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Relationship Between the Limonoid Content in Different Parts of the Sour Orange (*Citrus aurantium* L.) and the Ligand Activity of a Bile Acid Receptor, TGR5

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Limonoids, a group of highly oxygenated triterpenoids mostly found in the Rutaceae and Meliaceae families, have many biological and physiological activities, such as anti-cancer, anti-microbial, and insecticidal ones. Recent studies suggest that some types of limonoids bind to a bile acid receptor, TGR5 (Takeda G protein–coupled receptor 5), and confer anti-obesity and anti-hyperglycemic effects. TGR5, also known as a G protein–coupled bile acid receptor 1 (GPBAR1), is a vital member of the membrane-bound G protein–coupled receptor (GPCR) family. In this study, we revealed the content of four types of limonoids (limonin, nomilin, obacunone, and limonin glucoside) and TGR5 ligand activity in a sour orange extract. The total concentration of the four limonoids was highest in the extract of ethyl acetate, followed by methanol and hexane in sour orange seeds. On the other hand, a luciferase assay using CHO cells transfected with a TGR5 confirmed that TGR5 ligand activity in the ethyl acetate extract of the seeds was as high as that in 50 µM nomilin, followed by that in the methanol extract of the seeds. The correlation coefficient between the limonoid content and the TGR5 ligand activity showed the highest value ($r = 0.867$) for nomilin, which supported a previous report that the TGR5 ligand activity of nomilin is higher than that of limonin or obacunone. However, the activity of the extract could not be explained by the nomilin content alone because the nomilin concentration in the extract used for the TGR5 assay was 3.9 µM, much lower than that in the control (50 µM nomilin), suggesting unknown compounds with higher TGR5 ligand activities in the seed extracts. In addition, the extract from cotyledons or germinated seeds showed higher TGR5 activity. Taken together, these results indicate that the seeds of citrus, such as the sour orange, may be a source of compounds that prevent obesity and metabolic disorders. In a future study, it will be necessary to comprehensively investigate citrus seed extracts to identify unidentified agonists for the TGR5 receptor.

Key Words: citrus, HPLC, luciferase assay, nomilin, seed.

Introduction

Limonoids represent a group of highly oxygenated triterpenoids that are mostly found in the Rutaceae and Meliaceae families (Hasegawa et al., 1999). Citrus plant tissues and organs such as the stem, leaf, seed, and fruit (peel and juice sac) accumulate limonoids at a significant level as aglycones or glucosides (Gualdani et al., 2016; Herman et al., 1989; Li et al., 2014; Rouseff and Nagy, 1982). The major neutral limonoids of citrus are limonin, nomilin, obacunone, and deacetylnomilin. Thirty-nine limonoid aglycones and 17 limonoid glucosides have been identified in the *Citrus* species and its

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hybrids (Jayaprakasha et al., 2008; Patil et al., 2009). Limonoid aglycones are found predominantly in citrus seeds and peels, while limonoid glucosides are the major components in the juice sac (Hasegawa et al., 1980, 1989; Russo et al., 2016; Vikram et al., 2007). Limonin and nomilin are the two major limonoids in citrus and are responsible for the bitter taste in citrus fruits (Dea et al., 2013; Endo et al., 2002; Hasegawa et al., 1973; Higby, 1938). Many studies have demonstrated the significant biological and physiological impacts of limonoids related to human health, such as anti-cancer (Jacob et al., 2000; Lam et al., 1994; Miller et al., 2004) and anti-microbial (Chowdhury et al., 2003; Govindachari et al., 2000) activities.

TGR5 (Takeda G protein–coupled receptor 5), also known as novel G protein–coupled bile acid receptor 1 (GPBAR1), is a vital member of the membrane-bound G protein–coupled receptor (GPCR) family (Kawamata et al., 2003; Maruyama et al., 2002). TGR5 is expressed in various tissues and organs throughout the body, such as the heart, liver, lung, spleen, kidney, placenta, stomach, gallbladder, intestine, brown adipose tissue, and endocrine glands and is recognized and activated by the binding of bile acids (BAs) as its endogenous ligands (Duboc et al., 2014; Kawamata et al., 2003; Maruyama et al., 2002; Pols et al., 2011). Maruyama et al. (2006) reported that *TGR5*/*GPBAR1*-disrupted mice fed a high-fat diet showed significant fat accumulation with body weight gain as compared with wild-type mice, suggesting a potential role of the gene in energy homeostasis. Notably, Watanabe et al. (2006) showed that the binding of bile acid with TGR5 induced energy expenditure by promoting thyroid hormone activation. Since then, TGR5 has attracted the attention of researchers as an important target for the potential treatment of several metabolic disorders (Tiwari and Maiti, 2009; van Nierop et al., 2017). Recently, TGR5 was found to be involved in glucose uptake into cells and in the remodeling of energy-storing white fat into energy-expending beige fat, suggesting its potential anti-obesity and anti-hyperglycemic effects (Lo et al., 2016; Velazquez-Villegas et al., 2018).

As naturally occurring TGR5 agonists, oleanolic acid from the olive (*Olea europaea* L.) and limonoids, such as the nomilin and obacunone abundant in citrus fruits, have been discovered and investigated in detail (Genet et al., 2010; Horiba et al., 2015; Ono et al., 2011; Sato et al., 2007). These compounds derived from agricultural crops lowered serum glucose and/or insulin levels in mice fed with a high-fat diet (Horiba et al., 2015; Ono et al., 2011; Sato et al., 2007). Based on these reports, such TGR5 activators contained in agricultural crops like olive and citrus could be expected to bind to the TGR5 receptor expressed in the stomach and small intestine and also exert effects such as anti-obesity activity after ingestion by humans. In recent years, obesity has become a major problem in developed countries due to nutritional bias in the diet and lack of exercise. In the future, it will be important to make more use of health-functional ingredients contained in foods and agricultural products that are locally available.

In the context described above, we preliminarily screened the seed extracts of seven citrus plants for TGR5 ligand activity and found the sour orange to have the highest activity. Several previous studies reported the limonoid content in sour orange fruit and seed extracts (Bennett et al., 1991; Dandekar et al., 2008; Matsumoto et al., 2008; Miyake et al., 1992; Ozaki et al., 1991; Vikram et al., 2007). However, no studies have considered the relationship between limonoid content and TGR5 ligand activity in citrus extracts. Therefore, in this study we aimed to clarify the relationship between the content of four major limonoids and TGR5 ligand activity in sour orange fruit by using high-performance liquid chromatography (HPLC) and a luciferase assay system.

Materials and Methods

Plant materials

The sour orange (*Citrus aurantium*) fruit used in this study was introduced from Italy and was collected in February 2017 and April 2019 from the field of the Faculty of Agriculture at Saga University (Fig. 1). The fruit was separated into seeds, juice sac, and peel. Subsequently, a single seed was divided into the seed coat and cotyledon. Sour orange seeds without seed coats were germinated on a glass petri dish containing moistened blotting paper and grown under dark conditions at 25°C in a growth chamber. Ten-day-old germinated seeds were transplanted from the petri dish to moistened potting soil composed of vermiculite and grown in a greenhouse under natural light conditions. In this study, the peel, juice sac, seeds, seed coat, cotyledon, germinated seeds, and 2- and 4-week-old seedlings were used as plant materials.

Preparation of plant extracts

Freeze-dried tissue samples were ground to a fine powder with a multi-bead shocker (Yasui Kikai Corporation., Osaka, Japan). The powder was subjected to

Fig. 1. The sour orange fruit used in this study. Scale bar, 3 cm.

solvent extraction prior to HPLC analysis.

Two grams of freeze-dried powder of the seed, peel, and juice sac (sampled in February 2017) was placed in a Soxhlet extractor (TGK Co., Ltd., Tokyo, Japan) and extracted with 100 mL hexane at 60–70°C for 2 h. Then, it was further extracted with 100 mL of ethyl acetate at the same temperature as above for 4 h. The ethyl acetate extract was transferred to a flask, and 25 mL of methanol was added to the residues. After ultrasonication for 10 min, the extracts were kept at room temperature for 1 h, followed by filtration. The methanol extraction procedure was repeated twice. The solvent was completely evaporated with a rotary evaporator (Tokyo Rikakikai Co., Ltd., Tokyo, Japan) at 100 rpm and 40°C, then diluted with appropriate solvents to be used for HPLC analysis. For the intact seed, cotyledon, seed coat, germinated seed (10 days under dark conditions at 25°C after sowing), and 2- and 4-week-old seedlings (derived from the seeds sampled in April 2019), 1 g of freeze-dried powder was extracted with 10 mL of 70% acetone three times. A total of 30 mL of the extract was evaporated with a rotary evaporator (Tokyo Rikakikai) at 40°C under low pressure to remove the acetone. The residual water was transferred into a tube. After 10 mL of ethyl acetate was added, the tube was vortexed and centrifuged at 500 rpm for 10 min, and then the ethyl acetate layer was removed to a new tube (this step was repeated thrice). Sodium sulfate was added to the ethyl acetate layer, followed by filtration. Then, the ethyl acetate was evaporated to dryness with a rotary evaporator (Tokyo Rikakikai). Each concentrated extract derived from 1 g of freeze-dried powder was dissolved in 1 mL of dimethyl sulfoxide (DMSO) as a stock solution for HPLC analysis and a luciferase assay.

Quantitative and qualitative analysis of limonoids using HPLC

Quantitative and qualitative analysis of limonoids was performed using an HPLC system (JASCO Corp., Tokyo, Japan) with a UV detector (UV-1570; JASCO) and an Inertsil ODS-3 column (3 μm, 4.6 × 100 mm; GL Sciences Inc., Tokyo, Japan) at 40°C. The mobile phases were 10% acetonitrile containing 3 mM phosphoric acid (solvent A) and 50% acetonitrile containing 3 mM phosphoric acid (solvent B). The elution of a binary solvent was conducted in a gradient fashion, starting at 100/0 (A/B), changing to 15/85 (A/B) for 50 min, and maintained at 0/100 (A/B) for 5 min, with a flow rate of 1.2 mL·min^{-1}. Five microliters of each sample was injected, and an absorbance of 210 nm was measured using the UV detector. Quantitative determinations were carried out based on the 25, 50, and 100 ppm standards of limonin (Sigma-Aldrich, St. Louis, MO, USA), nomilin (LKT Laboratories, Inc., St. Paul, MN, USA), obacunone (provided by Dr. Hasegawa of the Fruit and Vegetable Chemistry Laboratory, Agricultural Research Service, United States Department of Agriculture), and limonin glucoside (LKT Laboratories) using a chromatography data system (ChromNAV 1.0; JASCO). For qualitative analysis, four (limonin, nomilin, obacunone, and limonin glucoside; used in quantitative analysis) and five (ichangin, deoxylimonin, deacetylnomilin, limonol, and limonin methoxime; provided by Dr. Hasegawa) standard limonoids were used.

Cell culture

CHO (Chinese hamster ovary) cells were cultured in MEMα (alpha-modified minimum essential medium) (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) supplemented with 1% penicillin/streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS, Gibco®; Thermo Fisher Scientific, Waltham, MA, USA). The cells were cultured in an incubator (MCO-5AC; SANYO Electric Co., Ltd., Osaka, Japan) set at 37°C in a humidified atmosphere of 5% CO_2. Cells were subcultured every three days.

Assay for TGR5 ligand activity

To measure TGR5 ligand activity, a luciferase assay system was used in this study. p3 × FLAG-hTGR5 was constructed by cloning a polymerase chain reaction (PCR) fragment of human TGR5 cDNA derived from IRAK064N14 (RIKEN Gene Bank, Tsukuba, Japan) into a p3×FLAG-CMV vector (Sigma-Aldrich). First, TGR5 genes (p3 × FLAG-hTGR5), CRE (cyclic AMP response element) reporter genes [pGL4.29 (luc2P/CRE/Hygro); Promega Corp., Madison, WI, USA], and polyethylenimine max (Polysciences Inc., Warrington, PA, USA) were placed in a 96-well plate. Second, CHO cells were placed at a density of 6×10^4 cells/well in 96-well plate for transfection. After a 4 h incubation of the CHO cells, the medium was replaced with MEMα containing 10% charcoal-stripped FBS. Samples [stock solutions for each extract, positive controls, and dimethyl sulfoxide (DMSO)] diluted with the medium (1:250) were added at a volume of 100 μL/well 24 h after transfection. Five hours after the samples were added, a solution of Bright-Glo™ (Promega Corp.) was added to each well of the plate. Chenodeoxycholic acid (CDC, a bile acid; FUJIFILM Wako Pure Chemical), limonin (Sigma-Aldrich), and nomilin (LKT Laboratories) were used as controls at a final concentration of 50 μM. TGR5 ligand activity was measured by detecting luciferase activity through luminescence with a luminometer (Luminoskan Ascent FL; Thermo Fisher Scientific). In an assay of the extracts, the stock solution (each sample derived from 1 g of starting materials was dissolved in 1 mL of DMSO) was finally diluted 250 times in the medium as a 1/250 diluted sample (a sample derived from 4 mg of starting materials per mL). The sample solution was further diluted in the medium and used for a dose-dependent assay with 1/1250, 1/2500, and 1/5000 diluted samples.

Statistical analysis

Data were analyzed using a one-way ANOVA and the differences were contrasted using Tukey's multiple comparison test. The relationship between the limonoid content and TGR5 ligand activity was investigated using Pearson's correlation analysis. All statistical analyses were performed at a significance level of $P < 0.05$ using R-3.2.0 (The R Project for Statistical Computing, http://www.R-project.org/, January 7, 2020).

Results

Limonoid content in the sour orange

HPLC was used to determine the limonoid content in the sour orange seed, peel, and juice sac sequentially extracted using three solvents (hexane, ethyl acetate, and methanol). As shown in Figure 2, four limonoids (limonin, nomilin, obacunone, and limonin glucoside) were found in the seed and peel, while only two limonoids (nomilin and limonin glucoside) were found in the juice sac. The total concentration of the four limonoids was highest in the ethyl acetate extract of the seed (1201 $\mu g \cdot g^{-1}$ DW), followed by the methanol extract of the seed (627 $\mu g \cdot g^{-1}$ DW) and the ethyl acetate extract of the peel (230 $\mu g \cdot g^{-1}$ DW) (Table S1; Fig. 2). In seeds, the total limonin, nomilin, obacunone, and limonin glucoside contents were 948, 872, 40, and 161 $\mu g \cdot g^{-1}$ DW, respectively (Table S1; Fig. 2).

Correlation of limonoid content and TGR5 ligand activity

TGR5 ligand activity in the extract from the seed, peel, and juice sac was measured using a luciferase assay system. The ethyl acetate extract of the seed showed a level of TGR5 ligand activity (relative value of luminescence = 3.3) similar to that (relative value of luminescence = 3.1) of 50 μM CDC and nomilin (Fig. 3A). However, the final concentration of the total limonoids (limonin, nomilin, and obacunone) in the extract was 9.9 μM (5.6, 3.9, and 0.4 μM, respectively), which was much lower than the 50 μM nomilin control (Table S1; Fig. 3A). The hexane and methanol extract of the seed and the hexane extract of the peel also showed significantly higher activity than the blank (DMSO). In addition, a dose-dependence effect was observed in the assay in which the concentration of the seed extract was changed from 1/250 to 1/5000 of the first stock solution of the samples (Fig. 3B).

A positive correlation between TGR5 ligand activity and limonoid content was demonstrated because the

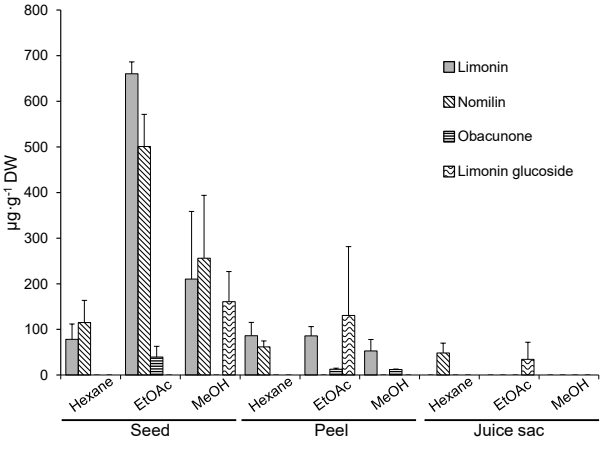

Fig. 2. The concentrations of four limonoids (limonin, nomilin, obacunone, and limonin glucoside) extracted from the seed, peel, and juice sac of the sour orange using a Soxhlet extractor with three different solvents (hexane, ethyl acetate, and methanol) sequentially. All of the tissues were freeze-dried before extraction. Each extract derived from 1 g of the freeze-dried sample was dissolved in 1 mL of DMSO, and then a 1/250 dilution of the first prepared extract was used for the assay. The values are the mean of results from three biological replicates. The error bars indicate the standard deviation (SD).

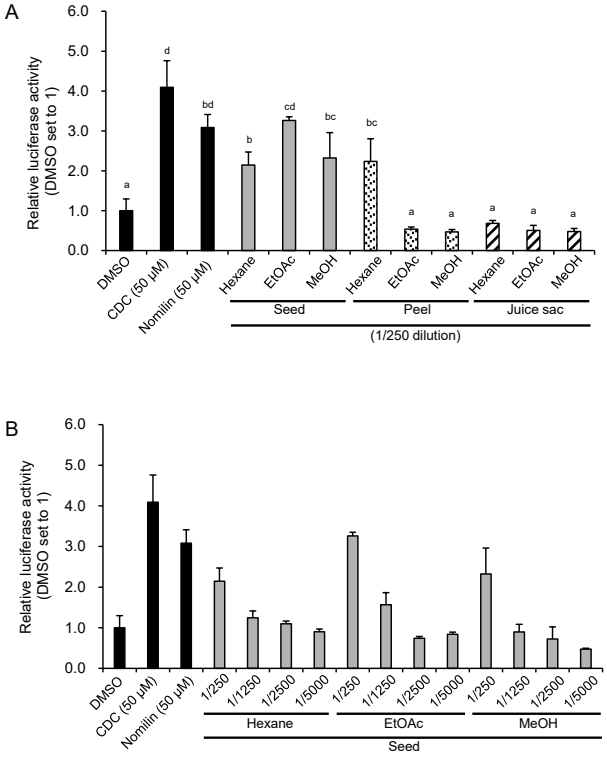

Fig. 3. TGR5 ligand activity assay. (A) Luciferase assay for TGR5 ligand activity in extracts from the seed, peel, and juice sac of the sour orange. CDC (50 μM), nomilin (50 μM), and each extract (using hexane, ethyl acetate, and methanol) of the sour orange seed, peel, and juice sac (1/250 dilution of the first prepared extract) were subjected to the assay. Different letters at the top of each bar indicate significant differences among the samples contrasted by Tukey's multiple comparison test ($P < 0.05$). (B) Luciferase assay for TGR5 ligand activity in different dilutions of the seed extract of sour oranges. Different dilutions (1/250, 1/1250, 1/2500, and 1/5000) of the first prepared extract (using hexane, ethyl acetate, and methanol) of sour orange seeds were subjected to the assay. The values were expressed as a relative luciferase activity (DMSO used as a blank was set to 1). Data are represented as the mean ± SD of five technical replicates.

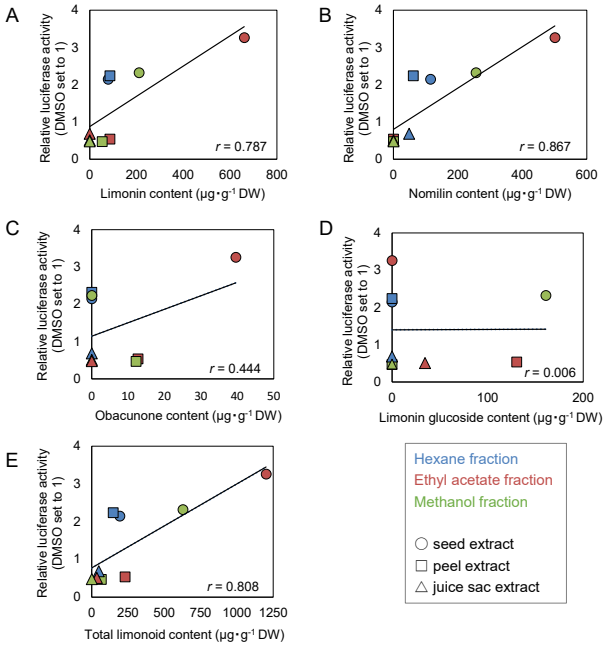

Fig. 4. Scatter plots between the limonoid content (μg·g^{-1} DW) and the values expressed as relative luciferase activity (DMSO was set to 1). (A) Limonin, (B) nomilin, (C) obacunone, (D) limonin glucoside, and (E) total limonoids. Each panel shows the plot of the values. The correlation coefficient was calculated using Pearson's correlation analysis.

order of the three extracts in the TGR5 ligand activity was almost the same as that of the limonoid content detected by HPLC (Fig. 4). Scatter plots of the limonoid content (limonin, nomilin, obacunone, limonin glucoside, and total limonoids) and luciferase activity measured for each extract showed a positive correlation with a high correlation coefficient of 0.867 (Fig. 4B), 0.808 (Fig. 4E), and 0.787 (Fig. 4A) for nomilin, total limonoids, and limonin, respectively.

Limonoid content and TGR5 ligand activity in cotyledons and seed coats

Seeds were investigated in more detail by separating them into the cotyledon (seed without the seed coat) and the seed coat. The intact seed and cotyledon of the sour orange contained two major limonoids, limonin and nomilin, whereas the seed coat contained those limonoids a lower levels than the intact seed and cotyledon (Fig. 5A). The limonin and nomilin contents varied depending on the tissues and showed a significant difference between them. The highest level of limonin was determined in the cotyledon (1090 μg·g^{-1} DW), followed by the intact seed (720 μg·g^{-1} DW) and seed coat (300 μg·g^{-1} DW). The nomilin concentration was significantly higher in the cotyledon (770 μg·g^{-1} DW) than in the intact seed (270 μg·g^{-1} DW) and seed coat (very small amount). The TGR5 ligand activity of the intact seed-, cotyledon-, and seed coat–derived extract was measured using a luciferase assay system (Fig. 5B). The extracts obtained from the intact seed, cotyledon and seed coat showed significantly higher luciferase activity than the blank (DMSO). The cotyledon extract showed the maximum luciferase activity, as compared to the intact seed and seed coat, whereas the intact seed and seed coat extracts showed similar luciferase activities (Fig. 5B).

Limonoid content and TGR5 ligand activity in seedlings at early developmental stages

Limonoid content was also determined via HPLC in the germinated seeds (10 days under dark conditions at 25°C after sowing) and 2- and 4-week-old sour orange seedlings (Fig. 5C). Limonin and nomilin were present in significant amounts in all of the tissues. The limonin content was found to be highest (1460 μg·g^{-1} DW) in the germinated seeds, followed by 2-week-old (710 μg·g^{-1} DW) and 4-week-old (620 μg·g^{-1} DW) seedlings. The nomilin concentrations were similar in the germinated seeds (450 μg·g^{-1} DW) and 2-week-old (530 μg·g^{-1} DW) and 4-week-old (620 μg·g^{-1} DW) seedlings. TGR5 ligand activity was also measured in the extracts of the germinated seeds and the 2- and 4-week-old seedlings using a luciferase assay system. All extracts exhibited significant activity for TGR5 (Fig. 5D). As expected, the germinated seed extract exhibited higher ligand activity, followed by the extract derived from the 2- and 4-week-old seedlings.

TGR5 ligand activity in seven fractions of the ethyl acetate extract of intact seeds

An aliquot of the ethyl acetate extracts of intact seeds were separated into seven fractions via HPLC (Fig. 6A). After drying, each fraction was dissolved in the same volume of DMSO as that injected for fractionation to adjust the concentration of each sample for the TGR5 assay. The result showed that there were higher activities in fractions 6 and 7, but lower or no activities in the other fractions (Fig. 6B). To identify the peaks in the chromatogram in Figure 6A, nine standard limonoids often detected in citrus were analyzed together with an extract of intact seeds and a blank sample (50% MeOH) via HPLC with a C_{18} ODS column at 210 nm. Limonin glucoside was eluted first at a retention time of 13.21 min, followed by ichangin, deoxylimonin, deacetylnomilin, limonol, limonin, nomilin, obacunone, and limonin methoxime at retention times of 24.60, 24.75, 26.81, 27.06, 28.21, 31.21, 34.17, and 34.48, respectively (Fig. 7). Two broad peaks eluted later than 36 min were derived from mobile phases.

Discussion

Limonoid content in the sour orange

We confirmed the distribution of major limonoids (limonin, nomilin, obacunone, and limonin glucoside) in sour orange fruit by extracting these compounds

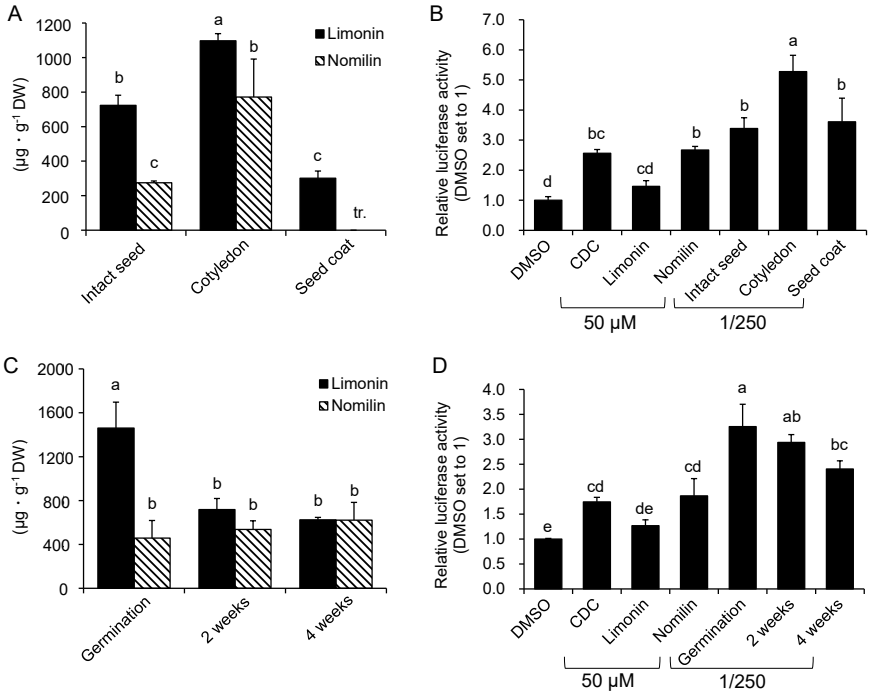

Fig. 5. Limonoid content and TGR5 ligand activity in seeds, germinated seeds, and seedlings. (A) The concentration of limonin and nomilin in ethyl acetate extracts from the intact seed, cotyledon (seed without seed coat), and seed coat of the sour orange; tr. = trace amount. (B) Luciferase assay for TGR5 ligand activity in extracts from the intact seed, cotyledon, and seed coat of the sour orange. (C) The concentration of limonin and nomilin in ethyl acetate extracts from the germinated seed (10 days under dark conditions at 25°C after germination) and 2- and 4-week-old seedlings (germinated seeds were transplanted into pots containing vermiculite in a greenhouse) of the sour orange. (D) Luciferase assay for TGR5 ligand activity in extracts from the germinated seed and 2- and 4-week-old seedlings of the sour orange. CDC (50 μM), limonin (50 μM), nomilin (50 μM), and extracts (1/250 dilution of the first prepared extracts) from the intact seed, cotyledon, seed coat, germinated seed, and 2- and 4-week-old seedlings of the sour orange were subjected to the assay. The ethyl acetate extracts were prepared by extraction with ethyl acetate from the aqueous layer after concentration of 70% acetone extracts from each tissue. The values (B, D) are expressed as a relative luciferase activity (DMSO was set to 1). Data are represented as the mean ± SD of three biological (A, C) and five technical (B, D) replicates. Different letters on each bar indicate significant differences among the samples contrasted by Tukey's multiple comparison test ($P < 0.05$).

sequentially with three different solvents. Overall, the four limonoids accumulated mainly in the seeds (2021 μg·g^{-1} DW), followed in order by the peel (443 μg·g^{-1} DW) and juice sac (83 μg·g^{-1} DW) (Table S1; Fig. 2). These results were consistent with those of previous reports showing that the distribution of limonoids in citrus fruit is tissue-specific, as the concentration was different in each tissue, and that the seed is the source of abundant limonoids in fruit (Hasegawa et al., 1980; Miyake et al., 1992; Ohta and Hasegawa, 1995; Rouseff and Nagy, 1982; Sun et al., 2005; Wang et al., 2016).

In the sour orange seeds used in this study, the concentrations of limonin, nomilin, obacunone, and limonin glucoside were 948, 872, 40, and 161 μg·g^{-1} DW, respectively (Table S1; Fig. 2). Rouseff and Nagy (1982) reported the concentrations of limonin, nomilin, and obacunone to be 1256, 242, and 104 μg·g^{-1} DW, respectively, in the common sour orange. Miyake et al. (1992) reported a concentration of obacunone (32 μg·g^{-1} DW) in sour orange seeds that was similar to that in our study, although those of limonin, nomilin, and limonin glucoside (2470, 178, and 610 μg·g^{-1} DW, respectively) were different from those in our study. On the other hand, Vikram et al. (2007) detected no nomilin in a sour orange seed extract. Considering reports that the limonoid content depends on the maturation stage or cultivar (Matsumoto et al., 2008; Sun et al., 2005; Wang et al., 2016), inconsistencies in the reported limonoid concentration seem to be due to differences in the season of sampling, cultivar/strain of the sour orange, or method of sample preparation.

Correlation of limonoid content and TGR5 ligand activity

The ligand activity of TGR5 was significantly higher in the extract from seeds, which contained a higher concentration of limonoids, than in extracts from other organs, except for the hexane extract from the peels (Fig. 3A). All of the samples that showed a value significantly higher than the blank (DMSO) contained nomilin to some extent (Figs. 2 and 3A). The effect of dose dependence on ligand activity indicated that the extract definitely contained compounds that highly activated TGR5 (Fig. 3B). It is noteworthy that the correlation coefficient between TGR5 ligand activity and

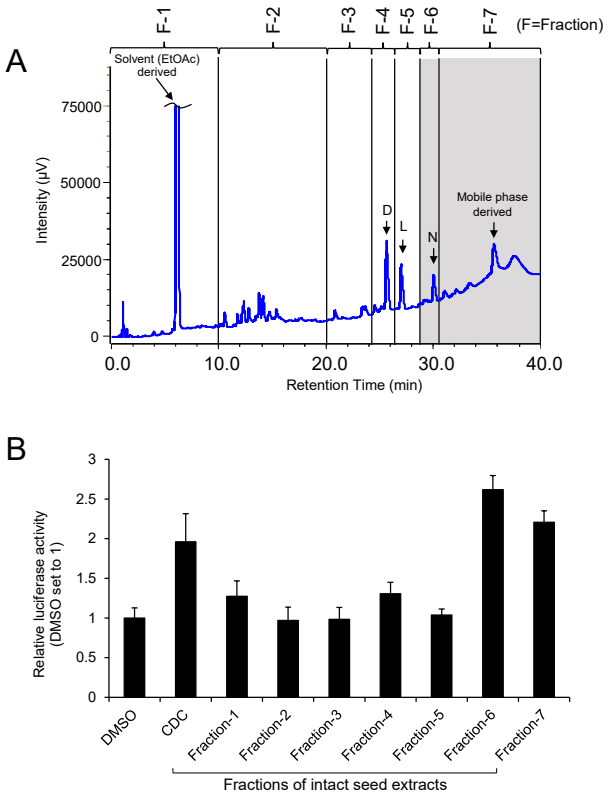

Fig. 6. TGR5 ligand activity in fractions of the ethyl acetate extract of the sour orange seed. (A) Representative HPLC profile of the ethyl acetate extract of the sour orange seed at 210 nm. The ethyl acetate extract was prepared in the same way as shown in Figure 5. LG, limonin glucoside; L, limonin; D, deacetylnomilin; N, nomilin. Gray boxes show the fractions with higher ligand activity for TGR5. (B) TGR5 ligand activity in the fractions of seed extract separated via high performance liquid chromatography as shown in (A). CDC (50 μM) was used as a positive control. The values in (B) are expressed as a relative luciferase activity (DMSO was set to 1). Data are represented as the mean ± SD of five technical replicates.

nomilin content was highest among the four limonoids analyzed in this study (Fig. 4B). On the other hand, there was no correlation ($r = 0.006$) between TGR5 ligand activity and limonin glucoside content, suggesting that limonin glucoside did not contribute to TGR5 receptor activation (Fig. 4D). Our results revealed that nomilin in the sour orange extracts effectively activated the TGR5 receptor. These results are consistent with the report of Ono et al. (2011), in which the TGR5 ligand activity of nomilin was higher than that of limonin or obacunone. However, the ethyl acetate extract of the seeds showed activity equivalent to that of a positive control of 50 μM nomilin despite the fact that the final limonoid concentration (limonin: 5.6 μM; nomilin: 3.9 μM; obacunone: 0.4 μM) derived from the ethyl acetate extract in the assay was much lower than 50 μM (Figs. 2 and 3). This result suggests that some compounds may have higher TGR5 ligand activity than nomilin, which was expected to be a major compound for activating TGR5 in the extracts.

Limonoid content and TGR5 ligand activity in seeds and seedlings

Because higher TGR5 ligand activity was found in the seed extract, we investigated both the limonoid content and the TGR5 ligand activity in more detail in the seeds and seedlings. It was found that limonin and nomilin accumulated more in the cotyledons than in the seed coats (Fig. 5A). As expected, the TGR5 ligand activity of the cotyledons was higher than that of the seed coats. However, the TGR5 ligand activity of the seed coats showed a level similar to that of the intact seeds, suggesting that some seed coat compounds other than nomilin activate the TGR5 receptor because only a trace amount of nomilin was detected in the seed coats (Fig. 5).

Germinated seeds (10 days under dark conditions after sowing) had a higher limonin content (1460 $\mu g \cdot g^{-1}$ DW) than cotyledons (seeds without seed coats) (1097 $\mu g \cdot g^{-1}$ DW), but a lower nomilin content (459 $\mu g \cdot g^{-1}$ DW) than cotyledons (771 $\mu g \cdot g^{-1}$ DW) (Fig. 5C). The total limonoid content (limonin + nomilin) before (1868 $\mu g \cdot g^{-1}$ DW in cotyledon) and after (1919 $\mu g \cdot g^{-1}$ DW in the germinated seed) germination was not greatly changed. This could be due to the metabolism of nomilin to limonin through ichangin in the sour orange biosynthetic pathway (Miyake et al., 1992). In contrast, Ariza et al. (2015) reported that a higher amount of limonoids, such as limonin, nomilin, and ichangin, was present in germinating seeds than in dormant seeds of *Citrus aurantium*. After germination (two and four weeks after transplanting to pots in a greenhouse), the limonin content decreased gradually, whereas the nomilin content did not vary significantly from germination to four weeks after transplanting (Fig. 5C). As expected, the TGR5 ligand activity was higher in the germination stage, followed by that in 2- and 4-week-old seedlings (Fig. 5D). The differences in limonin content reflect those in TGR5 activity because the nomilin content was almost the same among the three samples.

In the seeds of citruses, including the sour orange, a large amount of limonoid glucosides is accumulated with concentrations of 0.31 to 0.87% of the dry weight (Miyake et al., 1992; Ozaki et al., 1991), whereas young seedlings contain practically no limonoid glucosides, suggesting the presence of β-glucosidase activity in seeds (Ronneberg et al., 1995). On the other hand, nomilin is synthesized in stem and root tissues, not in leaves, fruits, or seeds, in *Citrus limon* (Hasegawa et al., 1986). In light of these facts, limonoid aglycones, such as limonin and nomilin, in germinated seeds and seedlings may be derived partly from these glucosides and partly from de novo synthesis in the stem and roots. Because the concentration of nomilin did not greatly change until at least four weeks after germination, the total amount of nomilin per plant increases during the growth of the seedlings. Although the enzymatic libera-

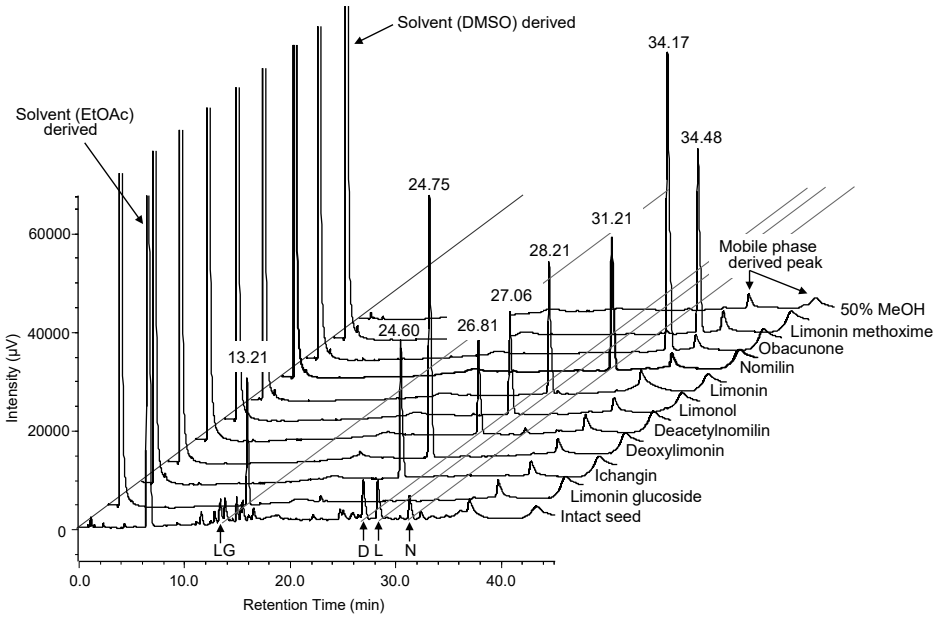

Fig. 7. Chromatograms of nine standard limonoids and the ethyl acetate extract of intact seeds separated via high performance liquid chromatography with a C_{18} ODS column detected at 210 nm. The ethyl acetate extract was prepared in the same way as shown in Figure 5. Methanol (MeOH, 50%) was used as a blank. Nine standard limonoids (limonin glucoside, ichangin, deoxylimonin, deacetylnomilin, limonol, limonin, nomilin, obacunone, and limonin methoxime) were subjected to analysis. The value on each peak represents the retention time (min) of each of the nine compounds. LG, limonin glucoside; D, deacetylnomilin; L, limonin; N, nomilin.

tion of limonin and nomilin from their glucosides could be a problem in the juice industry because of their bitterness, it may contribute to the creation of a source of bioactive limonoid aglycones.

Substances with TGR5 ligand activity in citrus seed extracts

Our study suggested that the citrus extracts may also contain compound(s) other than nomilin with TGR5 ligand activity. To investigate these unidentified active substances, we separated the ethyl acetate extracts of the seeds into seven fractions via HPLC (Fig. 6A), since the aqueous phase after extraction with ethyl acetate showed no activity in the TGR5 ligand assay (data not shown). This result revealed that the two fractions contained higher activity, as shown in Figure 6B (F-6 and F-7, shaded gray). As expected, fraction 6 (F-6), which contained nomilin (N), showed high activity similar to that of 50 μM CDC. In addition, fraction 7 (F-7) showed activity at the same level as 50 μM CDC (positive control). To search for known limonoids that could be eluted around F-7, nine standard limonoids were separated via HPLC together with the ethyl acetate extract and methanol as a blank. According to the chromatogram shown in Figure 7, limonin glucoside (LG), ichangin and deoxylimonin, deacetylnomilin (D) and limonol, and limonin (L) should be contained in F-2, F-3, F-4, and F-5, respectively, which had no, or much less, activity as compared to the positive control. On the other hand, obacunone and limonin methoxime should be contained in F-7, but those limonoids were eluted after nomilin, where there were only small or broad peaks detected at 210 nm for the seed extracts (Figs. 6A and 7).

Bennett and Hasegawa (1980) and Miyake et al. (1992) reported that sour orange seeds contained ichangin, isolimonic acid, and several kinds of limonoid glucosides, in addition to limonoid aglycones including the four examined in this study. TGR5 binds certain limonoid aglycones, such as nomilin and obacunone, but does not seem to bind their glucosides effectively. However, water-soluble limonoid glucosides could be utilized as TGR5 agonists after the liberation of their aglycones by hydrolysis in humans, considering a report that limonin glucoside is absorbed and metabolized to produce limonin in humans (Manners et al., 2003). Recently, Sasaki et al. (2017) constructed an hTGR5–nomilin binding model and demonstrated that four hydrophilic hydrogen-bonding interactions occurred between four oxygen atoms of nomilin and three amino acid residues of hTGR5. The binding mechanism of nomilin to TGR5 is similar to that of obacunone, but different from that of taurolithocholic acid (TLCA), a kind of bile acid (Sasaki et al., 2017). Based on their results and ours, novel agonists with higher activity for TGR5 could be found in citrus fruits, such as the sour orange, by considering compounds with some heteroatoms which could form hydrogen bonds with specific amino acid residues of TGR5, like nomilin and obacunone.

Conclusion

In this study, we revealed the content of four kinds of limonoids and theTGR5 ligand activity in extracts obtained via sequential extraction using three different solvents from the seed, peel, and juice sac of the sour orange. In addition, we confirmed a high correlation between nomilin content and TGR5 ligand activity, although the activity could not be explained by the nomilin content in the seed extract alone. Therefore, sour orange seeds may contain components other than nomilin with TGR5 ligand activity. The seed or germinated seed extracts of citruses such as the sour orange, may be a source of compounds that prevent obesity and metabolic disorders. To the best of our knowledge, this is the first study to report on the TGR5 activity in crude extracts from citrus tissues. In a future study, it will be necessary to comprehensively investigate citrus seed extracts for unidentified agonists for the TGR5 receptor.

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