List of Research Publications


Immunomodulatory studies of a bioactive fraction from the fruit of *Prunus cerasus* in BALB/c mice

Sheikh Abida, Anamika Khajurib,⁎, Qazi Parvaiz a, Tabasum Sidiqb, Aruna Bhatiac, Surjeet Singhbd, Shabir Ahmadb, M.K. Randhawac, N.K. Sattid, Prabhu Duttbd

a Dept. of Biotechnology, Indian Institute of Integrative Medicine (CSIR), Srinagar-190005, India
b Division of Pharmacology, Indian Institute of Integrative Medicine (CSIR), Jammu-180001, India
c Department of Biotechnology, Punjabi University, Patiala, Punjab-147002, India
d Division of Natural Products Chemistry, Indian Institute of Integrative Medicine (CSIR), Jammu-180001, India

**Abstract**

In order to evaluate the role of ethyl acetate fraction (PNRS-EtOAC) obtained from the *Prunus cerasus* fruit in the modulation of immune responses, detailed studies were carried out using a panel of in vivo assays. Oral administration of PNRS-EtOAC (25–100 mg/kg) stimulated the IgM and IgG titre expressed in the form of hemagglutination antibody (HA) titre. Further, it elicited a dose related increase in the delayed type hypersensitivity reaction (DTH) after 24 and 48 h in BALB/c mice. Besides augmenting the humoral and cell mediated immune response, the concentration of cytokines (IFN-γ, IL-4, and TNF-α) in serum with respect to T cell interactions, i.e. the proliferation of lymphocytes were significantly increased at 50 mg/kg compared with the control. The results in these studies demonstrated the immunostimulatory effect of PNRS-EtOAC in a dose-dependent manner with respect to the macrophage activation possibly expressing the phagocytosis and nitrite production by the enhancement of TNF-α production as a mode of action.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

The immune system is known to be involved in the pathologic mechanism of many diseases. In recent days, lot of medicines, chemicals, as well as natural products have been introduced in order to stimulate the non-specific defense mechanism as well as specific immune response. For example, phytosterols and polyphenols from plants are known to be involved in improving immune response [1]. It has been found that many plant and animal products that are rich in polyphenols, such as red wine, black tea, Prunus cerasus, and black cherries, have been found to have beneficial properties in improving immune responses [2]. In recent time, focus on plant research has been intensified all over the world and a large number of evidence has been collected to show immense potential of medicinal plants and fruits used in various traditional system of medicine [3]. Researchers continue to explore the benefits of “superfoods”, a unique group nutrient-rich fruits that contain natural compounds shown to have potential disease-fighting properties. Few fruits fall in this category and emerging science shows sour cherries isolated from *P. cerasus* are one among them. Cherries are rich in anthocyanins with a strong neurodegenerative activity, and thus they can serve as a good source of bio-functional phytochemicals in our diet, providing health-promising effects in humans [4]. The principal nutrients thought to provide the protection afforded by fruits and vegetables are antioxidants such as vitamin C, vitamin E, β-carotene, and flavonoids (including flavones, isoflavones, and anthocyanins). Sour cherries are considered as good sources of both flavonoids and phenolic acids like anthocyanins, etc. [5,6]. Convincing phytochemical research studies show that sour cherries are one of the few known food sources that are a rich source of powerful antioxidants including melatonin, quercetin, kaempferol, chlorogenic acid, p-coumaric acid, gallic acid, perillyl alcohol and ellagic acid. Melatonin is a powerful antioxidant considered more potent than vitamins C, E, and A, because it is soluble both in fat and water [7]. A recent study published in the American Journal of Clinical Nutrition found that sour cherries ranked 14 in the top 50 foods for highest antioxidant content per serving size-surpassing well known leaders such as red wine, prunes, dark chocolate and orange juice [8]. However the immunostimulatory potential of *P. cerasus* on immune system has not yet been explored.

Therefore, the objective of the present study was to study the immunomodulatory activity of PNRS-EtOAC fraction isolated from the fruits of *P. cerasus*. In this attempt, the effects of PNRS-EtOAC on humoral immunity keeping neutralizing antibodies in mind, cellular immune responses via delayed type hypersensitivity reaction, lymphocyte proliferation, macrophage phagocytosis, release of NO by the activated macrophages, cytokine profile, lymphocyte phenotyping and costimulatory molecules were investigated.

⁎ Corresponding author. Tel.: +91 2574425, 9906120729.
E-mail address: anamikakhajurias@yahoo.com (A. Khajuria).

1567-5769/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2012.02.001
2. Materials and methods

2.1. Reagents

Medium RPMI 1640 (Himedia, Bombay, India), 96 V well microtitration plates and micro-tissue culture plates (96 U well) from Tarsan, trypan blue (Microlabs, Bombay), fetal calf serum (FCS), concanavalin-A (Con-A), lipopolysaccharide (LPS, E. coli 055 B5), gum acacia, dimethylsulphoxide (DMSO), Hank’s balanced salt solution (HBSS), HEPES, 2-mercaptoethanol, penicillin, streptomyacin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2,5-dimethyltetrazolium bromide) from Sigma were used. Assay kits for all cytokines IFN-γ, IL-4 and TNF-α were purchased from BD, USA. *Candida albicans* were procured from Biological Laboratories, USA.

2.2. Plant material and extraction

2.2.1. Collection of plant material

The sour cherry (Prunus cerasus L.) fruits (70 kg) were collected in the field research center of the Pomology division of Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir (SKUAST-K) Srinagar J & K, India. The cherries were then deprived of stalk and destoned. Pitted fruit was lyophilized to get a gummy residue of 10.3 kg. The frozen cherries were flushed with nitrogen in freezer bags prior to their storage at −20 °C.

2.2.2. Equipment

The Water HPLC system comprising of two waters 515 HPLC pumps, automatic sampling unit (waters 717 plus auto sampler), column oven, photodiode array detector (waters 2996), Merck Rp-18 column (5 μm, 250×4.00 mm ID), temperature control module II and Waters Empower software was used for data analysis and data processing.

2.2.3. Experimental conditions

The HPLC analysis of ethyl acetate fraction and the pure compounds (Figs. 1 and 2) was performed at a flow rate of 1.0 mL/min using mobile phase consisting of 0.05% TFA in ACN: 0.05% TFA in water (gradient). The photodiode array detector was set at wavelength of 340 nm.

2.2.4. Extraction of cherries

Lyophilized fruit (2 kg) was ground and extracted with methanol (5 L). The fruit material was mechanically stirred for 4 h and methanol was evaporated under reduced pressure at 40±5 °C to yield a crude extract. The extraction process was repeated three times more under similar conditions. Total of 5 batches (each of 2 kg) of lyophilized fruit were extracted with methanol similarly. The total combined extract was clarified by centrifugation and the clear extract was concentrated on rotavapour to get a red methanolic fruit extract (5.6 kg). This extract on preliminary screening showed very promising immunomodulatory activity. In order to pinpoint the activity in the extract, bioactivity guided fractionation was carried out by fractionating it into polar and non-polar fractions. 1.1 kg of the methanolic extract was dissolved in distilled water (3 L) and extracted with ethyl acetate (3 L×7) till no yellow coloration was observed in the upper layer of the ethyl acetate. Total 5 batches (each of 1.1 kg methanol extract) were extracted with ethyl acetate under similar conditions. Pooled ethyl acetate fraction was concentrated on rotavapour to obtain a gummy extract of 470 g. The water fraction was lyophilized. Preliminary bio-evaluation (immunomodulatory) study of both the fractions showed that ethyl acetate fraction was more immunonopotent. The ethyl acetate fraction was taken up for the isolation of chemical constituents.

2.2.5. Isolation of bioactive compounds

The ethyl acetate fraction (450 g) was subjected to silica gel (60–120 mesh) column chromatography. The column was eluted using a gradient of CHCl₃–CH₃OH (100:0–0:100) to afford 350 fractions. All the 350 fractions were checked on TLC (n-Butanol:Acetic acid::Water::4:1:5), spots were visualized by spraying the TLC plate with freshly prepared PEG-borinate solution (2-amoethoxy diphenylborinate, 1% in CH₃OH: polyethylene glycol-4000, 5% in C₆H₅OH, 1:1:v/v). Out of 350 fractions, four fractions, fraction-1 (eluted in 2% CH₃OH in CHCl₃), fraction-2 (eluted in 8% CH₃OH in CHCl₃), fraction-3 (eluted in 15% CH₃OH in CHCl₃) and fraction-4 (eluted in 20% CH₃OH in CHCl₃) each showed one major spot in TLC. These four fractions were subjected to repeated column chromatography on silica gel to obtain four compounds. The compounds were identified as quercitin₁ (from fraction-1), daidzin² (from fraction-2), rutin³ (from fraction-3) and chlorogenic acid⁴ (from fraction-4). Compounds were finally purified by crystallization and identified with the help of ¹H,¹³C NMR.

2.2.6. Sample preparation and chemical standardization of bioactive fraction

The accurately weighed quantity of the dried ethyl acetate fraction (28.2 mg) was taken and was dissolved in 2 ml methanol HPLC grade. Pure compounds chlorogenic acid (1.2 mg/5 mL), daidzin (4 mg/5 mL), rutin (1 mg/5 mL) and quercitin (3.2 mg/5 mL) were dissolved in methanol HPLC grade. The samples were centrifuged and filtered through Millipore micro filter (0.45 μm) and were used for analysis. For markers, working solutions in the concentration range of

Fig. 1. Chromatogram of ethyl acetate fraction showing presence of compounds. The HPLC analysis of ethyl acetate fraction was performed at a flow rate of 1.0 mL/min using mobile phase consisting of 0.05% TFA in ACN: 0.05% TFA in water (gradient). The photodiode array detector was set at wavelength of 340 nm.
200–800 μg/mL were prepared by diluting with methanol. These working solutions of all the marker compounds were mixed together in the ratio (chlorogenic acid: daidzin: rutin: quercitin: 2:4:3:1) and were injected in different concentrations (5 μL, 10 μL, 15 μL, 20 μL, 25 μL). The calibration curve of each marker compound in the mixture was plotted using five levels of concentrations and linearity of each marker compound was observed in the concentration range 48 μg–240 μg for chlorogenic acid, 320 μg–1600 μg for daidzin, 60 μg–300 μg for rutin and 64 μg–320 μg for quercitin. The marker compounds in the ethyl acetate fraction were quantified using these calibration curves (Table 1). Structures of isolated compounds from PNRS-EtOAC are shown in Fig. 3.

### Table 1

Quantities of marker compounds estimated using standard curve.

<table>
<thead>
<tr>
<th>Marker Compounds (g/100 g)</th>
<th>Ethyl acetate fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorogenic acid</td>
<td>0.364</td>
</tr>
<tr>
<td>Daidzin</td>
<td>0.914</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.109</td>
</tr>
<tr>
<td>Quercitin</td>
<td>0.636</td>
</tr>
</tbody>
</table>

2.3. Experimental animals

The study was conducted on male BALB/c mice (18–22 g). The ethical committee of the Indian Institute of Integrative Medicine, Jammu (CSIR, India) instituted for animal handling approved all the protocols. The animals were bred and maintained under standard laboratory conditions: temperature (25 ± 2 °C) and a photoperiod of 12 h. Commercial pellet diet and water were given ad libitum.

2.4. Immunization schedule

Sheep red blood cells (SRBC) were used as a source of T-dependent antigen. For this purpose the blood was withdrawn from a healthy sheep in Alsever’s solution [9]. SRBC used for immunization were prepared in pyrogen free normal saline. Mice were divided into eight groups, each consisting of six animals. PNRS-EtOAC at 25 mg, 50 mg, and 100 mg/kg (in 200 μL of normal saline) was administered orally by gavage for 15 days, daily. The dose volume was 0.2 mL. Control group received normal saline. Levamisole, a known immunostimulator reported to augment the antibody response [10] was given orally as positive control, at a dose of 2.5 mg/kg body weight. All groups were immunized with 0.2 mL of SRBC (5 × 10^9) per mouse intraperitoneally (i.p.) on day 0 of drug treatment. Additional three immunized groups, challenged on day 7 with SRBC were used for DTH and different immunoglobulin and phagocytic assays.

2.5. Hemagglutination antibody (HA) titre

The animals were immunized by injecting 0.2 mL of 10% of fresh SRBC suspension intraperitoneally on day 0. Blood samples were collected in micro-centrifuge tubes from individual animals by retro-orbital plexus on day 7 for primary antibody titre and day 14 for secondary antibody titre. Serum was separated and antibody levels were determined by the hemagglutination technique [11]. Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25 μL volumes of normal saline in a micro-titration plate to which were added 25 μL of 1% suspension of SRBC in saline. After mixing, the plates were incubated at room temperature for 1 h and examined for hemagglutination under the microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

2.6. Delayed type hypersensitivity (DTH)

PNRS-EtOAC was administered 2 h after SRBC injection and once daily on consecutive days. Six days later, the thickness of the left hind
footpad was measured with a spherometer (pitch, 0.01 mm) and was considered as a control. The mice were then challenged by injecting 20 μL of 5 × 10^6 SRBC/mL intradermally into the left hind footpad. The footpad thickness was measured again after 24 and 48 h [11].

2.7. Spleen cell proliferation assay (ex-vivo)

Spleen collected under aseptic conditions in HBSS, was minced using a pair of scissors and passed through a fine mesh steel to obtain a homogenous cell suspension and the erythrocytes were lysed with ammonium chloride (0.8%, w/v). After centrifugation (380 × g for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium [RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/ mL penicillin, 100 μg/mL streptomycin and 10% FCS]. The cell number was counted with a hemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95% [12].

To evaluate the effect of PNRS-EtOAC on the proliferation of splenic lymphocytes collected from PNRS-EtOAC treated mice, the spleen cell suspension (1 × 10^7 cells/mL) was pipetted into 96-well plates (200 μL/well) and cultured at 37 °C for 72 h in a humidified, 5% CO₂ atmosphere containing 5% CO₂ in the presence of Con-A (5 μg/mL) and LPS (10 μg/mL). After 72 h, 20 μL of MTT solution (5 mg/mL) was added to each well and the plates were incubated for 4 h. Thereafter, plates were centrifuged (1400 × g, 5 min) and the untransformed MTT was removed carefully by pipetting. To each well, 100 μL of a DMSO working solution (192 μL DMSO with 810 μL 1 M HCl) was added and the absorbance was evaluated in an ELISA reader at 570 nm after 15 min.

2.8. Phagocytosis

The peritoneal macrophages of PNRS-EtOAC (25–100 mg/kg) treated mice were harvested by flushing the cavity with 5 mL of RPMI 1640 medium, pelleted by centrifugation at 1100 rpm for 10 min, resuspended in RPMI 1640 containing 10% heat activated fetal bovine serum, 4 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and 100 mM sodium pyruvate. The viable cell suspension (2 × 10^6 cells/mL) was allowed to adhere to glass slides for 2 h at 37 °C in a humidified CO₂ incubator. The glass slides were washed thoroughly to remove non-adherent cells. 100 μL of opsonized Candida cells (100 °C, 30 min) was then spread over the adherent cells. The slides were incubated for 15 min in a humidified CO₂ incubator and stained with trypan–eosin after thorough washing with PBS to determine the adherent cells containing yeast cells microscopically [13]. The number of yeast cells ingested per macrophage was taken as the phagocytic index. One hundred adherent cells were counted to determine percent phagocytosis.

2.9. Collection of peritoneal macrophages, nitrite and cytokine assay

A volume of 10 mL of RPMI-1640 was injected into the peritoneal cavity of challenged mice on day 15. After 5 min, the medium was taken out and centrifuged at 1800 × g for 10 min at 4 °C. The cell pellet was resuspended in RPMI 1640 medium. Macrophages (3 × 10^6) were seeded in 24-well culture plate in a CO₂ incubator for 3 h. At the end of incubation period, non-adherent cells were removed and plates were further incubated for 48 h in the presence of LPS (1 μg/mL). Supernatants were collected after centrifugation and kept at −80 °C for the measurement of cytokines. For the NO₂⁻ assay (nitrite content), 100 μL of culture media was incubated with 150 μL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine 2.5% phosphoric acid solution) at room temperature for 10 min in 96-well micro plate [13]. Absorbance at 540 nm was read using an ELISA plate reader. Standard calibration curves were prepared using sodium nitrite as standard. Measurement of cytokine (TNF-α) was carried out using commercial kits (BD Opt EIA set) as per the manufacturer's instructions.

2.10. Determination of IFN-γ, TNF-α and IL-4 in mice sera by ELISA

Serum was collected 4 h after the final oral administration of PNRS-EtOAC (25, 50 and 100 mg/kg). Interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and IL-4 concentration were measured by enzyme-linked immunosorbent assay (ELISA kit, BD Opt EIA set) according to the instructions of the manufacturer [14].

2.11. Lymphocyte phenotyping in spleen

The spleen (1/3 of the organ) was placed in PBS buffer (without Mg²⁺ and Ca²⁺) and stored on ice prior to preparation of single cell suspension. Splenic erythrocytes were lysed with red blood cell lysis buffer (BD Pharmingen). Cell suspensions were refrigerated (ca. 4 °C) pending staining with antibodies. All reagents were purchased from BD Pharmingen. For each sample, 2 × 10⁶ cells were stained with conjugated anti-CD4 FITC and anti-CD8 PE antibodies. After staining with antibodies, cells were washed and resuspended in PBS for flow cytometric analysis which was performed on a FACS Calibur flow cytometer equipped with Cell Quest software (Becton Dickinson) [11].

2.12. Phenotypic analysis of co-stimulatory molecules (CD80 and CD86)

Splenic cells from PNRS-EtOAC treated and untreated BALB/c mice were suspended in RPMI-1640 medium after removing the red blood cells by RBC lysis buffer. Cells (1 × 10^7 cells/mL) were washed thrice and incubated for 1 h at 37 °C on plastic petri plates. After removing the floating cells (T and B cells), adherent cells (macrophages) were collected and washed with PBS. Thereafter, 2 × 10⁶/mL of macrophages were suspended in cell staining solution (BD Pharmingen) in 24 well plate. To quantify the expression of co-stimulatory molecules, macrophages were stained with FITC-labeled anti-CD80 (B7-1) and anti-CD86 (B7-2) mAbs on ice for 30 min and washed with PBS. Ten thousand cells were collected for each sample, and the data were analyzed with FACScan flow cytometer [15].

2.13. Statistical analysis

Data are expressed as Mean±S.E.M. and statistical analysis was carried out using one-way ANOVA (Bonferroni correction multiple comparison test). Dunnett's test was used to analyze the different variables in the same subject and P values less than 0.05 were being taken as statistically significant.

3. Results

3.1. Effect of PNRS-EtOAC on anti-SRBC antibody titre

Anti-SRBC antibody (IgM and IgG) titres were measured in mice sera of different groups, collected retro-orbitally on 7 and 14 days after immunization and treatment. Anti-SRBC antibody titres increased in mice treated with three doses of PNRS-EtOAC (25, 50, and 100 mg/kg) after seven days when compared with control. A similar profile was obtained after 14 days, with IgG predominating over IgM (Table 2). The maximum effect was observed at 50 mg/kg in both primary and secondary antibody titre (P < 0.01). Further increase in dose (100 mg/kg) showed a decreased response. Administration of levamisole (2.5 mg/kg, p.o.), used as a positive control resulted in a significant increase in the humoral antibody titre compared with the control animals.
3.2. Effect of PNRS-EtOAC on delayed type hypersensitivity (DTH) reaction in mice

In order to assess the cell-mediated immune response, DTH reaction to SRBC was measured as given in Table 2, in which data are expressed in terms of the swelling of the footpad. After administration of the PNRS-EtOAC (25–100 mg/kg, p.o.), a significant increase (P<0.01) in footpad thickness was found at 24 and 48 h as compared with the control group: maximum increase being observed at 50 mg/kg. Further increase in dose (100 mg/kg) showed a decreased response.

3.3. Effect of PNRS-EtOAC on splenocyte proliferation assay (ex-vivo)

The effect of PNRS-EtOAC on Con-A and LPS-stimulated splenocyte proliferation in immunized mice is shown in Fig. 4. Lymphocyte proliferation was studied by MTT assay. PNRS-EtOAC caused profound lymphocyte activation which triggered significant (P<0.01) and concentration-dependent proliferation of naïve murine splenocytes. Con-A (5 μg/mL) and LPS (10 μg/mL) stimulated splenocyte proliferation was significantly enhanced by PNRS-EtOAC with the maximum effect at 50 mg/kg dose and this cellular proliferation was increased up to two fold in Con-A and LPS treated cells respectively, compared to the control. Further increase in dose (100 mg/kg) showed a decreased response.

3.4. Effect of PNRS-EtOAC on macrophage phagocytosis

Pre-incubation of macrophages with PNRS-EtOAC enhanced the phagocytic function of the adherent macrophages. A significant increase in the number of cells phagocytizing the yeast cells ingested per adherent cell (phagocytic index) was observed with in vivo doses ranging from 25 to 50 mg/kg compared with the control group: maximum increase being at 50 mg/kg dose (Fig. 5). Further increase in the dose (100 mg/kg) decreased the effect.

3.5. Effect of PNRS-EtOAC on nitric oxide and TNF-α release by macrophages

The effect of PNRS-EtOAC on macrophage function was assessed by measuring the amount of NO and TNF-α produced from peritoneal macrophages of PNRS-EtOAC treated mice. Griess reagent was used to measure the nitrite levels, the stable end-product of NO metabolism. Macrophages were cultured in RPMI-FBS (10%) with LPS (1 μg/mL) and contents of nitrite and TNF-α were measured in the supernatants. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve and the results are expressed in μM. The effect of PNRS-EtOAC (25, 50 and 100 mg/kg) on nitric oxide production is shown in Fig. 6. Increasing doses of PNRS-EtOAC (25–50 mg/kg) significantly enhanced the nitrite content in peritoneal macrophages. The maximum effect was observed at 50 mg/kg dose compared with the control group.

Furthermore, increasing doses of PNRS-EtOAC registered a significant increase in TNF-α release from peritoneal macrophages (Fig. 7), maximum increase being at 50 mg/kg dose of PNRS-EtOAC (P<0.01) treated group compared with control. Further increase in dose (100 mg/kg) showed a decreased response.

3.6. Effect of PNRS-EtOAC on IFN-γ, TNF-α and IL-4 in mice sera by ELISA

In order to establish that Th1 and Th2 cytokines were involved in the immunostimulatory activity of PNRS-EtOAC, cytokine secretion patterns were analyzed in the sera of immunized mice. PNRS-EtOAC caused a significant (P<0.01) dose dependent up-regulation of the

---

**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Antibody response</th>
<th>DTH response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Log-2 titre (Mean±S.E.)</td>
<td>Paw edema (mm) (Mean±S.E.)</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>6.10±0.43</td>
<td>1.82±0.03</td>
</tr>
<tr>
<td>Levamisole</td>
<td>2.5</td>
<td>8.4±0.35*</td>
<td>2.20±0.05*</td>
</tr>
<tr>
<td>PNRS-EtOAC</td>
<td>25</td>
<td>9.3±0.23*</td>
<td>2.35±0.03*</td>
</tr>
<tr>
<td>PNRS-EtOAC</td>
<td>50</td>
<td>11.2±0.31**</td>
<td>2.54±0.02**</td>
</tr>
<tr>
<td>PNRS-EtOAC</td>
<td>100</td>
<td>7.8±0.21</td>
<td>2.18±0.03</td>
</tr>
</tbody>
</table>

Antibody titres (IgM and IgG) in mice sera were measured on day 7 and day 14 after immunization. DTH response was determined in SRBC immunized, PNRS-EtOAC treated mice at 24 and 48 h after antigen challenge. Data are mean±S.E. of six animals. *P<0.05 and **P<0.01 compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test). For experimental details refer to Materials and methods section.
Th1 (IFN-γ, TNF-α) and Th2 (IL-4) cytokines at the doses of 25 and 50 mg/kg: maximum response being at 50 mg/kg dose compared with the control group (Fig. 8a, b and c). Further increase in the dose (100 mg/kg) showed a decreased response.

3.7. Effect of PNRS-EtOAC on lymphocyte phenotyping in spleen

The effect of PNRS-EtOAC on the population of cell surface markers like CD4 and CD8 populations was determined in splenocytes prepared from spleen of mice collected after 14 days of oral treatment of PNRS-EtOAC. Results are depicted in Fig. 9. PNRS-EtOAC caused a significant (P < 0.01) dose dependent increase in both CD4 and CD8 population at the doses of 25 and 50 mg/kg: maximum response being at 50 mg/kg dose compared with the control group. Further increase in the dose (100 mg/kg) showed a decreased response.

3.8. Effect of PNRS-EtOAC on the expression of CD80 and CD86

CD80 and CD86 present on antigen presenting cells are compulsory for the activation of lymphocytes and the expression of cytokines. It was observed that PNRS-EtOAC significantly up-regulated the expression of both CD80 and CD86 on splenic macrophages in a dose-dependent manner. PNRS-EtOAC at the dose of 50 mg/kg induced optimum enhancement of 2-fold in the number of cells expressing CD80/CD86 over the control animals (Fig. 10). Further increase in the dose (100 mg/kg) showed a decreased response.
4. Discussion

Immunomodulation using fruits and medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when host defense mechanism has to be activated under the conditions of impaired immune response or when a selective immunosuppression is desired in situations like autoimmune disorders. There are a number of diseases where immunostimulant drugs are required to overcome the immunosuppression induced by drugs or environmental factors and immunosuppressants are required where there is undesired immunopotentiation. There is strong requirement of the drugs which can boost immune system to combat the immunosuppressive consequences caused by stress, chronic diseases like tuberculosis, conditions of impaired immune responsiveness (e.g. AIDS), etc. [16]. The benefits of “superfruits”, a unique group nutrient-rich fruits containing natural compounds are shown to have potential disease-fighting properties. Some of fruits fall in this category and emerging science shows sour cherries (P. cerasus) are one among them. Convincing phytochemical research studies show that sour cherries are one of the few known food sources that are a rich source of powerful agents especially anthocyanins and flavonoids. Anthocyanins convey marked antioxidant activity via the donation of electrons or hydrogen atoms from hydroxyl moieties to free radicals [17,18]. Numerous disease processes are believed to have a free radical component, such as inflammation, cancer, stroke, Alzheimer’s disease, etc. [19]. In various pathologies (atherosclerosis, apoptosis, aging, diabetes), cell oxygen radical-related damage can be protected by the antioxidant potential of anthocyanins. Early studies revealed that anthocyanins are related to the quality index of sour cherries and found that sour cherry extracts reduce inflammation, alleviate the pain of gout and arthritis [20,21]. There is literature data supporting the antioxidant activity of anthocyanins isolated from sour cherries in vitro [22]. Literature data of Seeram et al., [23] and Tall et al., [21] have shown that orally administered pure anthocyanins extracted from sour cherries contain dose-dependently powerful antioxidant and anti-inflammatory properties. Flavonoids play some important pharmacological roles against diseases, such as cardiovascular disease, cancer, inflammation and allergy [24,25]. Isoflavone daidzein found in sour cherries could stimulate murinenon-specific immunity, activate humoral immunity and enhance cell-mediated immunity [26]. Various experiments have been conducted reporting the immunomodulatory action of citrus fruits on macrophage and lymphocyte functions in BALB/c mice [27,28]. In the past a number of drugs with plant or mineral origin have been advocated as means of immunomodulation for various diseased conditions in humans [29]. Side effects associated with allopathic drugs along with their high cost have enforced the need for search of alternative drugs with least or no side effects, especially those belonging to the traditional system of medicine like Ayurveda. Most of the plants so far reported with immunostimulatory action have major effect on the non-specific arm of immunity especially on macrophage functions [30]. Although fruit plants have been investigated for varied pharmacologic activities, the immunostimulatory potential of P. cerasus still remains unexplored. This investigation deals with the PNRS-EtOAC fraction which was exploited for its immunomodulatory activity. The fraction was found to be a pronounced immunostimulator at the tried doses of 25 and 50 mg/kg in BALB/c mice in a dose-dependent manner with maximum stimulation observed at 50 mg/kg dose. Levamisole, which is attracting more attention owing to its use as an immunomodulator, in supporting anti-carcinogenic drugs, in the treatment of skin diseases and in improving weight gain in animals, was used as a reference standard in this study [31]. Levamisole was used at 2.5 mg/kg in this investigation and this dose was selected out of the several doses tried in our lab earlier to optimally stimulate the various humoral and cellular immune parameters of mice.

In the present study, immunomodulatory potential of PNRS-EtOAC was explored extensively on the modulation of both T and B-cells in relation to serum immunoglobulins IgM and IgG to T-dependent antigen SRBC. Primarily, the antibody response to SRBC was observed by the hemagglutination titre. The augmentation of humoral antibody response to T-dependent antigen (SRBC) reveals the increased responsiveness of macrophages since the antibody production is closely associated with the co-operation of macrophages, T and B lymphocyte responsiveness [32]. The T cells in turn participate in the expression of cell mediated immunity contributing to DTH. A DTH reaction is an expression of cell-mediated immunity and plays a role in many inflammatory disorders [33]. Treatment with PNRS-EtOAC enhanced the DTH reaction, as reflected by the increased footpad thickness compared to the control group, suggesting heightened infiltration of macrophages to the inflammatory site. Moreover, PNRS-EtOAC stimulated phagocytosis and augmented Con-A and LPS induced splenocyte proliferation.

It is accepted that cytokines are major factors involved in regulation of the immune response to antigens and infectious agents. Th1 cells are able to produce IL-2 and IFN-γ whereas Th2 cells can produce IL-4. The augmentation of T and B cells with PNRS-EtOAC may be due to a cytokine-mediated mechanism. Since IFN-γ is upregulated in PNRS-EtOAC treated groups, it is an important immunoregulatory molecule which protects against viral infections, induces the generation of T cells, activates macrophages and regulates crossth1 and Th2 cells.
Both TNF-α and IFN-γ can enhance immunoregulatory ability. IL-4, known to activate monocytes or macrophages, is produced by T helper (Th2) cells or mast cells. Investigation of the balance of Th1 and Th2 cytokine production should be helpful to understanding the outcomes of different immunologically deregulated states [34]. PNRS-EtOAC upregulated the production of IL-4 with a significant release of IFN-γ and TNF-α thus regulating the Th1 and Th2 balance.

Macrophages are important cells for the immune system which play an important role in host defense mechanisms for protection from microbial invaders. When macrophages are stimulated with foreign substances, a variety of cytokines and chemicals are released to induce fundamental defense systems. Among them, TNF-α is a representative cytokine secreted by macrophages that plays a key role in the cytokine network, e.g. T cell and NK cell activation [28]. Also, TNF-α is a major pro-inflammatory cytokine characteristically produced at sites of inflammation by macrophages and is considered to help in eliminating certain invaders. Its levels in plasma are directly correlated with the ability of phagocytes to generate superoxide and to increment the activity of iNOS and, thus, NO levels [35]. Furthermore, TNF-α produced by activated macrophages, enhances the cytokotic action of macrophages. We observed that PNRS-EtOAC strongly showed a significant increase in the phagocytic activity of macrophages against Candida.

To further elucidate the mechanism of PNRS-EtOAC as an immunomodulator, the effects of PNRS-EtOAC on both CD4+ and CD8+ cells or mast cells. PNRS-EtOAC showed a significant increase in the phagocytic activity of macrophages against Candida.

References


