Feeding deterrents from *Momordica charantia* leaves to cucurbitaceous feeding beetle species

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Abstract

Cucurbitaceous feeding beetle species, *Aulacophora femoralis*, *A. nigripennis*, *Epilachna admirabilis* and *E. boisduvali* seldom attack *Momordica charantia*. The methanol extract of *M. charantia* leaves strongly deterred four beetle species from feeding. The methanol extract was partitioned between organic solvent and water, and the chloroform fraction, which showed a strong feeding detergency, was obtained. The chloroform fraction was chromatographed with a silica gel column, and momordicines I and II were isolated. *A. nigripennis* was strongly deterred from feeding by momordicines I and II at lower concentrations than contained in *M. charantia* leaves. *A. femoralis* was deterred from feeding by momordicine II at a high concentration. The feeding of *E. admirabilis* and *E. boisduvali* was not affected by momordicines I and II alone. However, *E. admirabilis* and *E. boisduvali* were strongly deterred from feeding by mixtures of momordicines I and II, and momordicine II and other components.

Key words: *Momordica charantia*, *Aulacophora femoralis*, *Aulacophora nigripennis*, *Epilachna admirabilis*, *Epilachna boisduvali*

INTRODUCTION

The host range of phytophagous insects is restricted by various factors present in plants. It is well known that plant chemicals play an important role in host plant selection. Cucurbitaceous plants, especially cultivated species such as cucumber, melon and pumpkin, are attacked by various herbivorous insects. Among cucurbitaceous plants, bitter melon, *Momordica charantia* L., is rarely attacked by phytophagous insects because of the presence of feeding deterrents, momordicine II and momordicine derivatives (Chandravadana and Pal, 1983; Chandravadana, 1987; Yasui et al., 1998). The leaf beetle species, *Aulacophora femoralis* (Motschulsky) and *A. nigripennis* Motschulsky, and the lady beetle species, *Epilachna admirabilis* Crotch and *E. boisduvali* Mulsant feed on cucurbitaceous plants and have different host plant ranges, respectively. For reasons as yet unknown, these beetle species do not feed on *M. charantia* leaves. It is important for an understanding of insect-plant interactions to investigate plant chemicals which are related to feeding

behavior. In this study, we investigated the feeding deterrent(s) of these cucurbitaceous feeding beetle species from *M. charantia* leaves and revealed the role of momordicines in their feeding behavior.

MATERIALS AND METHODS

Insects and plants. *E. admirabilis* and *A. nigripennis* were collected in an experimental field of the Faculty of Agriculture, Tohoku University. *E. boisduvali* and *A. femoralis* was collected on Ishigaki Island, Okinawa Prefecture. The beetles were reared at $24 \pm 1^\circ\text{C}$ under a 16L–8D photoregime. The host plant leaves were provided as food. Plants were cultivated in the experimental field of the Faculty of Agriculture, at Tohoku University.

Bioassay. The feeding preference test was conducted using leaves of *M. charantia* or bitter melon, *Sicyos angulatus* L., in a no-choice test. Disks (2 cm diameter) were punched out of leaves with a cork borer. A filter paper (Toyo, 1, 7 cm d.) was placed at the bottom of a petri dish (6 cm d.) and distilled water (0.3 ml) was added to maintain humidity. Two disks were weighed

and then placed in the bottom of the petri dish. Three adults, of mixed gender, starved for 24 h, were released in the petri dish and allowed to feed on the disks freely for 2 h under $24 \pm 1^\circ\text{C}$. Each test was replicated five times. After each test was finished, the leaf disks were removed and weighed again. In this test, the amount fed on was evaluated by the weight consumed instead of the area eaten because *M. charantia* leaves were different in thickness from *S. angulatus* leaves.

To estimate the feeding deterrent activity of *M. charantia* leaf extracts, leaf disk assays were conducted using *S. angulatus* leaves. The leaves were punched out with a cork-borer (2 cm d.). An ethanol solution (0.03 ml) of a test sample was applied uniformly on the upper surface of leaf disks. The solvent was removed quickly by air drying. Concentration of the test solution was 1 g fresh leaf equivalent/ml ethanol. A leaf disk treated with ethanol only was used as the control. A filter paper was set in a petri dish (6 cm d.) and moistened with distilled water (0.3 ml) to maintain humidity. Treated or control disks were then placed individually in petri dishes exposing their upper surfaces. Three adults of mixed gender, starved for 24 h were released in the petri dish and allowed to feed on the leaf disk for 2 h under $24 \pm 1^\circ\text{C}$. The extent of feeding was assessed by measuring the area of the leaf disk consumed using a graph sheet. The feeding deterrent activity was estimated by Mann-Whitney test. The data was translated to feeding inhibition (FI) by the following formula:

$$\text{FI} = 100 \times (\Sigma \text{area consumed on control disk} - \Sigma \text{area consumed on treated disk}) / \Sigma \text{area consumed on control disk}.$$

Extraction and fractionation of active substances. Fresh leaves of *M. charantia* (1208.8 g) were extracted with methanol (12 l $\times$ 3) at room temperature. The extracts were filtered and then concentrated under reduced pressure with a rotary evaporator to dryness. The methanol extract was dissolved in water (1 l) and successively partitioned with chloroform (1 l $\times$ 3), ethyl acetate (1 l $\times$ 3), and water saturated *n*-butanol (1 l $\times$ 3). Each fraction was dried under reduced pressure. A portion of the chloroform extract was chromatographed on a silica gel

column (Wako-gel® C-200, Wako Pure Chemical Industries Ltd., 120 g, 450×28 mm i.d.) and successively eluted with hexane:ethyl acetate = 4:1 (fr. 1), 1:1 (fr. 2), 1:9 (fr. 3), ethyl acetate:methanol = 24:1 (fr. 4), 9:1 (fr. 5), and methanol (fr. 6). Each eluent was 1 l. The active fractions were chromatographed further.

Isolation and identification of momordicines. The fraction containing momordicine I was chromatographed further on a silica gel column (Wako-gel® C-300, 17 g, 500×10 mm i.d.), and eluted successively with chloroform, 1% methanol in chloroform, and 5% methanol in chloroform (50 ml each). These eluates were collected 2 ml each. The fraction containing momordicine II was chromatographed on an ODS column (YMC-gel® ODS-A 60-60/30, YMC Co. Ltd., 30 g, 235×19 mm i.d.), and eluted with 80% aqueous methanol, 90% aqueous methanol, and methanol (200 ml each). These eluates were collected 20 ml each. These eluates were monitored with TLC (Silica gel 60 F₂₅₄, 0.25 mm, 10×20 cm, Merck) using ethyl acetate:methanol:water (16:1:1) as the developing solvent. Visualization was achieved by heating at 115°C after spraying the reagent (10% H₂SO₄ in water, Libermann-Burchard and vanillin-phosphoric acid reagents), and then the TLC plate was inspected at UV-365 nm. Momordicine I was finally recrystallized from ethanol. Momordicine II was further purified by HPLC. A Toso CCMP pump and a UV-8000 detector were used in the HPLC system. The UV detector was set at 210 nm. Purification was performed with a column of TSKgel® ODS-80 T_M (Toso, 300×21.5 mm i.d.). The solvent was 85% methanol in water, and the flow rate was 5 ml/min.

Quantification of momordicines. Chloroform fraction (20 mg) was separated with a Sep-pak® C-18 cartridge (80% methanol in water, 3 ml). The eluate was dried and then subjected to HPLC analysis. Quantification of momordicines was performed with a column of TSKgel® ODS-80 T_s (Toso, 150×4.6 mm i.d.). The gradient separation was achieved using acetonitrile:water = 2:8 (solvent A) and acetonitrile:water = 6:4 (solvent B). The mixture ratio of solvent A:B was from 1:0 to 0:1 for 50 min and the flow rate was 1 ml/min.

Instruments. ^1H -NMR, ^{13}C -NMR, ^1H - ^{13}C HSCOSY, ^{13}C - ^1H HSCOSY and DEPT spectra were recorded on a Varian Unity INOVA 600 spectrometer in pyridine- d_5 with TMS as the internal standard. FAB-MS spectra were measured on a JEOL JMS HX-105 MS spectrometer.

RESULTS

Feeding response of beetle species to plant leaves

The amount of *M. charantia* leaves consumed by the four beetle species was much less than those of *S. angulatus* leaves. Although *E. admirabilis* fed slightly on *M. charantia* leaves, no obvious bites were observed on *M. charantia* leaves by the three other beetle species (Fig. 1).

Bioassay for feeding deterrents

The methanol extract of *M. charantia* leaves had a strong feeding detergency to the four beetle species. Among the fractions obtained from the methanol extract, the chloroform fraction showed the strongest feeding detergency against these four species, and the ethyl acetate fraction showed strong feeding deterrent activity only to *A. nigripennis* (Fig. 2). The chloroform fraction was separated into six fractions with silica gel column chromatography. Among the six fractions, fraction 4 ex-

Fig. 1. Feeding responses of the four beetle species to *Sicyos angulatus* and *Momordica charantia* leaves. ** Significant difference at $p < 0.01$ (Mann-Whitney test).

hibited the strongest feeding deterrent activity to the four beetle species. However, the deterrent activity against 3 of the 4 beetle species, but not for *A. nigripennis*, was much weaker than that of the chloroform extract. *A. nigripennis* was deterred from feeding by fractions 3, 4, 5 and 6, as intensely as the chloroform fraction (Fig. 3). Further purification was performed for fraction 3 which deterred the feeding of only *A. nigripennis*, and fraction 4 which was a strong feeding deterrent to the four beetle species.

Fig. 2. Feeding responses of the four beetle species to each fraction. Inhibition (%) = $100 \times (\Sigma \text{area consumed on control disks} - \Sigma \text{area consumed on treated disks}) / \Sigma \text{area consumed on control disks}$. Replications are: *A. femoralis*: 6, *A. nigripennis*: 8, *E. admirabilis*: 8, *E. boisduvali*: 6, respectively.

Fig. 3. Feeding responses of the four beetle species to column chromatography fractions of chloroform extract. Inhibition (%) was calculated by the formula described in Fig. 2. Each test was replicated six times.

Isolation and identification of active compounds

The TLC analysis of fractions 3 and 4 revealed two main spots (fraction 3: $R_f=0.69$, fraction 4: $R_f=0.2$, respectively) by H_2SO_4 reagent. These compounds were positive against Libermann-Burchard and vanillin-phosphoric acid reagents (fraction 3: yellow-orange, fraction 4: orange-brown fluorescence in UV-365 nm, respectively) on TLC. Therefore, these substances were considered as steroidal compounds. The compound in fraction 3 was separated with silica gel column chromatography and finally purified by recrystallization from ethanol (compound A). The substances in fraction 4 were separated with ODS column chromatography and finally purified with HPLC (compound B).

An 1H -NMR spectrum of compound A showed the presence of five methyl groups (δ 0.90, 0.95, 1.16, 1.18 and 1.50, each 3H, s), and two methyl groups on olefinic carbon (δ 1.71 and 1.73, each 3H, s), olefin (δ 5.63 and 6.30, each 1H, d), and formyl (δ 10.68, 1H, s). The ^{13}C -NMR spectrum showed signals of seven methyl carbons (δ 15.01, 18.12, 18.26, 19.13, 25.82, 26.28 and 27.37), seven methylene carbons (δ 21.80, 22.75, 28.13, 29.63, 29.96, 34.93 and 45.48), four methine carbons (δ 32.94, 36.92,

50.64 and 51.24), four quaternary carbons (δ 41.83, 45.96, 48.39 and 50.73), three hydroxymethine carbons (δ 65.16, 65.75 and 75.71), four olefinic carbons (δ 124.35, 130.84, 131.95 and 145.73) and a formyl carbon (δ 207.89). Signals of 1H and ^{13}C -NMR spectra were assigned by DEPT, 1H - 1H COSY and 1H - ^{13}C COSY spectra. The FAB-MS spectrum showed molecular ion at m/z 495 $[M+Na]^+$ and at m/z 511 $[M+K]^+$. These spectral data indicated that compound A was a tetracyclic triterpene, and it was identified as momordicine I (3,7,23-trihydroxycucurbita-5,24-dien-19-al).

1H -NMR and ^{13}C -NMR spectra of compound B showed similar signals to those of compound A, but showed the presence of the β -glucopyranosyl group (1H -NMR: δ 3.89, 4.01, 4.20, 4.34, 4.45, each 1H, and 4.95, 1H, d, $J=7.4$ Hz. ^{13}C -NMR: δ 104.16, 78.90, 78.28, 75.62, 71.75 and 62.92). The glycosylation downfield shift was observed for the signal due to C-23 (δ 65.16 to δ 75.25). The FAB-MS spectrum showed molecular ions at m/z 657 $[M+Na]^+$ and at m/z 673 $[M+K]^+$. Based on these spectral data, it was identified as momordicine II, the glucoside of momordicine I, 23- O - β -glucopyranoside of 3,7,23-trihydroxycucurbita-5,24-dien-19-al. Spectral data of momordicines I and II were previously reported in literature (Yasuda

et al., 1984; Yasui et al., 1998), and our results corresponded with those data.

The quantitative analysis by HPLC revealed that the concentrations of momordicines I and II were 1.2 mg and 3.5 mg/g fresh leaves (average of three replicates), respectively.

Feeding response of the beetle species to momordicines

A. nigripennis was strongly deterred from feeding by momordicine I at a higher concentration of 0.05 mg/ml (Fig. 4a), whereas the other three beetle species were not deterred from feeding by momordicine I even at a concentration of 2 mg/ml (Fig. 5a). *A. nigripennis* was deterred from feeding by momordicine II at a higher dose of 0.5 mg/ml (Fig. 4b). *A. femoralis* was also deterred from feeding at a concentration of 5 mg/ml, while the feeding of two other lady beetles was not affected by momordicine II at a dose of 2 mg/ml (Fig. 5b).

Fig. 4. Feeding responses of *A. nigripennis* to momordicines I (a) and II (b) with five different concentrations. Each test was replicated five times. **Significant difference between control and treatment at $p < 0.01$ (Mann-Whitney test).

Feeding response of lady beetles to mixtures of fractions and momordicines

Since two lady beetle species were not deterred from feeding by momordicines alone, fraction 4, which was a strong deterrent to all four beetle species, and fractions 3, 5 and 6, which were weak feeding deterrents, were mixed and used for feeding assays. These results showed that both *E. admirabilis* and *E. boisduvali* were deterred from feeding by the mixture of fraction 4 and other fractions. To investigate momordicine II, which is the main component of fraction 4, acting as a synergistic feeding deterrent with other fractions, a feeding test was conducted using a mixture of momordicine II and other fractions. These results revealed that *E. admirabilis* was strongly deterred from feeding by a mixture of momordicine II and fraction 3, whereas *E. boisduvali* was strongly deterred from feeding by a mixture of momordicine II and fraction 6. A mixture of momordicine I and momordicine II was also

Fig. 5. Feeding responses of *A. femoralis*, *E. admirabilis* and *E. boisduvali* to momordicines I (a) and II (b) with different concentrations. Each test was replicated five times. **Significant difference between control and treatment at $p < 0.01$ (Mann-Whitney test).

Fig. 6. Feeding responses of *E. admirabilis* and *E. boisduvali* to the mixture of fractions (Fr.) and momordicines. MD 1 and MD 2 indicate momordicine I and momordicine II, respectively. Each test was replicated five times. Inhibition (%) was calculated by the formula described in Fig. 2.

used in a feeding test. The result showed that the mixture of momordicines deterred both species from feeding, but the deterency was weaker than that of the mixture of momordicine II and other fractions (Fig. 6).

DISCUSSION

In the host plant selection by phytophagous insects, not only chemical constituents but also physical properties such as toughness and trichomes are important. *M. charantia* leaves have soft and smooth surfaces compared with *S. angulatus* leaves. Therefore, the unpalatability of *M. charantia* leaves was attributed to some chemicals in the leaves. Previous studies also indicated that the resistance of *M. charantia* to herbivorous insects was caused by the presence of feeding deterrents such as momordicine II (Chandravadana, 1987; Yasui et al., 1998). Our results revealed that momordicine I as well as momordicine II strongly deterred the feeding of *A. nigripennis*. Moreover, momordicine I deterred feeding by *A. nigripennis* at lower concentrations than momordicine II. This is the first report demonstrating that momordicine I acts as a feeding deterrent and the activity is stronger than that of momordicine II.

A. femoralis was deterred from feeding only by momordicine II at a concentration of 5

mg/ml. This is a higher amount than is present in *M. charantia* leaves. Because the leaves of *S. angulatus* contain some feeding stimulant(s) for beetle species, it can possibly reduce the feeding deterency of momordicines to *A. femoralis*. Among cucurbitaceous feeding leaf beetle species, *A. femoralis* has a wide host range and opportunity to contact various plant chemicals, whereas *A. nigripennis* has a narrow host plant range and usually depends on the genus *Trichosanthes* as hosts. Therefore, *A. femoralis* seems to be less affected by plant chemicals such as momordicines than *A. nigripennis*. Since *A. nigripennis* was also deterred from feeding by ethyl acetate fraction and silica gel column fractions, *A. nigripennis* is more sensitive to feeding deterrents in *M. charantia* leaves than the other three species.

On the other hand, *E. admirabilis* and *E. boisduvali* were not deterred from feeding by momordicines I and II at the higher concentration present in *M. charantia* leaves. Although the chloroform fraction was a strong feeding deterrent against these beetle species, the feeding deterrent activity decreased after fractionation by silica gel column chromatography. *E. admirabilis* was strongly deterred from feeding by a mixture of momordicine II and fraction 3,

while *E. boisduvali* was strongly deterred from feeding by a mixture of momordicine II and fraction 6. Although the mixture of momordicines I and II also deterred feeding of both species, the activity was weaker than that of the mixture of momordicine II and other fractions.

These results indicate that synergistic feeding deterrent(s) with momordicine II are present in *M. charantia* leaves and act as multiple feeding deterrents to the lady beetle species. Momordicine I is thought to be a synergist. *M. charantia* leaves contain at least six cucurbitane triterpenoids including momordicines I and II (Yasuda et al., 1984; Fatope et al., 1990). It is unknown whether the other compounds act as feeding deterrents. In conclusion, it appears that *M. charantia* leaves can avoid being attacked by many phytophagous insects due to the presence of many feeding deterrents, especially momordicines I and II.

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