Antifeedants of Indian Barnyard Millet, *Echinochloa frumentacea* Link, against Brown Planthopper, *Nilaparvata lugens* (Stål)

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Eight compounds isolated from Indian barnyard millet have been identified as l-malic acid, trans-aconitic acid, (+)-isocitric acid, 5-O-caffeoylquinic acid, 4-O-caffeoylquinic acid, isocarlinoside, 2'-(2'O-glucuronosyl)glucuronosyltricin, respectively. These compounds showed high antifeeding activity against brown planthopper only when they were combined.

**Key words: Nilaparvata lugens, Antifeedant, Echinochloa frumentacea**

**Introduction**

Three species of planthopper, brown planthopper [*Nilaparvata lugens* (Stål)], white-black planthopper [*Sogatella furcifera* (Horváth)], and smaller brown planthopper [*Laodelphax striatellus* (Fallén)], are well known as notorious rice pests in many Asian countries including Korea, China, and Japan. Continuous study of the feeding behaviour of these planthoppers led to find out that Indian barnyard millet, *Echinochloa frumentacea* Link, is resistant to *N. lugens* and *S. furcifera*, but susceptible to *L. striatellus* (Kim et al., 1994a). Through biological and chemical studies, we have come to the conclusion that resistance of this millet to each planthopper species is due mainly to the presence of antifeedants (Kim et al., 1994a). This paper deals with isolation and identification of antifeedants of *N. lugens* in Indian barnyard millet and presents feeding responses to the isolated compounds.

**Materials and Methods**

**Insect**

The brown planthopper has been reared successively on rice seedlings at 25 °C, under 14 h illumination.

**Plant**

Indian barnyard millet was grown in a greenhouse with no pesticides.

**Separation of the antifeedants of *N. lugens***

Fresh stems and leaves of Indian barnyard millet (4.8 kg) were cut in rather large pieces (about 10 cm long), immersed in methanol (7 l) for 4 d, and decanted. This procedure was repeated three times. The combined methanol extract was evaporated under reduced pressure. The residue was dissolved in water (2.5 l) and washed three times with hexane (1.5 l × 3). The aqueous phase was evaporated to dryness, leaving a brownish oil (108.6 g). This oil (51.6 g) was separated into neutral (16.35 g), basic (4.82 g), and acidic (10.09 g) fractions by column chromatography on cation exchange resin (Dowex 50W X 8, H⁺ form, 200–400 mesh) eluted with 2 n NH₄OH and subsequently on anion exchange resin (Dowex 1 X 8, formate form, 200–4000 mesh) eluted with 20 n formic acid. The acidic fraction was then chromatographed on a reverse phase open column (ODS, 100–200 mesh, Fuji Silysia Chemical Ltd.) eluted with water, 20% aqueous methanol, 40% aqueous methanol, and methanol to obtain ODS H₂O (2.84 g), ODS 20% MeOH (0.58 g), ODS 40% MeOH (0.33 g), and ODS MeOH (0.08 g) fractions.

**Instruments**

SI-, EI-, and GC-mass spectra were recorded with a Hitachi M-80 mass spectrometer. SIMS data were measured at 8 kV with Xe as the primary beam gas, and the samples were put on a silver sample stage mixed with glycerol. GC-MS was
measured at 70 eV. GLC analyses were done with a Hewlett Packard 5790A instrument with a fused silica column (25 m × 0.2 mm i.d.) coated with OV-101 (0.25 mm thickness), programmed from 70 °C (2 min holding) to 300 °C at a rate of 4 °C/min. The 20% aqueous methanol fraction was analyzed by HPLC (column, Cosmosil 5Ph, 250 mm × 10 mm i.d.; flow rate, 2 ml/min; detection at 254 nm) eluted with a mixture of water, methanol, acetonitrile, and acetic acid (85:10:5:1 v/v/v). The 40% aqueous methanol fraction was analyzed by HPLC (column, Cosmosil 5Ph, 250 mm × 10 mm i.d.; flow rate, 2 ml/min; detection at 254 nm) eluted with a mixture of water, methanol, acetonitrile, and acetic acid (70:20:10:1 v/v/v). Optical rotation was measured with a JASCO ORD Model J-5 spectropolarimeter. 1H NMR and 13C NMR spectra including two-dimensional correlation spectra were measured with a JNM AL400 (400 MHz) instrument. TMS was used as an internal standard. Letters (br.), s, d, t, q, and m represent (broad)singlet, doublet, triplet, quartet, and multiple, respectively, and coupling constants are given in Hz.

**l-Malic acid (1):** $t_R = 21.6$ min. – $[\alpha]_D^{21} = -6.0^\circ$ (c 10, MeOH). – 1H NMR (D2O): $\delta = 4.66$ (1H, t, $J = 5.8$), 2.95 (2H, d, $J = 5.8$). – GC-MS (as methyl ester): $m/z$ (%) = 130(40), 103(100), 71(13), 59(25), 43(56).

**trans-Aconitic acid (2):** $t_R = 26.7$ min. – 1H NMR (DMSO-d6 + D2O): $\delta = 2.49$ (2H, s), 6.70 (1H, s). – 13C NMR (DMSO-d6 + D2O): $\delta = 32.8$ (t), 128.9 (d), 140.3 (s), 166.6 (s), 167.4 (s), 171.1 (s). – GC-MS (as methyl ester): $m/z$ (%) = 184(98), 157(45), 156(94), 153(100), 125(53), 113(29), 59(80).

**(-)-Isocitric acid (3):** $t_R = 34.2$ min. – $[\alpha]_D^{24} +125^\circ$ (c 1.0, MeOH). – 13C NMR (CDCl3 as methyl ester): $\delta = 32.2$ (t), 45.0 (d), 52.0 (q), 52.3 (q), 52.9 (q), 70.7 (d), 171.3 (s), 172.1 (s), 173.4 (s). – GC-MS (as methyl ester): $m/z$ (%) = 175(20), 146(16), 143(66), 115(100), 83(17), 59(21).

**5-O-Caffeoylquinic acid (4):** SIMS: $m/z$ (%) = 355 (M+H+, 38), 185 (matrix peaks, 2×glycerol+H+, 100), 163(20). – 1H NMR (DMSO-d6 + D2O): $\delta = 7.47$ (Ca-3, 1H, d, $J = 16.0$), 7.03 (Ca-2’, 1H, d, $J = 1.6$), 6.98 (Ca-6’, 1H, dd, $J = 1.6, 8.1$), 6.77 (Ca-5’, 1H, d, $J = 8.1$), 6.21 (Ca-2, d, $J = 16.0$), 5.19 (Q-5, 1H, m), 3.86 (Q-3, 1H, m), 3.55 (Q-4, 1H, m), 2.2 ~ 1.7 (Q-2, 6, 4H, m). – 13C NMR (DMSO-d6 + D2O): $\delta = 175.2$ (Q-COOH, s), 169.1 (Ca-COO-, s), 149.4 (Ca-4’, s), 146.9 (Ca-3, d), 146.8 (Ca-3’, s), 128.1 (Ca-1’, s), 122.9 (Ca-6’, d), 116.5 (Ca-5’, d), 115.9 (Ca-2’, d), 115.3 (Ca-2, d), 75.5 (Q-1, s), 74.9 (Q-4, d), 73.0 (Q-5, d), 68.4 (Q-3, d), 41.5 (Q-2, t), 36.8 (Q-6, t) (Ca, caffeic acid; Q, quinic acid).

**4-O-Caffeoylquinic acid (5):** SIMS: $m/z$ (%) = 355 (M+H+, 8.7), 185 (matrix peaks, 2×glycerol+H+, 41), 163(100). – 1H NMR (DMSO-d6 + D2O): $\delta = 7.51$ (Ca-3, 1H, d, $J = 16.0$), 7.05 (Ca-2’, 1H, d, $J = 12$), 7.01 (Ca-6’, 1H, dd, $J = 1.2, 8.1$), 6.78 (Ca-5’, 1H, d, $J = 8.1$), 6.29 (Ca-2, d, $J = 16.0$). – 13C NMR (DMSO-d6 + D2O): $\delta = 175.2$ (Q-COOH, s), 169.1 (Ca-COO-, s), 149.4 (Ca-4’, s), 146.9 (Ca-3, d), 146.8 (Ca-3’, s), 128.1 (Ca-1’, s), 122.9 (Ca-6’, d), 116.5 (Ca-5’, d), 115.9 (Ca-2’, d), 115.3 (Ca-2, d), 76.4 (Q-1, s), 79.3 (Q-4, d), 69.7 (Q-5, d), 65.1 (Q-3, d), 42.7 (Q-2, t), 38.5 (Q-6, t) (Ca, caffeic acid; Q, quinic acid).

**Isocarlinoside (6-C-α-Larabinopyranosyl-8-C-β-D-glucopyranosyl-2β-equinol) (6):** SIMS: $m/z$ (%) = 581 (M+H+, 1.0), 505 (1.0), 185 (matrix peaks, 2×glycerol+H+, 100), 131(5.0). – 1H NMR (DMSO-d6 + D2O): $\delta = 7.41$ (Lu-2’, 6’, 2H, m), 6.94 (Lu-5’, 1H, d, $J = 8.4$), 6.61 (Lu-3, 1H, s), 5.0 – 3.2 (G-1 ~ 6 and A-1 ~ 5, 11H, m). – 13C NMR: $\delta = 182.0$ (Lu-4, s), 164.1 (Lu-2, s), 164.7 (Lu-7, s), 158.0 (Lu-5, s), 154.9 (Lu-9, s), 149.6 (Lu-4’, s), 145.7 (Lu-3’, s), 121.8 (Lu-6’, d), 119.3 (Lu-1’, s), 115.5 (Lu-5’, d), 114.0 (Lu-2’, d), 103.6 (Lu-10, s), 102.5 (Lu-3, d), 108.0 (Lu-6, s), 104.9 (Lu-8, s), 73.2 (G-1, d), 70.7 (G-2, d), 78.9 (G-3, d), 70.1 (G-4, d), 82.0 (G-5, d), 61.5 (G-6, t), 73.8 (A-1, d), 68.4 (A-2, d), 74.1 (A-3, d), 69.5 (A-4, d), 70.8 (A-5, t) (Lu, luteolin; G, glucose; A, arabinose).

**2-O-Rhamnosylisoorientin (7):** SIMS: $m/z$ (%) = 595 (M+H+, 14), 185 (matrix peaks, 2×glycerol+H+, 100), 131 (1.0). – 1H NMR (DMSO-d6 + D2O): $\delta = 7.41$ (Lu-2’, 6’, 2H, m), 6.96 (Lu-5’, 1H, d, $J = 8.8$), 6.63 (Lu-3, 1H, s), 6.56 (Lu-8, 1H, s), 5.07 (R-1, 1H, br.s), 4.75 (G-1, 1H, d, $J = 9.8$), 4.25 (G-2, 1H, m), 3.2 ~ 3.7 (G-3, 4.5, 5H, m), 2.43 (R-5, 1H, dq, $J = 9.4, 6.0$), 0.62 (R-6, 3H, d, $J = 9.4$). – 13C NMR: $\delta = 181.9$ (Lu-4, s),
163.4 (Lu-2, s), 162.7 (Lu-7, s), 161.4 (Lu-5, s), 156.6 (Lu-9, s), 149.4 (Lu-4, s), 145.5 (Lu-3', s), 121.7 (Lu-1', s), 119.1 (Lu-6', d), 116.2 (Lu-5', d), 113.3 (Lu-2', d), 108.6 (Lu-6, s), 103.7 (Lu-10, s), 103.0 (Lu-3, d), 93.9 (Lu-8, d), 80.9 (G-5, d), 79.5 (G-3, d), 75.7 (G-2, d), 71.6 (G-1, d), 70.5 (G-4, d), 61.3 (G-6, t), 100.6 (R-1, d), 71.7 (R-4, d), 70.5 (R-2, d), 70.4 (R-3, d), 68.2 (R-5, d), 17.4 (R-6, q) (Lu, luteolin; G, glucose; R, rhamnose).

7-O-(2"O-Glucuronosyl)glucuronosyltricin (8): SIMS: m/z (%) = 683 (M+H+, 14), 329(100), 229(45), 185 (matrix peaks, 2× glycerol+H+, 100). – 1H NMR (DMSO-d6 + D2O): δ = 7.26 (T-2', 6', 2H, s), 6.82 (T-6, 8, 2H, m), 6.38 (T-3, 1H, s), 3.93 (OMe-3', 5', 6H, s), 6.38 (Gl-1, 1H, d, J = 6.7), 4.03 (Gl-5, 1H, d, J = 9.5), 3.50 (Gl-2', 3', 2H, m), 3.42 (Gl-4, 1H, t, J = 9.5), 4.54 (Gl-1', 1H, d, J = 7.8), 3.65 (Gl-5', d, J = 9.3), 3.2 – 3.3 (Gl-3', 4', 2H, m), 3.00 (Gl-2', 1H, dq, J = 7.8, 8.3). – 13C NMR: δ = 182.0 (T-4, s), 164.1 (T-2, s), 162.1 (T-7, s), 160.8 (T-5, s), 156.8 (T-9, s), 148.0 (T-3', 5', s), 139.6 (T-4', s), 120.3 (T-1', s), 105.4 (T-10, s), 104.3 (T-2', 6', d), 103.8 (T-3, d), 99.0 (T-6, d), 95.2 (T-8, d), 56.2 (T-3', 5'-OMe, q), 170.0 (Gl-6, s), 97.8 (Gl-1, d), 82.3 (Gl-2, d), 74.6 (Gl-3, d), 74.6 (Gl-5, d), 70.5 (Gl-4, d), 170.0 (Gl-6', s), 104.2 (Gl-1', d), 75.5 (Gl-5', d), 75.2 (Gl-3', d), 73.8 (Gl-2', d), 71.4 (Gl-4', d) (T, tricin; Gl, glucuronic acid).

Hydrolysis of compound 8

Compound 8 (500 mg) was mixed with 1 ml of 5% hydrochloric acid in water, and was heated at 80°C for 1 h. The solution was passed through a Sep-pak C18 cartridge (Waters) eluted with 10 ml of water, and then with 10 ml of 40% aqueous methanol. Glucuronic acid was recovered from the water eluent and tricin was obtained from the 40% aqueous methanol eluent.

Results and Discussion

The aqueous phase showed high antifeeding activity against N. lugens after the methanol extract of Indian barnyard millet stems and leaves was defatted with hexane. This phase was separated into acidic, neutral, and basic fractions by column chromatography on cation and anion exchange resins. Of these fractions, only the acidic fraction had activity as feeding deterrent to N. lugens (Fig. 1A).

The acidic fraction was then chromatographed on a reverse phase open column (ODS, 100–200 mesh) eluted with water, 20% and 40% aqueous methanol, and methanol, successively. The bioassay showed that the combined fraction of water, 20% and 40% aqueous methanol eluents had the same level of activity as the original acidic fraction, while each fraction alone was much less active. This indicates that the inhibitory activity for feeding is not attributable to a single component but to several components combined (Fig. 1A).

The water fraction, mainly composed of organic acids, was analyzed by capillary GLC after methylation using methanolic hydrogen chloride. The 20% and 40% aqueous methanol fractions were analyzed by HPLC (column, Cosmosil 5Ph, 250 mm × 10 mm i.d.; flow rate, 2 ml/min; detection at 254 nm) eluted with a mixture of water, methanol, acetomitrile, and acetic acid. Rice plant was also treated through the same procedure, and the corresponding water, 20% and 40% aqueous methanol fractions of the acidic part were obtained. All analyses were done with a concentration equivalent to a 10% solution of the original extract, but a two-fold concentration was used in the GLC analysis of the water fraction of the rice plant extract. Repeated bioassays guided to isolate compounds 1–8 (Fig. 2) as antifeedants against N.
lugens from the Indian barnyard millet extract, most of which were not detected or were at low levels in the rice plant extract.

Compounds 1, 2, and 3 in the water eluate were identified as l-malic acid, trans-aconitic acid, and (+)-isocitric acid, respectively, by comparing $^1$H and $^{13}$C NMR and mass spectra, and optical rotation values with those of authentic samples after methylation by using 5% hydrogen chloride in methanol or being intact. l-Malic acid and (+)-isocitric acid were reported as a part of the antifeedants against N. lugens from finger millet, Eleusine coracana Gaertn. (Kim et al., 1994b). trans-Aconitic acid was also reported as an antifeedant against N. lugens from barnyard grass, Echinochloa oryzicola Vasing (Kim et al., 1975, 1976).

The $^1$H and $^{13}$C NMR, and secondary ion mass spectra (SIMS) of compounds 4 and 5 gave very similar patterns, and the molecular weights of compounds 4 and 5 were estimated from the SIMS spectra to be the same (M+H$,^+$, 355). The $^1$H NMR spectra of 4 and 5 had characteristic 1,3,4-substituted benzene ring signals, trans-olefinic signals with the coupling constant $J = 16.0$ Hz, and quinic acid moieties at $\delta$ 3.86 ~ $\delta$ 5.19 (compound 4) and $\delta$ 3.86 ~ $\delta$ 4.71 (compound 5), respectively. The methine proton of position 5 in the quinic acid moiety of compound 4 and that of position 4 of compound 5 were shifted downfield in the $^1$H NMR spectra. These downfield shifts clearly indicate the typical acylation effect on these positions, respectively (Couperus et al., 1978; Wenkert et al.,...
1978). Compounds 4 and 5 were, therefore, identified as 5-O-cafeoylquinic acid and 4-O-cafeoylquinic acid by comparing the spectral data of compounds 4 and 5 with those of authentic chlorogenic acid (3-O-cafeoylquinic acid). Chlorogenic acid is well known as one of the widely distributed common phenylpropanoid derivatives in many plants, but could not be detected in either rice plant or Indian barnyard millet.

Compounds 6, 7, and 8 were expected from their $^1$H NMR and $^{13}$C NMR spectra in the field of aromatic and oxygen-attached regions as flavonoid glycosides. The molecular weights of compounds 6, 7, and 8 were guessed from the SIMS spectra to be 580, 594, and 682, respectively. Compounds 6 and 7 were identified as isocarlinoside and 2′-O-rhamnosylisorientin by comparing the spectral data of compounds 6 (Wenkert et al., 1978) and 7 (Agrawal et al., 1989). The two-dimensional NMR (H-H COSY, C-H COSY, and HMQC) data of these compounds also suggested these identifications.

Compound 8 has a trinic moiety as an aglycon as judged from the $^1$H and $^{13}$C NMR spectra in the aromatic field and is an O-glycoside because three protons at positions 3, 6, and 8 in the aglycon were observed. Two mole of the glucuronic acid were obtained after acid hydrolysis. The carbon atom at position 2 in one of the glucuronic acid moieties was shifted downfield in the $^{13}$C NMR spectrum, and correlations were observed between the anomeric proton and the methine carbon atom at position 2 and between the other anomeric proton and the carbon atom at position 7 in the aglycon by two-dimensional NMR spectroscopy, respectively. Compound 8 was, therefore, identified as 7-O-(2′-O-glucuronosyl)glucuronosyltricin. This compound is a very rare flavonoid glycoside, which has been only found in alfalfa, *Medicago sativa* L. (Stochmal et al., 2001).

Contents of these isolated compounds contained in the 10% solution of each original plant extract were estimated by HPLC, using an authentic specimen or an isolated compound as an internal standard, as shown in Table I. As far as l-malic acid (1) and (+)-isocitric acid (3) are concerned, these compounds were also contained in the rice plant extract, but the amounts were far less than those in the Indian barnyard millet extract as shown in Table I.

The combined solution of the identified compounds, as listed in Table I, and the original acidic fraction of Indian barnyard millet were neutralized with KOH and subjected to a bioassay as described in our previous paper (Kim et al., 1994a).

As shown in Fig. 1B, the eight isolated compounds showed the same level of activity on feeding of *N. lugens* as the original acidic fraction of the Indian barnyard millet extract only when all of them were combined.

It is characteristic to note that trans-aconitic acid (2), the antifeedant of barnyard grass against *N. lugens* (Kim et al., 1975, 1976), was also detectable in the Indian barnyard millet extract but not in the rice plant extract. The amount of 2 in Indian barnyard millet seems to be enough to show anti-feeding activity against *N. lugens* because it is almost the same amount as that in barnyard grass (Kim et al., 1975, 1976). Though the water eluate of Indian barnyard millet contains enough amount of trans-aconitic acid, the fraction did not show strong activity alone. This result clearly indicates that the antifeedant of barnyard grass against *N. lugens* must be reinvestigated and that the participation of components other than trans-aconitic acid must be evaluated, though this acid has been shown to be common in many species of plants including gramineous plants (Burau, 1969; Clark, 1969; Stout et al., 1967) and may be still one of the key active compounds involved in the resistance of gramineous plants against *N. lugens*.

In our previous report (Kim et al., 1994b), we isolated and identified nine compounds as anti-feeding against *N. lugens* from finger millet, i.e., l-malic acid and (+)-isocitric acid were isolated from the water eluate of finger millet extract, five derivatives of phenylpropanoid and benzoic acid from the 20% aqueous methanol eluate, and two

<table>
<thead>
<tr>
<th>Compound</th>
<th>Indian barnyard millet</th>
<th>Rice plant</th>
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<tbody>
<tr>
<td>1-Malic acid (1)</td>
<td>2950</td>
<td>1380</td>
</tr>
<tr>
<td>trans-Aconitic acid (2)</td>
<td>6800</td>
<td>ND</td>
</tr>
<tr>
<td>(+)-Isocitric acid (3)</td>
<td>3420</td>
<td>570</td>
</tr>
<tr>
<td>5-O-Caffeoylquinic acid (4)</td>
<td>270</td>
<td>ND</td>
</tr>
<tr>
<td>4-O-Caffeoylquinic acid (5)</td>
<td>220</td>
<td>ND</td>
</tr>
<tr>
<td>Isocarlinoside (6)</td>
<td>50</td>
<td>ND</td>
</tr>
<tr>
<td>2″-O-Rhamnosylisorientin (7)</td>
<td>70</td>
<td>ND</td>
</tr>
<tr>
<td>7-O-(2″-O-Glucuronosyl)glucuronosyltricin (8)</td>
<td>130</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detectable.
flavonoid glycosides from the 40% aqueous methanol eluate. These compounds show high antifeeding activity against *N. lugens* only when they are combined, but each of these compounds does not show strong activity like those of Indian barnyard millet. Antifeedants against *N. lugens* isolated from finger millet and those from Indian barnyard millet are quite different except for L-malic acid and (+)-isocitric acid. Both results, however, may indicate that the antifeeding activity against this insect species expresses only when enough amounts of organic acid and appropriate phenyl-

propanoid derivatives and flavonoid glycosides exist and all of them are combined.

In near future, we will try to elucidate the resistance of Japanese barnyard millet, *Echinochloa ute-
lis* Ohwi et Yabuno, which is resistant to *N. lugens*, but susceptible to *S. furucifera*, and the resistances of Indian barnyard millet and finger millet against *S. furucifera*, respectively.

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Burau R. G. (1969), Polarographic estimation of aconi-


