Feeding Stimulative Activity of Steroidal and Secoiridoid Glucosides and Their Hydrolysed Derivatives toward the Olive Weevil (*Dyscerus perforatus*)

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β-Sitosteryl-D-glucoside and oleuropein isolated from the olive tree (*Olea europaea*) and their hydrolysed derivatives were tested by a feeding stimulative activity bioassay using the olive weevil (*Dyscerus perforatus*). Although the steroidal glucoside showed potent feeding stimulative activity, the activity of the aglycone (β-sitosterol) was significantly lower than that of the glucoside. On the other hand, the difference in the activity between oleuropein, a secoiridoid glucoside, and the hydrolysed derivatives was not significant.

Key words: Olive Weevil, Olive Tree, Feeding Stimulants

Introduction

The olive weevil [*Dyscerus perforatus* (ROELOFS); Coleopetera; Curculionidae], a native species in Japan, is the most serious pest of the olive trees. The adult weevils can survive for over 900 days in a room (Matsuzawa *et al.*, 1958) and the females have a long egg-laying period from spring to autumn. Moreover, the larvae attack the bark and trunk of olive trees, weakening and withering even adult trees as the assault progresses. Before the introduction of the olive to Japan in 1908, *Ligustrum japonicum* Thunb., and *L. obtusifolium* Sieb. et Zucc, belonging to the same Oleaceae family as the olive, were considered as the host plants for this weevil. However, the insect went away and left these two species thereafter and colonized the olive where it attained much higher population densities than in the former plants and thereby the assault becomes seriously damaging for the olive trees.

During the course of our study on the relationship between the olive tree and olive weevil, we have been interested in the possible chemical constituents of this plant that are responsible for their host selection and attraction of the insect.

We previously reported that a secoiridoid glucoside, oleuropein, (Nakajima *et al.*, 1995) and two minor lignans, (–)-olivil and (+)-1-acetoxypinoresinol (Kadowaki *et al.*, 2003) isolated from olive tree stimulated the feeding habit of the weevil. Here, we describe the feeding stimulative activity of β-sitosteryl-D-glucoside as an additional feeding stimulant from the olive and its aglycone along with the activity of secoiridoid glucoside and its hydrolysed derivatives.

Materials and Methods

General experimental procedures

Silica gel 60 (Nacalai Tesque, 230–400 mesh) was used for column chromatography. All the NMR experiments were conducted with a Varian VXR500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C). GC-MS (Automass 20, JEOL) analyses in the electron impact ionization (EI, 70e V) were performed on a DB-1 column (0.25 mm Ø, 30 m), using a temperature programme from 70 °C (3 min) to 250 °C (70 min) at 10 °C min^{-1}. GC-FID (G-3000, HITACHI) was performed on the TC-1701 column (0.25 mm Ø, 30 m), using a temperature programme from 110 °C to 280 °C (5 min) at 5 °C min^{-1}. Mass spectra were recorded on an API III triple quadrupole mass spectrometer (PE-Scix; Thorn Hill, ON, Canada) equipped with an electrospray (ES) interface. Elemental analysis was performed on PE 2400 II IR (KBr) spectrum

was measured on a JASCO FT/IR-5000 spectrometer. Optical rotation was taken on a JASCO Dip-360 digital polarimeter.

Insect and plant materials

The weevils were field-collected as newly emerged adults from infested olive trees from July to September in 2002. Male and female weevils were separately reared in plastic containers (27 cm × 20 cm × 13 cm) with a piece of a young branch from an olive tree (*ca.* 20 cm length, 5 mm Ø) and wet cotton under gregarious conditions at 25 °C with a 12L:12D photoperiod.

The olive tree (*Nevadillo Blanco*) was obtained from our University and Nippon Olive Co., Ltd. in April 2001 and was cut into log pieces wihout their leaves and fruits for the extraction of feeding stimulants.

Feeding bioassay

The no choice paper disk method reported before (Kadowaki *et al.*, 2003) was used for bioassay.

Preparation of feeding stimulants and their hydrolysed derivatives

The methanolic extract (26.4 liters) of olive logs (9.1 kg) was concentrated under reduced pressure and partitioned first with hexane (500 ml × 3) and then with ethyl acetate (500 ml × 3). The ethyl acetate soluble fraction (160 g) was separated by column chromatography on silica gel, successively eluting with hexane (100%), hexane: ethyl acetate = 7:3 and 1:1 (v/v), ethyl acetate (100%) and methanol (100%). The methanol fraction was further separated in the silica gel column, eluting with $CHCl_3$: methanol = 4:1 (v/v), followed by another silica gel column, eluting with $CHCl_3$: methanol = 10:0.5 and 10:1(v/v), to afford an active fraction (220 mg). After evaporation of the solvent to near dryness and recrystallization from ethyl acetate, β-sitosteryl-D-glucoside was obtained from the fraction as colorless needles (35 mg). *Anal.* Calcd. for $C_{35}H_{60}O_6$: C, 72.87%; H, 10.48%. Found. C, 72.35%; H, 10.41%; ^{1}H-NMR δ_H (500 MHz, *pyridine-d_5*); 0.66 (3H, s, H-18), 0.86 (3H, d, J = 6.0 Hz, H-26), 0.87 (3H, d, J = 6.0 Hz, H-27), 089 (3H, t, H-29), 0.92 (2H, m, H-9, 14), 0.93 (3H, s, H-19), 0.98 (5H, m, H-1, 21, 24), 1.10 (2H, m, H-12, 17), 1.23 (2H, m, H-23), 1.26 (4H, m, H-16, 28), 1.38 (5H, m, H-11, 20, 22), 1.50 (1H, m, H-8), 1.54 (2H, m, H-15), 1.68 (1H, m, H-25), 1.71 (1H, m, H-1), 1.73 (1H, m, H-2), 1.87 (2H, m, H-7), 1.97 (1H, br, d, J = 12.5 Hz, H-12), 2.13 (1H, m, H-2), 2.47 (1H, t, J = 12.0 Hz, H-4), 2.74 (1H, dt, J = 12.0, 2.1 Hz, H-4), 3.97 (2H, m, H-3, 5′), 4.07(1H, t, J = 7.6, 7.6 Hz, H-2′), 4.30 (2H, m, H-3′,4′), 4.43 (1H, dd, J = 11.6, 5.1 Hz, H-6′a), 4.58 (1H, d, J = 11.6 Hz, H-6′b), 5.06 (1H, d, J = 7.6 Hz, H-1′), 5.35 (1H, t, J = 3.0 Hz, H-6); ^{13}C-NMR δ_C (125 MHz, *pyridine-d_5*); 12.0 (C-29), 12.2 (C-18), 19.0 (C-21), 19.2 (C-26), 19.4 (C-19), 20.0 (C-27), 21.3 (C-11), 23.4 (C-28), 24.5 (C-15), 26.4 (C-23), 28.5 (C-16), 29.4 (C-25), 30.2 (C-2), 32.0 (C-7), 32.2 (C-8), 34.2 (C-22), 36.4 (C-20), 36.9 (C-10), 37.5 (C-1), 39.3 (C-4), 39.9 (C-12), 42.5 (C-13), 46.0 (C-24), 50.3 (C-9), 56.2 (C-17), 56.8 (C-14), 62.8 (C-6′), 71.7 (C-4′), 75.4 (C-2′), 78.1 (C-5′), 78.5 (C-3), 78.6 (C-3′), 102.6 (C-1′), 121.9 (C-6), 140.9 (C-5)

Acidic hydrolysis of β-sitosteryl-D-glucoside

β-Sitosteryl-D-glucoside (10 mg) was hydrolysed with 1 N HCl in methanol at 60 °C for 7 h, then the solvent was evaporated, and the residues suspended in 1 N HCl followed by heating at 60 °C for 15 h. After neutralization with $NaHCO_3$, the products were distributed between H_2O-$CHCl_3$. The aqueous layer was freeze-dried, and trimethylsilylated with Tri-Sil Reagent (PIERCE, Rockford, IL, USA, 100 μl) for 5 min at room temperature. After concentration under N_2 stream, the residue was dissolved in *n*-hexane and analysed by GC under the condition as described above, The retention time of the trimethylsilylated sugar from β-sitosteryl-D-glucoside coincided with that of an authentic sample (trimethylsilylated β-D-glucose, t_R: 20.6 min). The $CHCl_3$ layer described above was purified by column chromatograply using silica gel 60, eluting with hexane: ethyl acetate = 4:1 (v/v). The combined fractions gave 4.9 mg of the aglycone (β-sitosterol) which was analysed by GC-MS. GCEIMS *m/z* (rel.int.): 414 [M]+ (23), 381 (25), 303 (39), 255 (38), 213 (100). ^{13}C-NMR δ_C (125 MHz, $CDCl_3$); 11.8 (C-29), 12.0 (C-18), 18.8 (C-21), 19.0 (C-26), 19.4 (C-19), 19.8 (C-27), 21.1 (C-11), 23.1 (C-28), 24.3 (C-15), 26.0 (C-23), 28.2 (C-16), 29.1 (C-25), 31.6 (C-2), 31.9 (C-7), 33.9 (C-22), 36.1 (C-20), 36.5 (C-10), 37.2 (C-1), 39.8 (C-

12), 42.3 (C-4), 42.3 (C-13), 45.8 (C-24), 50.1 (C-9), 56.0 (C-17), 56.8 (C-14), 71.8 (C-3), 121.7 (C-6), 140.7 (C-5).

Oleuropein

Oleuropein (96 mg) was isolated from olive logs (480 g) as previously reported (Nakajima *et al.*, 1995). ^{1}H-NMR δ_H (500 MHz, CD_3OD); 1.65 (3H, d, J = 7.0 Hz, H-10), 2.43 (1H, dd, J = 14.0, 9.2 Hz, H-6a), 2.70 (1H, dd, J = 14.0, 4.6 Hz, H-6b), 2.75 (2H, t, J = 7.0 Hz, H-2′), 3.32 (1H, m, H-2″), 3.35 (1H, m, H-4″), 3.36 (1H, m, H-3″), 3.44 (1H, m, H-5″), 3.67 (1H, dd, J = 11.6, 1.8 Hz, H-6″a), 3.70 (3H, s, CH_3O-), 3.88 (1H, dd, J = 11.6, 1.8 Hz, H-6″b), 3.96 (1H, dd, J = 9.2, 4.6 Hz, H-5), 4.1 (1H, dt, J = 10.7, 7.0 Hz, H-1′a), 4.2 (1H, dt, J = 10.7, 7.0 Hz, H-1′b), 4.82 (1H, d, J = 7.9, H-1″), 5.90 (1H, s, H-1), 6.07 (1H, q, J = 7.0 Hz, H-8), 6.55 (1H, dd, J = 7.9, 2.1 Hz, H-8′), 6.70 (1H, d, J = 2.1 Hz, H-4′), 6.70 (1H, d, J = 7.9 Hz, H-7′), 7.50 (1H, s, H-3); ^{13}C-NMR δ_C (125 MHz, CD_3OD); 13.6 (C-10), 31.8 (C-5), 35.3 (C-2′), 41.2 (C-6), 51.9 (**C**H_3O-), 62.7 (C-6″), 66.9 (C-1′), 71.4 (C-4″), 74.7 (C-2″), 77.9 (C-5″), 78.3 (C-3″), 95.2 (C-1), 100.8 (C-1″), 109.3 (C-4), 116.4 (C-4′), 117.0 (C-7′), 121.3 (C-8′), 124.9 (C-8), 130.4 (C-9), 130.7 (C-3′), 144.8 (C-6′), 146.2 (C-5′), 155.2 (C-3), 168.7 (C-11), 173.2 (C-7).

Enzymatic hydrolysis of oleuropein

Oleuropein (90 mg) was hydrolysed with β-glucosidase (SIGMA, 50 mg) in distilled water at room temperature for 20 h. After extraction of the reaction mixture with ethyl acetate, the ethyl acetate soluble fraction (46 mg) was purified by silica gel column chromatography, eluting with hexane: ethyl acetate = 7:3, 1:1 and 3:7 (v/v). The mixture of diastereomers of 3,4-(dihydroxyphenyl) ethanol elenolic acid ester (4.6 mg, 8S, 9R: 8R, 9R = 5:1, Bianco *et al.*, 1999) transformed from the aglycone of oleuropein was obtained from the fraction of 7:3. ES-MS: *m/z* 379.0 for $[C_{19}H_{22}O_8+H^+]$; ^{13}C-NMR ì$_C$ (125 MHz, $CDCl_3$); 19.3 (C-10), 26.9 (C-5), 34.1 (C-2′), 37.1 (C-6), 51.7 (COO**C**H_3), 54.4 (C-9), 65.2 (C-1′), 70.7 (C-8), 106.3 (C-3′), 115.1 (C-4′), 116.3 (C-7′), 121.2 (C-8′), 130.4 (C-4), 143.0 (C-6′), 143.3 (C-5′), 155.9 (C-3), 167.7 (C-11), 171.8 (C-7), 200.1 (C-1) for a diastereomer. 19.3 (C-10), 26.9 (C-5), 34.1 (C-2′), 37.1 (C-6), 50.9 (C-9), 51.7 (COO**C**H_3), 65.2 (C-1′), 69.6 (C-8), 106.3 (C-3′), 115.1 (C-4′), 116.3 (C-7′), 121.2 (C-8′), 130.4 (C-4), 143.0 (C-6′), 143.3 (C-5′), 155.9 (C-3), 167.7 (C-11), 171.8 (C-7), 200.1 (C-1) for another one.

Results and Discussion

The isolation of β-sitosteryl-D-glucoside (Fig. 1) from the methanolic extract of olive logs was guided by a feeding stimulative activity test using the olive weevil. Structural elucidation of the steroidal glucoside was mainly done by spectral analysis. Oleuropein (Fig. 1) was also isolated from olive logs according to the procedure previously reported (Nakajima *et al.*, 1995).

Figure 2 shows the feeding stimulative activities of β-sitosteryl-D-glucoside and its aglycone toward male and female olive weevils. β-Sitosteryl-D-glucoside showed a 20–25% feeding stimulative activity for both sexes. On the other hand, the aglycone, β-sitosterol, did not exhibit any stimulative activity in the case of the males, but showed weak activity for the females (+ 10%). The negative value (*ca.* – 10%) for the male might be considered as a possible consequence of the antifeeding effect of β-sitosteryl-D-glucoside. Further experiments should be performed carefully for discussing this possibility. The difference in activity of β-sitosteryl-D-glucoside and its aglycone was significant ($p < 0.05$, t-test) for both sexes. However, the feeding responses between males and females were not significantly different to the glucoside.

When the glycosidic bond in oleuropein was hydrolysed by β-glucosidase, the liberated aglycone was reported to cause a fast irreversible molecular transformation in the acetal moiety affording the derivative, 3,4-(dihydroxyphenyl) ethanol elenolic acid ester (3,4-DHPEA-EA) (Bianco *et al.*, 1999) and two diastereomers of 3,4-DHPEA-EA were isolated from an extract of olive leaves (Gariboldi *et al.*, 1986). In our study, we could not obtain the aglycone, but the mixture of such diastereomers by enzymatic reaction of oleuropein using β-glucosidase. Therefore, the feeding stimulative activity of the diastereomers of 3,4-DHPEA-EA was tested instead of assaying the aglycone itself. Figure 2 exhibits the feeding stimulant activities of oleuropein and the diastereo mixture toward the male and female olive weevil. Oleuropein showed

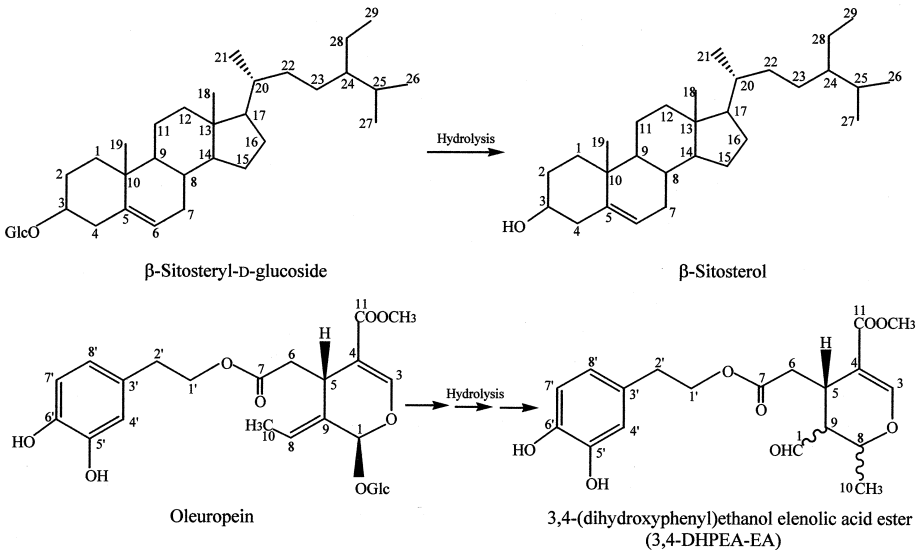

Fig. 1. Structures of feeding stimulants and their derivatives.

ca. 12% activity for the male and *ca*. 13% for the female, respectively. In addition, the diastereo mixture of 3,4-DHPEA-EA also showed activity of *ca*. 15% for both sexes. It is interesting that the difference in activity between oleuropein and the derivatives was not significant.

β-Sitosterol is known as one of the biting factor for silkworms (Hamamura *et al.*, 1961) and it is also an important intermediate in cholesterol biosynthesis for insects. In our present study, β-sitosteryl-D-glucoside was found to be a potent feeding stimulant toward the olive weevil, but its aglycone (β-sitosterol) was not. On the other hand, the possibility was reported that the olive tree is resistant to insect attack and that the secoiridoid glucosides, oleuropein and ligstroside, are involved in the defense mechanism (Kubo *et al.*, 1985). Our study, however, has revealed oleuropein and the derivatives to be feeding stimulants toward the olive weevil.

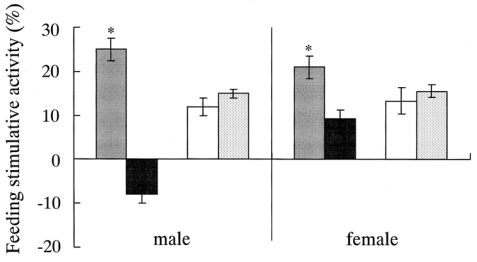

Fig. 2. Feeding stimulative activities of β-sitosteryl-D-glucoside, oleuropein and their hydrolysed derivatives at 10 μg/disk toward the male and female weevils.
Bars are mean ± SE (n = 50 for β-sitosteryl-D-glucoside and its aglycone and n = 20 for oleuropein and diastereomers of 3,4-DHPEA-EA). * Significantly different from the aglycone by Student's t-test ($p < 0.05$). ▨ β-Sitosteryl-D-glucoside; ■ aglycone; □ oleuropein; ▦ diastereomers of 3,4-DHPEA-EA.

It is not clear so far what the difference in feeding stimulative activity of these compounds is implying, but suggested that they may play important roles for specific plant-insect interactions, host selection and attraction in the natural ecosystem. Furthermore, our study may contribute to developing a pest control and integrated pest management.

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