

Note

Limonoids in Seeds of Iyo Tangor (*Citrus iyo hort. ex Tanaka*)

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Iyo tangor (*Citrus iyo hort. ex Tanaka*) seeds contained limonin, nomilin, obacunone and deacetylnomilin, in order of decreasing concentration. They also contained the 17- β -D-glucopyranosides of nomilin, obacunone, limonin, deacetylnomilin and nomilinic acid. Total limonoid aglycone concentration in the seeds was 873 mg per 100 g on a dry weight basis and total limonoid glucoside concentration was 446 mg. The composition and relative concentration of each limonoid aglycone and glucoside in Iyo tangor seeds were very similar to those of other citrus species distributed widely in western Japan such as Valencia orange (*C. sinensis* Osbeck), Sanbokan (*C. sulcata hort. ex Tanaka*) and Hyuganatsu (*C. tamurana hort. ex Tanaka*). These data reveal that Iyo tangor taxonomically belongs to the same group as those citrus species.

Keywords: Iyo tangor, Iyo-mikan, Iyokan, *Citrus iyo hort. ex Tanaka*, limonoids, limonoid glucosides, HPLC, citrus taxonomy

Limonoids are a group of chemically related triterpenoid derivatives found in the Rutaceae and Meliaceae families. Among the limonoid compounds isolated from citrus and its hybrids, limonin is the predominant limonoid aglycone in fruit tissues and seeds (Hasegawa *et al.*, 1980). The limonoid aglycones such as limonin and nomilin cause bitterness in a variety of citrus fruits and lower the economic value of their juices. Citrus limonoids, however, have been shown to have biological activities. They induce increased activity of a detoxifying enzyme, glutathione *S*-transferase, and inhibit the formation of tumors in laboratory animals (Lam & Hasegawa, 1989; Lam *et al.*, 1989; Miller *et al.*, 1989).

Limonoids are present in mature citrus fruit tissue and seeds as both aglycones and glucoside derivatives (Hasegawa *et al.*, 1989). Limonin 17- β -D-glucopyranoside has also been shown to possess anticancer activity in hamsters (Miller *et al.*, 1992). Limonoid glucosides are practically tasteless and soluble in water, making them ideal natural food additives for cancer prevention, and markedly increasing the demand for them in recent years. Citrus seeds have been shown to contain a high concentration of limonoid aglycones and glucosides (Hasegawa *et al.*, 1980; Ozaki *et al.*, 1991; Ohta *et al.*, 1992, 1995; Ohta & Hasegawa, 1995). We are continuously searching for new sources of limonoids.

The taxonomic characterization of citrus species and their relatives is also of interest. Limonoids are excellent taxonomic markers because they are specific for the Rutaceae and Meliaceae plants. Since certain limonoid biosynthetic pathways are unique to a specific species, the identity of these compounds can be easily determined (Ozaki *et al.*, 1991; Ohta *et al.*, 1992; Berhow *et al.*, 1994).

Iyo tangor (*Citrus iyo hort. ex Tanaka*) is a very popular citrus due to its unique sweet flavor, and it occupies the second position in citrus fruit and juice production in Japan. This means that the residue of Iyo tangor fruit juice is a potentially significant resource from which to extract and recover the limonoids. The aims of this study were to examine the limonoids in seeds of Iyo tangor, and to discuss its taxonomic group based on limonoid levels.

Materials and Methods

Materials Iyo tangor (*Citrus iyo hort. ex Tanaka*) was collected at the beginning of November 1996, at the Fruit Tree Research Station of Saga Prefecture, Japan. The seeds were ground with a Retsch mill (Brinkmann, Westbury, NY) after drying at 60°C for 3 days.

Chemicals and apparatus A preparative high-performance liquid chromatographic (HPLC) column, C₁₈ reversed phase, partisol ODS-3, 2.2×25 cm, particle size 10 μ m, and an analytical HPLC column, 4.6×250 mm, particle size 5 μ m, were obtained from Analtech (Newark, DE). Hesperidinase and naringinase were purchased from Sigma (St. Louis, MO). Sep-Pak silica cartridges were obtained from Millipore (Tokyo). DEAE-Sephacel and XAD-2 resin (20–60 mesh) were also purchased from Sigma. NMR spectra were recorded with a JEOL EX400 at 400 MHz for ¹H and 100 MHz for ¹³C. The limonoid aglycones limonin, nomilin, deacetylnomilin and obacunone, and the 17- β -D-glucopyranosides of limonin, deacetylnomilin, nomilinic acid and obacunone were used as standards in this study. These were isolated from various citrus seeds and characterized by NMR spectrometry at the Western Regional Research Center, USDA, ARS, Albany, California, USA.

Extraction Limonoid extraction was performed by the method described previously (Ohta *et al.*, 1995). An aliquot of

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100 g of ground seed meal was placed in a Soxhlet extractor, washed thoroughly with hexane to remove oily materials, extracted with acetone and subsequently extracted with methanol. The acetone extract contained most of the limonoid aglycones, and the methanol extract contained the limonoid glucosides and the residue of aglycones. The latter fraction was evaporated to dryness, and the residue was extracted with a mixture of dichloromethane and water (1:1). The water fraction contained limonoid glucosides and the dichloromethane fraction contained limonoid aglycones.

Isolation and identification of limonoid glucosides The limonoid glucoside fraction (water fraction), after adjusting its pH to 6.5 with 1 M NaOH, was transferred to the top of a column (2.5×25 cm) packed with DEAE-Sephacel and eluted with a linear gradient system with increasing NaCl content in water. The glucoside fraction was then desalted through a Dowex 50 column (H⁺ form, 1.5×20 cm), and refractionated on an XAD-2 column (2.5×75 cm). The column was eluted with methanol (Ohta *et al.*, 1992). After the solvent was evaporated, the residue was dissolved in water and fractionated on a preparative HPLC column. The column was eluted at 6 ml/min with a linear gradient starting with 15% and ending with 55% methanol over 75 min. Isolates containing each limonoid glucoside were characterized by NMR and HPLC analyses.

Quantitative analyses of limonoid aglycones and glucosides

The acetone and dichloromethane fractions were combined and evaporated to identify and analyze the limonoid aglycones. The dried material was resuspended in methanol and a portion (20 μl) was injected into a C₁₈ reversed phase column, the column being eluted isocratically with acetonitrile-methanol-water (10:41:49). The limonoid aglycones were separated and identified by monitoring the UV absorption at 210 nm as described previously (Ozaki *et al.*, 1991; Ohta *et al.*, 1995).

The limonoid glucoside content was also determined by HPLC methods as described previously (Ozaki *et al.*, 1991; Ohta *et al.*, 1995). A portion of the water extract was treated with hesperidinase and naringinase in a 0.1 M sodium formate buffer solution at pH 3.8 for 20 h at room temperature. This treatment was necessary to obtain good peak resolution by cleaving the sugars from interfering flavonoids. The sample was loaded on a Sep-Pak cartridge, washed with water and eluted with methanol. This methanol fraction was evaporated in a test tube, then dis-

solved in 250 μl of 15% acetonitrile containing 3 mM phosphoric acid. Duplicate 100 μl injections were made into a C₁₈ reversed phase-HPLC column, the column being eluted at 1 ml/min with a linear gradient system, starting with 15% acetonitrile in 3 mM phosphoric acid and ending with 26% acetonitrile over 33 min. The elution was monitored by UV absorption at 210 nm and a standard curve was run of each limonoid glucoside; its peak area quantified each compound. All treatments were performed in duplicate and average values are shown.

Results and Discussion

Table 1 shows the limonoid aglycone and limonoid glucoside content in the seeds of Iyo tangor. HPLC analysis showed the presence of four limonoid aglycones (Fig. 1a): limonin, nomilin, obacunone and deacetylnomilin in order of decreasing concentration. Like many other citrus (Ozaki *et al.*, 1991; Ohta *et al.*, 1995; Ohta & Hasegawa, 1995), limonin was the predominant limonoid in the seed of Iyo tangor. The total limonoid aglycone content was 873 mg per 100 g on a dry weight basis of seed. Five limonoid glucosides from the seeds of Iyo tangor were isolated by preparative HPLC and characterized by NMR analysis. They were the 17-β-D-glucopyranosides of nomilin, obacunone, limo-

Table 1. Limonoid aglycones and glucosides in seeds of Iyo tangor (*Citrus iyo* hort. ex Tanaka)^{a)}.

Limonoids	T _R (min)	Content (mg/100 g dry seed)
Aglycone of		
Limonin	15.1	457
Deacetylnomilin	18.8	72
Nomilin	26.3	253
Obacunone	43.2	91
Total		873
Glucoside of		
Limonin	16.2	53
Deacetylnomilin	23.3	48
Nomilin	29.6	224
Nomilinic acid	30.5	34
Obacunone	33.8	87
Total		446

^{a)} See text for analytical conditions.

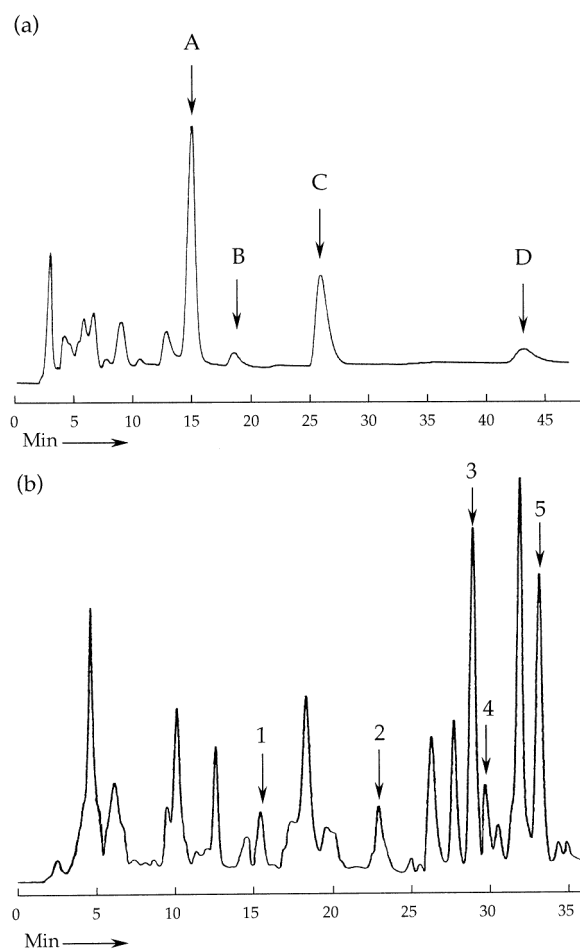


Fig. 1. High-performance liquid chromatograms of acetone and dichloromethane extracts (a), and water extract (b) from Iyo tangor seeds. Peaks (a) limonin (A), deacetylnomilin (B), nomilin (C) and obacunone (D). (b) 17-β-D-glucopyranosides of limonin (1), deacetylnomilin (2), nomilin (3), nomilinic acid (4) and obacunone (5). See text for the HPLC conditions.

nin, deacetylnomilin and nomilinic acid. Quantitative analysis by HPLC showed the seeds contained a relatively high concentration of the nomilin glucoside (Fig. 1b); 50% of the total glucosides. Total limonoid glucoside concentration was 446 mg.

In citrus taxonomic classification according to Tanaka (1969), Iyo tangor is classified into the same group V as other citrus species such as Valencia orange (*C. sinensis* Osbeck), Sanbokan (*C. sulcata* hort. ex Tanaka) and Hyuganatsu (*C. tamurana* hort. ex Tanaka). The composition and relative concentration of each limonoid aglycone and glucoside in Iyo tangor is very similar to those of the common citrus species mentioned above (Ozaki *et al.*, 1991). These support that Iyo tangor belongs to group V. Like others, the ratio of aglycones to glucosides was also approximately 2 in the Iyo tangor seeds. This supports the previous suggestion that the limonoid and limonoid glucosides in seeds are biosynthesized there, independent from the biosynthesis occurring in the fruit tissue (Fong *et al.*, 1991).

The conversion of open D-ring limonoid aglycones to their corresponding glucosides is catalyzed by UDP-D-glucose transferase, which has been isolated from the navel orange tissue (Hasegawa *et al.*, 1997). The glucosidation of aglycones in the seeds begins during the late stage of fruit growth and continues during maturation until the fruit is harvested (Hasegawa *et al.*, 1991). Iyo tangor seeds also contained limonoid glucosides. Normally, fruits of Iyo tangor are harvested in January to February; the fruits used in this study were sampled during the early season (November). Therefore, fruit sampled during the late season would have a higher concentration of limonoid glucosides. This study demonstrated that Iyo tangor seeds are also useful sources of limonoid aglycones and limonoid glucosides.

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