Preliminary Screening of Six Popular Fruits of Bangladesh for *in vitro* IgM Production and Proliferation of Splenocytes

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Abstract

Methanolic extracts of whole fruits or juice of six popular fruits of Bangladesh were evaluated for immunostimulating activity based on immunoglobulin M production and proliferation of murine splenic cells in culture medium. The production of IgM in cultured supernatants was determined by an Enzyme-Linked Immunosorbent Assay (ELISA) and the proliferation of spleen cells was measured by 3-(4,5-dimethylthiazol-2-y)-2,5-diphenylterazolium bromide (MTT) . Of the six crude extracts assayed, methanol extracts of *Spondias dulcis* (L.) and *Dillenia indica* (L.) at 200 µg/ml enhanced polyclonal IgM production by 108.33% and 90.72%, respectively when compared to the control. Treatment of splenocytes with the juice extracts of *Ananas comosus* (L.) Merr. and *Citrus medica* (L.) at 200 µg/ml increased IgM productions by 155% and 103.85%, respectively in comparison to untreated cells. No dose of the methanol extract of *Phyllanthus embelica* (L.) and juice extract of *Citrus grandis* (L.) could increase IgM level in cultured supernatants. The increment of IgM production was observed to be 190% higher than the control which was produced by the juice extract of *Ananas comosus* (L.) Merr. at a dose of 2 mg/ml. None of the extract could stimulate the proliferation of cultured splenocytes. Thus, the present findings suggest that *S. dulcis*, *D. indica*, *A. comosus* and *C. medica* possess moderate immunostimulating activities through the stimulation of B cells for IgM production and hence may be useful for enhancing the immunity of the body. However, comprehensive studies are needed to ascertain their immunopotentiality through both of cellular and humoral arms of immunity. To the best of our knowledge, this is the first report on the immunostimulating activity of the above mentioned fruits of Bangladesh.

Key words: Fruits, Bangladesh, IgM, proliferation, antibody, immunostimulant, splenocytes, mice.

Introduction

Epidemiological studies have shown that an inverse relationship exists between consumption of dietary flavonoids and cardiovascular diseases (Tripoli *et al.*, 2007). Different scientific studies reported that consumption of fruits reduces the risk of cancers (Kris-Etherton *et al.*, 2002), neurogenerative diseases, e.g., Parkinson’s and Alzheimer’s diseases (Di Matteo and Esposito, 2003), inflammation and aging (Ames *et al.*, 1993). Fruits are important sources of macro-and micro-nutrients; they can be used for both the prevention and cure of diseases only if their therapeutic and nutritional values are known. Since plants secondary metabolites are subject to variation in relation to the environment of cultivation, the secondary metabolites in the fruits growing in Bangladesh may not be identical to those of the same fruits grown in other countries (Hossain *et al.*, 2008). Despite the huge consumption of fruits, scientific data regarding their pharmacological and immunological properties are relatively scarce. External immunostimulants are very helpful to induce immune responses against microbes to prevent infections in immune-compromised conditions, such as aging, cancers, sepsis, diabetes, etc. (Goto *et al.*, 2010). Thus, the present investigation was aimed to assess some commonly eaten fruits of Bangladesh for their immunostimulating activities by determining the *in vitro* IgM production and proliferations of murine spleen cells. The traditional use and pharmacological reports of the currently investigating fruits are summarized in Table 1.
Materials and Methods

Collection of sample and preparation of extract: Raw and green fruits of Amra (S. dulcis), Jambura (C. grandis), Amloki (P. embelica), Chalta (D. indica), Anaras (A. comosus) and Jamir (C. medica) were purchased from commercial stores at Dhaka, Bangladesh. They were authenticated by a Botanist at the Department of Botany, Jahangirnagar University, Savar, Dhaka, Bangladesh, and the voucher specimens were preserved accordingly. Fruits of S. dulcis, P. embelica and D. indica were sliced into small pieces and extracted with 70% methanol at room temperature with occasional shaking for 14 days. The juices were similarly extracted from C. grandis, A. comosus and C. medica. The extract was decanted and the solvent was removed with a rotary evaporator followed by freeze-drying to obtain the crude extract.

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 Kirkegaard & Perry Laboratories (Gaithersburg, MD, USA), respectively. MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylerazolium bromide] reagent was purchased from Sigma Chemical Co., Ltd. (Tokyo, Japan).

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 ages varied

### Table 1. Traditional uses and pharmacological activities of some common fruits of Bangladesh

<table>
<thead>
<tr>
<th>Plant name (Family)</th>
<th>Local name/ common name</th>
<th>Traditional use(s)</th>
<th>Reported phytochemical/ pharmacological activities</th>
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<tr>
<td><em>Spondias dulcis</em> L. (Anacardiaceae)</td>
<td>Amra/AMBarella</td>
<td>Enhancement of eyegight and treatment of eye infections (Rahmatullah et al., 2009a).</td>
<td>Polysaccharide has been identified from the fruits pulp and it has eliciting activity on peritoneal macrophages (Iaconini et al., 2005).</td>
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<tr>
<td><em>Citrus grandis</em> L. Osbeck (Rutaceae)</td>
<td>Jambura/grapefruit</td>
<td>Treatment of skin diseases, such as, scabies, eczema, itches, etc. (Rahmatullah et al., 2009b); treatment of fever (Rahmatullah et al., 2010), and as carminative (Rahmatullah et al., 2009b).</td>
<td>Juice has antioxidant and free radical scavenging abilities (Tsai et al., 2007).</td>
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<tr>
<td><em>Phyllanthus embelica</em> L. (Euphorbiaceae)</td>
<td>Amloki/Amla</td>
<td>To maintain healthy conditions of body (Rahmatullah et al., 2010), treatment of erectile dysfunction, diabetes (Rahmatullah et al., 2009b).</td>
<td>Fruit extract exhibited anti-diarrheal (Perianayagam et al., 2005), potent antioxidant (sometimes stronger than quercetin and BHA) (Liu et al., 2008; Polanors et al., 2009; Scartezzini et al., 2006; Rao et al., 2005), anti-tussive (Nosalova et al., 2003), antibacterial (Dhale and Mogle, 2011) and anti-diabetic (Krishnaraju et al., 2005) activities. Phenolics purified from fruits reported to possess antioxidant (Bagai et al., 2005) and anti-proliferative capacities (Luo et al., 2011; Zhang et al., 2004).</td>
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<tr>
<td><em>Dillenia indica</em> L. (Dilleniaceae)</td>
<td>Chalta/Chulta/Ouau</td>
<td>Treatment of dysentery (Rahmatullah et al., 2009c), abdominal pain, regulation of body’s heat, as expectorant in cough mixture, tonic, laxative and astringent (Maniruzzaman, 1993; Shendge et al., 2011). The fruit mixed with sugar and water is used as a cooling beverage for the treatment of fever, to remove fatigue. The fruit juice is used as cardio-tonic (Shome et al., 1980).</td>
<td>Phytochemical studies showed the presence of flavonoids, such as, myricetin, kaempferol, quercetin, isorhamnetin, naringenin; and phenolic materials (Banerji et al., 1975; Pavanasasivam &amp; Suktambawa, 1975). Fruit extract exhibited antioxidant activity (Abdille et al., 2005).</td>
</tr>
<tr>
<td><em>Ananas comosus</em> (L.) Merr (Bromeliaceae)</td>
<td>Anaras/Pineapple</td>
<td>Treatment of fever, belminthiasis (Rahmatullah et al., 2009a).</td>
<td>Cysteine proteinases (major one identified as bromelain) are present in pineapple and they have anti-edematous, anti-inflammatory, anti-thrombotic and fibrinolytic activities (Larocca et al., 2010).</td>
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<tr>
<td><em>Citrus medica</em> L. (Rutaceae)</td>
<td>Jamir/Citron</td>
<td>As cardiac tonic in palpitation (Krishnaraju et al., 2006), anti-toxic, pulmonary sedative, appetizer and stomachic (Arias and Ramon-Laca, 2005).</td>
<td>Remedy against febrile illnesses (Ajaiyeoba et al, 2003) as anti-cancer (Tian et al., 2001) and anti-oxidant (Jayaprakash and Patal, 2007).</td>
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</table>
between 8 to 12 weeks. 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. Procedures involved were approved by the Animal Research Control Committee of Okayama University, Japan.

Preparation of murine spleen cells: Spleen cells from BALB/c female mice, void of erythrocytes, were prepared by lysis of erythrocytes with ammonium chloride as described previously (Sarker et al., 2011). Mice were killed and the spleens were collected aseptically and mashed with spatula through the strainer to pass the cells into MEM (pH adjusted to around 7.0 with 1/N NaOH) in a petridish. 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 NH₄Cl, 10 mM KHCO₃, 0.1 mM Na₂EDTA; pH 7.2) was added to the cells in the centrifuge tube for the lysis of 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 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: Spleen cells which were obtained as described previously 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 × 10⁵ 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. 50 µl of 2-mercaptoethanol (2-ME) (0.2 mM) was added into each well and the plates were incubated for 1 h at room temperature. After washing the plates 100 µl/well of cultural supernatants (diluted with 1% BSA-PBS-Tween 20, accordingly) or standard mouse-IgM were added into each well and the plates were incubated for 2 hours at room temperature. The plates were again washed three times by wash buffer (200µl/well). Fifty µl per well of horseradish peroxidase-conjugated goat anti-mouse IgM antibody (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 incubated for 10 min at room temperature and the optical densities at 405 nm were measured using an automatic plate reader (Bio-Rad Laboratories, USA).

Results and Discussion

Effect of Bangladeshi fruit extracts on the production of polyclonal IgM in vitro: Traditional uses and updated scientific reports six of the fruits under investigation were noted in Table 1. BALB/c female mice spleen cells were sub-cultured with or without the extracts of different fruits
for 5 days and the amount of IgM production in the cultural supernatants were determined by an IgM-ELISA as previously mentioned in the experimental section. LPS was used as a positive control. As shown in the Fig.1, cultured splenocytes treated with 200µg/ml of 70% methanol extract of *S. dulcis* (Amra) and *D. indica* (Chalta) enhanced IgM production by 108.33% (Fig. 1A) and 90.72% (Fig. 1D) in comparison to untreated cells, respectively. Similarly, juice extract of *C. medica* (Jamir) augmented IgM production level by 103.85% when compared to the control (Fig. 1F). Juice extract of *A. comosus* (Anaras) at the doses of 20 µg/ml, 200 µg/ml and 2 mg/ml increased IgM level in cultured supernatants by 123.33%, 155% and 190% respectively (Fig. 1E). On the other hand, juice extract of *C. grandis* (Jambura) and methanol extract of *P. embelica* (Amloki) failed to enhance IgM level regardless to the dose applied (Fig. 1B & C).

**Evaluation of six fruit extracts for the proliferation of murine splenocytes:** BALB/c female mice whole spleen cells were sub-cultured with or without the indicated doses of the selected fruit extracts for 5 days and the proliferation of cells were measured by MTT assay. The investigations showed that no dose of any extracts could remarkably stimulate the proliferation of cultured cells (Fig. 2). Instead, in some cases, the proliferations of cells were suppressed when treated with extracts (Fig. 2 E & F).

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**Figure 1.** Evaluation of different fruit extracts for the production of polyclonal 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 extracts of six Bangladeshi fruits at 37°C in the 5% CO₂ incubator for 5 days. The IgM levels in the cultured supernatants were determined by an IgM-ELISA. The data are means ± S.D. of triplicate cultures.
Figure 2. Evaluation of crude extracts of six Bangladeshi fruits for 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 different extracts at 37ºC in the 5% CO2 incubator for 5 days. The proliferations of cells were measured by MTT method. The data are means ± S.D. of triplicate cultures.

The investigation resulted in moderate immunostimulating properties in four of the six crude extracts of *S. dulcis*, *D. indica*, *A. comosus* and *C. medica*. Enhanced antibody production demonstrated that the extracts of these fruits promoted the differentiation of B cells to the antibody secreting plasma cells. It is important to note here that although the crude extract of *A. comosus* promoted IgM production (Fig. 1E), it suppressed the proliferation of cultured cells (Fig. 2E), the exact reason of which is not known.

Lacomini et al., (2005) reported the eliciting activity of *S. dulcis* on peritoneal macrophage. The present report would be an addition to this on the immunomodulatory activities of *S. dulcis* for future investigations. Many reports were published on the antioxidant (Liu et al., 2008; Poltanov et al., 2009) and anti-cancer properties (Luo et al., 2011; Zhang et al., 2004) of *P. embelica*. In the present study, crude methanol extract of this fruit could neither enhance antibody (IgM) production nor stimulate splenocytes proliferation *in vitro*.

It has been reported that cysteine proteinases, which is present in pineapple, has anti-thrombotic, anti-inflammatory and fibrinolytic activities (Larocca et al., 2010). *C. medica* fruit was reported to possess anticancer (Tian et al., 2001) and antioxidant properties (Jayaprakash and Patil, 2007). The present study found that the crude extracts of the two fruits exhibited immunoenhancing capacity through the enhancement of IgM production (Fig. 1E & F). Thus, these fruits can be used for the prevention and treatment of cancer, inflammation and body defense.
Conclusion

This preliminary study reports that four out of six screened fruits possess immunostimulating activities promoting humoral immune response through the enhancement of IgM production. This report may guide for further investigations. This study will help the people to select fruit(s) if they need external immunostimulants through food intake in case of body’s impaired immune system. As this is a preliminary report, additional cellular and humoral scientific investigations are recommended.

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