activate humoral immunity and enhance cell- mediated immunity.[27] Various experiments have been conducted reporting the immunomodulatory action of citrus

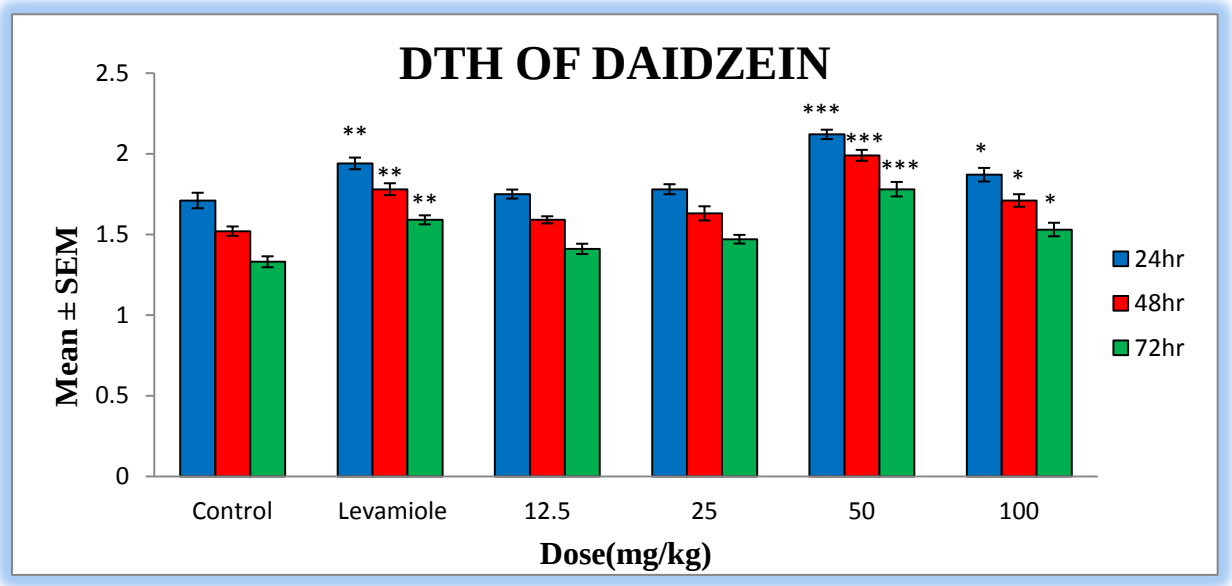

Figure2: Effect of Daidzein on delayed type hypersensitivity reaction

fruits on macrophage and lymphocyte In the past a number of drugs with plant or mineral origin have been advocated as means of immunomodulation for various diseased conditions in humans[30] Side effects associated with allopathic drugs along with their high cost have enforced the need for search of alternative drugs with least or no side effects, especially those belonging to the traditional system of medicine like Ayurveda.

Most of the plants so far reported with immunostimulatory action have major effect on the non-specific arm of immunity especially on macrophage functions.[31] This investigation deals with the isolated Diadzein which was exploited for its immunostimulatory activity. Diadzein was found to be a pronounced immunostimulator at the tried doses of 50 and 100 mg/kg in BALB/c mice in a dose dependent manner with maximum stimulation observed at 50 mg/kg dose. Levamisole, which is attracting more attention owing to its use as an immunomodulator, in supporting anti-carcinogenic drugs, in the treatment of skin diseases and in improving weight gain in animals, was used as a reference standard in this functions in BALB/c mice.[28,29] study.[32] Levamisole was used at 2.5 mg/kg this investigation and this dose was selected out of the several doses tried in our lab earlier to optimally stimulate the various humoral and cellular immune parameters of mice. In the present study, immunomodulatory potential of Diadzein was explored extensively on the modulation of both T and B-cells in relation to serum immunoglobulins IgM and IgG to T-dependent antigen SRBC. Primarily, the antibody response to SRBC was observed by the hemagglutination titre. The augmentation of humoral antibody response to T-dependent antigen (SRBC) reveals the increased responsiveness of macrophages since the antibody production is closely associated with the co-operation of macrophages, T and B lymphocyte responsiveness.[33] The T cells in turn participate in the expression of cell mediated immunity contributing to DTH. A DTH reaction is an expression of cell-mediated immunity and plays a role in many inflammatory disorders.[34] Treatment with Diadzein enhanced the DTH reaction, as reflected by the increased foot- pad thickness compared to the

control group, suggesting heightened infiltration of macrophages to the inflammatory site. It is clear from this study that Diadzein played an important role in the modulation of the immune response and thus may have applications in combating various life-threatening infections. Therefore, it could be a drug of choice, effective in treating the diseases where the underlying defect is a T-cell and B-cell deficiency or phagocytic dysfunction.

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