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Original Article

IN VITRO ASSESSMENT OF PRASARANI SANDHAN, A TRADITIONAL POLYHERBAL AYURVEDIC MEDICINE, FOR IMMUNOSTIMULATING ACTIVITY IN SPLENIC CELLS OF BALB/C MICE

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ABSTRACT

Objective: The objective of the present study was to evaluate the immunostimulating potential of an Ayurvedic preparation Prasarani Sandhan (PRS) by measuring immunoglobulinM (IgM) production and splenocytes proliferation *in vitro*.

Methods: Freshly prepared BALB/c mice splenocytes were treated with 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4% (v/v) of PRS and the cells were sub-cultured at 37°C, humidified atmosphere containing 5% CO_2 for 120 hours. IgM production and cells proliferation were determined by enzyme-linked immunosorbent assay (ELISA) and 3-(4,5-dimethylthiazol-2-y)-2,5-diphenylterazolium bromide (MTT) methods, respectively. The presence of endotoxin was determined by treating PRS with polymyxin B in culture.

Results: PRS at the doses of 0.25, 0.5, 0.75, 1, 1.5% (v/v) significantly promoted polyclonal IgM productions (1.847, 2.024, 1.986, 1.958, and 1.465 µg/mL, respectively) compared to control (0.358 µg/mL). Similarly, 0.25, 0.5, 0.75, and 1% (v/v) of the preparation significantly stimulated splenocytes proliferation (absorbance 0.346, 0.394, 0.385, and 0.378 respectively, at 570 nm) compared to control (Abs. 0.156). The highest increment of IgM production and splenocytes proliferation by 0.50% PRS was 5.65 and 2.52-times than control. Polymixin B treated PRS significantly reduced its ability for IgM production and cells proliferation by 51.5% and 30%, respectively. Thus, the findings suggest that the apparently observed enhancement of IgM by PRS was due to the contamination of bacterial endotoxin, PRS has no immunostimulating property.

Conclusion: This is the first report on the immunostimulating activity of PRS. Prasarani Sandhan was contaminated with bacterial endotoxin and the preparation failed to exhibit immunostimulating activity *in vitro*.

Keywords: Prasarani Sandhan, Ayurvedic medicine, Immunostimulant, Splenocytes, Immunoglobulin M, Differentiation, Proliferation, BALB/c mice.

INTRODUCTION

Although the Ayurvedic medicines are the most popular form of alternative medicines being practiced mostly in Asian developing countries including Bangladesh, scientific research are very negligible on it compared to Allophathic or modern medicines. The popularity of herbal medicine is increasing due to the tremendous side-effects of synthetic or modern medicines[1]. Besides, negative feedbacks of several commercially available drugs lead to the investigations of new therapeutic approaches from natural sources and alternative medicines[2].

Recently, it has been given great emphasis on the scientific evaluation of traditional complementary and alternative medicines for the justification of their traditional use as well as for the assessment of new therapeutic value that has not yet been explored scientifically. WHO has recently focused its attention to the traditional, complementary and alternative medicines to include them in the national healthcare system, and suggests researches on these medicines in order to ensure their safety, efficacy and quality[3]. The national health policy of Bangladesh has the similar objective to encourage the systemic improvement of the practice of indigenous medicines and emphasized scientific evaluation of those indigenous and herbal medicines[4].

ImmunostimLants are important for the protection of our body from possible infections when body's defensive capacity is attenuated. Immune system is impaired in many conditions like, diabetes, HIV infections, consumption of glucocorticoids, sepsis, and cancer[5]. Ayurvedic medicines enlisted many medicinal plants that are believed to be human immunomodulators. Prasarani Sandhan (PRS) is an Ayurvedic formulated medicine composed of 6 plant ingredients[6], listed in the Table 1 in which the root of *Paederia foetida* L. Being the main ingredients. PRS is traditionally indicated for the treatment of rheumatoid arthritis and all sorts of rheumatic diseases[7-8].

Only few researches have conducted the scientific study of PRS which include hetamological effects[9], safety [8], and anti-inflammatory activity[10]of PRS. However, so far immunomodulatory study of PRS has not been conducted by any researcher or no report is available. Ayurved medicine enlisted many medicinal plants that are believed to act as immune system modulators/activators and formulations based on such plants play an important role in modern healthcare, especially on managing immune related problems as well as unavailability of effective and/or safer treatment regime[11-12].

Thus, the present study was aimed to evaluate the effect of PRS on the promotion of humoral immunity by measuring the production of Immunoglobulin M (IgM) and proliferation of cells in cultured murine splenocytes *in vitro*.

MATERIALS AND METHODS

Collection of sample

PRS used in this study was collected from the Sree Kundeshwari Aushadhalay, Chittagong, Bangladesh, and was prepared according to Bangladesh National Formulary of Ayurvedic Medicine[3]. The in-process and quality control for the preparation was strictly controlled and monitored by the experienced officials of Sree Kundeshwari Aushadhalay. The ingredients of Prasarani Sandhan are listed in Table 1.

Chemicals and reagents

Roswell park memorial institute (RPMI)-1640 and Eagle's minimum essential medium (MEM) were purchased from ICN Biomedicals (Irvine, CA, USA) and Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, bovine serum albumin (BSA) (Fraction V), Tween 20, and fetal calf serum (FCS) were purchased from Sigma-Aldrich (Japan). Purified mouse IgM, goat anti-mouse IgM antibody (Ab), and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgM Ab were obtained from Zymed Laboratories Inc (San Francisco, CA, USA), Organon Teknika Corporation (Durham, NC, USA), and Kirkegaad & Perry Laboratories (Gaithersburg, MD, USA), respectively.

Mice

BALB/c female mice were purchased from Charles River Japan (Yokohama, Japan). They were maintained under specific pathogen-free conditions in the animal facility of Okayama University and used between 8 and 12 weeks of age. All experimental procedures concerned with mice were performed according to the guidelines established by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and to the Guidelines for Animal Experiments at Okayama University and were approved by the Animal Research Control Committee of Okayama University, Japan.

Preparation of murine spleen cells

Spleen cells from BALB/c female mice, depleted of erythrocytes, were prepared by lysis of erythrocytes with ammonium chloride as previously mentioned by Sarker et al[13-14]. Mice were killed and spleens were collected aseptically. The spleens were mashed with spatula through the strainer to pass the cells into MEM (pH adjusted to around 7.0 with 1N NaOH) in a Petri dish (Iwaki, Japan). Cells were suspended by Pasteur pipette and screened by passing through a mesh into the centrifuge tube. The cell suspensions were then centrifuged for 5 min at 4°C and 2000 rpm. The supernatants were removed and ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.2) was added to the cells into the centrifuge tube for lysing the erythrocytes for 5 min at room temp. MEM was added to the centrifuge tube, suspended and centrifuged for 5 min at 4°C and 2000 rpm. The supernatants were removed and the cell pellets were washed twice with MEM. The cells were re-suspended in MEM and passed through a mesh into another centrifuge tube to collect the spleen cells. The viability of the prepared splenocytes was determined by the Trypan-blue exclusion technique and cells having viability higher than 70% were used for the experiments.

Cell culture

Cell culture was done as described by Sarker et al.[15]. Freshly prepared spleen cells were suspended in basal culture medium (RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL of penicillin G and 100 µg/mL of streptomycin). The cells (2.5×10^5 cells/100 µL/well) were plated in 96-well U-bottom plates (Nunc, Roskilde, Denmark) and incubated for 30 min at 37°C in a fully humidified atmosphere containing 5% CO_2. Fifty µL of 2-mercaptoethanol (2-ME) (0.2 mM), diluted with the basal culture medium, was added into each well and plates were incubated for 5 days with or without the addition of PRS and LPS, diluted with the basal medium, at 37°C in the CO_2 incubator. The cultured supernatants were then collected and frozen at −30°C for IgM-ELISA and the cells pellets were used for MTT assay.

Table 1: List of plant materials used in Prasarani Sandhan according to the formula of Bangladesh National Formulary of Ayurvedic Medicine[6]

S. No.	Plants` scientific (and local name) with family	Plant`s parts used
1	*Paederia foetida* L. (Prasarani/Gandhavadule) (f. Rubiaceae)	Root
2	*Piper longum* L. (Pippoli) (f. Piperaceae)	Seed
3	*Piper chaba* Hunter (Chabya) (f. Piperaceae)	Root
4	*Plumbago zeylanica* L. (Chita) (f. Plumbaginaceae)	Root
5	*Zingiber officinale* Roscoe (Ada) (f. Zingiberaceae)	Rhizome/Flower
6	*Allium sativum* L. (f. Amaryllidaceae)	Bulb

All the experiments were carried out *in vitro* in the Laboratory of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

Measurement of splenocytes proliferation by MTT assay

The growth of cultured cells was determined by MTT assay as previously described[16-18]. Briefly, at the end of incubation of splenocytes for 120 h, 160 µL supernatants were removed. Sixty µL of fresh medium and 25 µL of MTT solution were added in each well and the plate was incubated for 2 h. After addition of 100 µL stop solution in each well, the plate was incubated overnight in dark at 37°C and the absorbance was measured at 570 nm by using a plate reader (Bio-Rad Laboratories, USA).

ELISA for the determination of IgM production in cultured supernatants

The IgM antibody production level in the serum was measured by a sandwich ELISA as described earlier[19-20]. Briefly, each well of 96-well microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 50 µL/well of goat anti-mouse IgM (10 µg/mL) diluted with phosphate buffer saline (PBS), and incubated the plates overnight in the dark at 4°C.

The plates were washed three times by PBS containing 0.05% Tween 20 (wash-buffer) (200 µL/well). The wells were blocked with 200 µL of 1% bovine serum albumin (BSA) in PBS for 2 h at room temp. After washing the plates, 100 µL/well of cultural supernatants (diluted with 1% BSA-PBS-Tween 20) or standard mouse-IgM were added into each well, and the plates were incubated for 2 h at room temp. The plates were again washed three times by wash buffer (200 µL/well). Fifty µL per well of horseradish peroxidase-conjugated goat anti-mouse IgM (0.2 µg/mL) was added into each well and the plates were incubated for 1 h at room temp. After washing the plates 100 µL/well of 0.1 M citrate buffer (pH 4.0) containing 2.5 mM 2,2 -azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.17% H_2O_2 were added. The plates were then incubated for 10 min at room temp and the optical densities at 405 nm were measured using an automatic plate reader (Bio-Rad Laboratories, USA).

Statistical analysis of data

The experimental results are expressed as means ± S. E. M. (standard error of the mean) of three independent experiments. The differences between the control and treated groups were analyzed by Dunnett's T3, Tukey HSD, and Student's *t*-tests and *P* values less than 5% were regarded as significant.

RESULTS

Effect of Prasarani Sandhan (PRS) on the production of polyclonal IgM

BALB/c female mice spleen cells were sub-cultured with or without PRS for 5 days and the quantity of IgM produced in the cultured supernatants was determined by an IgM-ELISA[19-20]. Lipopolysaccharide (LPS) was used as a positive control. Our data (Fig. 1) showed that PRS at the doses of 0.25, 0.5, 0.75, 1, and 1.5% augmented IgM production by 5.16, 5.65, 5.55, 5.45 and 4.17 folds higher than that of control, respectively. The highest IgM enhancement by 0.50% of PRS was observed to be 5.65 times greater than the control. LPS (0.1 µg/mL) was used as a positive

control which also increased IgM production 9-fold higher than control. Therefore, our data indicates that the result of the present study is strongly comparable with the effect of the LPS. The concentration of PRS higher than 2% significantly degraded the production of IgM level, hence those concentrations were considered to be toxic to cultured cells.

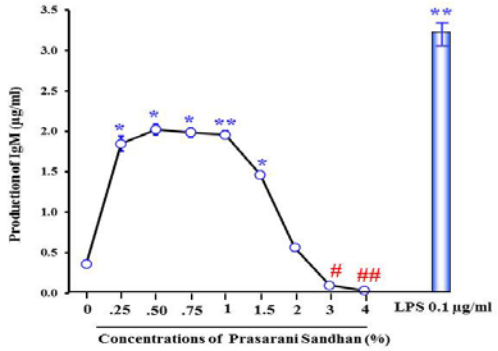

Fig. 1: Effect of Prasarani Sandhan (PRS) on the production of Immunoglobulin M (IgM) in cultural supernatants of murine splenocytes.

BALB/c mice whole spleen cells (2.5 ×10^5 cells/well) were incubated with the indicated concentrations of PRS at 37°C in the 5% CO_2 incubator for 5 days. The IgM level in the supernatants was determined by an ELISA. The data are means ± S. E. M. of three independent experiments. **P*<0.05, ***P*<0.01, # *P*<0.05, and ## *P*<0.01, as compared with the control (Dunnett's T3 test).

Effect of Prasarani Sandhan (PRS) on the proliferation of murine splenocytes:

BALB/c female mice spleen cells were sub-cultured with or without varying doses of PRS for 5 days and the proliferation of cells was measured by MTT assay**[16-18]**. As shown in the Fig. 2, PRS at the concentrations of 0.25, 0.50, 0.75, and 1% significantly stimulated the proliferation of splenocytes by 2.22, 2.52, 2.47, and 2.42-folds, respectively; the highest enhancement of proliferation was exhibited by 0.50% of PRS which was 2.52 times higher than that of control.

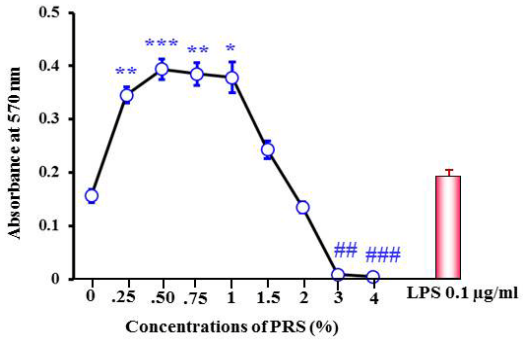

Fig. 2: Effect of Prasarani Sandhan (PRS) on the proliferation of murine splenocytes in culture.

BALB/c mice whole spleen cells (2.5 ×10^5 cells/well) were incubated with the indicated concentrations of PRS at 37º C in the 5% CO_2 incubator for 5 days. The proliferations of cells were determined by MTT assay. The data are means ± S. E. M. of three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, ## *P*<0.01, and ### *P*<0.001, as compared with the control (Tukey HSD test).

Suppression of Immunostimulating activity of PRS by Polymyxin B (PMB):

There was a possibility of endotoxin contamination, such as LPS, in the PRS during the processing of the ingredients, in fermentation stage of manufacturing, etc. In order to exclude the endotoxin contamination possibility in PRS and to justify whether the activity exhibited by PRS preparation is due to its chemical properties or bacterial contamination, we treated PRS with polymixin B (PMB) antibiotic in the culture. Treatment of PRS drastically reduced the activity of PRS for the enhancement of IgM production and proliferation of cells. The presence of polymixin B (PMB) antibiotic inhibits the stimulating activity of LPS. The investigation resulted that treatment of PRS (0.50%) by PMB inhibited the IgM production ability of this preparation by 51.5% compared to untreated cells (Fig 3A). Similarly, activity of PRS for the proliferation of splenocytes was 30 % degraded in PMB-treated cells compared to control data (Fig 3B).

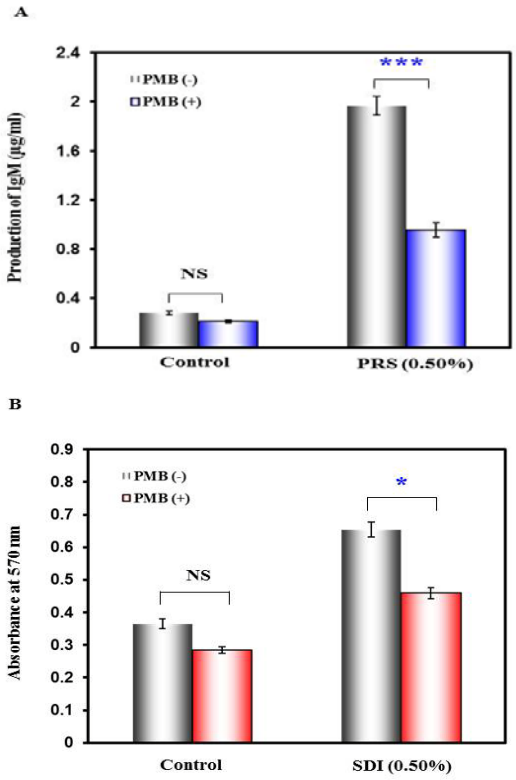

Fig. 3: Assessment of bacterial endotoxicity in Prasarani Sandhan (PRS) by determining IgM production in cultured supernatants (A) and proliferation of splenocytes in the presence and absence of Polymyxin B (PMB).

BALB/c mice splenocytes (2.5×10^5 cells/well) were incubated with the optimum concentration of PRS (0.50%) and with or without PMB at 37°C in the 5% CO_2 incubator for 5 days. Production of IgM in cultured supernatants was measured by an ELISA (A). The proliferations of cells were determined by MTT assay (B). The data are means ± S. E. M. of three independent experiments (Student's *t*-test).

DISCUSSION

Our result showed that different doses of Prasarani Sandhan (PRS) potentially enhanced the levels of IgM production (Fig. 1) and proliferation of murine splenocytes (Fig. 2). These two experimental results initially indicated the immunostimulating potential of PRS. However, treatment of PRS by PMB remarkably reduced the level of IgM production in cultured cells compared to PMB-untreated PRS (Fig 3). PMB binds to the lipid portion of bacterial LPS and thus inhibits the activity of LPS[21]. Therefore, we can say that PRS contained bacterial LPS as contaminant and PRS exhibited immunostimulating activities because of the presence of that LPS, not due to its active chemical(s). From our study, we can reach to a conclusion that PRS does not possess any ability to enhance humoral immunity and this Ayurvedic preparation was contaminated with

bacteria or bacterial endotoxin which may come from the formulating ingredients or may be incorporated during its manufacturing process. Previously, we reported the immunostimulating activities of two other Ayurvedic preparations: Kanakasav and Chandanasav. Kanakasav was not found to contaminate with bacterial endotoxin[10] but Chandanasav was reported to be contaminated with bacterial LPS[3], similarly with this study. Ayurved medicines are natural medicines that are prepared from natural plants mainly; hence, their production cost is very cheap. Besides, these medicines have less or least or no side-effects compared to modern Allopathic medicines. Yet, negligence of modern research on Ayurved medicines, lack of prominent scientific advancement in its preparation techniques, and, above all, microbial contamination of these preparations during the manufacturing process are still considered as great problems, especially in Bangladesh.

CONCLUSION

On the basis of present investigations, it can be reported that Prasarani Sandhan does not possess immunostimulating activity in respect of the humoral immune response and the preparation was found to be contaminated by bacterial endotoxin (LPS). So, it should be very careful in the preparation of not only PRS but also all kinds of Ayurvedic medicines.

CONFLICT OF INTERESTS

Declared None

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