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THE EXTRACTION, ISOLATION, AND ANTIOXIDANT ANALYSIS OF ANNONACIN FROM THE FRUIT OF NORTH AMERICAN PAWPAW (*Asimina triloba*)

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Abstract

Soxhlet extractions using ethanol as a solvent, were performed to extract Annonaceous acetogenins (annonacin) from the fruit of the North American Pawpaw (*Asimina triloba*). Extracts were then analyzed using ultraviolet spectrophotometry to identify annonacin and any other acetogenin-like compounds present in the samples. Determined concentrations of annonacin present in fruit samples were then analyzed and compared to known antioxidants through formation and detection of reactive oxygen species using visible spectroscopy in a 96 well plate reader. Putrescine and synthesized polyamine compounds B3HP, B4HP, B5HP had comparable antioxidant activity to the standard, ascorbic acid, while annonacin extracts had less antioxidant activity.

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Introduction

The Annonaceae family contains over 2,500 known species of plants, and 135 genera, many of which are grown for their fragrant flowers, edible fruit, and medicinal properties (1). Plants of the Annona genus originate in the American tropics. Many of the species have important historical significance to the indigenous peoples of South America such as the Incas, who used it for medicinal purposes and as a food crop (2). An infusion of the seeds and leaves is currently used medicinally in some cultures to treat intestinal parasites, head lice, and a variety of other ailments. Additionally, the extracts of the seeds and leaves are used as a natural insecticide in areas where species of Annona are widely grown (1). Some of the most commonly cultivated species are the (*Annona cherimoya cherimola*) cherimoya, (*Annona muricata*) guanabana, and (*Annona x atemoya*) atemoya. The manufacture of pawpaw (*Asimina triloba*) products has started to increase in popularity in the Midwestern United States (2, 3). The Annonaceae family hosts a class of unique compounds classified as Annonaceous acetogenins, which are polyketide long-chain fatty acids. The compound annonacin $C_{35}H_{64}O_7$ (Figure 1) is one example (2, 4). Annonacin is a derivative of a long-chain fatty acid that contains disubstitued tetrahydrofuran rings, a terminal butenolide and alkyl groups (3). These compounds are known to be potent inhibitors of mitochondrial complex I (NADH-dehydrogenase) (5). This enzyme functions by recycling NADH to NAD^+ in the oxidative phosphorylation metabolic pathway. The oxidative phosphorylation process creates a proton gradient across the mitochondrial inner membrane to drive production of ATP for the cell (5). Acetogenins such as annonacin selectively bind to mitochondrial complex I (NADH-dehydrogenase) to inhibit its function. This inhibition causes the enzyme to be less capable of recycling NADH back to NAD^+, and therefore, makes oxidative phosphorylation more difficult for the cell (6). This can result in a cell deficit of ATP and can later cause apoptosis or necrosis (6). In previous studies, where annonacin was introduced to model rats it was reported that in sufficient doses, brain cells experienced decreased ATP levels and neuronal death (6). Multiple studies are now proposing annonacin as a potential candidate for cancer treatment because of its cytotoxicity (7-9).

Another series of studies have found, high levels of annonacin caused cell death in brain cells that produce dopamine (10). Annonacin contributed to the neurodegenerative condition Parkinsonism caused in part by the depletion of neurons responsible for producing this neurotransmitter (11, 12). In populations where species of Annona are widely grown and consumed such as Guadeloupe, there were significantly higher than normal cases of atypical Parkinsonism which has been correlated to prolonged high exposure to annonacin (13). Several studies in Guadeloupe, found many of the individuals who were diagnosed with atypical Parkinsonism also regularly consumed Annona fruits or herbal teas (12, 14).

Antioxidants are compounds that neutralize free radicals in the body that can cause harmful effects, including heart disease and cancers (15). Further studies suggest, the acetogenins found in the root, twigs and seeds of the pawpaw tree are strong inhibitors of cancer cells (16, 17). One study found that the root, seeds, leaves and fruit of the pawpaw tree had a greater antioxidant activity due to the high concentration of annonacin's hydroxyl groups (18). A separate study found that the extraction of the enzyme papain from the pawpaw fruit was used in rejuvenation skin products (19).

Based on previous studies, our research will focus on Soxhlet extraction of annonacin of the pawpaw fruit and seeds acquired from Integration Acres in Albany, Ohio. After extraction the presents of annonacin was measured by absorbance at annonacin's lambda maximum 214 nm on an ultraviolet (UV) spectrophotometer (20). This study will also compare the antioxidant activity of the pawpaw flesh and seed extracts, putrescine, and synthesized

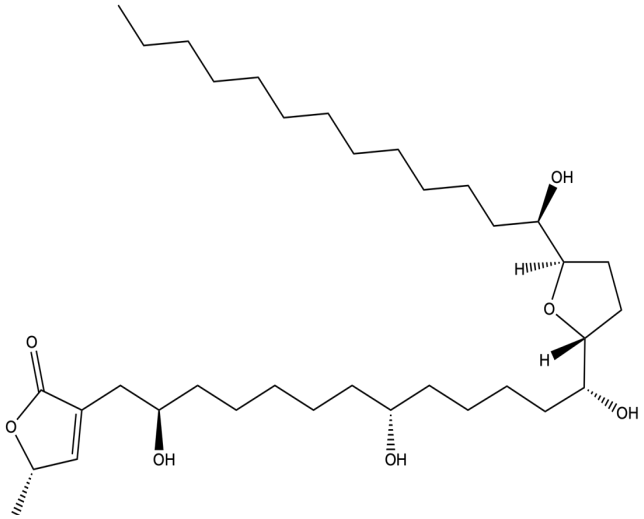

Figure 1. **:** Annonacin structure (2S)-2-methyl-4-[(2R,8R,13R)-2,8,13-trihydroxy-13-[(2R,5R)-5-[(1R)-1-hydroxytridecyl]oxolan-2-yl]tridecyl]-2H-furan-5-one

polyamine compounds (21) to the ascorbic acid standard (22, 23) using the FRAP assay. Previously putrescine has shown antioxidant activity when added to extracts of Moringa leaves from Jojoba plants (24).

The synthesized systems used in this study were putrescine analogs that were produced in our laboratory. These analogs were synthesized through a disubstitution reaction on both terminal nitrogens of the lead compound putrescine (1, 4-diaminobutane) by alkyl hydroxyl chlorides. The synthesized analogs included; N1, N4-bis(3-hydroxypropyl)putrescine (B3HP), N1, N4-bis(4-hydroxybutyl)putrescine (B4HP), and N1, N4-bis(5-hydroxypentyl) putrescine (B5HP) (21).The ferric-reducing antioxidant power (FRAP) assay is an electron transport-based method that measures the reduction of ferric ion (Fe^{3+}) ligand complex to the blue colored ferrous (Fe^{2+}) complex by antioxidants (25). When using a FRAP assay, the higher concentration of Fe^{3+} suggests less antioxidant properties (26)

Methods

Samples of pawpaw fruit were obtained from Integration Acres in Albany, Ohio in the fall of 2020. Samples of fresh pawpaw pulp, fresh seeds, and frozen pawpaw pulp were dried in a convection oven set at 40°C for a 48-hour period. Ten gram portions of the dried samples were placed in a Soxhlet extraction thimble (n = 3). These portions underwent a 72-hour Soxhlet extraction using 200 ml of 100% ethanol as a solvent (CAS number 64-17-5). Extracts were kept in a refrigerator at 4ºC until each sample was identified using an UV spectrophotometer (Perkin-Elmer Lambda 25, Pittsburgh, PA) measuring absorbance at 214 nm. Antioxidant activity was analyzed using a ferric-reducing antioxidant power assay (BioAssay Systems, FRAP Assay Kit, Hayward, CA) with a 96 well plate reader (Thermo Scientific MCC/340) at 595 nm. The concentration for each of the samples measured using the FRAP assay were 0.5 µM aqueous solutions, as compared with a 0.5 µM ascorbic acid standard. All solvents and reagents were from Sigma Aldrich; St. Louis, MO.

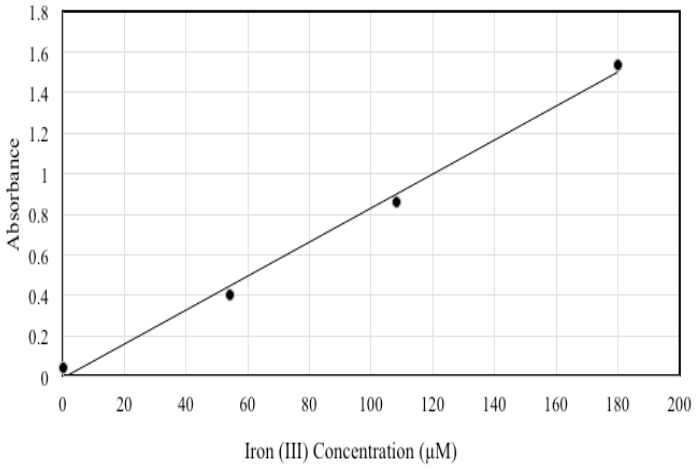

Figure 2. Standard curve of antioxidant activity of absorbance versus iron (III) concentration

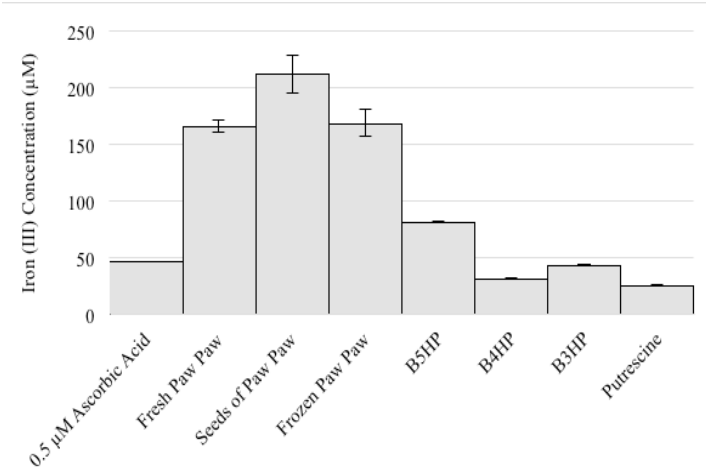

Figure 3. Antioxidant ability of varying natural and synthesized compounds

Discussion

After running the FRAP assay on iron (III) controls, a standard curve was constructed (figure 2). The standard curve was used to calculate ferric ion concentration for each sample. Figure 3 was constructed to compare the antioxidant properties of the standard ascorbic acid, annonacin extracts, synthesized putrescine analogs, and putrescine. The standard ascorbic acid had a Fe^{3+} concentration of 46.1 µM. Putrescine and the synthesized analogs all had comparable antioxidant properties to ascorbic acid. Putrescine had the lowest Fe^{3+} concentration at 24.7 µM, followed by B4HP at 31.2 µM, B3HP 42.9 µM and B5HP 80.9 µM. The annonacin extracts had higher Fe^{3+} concentrations when compared with the ascorbic acid standard. They were as follows; dried pawpaw fresh flesh 165.9 µM, dried seeds 211.9 µM and dried frozen flesh 168.7 µM. These results suggest putrescine and two synthesized analogs, B4HP and B3HP, show comparable antioxidant activity to ascorbic acid. The pawpaw extracts had almost quadruple the concentration of Fe^{3+} when compared with ascorbic acid. Based on previous work, it was expected that the annonacin extracts would have greater antioxidant activity then the results suggest. One explanation from a previous study found, the longer a fruit or vegetable remains in the refrigerator, the antioxidant activity continues to decrease (26). The annonacin extracts were stored in the refrigerator for 5-7 months before running the FRAP assay. Another explanation for the decreased antioxidant activity could be that the extracts were analyzed dissolved in their extraction solvent. Evaporation of the ethanol solvent to dryness, may increase the solid extracts antioxidant activity.

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