International Immunopharmacology 16 (2013) 165–170

Contents lists available at SciVerse ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Preliminary report

Anti-inflammatory effects of phenolic extracts from strawberry and mulberry fruits on cytokine secretion profiles using mouse primary splenocytes and peritoneal macrophages

Chieh-Jung Liu, Jin-Yuarn Lin *

Department of Food Science and Biotechnology, National Chung Hsing University, 250 Kuokuang Road, Taichung 40227, Taiwan, ROC

ARTICLE INFO

Article history:
Received 2 February 2013
Received in revised form 25 March 2013
Accepted 31 March 2013
Available online 13 April 2013

Keywords:
Cytokines
Mulberry fruit
Peritoneal macrophages
Phenolic extracts
Splenocytes
Strawberry fruit

ABSTRACT

This study isolated phenolic-rich extracts from strawberry (ES) and mulberry (EM) fruit juice using 70% ethanol, analyzed the individual phenolics including four flavonoid components using HPLC and assessed their cytokine secretion regulatory activities using murine primary splenocytes and peritoneal macrophages. The results showed that EM was rich in *p*-coumaric acid (20798 ± 719 μg/g dry weight), rutin (1992 ± 26 μg/g dry weight) and quercetin (81 ± 5 μg/g dry weight), but ES was relatively rich in *p*-coumaric acid (7475 ± 1219 μg/g dry weight), morin (101 ± 68 μg/g dry weight) and quercetin (72 ± 42 μg/g dry weight). ES and EM administration significantly decreased splenocytes' (IFN-γ + IL-2 + IL-12)/IL-10 (Th1/Th2) cytokine secretion ratios in the absence or presence of lipopolysaccharide (LPS) and TNF-α/IL-10 (pro-/anti-inflammatory) cytokine secretion ratios in the presence of LPS in dose-dependent manners. Our results suggest that ES and EM that are rich in *p*-coumaric acid, rutin, morin or quercetin, may have strong immunomodulatory effects on splenocytes, *via* decreasing Th1/Th2 and pro-/anti-inflammatory cytokine secretion ratios.

1. Introduction

There is a negative correlation between the consumption of diets rich in fruits and vegetables and the risks for chronic angiogenic diseases, such as cardiovascular diseases, arthritis, chronic inflammation and cancers [1–4]. The physiological functions of fruits and vegetables are partly attributed to their abundance of phenolic and flavonoid compounds [5]. Phenolic and flavonoid compounds that are ubiquitous in plants are of considerable interest due to their bioactive functions including anti-microbial, anti-viral and anti-inflammatory activities along with their high anti-oxidant ability [6]. Among fruits, strawberry and mulberry juice is rich in phenolic compounds, including some flavonoids, with immuno-modulatory potential *via* stimulating murine splenocyte proliferation and cytokine secretion, as well as exhibiting prophylactic effects on LPS-induced inflammation in peritoneal macrophages [5,7,8]. Strawberry fruit supplementation was applied for short-term improved dyslipidemia and circulating adhesion molecules in subjects with metabolic syndrome [9]. Mulberry fruit was observed to show considerable high nutritional value and antioxidant activity which could be developed for functional food that benefits human health [10]. Distinctly, the dietary fruits have a direct effect on human nutrition and health. Strawberry and mulberry fruits seem to have strong beneficial effects on metabolic syndrome. However, the immuno-modulatory active components in mulberry and strawberry fruits are still seldom reported.

Cytokines, small proteins acting in paracrine, autocrine and endocrine manners, can be secreted by different cells and play an important role as hormonal mediators for host defence, growth and repair processes within injured tissues [11]. There are two $CD4^+$ T-helper type 1 (Th1) and Th2 lymphocytes that produce some common cytokine products; others are mutually exclusive. Th1 cells may produce interferon (IFN)-γ, tumor necrosis factor (TNF)-α/β, interleukin (IL)-2, IL-3, IL-6, IL-10, IL-12, IL-20, IL-24, IL-32 and granulocyte–macrophage colony-stimulating factor (GM-CSF); however Th2 lymphocytes synthesize IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-13, IL-16, IL-21, IL-25, IL-31 and GM-CSF [12,13]. Among cytokines, some cytokines such as IL-1, TNF, and IL-6 can be roughly classified as pro-inflammatory cytokines because they may highlight the way and trigger local inflammation within injured tissues [11]. In contrast, some Th2 cytokines, particularly IL-10, are recognized as anti-inflammatory cytokines that are produced by Th2 cells, T regulatory cells (Th3 cells), macrophages and some B cells to inhibit the synthesis of Th1 and other cytokines and macrophage functions during the late inflammation phase [14]. Generally, Th1 cytokines tend to induce pro-inflammatory responses, however Th2 cytokines if secreted at the right moment may inhibit Th1 cytokine production and exert potent anti-inflammatory functions. Undoubtedly, an imbalance in Th1/Th2 patterns, and pro-/anti-inflammatory cytokines generally accompanied with stress hormones may cause differential diseases, such as persistent infections, severe immuno-suppression, autoimmunity, allergy/atopy, tumor growth and chronic graft-versus-host disease [15]. An appropriate

* Corresponding author. Tel./fax: +886 4 22851857.
E-mail address: jinlin@nchu.edu.tw (J.-Y. Lin).

1567-5769/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.intimp.2013.03.032

regulation of the Th1/Th2 imbalance and pro-/anti-inflammatory cytokine expression in immune cells may avoid immune disorder diseases.

Several Korean traditional medicinal herbs, such as *Juglans sinensis*, *Psoralea corylifolia*, and Cheong-a-hwan (herbal prescription composed of *J. sinensis* and *P. corylifolia*) extracts, show potential as novel therapeutic agent for asthma by modulating the relationship between Th1/Th2 cytokine balance [16]. The anti-inflammatory effects of dietary phenolic compounds including genistein and (−)-epigallocatechin-3-gallate (ECCG) on inflamed intestinal epithelial Caco-2 cells *via* down regulating the inflammatory response by a pathway implicating a largely post-transcriptional regulatory mechanism have been reported [17]. Potential phyto-chemicals, especially phenolics including some flavonoids, from different food materials or herbs shed a light on immuno-modulation and may be beneficial for the corresponding human diseases.

We hypothesized that strawberry and mulberry fruit phenolic compounds including some flavonoids, that are ethanol-soluble components, have great immuno-modulation potential. To validate this assumption, ethanolic extracts from strawberry and mulberry fruit juice were isolated, characterized and their modulating effects on cytokine secretion profiles evaluated using mouse primary splenocytes and peritoneal macrophages in the absence or presence of lipopolysaccharide (LPS). Both Th1/Th2 and pro-/anti-inflammatory cytokine secretion profiles were determined using enzyme-linked immuno-sorbent assay (ELISA) in the present study.

2. Materials and methods

2.1. Preparation of ethanolic extracts from strawberry and mulberry fruit juice

Strawberry (*Fragaria ananassa*) and mulberry (*Morus alba*) fruits were purchased from a local supermarket in Taichung, Taiwan. The edible portions were weighed, washed and chopped to squeeze fruit juice using a manual stainless screw squeezer (Vegetable & Fruit Grinder, manual type, Mei-Er-Then Co., Ltd, Taipei, Taiwan, ROC). The juice was centrifuged at 10,000 ×*g* (4 °C) for 30 min and the supernatant collected using suction filtration through filter papers (Toyo No. 5B). The filtrate was measured, lyophilized and stored at −30 °C for future use [8]. To extract the phenolic-rich including flavonoid-rich components from the fruit juice, an aliquot of one gram of the lyophilized fruit juice was added with 12 ml of deionized water to dissolve the juice sample. The juice was added with 3 volumes of 95% ethyl alcohol to achieve a mixture with a final concentration of 70% ethyl alcohol [18]. The mixture was allowed to stand with slow stirring at 4 °C for 48 h to extract the phenolic-rich components. The resultant mixture was centrifuged at room temperature, 5500 ×*g* for 20 min to separate the ethanol-soluble extracts, namely phenolic-rich components. The pellet was extracted using 70% ethyl alcohol again. The supernatant was collected and the solvent removed using a rotary vacuum evaporator (EYELA, Tokyo Rikakika Co., Ltd, Bohemia, NY). The residues were lyophilized and stored at −30 °C until use. The ethanolic extract yield from the strawberry and mulberry fruit juice lyophilized powder was 67.2 ± 7.4 % (w/w) and 78.7 ± 1.7 % (w/w), respectively. The individual phenolics including some flavonoid component levels, including *p*-coumaric acid, rutin, morin, quercetin, and isorhamnetin, in the ethanolic extracts were determined using gradient high performance liquid chromatography (HPLC).

2.2. HPLC analysis of phenolics including some flavonoids in ethanolic extracts from strawberry and mulberry fruit juice

An aliquot of 0.05 g of isolated strawberry fruit juice ethanolic extract (ES) or mulberry fruit juice ethanolic extract (EM) was re-suspended in 1 ml of methanol (HPLC-grade, Tedia Co. Inc., Fairfield, OH) and filtered through a 0.22 μm filter (Minisar SRP4, PTFE membrane, Sartorius, Goettingen, Germany). The filtrate sample was stored at −30 °C until use. For HPLC analysis, the sample solution was ultrasonically degassed before use. The mobile phase A (double distilled water: tetrahydrofuran (ECHO, Miaoli, Taiwan): trifluoroacetic acid (Sigma) = 98:2:0.1 (v/v/v)) and mobile phase B (acetonitrile, HPLC grade, ECHO, Miaoli, Taiwan) were filtered through a 0.45 μm filter (Durapore, Millipore, MA, USA) under vacuum and ultrasonically degassed before use. The mobile phase flow rate was 1 ml/min. Aliquots of 20 μl sample solution were subjected to HPLC analysis. The pump (L-2131, Hitachi, Tokyo, Japan), UV–Visible detector (L-2400, Hitachi) and chromatographic separation column (250 × 4.6 mm, 5 μm; Mightsil RP-18 GP250, Kanto Chemical Co., Inc., Tokyo, Japan) were used. The pump was controlled under the program (D-2000 Elite, Hitachi) a gradient elution starting at mobile phase A 83% and mobile phase B 17% for 7 min, then mobile phase A 75% and mobile phase B 25% for 8 min, mobile phase A 65% and mobile phase B 35% for 5 min, mobile phase A 50% and mobile phase B 50% for 7 min, mobile phase A 0% and mobile phase B 100% for 8 min and ending at mobile phase A 83% and mobile phase B 17% for 5 min. The detection was at 370 nm. Four flavonoids including rutin, morin, quercetin, and isorhamnetin, and one phenolic acid *p*-coumaric acid which all are phenolic compounds were selected as standards. The retention time (RT) of rutin, *p*-coumaric acid, morin, quercetin, and isorhamnetin standards was found at 10.6, 11.7, 18, 20, and 23.5 min, respectively. Quantification of individual phenolic compound levels was based on the peaks integral area ratio between the standard and sample.

2.3. Isolation and cultures of peritoneal macrophages

The animal use protocol for these experiments was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), National Chung Hsing University, Taiwan, ROC. Female BALB/c strain mice (10 weeks old) weighting 20–25 g were used throughout the experiment. The primary peritoneal macrophages from mice were collected according to the method described by Lin et al. [8]. The peritoneal adherent cells (>90% of macrophages) from each animal were adjusted to 2×10^6 cells/ml in TCM medium with a hemocytometer using the trypan blue dye exclusion method. The peritoneal macrophages in the absence or presence of stimulus lipopolysaccharide (LPS, L-2654, Sigma-Aldrich Co., St. Louis, MO; at the final concentration of 5 μg/ml) and different administration concentrations of ES and EM were co-plated in 48-well plates. The plates were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air for up to 48 h. The plates were then centrifuged at 400 ×*g* for 10 min to obtain the cell culture supernatants and cell pellets. The cell culture supernatants were collected for cytokine assay using ELISA. Lipopolysaccharides (LPS) are large molecules consisting of a lipid and a polysaccharide joined by a covalent bond and induce strong immune responses in immune cells. Unfortunately, the concentration of LPS in the samples was not measured in this study. However, our data in our previous studies indicated that LPS did not contaminate samples in the isolation procedure using the same method [18].

2.4. Isolation and cultures of mouse primary splenocytes

Immediately after the mice primary peritoneal macrophages were collected, the splenocytes were prepared by aseptically removing the spleens from the BALB/c mice. The spleens were homogenized in TCM medium with help of a syringe piston. Single spleen cells were collected and treated by lysing the red blood cells with RBC lysis buffer (0.017 M Trizma base (Sigma-Aldrich Co.), 0.144 M ammonium chloride (Sigma-Aldrich Co.), pH 7.4, 0.20 μm filtered). Splenocytes were isolated from each animal and adjusted to a concentration of 1×10^7 cells/ml in TCM medium with a hemocytometer using the trypan blue dye exclusion method [5]. The splenocytes in the absence or presence of stimulus (LPS at the final concentration of 5 μg/ml) and different administration concentrations of ES and EM were co-plated