Biological activities of fructooligosaccharide (FOS)-containing *Coix lachryma-jobi* Linn. extract

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Abstract Fructooligosaccharide (FOS), a prebiotic was extracted from the grain of *Coix lachryma-jobi* Linn. (Job's tears) by hot water extraction at 60 °C for 1 h. The resulting dried powder extract was assayed for FOS content of 1-kestose (GF₂), nystose (GF₃) and 1-β-D-fructofuranosyl-nystose (GF₄) using HPLC equipped with RI detector. Total FOS content of the extract was 24.98±7.48% (g/100 g crude extract). The biological activity including antioxidant and cytotoxicity of the FOS-containing extract was determined. The antioxidant activity by DPPH free radical scavenging of FOS-containing extract was comparable to vitamin C (0.97 fold of vitamin C) with a slight lipid peroxidation inhibition activity. The extract exhibited no cytotoxic effect on normal human skin fibroblast. These results have confirmed not only the source of FOS from Job's tears extract but also its potential application as antioxidant in food or cosmetic products.

Keywords Fructooligosaccharide · *Coix lachryma-jobi* Linn. · Job's tears · Antioxidant

Introduction

Fructooligosaccharides (FOS) can be classified as non-digestible oligosaccharides since the β-linkages between fructose monomers cannot be hydrolyzed by enzymes of endogenous origin (Xu et al. 2003). FOS consist mainly of 1-kestose (GF₂), nystose (GF₃) and 1-β-D-fructofuranosyl-nystose (GF₄) which have 1–3 units of fructose, bond to the β, 2–1 position of sucrose, respectively. The FOS has been shown to enhance the growth of *Bifidobacterium* and *Lactobacillus* but inhibited *Escherichia coli* and *Salmonella* in the large intestine (Bunce et al. 1995; Choi et al. 1994; Fukata et al. 1999; Hidaka et al. 1986; Roberfroid et al. 1998; Xu et al. 2002). FOS could have full or partial degradation to fructose or formation of other compounds like caramelization products, possessing new biological properties such as antioxidant properties of the latter compounds (Huebner et al. 2008). *Coix lachryma-jobi* Linn. (Job's tears), a native plant of Southeast Asia exists in the wild and cultivated. It is also found wild in Africa and Asia and warmer parts of the Mediterranean. The cultivated races in Southeast Asia have been selected for grain that is easily husked. The grain is prepared by roasting and may be eaten dry, used as porridge, or processed into flour (Jideani and Jideani 2011). The major ingredients of Job's tears extract are fatty acid and oligosaccharides. Fatty acids, such as hexadecanoic acid, octadecanoic acid, octadecadienoic acid, palmitic acid, stearic acid, oleic acid and linoleic acid are presented in the Job's tears seeds. Stigmasterol, coixenolide and coixol are also found in this plant (Jiang and Xiao 1986; Li 2006). For the in vitro cytotoxicity study, normal human skin fibroblast has been widely used (Lee et al. 2000; Iordăchescu et al. 2002; Dechsakulthorn et al. 2007;

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Manosroi et al. 2011). Skin fibroblast is a suitable cell for in vitro cytotoxicity test for the prediction of skin irritation in vivo since the close relationship was found between Alamar blue (AB) assay with human skin fibroblasts and human patch test ($r=0.867$). There was also a relatively good correlation between neutral red uptake (NRU) and in vivo patch test ($r=0.648$) (Lee et al. 2000). The objective of this study was to evaluate the biological activities of FOS from Job's tears extract including antioxidative activity by DPPH free radical scavenging, lipid peroxidation inhibition and metal ion chelating activity, and the cytotoxicity on normal human skin fibroblast. The results from these studies can be applied for the further development of food supplements as a multifunctional ingredient.

Materials and methods

Materials

Coix lachryma-jobi Linn. (Job's tears) of Rai Thip® brand were purchased from Thai Cereals World (Nonthaburi, Thailand). Vitamin C (L-(+)-ascorbic acid), α -tocopherol, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical were from Sigma Chemical Co. (St. Louis, MO, USA). Linoleic acid was purchased from Fluka, Switzerland. Standard 1-kestose, nystose and 1- β -D-fructofuranosyl nystose were purchased from Wako, Osaka, Japan. All other chemicals and reagents were analytical grade.

FOS extraction

The FOS was extracted from Job's tears grain by hot water extraction method. Briefly, the grain was ground and mixed with distilled water at the ratio of grain/water of 1:4 w/v. The mixture was warmed in a water bath at 60 °C with stirring every 15 min for 1 h. After 1 h, the mixture was cooled down to room temperature (25 ± 2 °C) and filtered through a Whatman filter No. 1 (Whatman®, Germany). The filtrate was collected and concentrated by using rotary evaporator (R-124 Buchi, Switzerland) and dried by lyophilizer (Alpha 1–2 LD; Christ, Germany).

Determination of FOS contents by HPLC with Refractive Index (RI) detector

The FOS contents in Job's tears extract were determined by HPLC equipped with RI detector using the standards 1-kestose (GF₂), nystose (GF₃) and 1- β -D-fructofuranosyl nystose (GF₄) were used. The FOS contents were analyzed by HPLC (Chromato-Pro Ver. 3.00, Senshu Scientific, Japan) equipped with RI detector (Chromato-Pro Ver. 3.00, Senshu Scientific, Japan). An amino column (Supelcosil™ LC-NH₂,

25 cm×4.6 mm, 5 μ m) was used with 70% acetonitrile as a mobile phase. The column temperature was controlled at 30 °C and the flow rate was constantly 1.5 ml/min for 20 min. The amount of each form of FOS was determined from the standards curves of GF₂, GF₃ and GF₄.

Antioxidative activities of FOS-containing Job's tears extract

DPPH radical scavenging activity

Free radical scavenging activities of the FOS-containing extract, standard antioxidant (vitamin C) and standard FOS (GF₂, GF₃ and GF₄) were determined by a modified DPPH assay (Brand-Williams et al. 1995; Tachibana et al. 2001; Manosroi et al. 2010). Briefly, 50 μ l of five serial concentrations (0.001–10 mg/ml) of the extract or the standard FOS, and 50 μ l of ethanolic solution of DPPH were added into each well of a 96-well microplate (Nalge Nunc International, NY, USA). The reaction mixtures were allowed to stand for 30 min at 25 ± 2 °C, and the absorbance was measured at 515 nm by a well reader (Bio-Rad, Model 680 Microplate Reader, USA). Vitamin C (0.001–10 mg/ml) was used as a positive control. The experiment was done in triplicate. The percentages of radical scavenging activity were calculated as the following equation;

$$\text{Scavenging (\%)} = [(A - B)/A] \times 100$$

where A was the absorbance of the control and B was the absorbance of the sample. The sample concentration providing 50% of scavenging (SC₅₀) was calculated from the graph plotted between the percentages of scavenging and the sample concentrations.

Lipid peroxidation activity

The antioxidative activity of the FOS-containing extract was assayed by modified Ferric-thiocyanate method (Osawa and Namiki 1981; Sun et al. 2011). An amount of 50 μ l of five serial concentrations of the extract and the standard FOS at 0.001–10 mg/ml in DMSO was added to 50 μ l of linoleic acid in 50% (v/v) DMSO in each of a 96-well microplate. The reaction was initiated by the addition of 50 μ l of NH₄SCN (5 mM) and 50 μ l of FeCl₂ (2 mM). The mixture was incubated at 37 ± 2 °C for 1 h. During the oxidation of linoleic acid, peroxides are formed leading to the oxidation of Fe²⁺ to Fe³⁺. The latter ions form a complex with thiocyanate which can be detected at 490 nm. The dissolving solution without the sample was used as negative control. Vitamin E at 0.001–10 mg/ml was used as a positive control. All experiments were performed in triplicate. The inhibition percentages of lipid

Fig. 1 Chromatogram of FOS from *Coix lachryma-jobi* Linn. (Job's tears) grain extract (GF₂, GF₃ and GF₄ indicated 1-kestose, nystose and 1-β-D-fructofuranosylnystose, respectively)

peroxidation of linoleic acid were calculated as the following;

$$\text{Inhibition of lipid peroxidation (\%)} = [(A - B)/A] \times 100$$

where A was the absorbance of the control and B was the absorbance of the sample. The sample concentration providing 50% inhibition of lipid peroxidation (LC₅₀) was calculated from the graph plotted between the percentages of lipid peroxidation inhibition and the sample concentrations.

Metal ion chelating activity

The metal ion chelating activity of the FOS-containing extract was assayed by the modified ferrous ion chelating method (Decker and Welch 1990). Briefly, 100 µl of five serial concentrations of the extract or the standard FOS at 0.001–10 mg/ml were added to 50 µl of 2 mM FeCl₂ solution. The reaction was initiated by the addition of 50 µl of 5 mM ferrozine and the total volume was adjusted to 300 µl by distilled water. The mixture was left at 25±2 °C for 15 min. Absorbance of the resulting solution was then measured at 570 nm by a microplate reader. EDTA (0.001–10 mg/ml) was

used as a positive control. The negative control contained FeCl₂ and ferrozine. All experiments were performed in triplicate. The inhibition percentages of ferrozine-Fe²⁺ complex formation were calculated by the following equation;

$$\text{Metal chelating activity (\%)} = [(A - B)/A] \times 100$$

where A was the absorbance of the control and B was the absorbance of the sample. The sample concentration providing 50% metal chelating activity (MC₅₀) was calculated from the graph plotted between the percentages of metal chelating activity and the sample concentrations.

Cytotoxicity on normal human skin fibroblast

Cell culture

Normal human skin fibroblast cell line was provided by Dr. Natthanej Luplertlop, Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Thailand. This cell line has been widely used in the in vitro cytotoxicity study (Lee et al. 2000; Iordăchescu et al. 2002; Dechsakulthorn et al. 2007; Manosroi et al. 2011). The fibroblasts were derived from the tissue by explants techniques in 30-mm diameter

Table 1 Antioxidant activity of standard FOS and Job's tears extract

Sample	Antioxidant activity (mg/ml)					
	SC ₅₀	Fold	LC ₅₀	Fold	MC ₅₀	Fold
Standard vitamin C	0.31±0.07	1	—	—	—	—
Standard vitamin E	—	—	1.01±0.59	1	—	—
Standard EDTA	—	—	—	—	10.73±5.68	1
1-Kestose (GF ₂)	>500	<0.001	>500	<0.002	>500	<0.021
Nystose (GF ₃)	>500	<0.001	>500	<0.002	ND ^a	ND ^a
1-β-D-Fructofuranosylnystose (GF ₄)	>500	<0.001	>500	<0.002	>500	<0.021
Job's tears extract	0.32±0.19	0.97	156.00±9.01	0.01	ND ^a	ND ^a

Values represented as mean±SE (n=3)

^a ND = Not detected, indicated no antioxidant activity

Fig. 2 Percentages of cell growth (% G) of the normal human skin fibroblast treated with standard vitamin C, standard FOS and FOS-containing *Coix lachryma-jobi* Linn. (Job's tears) extract determined by SRB assay ($n=3$)

tissue culture dishes. They were cultured under standard conditions in the complete culture medium containing α -Modified Eagles culture medium (MEM-Alpha, Hyclone, Utah, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Hyclone, Utah, USA), penicillin (100 U/ml; Hyclone, Utah, USA) and streptomycin (100 mg/ml; Hyclone, Utah, USA). Cells were incubated in a temperature-controlled, humidified incubator (Shel Lab, model 2123TC, USA) with 5% CO₂ at 37 °C and subcultured every 5–7 days. The cells of 8th to 10th generation were used in this study.

Cytotoxicity study by SRB assay

The standard FOS, FOS-containing Job's tears extract and the standard ascorbic acid were diluted in α -Modified Eagles culture medium to final concentrations of 0.001–10 mg/ml and assayed for cell proliferation activity on normal human skin fibroblast by the SRB assay. The cells were plated at a density of 1.0×10^5 cells/ml in 96-well plates and left for cell attachment overnight at 37 °C in 5% CO₂ incubator. Cells were then exposed to the extract for 24 h. After incubation, the adherant cells were fixed in situ with 50% trichloroacetic acid, washed and dyed with 0.4% SRB. The bound dye was solubilized by Tris solution and the absorbance was measured at 540 nm in a microplate reader (Bio-Rad, Model 680 Microplate Reader, USA). The results were presented in

the percentages of cell growth (% G) which were calculated according to the following equation:

$$\% \text{ Cell Growth (\%G)} = \frac{T_{\text{treat}} - T_0}{T_{\text{control}} - T_0} \times 100$$

where T_{treat} indicated the OD₅₄₀ of the treated cells after 24 h treatment, T_{control} indicated the OD₅₄₀ of the control and T_0 indicated the OD₅₄₀ of the treated cells at initial.

Results and discussion

Physical properties and FOS content of Job's tears extract

The lyophilized Job's tears extract was brown-dark brown powder with percentage yield of crude extract of $2.83 \pm 0.69\%$. HPLC chromatogram of standard FOS exhibited the retention time of 1-kestose (GF₂), nystose (GF₃) and 1-β-D-fructofuranosylnystose (GF₄) at 5.96, 6.53 and 7.18 min, respectively (data not shown). According to the standard GF₂, GF₃ and GF₄, the Job's tears extract showed the FOS peak at retention time of 5.50, 6.21 and 7.17 min, respectively (Fig. 1). The FOS contents of the extracted were calculated by comparing with the calibration curve of standard FOS. Total FOS content (GF₂+GF₃+GF₄) of Job's tears extract was $24.98 \pm 7.48\%$ (g/100 g crude extract). The

percentage yield of crude extract and total FOS were present as mean±SE.

Bioactivities of job's tears extract

The antioxidant activities by DPPH free radical scavenging, lipid peroxidation inhibition and metal ion chelating activity of standard FOS and FOS-containing Job's tears extract were investigated. The standard FOS (GF₂, GF₃ and GF₄) demonstrated very low antioxidant activity as shown in Table 1. The antioxidation by free radical scavenging and lipid peroxidation inhibition activities was observed from standard GF₂, GF₃ and GF₄. The metal ion chelating activity was observed only from standard GF₂ and GF₄. The FOS-containing Job's tears extract exhibited antioxidant activity by free radical scavenging and lipid peroxidation inhibition of 0.97 and 0.01 fold of standard vitamin C and E respectively, while the metal ion chelating activity was not observed. The antioxidant activity of the Job's tears extract was similar to several previous studies (Kwak et al. 2004; Tseng et al. 2006; Lakkham et al. 2009). In this study, the DPPH radical scavenging activity of Job's tears water extract (SC₅₀ 0.32±0.19 mg/ml) was higher than methanolic extract (SC₅₀ 4.92±0.06 mg/ml) in the previous study (Tseng et al. 2006). This antioxidant capacity might be due to 6 phenolic compounds found in Job's tears seed which were coniferyl alcohol, syringic acid, ferulic acid, syringaresinol, 4-ketopinoresinol and mayuenolide (Kuo et al. 2001). Furthermore, the free radical scavenging and lipid peroxidation inhibition activities of the FOS-containing extract over the standard FOS might be due to the thermal treatment during the extraction procedure. The partial thermal degradation of FOS to fructose or other compounds like caramelization products could induce the formation of new antioxidant (Tsai et al. 2004) such heterocyclic compounds including furans (HMF). Mesa et al. (2008) also suggested that FOS caramels with molecular masses lower or equal to 10 kDa is an important source of antioxidants that are able to scavenge peroxy radicals and to prevent in vivo LDL oxidation. Cytotoxicity of standard FOS and FOS-containing Job's tears extract on normal human skin fibroblast was also determined and demonstrated in Fig. 2. All standard FOS and FOS-containing Job's tears extract at the range of 0.01–1.00 mg/ml concentration exhibited very low or no cytotoxic effect on the treated cells with % G was over 86%.

Conclusions

FOS-containing *Coix lachryma-jobi* Linn. (Job's tears) extract demonstrated antioxidant property by DPPH free radical scavenging and lipid peroxidation activities of 0.97 and 0.01 fold of standard vitamin C and vitamin E,

respectively. FOS-containing Job's tears extract showed no cytotoxic effect on normal human skin fibroblast. These results have confirmed not only the source of FOS from Job's tears but also the application as a multifunctional food supplement and cosmetic ingredient.

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