Original Article

Study the Immunomodulatory Effects of Combined Extracts of *Sesbania grandiflora* Flowers and *Cocculus hirsutus* Leaves on the Circulating Antibody Response

Mallik Arunabha* **and Nayak Satish**

Department of Pharmacology, Bansal College of Pharmacy, Bhopal (M.P.), India

Address for Correspondence

Department of Pharmacology, Bansal College of Pharmacy, Bhopal (M.P.), India
E-mail: mallikpharmacology@gmail.com

ABSTRACT

Polyherbal therapy is said to be a current pharmacological principle having the advantage of producing maximum therapeutic efficacy with minimum side effects. We assessed the Immunomodulatory effects of a combination of extracts from Sesbania *grandiflora* flowers and *Cocculus hirsutus* leaves. In this work we detected the IgM and IgG levels in sera from mice treated with the HP-I, HP-II and HP-III against SRBC in ELISA methods at 0, 4, 7 and 14 days. In addition, we analyzed the phytochemical properties of the different herbal preparations. After oral administration of the dose of combined extract (100-400 mg/ kg b.w.) increase the serum antibody titers after four and seven days, suggesting immunostimulatory activity through modulation of B lymphocyte functions. These results suggested that HP-I from 1:1 of both plants contained several chemical compounds that possess positive modulator effects on the immune system.

Keywords: *Sesbania grandiflora*, *Cocculus hirsutus*, IgG, IgM, ELISA.

INTRODUCTION

Drug formulation in *Ayurveda* is based on two principles: Use as a single drug and use of more than one drug, in which the latter is known as PHF. This key traditional therapeutic herbal strategy exploits the combining of several medicinal herbs to achieve extra therapeutic effectiveness, usually known as polypharmacy or polyherbalism[1]. The *Ayurvedic* literature "*Sarangdhar Samhita*" dated centuries ago in 1300 A. D. Has highlighted the concept of polyherbalism in this ancient medical system[2]. In the traditional system of Indian medicine, plant formulations and the combined extracts of plants are chosen rather than individual

ones. It is known that *Ayurvedic* herbs are prepared in a number of dosage forms, in which mostly all of them are PHF[3]. Moreover, polyherbal therapies the combination of various types of agents from different plant sources, can be used to enhance efficacy. Polyherbal therapies have the synergistic, potentiative, agonistic/ antagonistic pharmacological agents within themselves that work together in a dynamic way to produce therapeutic efficacy with minimum side effects.[4]

Sesbania grandiflora(L.) Pers. (Fabaceae), known as Agasti or swamp pea, is an important medicinal plant and native to many Asian countries including India. The bark, leaves, gums, and flowers are considered medicinal. They are used as diuretic, emetic, febrifuge, laxative, and tonic.[5] In *Rasashastra*, it is used for processing of various formulations. The tender leaves, green fruit and flowers are eaten alone as a vegetable or mixed into curries or salads in various parts of South Asia.[6] A tea made from the leaves is believed to have antibiotic, anthelmintic, antitumor, and contraceptive properties.[7] The bark is considered as a tonic and an antipyretic, and a remedy for gastric troubles and diabetes. The principal medicinal effects are due to the tree's astringency; hence it is used against inflammation, venom and other poisons, bacterial infections and tumour.[8]

Sesbania grandiflora L flower and other parts of plants contains proteins, tannins, oleanolic acid, keampferol, grandifloral, cystine, isolucine, aspargine, phenylalamine, valine, nicotinic acid and vitamine C.[9] These chemical constituents are well known for their potential health benefits and have been reported to possess valuable biological ctivities such as antibacterial and antifungal[10], antioxidant[11], anticonvulsant and anxiolytic[12], and hepatoprotective properties[13] and hypo-lipidemic effect. In a more recent study, it was found that the of Sesbania grandiflora leaves could also afford a significant hypolipidemic effect against Triton induced hyperlipidemia in rats.[14]

Cocculus hirsutus (L.) belonging to family Menispermaceae is widely growing plants found in tropical and subtropical region in India in dry localities and used medicinally by the Indian tribes for wide range ailments including constipation and kidney problem.[15,16]

It is believed to be *Cocculus hirsutus* is useful in treating 'Kapha' and 'Vata' conditions, poisonous bites, leprosy, skin diseases, pruritis, dyspepsia, bronchitis, gout, spermatorrhoea and hypertension.[17,18] Leaves are used in the treatment[19], hyperglycaemic[20], neuralgia and anti-inflammatory.[21] Roots are used for the treatment of anti-inflammatory and analgesic[22] rheumatism, dyspepsia, skin diseases, impotency[23] and constipation.[24]

The plant of *Cocculus hirsutus* has been reported to contain essential oil, β-sitosterol, ginnol, glycosides, sterols and alkaloids.[25,26] Preliminary phytochemical analysis of the leaves showed presence of alkaloids, phenolic compounds, flavonoids, glycosides, and carbohydrates.[27] The phytochemical studies show the presence of bis-benzyl isoquinoline alkaloids; viz. shaheenine, cohirsinine, hirsutine, jamtinine, jamitine- N -oxide, cohirsine , cohirsitine and haiderine.[28]

An earlier study in our laboratory compared the effect of extracts from the *Sesbania grandiflora* [29] and *Cocculus hirsutus*[30] separately on humoral and cell-mediated immunity. The present study was designed as to find out the potential combinations for the circulating antibody response and with a conventional Immunomodulatory, Levamisole.

MATERIALS AND METHODS

Plant Materials

The flowers of *Sesbania grandiflora* and *Cocculus hirsutus* leaves were collected from Bhopal District, Madhya Pradesh, India. The Prof. Madhuri Modak, Professor, Department of Botany, M.V.M. College, Bhopal, Madhya Pradesh, India, conducted further taxonomic identification.

Preparation of the extract

The individual *Sesbania grandiflora* and *Cocculus hirsutus* powdered drug was taken and subjected for successive solvent extraction. The extraction was carried out for 18 hrs with the following solvents with a ratio 1:4 w/v, in the increasing order of the polarity i.e. Petroleum ether, chloroform, methanol.

Preparation of combination of herbal preparation

Combination of methanolic extracts of two herbals *Sesbania grandiflora* flowers and *Cocculus hirsutus* leaves have been taken in different ratios to prepare different three herbal extract combinations by trial and error method as HP-I (1:1), HP-II(1:2) and HP-III(2:1). These three combinations were used in further experiments.

Qualitative Chemical Examination of Extracts

To identify the essential constituents of the Combined extracts of *Sesbania grandiflora* flowers and *Cocculus hirsutus* leaves such as alkaloids, terpenes and steroids, saponins, flavonoids, poly-saccharides and tannins, a preliminary phytochemical screening was carried out using various test methods of Dragendroff's and Mayer's test, Liebermann–Burchard test, foam formation test, lead acetate test, Molish's and Fehling's test and ferric chloride test.[31]

Acute Toxicity Study

Healthy female albino mice weighing 25-30g, maintained under controlled conditions of temperature (20 - 25^{o}C) and humidity (55%) were used for toxicity study as per the internationally accepted protocol drawn under the OECD guidelines 423. The over night fasted animals were administered orally at the dose level of 2000 mg/kg body weight by gastric intubation and were observed for toxic symptoms such as behavioural change, locomotion, convulsion and mortality for 48 hours. Based on the study the doses were selected for the evaluation of immunomodulator activity.[32]

Preparation of SRBC membrane antigens for ELISA

Briefly, sheep blood was collected in Alsever's solution and cells were isolated by natural process at 1000 revolutions per minute for fifteen min. Once removal of plasma and therefore the buffy coat, the cells were washed with 5 volumes of 0.9% NaCl 3 times. The washed pellets were suspended in approximately two volumes of 0.05 M Tris-HCl with 0.1 mM EDTA (pH 7.6), were mixed completely, and were centrifuged at 25000 revolutions per minute for thirty min. This method was recurrent as necessary until the supernatant became clear. The pellet suspension was more filtered through 3 layers of gauze, centrifuged once more and resuspended in 0.1% sodium dodecyl sulphate (SDS) with 0.02% sodium azide (three times pellet volume). The solubilized membrane antigens were dialyzed against 0.1% SDS in PBS and hold on at -20^{o}C.[33]

Experimental design

The mice were divided into different groups, each consisting of 6 animals.

Control group of animals received 1 mL 1% SCMC for 14 days , Standard group

of animals received Levamisole at 50 mg/kg b.w. for 14 days and the test groups administered at dose 100, 200 and 400 mg/kg b.w. respectively for 14 days.

Mouse blood sample were obtained at the time of immunization (day 0) and 4, 7 and 14 days. Anti-SRBC circulating antibody titers of mice were determined by ELISA method. SRBC antigen, in aliquots of a 5 µg/mL suspension in PBS, pH 7.2 (125 µL/well), was incubated during the night at 4ºC in high-binding micro plates. Before every subsequent step, plates were washed 3 times with PBS-T. Unbound sites were blocked with 3% powdered milk powder in PBS (200 µL/well) for two h at room temperature (RT). Serum from experimental animals diluted 1:8 in PBS-T was added to the wells (125 µL/well) followed, once one h incubation at RT, by peroxidase conjugated anti-mouse IgG (1:1000) and anti-mouse IgM (1:2000) diluted in PBS-T (125 µL/well). Once another hour at RT, OPD substrate was added (200 µL/well) and therefore the plates were incubated within the dark for twenty min at RT. The reaction was stopped with 2M H_2SO4 (75 µL/well) and therefore the color every well was evaluated at 492 nm on ELISA reader.[34]

RESULTS

In the qualitative phytochemical analysis, HP-I, HP-II and HP-III were found to show positive results for the presence of alkaloids, glycosides, saponins, phenolic compounds and tannins, amino acid, and carbohydrates. These details of the results are summarized in **Table 1**.

As per GHS classification, HP-I, HP-II and HP-III were found to be of class 4 (>300 to 2000 mg/kg, b.w.); However, based on mortality in either steps of acute oral toxicity test guidelines 423, different LD50 cut-off dose (in mg/kg, b.w.) were determined. Accordingly, the individual effective lower dose Median dose and higher dose were determined (in mg/kg, b.w.) as 100, 200 and 300 mg/kg, b.w.

A significant IgM titer increase was observed for doses, 100, 200 and 400 mg/kg of HP-I and in comparison to the positive control, four days after immunization and treatment. The increase was also observed in animals immunized and treated with HP-I, 100-400 mg/kg ($p < 0.01$) compared to positive controls seven days after treatment. IgG titers also increased significantly in mice treated with all doses of HP-I ($p < 0.001$) after seven days when compared with immunized only mice (positive control). A similar profile was obtained after 14 days. See (**fig. 1)**

IgM titer increase was observed for doses, 200 and 400 mg/kg ($p < 0.01$and $p < 0.001$ respectively) of HP-II in comparison to the positive control, four days after immunization and treatment. IgG titers also increased significantly in mice treated with 200 and 400 mg/kg of HP-II ($p < 0.001$) after seven days when compared with immunized only mice (positive control). A significant also observed with 400 mg/kg ($p < 0.05$) after fourteen days. See (**fig. 2)**

A significant IgM titer increase was observed for doses, 100, 200 and 400 mg/kg ($p < 0.05$, $p < 0.001$ and $p < 0.001$ respectively) of HP-III in comparison to the positive control, four days after immunization and treatment. The increase was also observed in animals immunized and treated with HP-III, but less significance for doses 200 and 400 mg/kg ($p < 0.05$ and $p < 0.01$ respectively). IgG titers also increased significantly in mice treated with 200 and 400 mg/kg of HP-I ($p < 0.001$) after seven days when compared with immunized only mice (positive control).A significant also observed with 200 and 400 mg/kg ($p < 0.05$) after fourteen days. See **(fig. 3)**

DISCUSSION

The humoral immune response can be measured as an increase levels of total antibodies or of specific antibodies against a non-pathogenic antigen such as SRBCs. The level of a specific antibody in the serum can also be used as a measure of the functional status of humoral immune-antigen recognition, activation, and expression. The integrity of the antibody mediated (humoral) immune response depends upon a competent population of B-lymphocytes, which have the functional capacity to develop into antibody producing plasma cells. Interpretation of the antibody response to SRBCs is complicated by the fact the blood cells are antigenically complex. Animal can make antibodies against foreign blood cells. These pre existing or natural antibodies are not derived from prior contract with foreign RBC, but result from exposure to similar or identical epitopes that commonly occurs in nature. Because of the antigenic complexcity of SRBCs, it is not known against which particular antigens the antibodies are being raised. Therefore, ELISA method was used to detect specific antibodies produced against certain antigens.[35] In this study the methanolic extracts of *Sesbania grandiflora* and *Cocculus hirsutus* both dose significantly increase or activate the secretion of immunoglobins such as IgM and IgG.

In our study emphasized that the combining ratios of the polyherbal Immunomodulatory remedies are equally important. Combining *Sesbania grandiflora* and *Cocculus hirsutus* in the ratio of 1:1 of 100-400mg/kg gives good immuno-mudulatory activity compared with 1:2 and 2:1 ratios.

The individual properties of these extracts provide cell mediated (by increasing DTH reaction) humoral (by increasing antibody trite level in heametaglutuniation reaction) phagocytises and protected the suppressive effect of cyclophosphamide.[29,30]

The combination of the *Sesbania grandiflora* and *Cocculus hirsutus* methanolic extract provides a more holistic efficacy desired in the stimulant of circulating antibody response at low dose. However, the result of the study showed that combination of both plants provides the best immunostimulant effect when the ratio of *Sesbania grandiflora* and *Cocculus hirsutus* are equal or when *Sesbania grandiflora* is more in the combined product. That might be possible by the pharmacodynamic and pharmacokinetic interaction of combined extract (herbal preparation).

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Table 1. Phytochemical investigation of Herbal Preparation

Chemical tests	HP-I	HP-II	HP-III
Alkaloids	++	++	+
Glycosides	+++	+	++
Saponins	+++	++	++
Phytosterols	-	-	-
Phenolic compound Tannins	+++	++	+++
Proteins and Amino acids	+	+	+
Carbohydrates	+++	+	++

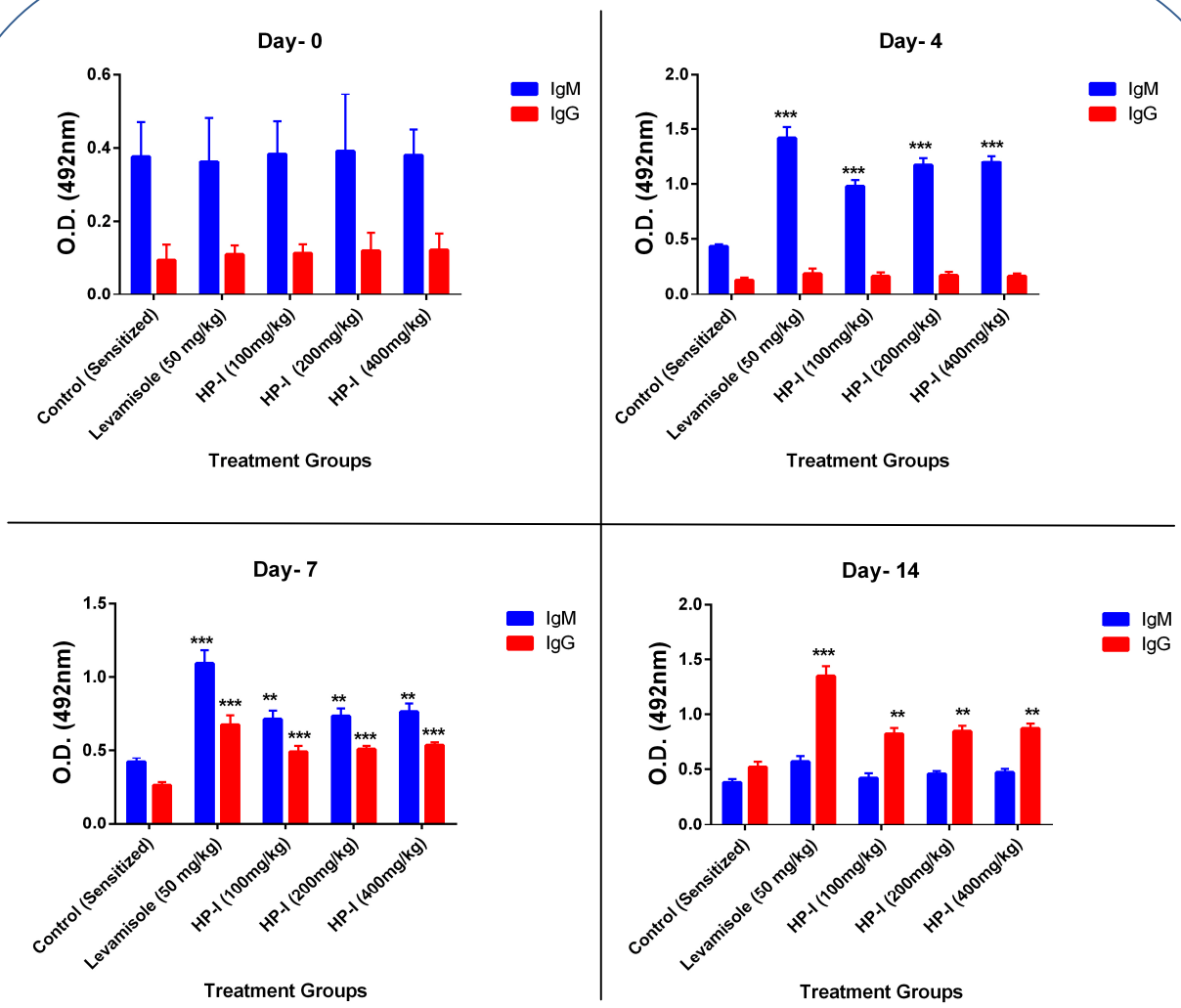

Figure 1. Detection of IgM and IgG levels in sera from mice treated with the HP-I against SRBC in ELISA. Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P<0.05$, **: $P<0.01$***: $P<0.001$ comparing with the control group.

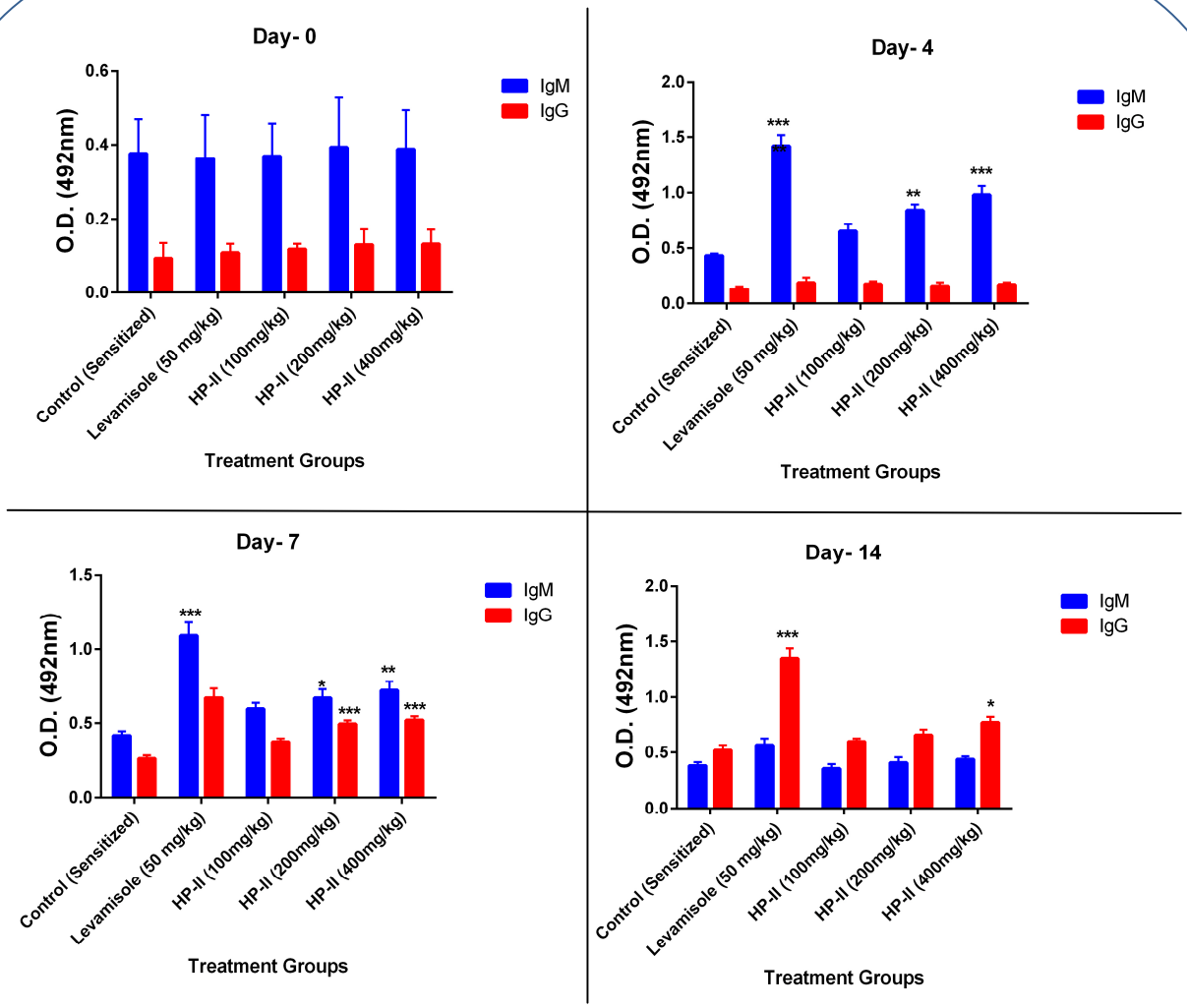

Figure 2. Detection of IgM and IgG levels in sera from mice treated with the HP-II against SRBC in ELISA. Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P<0.05$, **: $P<0.01$***: $P<0.001$ comparing with the control group.

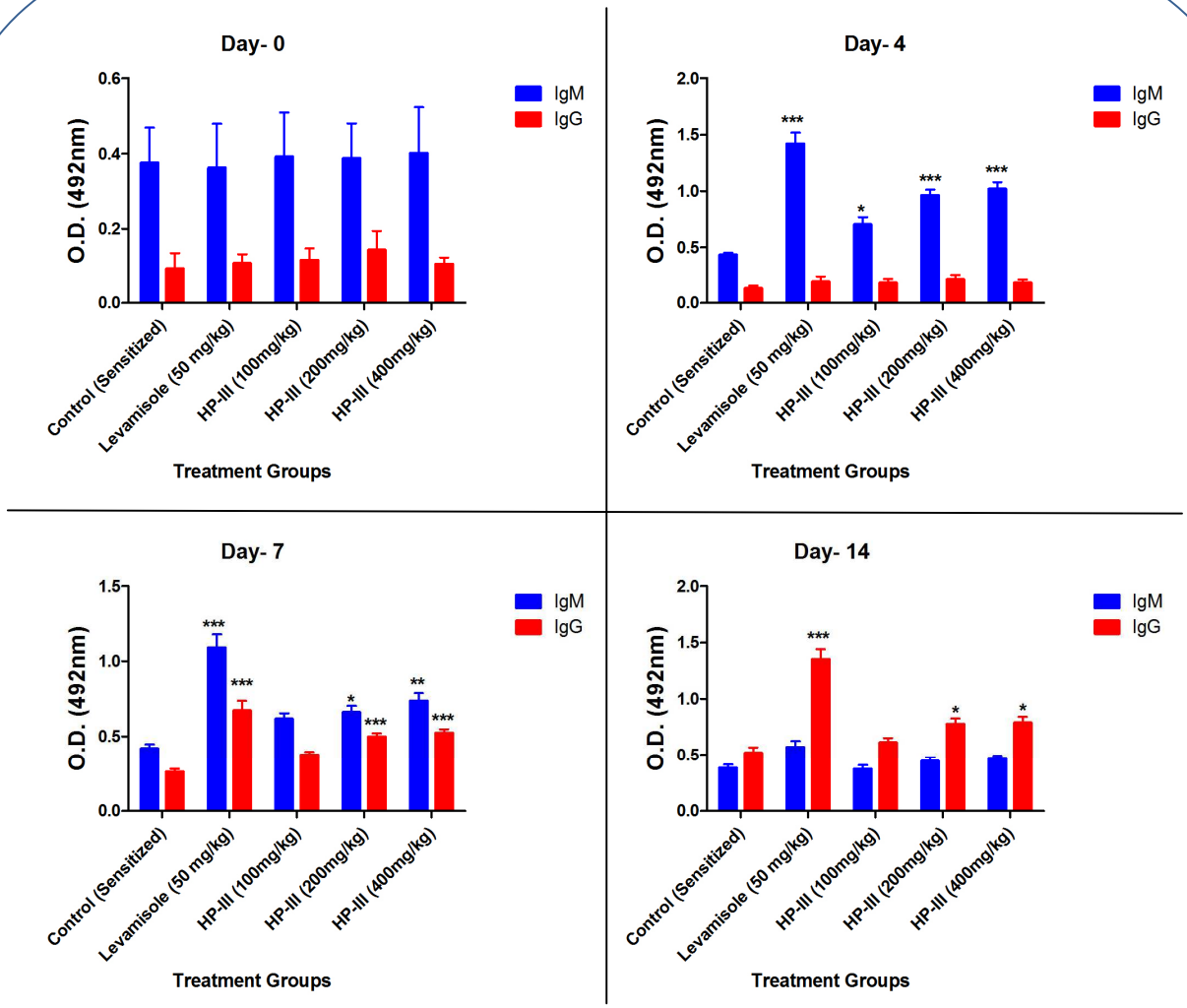

Figure 3. Detection of IgM and IgG levels in sera from mice treated with the HP-III against SRBC in ELISA. Statistical analysis was carried out employing the ANOVA followed by Dunnett test *: $P<0.05$, **: $P<0.01$***: $P<0.001$ comparing with the control group.