Bioscience, Biotechnology, and Biochemistry, 2021, Vol. 85, No. 7, 1594-1601

doi: 10.1093/bbb/zbab077
Advance access publication date: 4 May 2021
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Triterpenes induced by young apple fruits in response to herbivore attack

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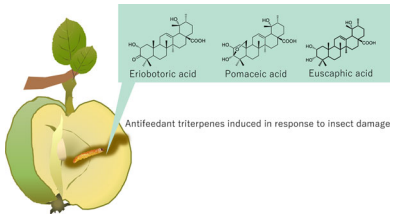
ABSTRACT

Apples *Malus domestica*, known as a rich source of triterpene acids, induced more variety and quantity of triterpene acids in response to herbivory or mechanical damage. There were 3 major induced compounds: pomaceic acid and euscaphic acid, both of which are known apple triterpene acids, and 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid (named eriobotoric acid), which was first identified in apples. In this study, the 3 compounds' induction curves after damage, varietal differences in induction amounts, and physiological roles against pest insects were further investigated. Eriobotoric acid showed clear antifeedant activity against lepidopteran insect *Spodoptera litura* but not against apple pests.

Graphical Abstract



Three apple triterpenes induced by insect feeding showed antifeedant activity against some lepidopteran insects but not against apple fruit feeders such as *Carposina sasakii*.

Keywords: triterpenes, annucoic acid, pomaceic acid, euscaphic acid, *Carposina sasakii*

Triterpenes are a diverse group of plant products. From components of plant surface waxes to signaling molecules, a wide range of compounds have been identified and summarized in several reviews (Thimmappa *et al.* 2014; Hill and Connolly 2018; Cárdenas, Almeida and Bak 2019). They play roles in plant defense and development, suggesting various bioactivities potentially applicable to food and pharmaceutical products. Various pharmacological activities have been reported, (Dzubak *et al.* 2006) such as anticancer, anti-inflammatory, antiulcerogenic, antimicrobial, antiplasmodial, anticariogenic,

Received: 17 February 2021; **Accepted:** 23 April 2021
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and antiviral. Although limited compared to such pharmacological studies, there have been several reports about antipest activities (González-Coloma *et al*. 2011). These anti-insect properties include the growth inhibition and chemosterilant activity of β-amyrin palmitate (Shankaranarayana, Ayyar and Rao 1980), feeding deterrent activity of oleanolic acid derivatives against *Leptinotarsa decemlineata* (Kubo *et al*. 1990), and insecticidal activity of nortriterpene quinone methides against the codling moth (*Cydia pomonella*) (Avilla *et al*. 2000). Considering the number of triterpenes identified so far, there are expected to be more unidentified plant defense substances.

Apples are among the most consumed fruits in the world, and there have been several studies on apple triterpenes (Cargnin and Gnoatto 2017; Butkevičiūtė *et al*. 2018; Nile *et al*. 2019). Triterpene acids are present particularly in the cuticular wax of peels, represented by ursolic and oleanolic acids (He and Liu 2007; Jäger *et al*. 2009). Apple triterpenes have attracted attention due to the beneficial uses of apple by-products (pomace, peel, fruit pulp) for health promotion. Thus, most research has focused on mature apple fruits, and few analytical data are available on young fruits. The defensive properties of apple triterpenes against pests and diseases remain unknown. Therefore, in this study, we analyze apple triterpenes, especially those induced in young fruits in response to herbivory infection and mechanical damage. The identified triterpenes are further explored for any defensive activities against pest/nonpest lepidopteran insects by feeding assays.

Materials and methods

Chemicals

Methanol (MeOH), hexane, ethyl acetate (EtOAc), acetonitrile (MeCN), and distilled water were obtained from Nacalai Tesque (Kyoto, Japan). The AcOH was obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). The ursolic acid (purity = 90%) and glycyrrhetinic acid (purity = 97%) were from Tokyo Chemical Industry (Tokyo, Japan).

Plant materials

Apple fruits ("Fuji") were cultivated without pesticides and harvested from the Apple Research Institute, Aomori Prefectural Industrial Technology Research Center (Aomori, Japan), in July 2017 and 2018. Ten apple cultivars ("Kotoku," "Jonathan," "Shinano Gold," "Ambitious," "Sensyu," "Jonagold," "Miki Life," "Indo," "Orin," and "Toki") were obtained from the same orchard, harvested in July 2017. The fruits were stored at 4 °C. Loquat *Eriobotrya japonica* fruits ("Mogi") were commercially available and harvested in May 2020; they were stored at 4 °C.

Insects

Spodoptera litura and *Helicoverpa armigera*, both widely known as general pests fed on various vegetables, and *Adoxophyes orana fasciata* larvae, known as a pest of apple leaves, were reared on an artificial diet (Insecta-LFS; Nihon Nosan Kogyo Ltd.). *Grapholita molesta* larvae (apple fruit pest), were reared on an artificial diet (SilkMate 2S, Nihon Nosan Kogyo Ltd.). *Carposina sasakii* larvae, known as the important pest of apple fruit in Japan, were reared on the young apples obtained from the Apple Research Institute, Aomori Prefectural Industrial Technology Research Center. Newly emerged female and male moths were kept in a plastic container, in which females laid their eggs on paraffin wax paper or apple fruits. They were reared at 24 ± 1 °C, 50 ± 10% RH, and 16L/8D photoperiod.

Quantitative time course analysis of triterpenes

Apple fruits "Fuji" were mechanically damaged by drilling a rough hole into them with a twisted stainless wire, imitating larval infestation. The damaged and peripheral parts of the fruits were cut out with a knife at predetermined times, that is, 10, 28, 34, 48, 72, 96, 127, and 148 h after the mechanical damage.

Fruit extraction and preparation

Pieces of fruit were crushed with 1.0 mL/g flesh weight (f.w.) MeOH with metal beads (ø 5 mm) using the Beads Crusher μT-12 (TAITEC Co., Saitama, Japan). The MeOH extracts were centrifugated (7,400 g, 10 min; 13,000 g, 5 min) and filtered out using a syringe filter (ADVANTEC®, DISMIC®-13HP, Toyo Roshi Kaisha Ltd., Tokyo, Japan) to obtain supernatant for the liquid chromatography–mass spectrometry (LCMS) analyses.

Purification of triterpenoids from apple fruits

Damaged young apple fruits (flesh of fruits, 320 g), previously used as feed for the lab colony of *C. sasakii*, were diced and crushed with MeOH using a food processor (SKF-A100, TIGER, Osaka, Japan). The MeOH extract was filtered through gauze and centrifugated at 13,000 g for 10 min. After evaporation under a vacuum, the dried MeOH extract (23.0 g) was diluted with EtOAc and washed with H_2O. The EtOAc layer was dried with Na_2SO_4 and concentrated to obtain brown residue (596 mg). The residue was subjected to column chromatography on silica gel (Wakogel® C-200, FUJIFILM Wako Pure Chemical Corporation, 15 g) and eluted by 30%-100% (v/v) EtOAc in hexane. Compound **1** was in 30% fraction (108.5 mg), and compound **2** and **3** were in 40% fraction (99.3 mg). These fractions were further purified by high-performance liquid chromatography (HPLC) (Shimadzu LC 10AS) connected to an RP-C18 column (Mightysil RP-18 GP Aqua 250-4.6 i.d., Kanto Chemical Co., Inc., Tokyo, Japan) and the ultraviolet–visible spectroscopy (UV–vis) detector (SPD-M10A, Shimadzu) to monitor the peaks at 210 nm. The operation conditions were as follows: isocratic conditions using 65% aq. MeCN containing 0.1% (v/v) AcOH for 15 min at a flow rate of 1.0 mL/min. After purification, compound **1** (87 mg), compound **2** (15 mg) and compound **3** (4 mg) were obtained. The triterpene mixture for bioassay was prepared from the EtOAc residue as described above. The EtOAc residue was partially purified by normal-phase flash chromatography (Combi Flash Rf+, normal-phase disposable column, 40 g, Teledyne Isco, Inc.), eluting 0%, 80%, and 100% (v/v) EtOAc in hexane. The 80% fraction which contained all the induced triterpenes was used, as triterpene fraction (TPPf), in the feeding assay.

Purification of eriobotoric acid from loquat fruits

Terpenoids from the loquat fruits (flesh of fruits, 820 g) were extracted as described above to obtain brown residue (1.3 g). The residue was fractionated by normal-phase flash chromatography (normal-phase disposable column, 40 g, Teledyne Isco, Inc.), eluting 30%-100% (v/v) EtOAc in hexane at 40 mL/min for 15 min in each concentration. The fraction containing eriobotoric acid (**1a**) was eluted to 30% fraction (142 mg). The fraction was purified by reversed-phase chromatography in an

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ODS column (SepPak, 5 g, Waters), eluting MeCN-H_2O containing 0.1% AcOH. After purification, eriobotoric acid (**1a**, 6 mg) was obtained.

LCMS analysis

Positive and negative electrospray ionization (ESI) mass spectral measurements were carried out with an LCMS-2020A instrument (Shimadzu) combined with an HPLC system (LC-20ADvp pump, CTO-20ACvp column oven, and SCL-20ACvp system controller, Shimadzu). A reversed-phase column (Mightysil RP-18 GP 50 × 2.0 mm i.d., Kanto Chemical Co., Inc.) was eluted (0.2 mL/min) with a solvent gradient of 2%-97% MeCN containing 0.08% AcOH in water containing 0.05% AcOH for 12 min and then with 97% MeCN containing 0.08% AcOH in water containing 0.05% AcOH for 4 min. The column temperature was maintained at 60 °C (CTO-10Avp column oven; Shimadzu).

Compounds **1**-**3** in the apple fruits were quantified by LCMS analysis. These compounds were diluted in MeOH with glycyrrhetinic acid added as the internal standard (10 μg/mL), and calibration curves were created to quantify them. The compounds were identified by comparing their retention time and MS information.

The sequential mass spectrometry (MS^n) analysis to obtain structural information was carried out with an LCMS combined with ion trap and time-of-flight instrument (LCMS-IT-TOF) (Shimadzu) equipped with atmospheric pressure chemical ionization (APCI) negative mode. A reversed-phase column (Mightysil RP-18 GP 50 × 2.0 mm i.d., Kanto Chemical Co., Inc.) was eluted (0.2 mL/min) under the same conditions as the LCMS analysis. The MS system was operated with a probe voltage of 1.77 kV, curved desolvation line (CDL) temperature of 200 °C, block heater temperature of 200 °C, nebulizer gas flow of 1.50 L/min, ion accumulation time of 10 ms, MS range of *m/z* 55-600, MS^2 range of *m/z* 139-600, and collision induced dissociation (CID) parameter of 50% energy and 50% collision gas. The MS data were processed with LCMS solution version 3.4 software (Shimadzu).

Nuclear magnetic resonance

The spectra were measured on an AV-III 400 NMR spectrometer (Bruker, Billerica, USA). All measurements were performed on a solution in fully deuterated methanol (MeOD, Eurisotop, Saclay, France), with tetramethylsilane (TMS) as an internal standard. The resonance frequency for ^1H NMR was 400.23 MHz, and that for ^{13}C NMR was 100.64 MHz.

Consumption and growth assay

The effect of eriobotoric acid (**1**) and pomaceic acid (**2**) on diet consumption and larval growth was assessed using early third-instar larvae (*S. litura*, *A. orana fasciata*, *G. molesta*, and *C. sasakii*). Purified euscaphic acid (**3**) was not tested because it was not enough for this assay. The assays were conducted in petri dishes (4 × 1 cm) in which a larva and agar-based diet were placed. The agar-based diet comprised agar (Agar Powder, Nacalai Tesque, 80 mg), artificial diet (Insecta-LFS, 0.25 g), distilled water (2 mL), and any one of eriobotoric acid (0, 1, and 3 mg/g diet), pomaceic acid (0, 100, and 300 μg/g diet), or the triterpene fraction (TTPf, 9 mg/g diet). MeOH solutions of the triterpenes or triterpene fraction residue were mixed with agar powder and evaporated to dryness. The dried powder was further suspended with distilled water and artificial diet, and heated up in a microwave (RE-SD10, SHARP Co., Osaka, Japan, 500 W) for 10 s. After cooled in refrigerator, it was cut into pieces (1.5 × 1.5 × 0.5 cm) and fed to the larvae at 24 ± 1 °C. Every 24 h, the larval weight and diet consumption weight were recorded.

Statistical analysis

The data obtained for diet consumption and growth were subjected to an analysis of variance (ANOVA), followed by the Tukey–Kramer test.

Ethics approval

No ethics approval is necessary for work with insects.

Results

Identification of triterpenes induced by feeding damage

Compounds **1**, **2**, and **3** were isolated from apple fruits infested by *C. sasakii* larvae using silica gel column chromatography and reversed-phase HPLC. The ^1H-NMR and ^{13}C-NMR spectrum of each compound exhibited signals of an olefinic proton and 7 methyl groups, 6 of which were singlet and 1 doublet, characteristic of the ursene skeleton (Section S2). The 3 compounds were structurally determined (Section S3). Compound **1** was assumed to be 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid upon comparing its spectral data to those reported for a compound isolated from *Geum japonicum* (Xu *et al.* 1996) and *Eriobotrya japonica* (Taniguchi *et al.* 2002) (Figure 1, **1**). Compound **2** was identified as 3β,25-epoxy-2,3α,19α-trihydroxyurs-12-en-28-oic acid (pomaceic acid) upon comparing it to the spectral data reported for a compound isolated from apple pomace (Waldbauer *et al.* 2016) (Figure 1, **2**). According to the report, hydroxy group at C-2 of pomaceic acid is determined to be β-position. Although we got nuclear magnetic resonance (NMR) data identical to their report, we concluded that the data was not enough to determine α or β-position (Section S4). Compound **3** was also identified as 2α,3α,19α-trihydroxyurs-12-en-28-oic acid (euscaphic acid) upon comparing its spectral data to those reported for a compound isolated from *Euscaphis japonica* (Takahashi *et al.* 1974) (Figure 1, **3**).

Structural comparison between compound 1 and 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid reported from loquat

In previous research, compound **1** (tentatively identified in our study as 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid) was not found in apples. Instead, annurcoic acid (1α,19α-dihydroxy-3-oxours-12-en-28-oic acid), which has similar mass spectra with our observed data (Cefarelli *et al.* 2006; D'Abrosca *et al.* 2006), is reported as apple triterpenes. In our study, using the MeOH extract of the loquat fruits, we examined whether compound **1** from the apple extract had an identical spectrum as the 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid reported from the loquat in the NMR analysis and MS^n analysis by LCMS-IT-TOF. Similar retention times and MS/MS fragments were observed at a peak of *m/z* 487 ($[M + H]^+$) in both the apple and loquat extracts, and no differences were observed (Section S5). The corresponding compound (**1a**) from the loquat fruit was purified as described, and the NMR spectra were compared. Here ^1H-NMR and ^{13}C-NMR were perfectly identical, and no differences were confirmed in COSY and NOESY. Therefore, the compound from

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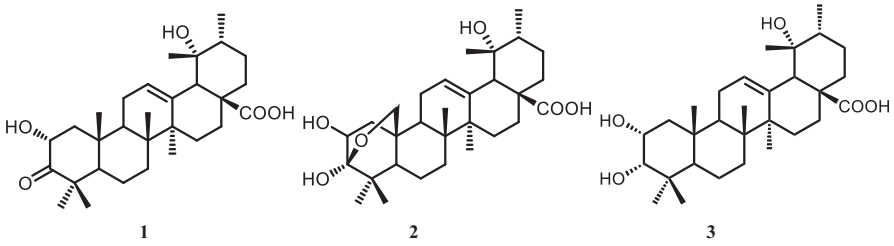

Figure 1. Structure of triterpenes induced by mechanical damage in apple fruits.

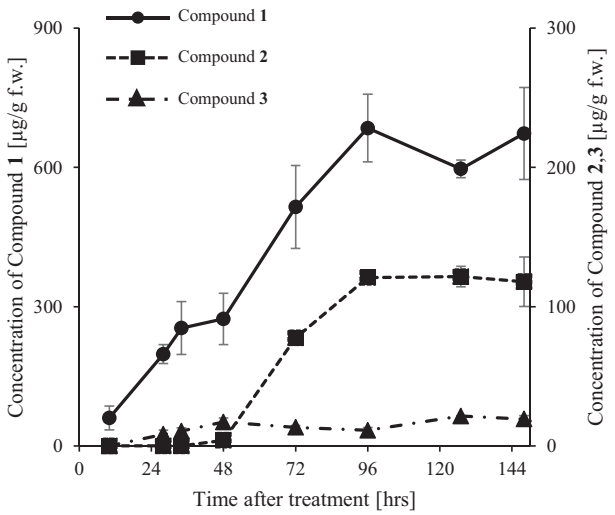

Figure 2. Quantitative time course analysis of compounds **1**-**3** at mechanically damaged area in apple fruit ("Fuji"). Apple fruits were mechanically damaged (drilled into with twisted steel wire) and the compound concentration in fruits were quantified by LCMS analysis. Each value is presented as the mean ± SE; $n = 5$.

the apples was clearly identified as 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid.

Quantification of triterpenes in apple fruits induced by infestation of *C. sasakii* larvae or mechanical damage

Apple fruits ("Fuji") infested by *C. sasakii* larvae and intact fruits were extracted by MeOH. These extractions were analyzed by LCMS. Compounds **1**-**3** were not detected in the intact fruits. However, in the infested fruits, triterpenes were detected at the damaged feeding sites specifically; a negligible amount was detected at the intact sites. In addition, mechanical damage instead of insect infestation also induced the triterpenes in the same pattern. The concentration at the feeding damaged sites was 500-1000 µg/g f.w. for compound **1** and 80-100 µg/g f.w. for compound **2**. Then, 10 apple cultivars in addition to "Fuji" were mechanically damaged, and the triterpenes at the damaged sites were quantified (Table 1). The concentration of compound **1** in "Fuji" was highest, followed by "Orin" and "Indo," while "Jonathan" had the lowest among the 11 cultivars. The concentration of compound **2** in "Orin" was highest, followed by "Jonagold" and "Toki"; the others presented similarly low concentrations.

Quantitative time course analysis of triterpenes

Apple fruits ("Fuji") were drilled into with twisted steel wire to imitate larval infestation, and 34 h after the mechanical damage, the flesh, including that in the damaged areas, was extracted by MeOH. To examine whether the mechanical damage was comparable with the insect damage, the amount of triterpene acids induced by the mechanical damage and insect damage were analyzed and compared. An LCMS analysis showed that the mechanical damage mimicked by the wire was effective enough to induce the same amounts of triterpenes. The quantitative time course analysis of compounds **1**, **2**, and **3** induced at the mechanically damaged areas in a fruit showed different patterns (Figure 2). Compounds **1** and **3** were induced within 24 h and increased linearly within 24-96 h, while compound **2** was induced after 48 h and increased linearly within 48-96 h.

Consumption and growth assay

While *A. orana fasciata* lives on apple leaves, *C. sasakii* and *G. molesta* are important apple fruit pests. As broadly polyphagous agricultural pests, *S. litura* and *H. armigera* were also used herein to see if the induced triterpenes had any general defensive activity against nonadapted herbivorous insects. All insect larvae were fed an agar-based diet, to which the triterpene fraction (TTPf) from *C. sasakii*–infested or intact fruits was added. The weight growth (Figure 3a-c) and diet consumption (Figure 4a-c) of the *S. litura*, *H. armigera*, and *A. orana fasciata* larvae decreased on the TTPf (infested) diet compared to on the control diet. The control (TTPf intact) data were not available (Figure 4b) because of the shortage of the insects. In contrast, there were no significant differences in the weight growth of the *C. sasakii* larvae on the TTPf (infested) diet (Figure 3e). Since the *C. sasakii* larvae excreted watery stool that was impossible to separate from the diet, their diet consumption was unmeasurable. Similar results were shown by the other apple-fruit pest, *G. molesta* larvae. The larvae fed on both TTPf (infested) diet and TTPf (intact) diet, yielding no significant differences in the weight growth (Figure 3d) and the diet consumption (Figure 4d). The LCMS analysis showed that the TTPf from the infested fruits contained several triterpenes, including eriobotoric acid and pomaceic acid, while few triterpenes were found in the TTPf from intact fruits. Another bioassay was repeated against *S. litura*, *A. orana fasciata*, and *C. sasakii*, serving an agar-based diet enriched with purified eriobotoric acid and pomaceic acid instead of TTPf. The eriobotoric acid had clear antifeedant activity against *S. litura* but not against *A. orana fasciata* or *C. sasakii* (Figure 5). The pomaceic acid had no such antifeedant activity against any of these insects (Figure 6). In this assay, euscaphic acid was not tested because of a shortage of the purified compound.

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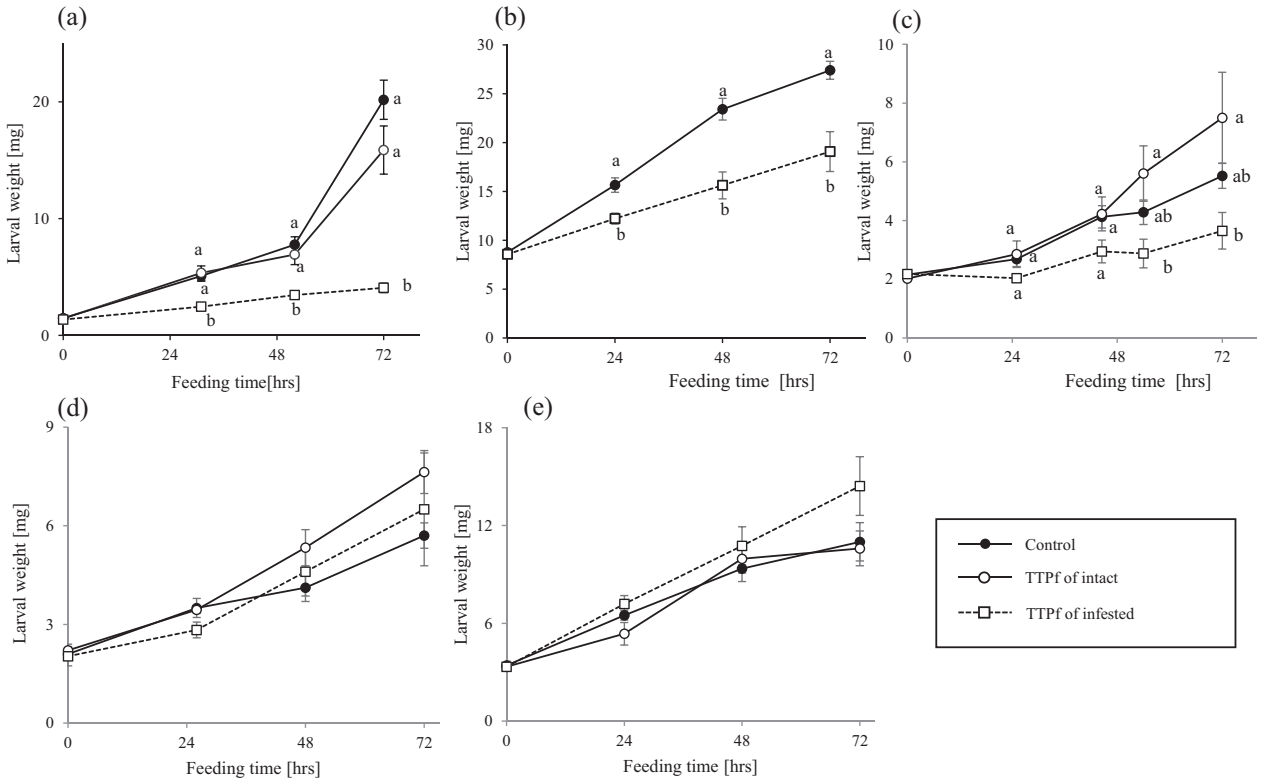

Figure 3. Effect of triterpene fraction from apple fruits infested by *C. sasakii* larvae on larval growth. *S. litura* (a), *H. armigera* (b), *A. orana fasciata* (c), *G. molesta* (d), and *C. sasakii* (e) larvae were fed on agar-based diet with triterpene fraction (TTPf) of infested fruits by *C. sasakii* or that of intact fruits, and weighed every 24 h for 72 h. Each value is presented as the mean ± SE; $n = 5$. Different letters represent significant difference ($P < .05$) by Tukey–Kramer test.

Table 1. Induced concentration of compounds **1** and **2** in fruit flesh (μg/g of fresh weight) and cultivar comparison ("Kotoku," "Jonathan," "Shinano Gold," "Ambitious," "Sensyu," "Jonagold," "Miki Life," "Indo," "Orin," "Toki," and "Fuji"). The data on compound **3** was not obtained because it was out of the target range of LCMS analysis.

Cultivar		Compound 1 (μg/g FW)	Compound 2 (μg/g FW)
Mechanically damaged	Kotoku	40.6 ± 24.5	4.9 ± 1.3
	Jonathan	29.0 ± 4.5	6.5 ± 0.3
	Shinano Gold	84.3 ± 17.2	17.4 ± 13.9
	Ambitious	94.3 ± 29.3	20.1 ± 8.1
	Sensyu	42.4 ± 5.5	23.3 ± 22.3
	Jonagold	328.8 ± 127.1	261.6 ± 84.3
	Miki Life	76.5 ± 42.8	37.4 ± 32.3
	Indo	336.5 ± 100.0	54.9 ± 38.4
	Orin	346.3 ± 140.2	327.5 ± 205.6
	Toki	134.6 ± 64.3	119.4 ± 54.5
	Fuji	514.8 ± 186.3	77.5 ± 9.3
Infested by *C. sasakii*	Fuji	469.0 ± 99.3	96.3 ± 33.6
Intact	Fuji	n.d.	n.d.

Data shown as mean ± SD, $n = 3$-5, n.d.: not detected.

Discussion

Despite the interest in apple secondary metabolites, relatively few investigations have focused on triterpene acids, probably due to analytical issues (Sut *et al.* 2018). Due to new analytical techniques using LCMS, the number of publications concerning apple triterpenes has risen sharply over the last few years. However, LCMS/MS-based identification relying too much on earlier research has a potential disadvantage of copying misinformation. The 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid (named eriobotoric acid) induced in our experiments was first identified in loquat fruits but had never been reported in apple fruits. Instead, 1α,19α-dihydroxy-3-oxours-12-en-28-oic acid (named annurcoic acid), a triterpene with a confusingly similar structure, has been reported (Cefarelli *et al.* 2006; D'Abrosca *et al.* 2006). Both apple and loquat fruits have many triterpenes in common, including ursolic acid, euscaphic acid, oleanolic acid, maslinic acid, tormentic acid, and corosolic acid (Liu *et al.* 2016; Waldbauer *et al.* 2016), but annurcoic acid is unique to apples. To examine the possibility that annurcoic acid is a misidentification of eriobotoric acid, we purified eriobotoric acid (**1a**) from loquat fruits and compared the analytical signal with that of compound **1** and annurcoic acid data reported in previous papers. Compounds **1** and **1a** had peaks at the same retention time with the same mass fragmentation pattern. The reported LCMS/MS fragmentation pattern of annurcoic acid was almost the same, and we could not tell one from the other. The NMR spectra of the compound **1** and **1a** were clearly identical, suggesting compound **1** was the 2α,19α-dihydroxy-3-oxours-12-en-28-oic acid. The NMR data of annurcoic acid reported by D'Abrosca *et al.* (2006), were also identical to the data of eriobotoric acid (**1**, **1a**), suggesting a misinterpretation had occurred. In D'Abrosca *et al.*'s paper, the hydroxy group was said to be located at C-1 in the axial position. If that were true, the common coupling J values between the proton located at C-1 (4.59 ppm) and protons at C2 would be 4-5 Hz (eq-ax) and 1-3 Hz (eq-eq), respectively. This did not fit to our observed data (6.3 and 12.5 Hz, respectively). The

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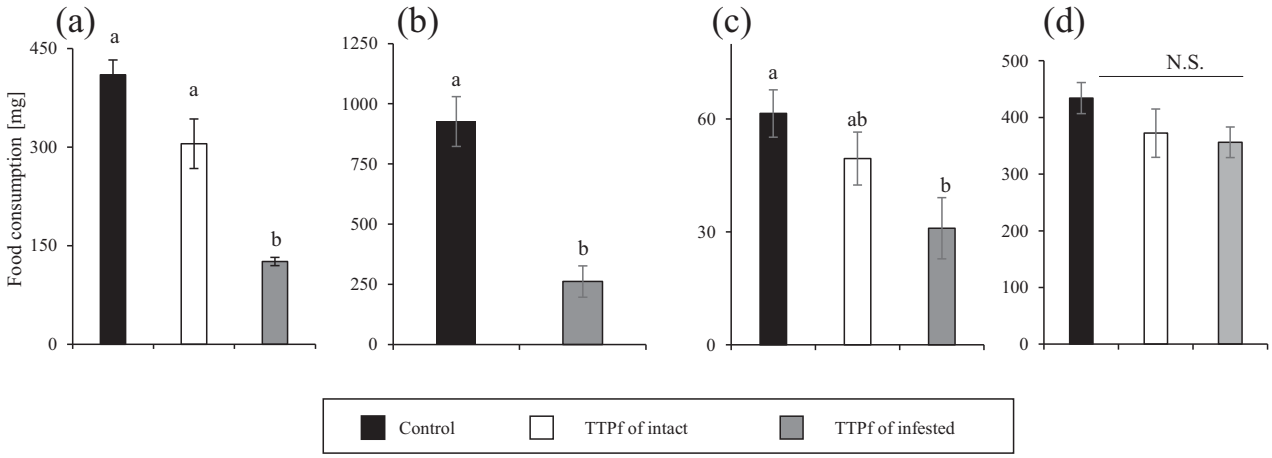

Figure 4. Effect of triterpene fraction (TTPf) from apple fruits infested by *C. sasakii* larvae on food consumption by *S. litura* (a), *H. armigera* (b), *A. orana fasciata* (c), and *G. molesta* (d) larvae. Each value is presented as the mean ± SE; $n = 5$. Different letters represent significant difference ($P < .05$) by Tukey–Kramer test. N.S.: not significant.

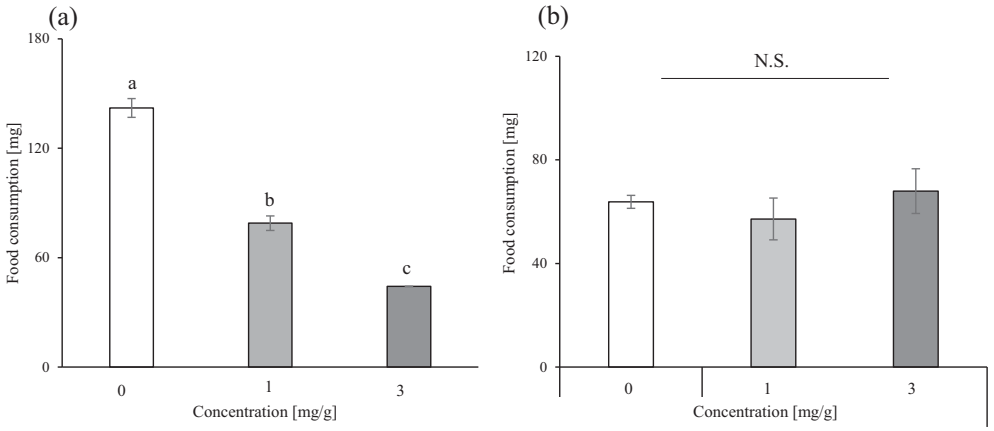

Figure 5. Effect of eriobotoric acid on food consumption by *S. litura* (a) and *A. orana fasciata* (b) larvae. The concentration of eriobotoric acid in the assay diet was calibrated based on its natural concentration in damaged fruits (1 mg/g) and 3 times enriched (3 mg/g). Food consumption for 72 h was weighed. Each value is presented as the mean ± SE; $n = 4$-5. Different letters represent significant difference ($P < .05$) by Tukey–Kramer test. N.S.: not significant.

observed signal supported our proposal that the calculated coupling J values should be 4-5 Hz (ax-eq) and 11-12 Hz (ax-ax), respectively (Section S6). Also, the key data to determine the structure of annurcoic acid was the correlation of H-5/C-1 in HMBC analysis (D'Abrosca *et al.* 2006). However, the signal for H-5 (1.18) and H-2 (1.17) are overlapped and can be confused, which may lead to wrong conclusion. The rest of the HMBC correlations in their data were also acceptable to conclude the structure as 2-hydroxy type. From these data, we report eriobotoric acid as an apple triterpene acid and propose the correction.

A recent paper reported a new triterpene discovered from apple pomace, that is, pomaceic acid (Waldbauer *et al.* 2016). According to the report, the compound was the most abundant triterpene acid in the tested apple pomace sample. Another paper reported the compound as minor compared with the other triterpene acids in apple peel extracts (Sut *et al.* 2018). There have only been a few papers concerning both pomaceic acid and annurcoic acid. One such paper showed that the amount of pomaceic acid was greater than that of annurcoic acid (Waldbauer *et al.* 2016), although the origin of the sample was not clear. Another study suggested that both compounds are abundant in an ancient apple variety but negligible in commercial varieties such as "Granny Smith," "Red delicious," "Golden delicious," and "Royal gala" (Sut *et al.* 2019). In these commercial varieties, the major triterpene acid components are ursolic acid and oleanolic acid in both pulps and peels. A study concerning "Fuji" reported that annurcoic acid was one of the major triterpene acids in the tested apple peels (McGhile *et al.* 2012). However, the data in that paper were unique compared to previous studies (Belding *et al.* 1998; Frighetto *et al.* 2008). The authors attributed the difference to the extraction procedure or difference of the relative concentrations of ursenoic acids between the epicuticular waxes and cell layers of the apple peels. In our case, eriobotoric acid (formerly identified as annurcoic acid) and pomaceic acid were the main components in the fruit flesh only after damage but negligible in intact fruits. The damage-induced accumulation may explain the differences in the concentrations of these compounds between our research and previous studies because the latter may not have been careful enough to check whether the apple sample materials were intact. The induction was also triggered by mechanical damage, and the induced amount was comparable to that of herbivory induction, suggesting the effect of elicitors (if any) from insect saliva is negligible in this induction response. We did not find any candidate glycosides of these triterpenes in our LCMS analysis, and acidic hydrolyzation of the homogenized tissues did not yield these triterpene acids;

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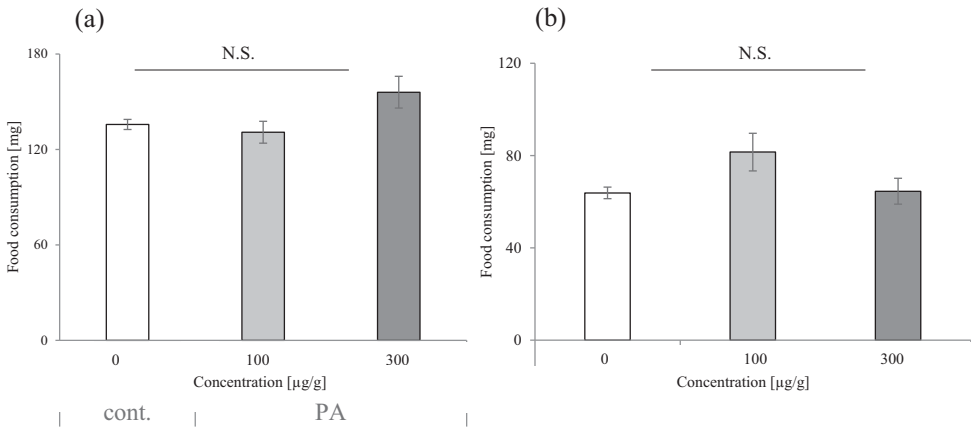

Figure 6. Effect of pomaceic acid on food consumption by *S. litura* (a) and *A. orana fasciata* (b) Larvae. The concentration of eriobotoric acid in the assay diet was calibrated based on its natural concentration in damaged fruits (100 μg/g) and 3 times enriched (300 μg/g). Food consumption for 72 h was weighed. Each value is presented as the mean ± SE; $n = 4$-5. N.S.: not significant by Tukey–Kramer test.

however, we cannot rule out the possibility that the hydrolyzation condition was not thoroughly optimized. The concentration of the 2 compounds in the damaged fruits increased as time passed and peaked around 96 h after the mechanical damage. However, leaving homogenized apple pulps at room temperature for several days did not yield the compounds. These results suggest that the 2 triterpene acids are synthesized *de novo* in living cells, which may explain why annurcoic acid and pomaceic acid are not the main components in some apple products that are usually processed while still fresh.

Table 1 shows the mechanically induced amounts of eriobotoric acid and pomaceic acid in the tested cultivars. The amount of induction varied depending on the cultivar. Pomaceic acid showed high induction in "Fuji," "Indo," "Orin," and "Toki" and low induction in "Kotoku" and "Jonathan." The amount of induced eriobotoric acid was particularly high in "Fuji" and "Indo." According to the apple varieties' genealogy, "Orin" and "Toki" were derived from "Indo." Thus, the characteristic of high pomaceic acid induction may reflect the phyletic lineage.

Although various physiological activities, such as enhancement of endothelial nitric oxide synthase (Waldbauer *et al.* 2016) and antioxidation (D'Abrosca *et al.* 2006), are known for inducing triterpenes in physically injured apple fruits, the physiological significance of these triterpenes is unknown. In this experiment, we focused on their physiological activity against pest insects. Insect damage is a physical injury, and it is well known that the responses induced against herbivores are often caused by mechanical damage (Mattiacci, Dicke and Posthumus 1994; Bolter *et al.* 1997). We examined the possibility that the induced triterpene acids function as defensive substances. Among the tested insects, the *S. litura*, *H. armigera*, and *A. orana fasciata* larvae were sensitive to the TTPf fraction of damaged fruits. In particular, eriobotoric acid clearly showed antifeedant activity against *S. litura* larvae (*H. armigera* was not tested). However, *A. orana fasciata*, known as a leaf-eating apple pest that occasionally bites fruits, was not sensitive to the purified eriobotoric acid, suggesting other triterpene acids (euscaphic acid or minor unidentified triterpene acids) may have been active. On-site induction of triterpene acids in the fruits in response to the herbivory may have precluded further damage to the fruits and driven the insects back to the leaves. As *S. litura* and *H. armigera* are serious agricultural pests known for their considerably broad host range, from vegetable to flowering plants, there are constant reports of them damaging new host plants. Recently, *H. armigera* were found in apple orchards, and a few apple fruits were found to have been invaded by the larvae, although the damage was very limited (Ishiguri 2021). This highlights the important function of induced triterpene acids in apples against potential pest insects. However, the eriobotoric acid and other induced triterpenes exhibit no defensive activity against *C. sasakii* or *G. molesta*. Since both insects have already adapted to apples as feed, they have likely overcome these triterpenes in some way. Other induced triterpenes, such as pomaceic acid and euscaphic acid, showed little resistance against the tested insects. However, there remains a possibility that these triterpenes may possess protective properties against apple diseases commonly mediated by larval feeding. Further research is required to thoroughly understand the physiological function of triterpene acids in apples.

Acknowledgments

The authors thank Japan Agricultural Cooperatives (JA) Kagawa and Shigeki Shinkai, Toka Osaka Co., Ltd, for kind supply of fruit materials.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

All data are provided in the manuscript and its Supporting Information files.

Author contribution

Y.O. and S.M. isolated and determined the compounds. Y.O. and Y.T. analyzed and evaluated the biological activities. Y.I. supplied the plant and insect materials. Y.O., N.M., Y.I., and N.Y. designed the research. Y.O. wrote the paper.

Funding

This work was financially supported by JSPS KAKENHI under Grant Numbers 26450068 and 17K07684.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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