SHORT COMMUNICATION

Preliminary study of the immunostimulating activity of an ayurvedic preparation, Kanakasava, on the splenic cells of BALB/c mice in vitro

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Abstract

Context: Immunostimulant plays an important role to prevent infections when defensive capacity of body is impaired, commonly occur with aging, cancer, diabetes, and sepsis. Kanakasava (KNK) is a polyherbal ayurvedic preparation used since ancient times for the treatment of respiratory diseases and to improve immunity.

Objective: The present study evaluated the immunostimulating potential of KNK.

Materials and methods: The immunostimulating activity of KNK was evaluated by measuring immunoglobulin M (IgM) production and splenocyte proliferation in vitro. BALB/c mice splenocytes were treated with 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, and 4% (v/v) of KNK and the cells were subcultured at 37°C, humidified atmosphere containing 5% CO2 for 120 h. The production of IgM in cultured supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) and the proliferations of cells were measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) method.

Results and discussion: KNK at the doses of 0.25, 0.5, 0.75, 1, and 1.5% (v/v) significantly augmented polyclonal IgM production (1.211, 1.260, 1.274, 1.180, and 1.028 µg/mL, respectively) compared to control (0.246 µg/mL). Similarly, the same doses stimulated the proliferation of splenocytes as well (Abs. 0.270, 0.281, 0.368, 0.328, and 0.301, respectively, measured at 570 nm) compared to untreated cells (Abs. 0.137). The activity of KNK was not retarded by the treatment of cells with polymixin B. Thus, our results demonstrate that KNK possesses immunostimulating potential that acts through the induction of lymphocytes for proliferation and IgM production.

Conclusion: KNK may be useful for strengthening immune responses in case of insufficient or impaired immunity.

Keywords: Kanakasava, Ayurvedic medicine, immunostimulant, splenocytes, IgM, differentiation, proliferation

Introduction

The immune system of the human body fights against bacteria, viruses, other parasites, and cancers (Goto et al., 2010). The defense of the immune system may be impaired and/or suppressed with age (Grubeck-Loebenstein, 1997), in conditions like diabetes, cancer, burns, sepsis, (Wustrow & Mahnke, 1996), and stress (Aubin, 2003). Besides, immunity is greatly suppressed in patients receiving chemotherapy and radiotherapy (Goto et al., 2010). External immunostimulating agents may be
useful for the upregulation of the immune system to prevent infections under those immune-compromised conditions.

Ayurvedic medicine enlisted many medicinal plants that are believed to be human immune system modulators/activators and formulations based on such plants playing an important role in modern healthcare, especially on managing immune-related problems, as well as fulfilling unmet needs for effective and/or safer treatment regimes (Khan & Balick, 2001; Patwardhan & Chorghade, 2004). Kanakasava (KNK) is a polyherbal ayurvedic preparation widely used mainly for the treatment of respiratory tract diseases, such as asthmatic cough, chronic bronchitis, etc. The preparation is also indicated to improve immunity. Previous studies reported the antibacterial and anthelmintic properties of KNK (Praveen et al., 2010). Although some of the constituents of this preparation were reported to possess immunomodulatory activities (Utsunomiya et al., 1997; Sunila & Kuttan, 2004; Jatav et al., 2011), no scientific study was conducted for the evaluation of immune-related properties of KNK. Therefore, the present investigation was designed to determine its immunostimulating activity on the basis of the production of immunoglobulin M (IgM) responsible for humoral immunity and the proliferation of splenic cells in vitro.

Materials and methods
Preparation and collection of sample
KNK, used in this study, was prepared according to the formulation and procedure mentioned in the Ayurvedic Pharmacopoeia of India (Anonymous, 2003). The ingredient of KNK are listed in Table 1. All the ingredients were purchased from reputed herbal medical ingredient suppliers in Dhaka, Bangladesh. The materials were confirmed with their morphological features by Dr. Saleh Ahmed Khan, associate professor and taxonomist, Department of Botany, Jahangirnagar University, Savar, Dhaka, Bangladesh and voucher specimens were preserved at the department. The preparation of the drug was done strictly under the supervision of an ayurvedic physician in the Ayurvedic Research Laboratory of the Department of Pharmacy, Faculty of Biological Sciences, Jahangirnagar University, Savar, Dhaka, Bangladesh. The sample was placed in centrifuge tubes and filtered using 0.22 µm microfilter just before use. 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) 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 (Tokyo, 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), Organon Teknika Corporation (Durham, NC), and Kirkegaard & Perry Laboratories (Gaithersburg, MD), respectively.

Mice
Female BALB/c 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 (Aoyama et al., 2005).

Mice were killed and spleens were collected aseptically. The spleens were mashed with a spatula through a strainer to pass the cells into MEM (pH adjusted to around 7.0 with 1 N NaOH) in a Petri dish (Iwaki, Japan). Cells were suspended with a Pasteur pipette and screened by passing through a mesh in 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 NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA; pH 7.2) was added to the cells in the centrifuge tube for lysing the erythrocytes for 5 min at room temperature. 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 resuspended in MEM and passed through a mesh into another centrifuge tube to collect the spleen cells.

<table>
<thead>
<tr>
<th>No.</th>
<th>Plant name</th>
<th>Parts used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Datura metel</em> L.</td>
<td>Whole plant</td>
</tr>
<tr>
<td>2</td>
<td><em>Addhatoda vasica</em> Nees</td>
<td>Root and bark</td>
</tr>
<tr>
<td>3</td>
<td><em>Glycerrha glabra</em> L.</td>
<td>Root</td>
</tr>
<tr>
<td>4</td>
<td><em>Piper longum</em> L.</td>
<td>Flower</td>
</tr>
<tr>
<td>5</td>
<td><em>Solanum surattense</em> Burm. f.</td>
<td>Whole plant</td>
</tr>
<tr>
<td>6</td>
<td><em>Mesua nagassarium</em> (Burm. f.) Kosterm</td>
<td>Flower</td>
</tr>
<tr>
<td>7</td>
<td><em>Zingiber officinalis</em> Roxb.</td>
<td>Rhizome</td>
</tr>
<tr>
<td>8</td>
<td><em>Clerodendrum serratum</em> (L.) Moon</td>
<td>Root</td>
</tr>
<tr>
<td>9</td>
<td><em>Abies spectabilis</em> (D.Don) G.Don</td>
<td>Leaf</td>
</tr>
<tr>
<td>10</td>
<td><em>Woodfordia fruticosas</em> (L.) Kurz</td>
<td>Inflorescence</td>
</tr>
<tr>
<td>11</td>
<td><em>Vitis vinifera</em> L.</td>
<td>Dried flower</td>
</tr>
</tbody>
</table>
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
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 x 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% CO₂. A 50 µL aliquot 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 KNK and LPS, diluted with the basal medium, at 37°C in the CO₂ incubator. The cultured supernatants were then collected and frozen at 30°C for IgM and IgG enzyme-linked immunosorbent assays (ELISA) and the cells pellets were used for MTT assay.

Cells proliferation study
The growth of cultured cells was determined by the MTT assay as described previously (Hansen et al., 1989). Briefly, at the end of incubation of splenocytes for 120 h, 160 µL of the supernatants were removed. A 60 µL aliquot 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 the dark at 37°C and the absorbance was measured at 570 nm by using a plate reader (Bio-Rad Laboratories, Hercules, CA).

ELISA for the determination of IgM production
The IgM antibody production level was measured by a sandwich ELISA as described earlier (Goto et al., 2010) with some modifications. 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 with PBS containing 0.05% Tween 20 (wash buffer) (200 µL/well). The wells were blocked with 200 µL of 1% BSA in PBS for 2 h at room temperature. 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 temperature. The plates were again washed three times with wash buffer (200 µL/well). Fifty microliter per well of HRP-conjugated goat anti-mouse IgM (0.2 µg/mL) was added into each well and the plates were incubated for 1 h at room temperature. 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% H₂O₂ were added. The plates were then incubated for 10 min at room temperature and the optical densities at 405 nm were measured using an automatic plate reader (Bio-Rad Laboratories).

Statistical analysis of data
The experimental results are expressed as means ± SEM. of three independent experiments. The differences between the control and treated groups were analyzed by Tukey HSD test, Student’s t-test, and Dunnett’s T3 test. p values less than 5% were regarded as significant.

Results and discussion
Enhancement of polyclonal IgM production by KNK
BALB/c female mice spleen cells were subcultured with or without KNK for 5 days and the amount of IgM production in the cultural supernatants were determined by an IgM-ELISA. LPS was used as a positive control. Our data (Figure 1) showed that KNK significantly enhanced polyclonal IgM production at the doses of 0.25, 0.5, 0.75, 1, and 1.5%, compared to control, and then declined. The highest IgM enhancement by KNK at a dose of 0.75% was found to be five fold greater than control. The concentration of KNK higher than 1.5% could not increase IgM level or those concentrations were toxic to the cells. Another study in our laboratory showed six times enhancement of IgG production in cultural supernatant by 1% KNK (v/v) comparing to untreated cells (unpublished data). The enhanced antibody production is an indication of the differentiation of B cells to antibody secreting plasma cells (Goto et al., 2010). Thus, the result demonstrated that KNK promoted the differentiation of B cells to antibody secreting plasma cells. Although the production of antibody was promoted by KNK at lower concentrations (0.25–1.5%), the higher doses, 3 and 4% of KNK, were found to suppress the
production of both IgM (Figure 1) and IgG antibodies (data not shown), suggesting the preparation contains an inhibitory substance(s) which is effective at higher concentrations. Gharete et al. (2011) determined the pH of different batches of KNK at different time intervals and reported mean pH to be around 4 in all cases. Therefore, the higher concentrations of KNK (any conc. higher than 3%) with its acidic pH may be cytotoxic for cultural splenic cells; hence, cells were supposed to die during culture with high concentrations. This may be another reason for the reduced production of antibodies in cultural supernatants of cells treated with higher concentrations of KNK.

**KNK induced the proliferation of murine spleen cells**

Spleen cells of BALB/c female mice were subcultured with or without varying doses of KNK for 5 days and the proliferation of cells were measured by the MTT assay (Aoyama et al., 2005). As shown in the Figure 2, KNK, at concentrations of 0.25, 0.50, 0.75, 1, and 1.5%, significantly stimulated the proliferation of murine splenocytes; the highest enhancement of proliferation was 2.5 times exhibited by 0.75% of KNK. Increasing proliferation of cells is an indicator of immunostimulation (Sarker, 2010). Similar to antibody production, the drug at higher doses (>2% v/v) was observed to be cytotoxic, hence suppressing the proliferation of splenocytes.

**Immunostimulating activity of KNK was not significantly reduced when treated with polymyxin B**

There was a possibility of endotoxin contamination, such as LPS, in the KNK during the processing of the ingredients, in the fermentation stage of manufacturing, for example. In order to exclude the endotoxin contamination possibility in KNK, its ability for the production of IgM and influence on proliferation was evaluated in cells treated with polymyxin B (PMB). The investigation resulted significant enhancement of IgM production in PMB-treated spleen cells as well (Figure 3A). KNK resulted in 436 and 570% increase in the production of IgM in PMB-untreated and PMB-treated spleen cells, respectively, compared to control data. There was no significant difference in the IgM production between PMB-treated and untreated cells.

Similarly, no significant difference in the activity of KNK was observed on the proliferation of PMB-untreated and treated cells (Figure 3B). These data also clearly indicate that treatment of KNK by PMB has no influence on cell proliferation.

PMB binds to the lipid portion of bacterial LPS and thus inhibit LPS activity (Morrison & Jacobs, 1976). Our

![Figure 2. Effect of Kanakasava on the proliferation of splenocytes in culture. BALB/c mice splenocytes (2.5 x 10⁶ cells/well) were incubated with the indicated concentrations of KNK at 37°C in the 5% CO₂ incubator for 5 days. The proliferation of cells was determined by the MTT assay. The data are means ± SEM. of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001, as compared with the control (Tukey HSD test).](image)

![Figure 3. Assessment of bacterial endotoxicity in Kanakasava by determining IgM production in cultured supernatants and proliferation of splenocytes in the presence and absence of polymyxin B (PMB). BALB/c mice splenocytes (2.5 x 10⁶ cells/well) were incubated with the optimum concentration of KNK (0.75%) and with or without PMB at 37°C in the 5% CO₂ 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 are means ± SEM. of three independent experiments (Student’s t-test).](image)
results demonstrate that KNK did not contain bacterial LPS as contaminant. Therefore, it can be concluded that the immunostimulating ability of KNK on murine splenocytes is not due to the presence of LPS. The response was due to active chemical(s) existing in the preparation.

KNK is a polyherbal formulation containing 11 plant materials (Table 1). Each plant material possesses different pharmacological and biological activities. For example, it has been reported that leaf extracts of Mesua nagassarium (Burm. f.) exerts antimicrobial and cytotoxic properties (Sikder et al., 2011). Ethanol extract of the roots of Clerodendrum serratum L. exhibited antibacterial and anti-inflammatory activities (Narayanan et al., 1999). Studies indicate that Glycyrrhiza glabra L. possesses antibacterial, antioxidant, antimalarial, anti-inflammatory, antiviral, antifungal and immunostimulatory activities (Jatav et al., 2011). Glycerrhizin was reported to have a specific inhibitor of the cell-mediated immunological response. It stimulates T cells for the production of γ-interferon (Utsunomiya et al., 1997; Jatav et al., 2011). Moreover, immunomodulatory properties also exhibited by other plant ingredients of the KNK formulation, such as, Piper longum L. (Sunila & Kuttan, 2004; Zaveri et al., 2010), Zingiber officinale Roxb. (Carrasco et al., 2009), and Adhatoda vasica (Vinothapooshan & Sundar, 2011). Vitis vinifera L. leaves showed antibacterial, antifungal, antiviral, and antioxidant properties (Orhan et al., 2009). The methanol extract of leaves and flowers of Datura metel L. has been reported to exhibit antimicrobial activity (Britto & Gracelin, 2011). Thus, the immunostimulating property of KNK may be due to the 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 of KNK preparation.

KNK is an Asava (fermented infusion) product, which is prepared through a fermentation process mediated by microorganisms. Classical literature indicates that microbes involved in Asava fermentation mediate the process; enhance therapeutic properties, which may be due to the microbial biotransformation of the initial ingredients of Asava into more effective therapeutics as end products; and improve the extraction of drug molecules from the herbs by alcohol-aqueous milieu, which is also produced by microbes (Sekar & Mariappan, 2008). Besides, the metabolic products of microbes may be included in the ultimate product prepared by this process. This kind of microbial chemical contribution may result in the therapeutic activity of the product. Hence, the immunostimulating action of KNK 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 work needs to be performed to substantiate the claim.

Conclusion

On the basis of our findings, it can be suggested that KNK possesses immunostimulating activity due to its capability of enhancing antibody production and splenocyte proliferation. Moreover, we can conclude that this ayurvedic preparation may be useful in upregulating immune responses, and thereby its use as immunostimulant in ayurvedic medicine can be rationalized. However, as this is a preliminary study, further in vitro research work of the effect of KNK on polymorphonuclear cells and macrophages, and in vivo studies for humoral and cellular immune responses, are recommended.

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Declaration of interest

The authors have no conflict of interest.

References


