An Elsevier Indexed Journal ISSN-2230-7346

Journal of Global Trends in Pharmaceutical Sciences

Research Article

IMMUNOMODULATORY ACTIVITY OF FORMONOETIN-7-*O*-B-D-GLUCOPYRANOSIDE FROM *OPERCULINA TURPETHUM*

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ARTICLEINFO

Key words:

Operculina turpethum, Formonoetin-7-*O*-β-D-glucopyranoside, Neutrophil adhesion, Phagocytic activity, Antibody titre, Delayed-type hypersensitivity.

ABSTRACT

To investigate the immunomodulatory activity of isolated compound Formonoetin-7-*O*-β-D-glucopyranoside from Methanolic Extract of *Operculina turpethum*. The isolated compound was characterized by spectral techniques namely FTIR, ^{1}H NMR, ^{13}C NMR and mass. Due to the paucity of the compounds **1** and **2**, the compound (**3)** Formonoetin-7-*O*-β-D-glucopyranoside was subjected to evaluate the immunomodulatory activity by the neutrophil adhesion test, phagocytic activity, delayed type hypersensitivity response and antibody titre. Administration of (**3**) at the doses of 10, 20, 40, 50 mg/kg b. w. p. o. exhibited significant ($p<0.05$) increase in percent neutrophil adhesion to nylon fibers as well as a dose-dependent increase in antibody titre values and phagocytic activity, potentiated the delayed-type hypersensitivity reaction induced by sheep red blood cells. It is concluded that Formonoetin-7-*O*-β-D-glucopyranoside from the methanol extract of *Operculina turpethum* have produced a significant immunomodulatory activity and possess a promising therapeutic potential for the prevention of autoimmune diseases.

INTRODUCTION:

Plants have been used throughout the world as drugs and remedies for treatment of various diseases as they have great potential for producing new drugs of great benefit to humankind [1]. There are many approaches to search for new biologically active principles in higher plants.

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Natural flora has gained its attention in the treatment of common cold to dreadful diseases viz., AIDS, Cancer, etc, such plants are called as medicinal plants which have curative properties due to the presence of various complex chemical substances of different composition, viz., grouped as alkaloids, glycosides, corticosteroids, terpenoids, isoflavanoids, steroids etc. Plants based products have been in use for medicinal value or other purposes right from the dawn of civilization, the traditional remedies of the ancient world were all based on natural products[2].

Operculina turpethum is an perennial with milky juice; root long, slender, fleshy, much branched; stems very long, twining and much twisted together, angled and winged,

pubescent, tough and brown when old[3]. Flowering occurs mainly between August and October. In addition, fruiting from November to December[4]. This plant is widespred in old tropics from E. Africa to N. Australia, this plant common in Godavari in andhra pradesh. It is widely distributed in tropical Africa and Asia. In India it is found in damp and it occurs almost throughout India up to an altitude of about 1000 m, it is sometimes grown in gardens for its beautiful flowers[5]. It is rare on open sandy soils. In addition, it is occasionally cultivated in India. It is found throughout India in open Distributed habitats and in woody thickets and hedges to an altitude 900-1000 m. occasionally grown in gardens as an ornamental plant[6].

2.MATERIALS AND METHOD

2.1 Plant material

Operculina turpethum was collected from the herbal garden of Tirunelveli district of Tamilnadu during the month of December 2015. The plant material was identified and authenticated by Dr. J. Jayaraman, Director, Plant Anatomy Research Centre, West Tambaram, Chennai. Voucher specimen (No. PARC/2015/986) was preserved for future references.

2.2 Animals

The experiment was performed on healthy male and female Sprague Dawley (SD) rats of eight weeks old and body weight of 140-160 g. The female rats were nulliparous and non-pregnant. The rats were fed with standard laboratory diets, given water *ad libitum* and maintained under laboratory conditions of temperature 22°C (± 3°C), with 12 h light and 12 h dark cycle. All experimental procedures were carried out in strict accordance with the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experimentation on Animals (Regd. No. 1696/PO/a/13/CPCCSEA).

2.3 Drugs

All the reagents used were of analytical grade. Methanol, n-hexane, chloroform, sodium chloride, carboxymethyl cellulose were purchased from Molychem, Mumbai, India. Levamisole from H. Chandanmal & Co, Chennai, India. Alsever's solution was purchased from Sigma (St. Louis, MO, USA).

3. EXTRACTION AND ISOLATION OF COMPOUND

3.1 Collection of plant material and preparation of extract

Fresh plant material of *Operculina turpethum* was collected from the herbal garden of Tirunelveli, Tamilnadu. The dried plant material was extracted for 72 h with 7 L of 70 % methanol (v/v) at 50 °C. The extract was evaporated under reduced pressure to furnish a dark brown thick semi solid residue. The preliminary phytochemical investigations were carried out with methanolic extract of *Operculina turpethum* (MEOT) for qualitative identification of phytochemical constituents.

3.2 Isolation of the compounds

MEOT was subjected for fractionation to isolate a compounds using flash column, HPLC, sephadex LH-20. The isolated compounds were identified as Quercetin (**1**), Formonoetin (**2**), and Formonoetin-7-*O*-β-D-glucopyranoside (**3**) by spectral techniques. In this study, F-7-β-D-g (compound **3**) was used in the treatment protocol because of minimal yields of compounds **1** (0.75 mg) and **2** (0.82 mg).

4. PHARMACOLOGICAL EVALUATION

4.1 Acute Toxicity Studies

Acute toxicity study for compound **3** was performed in Wistar albino mice as per OECD guideline 425 (OECD. 2001). Animals were divided into 3 groups of *3 animals in a group.* Female, nulliparous and non-pregnant mice weighing between 18-22g was selected for this study. The animals were kept fasting overnight provided only with water. The dose progression study was carried out at three different dose levels of 100 mg/kg, 250 mg/kg and 500 mg/kg and observed for mortality during 48 h study period. The dose was administered only once for each group. The dose at which mortality was observed in two out of three mice was considered as toxic dose. All the animals were observed twice daily for health condition, morbidity and mortality for 14 days[7].

4.2 Selection of doses

The value of LD_{50} for compound **3** was calculated using acute oral toxicity study. As the lethal dose was found at 500 mg/kg b. w. p. o.,1/10th of the dose of 50 mg/kg b.w. p. o. was taken as the test dose for further study.

4.3 Treatment protocol

Wistar albino rats were divided into 6 groups of *6 animals in a group viz*. Group I served as control and received 0.5 ml of 2% w/v carboxymethyl cellulose suspension p.o, the treatment groups II - V were orally at four divided doses of isolated compound **3** (10, 20, 40 and 50 mg/kg). Group VI were given standard drug Levamisole (50 mg/kg, p.o) for 14 days.

4.4 Antigen

Fresh blood was collected in Alsver's solution in 1:1 proportion from local slaughter house where sheep are sacrificed. Sheep red blood cells (SRBCs) were separated by centrifugation and were washed three times in normal saline and adjusted to a concentration of 1×10^9 cells/ml for immunization and challenge[8].

4.5 Immunomodulatory activity – Neutrophil adhesion test

On the 14th day after drug treatment, bloodsamples were collected by puncturing the retro orbital plexus into heparanized vials and analyzed for total leucocyte count (TLC) by Sysmex cell counter and differential leucocyte count (DLC) by fixing blood smears and staining with Leishman's stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibers for 15 min at 37 °C. The incubated blood samples were again analyzed for TLC and DLC. The product of TLC and % neutrophil gives neutrophil index (NI) of blood sample. Finally percent neutrophil adhesion was calculated [9].

$$\text{Neutrophil adhesion (\%)} = \frac{NI_u - NI_t \times 100}{NI_u}$$

Where NIu = Neutrophil index of untreated blood samples and NI_t = Neutrophil index of treated blood samples

4.6 *In vivo* phagocytic activity by carbon clearance assay

The control group received vehicle whereas the treatment groups were administered with test drug orally daily for 20 days. Colloidal carbon solution, Rotring ink® (Hamburg, Germany) was diluted with normal saline (1:8), and injected (0.01 ml/g body weight) was via tail vein to each animal 24 h after last dose. Blood samples were drawn from retroorbital plexus under ether anesthesia at 2 and 15 min after injection. Blood (25µl) was mixed with 0.1 % sodium carbonate (2 ml) for the lysis of erythrocytes OD was recorded at 660 nm [10]. The phagocytic index (K) was calculated by using following equation: The phagocytic index (K) was calculated by using following equation:

$$K = (\ln OD1 - \ln OD2) / (T2 - T1),$$

where OD_1 and OD_2 are the optical densities at times T1 and T2, respectively.

4.7 Delayed type hypersensitivity response

This test was done to measure the cellular immunity. On day 0, all rats were immunized with 0.5×10^9 /ml of SRBC by S.C route. The rats were treated orally with test and standard drug for 14 days. On the day of termination, the thickness of right hind foot pad was measured at 24 h using Schnelltaster caliper (H.C. Kroplin Hessen, Schluchtern, Germany). Immediately after, the animals were challenged by S.C administration of 0.5×10^9 /ml SRBC into right hind foot pad and its DTH responses were measured after 24 h. The difference between pre and post challenge of foot pad thickness is expressed in mm which reveals the measure of delayed type of hypersensitivity[11].

4.8 Humoral Antibody (HA) Titre

Haemagglutination test measures the humoral immune response. Rats were administered with the assigned treatment for 14 days. On day 0, all rats were immunized with 0.5×10^9/ml of SRBC by i.p. route. Blood samples were collected by retro orbital puncture on 7th day for primary antibody titre and on 15th day for secondary antibody titre. Serum was separated by centrifugation at 1500 rpm for 5 min and the hemagglutination titre was determined using microtitre plates. Two fold dilutions of pooled serum samples were made in 25 µl of normal saline in 96 well micro titre plates and 25 µl of 1% suspension of 1×10^9/ml SRBC in saline was added. After

mixing, the plates were incubated at 37 ˚C for 2 h and examined for agglutination. The highest number of dilution of serum showing button formation at the bottom of the well was taken as haemagglutination titre [12].

4.9 Statistical analysis

The experiment results are expressed as means ± SE. Statistical analysis of the data obtained from the experiment was carried out using the one way analysis of variance (ANOVA) followed by Dunnett's multiple range test. P-values <0.05 was considered as statistically significant using SAS software -Version 6 (*SAS* Institute, Cary, North Carolina).

5. RESULT

5.1 Acute oral toxicity study

In acute oral toxicity study, Formonoetin-7-*O*-β-D-glucopyranoside (**3**) administration does not produce mortality and any clinical signs of toxicity within 48 h over a period of 14 days. LD_{50} dose of compound **3** was found to be 500 mg/kg b. w. p. o. and 1/10 of LD_{50} dose can be termed therapeutic dose.

5.2 Immunomodulatory activity - Neutrophil Adhesion Test

In control group, the percent neutrophil adhesion was found to be 28.21 while in treatment groups II – VI, administration of compound **3**. A statistical significant difference (P<0.05) was observed in the treatment group for neutrophil adhesion which is comparable with the control group (Table 1).

5.3 *In vivo* phagocytic activity by carbon clearance assay

Effect of compound **3** on the phagocytic activity by the carbon clearance test is shown in Table 2. Formonoetin-7-*O*-β-D-glucopyranoside (**3**) showed significant (p<0.01) increased in phagocytic index when compared to control group. This indicates stimulation of the reticuloendothelial system.

5.4 Delayed type hypersensitivity response

SRBC induced delayed type hypersensitivity was used to assess the effect of the compound on cell mediated immunity. In treatment group, administration of compound **3** produced the % inhibition of foot pad thickness as 17.3, 22.4, 40.5 and 45.6 at the dose of 10, 20, 40 and 50 mg/kg b.w. p.o whereas in the standard group, Levamisole produced 51.9 at the dose of 50 mg/kg, b.w. p.o. The control group does not possess the ability to decrease the foot pad thickness (Table 3).

5.5 Haemagglutinating Antibody (HA) Titre

The augmentation of the humoral immune response to SRBC by the compound **3** at the dose of 10, 20, 40 and 50 mg/kg, b.w.p.o and Levamisole 50 mg/kg, b.w.p.o. showed statistically significant difference (*P <0.05) when compared to control group (Table 4).

DISCUSSION

Immunomodulatory agents of the plant and animal origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system. Cytokines are secreted by activated immune cells for margination and extravasation of the phagocytes mainly polymorphonuclear neutrophils[13]. Significantly evoked increase in the adhesion of neutrophils to nylon fibers which correlates to the process of margination of cells in blood vessels. In the present study, AI evoked a significant increase in percent of neutrophils. This may help in increasing immunity of body against microbial infections. The role of phagocytosis is the removal of microorganisms and foreign bodies, dead or injured cells. The increase in the carbon clearance index reflects the enhancement of the phagocytic function of mononuclear macrophage and nonspecific immunity. Phagocytosis by macrophages is important against the smaller parasites and its effectiveness is markedly enhanced by the opsonisation of parasites with antibodies ans leading to a more rapid clearance of parasites from the blood[14]. Compound **3** appeared to enhance the phagocytic function by exhibiting a clearance rate of carbon by the cells of the reticulo-endothelium system.

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines).The augmentation of the humoral response was evidenced by an enhancement of antibody responsiveness to SRBC in albino rats as consequence of both

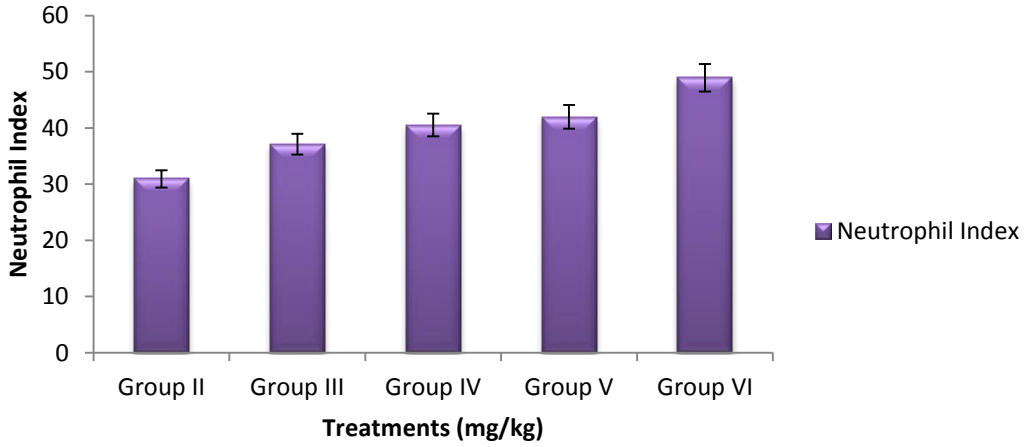
pre and post immunization protein treatment indicates the enhanced responsiveness of macrophages and b-lymphocytes subsets involved in antibody synthesis.

Table 1 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on Neutrophil adhesion in rats

treatment	Neutrophil Index		(%) Neutrophil Adhesion
	UB	FTB	
Group I	163.70 ± 60.21	112.13 ± 45.34	28.21 ± 1.41
Group II	199.60 ± 43.11	138.12 ± 54.29	30.93 ± 1.63*
Group III	230.15 ± 39.72	141.61 ± 30.47	37.13 ± 9.033*
Group IV	261.25 ± 48.22	154.62 ± 22.34	40.52 ± 10.5 *
Group V	269.28 ± 32.45	158.82 ± 36.23	41.97 ± 12.23*
Group VI	311.60 ± 33.72	155.36 ± 28.42	48.94 ± 20.84*

N= 6, values were expressed as Mean ± SEM, *p value <0.05 statistically significant; UB - untreated blood; FTB - Fiber Treated Blood. Group I ; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

Figure 1 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on Neutrophil adhesion in rats



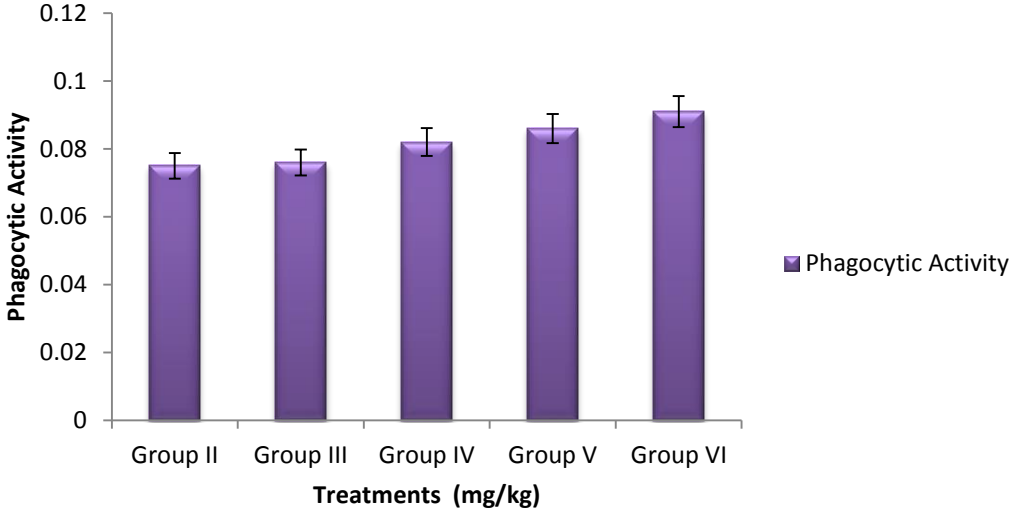
N= 6, values were expressed as Mean ± SEM, *p value <0.05 statistically significant; UB - untreated blood; FTB - Fiber Treated Blood. Group I; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

Table 2 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on *In vivo* phagocytic activity by carbon clearance assay

Treatment	Phagocytic activity
Group I	0.065 ± 0.052*
Group II	0.075 ± 0.036*
Group III	0.076 ± 0.041**
Group IV	0.082 ± 0.032**
Group V	0.086 ± 0.045*
Group VI	0.091 ± 0.025*

N= 6, values were expressed as Mean ± SEM, *p value <0.05, **p value <0.05, statistically significant; UB - untreated blood; FTB - Fiber Treated Blood. Group I; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

Figure 2 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on *In vivo* phagocytic activity by carbon clearance assay



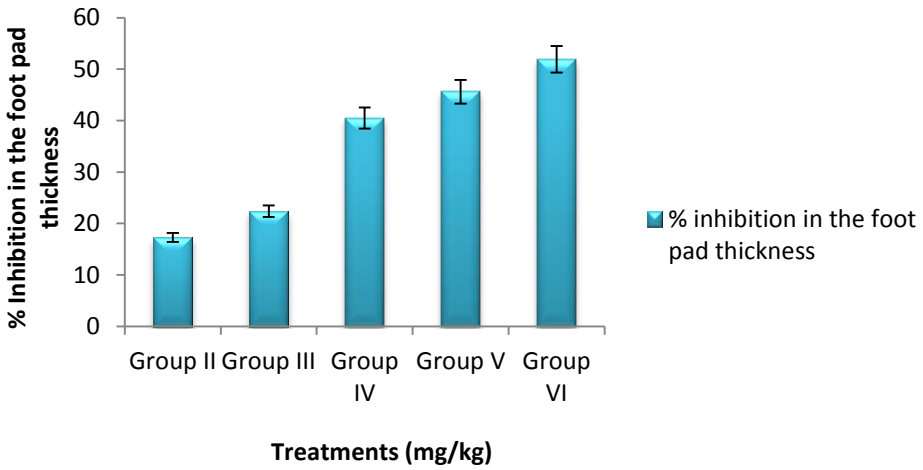
N= 6, values were expressed as Mean ± SEM, *p value <0.05, **p value <0.05, statistically significant; UB - untreated blood; FTB - Fiber Treated Blood. Group I; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

Table 3 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on Delayed type Hypersensitivity in rats

Treatment	DTH response (in mm)	% inhibition in the Foot pad thickness
Group I	0.51±0.02	-
Group II	0.49± 0.057*	17.3%
Group III	0.46 ± 0.183*	22.4%
Group IV	0.37 ± 0.147*	40.5%
Group V	0.30± 0.032**	45.6%
Group VI	0.25 ± 0.983*	51.9%

N= 6, values were expressed as Mean ± SEM, *p value <0.05, **p value <0.05 statistically significant, DTH response – delayed type hypersensitivity response, Group I; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

Figure 3 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on DTH in rats



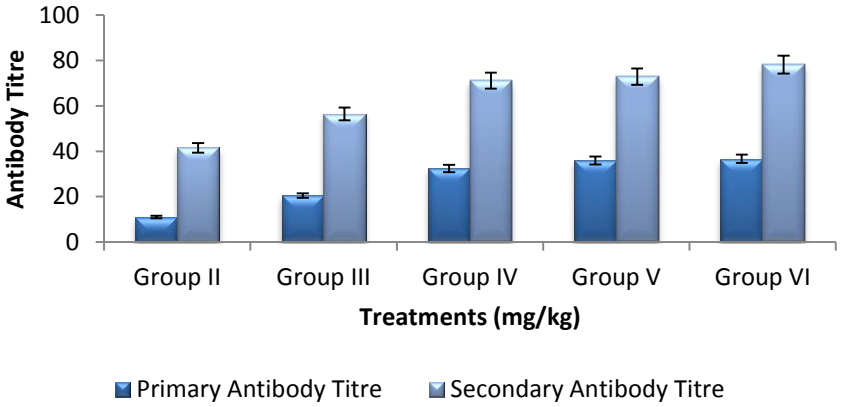
N= 6, values were expressed as Mean ± SEM, *p value <0.05, **p value <0.05 statistically significant, DTH response – delayed type hypersensitivity response, Group I; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

Table 4 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on Humoral Antibody Titre in rats

Treatment	Hemagglutination antibody titer	
	Primary Antibody Titre	**Secondary Antibody Titre**
Group I	9.07 ± 2.32	25.03 ± 11.21
Group II	11.02 ± 8.49*	41.52 ± 16.40*
Group III	20.51 ± 4.32*	56.42 ± 30.23*
Group IV	32.38 ± 5.33*	71.14 ± 45.22*
Group V	35.91 ± 6.14*	72.92 ± 45.89*
GroupVI	36.67 ± 3.82*	78.22 ± 49.29*

N= 6, values were expressed as Mean ± SEM, *p value <0.05 statistically significant; Group I; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

Figure 4 Effect of Formonoetin-7-*O*-β-D-glucopyranoside on Humoral Antibody Titre in rats



N= 6, values were expressed as Mean ± SEM, *p value <0.05 statistically significant; Group I; vehicle control group, Groups II, III, IV and V; treatment group, Group VI; standard group.

The DTH response, which is a direct correlate of Cell Mediated Immunity (CMI), was found to be increased by the administration of test compound. During CMI responses, sensitized T-lymphocytes, when challenged by the antigen, are converted to lymphoblasts and secrete lymphokines, attracting more scavenger cells to the site of

reaction[15]. The infiltrating cells are thus immobilized to promote defensive. In our studies, footpad volume was enhanced after test compound treatment suggests cell mediated enhancement. When SRBC added to the serum dilution, antibody is formed. This antibody bind to antigen and this will neutralize it or facilitate its elimination by cross linking to form latex that is more readily ingested by phagocytic cells. When the test animals pretreated, value of circulating antibody increases[16]. The test involves double dilution of serum sample and addition of SRBC. If the serum antibody react with SRBC then agglutination occurs because of formation of antibody which bridges with neighbouring erythrocytes and these settle at the bottom as latex[17]. Unagglutinted RBC as a button in cell bottom. If haemaglutination was detected in the serum wells a not in control wells then result is recorded as titre[18]. RBC at neutral pH possess negative ions that cloud which repel one another. SRBC induced inflammation is a biphasic phenomenon the first phase of edema is attributed to release of histamine and 5-Hydroxytryptamine[19]. Plateau phase is maintained by kinin like substances and second accelerating phase of swelling is attributed to prostaglandin like substances. The knowledge of these mediators involved in different phases is important for interpreting mode of drug action[20].

CONCLUSION

Immunomodulatory agents of the plant and animal origin enhance the immune responsiveness of an organism against a pathogen by activating the immune system. Administration of an isolated compound orally showed significant *invivo,* immunomodulatory activity and possibly exerts its effect through diverse mechanisms that may involve cellular pathways. Further studies are warranted for the understanding the exact mechanisms responsible for immunomodulation.

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