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Immunopotentiality of Ayurvedic polyherbal formulations "Saribadi" and "Anantamul Salsa" with augmentation of IgM production and lymphocytes proliferation: A preliminary study

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ABSTRACT

Objective: To assess the immunopotentiality of Ayurvedic polyherbal preparations, "Saribadi" and "Anantamul Salsa".

Methods: Freshly prepared BALB/c mice splenocytes were cultured with "Saribadi" or "Anantamul Salsa" treatment [doses of 0.25%, 0.50%, 0.75%, 1.00%, 1.50%, 2.00%, 3.00% and 4.00% (v/v)] at 37 °C for 5 days. The immunoglobulin M (IgM) production and lymphocytes proliferation were determined by ELISA and MTT methods, respectively. Endotoxin contamination was assessed by treating the preparations with polymyxin B.

Results: The doses of "Saribadi" [0.25%, 0.50%, 0.75% and 1.00% (v/v)] significantly increased IgM productions (0.966, 0.728, 0.695 and 0.615 μg/mL *vs*. control 0.265 μg/mL) and lymphocytes proliferation [absorbance 0.311, 0.394, 0.372 and 0.334 optical density (OD) *vs*. control 0.162 OD]. Similarly, the doses of "Anantamul Salsa" [0.50%, 0.75%, 1.00% and 1.50% (v/v)] promoted IgM productions (0.933, 0.919, 0.917 and 0.892 μg/mL *vs*. control 0.502 μg/mL) and the doses of "Anantamul Salsa" [0.50%, 0.75%, 1.00%, 1.50%, 2.00%, and 3.00% (v/v)] stimulated lymphocytes proliferation (absorbance 0.395, 0.326, 0.440, 0.398, 0.452 and 0.355 OD *vs*. control 0.199 OD). The activity of "Saribadi" and "Anantamul Salsa" was not retarded by the treatment of preparations with polymyxin B.

Conclusions: Immunomodulatory activity of "Saribadi" and "Anantamul Salsa" was unveiled for the first time. "Saribadi" and "Anantamul Salsa" possess immunostimulating potential acting through the induction of lymphocyte proliferation and IgM production. These preparations may be useful in strengthening immune responses. However, further cellular and *in vivo* studies are required.

1. Introduction

The defensive system of human body, composed of innate and adaptive immunological arms, protects it from possible infections from microorganisms and prevents the formation of cancerous cells and/or eliminates them as soon as they appear as an antigen [1,2]. In certain conditions, such as, diabetes, obesity, burns, sepsis, stress, insomnia, AIDS, lack of exercise, poor nutrition and aging, the immunity is greatly suppressed [2–5]. Immunity is also suppressed in case of

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The experiments concerned with the use of mice were approved by the ethical approval of Animal Research Control Committee of Okayama University.

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receiving radiotherapy, chemotherapy, and antibiotics [2,6,7]. The impaired immune system is greatly susceptible to several diseases including infections, cancers, allergy, asthma, ulcerative colitis and asthma [8,9]. Immunopotentiators play a vital role to upgrade the immunity to protect the body from possible infection and cancers under those immunocompromised conditions.

The popularity of herbal medicine is increasing due to the perceived tremendous side-effects of synthetic or allopathic medicines [10–12]. Herbal medicines are considered to be safe as it contains complementary ingredients that could neutralize the side-effects of one chemical from one to another. Although Ayurvedic medicines are one of the most popular forms of alternative medicines, scientific reports on the evaluation of those medicines are very negligible comparing to allopathic medicines [13]. Ayurvedic medicines are prepared from the multiple combination of a number of medicinal plants which are believed to act as an immunomodulating agent. Therefore, herbal medicines recently have gained attention for the evaluation of immunopotentiality and the treatment of immune disorders [8]. This investigation was designed for the preliminarily evaluation of the immunopotentiality of 20 Ayurvedic preparations, including "Amalaki Rasayan", "Anantamul Salsa", "Ashokarista", "Aswagandharista", "Basakarista", "Bolarista", "Kalomeghasav", "Kutajarista", "Rohitakarista", "Shirisharista", "Ushisharista", "Abhyarista", "Arabindasav", "Arjunarista", "Saribadi", "Dasamularista", "Debdarbadyarista", "Khadirarista", "Moha Draksharista" and "Amritarista". Among the 20 Ayurvedic preparations, only two preparations ("Saribadi" and "Anantamul Salsa") exhibited prospective immunopotentiating activities *in vitro*.

"Saribadi" is a polyherbal Ayurvedic preparation indicated for the treatment of gonorrhoea, syphilis, skin diseases and blood impurities [14]. No scientific literature has been reported on the pharmacological or therapeutic activity of "Saribadi". "Anantamul Salsa" is indicated for the treatment of gonorrhoea, syphilis, such as blood poisoning/purifier, all types of skin diseases including acne, skin-allergies dermatitis, boils, skin rashes and other skin disorders including gout and leprosy [14,15]. Furthermore, no scientific report is available on the pharmacological or therapeutic activities of "Anantamul Salsa". *Hemidesmus indicus* is the main plant of both herbal preparations, "Saribadi" and "Anantamul Salsa". This plant and its constituents were reported to possess antioxidant, anticancer, antidiabetic, antibacterial, antihyperlipidemic and cardioprotective effects [16–21].

Scientific report is completely absent for the evaluation of "Saribadi" and "Anantamul Salsa" on the immunomodulating activities. Therefore, the present investigation was designed for the determination of immunopotentiating activity of those preparations measuring the production of immunoglobulin M (IgM) antibody responsible for humoral immunity and the proliferation of splenic lymphocytes.

2. Materials and methods

2.1. Preparation of drug sample

All the drugs used in this study including "Saribadi" and "Anantamul Salsa" were prepared according to the formulation and procedure mentioned in Bangladesh National Formulary of Ayurvedic Medicine and the Ayurvedic Pharmacopoeia of India [22,23]. All the ingredients were purchased from the reputed herbal medical ingredients' suppliers in Dhaka, Bangladesh. The materials were confirmed with their morphological features by a senior taxonomist at the Botany Department of Jahangirnagar University, Bangladesh. The preparations of the drugs were done strictly under the supervision of an Ayurvedic physician in the Ayurvedic Research Laboratory of Pharmacy Department in Jahangirnagar University Bangladesh. The samples were filled in centrifuge tubes and filtered using 0.22 μm micro-filters just before its use. All the experiments were carried out in the Immunochemistry Laboratory of the Faculty of Pharmaceutical Sciences, Okayama University Japan.

2.2. Chemicals and reagents

RPMI 1640 was purchased from ICN Biomedicals, USA, and Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical Co., Ltd., Japan. Lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, bovine serum albumin, Sigma-Fast o-phenylenediamine, Tween-20, fetal calf serum (FCS) and MTT were purchased from Sigma Aldrich, Japan. Mouse IgM (purified), goat anti-mouse IgM antibody and horseradish peroxidase-conjugated goat anti-mouse IgM antibody were purchased from Zymed Laboratories Inc., USA, Organon Teknika Corporation, USA, and Kirkegaad and Perry Laboratories, USA, respectively.

2.3. Study animal

Female BALB/c mice were obtained from Charles River, Japan. The animals were housed at pathogen-free rooms, 12-light/12-dark cycle and at 20–25 °C temperature in the animal facility of Okayama University, Japan. The animals were used at the age of 8–12 weeks. The experiments concerned with the use of mice were approved by the ethical approval of Animal Research Control Committee of Okayama University.

2.4. Preparation of murine whole spleen cells

Splenocytes (depleted erythrocytes) were prepared from BALB/c mice by the process as described by Sarker *et al*. [24,25]. Mice were sacrificed to collect spleens aseptically. The spleens were mashed using spatula through the strainer to pass the cells into MEM in a Petri-dish (Iwaki, Japan). Cells were then suspended by Pasteur pipette and screened by passing through a mesh into the centrifuge tube. The cell suspensions were centrifuged for 5 min at 4 °C temperature and speed at 2 000 r/min. Cells supernatants were removed and ammonium chloride potassium lysis buffer (0.15 mol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L ethylenediaminetetraacetic acid disodium salt dihydrate; pH 7.2) was added to the cells inside the centrifuge tube for 5 min at room temperature for lysing the erythrocytes. With the addition of MEM into the centrifuge tube, cells were suspended and centrifuged at 2 000 r/min at 4 °C for 5 min. Cell pellets were washed two-times with the MEM after removal of the supernatants. Re-suspension of cells in MEM was performed and screened through mass to pass into another centrifuge tube to collect the splenocytes. Freshly prepared splenocytes viability was determined by trypan-blue exclusion method [26]. Cells that had viability more than 70% were used for the experiment purposes.

2.5. In vitro culture of lymphocytes

Lymphocytes were cultured according to the procedures described previously [26]. Freshly prepared spleen cells (mainly lymphocytes) were suspended in RPMI 1640 medium, supplemented with 10% heat-inactivated FCS, 2 mmol/L L-glutamine, 100 IU/mL penicillin G and 100 μg/mL streptomycin. Lymphocytes (2.5×10^5 cells/well) were plated in 96-well U-bottom plates (Nunc, Denmark) and cultured for 30 min at 37 °C and 5% CO_2 containing airflow with humidified atmosphere. With the addition of 50 μL 2-mercaptoethanol (concentration of 0.2 mmol/L) per well, the plate was incubated for 120 h at 37 °C in the CO_2 incubator with/without the treatment with "Saribadi" or "Anantamul Salsa" and LPS. The plates were centrifuged, and supernatants were collected from each well and frozen at −30 °C to perform IgM ELISA. After collecting the supernatants, the sediment cells pellets were used to perform MTT assay.

2.6. Measurement of lymphocytes proliferation by MTT method

The proliferation of lymphocytes in culture was measured by MTT method as mentioned earlier [27,28]. In brief, lymphocytes were cultured for 5 days as the condition mentioned above and 160 μL supernatant was removed from each well. Freshly prepared RPMI/FCS medium (60 μL) and MTT solution (25 μL) were incorporated into each well and again incubated for 2 h. With the addition of stop solution (100 μL/well), plates were again incubated overnight at 37 °C in the dark environment. The absorbance was measured at 570 nm by using a plate reader (Bio-Rad Laboratories, USA).

2.7. ELISA for the determination of IgM production

The IgM antibody production level was determined by a sandwich ELISA [29,30]. Briefly, 96-well microtiter plate (Maxisorp, Nunc, Denmark) was coated with goat anti-mouse IgM (10 μg/mL) for overnight incubation of the plate in the dark at 4 °C temperature. The plate was washed thrice with phosphate buffer saline containing 0.05% Tween 20 (wash-buffer). Blocking the plate wells with 1% bovine serum albumin for 2 h at room temperature, the plate was washed 3 times and 100 μL/well of cultural supernatants or standard mouse-IgM was added into each well and the plate was incubated at room temperature for 2 h. After washing the plates thrice with wash buffer, 50 μL of horseradish peroxidase-conjugated goat anti-mouse IgM (0.2 μg/mL) was added into each well and the plate was incubated at room temperature for 1 h. The plate was again washed and 100 μL of 0.1 mol/L citrate buffer (pH 4.0) containing 2.5 mmol/L 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and 0.17% H_2O_2 was added into each well. Finally, the plate was incubated for 10 min at room temperature and the optical density (405 nm) was measured using an automatic plate reader (Bio-Rad Laboratories, USA).

2.8. Statistical analysis of data

The experimental data were expressed as mean ± SEM of three independent experiments. The differences between the control and treated groups were analyzed by Tukey honest significant difference test, Student's *t*-test and Dunnett's T3 test. *P* values less than 0.05 were considered as significant.

3. Results

3.1. Potentiation of polyclonal IgM production by "Saribadi" and "Anantamul Salsa"

Murine splenic cells were cultured with or without "Saribadi" or "Anantamul Salsa" at 37 °C in a cell culture incubator for 5 days. The quantity of polyclonal IgM production in cultured supernatant was determined by an IgM-ELISA. LPS was used as a positive control. Our data (Figure 1A) showed that "Saribadi"

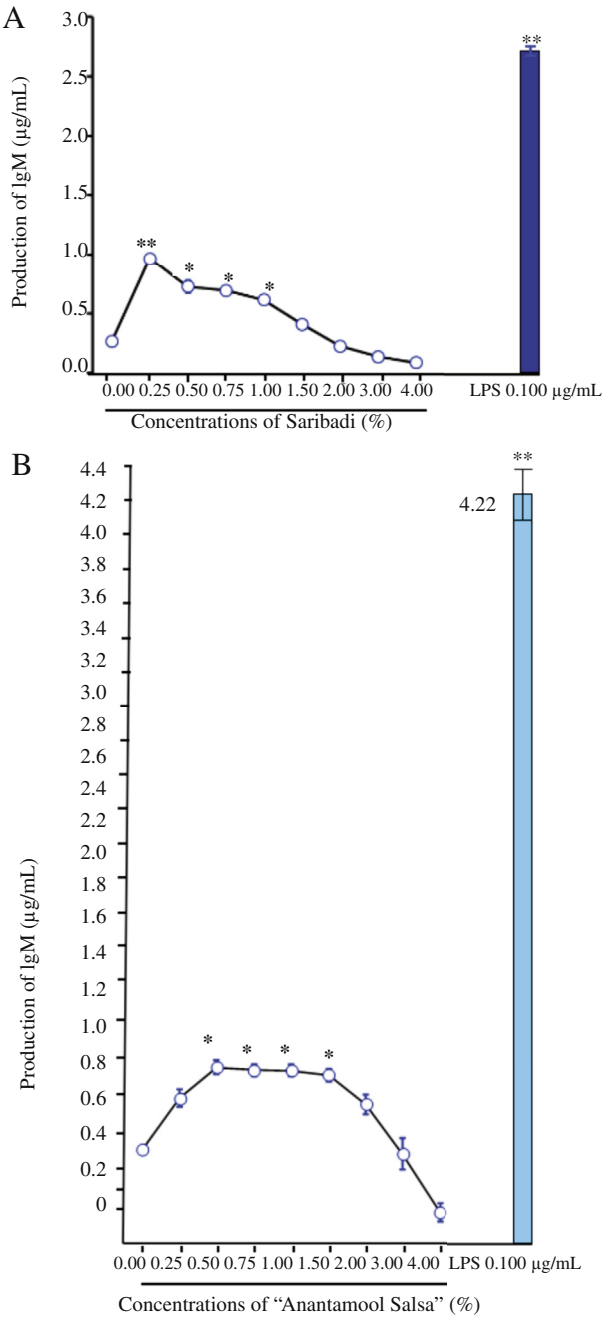

Figure 1. Effect of "Saribadi" and "Anantamul Salsa" on the production of IgM in cultural supernatants of murine lymphocytes.
BALB/c female mice whole spleen cells (2.5×10^5 cells/well) were cultured with the indicated concentrations of "Saribadi" (A) or "Anantamul Salsa" (B) at 37 °C in the incubator having 5% CO_2 airflow for 5 days. The level of IgM produced in the cultured supernatants was determined by an ELISA. The data were mean ± SEM of three independent experiments. *: $P < 0.05$; **: $P < 0.01$, as compared with the control (Dunnett's T3 test).

at the doses of 0.25%, 0.50%, 0.75%, and 1.00% significantly enhanced the amount of polyclonal IgM production in treated lymphocytes compared to untreated cells. Although the 1.50% dose of "Saribadi" enhanced the production of IgM, the data were not statistically significant, and then declined. The highest enhancement of IgM production by "Saribadi" at a dose of 0.25% was found to be 3.65 times greater than that of control, whereas LPS (0.1 μg/mL) caused 10.5 folds increment of IgM production. The concentration higher than 1.50% of "Saribadi" could not increase IgM level or those concentrations were toxic to the cells. Similarly, "Anantamul Salsa" at the doses of 0.50%, 0.75%, 1.00% and 1.50% significantly augmented the level of IgM production compared to untreated cells (Figure 1B). "Anantamul Salsa" at the doses of 0.25% and 2.00% increased statistically insignificant levels of antibody productions but the doses 3.00% and 4.00% resulted in reduced production of IgM. The highest level of IgM production was observed by 0.50% of "Anantamul Salsa" which was found to be 1.86 times higher than the control. LPS (0.1 μg/mL) promoted 8.4 folds IgM production comparing the untreated cells.

3.2. Induction of lymphocytes proliferation by "Saribadi" and "Anantamul Salsa"

The lymphocytes collected from BALB/c female mice were cultured with/without different doses of "Saribadi" or "Anantamul Salsa" for 120 h and lymphocytes proliferation was determined by MTT method [25]. As shown in Figure 2A, "Saribadi" at concentrations of 0.25%, 0.50%, 0.75% and 1.00% significantly stimulated the proliferation of murine lymphocytes and the highest enhancement of proliferation was 2.43 times, exhibited by 0.50% dose of "Saribadi". Although 1.50% dose of "Saribadi" slightly increased lymphocytes proliferation, 2.00%, 3.00% and 4.00% doses of the preparation were found to be cytotoxic as these doses remarkably reduced the number of lymphocytes in culture (Figure 2A). On the other hand, lymphocytes proliferation was significantly increased by 0.50%, 0.75%, 1.00%, 2.00% and 3.00% concentrations of "Anantamul Salsa" (Figure 2B). Only the highest used dose of "Anantamul Salsa" (4% v/v) was found to be cytotoxic which drastically reduced the number of lymphocytes in culture.

3.3. Immunopotential ability of "Saribadi" and "Anantamul Salsa" was not deteriorated with polymyxin B treatment

We evaluated the endotoxin contamination (LPS incorporation in the preparations) possibility in "Saribadi" and "Anantamul Salsa" during the processing of the ingredients, in fermentation stage of manufacturing, *etc*. In order to exclude the endotoxin contamination possibility in "Saribadi" and "Anantamul Salsa", the preparations were treated with polymyxin B (PMB) antibiotic during its incorporation in the culture of murine splenic cells. The investigation also resulted in a significant promotion of IgM production and lymphocytes proliferation by both "Saribadi" and "Anantamul Salsa" preparations in PMB-treated spleen cells as well (Figure 3A, B). The amount of IgM production stimulated by PMB-untreated "Saribadi" (0.25%) was 1.1 μg/mL, whereas PMB-treated "Saribadi" produced 0.8 μg/mL of IgM. Both the PMB-untreated and -treated "Saribadi" produced significantly high levels of IgM comparing to the control ("Saribadi"-untreated) group. Production of IgM by "Saribadi" was reduced by 20.96% in PMB-treated group compared to PMB-untreated group and this reduction was statistically insignificant. Besides, the IgM production was also reduced (25.34%) in PMB-treated control group comparing to PMB-untreated control group (Figure 3A). This comparison indicated that the slight reduction (20.96%) of IgM in PMB-treated "Saribadi" stimulated by cells was not due to the effect of PMB on "Saribadi" for the reduction of IgM production and it was only the effect of PMB itself on the cells.

In case of "Anantamul Salsa", the production of IgM in PMB-treated "Anantamul Salsa" group was increased by 7.80%, instead of decreasing, comparing to PMB-untreated "Anantamul Salsa" group (Figure 3A). This showed that the PMB treatment did not have any effect on the stimulating activity of "Anantamul Salsa" for the production of IgM.

Similarly, there was no significant difference in the activities of "Saribadi" and "Anantamul Salsa" for the proliferations of lymphocytes in PMB-untreated and PMB-treated groups (Figure 3B). The proliferation of lymphocytes was reduced by 20.00%, 21.98%, and 9.38% in PMB-treated control, "Saribadi" and "Anantamul Salsa" groups, respectively. Thus, the data also clearly indicated that the treatment of "Saribadi" and

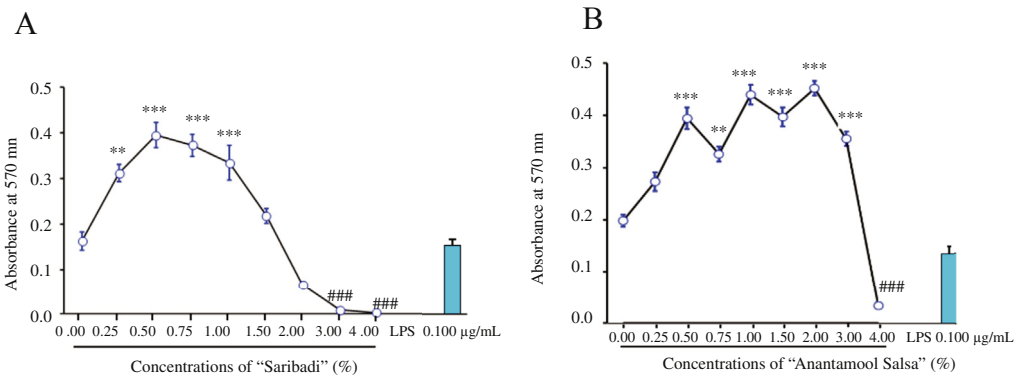

Figure 2. Effect of "Saribadi" and "Anantamul Salsa" on the proliferation of murine lymphocytes in culture.
BALB/c mice splenic lymphocytes (2.5×10^5 cells/well) were incubated with the indicated concentrations of "Saribadi" (A) or "Anantamul Salsa" (B) at 37 °C in the incubator having 5% CO_2 airflow for 5 days. The proliferations of cells were measured by MTT assay. The data were expressed as mean ± SEM of three independent experiments. **: $P < 0.01$ (increased level); ***: $P < 0.001$ (increased level); $^{###}$: $P < 0.001$, as compared with the control (Tukey honest significant difference test).

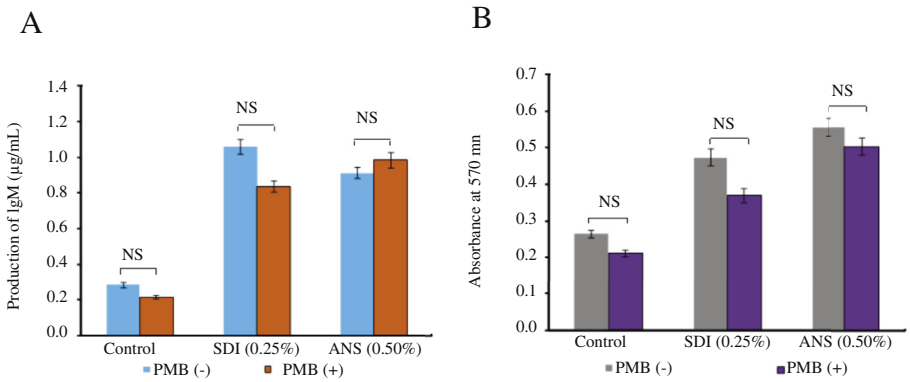

Figure 3. Evaluation of the contamination of "Saribadi" and "Anantamul Salsa" by bacterial endotoxin with the measurement of IgM production (A) and lymphocytes proliferation in the presence and absence of PMB.
BALB/c female mice lymphocytes (2.5×10^5 cells/well) were treated with the optimum concentration of "Saribadi" (0.25%) and "Anantamul Salsa" (0.5%) with or without PMB at 37 °C in the 5% CO_2 incubator for 5 days. A: Production of IgM in cultured supernatants was measured by an ELISA; B: The proliferations of cells were determined by MTT assay. The data were expressed as mean ± SEM of three independent experiments (Student's *t*-test).

"Anantamul Salsa" by PMB had no influence on the activity of "Saribadi" and "Anantamul Salsa" for the proliferation of lymphocytes in culture.

4. Discussion

The increase in the quantity of antibody production is an indicator of the differentiation of B cells to antibody secreting plasma cells [1,2]. Thus, the present investigation demonstrated that "Saribadi" and "Anantamul Salsa" promoted the differentiation of B cells to plasma B cells. Although the production of antibody was promoted by "Saribadi" and "Anantamul Salsa" at lower concentrations (0.25%–2.00%), the higher doses (3.00% and 4.00%) of the preparations were found to suppress the productions of IgM, suggesting that the preparations may contain an inhibitory substance which is effective at higher concentrations.

Increasing proliferation of cells is an indicator of immunostimulation [1]. Similar to antibody production manner, "Saribadi" at higher doses (> 2.00% v/v) were observed to be cytotoxic, whereas "Anantamul Salsa" dose (4.00%) was found to be cytotoxic, which suppressed the proliferation of lymphocytes. In both the cases of the evaluation of "Saribadi" and "Anantamul Salsa", the LPS at the concentration of 0.1 μg/mL could not significantly stimulate the proliferation of lymphocytes.

PMB binds to the lipid portion of bacterial LPS and thus inhibits the activity of LPS [26,31]. Thus, our results demonstrate that "Saribadi" and "Anantamul Salsa" did not contain bacterial LPS as contaminant. Therefore, it can be concluded that the immunostimulating ability of "Saribadi" and "Anantamul Salsa" on murine lymphocytes was not due to the presence of LPS, rather because of the active chemical(s) existing in the preparations.

Thus, the immunopotential property of "Saribadi" and "Anantamul Salsa" was supposed due to the presence of active constituent(s) from any of the plant materials having immunostimulatory or immunomodulatory properties or may be due to the combined immunostimulatory activities or synergistic effect of the immunostimulatory plant ingredients used in the preparations of "Saribadi" and "Anantamul Salsa".

A strong immune system is very much essential to protect our body from the generation of several diseases such as infections and cancer. An attenuated immune system is highly susceptible to infections and cancers. Natural immunopotentiating agents are believed to be promising means for the prevention and treatment of many diseases that occur due to insufficient immunity [8,32]. Therefore, immunostimulants have very good demand to improve the impaired immunity.

"Saribadi" and "Anantamul Salsa" are "Asava" (fermented infusion) products which are prepared through fermentation process mediated by microorganisms. Classical literature indicates that microorganisms involved in "Asava" fermentation not only mediate the process but also involve microbial biotransformation of the initial "Asava" ingredients into more effective therapeutic end-products [25]. Better therapeutic properties can also be obtained due to the improvement of the extraction of drug molecules from the herbs by alcohol-aqueous milieu which is also produced by microbes [25]. In addition, microbial metabolic products may be included in the final product prepared by "Asava". Such kind of microbial chemical contribution may result in the therapeutic activity of the product. Hence, the immunostimulating action of "Saribadi" and "Anantamul Salsa" found in this investigation may also be due to the metabolic products of microbes involved in the fermentation, or unknown chemical substance(s) formed because of the intimate contact of microbes with the herbal ingredients. However, more exhaustive investigations are needed to substantiate the claim.

From our preliminary findings, it can be concluded that Ayurvedic herbal preparations, "Saribadi" and "Anantamul Salsa" possess immunopotentiating activity due to its capability on enhancing antibody production and lymphocytes proliferation. These two preparations may be useful for the up-regulation of immune responses and/or in case of impaired immunity to combat infections. However, as this is a preliminary study report, further details *in vitro* studies on the effects of those two herbal preparations on macrophage, natural killer cells, T-cells, cytokines [tumor necrosis factor-α, interferon-γ, interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-12] productions as well as *in vivo* humoral and cellular immunological investigations are required to measure its actual immunomodulating potential and molecular mechanism of actions.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Goto T, Sarker MMR, Zhong M, Tanaka S, Gohda E. Enhancement of immunoglobulin M production in B cells by the extract of red bell pepper. *J Health Sci* 2010; **56**(3): 304-9.
[2] Sarker MMR, Gohda E. Promotion of anti-keyhole limpet hemocyanin IgM and IgG antibody productions *in vitro* by red bell pepper extract. *J Funct Foods* 2013; **5**(4): 1918-26.
[3] Godbout JP, Glaser R. Stress-induced immune dysregulation: implications for wound healing, infectious disease and cancer. *J Neuroimmune Pharmacol* 2006; **1**(4): 421-7.
[4] Keusch GT. The history of nutrition: malnutrition, infection and immunity. *J Nutr* 2003; **133**(1): 336S-40S.
[5] Sheridan PA, Paich HA, Handy J, Karlsson EA, Hudgens MG, Sammon AB, et al. Obesity is associated with impaired immune response to influenza vaccination in humans. *Int J Obes* 2012; **36**: 1072-7.
[6] Sarker MM, Nahar S, Shahriar M, Seraj S, Choudhuri MS. Preliminary study of the immunostimulating activity of an ayurvedic preparation, Kanakasava, on the splenic cells of BALB/c mice *in vitro*. *Pharm Biol* 2012; **50**(11): 1467-72.
[7] Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. Immunological aspects of cancer chemotherapy. *Nat Rev Immunol* 2008; **8**(1): 59-73.
[8] Ahmad W, Jantan I, Kumolosasi E, Bukhari SN. Immunostimulatory effects of the standardized extract of *Tinospora crispa* on innate immune responses in Wistar Kyoto rats. *Drug Des Devel Ther* 2015; **9**: 2961-73.
[9] Turvey SE, Broide DH. Innate immunity. *J Allergy Clin Immunol* 2010; **125**(2 Suppl 2): S24-32.
[10] Imam H, Mahbub NU, Khan MF, Hana HK, Sarker MM. Alpha amylase enzyme inhibitory and anti-inflammatory effect of *Lawsonia inermis*. *Pak J Biol Sci* 2013; **16**(23): 1796-800.
[11] Sarker MMR. Adulteration of herbal medicines and dietary supplements with undeclared synthetic drugs: dangerous for human health. *Int J Pharm Pharm Sci* 2014; **6**(4): 1-2.
[12] Sarker MMR, Imam H, Bhuiyan MS, Choudhuri MSK. *In vitro* assessment of Prasarani Sandhan, a traditional polyherbal Ayurvedic medicine, for immunostimulating activity in splenic cells of BALB/c mice. *Int J Pharm Pharm Sci* 2014; **6**(9): 531-4.
[13] Sarker MMR. Therapeutic efficacy and safety of traditional Ayurvedic medicines: demands for extensive research and publications. *J Homeopath Ayurveda Med* 2014; **3**: e113.
[14] Sree Kundeswari Aushadhalya Ltd. Asava and arista (liquid preparations). Chittagong: Sree Kundeswari Aushadhalya Ltd. [Online] Available from: http://kundeswari.com/ashava.htm [Accessed on 17th January, 2016]
[15] IndiaMART. Anantamul salsa syrup. Uttar Pradesh: IndiaMART. [Online] Available from: http://trade.indiamart.com/details.mp?offer=8966376673 [Accessed on 17th January, 2016]
[16] Jayaram S, Dharmesh SM. Assessment of antioxidant potentials of free and bound phenolics of *Hemidesmus indicus* (L) R.Br against oxidative damage. *Pharmacogn Res* 2011; **3**(4): 225-31.
[17] Ferruzzi L, Turrini E, Burattini S, Falcieri E, Poli F, Mandrone M, et al. *Hemidesmus indicus* induces apoptosis as well as differentiation in a human promyelocytic leukemic cell line. *J Ethnopharmacol* 2013; **147**(1): 84-91.
[18] Weissner W. Anantamul (*Hemidesmus indicus*): a review of biomedical studies and U.S. products. *Ayurveda J Health* 2014; **7**(4): 40-52.
[19] Gayathri M, Kannabiran K. Antimicrobial activity of *Hemidesmus indicus*, *Ficus bengalensis* and *Pterocarpus marsupium* roxb. *Indian J Pharm Sci* 2009; **71**(5): 578-81.
[20] Sowmia C, Kokilavani R. Antidiabetic and anti-hypercholesterolemic effect of *Hemidesmus indicus* Linn.R. root in alloxan induced diabetic rats. *Anc Sci Life* 2007; **26**(4): 4-10.
[21] Khandelwal VK, Balaraman R, Ondrejcáková M, Pancza D, Ravingerová T. Effect of *Hemidesmus indicus* on ischemia-reperfusion injury in the isolated rat heart. *Pharm Biol* 2010; **48**(6): 611-4.
[22] *Bangladesh National Formulary of Ayurvedic Medicine*. Dhaka: Bangladesh Unani and Ayurvedic Board; 1992.
[23] *The Ayurvedic pharmacopoeia of India*. New Delhi: Ministry of Health and Family Welfare, Government of India, Department of Indian Systems of Medicine and Homeopathy; 2007.
[24] Sarker MMR, Mazumder MEH, Rashid MH. *In vitro* enhancement of polyclonal IgM production by ethanolic extract of *Nigella sativa* L. seeds in whole spleen cells of female BALB/c mice. *Bangladesh Pharm J* 2011; **14**(1): 73-7.
[25] Sarker MMR, Nimmi I, Kawsar MH. Preliminary screening of six popular fruits of Bangladesh for *in vitro* IgM production and proliferation of splenocytes. *Bangladesh Pharm J* 2012; **15**(1): 31-7.
[26] Sarker MMR. Studies on the enhancement of antigen-specific antibody production in murine B cells by the extract of bell pepper flesh [dissertation]. Okayama: Okayama University; 2010.
[27] Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989; **119**(2): 203-10.
[28] Sarker MM, Zhong M. Keyhole limpet hemocyanin augmented the killing activity, cytokine production and proliferation of NK cells, and inhibited the proliferation of Meth A sarcoma cells *in vitro*. *Indian J Pharmacol* 2014; **46**(1): 40-5.
[29] Sarker MMR. Induction of humoral immunity through the enhancement of IgM production in murine splenic cells by ethanolic extract of seed of *Piper nigrum* L. *J Sci Res* 2012; **4**(3): 751-6.
[30] Sarker MMR, Choudhuri MSK, Zhong M. Effect of Chandanasav, an Ayurvedic formulation, on mice whole splenocytes for the production of polyclonal IgM and proliferation of cells: a preliminary study. *Int J Pharm Sci Res* 2012; **3**: 1294-9.
[31] Morrison DC, Jacobs DM. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry* 1976; **13**(10): 813-8.
[32] Beutler B. Innate immunity: an overview. *Mol Immunol* 2004; **40**(12): 845-59.