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

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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.

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, these are termed as immunostimulants. A number of fruits used in the traditional medical system of remedies in India, have been shown to possess immunostimulating activity acting at the different levels of the immune system [1]. *Prunus cerasus* is a medicinal fruit claimed to possess number of therapeutic uses including anti-inflammatory as well as immunological [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 "superfruits", 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, kaempherol, 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 co-stimulatory molecules were investigated.

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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 Tarson, 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, streptomycin and MTT (3-(4,5-dimethylthiazol-2yl)-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 (*P. cerasus* L.) fruits (70 kg) were collected in the special nylon bags from 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 $CHCl_3$–CH_3OH (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-aminoethyl diphenylborinate, 1% in CH_3OH: polyethylene glycol-4000, 5% in C_2H_5OH, 1:1.v/v). Out of 350 fractions, four fractions, fraction-1 (eluted in 2% CH_3OH in $CHCl_3$), fraction-2 (eluted in 8% CH_3OH in $CHCl_3$), fraction-3 (eluted in 15% CH_3OH in $CHCl_3$) and fraction-4 (eluted in 20% CH_3OH in $CHCl_3$) 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 quercitin[1] (from fraction-1), daidzin[2] (from fraction-2), rutin[3] (from fraction-3) and chlorogenic acid[4] (from fraction-4). Compounds were finally purified by crystallization and identified with the help of ^1H, ^{13}C 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

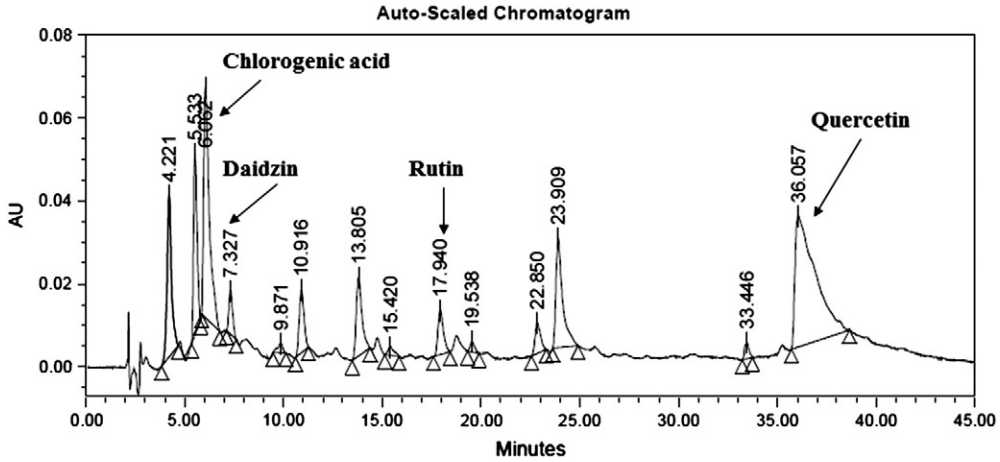

Fig. 1. Chromatogram of ethyl acetate fraction showing presence of compounds. The HPLC analysis of ethyl acetate fraction was performed at a flow rate of 1.0 mL/min using mobile phase consisting of 0.05% TFA in ACN: 0.05% TFA in water (gradient). The photodiode array detector was set at wavelength of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