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EFFECT OF CHANDANASAV, AN AYURVEDIC FORMULATION, ON MICE WHOLE SPLENOCYTES FOR THE PRODUCTION OF POLYCLONAL IgM AND PROLIFERATION OF CELLS: A PRELIMINARY STUDY

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ABSTRACT

In recent days, it has been given great emphasis on the scientific evaluation of traditional or complementary and alternative medicines in order to justify the traditional use, safety, efficacy, quality, and to know its therapeutic values that have not yet explored. Chandanasav, an ancient Ayurved formulation, is commonly used for the treatment of malnutrition, presence of semen in the urine, and treatment of heart and autoimmune diseases. Till now, no research information is available on the immuno-stimulatory activities of this preparation. The present study was designed to evaluate the preliminary immunostimulating potential of Chandanasav for polyclonal immunoglobulin M (IgM) production and proliferation of murine spleen cells. BALB/c mice splenocytes were treated with 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3 and 4% (v/v) of Chandanasav and the cells were sub-cultured at 37^{o}C in a humidified atmosphere containing 5% CO_2 for 120 h. IgM production in cultured supernatant was determined by Enzyme-Linked Immunosorbent Assay (ELISA) and splenocytes proliferation was measured by 3-(4, 5-dimethyl thiazol-2-y)-2, 5-diphenylterazolium bromide (MTT) assay. Chandanasav at the doses of 0.25, 0.5, 0.75, 1, 1.5 and 2% (v/v) remarkably augmented polyclonal IgM production in cultured supernatants, and those doses moderately enhanced the proliferation of splenocytes. The highest increment of IgM production, observed by 1% Chandanasav, was found to be 11.5-fold higher than that of untreated cells. On the other hand, the highest enhancement of splenocytes proliferation was observed to be 2.5 times higher than control. The IgM production capacity and cells proliferation ability of Chandanasav were reduced by 43 and 24% respectively in cells treated with polymixin B. Thus, our results suggest that the observed enhancement of IgM production treated with Chandanasav in this study was due to the contamination of bacterial endotoxin and hence, this preparation does not possess immunostimulating property. However, further research is recommended to ascertain this finding.

INTRODUCTION: Although the Ayurved medicines are the most popular form of alternative medicines being practiced in Bangladesh, scientific researches are very negligible on it compared to Allophathic or modern medicines. 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 [1]. The national health policy of Bangladesh has similar objective to encourage the systemic improvement of the practice of indigenous medicines and emphasized scientific evaluation of those indigenous and herbal medicines [2]. Chandanasav is an Ayurved formulated medicine composed of 24 plant ingredients, listed in the **Table 1**, *Santalam album* L. and *Pterocarpus santalinus* L. being the main ingredients [3]. This preparation is indicated for the treatment of malnutrition, presence of semen in urine, painful micturation, heart diseases, autoimmune diseases, loss of appetite and as astringent and carminative [3, 4]. Only few researches have done for the scientific evaluation of this preparation and no data is available on its immunostimulating activities. Ayurved medicine enlisted many medicinal plants that are believed to be human 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 [5, 6].

TABLE 1. LIST OF PLANT MATERIALS IN CHANDANASAV ACCORDING TO THE FORMULA OF BANGLADESH NATIONAL FORMULARY OF AYURVEDIC MEDICINES [3]

Sl. No.	Plant name with family	Parts used
1	*Santalam album* (Sandal wood) (f. Santalaceae)	Wood
2	*Pterocarpus santalinus* L. (f. Fabaceae)	Wood
3	*Pavonia odorata* (f. Malvaceae)	Root
4	*Cyperus rotundus* L. (f. Cyperaceae)	Rhizome
5	*Nymphaca stellosta* (f. Nymphaeaceae)	Rhizome/Flower
6	*Gmelina arborea* (Roxb.) (f. Lamiaceae)	Fruit
7	*Aglaia roxburghiana* (f. Maliaceae)	Seed
8	*Prunus cerasoides (* D. Don) (f. Rosaceae)	Wood
9	*Symplocos racemosa* (f. Symploocaceae)	Bark
10	*Rubia cordifolia* (f. Rubiaceae)	Root
11	*Stephania hernandifolia* (f. Menispermaceae)	Root/Stem
12	*Swertia chirata* (f. Gentianaceae)	Root/Stem
13	*Ficus bengalensis* L. (f. Moraceae)	Bark
14	*Ficus relegiosa* L. (f. Moraceae)	Bark
15	*Magnifera indica* L. (f. Anacardiaceae)	Bark
16	*Bauhinia variegata* L. (f. Fabaceae)	Bark
17	*Trichosanthes dioica* Roxb. (f. Cucurbitaceae)	Leaf
18	*Vanda roxburghii* (f. Orchidaceae)	Root
19	*Glycerrhiza glabra* L. (f. Legumonoceae)	Root
20	*Oldenlandia corymbosa* (f. Rubiaceae)	Whole plant
21	*Curcuma zedoaria* (f. Zingiberaceae)	Rhizome
22	*Salmalia malabarica* (f. Bombacaceae)	Exudate
23	*Woodfordia fruticosa* (f. Lythaceae)	Flower
24	*Vitis vinifera* L. (f. Vitaceae)	Fruit

Thus, the present study was aimed to determine the effect of Chandanasav on the enhancement of humoral immunity by measuring the production of IgM and cells proliferations in cultured murine splenocytes in order to evaluate its immunostimulating activity.

MATERIALS AND METHODS:

Collection of sample: Chandanasav, 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 Chandanasav are listed in Table 1.

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.

Chemicals and reagents: 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 mice 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 us [7, 8]. 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.,* 2011 [7]. 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 Chandanasav 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.

Measurement of splenocytes proliferation by MTT assay: The growth of cultured cells was determined by MTT assay as previously described [9]. 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 [7, 8]. 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 2h 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-ethylbenzthia zoline-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).

Data Analysis: The experimental results are expressed as means ± S.D. of triplicate culture.

RESULTS:

Effect of Chandanasav on the production of Polyclonal IgM: BALB/c female mice spleen cells were sub-cultured with or without Chandanasav for 5 days and the quantity of IgM produced in the cultured supernatants were determined by an IgM-ELISA. Lipopolysaccharide (LPS) was used as a positive control. Our data (**Fig. 1**) showed that Chandanasav at the doses of 0.25, 0.5, 0.75, 1, 1.5 and 2% enhanced IgM production by 7.5, 11, 10, 11.5, 10 and 7.5-times higher than that of control, respectively.

The highest IgM enhancement by 1% of Chandanasav was observed to be 11.5 times greater than the control. LPS (0.1 µg/ml) was used as a positive control which increased IgM production 7-fold higher than control. Therefore, our data demonstrates that the result of the present study is strongly comparable with the effect of the LPS. The concentration of Chandanasav higher than 2% could not increase IgM level or those concentrations were toxic to the cells.

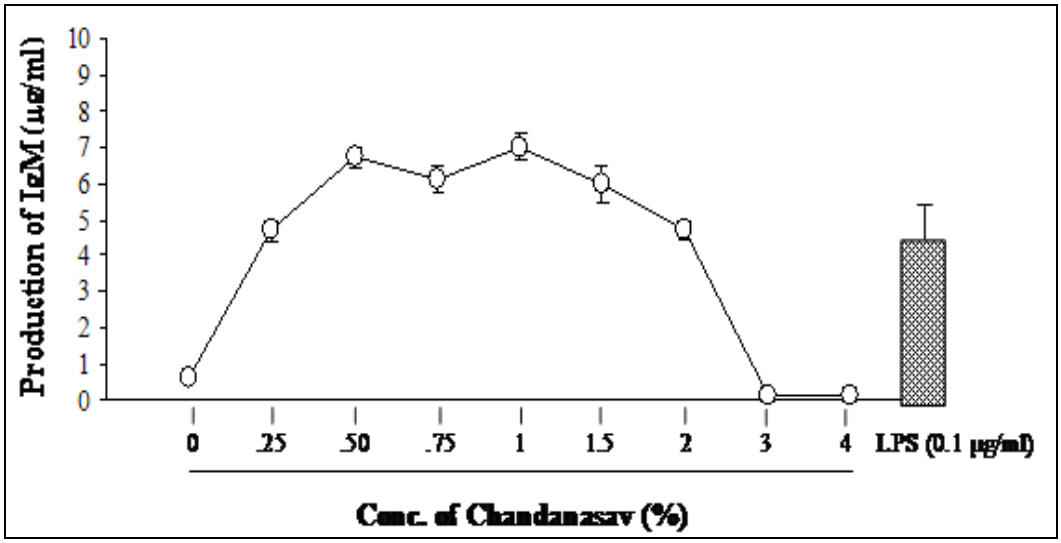

FIG. 1: EFFECT OF CHANDANASAV (CDV) ON THE PRODUCTION OF IgM IN CULTURED SUPERNATANTS OF MURINE SPLENOCYTES

BALB/c mice whole spleen cells (2.5 ×10^5 cells/well) were incubated with the indicated concentrations of CDV at 37^{O}C in the 5% CO_2 incubator for 5 days. The IgM levels in the supernatants were determined by an ELISA. The data are means ± S.D. of triplicate culture

Effect of Chandanasav on the proliferation of murine spleen cells: BALB/c female mice spleen cells were sub-cultured with or without varying doses of Chandanasav for 5 days and the proliferation of cells were measured by MTT assay [9]. As shown in **Fig. 2**, Chandanasav at concentrations of 0.25, 0.50, 0.75, 1, 1.5 and 2% stimulated the proliferation of splenocytes 1.52, 2, 1.71, 2.09, 2.25 and 2.56-folds respectively; the highest enhancement of proliferation was 2.56 times higher than that of control exhibited by 2% of Chandanasav.

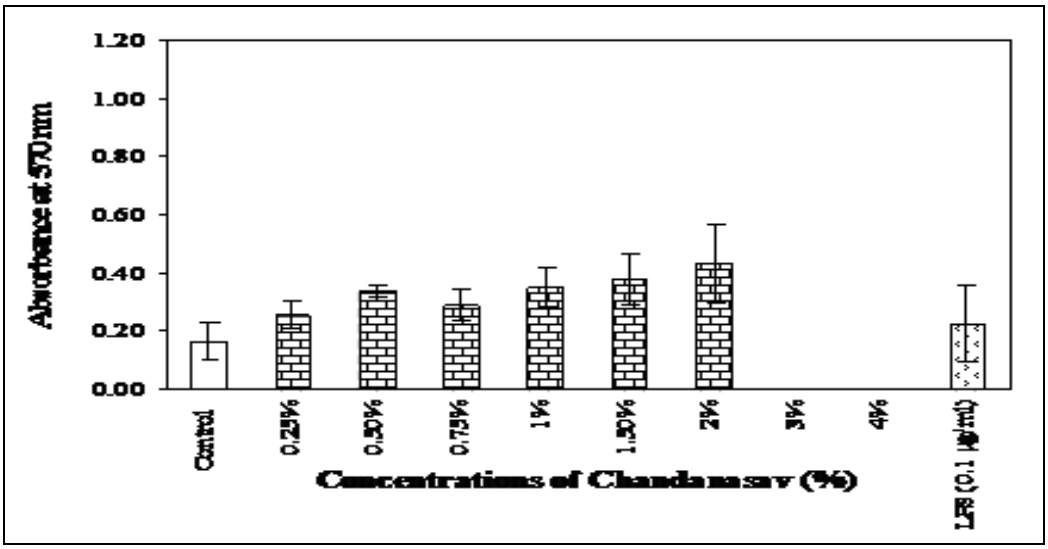

FIG. 2: EFFECT OF CHANDANASAV (CDV) ON THE PROLIFERATION OF SPLENOCYTES IN CULTURE

BALB/c mice whole spleen cells (2.5 ×10^5 cells/well) were incubated with the indicated concentrations of CDV at 37°C in the 5% CO_2 incubator for 5 days. The proliferations of cells were determined by MTT assay. The data are means ± S.D. of triplicate culture.

Polymyxin B (PMB) suppressed the activity of Chandanasav for the production of IgM and proliferation of cells: There was a possibility of endotoxin contamination, such as LPS, in the Chandanasav during the processing of the ingredients, in fermentation stage of manufacturing, etc. In order to exclude the endotoxin contamination possibility in Chandanasav, its ability for the production of IgM and influence on proliferation was evaluated in cells treated with an antibiotic polymixin B (PMB) which inhibits the activity of LPS. The investigation resulted that treatment of Chandanasav (1%) by PMB inhibited the IgM production ability of this preparation by 43.35% compared to untreated cells (**Fig. 3A**).

Similarly, activity of Chandanasav for the proliferation of murine splenocytes was 24.40% reduced in PMB-treated cells compared to Control (**Fig. 3B**).

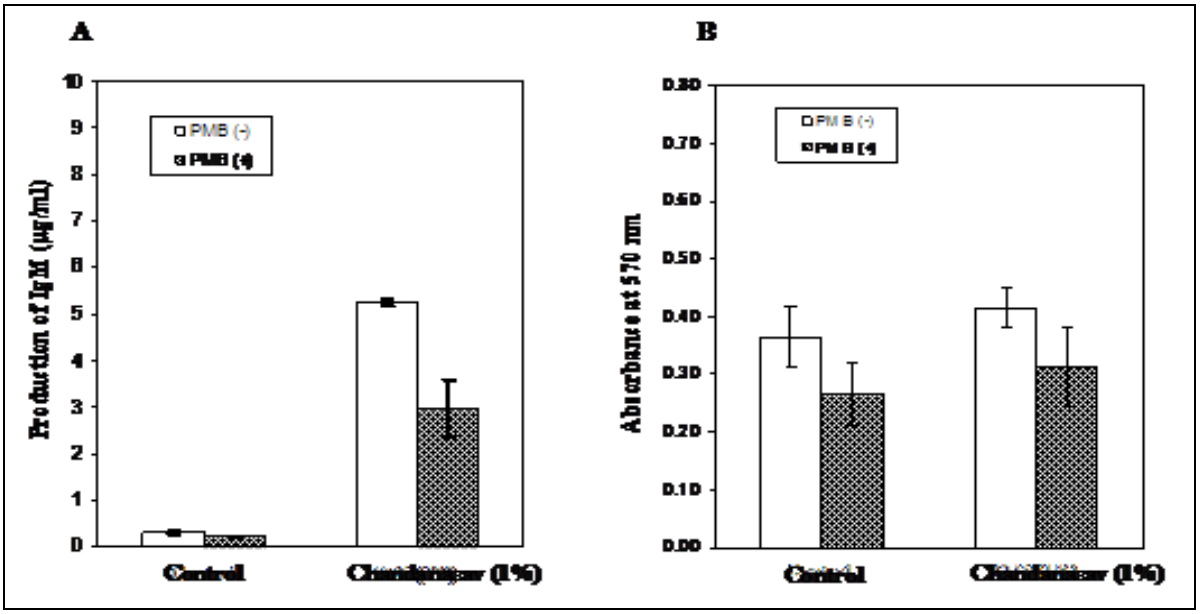

FIG. 3: ASSESSMENT OF BACTERIAL ENDOTOXICITY IN CHANDANASAV BY MEASURING IgM PRODUCED IN CULTURED SUPERNATANTS (A) AND PROLIFERATION OF SPLENOCYTES (B) IN THE PRESENCE AND ABSENCE OF PMB. BALB/c mice splenocytes (2.5×10^5 cells/well) were incubated with the optimum conc. of CDV (1%) and with or without 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 proliferation of cells were determined by MTT assay. The data are means ± S.D. of triplicate cultures.

DISCUSSION: Our result showed that different doses of Chandanasav potentially enhanced the level of IgM production (**Fig. 1**) and moderately increased the proliferation of murine splenocytes (**Fig. 2**). These two experimental results indicate the immunostimulating potential of Chandanasav. However, treatment of Chandanasav by PMB remarkably reduced the level of IgM production in cells compared to Chandanasav untreated by PMB. PMB binds to the lipid portion of bacterial LPS and thus inhibit LPS activity [12].

Thus, we can say that Chandanasav contained bacterial LPS as contaminant and, for that reason, the immunostimulating activities of Chandanasav (as shown in **Fig. 1** and **Fig. 2**) were drastically reduced (**Fig. 3**) when treated with PMB. Therefore, it can be concluded that Chandanasav does not possess potential ability to enhance humoral immunity; rather the elevated IgM production observed in this study was due to the contamination of bacterial LPS with Chandanasav in the formulating ingredients or during its manufacturing process.

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 Ayurvedic medicines, lack of modern advancement on its preparation techniques and, above all, microbial contamination of these preparations during the manufacturing process still great problems in Bangladesh.

CONCLUSION: On the basis of present investigations, it can be reported that Chandanasav does not possess immunostimulating activity in respect of humoral immune response and the preparation was contaminated by bacterial endotoxin (LPS). So, it should be very careful in the preparation of Chandanasav as well as during the manufacturing of Ayurved medicines.

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REFERENCES:

1. Karim MR: Comparative Pharmacological Studies of Ayurvedic Medicines Used in Renal Disorders. M. Pharm. thesis. Jahangirnagar University, Pharmacy dept., Bangladesh, 2002.
2. Chakraborty S, Rajia S, Choudhuri, MSK, Hossain MF, Sattar M and Shrestha, T: Some pharmacological findings of non therapeutic importance of an Ayurvedic preparation Chandanasava. Oriental Pharmacy and Experimental Medicine 2006; 6(2): 157-160.
3. Anonymous. *Bangladesh National Formulary of Ayurvedic Medicine*, National Unani and Ayurvedic Formulary Committee Bangladesh, Board of Unani and Ayurvedic System of Medicine, Bangabandhu Avenue, Dhaka, Bangladesh, 1992.
4. Shankar K and Amritpal S: Standardization of polyherbal Ayurvedic formulation: Chandanasava. International Journal of Research in Ayurveda & Pharmacy 2011; 2(2): 665-669.
5. Khan S and Balick MJ: Therapeutic plants of Ayurveda: a review of selected clinical and other studies for 166 species. Journal of Alternative and Complementary Medicine 2001; 7: 405-515.
6. Patwardhan B and Chorghade M: Ayurveda and natural products discovery. Current Science 2004; 86: 789-98.
7. Sarker MMR, Mazumder MEH and Rashid MHO: *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 Pharmaceutical Journal 2011; 14: 73-77.
8. Goto T, Sarker MMR, Zhong M, Tanaka S and Gohda E: Enhancement of immunoglobulin M Production in B cells by the Extract of Red Bell Pepper. Journal of Health Science 2010; 56: 304-09.
9. Hansen MB, Neilsen SE and Berg K: Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. Journal of Immunological Methods 1989; 119: 203-10.
10. Sarker MMR: Studies on the enhancement of antigen-specific antibody production in murine B cells by the extract of bell pepper flesh. PhD Thesis, Okayama Univeristy, Japan, 2010.
11. Yoshihara R, Aoyama E, Kadota Y, Kawai S, Goto T, Zhong M and Gohda E: Differentiation of murine B cells induced by chondroitin sulfate B. Cellular Immunology 2007; 250: 14-23.
12. Morrison DC and Jacobs DM: Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry 1976; 13: 813-18.

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