Osajin and Pomiferin, Two Isoflavones Purified from Osage Orange Fruits, Tested for Repellency to the Maize Weevil (Coleoptera: Curculionidae)

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ABSTRACT The fruit of the osage orange tree, *Maclura pomifera* (Raf.) Schneid (Moraceae), has long been thought to be repellent to insects. A preliminary study reported here confirmed repellency of fruit extracts to the maize weevil, *Sitophilus zeamais* Motschulsky. Two isoflavones, osajin and pomiferin, were isolated from the mature fruit of *M. pomifera* in high purity (≥95%). Testing of purified osajin and pomiferin failed to show repellency. Repellency is likely caused by factors other than isoflavones in the fruit.

KEY WORDS *Maclura pomifera*, *Sitophilus zeamais*, osajin, pomiferin, isoflavone, repellency

THE FRUIT OF the osage orange, *Maclura pomifera* (Raf.) Schneid (Moraceae), has been used as an insect repellent for many years. Pioneers placed the ripe fruit of this tree in cupboards to repel roaches and other insects (Sand 1991, Brandies 1979). Past research indicates that there may be scientific justification for this well-popularized use. Karr and Coats (1991) found fragments of the fruit, as well as its hexane and methanol extracts, to be significantly repellent to the German cockroach *Blattella germanica.* The wood of the osage orange tree resisted termites (Esenther 1977; Wolcott 1953, 1955, 1957) and had antifungal properties (Smith and Perino 1981). Antimicrobial activity has been found in extracts containing osajin or pomiferin (Mahmoud 1981), two of the major angular isoflavonoid components present in osage orange fruits (Fig. 1A) (Walter et al. 1938, Wolfrom and Mahan 1942). Osage orange fruit was also found to contain small amounts of the linear isoflavonoids scandenone and auriculasin, structural isomers of osajin and pomiferin, respectively (Fig. 1B) (Delle Monache et al. 1994).

In this study we report repellency from the extracts of *M. pomifera* fruit. Because osajin and pomiferin are well known to occur in this fruit, the extraction and purification of osajin and pomiferin is reported. The results of an assay that examined the behavioral response of the maize weevil *Sitophilus zeamais* Motschulsky to highly purified (≥95%) osajin and pomiferin are reported as well.

Materials and Methods

Extraction of Fruits for Preliminary Assay. Mature *M. pomifera* fruit was collected in Auburn, NE, in October 1995, and stored at −20°C for 8 mo. For both extraction types reported here, the fruits were divided into "rind" and "core" portions. The rind was defined as the portion of the fruit from the outer surface inward to the outer perimeter of the seed layer. The core was defined as the seed layer and extending inward. After thawing to room temperature, the fruit was cut into 250-g rind or 250-g core pieces and placed into separate soxhlet extraction apparatuses. For each fruit portion, rind or core, one soxhlet apparatus contained 500 ml hexane, another contained 500 ml dichloromethane, and a third contained 500 ml methanol. Each apparatus was heated to the boiling point of its respective solvent and allowed to cycle for 24 h. The solvent was collected, filtered to remove particulates by using Whatman (Hillsboro, OR) G8 glass fiber filters, and passed through anhydrous sodium sulfate to remove water. The solvents were removed by rotary evaporation at 500 mmHg vacuum and 30°C. Alternatively, 250 g of rind and core pieces were placed in 500 ml of either hexane, dichloromethane, or methanol and allowed to soak for 24 h. The particulates were removed by vacuum filtration, the water was removed with anhydrous sodium sulfate and the solvents were removed by rotary evaporation. The residues were redissolved in a minimum amount of solvent and used in the bioassays described later in this section. The extracts obtained from each method were tested in a preliminary round of testing (data not shown). Those extracts obtained by soaking extraction of the cores and rinds with hexane, by soxhlet extraction of the rind and core with dichloromethane, and by soxhlet extraction of the rind with methanol were retested

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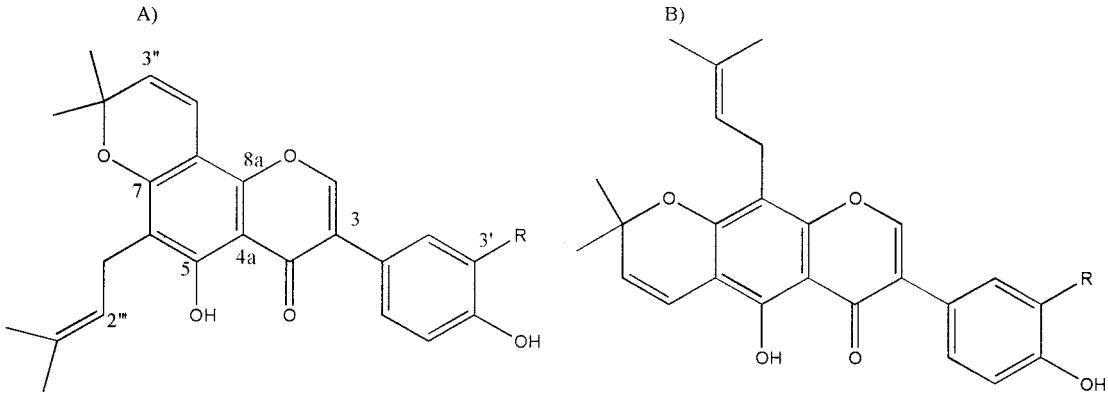

Fig. 1. Structures of (A) osajin (R = H) and pomiferin (R = OH) and (B) scandenone (R = H) and auriculasin (R = OH).

because they showed potential activity in the initial testing. The results of the second round of testing are those reported here. Repellency was measured at 0.5, 18, and 24 h.

Extraction of Osajin and Pomiferin. After thawing to room temperature, 12 fruits were cut into 2-cm^3 pieces and pressed by hand to obtain the juice, which contained high amounts of the components of interest. The collected juice was centrifuged for 30 min. Thin-layer chromatography detected no osajin or pomiferin in the water layer. The water layer was discarded, and the particulates were extracted three times with 200 ml dichloromethane (ACS certified grade, Fisher, Pittsburgh, PA). The dichloromethane solution was passed through anhydrous sodium sulfate, collected, and vacuum filtered. The pressed fruit pieces were extracted also with three 500-ml portions of dichloromethane, which were passed through anhydrous sodium sulfate to remove water and then vacuum filtered. The collected dichloromethane was combined with the dichloromethane extract of the juice. The dichloromethane was removed from the combined extracts via rotary evaporation. The residue was dissolved in acetone and passed through a large (5.5 by 40 cm) open silica gel column wet-packed with acetone. Acetone (100%, ACS certified grade, Fisher, Pittsburgh, PA) was used as the eluent, and washing continued until products were no longer observed in the eluate, as determined by spotting the eluate on thin-layer chromatography (TLC) plates, using 1:1 hexane:ethyl acetate as a solvent system.

The acetone was removed by rotary evaporation. The resultant mixture, containing osajin and pomiferin, as well as other components, was run through an open silica gel column with 9:1 hexane:ethyl acetate as an eluent. Fractions were collected in 25-ml portions until the fractions containing osajin began to contain pomiferin as well, evidenced by TLC. The solvent system was then changed to 4:1 hexane:ethyl acetate until the fractions began to contain pomiferin only. At that time, the solvent system was changed to 7:3 hexane:ethyl acetate until pomiferin was no longer detected in the fractions. Fractions containing a mixture of osajin and pomiferin were purified either on an identical column, or by preparatory thin-layer chromatography in 1:1 hexane:ethyl acetate. The purity of the resulting osajin and pomiferin samples was determined by high-pressure liquid chromatography (HPLC). The identities of osajin and pomiferin were confirmed by using solid-probe electron-impact mass spectrometry (EIMS) and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR).

Analysis and Identification. The melting range of each isolated compound was determined by using a Fisher-Johns Melting Point Apparatus (Pittsburgh, PA). Infrared spectra (IR) of these compounds were obtained by using a Beckman Acculab 2 (Fullerton, CA). High-pressure liquid chromatography was conducted by using a Hewlett–Packard Series 1100 HPLC (Palo Alto, CA) and a Waters μBondupak C_{18} column (Milford, MA) with a flow rate of 2 ml/min of 1:1 acetonitrile:water initially and ramped to 100% acetonitrile over 10 min. Peaks were detected at 254 nm by using a Kratos Spectraflow 757 variable-wavelength detector (Chestnut Ridge, NY). Proton nuclear magnetic resonance spectra (^{1}H-NMR) in deuteroacetone and carbon nuclear magnetic resonance spectra (^{13}C-NMR) in deuterochloroform (tetramethylsilane internal standard) were obtained on a Varian VXR-300 NMR (Palo Alto, CA). A Finnigan TSQ700 mass spectrometer (San Jose, CA) was used to obtain the mass spectra, with solid probe introduction of the sample, and electron impact of 70 eV.

Behavioral Bioassay of Osajin and Pomiferin. The repellency assay is based on that used for German cockroaches by Karr and Coats (1991). Maize weevil (*S. zeamais*) adults from a colony reared in the laboratory for over five years were used. The repellency tests were conducted in a choice apparatus, which consisted of two 35-ml jars connected at their mouths by transparent plastic tubing (7 cm by 1.5 cm i.d.). For the preliminary test, the residue from the extraction process was dissolved in a minimum amount of solvent, and 1 ml was applied to the filter paper. Appropriate solvent controls were conducted. Highly pure (≥95%) osajin or pomiferin was diluted in acetone to

Table 1. Repellency of five osage orange extracts to the maize weevil

Extraction method	% repelled from treated chamber (± SEM)		
	0.5 h	18 h	24 h
Soak: Hexane core	56.0 ± 6.0	41.0 ± 3.0	28.0 ± 3.0
Soak: Hexane rind	62.5 ± 12.5	59.5 ± 9.5	41.0 ± 3.0
Soxhlet: Dichloromethane core	40.5 ± 21.5	34.5 ± 3.5	72.0 ± 3.0
Soxhlet: Dichloromethane rind	90.5 ± 9.5	97.0 ± 3.0	84.5 ± 3.5
Soxhlet: Methanol rind	56.5 ± 12.5	50.0 ± 0.0	59.5 ± 15.5

50 mg/ml (approximately saturated), then serially diluted to 5 and 0.5 mg/ml. One milliliter of one of these solutions (treated side) or 1 ml of acetone (untreated side) was applied to a filter paper, and the acetone allowed to evaporate in a fume hood before the papers were placed in the choice-jar units. The set-up for the control groups was the same but both sides contained papers previously treated with 1 ml acetone. Each assay was conducted with 20 insects, starting with 10 per jar. The test had five replicates. Two series of randomizations were carried out; one for each replicate to determine the position of the choice-jar units in relation to one another, and a second to determine for each treatment unit the position of the treated side, either to the right or to the left. The distribution of the insects, in the treated side, the untreated side, and in the tube connecting the two sides was determined at 1, 17, 24, and 48 h. Any repellency of the test compounds would likely occur on contact because of the compounds' low volatility, therefore the assumption was made that the chemical repelled the insects in the connecting tube. The data were analyzed by using analysis of variance (ANOVA) to detect significance. The four time points were compared with each other by using least significant difference (LSD) (lsd, $\alpha = 0.05$).

Results and Discussion

Extraction of Fruit and Preliminary Assay. Upon removal of the solvent, 250-g fruit pieces yielded 1.14 g in hexane soaking extraction of the core, 0.77 g in hexane soaking extraction of the rind, 1.93 g in dichloromethane soxhlet extraction of the core, 1.84 g in dichloromethane soxhlet extraction of the rind, and 28.0 g in methanol soxhlet extraction of the rind. The methanol extraction resulted in a syrup, because starch, sugars, and water are likely to be soluble in hot methanol.

Table 1 reports the percentage of weevils repelled from the treated chamber (±SEM). ANOVA detected that significance caused by extraction method was not found at 0.5 h, but was found at 18 ($F = 25.06$; df = 4, 5; $P < 0.002$) and 24 h ($F = 9.32$; df = 4, 5; $P < 0.02$). In cases where significance was observed, the dichloromethane extraction of the rind was the most effective. Because osajin and pomiferin are well known to occur in the extracts of this plant, we decided to focus on these two compounds as the potential active ingredients.

Analysis and Identification. The purity of osajin was found by HPLC to be 94.6%, and the purity of pomiferin was 98.2%.

The melting point range for osajin was observed to be from 186 to 191°C, and past studies report a melting point of 190–192°C (Jain and Sharma 1974). Scandenone has a reported melting point range of 164–165°C (Delle Monache et al. 1994). Pomiferin melted at 172–176°C, at which temperature a color change occurred and the substance resolidified. This resolidified substance melted at 204–209°C. The product was recovered from the melting point apparatus with acetone, and the solution was run on TLC with 1:1 hexane: ethyl acetate. Visualization of the plate with potassium permanganate (0.1 M) spray revealed a spot identical in R_f value to the unheated sample ($R_f = 0.55$), and a lower spot that streaked from the origin to Rf = 0.30. Pomiferin has a reported melting point of 200.5°C at maximum purity, whereas auriculasin melts at 176–178°C (Delle Monache et al. 1994).

Infrared spectroscopy of osajin showed peaks at the following frequencies: 3,610 cm^{-1}, 3,060 cm^{-1} 1,650 cm^{-1}, 1,620 cm^{-1}, 1,590 cm^{-1}, and 1,520 cm^{-1}, 1,435 cm^{-1}, 1,380 cm^{-1}, 1,275 cm^{-1}, 1,230 cm^{-1}, and 1,180

Table 2. Repellency of osajin and pomiferin to the maize weevil

Compound	Dose (mg/chamber)	% repelled from treated chamber (±SEM)			
		1 h	17 h	24 h	48 h
Osajin	50	76 (3.3)a	59 (3.3)a	45 (3.9)a	47 (4.1)a
	5.0	68 (9.8)a	41 (4.8)b	48 (4.1)ab	48 (4.9)ab
	0.5	51 (8.0)a	38 (3.7)b	48 (2.6)ab	47 (4.6)ab
	0 (control)	61 (4.8)a	54 (11.7)a	42 (12.1)a	40 (4.7)a
Pomiferin	50	76 (7.0)a	55 (7.6)a	53 (4.6)a	58 (11.4)a
	5.0	70 (6.1)a	53 (4.6)a	57 (9.6)a	55 (8.5)a
	0.5	75 (4.2)a	62 (8.5)ab	57 (4.6)b	64 (4.0)ab
	0 (control)	61 (4.8)a	54 (11.7)a	42 (12.1)a	40 (4.7)a

Values followed by the same letter within a row are not significantly different according to LSD ($\alpha = 0.05$).

cm^{-1}. The reported structures of osajin (Wolfrom et al. 1946) and scandenone (Pelter and Stainton 1966) both contain bonds that may give rise to these observed peaks.

The mass spectra of osajin and pomiferin agreed with that reported by Delle Monache et al. (1994). Osajin and its isomer scandenone are indistinguishable from one another by mass spectrometry, as are pomiferin and auriculasin.

The spectrum of osajin obtained by ^{13}C-NMR in deuterochloroform has peaks corresponding to the carbons of osajin, as displayed in Fig. 1A. These chemical shifts should differ from those of Delle Monache et al. (1994), who report chemical shifts in deuteroacetone. The spectrum displayed the following peaks: δ 181.14 (C at 4), δ 159.26 (C at 5), δ 157.31 (C at 4′), δ 155.98 (C at 7), δ 152.37 (C at 2), δ 150.55 (C at 8a), δ 131.64 (C at 3‴), δ 130.34 (C at 2′ and 6′), δ 127.17 (C at 3″), δ 123.57 (C at 3), δ 122.98 (C at 1′), δ 121.90 (C at 2‴), δ 115.70 (C at 3′ and 5′), δ 114.94 (C at 4″), δ 112.90 (C at 6), δ 105.58 (C at 4a), δ 100.76 (C at 8), δ 77.09 (C at 2″), δ 28.12 (the two 2″ methyls), δ 25.80 (*trans*-3‴ methyl), δ 21.30 (C at 1‴, and δ 17.92 (*cis*-3″ methyl). See Fig. 1A for designation of carbon atoms. Determination of carbon positions was achieved through comparison with Delle Monache et al. (1994) and published ^{13}C-NMR spectra from similar compounds (Silverstein et al. 1991).

Delle Monache et al. (1994) reported ^{1}H-NMR spectrum for osajin and pomiferin in deuteroacetone. Our spectrum of osajin agreed with theirs to within δ 0.03 for all peaks, except for δ 8.50, which they report as δ 8.60. This proton is on a hydroxyl group, the chemical shift of which can vary more so than other protons. Delle Monache et al. (1994) reported diagnostic peaks occurring at δ 8.17 and 3.31 for osajin, and at δ 8.26 and 3.41 for scandenone. Our spectrum agreed with osajin. Our analysis of pomiferin by ^{1}H-NMR in deuteroacetone agreed with theirs to within δ 0.02, except for δ 8.00, which varied from theirs by δ 0.07. As was the case with osajin, this proton occurred on a hydroxyl group. Delle Monache et al. (1994) reported diagnostic peaks at δ 8.16 and 3.31 for pomiferin, and δ 8.24 and 3.40 for auriculasin. Our spectrum was consistent with pomiferin.

Scandenone is present in *M. pomifera* fruit at around 15% of the concentration of osajin, and auriculasin is present at 13% of pomiferin's concentration (Delle Monache et al. 1994). Though our MS, IR, and ^{13}C-NMR data are not conclusive, the observed melting point, ^{1}H-NMR, and the relative abundance in the plant reported in Delle Monache et al. (1994) indicate that we have isolated the angular isoflavones osajin and pomiferin by the processes outlined here. Small amounts of scandenone and auriculasin are present as minor contaminants, evidenced by minute peaks on ^{1}H-NMR corresponding to the diagnostic peaks of the linear isomers.

Behavioral Assay of Osajin and Pomiferin. The results of the behavioral assay are summarized in Table 2. The data are expressed as the mean percentage of insects repelled from the treated chamber (±SEM). ANOVA detected significance caused by time for osajin ($F = 7.73$; df = 3, 64; $P < 0.001$) and pomiferin ($F = 4.72$; df = 3, 64; $P < 0.005$). Significance caused by concentration was not seen for osajin or pomiferin, and the interaction between concentration and time was not significant for either compound. Although the number of insects repelled in 1 h was significantly more at 50 than at 0.5 mg osajin per chamber, it was not significantly different from the control, because there was no significance caused by concentration in the model. When significance was seen for a concentration (at $\alpha = 0.05$) among the various time intervals, the number of insects repelled decreased with increasing time in the apparatus. Because repellency caused by concentration was not significant, we concluded that factors other than osajin or pomiferin, unknown to us at this time, affected the distribution of the insects within the apparatus.

No significant repellency was observed when the weevils were exposed to purified osajin and pomiferin. We have thus concluded that neither osajin nor pomiferin is singularly responsible for *M. pomifera* repellency to *S. zeamais.* Many of the constituents in this plant are volatile, but were not examined in this study. Volatility is important for repellency over large areas, such as in homes or workplaces. Efforts are currently underway in our laboratory to identify volatile components from this fruit and to test the repellency of these volatile components in behavioral assays.

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