

## Identification of Annonaceous Acetogenins in the Ripe Fruit of the North American Pawpaw (*Asimina triloba*)

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The North American pawpaw [*Asimina triloba* (L.) Dunal] is a tree fruit in the early stages of commercial production in the United States. This plant contains annonaceous acetogenins in the twigs, unripe fruit, seeds, roots, and bark tissues, which display antitumor, pesticidal, antimalarial, anthelmintic, piscicidal, antiviral, and antimicrobial effects, suggesting many potentially useful applications. However, commercial development of these compounds, based on twig extracts, has been problematic due to limited availability of biomass for extraction. Additionally, acetogenin compounds contained in fruit of pawpaw relatives (soursop or *Annona muricata*) and tea made from the leaves of these plants may lead to an increased risk of atypical Parkinsonism later in life with overconsumption of these compounds. Therefore, the objectives of this study were (1) to determine if extracts of ripe pawpaw fruit pulp displayed acetogenin activity, (2) to identify potential acetogenin compounds in the fruit tissue, and (3) to determine if the acetogenin activity varied in diverse pawpaw genotypes and closely related *Annona* species. Extracts of ripe fruit had total extract weights and bioactivity using the brine shrimp bioassay similar to those from 'NC-1' pawpaw twig tissue. Pulp from soursop, cherimoya, and several additional pawpaw cultivars ('Mitchell', 'Overleese', 'NC-1', 'Zimmerman', 'Wells', and 'Sunflower') also displayed bioactivity, but peach or banana pulp did not. Ripe pawpaw pulp extract subjected to HPLC-MS analysis identified three prominent acetogenins: asimicin, bullatacin, and bullatalacin. This study points to pawpaw fruit pulp serving as a new biomass source for the extraction of acetogenin compounds for product development. An assessment of the potential human health risk of overconsumption of fruit and acetogenin bioavailability and degradation studies should be pursued.

**KEYWORDS:** Antitumor; pesticidal; antimalarial; anthelmintic; piscicidal; antiviral; antimicrobial; pawpaw cultivars

### INTRODUCTION

Annonaceous acetogenins are a large class of unique structurally homogeneous polyketide (C32 or C34 fatty acid) compounds found in the Annonaceae family, which includes both genera *Annona* and *Asimina* (1, 2). These compounds are potent inhibitors of mitochondrial (complex I) as well as cytoplasmic (anaerobic) production of adenosine triphosphate (ATP) and related nucleotides. Acetogenin compounds are powerful cytotoxins and display in vivo antitumor, pesticidal, antimalarial, anthelmintic, piscicidal, antiviral, and antimicrobial properties, suggesting many potentially useful applications (1–3). Commercial development of these compounds could range from pesticides to topical or dietary supplement products. Applications and commercial products include a shampoo for treating infestations of head lice, fleas, and ticks, a series of pesticidal sprays, and an

ointment for the treatment of oral herpes (HSV-1) and other skin afflictions (1).

Acetogenins, such as asimicin and bullatacin, may prove to be important compounds for combating specific cancers in the future. Oberlies et al. (4) evaluated 14 acetogenins against the MCF-7/Adr cell line to establish their structure–activity relationships (SARs); of the 14 acetogenins, 13 were generally more potent than all three of the standard drugs. Bullatacin was 258 times more cytotoxic against the MCF-7/Adr cell line than adriamycin. Asimicin, bullatacin, and the bullatacinones were also active in laboratory mice bearing implanted L-1210 leukemia (5). Certain acetogenins are often selectively cytotoxic to one or only a few cancer cell lines (6, 7). Recently, Cuendet et al. (8) tested pawpaw twig extracts containing bullatacin, asimicin, and trilobacin in the *N*-methyl-*N*-nitrosourea-induced mammary carcinogenesis model. With Sprague–Dawley rats given a diet containing pawpaw extract (1250 and 2500 mg/kg of diet; based on maximum tolerated dose studies), mammary tumor latency was increased from 55 to 66 days. Insecticides based on

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**Table 1.** Dried Weight of Extract and Brine Shrimp Mortality (LC<sub>50</sub>) Values for Extracts for the Pawpaw Cultivar 'NC-1' for Frozen Pulp, Dried Pulp, Dried Twig Tissue, and Frozen Peach and Fresh Banana Pulp<sup>a</sup>

sample	LC <sub>50</sub> (ppm)	wt of extract (mg/g of DW)	wt of tissue extracted (g)	actual dry wt (g)
'NC-1' dried twig	0.01 (±3.01)	9.3 a	2.5	2.5
'NC-1' dried pulp	1.09 (±3.72)	11.0 a	2.5	2.5
'NC-1' frozen pulp	0.02 (±2.63)	4.9 ab	9.0	2.5
peach frozen pulp	560 (±258)	1.8 b	9.0	2.5
banana fresh pulp	5204 (±294)	2.1 b	9.0	2.5

<sup>a</sup> Parentheses enclose standard error; any two means within a column not followed by the same letter are significantly different at  $P \leq 0.05$  using Fisher's protected LSD mean separation.

acetogenins serve as slow-acting toxins and are particularly effective against chewing insects (3). Acetogenin compounds are toxic to several pest insects including cockroaches, Colorado potato beetle, blowfly larvae, Mexican bean beetles and their larvae, bean leaf beetle, mosquito larvae, two-spotted spider mites, striped cucumber beetles, European corn borers, melon or cotton aphids, and a nematode (9–12).

The North American pawpaw [*Asimina triloba* (L.) Dunal] is a tree fruit in the initial stages of commercial production in the United States (13, 14). There are about 40 commercially available clonal pawpaw cultivars with excellent fruit quality that are propagated clonally by grafting or budding with scionwood from superior trees onto common seedling rootstock of diverse genetic origin (15). Previous studies have identified over 40 other bioactive acetogenins and other compounds in the crude extracts of twigs, unripe fruits, seeds, root, and bark of the North American pawpaw (16–18). Pawpaw twigs show seasonal variation in acetogenin bioactivity, displaying the highest bioactivity during the growing season, in May and June, with increased concentrations of asimicin, bullatacin, and trilobacin (18).

The identification of a larger biomass source of acetogenin compounds would be helpful in developing commercial products based on acetogenin compounds. Fruit biomass represents a potentially larger, about 35 lb of fruit fresh weight (9.9 lb of dry weight)/mature tree/year versus about 5 lb of fresh weight (1.8 lb of dry weight) of young twig tissue/mature tree/year, source for extractable acetogenin compounds than twig tissue for use as a botanical pesticide (19). Ripe pawpaw fruit has not been investigated as a source of these compounds. Recent scientific papers have suggested a possible link between the long-term frequent consumption of Annonaceae family fruit and tea in Guadeloupe (20–24), New Caledonia (25), and people of Caribbean origin (26) and a higher rate of atypical Parkinsonism in later life. Acetogenin compounds present in the fruit and leaves of these pawpaw relatives in the genus *Annona* [e.g., soursop (*Annona muricata*)] may be responsible for the development of atypical Parkinsonism in later life (27, 28). However, sensitivity to acetogenin compounds may be limited to those with a specific genetic predisposition (29). If the North American pawpaw also contains these compounds, issues of bioavailability of acetogenin compounds in fruit pulp and varietal differences in acetogenin concentration would become important research areas. Therefore, the objectives of this study were (1) to determine if extracts of ripe pawpaw fruit pulp displayed acetogenin activity, (2) to identify the major acetogenin compounds in the fruit tissue if acetogenin activity was present, and (3) to determine if the acetogenin activity varied in diverse pawpaw genotypes and closely related *Annona* species.

## MATERIALS AND METHODS

**Plant Material.** Ripe pawpaw fruit was collected from the cultivar 'NC-1' in September 2005. Pulp from at least five ripe fruits were separated from skin and seeds, homogenized in a food processor, placed in zip-lock

bags, and stored in a freezer at  $-15^{\circ}\text{C}$  until extraction. Three replicate bags of five ripe fruits each were collected. Fruit weight averaged 100 g, and approximately 50 g of pulp was extracted from each fruit prior to homogenization; there was a total of about 250 g of pulp per replicate bag. About 50 g of the well-homogenized pulp from each replicate bag was thawed and dried at  $50^{\circ}\text{C}$  for 4 days prior to being extracted. Actively growing twig tissue was collected in June from 'NC-1', dried at  $50^{\circ}\text{C}$  for 4 days, and milled for extraction. Three ripe cherimoya (*Annona cherimola*) and peach fruits were purchased at a local supermarket, and the pulp was cut into small pieces and frozen until extracted. Three ripe yellow bananas were also purchased, and the pulp was cut into small pieces and the fresh tissue extracted. Canned soursop fruit pulp in syrup (La Fe Foods Inc., Moonachie, NJ) was extracted. Ripe fruits were also collected from the pawpaw cultivars 'Mitchell', 'Overleese', 'Zimmerman', 'Wells', 'Sunflower', and 'NC-1', and the material was frozen as described above for later extraction. All pawpaw fruit were produced from the orchards located at the Kentucky State University Research and Demonstration Farm (Frankfort, KY) and produced without the application of any pesticides.

**Extraction and Brine Shrimp Testing (BST).** Tissues were extracted using either dried or thawed pulp or dried twig tissue with 25 mL of 95% ethanol and concentrated through a series of chloroform/water and methanol/hexane partitions as described by Ratnayake et al. (17, 30) with the exception that only one chloroform water partition was performed. Frozen pawpaw fruit tissue contains approximately 28% dry matter, and fresh current season growth twig tissue contains about 36% dry matter (unpublished data); therefore, a correspondingly larger amount of fresh tissue was used in relation to dried tissue for extraction. Extracts were obtained from 9.0 g of frozen pulp from the pawpaw cultivar 'NC-1', 2.5 g of dried pulp and dried twig tissue, and 9.0 g of peach and banana pulp. In a second experiment, extracts were obtained from 9.0 g of thawed peach and cherimoya fresh pulp, canned soursop pulp, and 'NC-1' and 'Sunflower' pawpaw thawed pulp. Twenty-five milligrams of extract, which was between 40 and 60% of the total dried extract weight, was removed from the final concentrated fraction and dissolved in 5 mL of methanol; 10, 100, and 1000  $\mu\text{L}$  amounts were transferred to 20 mL liquid scintillation vials corresponding to 10, 100, and 1000 ppm concentrations. Methanol was completely evaporated from the vials before 20 brine shrimp larvae, taken 48 h after initiation of hatching in artificial seawater, were added to each vial, and the final volume of each vial was adjusted to 5 mL using artificial seawater. After 24 h, survivors were counted and LC<sub>50</sub> values with standard error were computed using probit regression analysis and a log<sub>10</sub> transformation for extract concentration using the statistical software SPSS (SPSS Inc., Chicago, IL). At least three replicate extractions from individual zip-lock bags of pulp were examined for each treatment.

To evaluate the genetic variation in ripe fruit pulp for bioactivity among pawpaw cultivars, the LC<sub>50</sub> values were calculated for 95% ethanol extracts from 9 g of frozen pulp from the cultivars 'Mitchell', 'NC-1', 'Overleese', 'Zimmerman', 'Wells', and 'Sunflower' on the basis of a response curve of 0, 10, 100, and 1000 ppm from three replicate extractions.

**HPLC-MS Analysis.** HPLC-MS was completed on the basis of the method of Gu et al. (18) at Nature's Sunshine Products (Spanish Fork, UT) for determining the presence of acetogenin compounds in extracts from frozen pulp of ripe fruit of the pawpaw cultivar 'NC-1'.

## RESULTS AND DISCUSSION

Extracts of ripe fruit of the pawpaw cultivar 'NC-1' had similar bioactivity or brine shrimp mortality, as indicated by LC<sub>50</sub> values,

to 'NC-1' twig tissue (**Table 1**). Both frozen and dried pawpaw pulp extracts showed similar bioactivity levels. Extracts of banana and peach, which were included as additional controls, displayed bioactivity only at the highest extract concentration. The  $LC_{50}$  values were  $< 1$  ppm for extracts of 'NC-1' dried twig and pulp, as well as frozen pulp, indicating high bioactivity in these samples. It is possible that as the frozen pulp thawed and dried, some of the acetogenin compounds present may have been metabolized or degraded as indicated by a trend for higher  $LC_{50}$  values for dried pulp. Using the method of Ratnayake et al. (17, 30), the greatest dried extract weight per gram of dry weight (DW) of pulp or twig material was found for 'NC-1' dried pulp and twig (**Table 1**).

Bioactivity of pawpaw extracts was compared to extracts of fruit from the pawpaw relatives soursop and cherimoya. Soursop

**Table 2.** Weight of Extract and Brine Shrimp Mortality ( $LC_{50}$ ) Values for Extracts from Canned Soursop Pulp and Frozen Peach, Cherimoya, and 'NC-1' and 'Sunflower' Pawpaw Pulp<sup>a</sup>

sample	$LC_{50}$ (ppm)	wt of extract (mg/g of FW)
cherimoya	991 ( $\pm 153$ )	3.7 b
soursop	8.89 ( $\pm 13.03$ )	1.6 c
'NC-1' pawpaw	0.04 ( $\pm 1.59$ )	5.1 a
'Sunflower' pawpaw	4.47 ( $\pm 2.08$ )	1.8 c
peach	1813 ( $\pm 183$ )	1.3 c

<sup>a</sup> Parentheses enclose standard error; any two means within a column not followed by the same letter are significantly different at  $P \leq 0.05$  using Fisher's protected LSD mean separation.

**Table 3.** Brine Shrimp Mortality ( $LC_{50}$ ) Values for the Pawpaw Cultivars 'Mitchell', 'NC-1', 'Overleese', 'Zimmerman', 'Wells', and 'Sunflower' from Frozen Pulp of Ripe Fruit<sup>a</sup>

pawpaw cultivar	$LC_{50}$ (ppm)	pawpaw cultivar	$LC_{50}$ (ppm)
'Mitchell'	0.31 ( $\pm 1.02$ )	'Zimmerman'	4.09 ( $\pm 0.49$ )
'Overleese'	0.38 ( $\pm 1.48$ )	'Wells'	7.33 ( $\pm 1.27$ )
'NC-1'	0.89 ( $\pm 1.41$ )	'Sunflower'	8.60 ( $\pm 0.42$ )

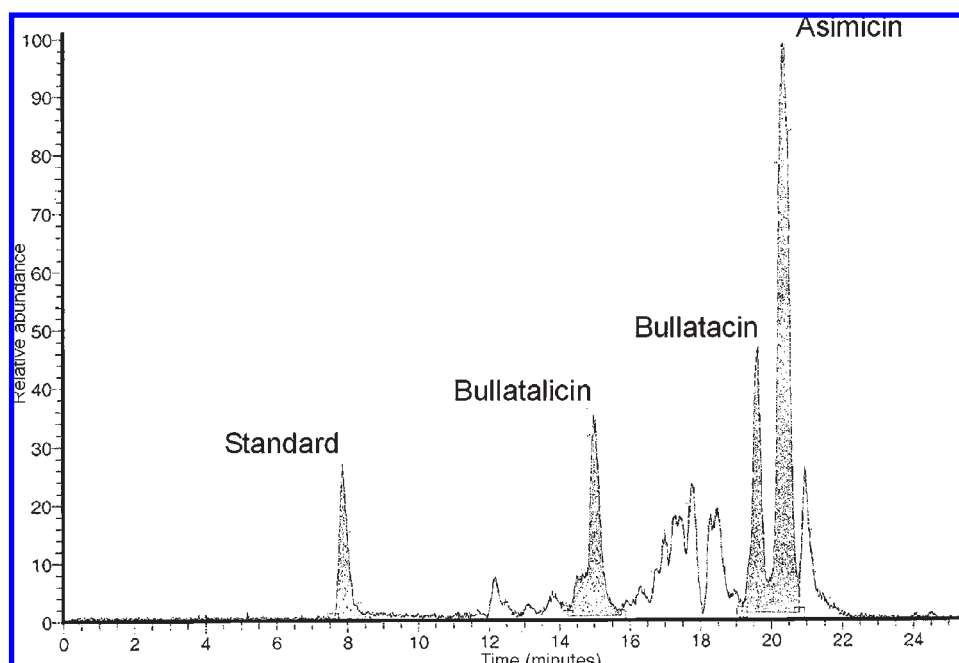
<sup>a</sup> Parentheses enclose standard error.

fruit contains acetogenin compounds (27, 28); however, acetogenin bioactivity has not been reported in cherimoya fruit. In terms of brine shrimp mortality, extracts obtained from ripe fruit pulp of the pawpaw cultivar 'NC-1' were again high in bioactivity; however, pulp from the pawpaw cultivar 'Sunflower' displayed lower bioactivity than observed for 'NC-1' (**Table 2**). Soursop displayed lower activity than either pawpaw cultivar, but higher bioactivity than peach or cherimoya. The  $LC_{50}$  values were  $< 1$  ppm for extracts of 'NC-1' pulp, followed by 'Sunflower' and soursop, with cherimoya and peach displaying little bioactivity (**Table 2**). Using the method of Ratnayake et al. (17, 30), the greatest total weight of extract per gram of FW of material was for 'NC-1' pulp, followed by soursop (**Table 2**).

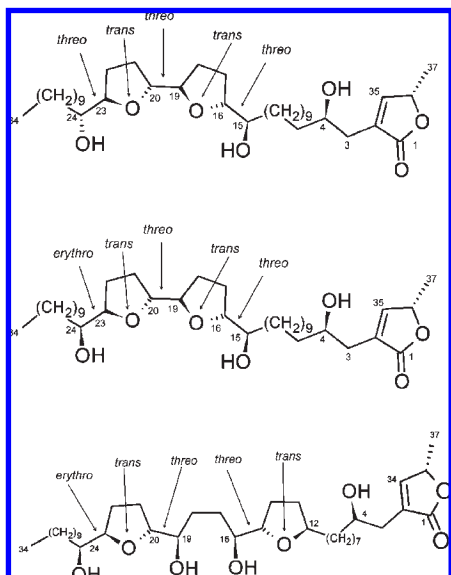
To determine whether bioactivity of frozen ripe fruit pulp varied by genotype, six commercially available pawpaw cultivars were evaluated for bioactivity. The  $LC_{50}$  values were highest ( $< 1.0$  ppm) in the cultivars 'Mitchell', 'NC-1', and 'Overleese', with 'Wells' and 'Sunflower' displaying lower activity ( $< 7.0$  ppm) and the cultivar 'Zimmerman' displaying intermediate activity (4.0 ppm; **Table 3**).

A sample of frozen ripe pawpaw pulp extract was obtained using the enrichment method of Ratnayake et al. (17, 30) and was subjected to HPLC-MS analysis. Three acetogenin compounds were identified at high levels in the sample: asimicin, bullatacin, and bullatalicin (**Figures 1 and 2**). The acetogenin compounds asimicin, bullatacin, and trilobacin have been reported at high levels in pawpaw twig samples previously by Gu et al. (18), and bullatalicin was reported by Martin et al. (31) in pawpaw twigs.

The methods used for tissue extraction likely yielded a mixture of compounds in addition to acetogenin compounds. Ripe pawpaw fruit pulp contains phenolic and antioxidant compounds that may have influenced extract efficacy in the bioassays (33). However, HPLC-MS analysis identified acetogenin compounds in the fruit pawpaw extracts, and the relative amount of acetogenin compounds in the extracted material was likely reflected in the bioassay. Some hydrophobic substances contained in the extracts could also display bioactivity. Acetogenin compounds have been identified in pawpaw twigs, bark, leaves, and unripe



**Figure 1.** Ripe pawpaw pulp extract from the cultivar 'NC-1' subjected to HPLC-MS analysis. The three largest peaks correspond to the acetogenin compounds asimicin, bullatacin, and bullatalicin.



**Figure 2.** Chemical structures for the three main annonaceous acetogenin compounds, asimicin (top), bullatacin (middle), and bullatalicin (bottom), identified in ripe pawpaw pulp.

fruit tissues (2, 3), but have not previously been reported in the ripe fruit pulp. Pawpaw fruit pulp could serve as a large renewable source of biomass for the extraction of acetogenin compounds. Fruit biomass of >35 lb of fruit fresh weight (9.9 lb of dry weight)/mature tree/year versus about 5 lb of fresh weight (1.8 lb of dry weight) of young twig tissue/mature tree/year (unpublished data) would be possible for some pawpaw genotypes. Additionally, Gu et al. (18) reported seasonal fluctuations in twig acetogenin levels that limited the harvest period for maximum yield of acetogenin compounds. Ripe fruit would provide a renewable source of biomass for extraction of these compounds, and predictable fruit harvest times would facilitate the procurement of large amounts of biomass for extraction. Therefore, ripe pawpaw fruit could represent a more cost-effective source than twigs for extractable acetogenin compounds for commercial products.

There are recent scientific papers suggesting a possible link between the long-term frequent consumption of Annonaceae family fruit and tea in Guadeloupe (20–24), New Caledonia (25), and Caribbean origin populations (26) and a higher rate of atypical Parkinsonism in later life. Acetogenin compounds in the fruit and leaves of these pawpaw relatives may be responsible for the development of atypical Parkinsonism in later life (27, 28). The bioactivity for pawpaw was higher than that of the canned soursop used in this study; however, the canning process may have reduced the bioactivity of the soursop pulp, and fresh fruit was unavailable for comparison.

The consumption of North American pawpaw has not been reported to cause atypical Parkinsonism. Occasional consumption of acetogenin compounds may not induce atypical Parkinsonism in later life (32). Sensitivity to acetogenin compounds may be limited to those with a specific genetic predisposition, and some tau, microtubule-associated proteins, variants may be more vulnerable than others to mitochondrial dysfunction (29). Limited consumption of pawpaw, which is primarily a short-term seasonal event, may avoid increased risk of atypical Parkinsonism. Pawpaw fruit pulp may serve as a new large biomass source for the extraction of acetogenin compounds and points to the need for an assessment of bioavailability and degradation studies in mammals.

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