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A Matured Fruit Extract of Date Palm Tree (*Phoenix dactylifera* L.) Stimulates the Cellular Immune System in Mice

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ABSTRACT: The immunomodulatory effects of a hot water extract from matured fruit of the date palm tree (*Phoenix dactylifera* L.) were investigated in comparison to those of prune and fig fruit in mice. The number of spleen IFN-γ^+CD4$^+$, IFN-γ^+CD49b$^+$ and IL-12$^+$CD11b$^+$ cells was highest in mice given the date extract-added diet. Polyphenols identified in the date extract, such as chlorogenic acid, caffeic acid, pelargonin and ferulic acid, stimulated IFN-γ mRNA expression significantly in mouse Peyer's patch cell cultures. Chlorogenic acid and caffeic acid also increased the number of IFN-γ^+CD4$^+$ cells significantly, while some polyphenols increased the number of IFN-γ^+CD49b$^+$ and IL-12$^+$CD11b$^+$ cells significantly. On the other hand, a 70% ethanol-insoluble date extract treated with trypsin increased the number of IFN-γ^+CD49b$^+$ and IL-12$^+$CD11b$^+$ cells significantly. These results indicate that some polyphenols and polysaccharides present in date fruit stimulate the cellular immune system in mice.

KEYWORDS: date fruit, immunostimulation, cellular immune system, mouse, Peyer's patch

INTRODUCTION

The fruit of the date palm tree (*Phoenix dactylifera* L.) has been harvested in the North Africa for at least 3,500 years,[1] and its cultivation has now spread to the Middle East, parts of Central and South America, and Southern Europe.[2,3] For the people of the Middle East, the date fruit has been considered to be an ideal food because of its high sugar content and as a good source of fiber, minerals and other nutrients.[4] Date fruit has also been used as a folk medicine for the prevention of various infectious diseases because of its antibacterial[5] and antifungal properties.[6]

On the other hand, the fruits of prune (*Prunus domestica*) and fig (*Ficus carica*) are more familiar in Japan. The prune originates from the Caucasus region in Western Asia,[7] and the fruit is now largely harvested in Pakistan, India,[8] and Eastern Europe.[9] Prune fruit has been used medicinally in India for the treatment of leucorrhea, irregular menstruation, and debility following miscarriage.[10] Similarly, fig is widely used in the eastern Mediterranean regions of Europe,[11] Africa, and southwestern Asia.[12] Fig has also been traditionally used in respiratory and antispasmodic remedies.[13] Date, prune and fig fruits are commonly consumed not only as fresh fruit but also dried and in traditional medicinal foods, and have potential functionality for improving lifestyle-related diseases.[2,14,15]

Immunities can be divided into two groups: innate and adaptive. The former represents the first line of defense against attacks by pathogens, and is mediated by humoral factors such as antimicrobial proteins and leukocytes such as macrophages, dendritic cells (DCs) and natural killer (NK) cells. The distinctive feature of innate immunity is to exert rapid effector function through limited kinds of germline-encoded receptors. In contrast, adaptive immunity develops later but clonally expresses a large variety of antigen receptors that are produced by site-specific somatic recombination.

The dominant components of dried date, prune and fig fruits are sugars, polysaccharides such as pectin and β-glucan, and polyphenols. Pectin is reported to possess certain immunomodulatory effects, including protection against *Streptococcus* infection in mice[16] and upregulation of interleukin (IL)-1β and interferon (IFN)-γ in rats,[17] while β-glucan is demonstrated to protect against bacterial and protozoan infections in experimental animals.[18,19] Moreover, a high intake of cocoa, which is rich in polyphenols, is reported to increase Th1 response and to decrease antibody response in rats.[20] However, there are few reports on the immunomodulatory activities of dried date, prune and fig fruits.

Thus, this study investigates the immunomodulatory effects of a hot water extract from matured fruit of the date palm tree in comparison to those of prune and fig fruit extracts in mice. Moreover, this study evaluates whether some polyphenols found in the date extract stimulate the cellular immune system in mice.

MATERIALS AND METHODS

Materials. Phycoerythrin (PE)-labeled anti-mouse IL-4 monoclonal antibody (mAb, clone 11B11), PE-labeled anti-mouse IFN-γ mAb (clone XMG1.2), PE-labeled anti-mouse IL-12 IL-23/p40 mAb (clone C15.6), biotin-labeled anti-mouse CD4 mAb (clone RM4−5), biotin-labeled anti-mouse CD49b mAb (clone DX5), biotin-labeled anti-mouse CD11b mAb (clone M1/70), and phycoerythrin/cyanine5 (PE/Cy5)-labeled streptavidin were obtained from BioLegend (San Diego, CA). Brefeldin A (BFA), ionomycin, streptomycin, and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). IntraPrep was purchased from Beckman Coulter (Marseille, France). Defined fetal bovine serum (FBS) was obtained from HyClone Laboratories (Logan, UT). Penicillin was obtained from MP Biomedicals (CostaMesa, CA). Roswell Park Memorial Institute (RPMI)-1640 was purchased from Nissui Pharmaceutical (Tokyo, Japan).

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Table 1. Composition of Diets

	composition (%)			
		extract-added diet		
	extract-free (control) diet	date	prune	fig
fruit extract	0.0000	10.0000	10.0000	10.0000
β-corn starch	46.5692	46.5692	46.5692	46.5692
α-corn starch	15.5000	15.5000	15.5000	15.5000
ovalbumin	14.0000	13.8720	13.7090	13.8530
sucrose	10.0000	0.2800	0.4000	0.2800
cellulose	5.0000	4.8480	4.8910	4.8670
soy oil	4.0000	4.0000	4.0000	4.0000
AIN-93M mineral mix[a]	3.5000	3.5000	3.5000	3.5000
AIN-93M vitamin mix[b]	1.0000	1.0000	1.0000	1.0000
L-cystine	0.1800	0.1800	0.1800	0.1800
choline bitartrate	0.2500	0.2500	0.2500	0.2500
TBHQ	0.0008	0.0008	0.0008	0.0008
total	100.0000	100.0000	100.0000	100.0000

[a] AIN-93M mineral mix: calcium, 0.52%; phosphorus, 0.23%; potassium, 0.38%; magnesium, 0.05%; sodium, 0.11%; iron, 34.25 ppm; zinc, 36.76 ppm; manganese, 11.09 ppm; copper, 6.73 ppm; cobalt, 0.02 ppm; iodine, 0.21 ppm. [b] AIN-93M vitamin mix: vitamin A, 4.00 IU/g; vitamin D3, 1.00 IU/g; α-tocopherol, 75.00 IU/kg; thiamine, 5.00 ppm; riboflavin, 6.00 ppm; niacin, 30.00 ppm; pantothenic acid, 15.00 ppm; choline, 1000.00 ppm; pyridoxine, 6.00 ppm; folic acid, 2.00 ppm; biotin, 0.20 ppm; vitamin B12, 25.00 μg/kg; vitamin K, 0.86 ppm.

TRIzol Reagent, dNTP, and M-MLV reverse transcriptase were purchased from Invitrogen Life Technologies (Carlsbad, CA). Two aliquots of SYBR premix Ex Taq mixture were obtained from Takara Bio (Shiga, Japan). Protocatechuic acid, pelargonin, caffeic acid, ferulic acid, and chlorogenic acid were purchased from Funakoshi Co., Ltd. (Tokyo, Japan), and syringic acid was obtained from Wako Pure Chemical Industries. ABEE labeling kit was purchased from J-Oil Mills, Inc. (Tokyo, Japan). All chemicals used in this study were of the highest analytical grade commercially available.

Preparation of Fruit Extracts. Dried date fruit harvested in the United Arab Emirates (UAE) was obtained from Marubeni Corporation (Tokyo, Japan). Dried prune and fig fruits were purchased from Shoei Foods Corporation (Tokyo, Japan). Each dried fruit (1,000 g) was cut into approximate 5 × 5 mm size pieces, including peel and pulp but not seeds, and boiled in 9,000 mL of hot distilled water (DW) for 2 h under reflux. The supernatant was collected by centrifugation (5000*g*, 30 min) and freeze-dried as date, prune and fig extracts. The weights of date, prune and fig extracts were 675 g, 593 g, and 525 g, respectively.

Fractionation of Date and Prune Extracts. The date (675 g) and prune (593 g) extracts were dissolved in 2,500 mL of DW, put in Spectra/Por CE dialysis tubing 100−500 molecular weight cutoff (MWCO) (Spectrum Laboratories, Inc., Rancho Dominguez, CA), and dialyzed for 16 h at 15 °C against DW. The ultrafiltration was carried out according to the procedure as described by Segura Campos et al.[21] The retentate in the dialysis tubing was ultrafiltrated successively with four MWCO membranes: 1,000 (YM-1), 5,000 (PLCC), 10,000 (PLGC), and 30,000 (YM-30) Da (Millipore, Billerica, MA) by means of a stirred ultrafiltration cell (model 8200; Amicon, Danvers, MA). The five ultrafiltered fractions were suspended in 10 mL of DW and freeze-dried, and designated as a fraction with molecular mass greater than 30,000 Da (30,000 Da retentate); from 10,000 Da to 30,000 Da (30,000 Da permeate−10,000 Da retentate); from 5,000 Da to 10,000 Da (10,000 Da permeate−5,000 Da retentate); from 1,000 Da to 5,000 Da (5,000 Da permeate−1,000 Da retentate); and from 500 Da to 1,000 Da (1,000 Da permeate). The yields of each fraction were 1.91 g, 1.48 g, 0.14 g, 0.59 g, and 1.80 g for the date extract, and 1.10 g, 0.20 g, 0.03 g, 0.12 g, and 2.96 g for the prune extract, respectively.

Next, the fraction with a molecular mass greater than 30,000 Da (0.5 g) was dissolved in 20 mL of 0.05 M sodium phosphate buffer (pH 7.2), with added ammonium sulfate at a concentration of 70% saturation, and incubated at 4 °C for 16 h. The sample was centrifuged (20000*g*, 30 min), and the precipitate was dissolved in 20 mL of the above buffer, put in Spectra/Por CE dialysis tubing 100−500 MWCO, and dialyzed for 16 h at 15 °C against the buffer. The retentate was then freeze-dried as a precipitate in 70%-saturated ammonium sulfate. The yield was 0.14 g. Similarly, the fraction with a molecular mass greater than 30,000 Da (0.5 g) was dissolved in 20 mL of 0.05 M sodium phosphate buffer (pH 7.6), with added trypsin at a concentration of 0.01% (w/w), and incubated at 25 °C for 16 h. The reaction was stopped with the addition of ethanol at a concentration of 70%, and incubated at 4 °C for 1 h. Then it was centrifuged (5000*g*, 20 min). The precipitate was freeze-dried as a precipitate in 70% ethanol. The yield was 0.31 g.

Oral Administration. Five-week-old male C3H/HeN mice were obtained from Japan SLC, Inc. (Shizuoka, Japan) and housed at 23 ± 2 °C under a standard 12 h light−dark cycle. After preliminary breeding for 1 week, the mice were divided into 4 groups in which they were given an AIN-93 M standard rodent diet (Clea Japan, Tokyo, Japan) as extract-free (control) diet, 10% date extract-added diet, 10% prune extract-added diet, and 10% fig extract-added diet. The amount of sugar and protein of the fruit extract-added diet conformed to the control diet. The detailed composition of each diet is shown in Table 1. Water was provided *ad libitum* from drinking bottles.

Each group consisted of 5 mice, and they were bred for 30 days. After the breeding, spleens and Peyer's patches were collected to investigate cell function. All animal experimentation undertaken during this study was conducted in accordance with the guidelines for the Regulation of Animal Experimentation at Shinshu University and according to Law No. 105 and Notification No. 6 of the Japanese government.

Peyer's Patch and Spleen Cell Suspensions, and Cell Cultures. The Peyer's patch and spleen cell suspensions were prepared as described previously.[22] The fractionated date and prune extracts were dissolved in phosphate-buffered saline (PBS) and added into the medium at a final concentration of 50 or 100 μg/mL. Individual standard polyphenols were dissolved in dimethyl sulfoxide (DMSO) and added

Table 2. Numbers of Immunocompetent Cells in Peyer's Patches and Spleens of Mice Given Date Extract-, Prune Extract-, and Fig Extract-Added Diets[a]

	no. of cells ($\times 10^4/10^6$ Peyer's patch or spleen cells)			
		extract-added diet		
immunocompetent cell	extract-free (control) diet	date	prune	fig
		Peyer's Patch		
IFN-γ^+CD4$^+$	4.60 ± 1.03 a	6.63 ± 0.70 b	7.10 ± 1.00 b	5.91 ± 0.22 b
IL-4$^+$CD4$^+$	4.94 ± 0.35 a	4.19 ± 0.66 a	5.36 ± 0.61 a	5.14 ± 0.88 a
IFN-γ^+CD49b$^+$	3.35 ± 0.53 a	4.15 ± 0.15 b	4.77 ± 0.50 b	3.64 ± 0.45 a
IL-12$^+$CD11b$^+$	5.97 ± 1.13 a	7.85 ± 0.23 b	7.23 ± 0.53 a	6.23 ± 0.85 a
		Spleen		
IFN-γ^+CD4$^+$	3.40 ± 0.24 a	4.83 ± 0.45 b	3.53 ± 0.18 a	3.28 ± 0.50 a
IL-4$^+$CD4$^+$	2.01 ± 0.37 a	1.94 ± 0.41 a	1.95 ± 0.24 a	1.97 ± 0.32 a
IFN-γ^+CD49b$^+$	2.25 ± 0.05 a	2.70 ± 0.32 a	2.28 ± 0.26 a	1.96 ± 0.24 a
IL-12$^+$CD11b$^+$	1.66 ± 0.26 a	2.50 ± 0.30 b	1.86 ± 0.26 a	1.29 ± 0.28 a

[a] Data are represented as the mean ± SD (n = 5). Items indicated with different letters (i.e., a, b) are significantly different ($P < 0.05$).

into the medium at a final concentration of 4 nmol/mL. The final concentration of DMSO was 0.01% and was determined to have no cytotoxicity. The cells were cultured at 37 °C in a humidified 5% CO_2 incubator for 48 h (for cell function analysis) or 24 h (for mRNA expression analysis).

Cell Functional Analysis. The cell surface markers and intracellular cytokines were labeled according to the procedure as described previously.[22] The cell number was determined using a Guava personal cell functional analyzer (Guava PCA, Guava Technologies, Hayward, CA).

Preparation of Total RNAs and Real-Time Reverse Transcription (RT) Polymerase Chain Reaction (PCR). The total RNAs from Peyer's patch cells were extracted as described previously.[22] Real-time RT-PCR was conducted using a Thermal Cycler Dice Real Time TP800 system (Takara Bio) using 2 × SYBR premix Ex Taq mixture. The primer sequences for amplifying IFN-γ were reported by Mizutani et al.[23] The primer sequences for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reported by Tobita et al.[24] The real-time RT-PCR reaction involved 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The relative amount of IFN-γ mRNA was normalized using GAPDH expression as an internal control. An expression index was calculated from the normalized relative amount in the absence of the extract/polyphenol to the normalized relative amount in the presence of the extract/polyphenol. This analysis was carried out in at least triplicate, and representative results are presented.

Ultraperformance Liquid Chromatography (UPLC) Analysis. The date extract (2 g) was dissolved in 40 mL of DW containing 0.1% formic acid, and applied onto a C-18 Sep-Pak (10 g) cartridge (Waters, Milford, MA) that was activated with methanol and equilibrated with DW containing 0.1% formic acid. Then, the unabsorbed components were washed out with DW containing 0.1% formic acid, and absorbed components were eluted with 50% methanol. The eluate was concentrated using a rotary evaporator and freeze-dried as a fraction with crude date polyphenols. The weight was 107.8 mg. The fraction was dissolved in methanol at a concentration of 200 mg/mL and analyzed by means of UPLC. UPLC analysis was performed using an ACQUITY UPLC system (Waters) and BEH C18 column (2.1 mm i.d. × 50 mm, 1.7 μm; Waters). The temperatures of the autosampler and the column were maintained at 20 and 40 °C, respectively. The separation was achieved with gradient elution using (A) DW containing 0.1% trifluoroacetic acid (TFA) and (B) acetonitrile containing 0.1% TFA as the mobile phase at a flow rate of 0.50 mL/min. Gradient condition of the mobile phase was as follows: B concentration, 5−15%, 0−10 min; B concentration, 15−95%, 10−12 min, and returned to the initial condition. The effluent was monitored at an absorbance of 254 nm.

Determination of Sugar Compositions. Determination of sugar compositions was carried out according to the procedure as described by Yasuno et al.[25] The fraction with a molecular mass greater than 30,000 Da (5 μg) was hydrolyzed and labeled using an ABEE labeling kit. The ABEE-converted monosaccharides were analyzed with high-performance liquid chromatography (HPLC). The column was an ODS-80Ts (4.6 mm i.d. × 150 mm; Tosoh, Tokyo, Japan). The HPLC was carried out according to the procedure as described by Yasuno et al.[25]

Statistical Analysis. Data is expressed as the mean ± standard deviation (SD). Statistical analyses were performed using the Dunnett's multiple comparison tests for one-way analysis of variance. Differences were considered significant when P values were less than 0.05.

RESULTS

Immunological Properties of Mice Given the Date Extract-, Prune Extract-, or Fig Extract-Added Diet. Six-week-old C3H/HeN mice were given the date extract-, prune extract-, or fig extract-added or the extract-free (control) diet for 30 days. No significant differences in the body weights were observed in mice given the four kinds of diets (data not shown).

As shown in Table 2, the number of IFN-γ^+CD4$^+$ cells in Peyer's patches was significantly higher in mice given the fruit extract-added diets than in those given the control diet, particularly the mice given the date extract- and prune extract-added diets. The number of Peyer's patch IFN-γ^+CD49b$^+$ cells was significantly higher in mice given the date extract- and prune extract-added diet than in those given the control diet and the fig extract-added diet, while that of IL-12$^+$CD11b$^+$ cells was significantly higher in mice given the date extract-added diet than in those given the control diet and the prune extract- and the fig extract-added diet. Similarly, the number of spleen IFN-γ^+CD4$^+$ and IL-12$^+$CD11b$^+$ cells was significantly higher in mice given the date extract-added diet than in those given the control, prune extract-added or fig extract-added diet.

Effect of Fractionated Date and Prune Extracts on IFN-γ Gene Expression in Mouse Peyer's Patch Cell Cultures. As shown in Figure 1, two date extract fractions with a molecular

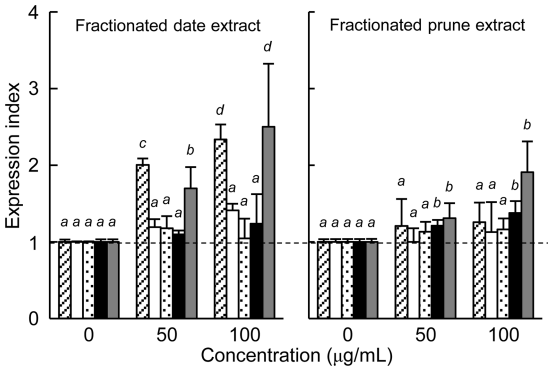

Figure 1. Effects of fractionated date and prune extracts on the expression of IFN-γ mRNA in mouse Peyer's patch cells. Peyer's patches were obtained from six-week-old mice bred with a commercially available standard diet, and the cells were cultured with 50 or 100 μg/mL of each fractionated extract for 24 h. Total RNAs from the cells were extracted and cDNAs were generated by RT reaction. The fraction with a molecular mass from 500 Da to 1,000 Da, the fraction with a molecular mass from 1,000 Da to 5,000 Da, the fraction with a molecular mass from 5,000 Da to 10,000 Da, the fraction with a molecular mass from 10,000 Da to 30,000 Da, and the fraction with a molecular mass greater than 30,000 Da are presented as the hatched, open, black-dotted, solid, and shaded bars, respectively. Data are represented as the mean ± SD (n = 3). Items indicated with different letters (i.e., *a*, *b*, *c*, *d*) are significantly different (P < 0.05).

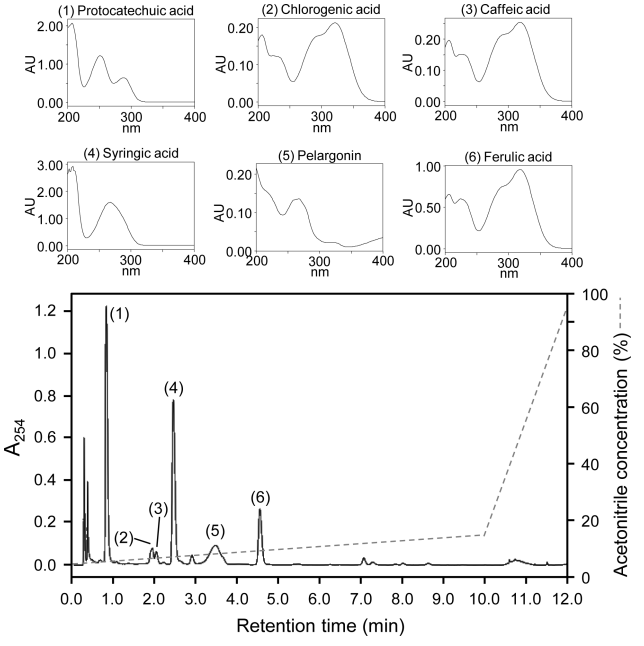

Figure 2. Spectra (upper) and chromatograms (lower) of the fraction with crude date polyphenols analyzed by UPLC: (1) protocatechuic acid, (2) chlorogenic acid, (3) caffeic acid, (4) syringic acid, (5) pelargonin, and (6) ferulic acid.

mass from 500 Da to 1,000 Da and greater than 30,000 Da significantly increased IFN-γ mRNA expression levels compared to the extract-free and the other date extract fractions, such as a molecular mass from 1,000 Da to 5,000 Da, 5,000 Da to 10,000 Da, and 10,000 Da to 30,000 Da. The prune extract fraction with a molecular mass from 10,000 Da to 30,000 Da and greater than

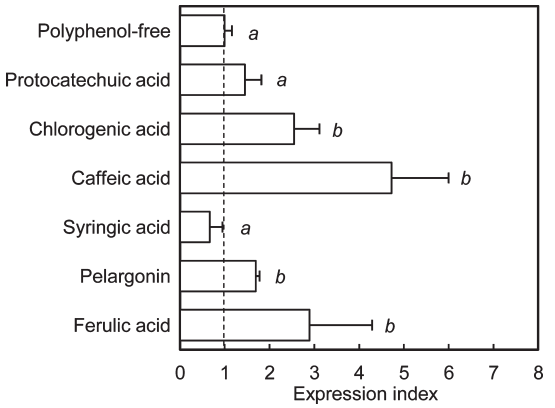

Figure 3. Effects of some polyphenols identified in the date extract on expression of IFN-γ mRNA in mouse Peyer's patch cell cultures. Peyer's patches were obtained from six-week-old mice bred with a commercially available standard diet, and the cells were cultured with 4 μM of each polyphenol for 24 h. Total RNAs from the cells were extracted and cDNAs were generated by RT reaction. Data are represented as the mean ± SD (n = 3). Items indicated with different letters (i.e., *a*, *b*) are significantly different (P < 0.05).

30,000 Da significantly increased IFN-γ mRNA expression levels compared to the other three prune extract fractions.

Identification of Polyphenols in the Date Extract by UPLC. As shown in Figure 2, more than 10 peaks were detected in the date extract on UPLC. The retention time and UV spectrum of these peaks were compared with those of standard polyphenols commercially available. Six major peaks were identified as follows: (1) protocatechuic acid, (2) chlorogenic acid, (3) caffeic acid, (4) syringic acid, (5) pelargonin, and (6) ferulic acid. Unfortunately, other peaks could not been identified.

Effects of Some Polyphenols Identified in the Date Extract on IFN-γ Gene Expression in Mouse Peyer's Patch Cell Cultures. Figure 3 shows the effect of six commercially available polyphenols identified in the date extract on IFN-γ mRNA expression in Peyer's patch cell cultures. Chlorogenic acid, caffeic acid, pelargonin, and ferulic acid increased IFN-γ mRNA expression significantly compared with polyphenol-free, protocatechuic acid and syringic acid.

Effects of Some Polyphenols Identified in the Date Extract on the Number of Immunocompetent Cells in Mouse Peyer's Patch Cell Cultures. Table 3 shows the numbers of immunocompetent cells in Peyer's patch cells cultured with four polyphenols that increased IFN-γ mRNA expression, as shown in Figure 3. Chlorogenic acid and caffeic acid increased the number of Peyer's patch IFN-γ^+CD4$^+$ cells significantly in comparison to polyphenol-free, pelargonin and ferulic acid. Chlorogenic acid, pelargonin, and ferulic acid increased the number of Peyer's patch IFN-γ^+CD49b$^+$ cells significantly in comparison to polyphenol-free and caffeic acid. Moreover, chlorogenic acid, caffeic acid, and ferulic acid enhanced the number of IL-12$^+$CD11b$^+$ cells significantly in comparison to polyphenol free and pelargonin.

Effects of Two Precipitates of the Date Extract Fraction with a Molecular Mass Greater than 30,000 Da on the Number of Immunocompetent Cells in Mouse Peyer's Patch Cell Cultures. Table 4 shows the numbers of immunocompetent cells in Peyer's patch cells cultured with either the ammonium sulfate or ethanol precipitate of the date extract fraction with a molecular mass greater than 30,000 Da. The ethanol precipitate increased the numbers of both IFN-γ^+CD49b$^+$ and IL-12$^+$CD11b$^+$ cells

Table 3. Numbers of Immunocompetent Cells in Peyer's Patch Cells Cultured with Four Polyphenols Identified in the Date Extract[a]

	no. of cells ($\times 10^4/10^6$ Peyer's patch cells)				
immunocompetent cell	free (control)	chlorogenic acid	caffeic acid	pelargonin	ferulic acid
IFN-γ^+CD4$^+$	3.83 ± 0.39 a	4.85 ± 0.09 b	4.81 ± 0.22 b	4.33 ± 0.21 a	4.33 ± 0.29 a
IFN-γ^+CD49b$^+$	3.30 ± 0.54 a	6.10 ± 0.80 c	3.63 ± 0.38 a	4.14 ± 0.15 b	5.60 ± 0.79 c
IL-12$^+$CD11b$^+$	5.15 ± 1.00 a	7.50 ± 0.91 b	6.68 ± 0.21 b	6.36 ± 0.15 a	7.38 ± 0.72 b

[a] Peyer's patches were obtained from six-week-old mice bred with a commercially available standard diet. Data are represented as the mean ± SD ($n = 4$). Items indicated with different letters (i.e., a, b, c) are significantly different ($P < 0.05$).

Table 4. Numbers of Immunocompetent Cells in Peyer's Patch Cells Cultured with 2 Kinds of Precipitates, in 70% Saturation of Ammonium Sulfate and in 70% Ethanol of the Date Extract Fraction with Molecular Mass Greater than 30,000 Da[a]

	no. of cells ($\times 10^4/10^6$ Peyer's patch cells)				
		ammonium sulfate precipitate		ethanol precipitate	
immunocompetent cell	free (control)	50 (μg/mL)	100 (μg/mL)	50 (μg/mL)	100 (μg/mL)
IFN-γ^+CD4$^+$	3.18 ± 0.28 a	3.64 ± 0.29 a	3.98 ± 0.29 a	3.99 ± 0.34 a	3.85 ± 0.32 a
IFN-γ^+CD49b$^+$	2.89 ± 0.05 a	2.91 ± 0.17 a	3.05 ± 0.15 a	3.26 ± 0.09 b	3.44 ± 0.15 b
IL-12$^+$CD11b$^+$	5.24 ± 0.63 a	5.61 ± 0.65 a	5.33 ± 0.56 a	7.35 ± 0.51 b	6.58 ± 0.41 b

[a] Peyer's patches were obtained from six-week-old mice bred with a commercially available standard diet. Data are represented as the mean ± SD ($n = 4$). Items indicated with different letters (i.e., a, b) are significantly different ($P < 0.05$).

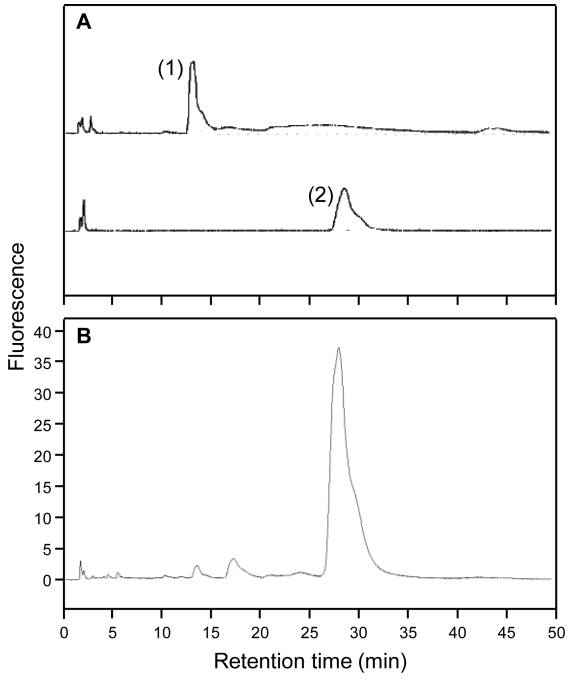

Figure 4. Chromatograms of ABEE-converted standard monosaccharides (A) and ABEE-converted degradation products of the date extract fraction with a molecular mass greater than 30,000 Da (B) on HPLC. Peaks: (1) ABEE-converted galacturonic acid, (2) ABEE-converted glucose.

significantly compared to precipitate-free. In contrast, the ammonium sulfate precipitate had little influence on the immunocompetent cells.

Determination of Sugar Compositions in the Date Extract Fraction with a Molecular Mass Greater than 30,000 Da. As shown in Figure 4B, three peaks were detected in the ABEE-converted degradation products of the date extract fraction with a molecular mass greater than 30,000 Da on HPLC. Retention times of two of these peaks were consistent with ABEE-converted galacturonic acid and ABEE-converted glucose (Figure 4A), although one minor peak could not been determined.

DISCUSSION

First, the number of Peyer's patch IFN-γ^+CD4$^+$, IFN-γ^+CD49b$^+$ and IL-12$^+$CD11b$^+$ cells was significantly increased in mice given the date extract-added diet than in those given the extract-free (control) diet. The number of Peyer's patch IFN-γ^+CD4$^+$ cells in mice given the prune or fig extract-added diet was increased, and the number of Peyer's patch IFN-γ^+CD49b$^+$ cells in those given the prune extract-added diet was stimulated significantly. However, the number of spleen IFN-γ^+CD4$^+$ and IL-12$^+$CD11b$^+$ cells was increased significantly only in mice given the date extract-added diet (Table 2). CD11b is a typical cell surface antigen of macrophages and DCs.[26] IL-12 is one of the major cytokines produced by these cells.[27] On the other hand, CD49b is a typical cell surface antigen of NK cells. IFN-γ is also the major cytokine produced by these cells and type 1-helper T (Th1) cells.[27] Macrophages and DCs form the first line of defense as components of the natural immune response in host defense. NK cells are also important accessory cells, which, in addition to macrophages and DCs, play a critical role in host defense to display cytotoxic effects on tumor cells.[28] The proliferation and activation of NK cells are induced by IL-12, and the activated NK cells produce IFN-γ.[27] Hence, it is known that an increase in the number of IFN-γ^+CD49b$^+$ cells is due to an

increase in the number of IL-12$^+$CD11b$^+$ cells. On the other hand, IFN-γ^+CD4$^+$ cells are Th1 cells, and the production of IFN-γ stimulates IL-12 produced from macrophages as well as NK cells and enhances the differentiation of naive T cells into Th1 cells.[29] These facts indicate that the date extract stimulates IL-12 produced by macrophages and/or DCs, suggesting that IL-12 activates NK cells in Peyer's patch, and differentiates naive T cells into Th1 cells in Peyer's patch and spleen. The tendency for increased Peyer's patch IL-12$^+$CD11b$^+$ cells indicates that the prune extract also activates NK cells and Th1 cells. Incidentally, it is suggested that the fig extract stimulates Th1 cells in a different way than the date and prune extracts, because the fig extract increases the number of Peyer's patch IFN-γ^+CD4$^+$ cells but does not increase the number of Peyer's patch IL-12$^+$CD11b$^+$ cells.

The two date extract fractions with a molecular mass from 500 Da to 1,000 Da and greater than 30,000 Da significantly increased IFN-γ mRNA expression levels compared to extract-free and the other fractions (Figure 1). This result suggests that at least two kinds of components in the date extract stimulate IFN-γ mRNA expression. Therefore, these date components might affect the increase of the number of Peyer's patch and spleen IFN-γ^+CD4$^+$ and Peyer's patch IFN-γ^+CD49b$^+$ cells in mice given the date extract-added diet.

It is generally considered that low molecular components present in fruits mainly are polyphenols. It is reported that date contains some polyphenols such as protocatechuic acid, caffeic acid, syringic acid, ferulic acid, procyanidins, and quercetin glycoside.[3,30] In this study, we identified six kinds of polyphenols in the date extract. In addition, we confirmed that chlorogenic acid and pelargonin were novel polyphenols in the date (Figure 2). The effects of these two polyphenols identified in the present study and some other polyphenols known to be included in date were examined on IFN-γ gene expression in mouse Peyer's patch cell cultures. Chlorogenic acid, caffeic acid, pelargonin, and ferulic acid increased IFN-γ mRNA expression significantly compared with polyphenol-free, protocatechuic acid and syringic acid (Figure 3). In addition, chlorogenic acid and caffeic acid increased the number of IFN-γ^+CD4$^+$ cells significantly compared to polyphenol-free, pelargonin and ferulic acid. Additionally, chlorogenic acid, pelargonin and ferulic acid enhanced the number of IFN-γ^+CD49b$^+$ cells significantly in comparison to polyphenol-free and caffeic acid. In the oral administration test, in mice given the date extract-added diet, the number of Peyer's patch IFN-γ^+CD4$^+$ and IFN-γ^+CD49b$^+$ and the number of spleen IFN-γ^+CD4$^+$ cells were significantly higher, and the number of spleen IFN-γ^+CD49b$^+$ cells tended to increase compared to those given the control diet. These results indicate that the increase in the number of spleen IFN-γ^+CD4$^+$ cells in mice given the date extract-added diet appears to be due to the action of chlorogenic acid and caffeic acid, while that in the number of spleen IFN-γ^+CD49b$^+$ cells in the mice may be due to the action of chlorogenic acid, pelargonin, and ferulic acid.

Generally, it is considered that high molecular components present in fruits are proteins and polysaccharides. We prepared two kinds of precipitates by the addition of ammonium sulfate or ethanol, following digestion with trypsin, into the date extract fraction with a molecular mass greater than 30,000 Da. The ethanol precipitate of the date extract, following digestion with trypsin, increased the numbers of both IFN-γ^+CD49b$^+$ and IL-12$^+$CD11b$^+$ cells significantly compared to precipitate-free, but the ammonium sulfate precipitate had little influence on the immunocompetent cells (Table 4). In addition, as shown by HPLC analysis, the date extract fraction with a molecular mass greater than 30,000 Da was mainly composed of glucose (Figure 4). It is reported that date contains β-D-glucan,[31] and the glucan is a polysaccharide containing glucose. These results suggest that the immunomodulatory functions of the date extract are also due to the β-D-glucan.

In conclusion, in this study, we demonstrated that date extract enhances the cellular immune system more strongly than prune and fig extracts. We propose that date may be used as an effective immunomodulator for preventing some diseases. In particular, we found that date extract stimulates IFN-γ^+CD4$^+$cells, or Th1 cells. It is well-known that type I allergy is caused by low Th1 cell levels.[32] Hence, we are investigating the effect of date extract-added diet on allergic symptoms of type I allergic model mice.

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ABBREVIATIONS USED

BFA, brefeldin A; DC, dendritic cell; DMSO, dimethyl sulfoxide; DW, distilled water; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Guava PCA, Guava personal cell function analyzer; HBSS, Hanks' balanced salt solution; HPLC, high-performance liquid chromatography; IFN, interferon; IL, interleukin; NK, natural killer; PBS, phosphate-buffered saline; PE, phycoerythrin; PE/Cy5, phycoerythrin/cyanine 5; PMA, phorbol 12-myristate 13-acetate; RPMI-1640, Roswell Park Memorial Institute-1640; RT-PCR, reverse transcription-polymerase chain reaction; TFA, trifluoroacetic acid; Th1, type 1 helper T; UAE, United Arab Emirates; UPLC, ultraperformance liquid chromatography

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