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Research Article

Neurotoxicity of Fruits, Seeds and Leaves of Plants in the Annonaceae Family

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Abstract

Fruits, seeds, twigs and leaves of several plants in the Annonaceae family contain neurotoxic compounds called acetogenins. Overconsumption of at least one of these fruits, graviola (Annona Muricata), caused an atypical form of Parkinson's disease on the islands of Guadeloupe, Guam and New Caledonia. It does not respond to the standard treatment with L-Dihydroxyphenylalanine (L-DOPA). This type of atypical Parkinsonism is similar to progressive supranuclear palsy, but with important differences. It is characterized by L-DOPA-resistant Parkinsonism, tremor, subcortical dementia and abnormal eye movements suggestive of Progressive Supranuclear Palsy (PSP). Patients also have hallucinations and dysautonomia, which are not characteristic of PSP. Furthermore, the oculomotor abnormalities and the tremor, which is jerky, differ from what is observed in classical PSP patients. The neurotoxicity is caused by mitochondrial dysfunction. A class of compounds called Acetogenins (ACGs) inhibits the mitochondrial NADH: Ubiquinone Oxidoreductase (complex-I of the respiratory chain). These compounds are lipophilic polyketides that are found in plants in the Annonaceae family. They have two toxicophores: a γ-Butyrolactone and one or more Tetrahydrofuran (THF) or Tetrahydropyran (THP) rings. There is also a long chain alkyl group on the other end of the molecule. Even though it strongly affects the physical-chemical properties of ACGs, it is not a toxicophores. Over 400 ACGs have been found in different plants in the Annonaceae family. They can be identified by Nuclear Magnetic Resonance (NMR) and liquid chromatography coupled to mass spectrometry (LC-MS). They form adducts with Ca^{2+} and Mg^{2+}, which helps to confirm their presence in samples.

Keywords: Parkinson's disease; Acetogenins; NADH: Ubiquinone Oxidoreductase; Graviola; Annona Muricata

Introduction

Fruits, seeds, twigs and leaves of several plants in the Annonaceae family contain neurotoxic compounds called acetogenins [1-3]. Epidemiological studies showed a link between the overconsumption of graviola (Annona Muricata) fruit and aqueous infusions of leaves (tea), and an atypical form of Parkinson's disease on the islands of Guadeloupe, Guam and New Caledonia, as well as the Afro-Caribbean and Indian population in London [4-15]. It does not respond to the standard treatment with L-Dihydroxyphenylalanine (L-DOPA) [4]. This atypical form of Parkinsonism is similar to progressive supranuclear palsy, but with important differences [4]. It is characterized by L-DOPA-resistant Parkinsonism, tremor, subcortical dementia and abnormal eye movements suggestive of Progressive Supranuclear Palsy (PSP) [11]. However, Patients also have hallucinations and dysautonomia, which are not characteristic of PSP. Furthermore, the oculomotor abnormalities and the tremor, which is jerky, differ from what is observed in classical PSP patients [11]. The neurotoxicity is caused (at least in part) by mitochondrial dysfunction [3,8,12-15]. Patients in Guadeloupe were separated into three different categories. Only one-third of them presented the classical features of idiopathic Parkinson disease. Another one-third had a syndrome resembling Progressive Supranuclear Palsy (PSP). The others were unclassifiable, according to criteria existing in 2007 [8].

A class of compounds called Acetogenins (ACGs) inhibits the mitochondrial NADH: Ubiquinone Oxidoreductase (complex I of the respiratory chain), which decreases ATP levels. This leads to neuronal cell loss and gliosis in the brain stem and basal ganglia [18-20]. However, other mechanisms of toxicity may be involved. This includes modulating histone H3 phosphorylation [16], complexation with Ca^{2+} ions [17] and somatodendritic redistribution of phosphorylated tau protein [12]. NADH: Ubiquinone Oxidoreductase is a mitochondrial enzyme present in not just neurons, but also cancer cells. As a result, there is a substantial body of literature on the anti-cancer properties of graviola, the North American pawpaw (Asimina triloba), acetogenins and acetogenin mimetics [19,21-25]. One recent article told how drinking a tea made from graviola leaves helped cure a patient who had metastatic negative breast cancer [23]. However, data on single individuals seldom means much to most oncologists. Still, it is worth noting that the dose of the major acetogenin in tea made graviola leaves (annonacin) is only 0.213% of the dose that one would receive from consuming the entire leaves that are sold as a dietary supplement [26]. That is, there are dietary supplements made from different parts of plants in the Annonaceae family that are sold as cures for cancer, even though they may be neurotoxic [1]. For graviola, "The

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Table 1: Genera and Species of Plants in the Annonaceae Family that have been Sources of Purified Acetogenins [18,30].

Genus and Species	Popular Names
Annona x *A. atemoya* (hybrid)	Atemoya, atemoia, A. squamosa x A. cherimola
Annona cherimolia	Custard apple, Chirimuya, cherimoya
Annona coriacea	Soursop, fruta-de-conde
Annona crassiflora	Marolo, Araticum do cerrado
Annona glabra	Pond apple alligator apple, cancer therapy
Annona jahnii	Manirito
Annona Montana	Mountain soursop
Annona muricata	Soursop, graviola, Brazilian pawpaw, guanábana
Annona nutans	Yaguá-nambí
Annona purpurea	Custard apple, soncoya, ox-heart
Annona reticulata	Wild sweetsop,
Annona senegalensis	African custard apple
Annona spraguei	Chirimoya; Negrito; Nonita de mono
Annona squamosa	Sugar apple, custard apple, sweetsop, fruta-do-conde, ata, pinha
Annona triloba	Pawpaw
Disepalum anomalum	Disepalum grandiflorum
Goniothalamus donnaiensis	Tian fang gu
Rollinia emarginata	Aratiku (fruit of the sky)
Rollinia mucosa	Biribá, wild sweetsop, anón cimarrón, cachiman
Rollinia sylvatica	Annona crassiflora Mart., Araticum do Mato
Uvaria boniana	Guang ye zi yu pan
Uvaria calamistrata	Ci guo zi yu pan
Uvaria grandiflora	Calabao
Uvaria narum	Neelavalli, Valeeshakhota (Ayurvedic medicine)
Uvaria tonkinensis	Dong jing zi yu pan
Xylopia aromitaca	Hemlock anon, malageuto, malageuto macho

estimated amounts of annonacin ingested in a year by eating one fruit or can of nectar a day were comparable to the dose (3.8 mg/kg/day) that induced widespread neurodegeneration in the basal ganglia and mesencephalon when infused intravenously into rats for 28 days (106 mg/kg)" [27]. More recently, it has been shown that annonacin can cross the blood-brain barrier and its bioavailability was about 3.2% of the orally ingested dose in rats [28].

The goals of this report are to review the literature on the neurotoxicities of the fruits, seeds and leaves of plants in the Annonaceae family and the biochemical toxins that are in them (acetogenins), as well as use Mass Spectrometry (MS) to find as many acetogenins as possible in the fruits of the North American pawpaw (Asimina triloba).

Plants in the Annonaceae Family

Acetogenins (ACGs) have been isolated from the fruit pulp, seeds, twigs, roots, stems, leaves and bark of many plants in the Annonaceae family, which contains about 2400 species in 108 genera, based on the sequences of nucleotides on multiple plastid DNA loci [29]. This is better than previous classifications based mostly on morphology. The new classification consists of four major clades, which were given the taxonomic rank of subfamily [29]. Some of the genera in the Annonoideae subfamily have acetogenins. They include Annona, Anonidium, Asimina, Boutiquea, Dasymaschalon, Diclinanona, Disepalum, Goniothalamus, and Neostenanthera. Even though many previous articles used the name Asimina triloba for the North American pawpaw, it is more properly called Annona triloba [29]. A partial list of plants in the Annonaceae family that have been sources of purified acetogenins is shown in Table 1 [16,17,30-32]. Note that some of them share the same common names. Also, many of them have uses in folk remedies and traditional medicine, including treating cancer. A book about the Annonaceae family was even published recently [33].

The most popular plant in the Annonaceae family is A. Muricata, also known as soursop in English and Guanabana in Spanish [15]. Soursop, Cherimoya (A. Cherimola), Sugar Apple (A. Squamosa) And Atemoya (A. Squamosa X A. Cherimola) are the most important in Brazil, where they are known as graviola, cherimólia, pinha and atemoia [34]. There is even a Worker's Nutrition program in Brazil, in which workers are encouraged to eat supposedly healthy fruits, including graviola [35]. Graviola is also recommended for oral rehydration therapy [36].

Acetogenins

Acetogenins from plants in the Annonaceae family are lipophilic polyketides [30]. Even though there is a review article that said that acetogenins are only found in this family [19], there is a report of acetogenins being found in the roots of a small tree in the genus Ampelocissus and family Vitaceae [37], and a different set of compounds also called acetogenins exist in red algae of the genus Laurencia as well as the sea hares (Dolabella Auricularia) that feed on them [38]. Also, some reports describe a set of compounds that are also called acetogenins, which are found in members of the Lauraceae family, including the popular avocado (Persea Americana) [39-41]. The so-called acetogenins from other families have very different structures and biological properties than those from the Annonaceae family [30]. Only the acetogenins from the Annonaceae family have been linked to atypical Parkinsonism [30].

Annonaceous Acetogenins (ACGs) have two pharmacophores: a γ-Butyrolactone and one or more Tetrahydrofuran (THF) or Tetrahydropyran (THP) rings. Like the acetogenins in avocados, there is also a long chain alkyl group on one end of the ACGs [42]. According to a recent review, over 400 ACGs have been found [42]. Figure 1 shows the generalized structures of the different classes of ACGs and Figure 2 shows some specific examples of ACGs [30]. They are derivatives of fatty acids and have 32 or 34 carbons, including a long chain alkyl group on one end and a monounsaturated or unsaturated lactones on the other end [30,42].

Figure 1 Structures of different types of anonaceous Acetogenins (ACGs), as described by others [19,30]. They have 32 or 34 carbons, including a long chain alkyl group on one end and a monounsaturated or unsaturated lactones on the other end. The R groups in LA, LB and LC can have 1-3 Tetrahydrofuran (THF) rings or a Tetrahydropyran (THP) ring. They also can contain different amounts of hydroxyl groups. There are many different types of R groups in the non-classical,

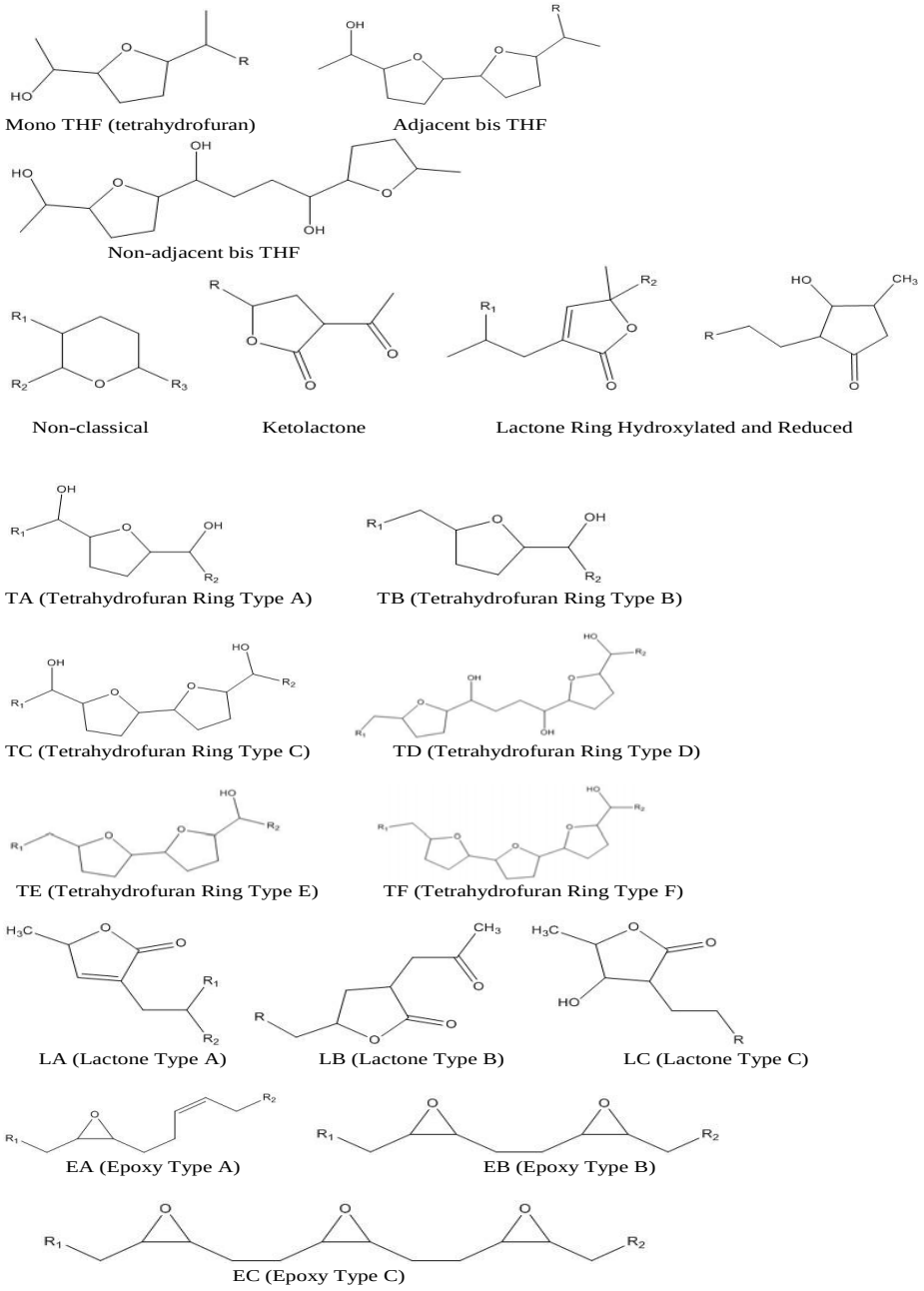
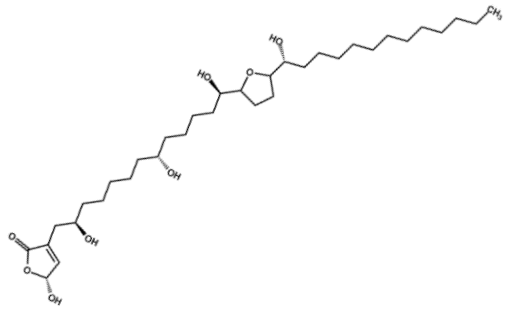


Figure 1:

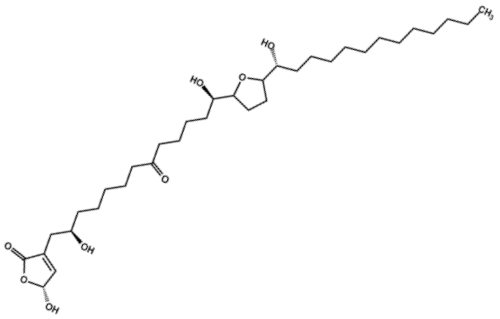
ketolactone, lactone and the hydroxylated and reduced lactones, but one of them ends in a long chain alkyl group. For example, the R_1 and R_2 in EA, EB and EC are aliphatic chains that end in a methyl on one end and a lactone on the other end

Figure 2 Structures of some of the known neurotoxic ACGs: annonacin (a mono THF ACG), annonacinone (a ketone), Squamocin, Bullatacin (di-THF ACGs), sootepensins A and sootepensins B (mono THF ACG) [22]. IC_{50}, EC_{50} death and EC_5 tau are the concentrations needed to cause 50% inhibition of the mitochondrial complex I, death in 50% of the cultured neurons and redistribution of the tau protein in 5% of the neurons, which is a standardized measure of the minimum concentration needed to cause tau pathologies [12]. Bullatacin and the sootepensins were not tested for neurotoxicity, but Bullatacin was reported to have an IC_{50} against MDR MCF-7/Adr breast cancer cells (0.0108 mg/mL) that was much lower than that of Adriamycin, which has been used for decades to help treat and cure breast cancer [45].

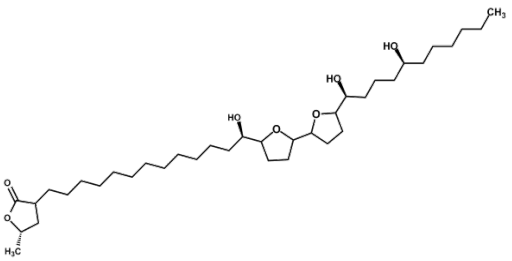
Several different parts of plants in the Annonaceae family have been analyzed for ACG content [1, 26-28, 43-51]. The methods used include Time of Flight Mass Spectrometry (TOF-MS), liquid chromatography coupled to mass spectrometry (LC-MS) and Nuclear Magnetic Resonance Spectrometry (NMR). Of special note is the ability to take the mass spectrum of a mass spectrum (MS/MS), in which a primary ion is generated in one quadrupole of the MS and then fragmented in another. In TOF-MS, analytes (such as acetogenins)



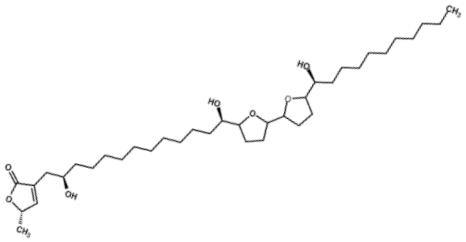
Annonacin $C_{35}H_{64}O_7$, MW 596.87846,
IUPAC name: (2S)-2-methyl-4-[(2R,8R,13R)-2,8,13-trihydroxy-13-[(2R,5R)-5-[(1R)-1-hydroxytridecyl] oxolan-2-yl]tridecyl]-2H-furan-5-one IC_{50} 55 nM, EC50 death 61 nM, EC_5 tau 44 nM



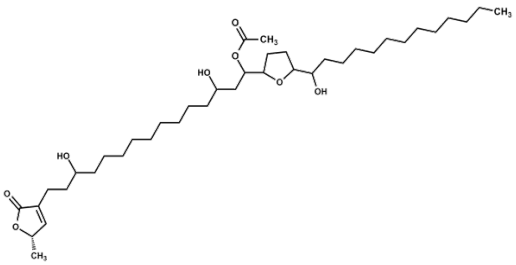
Annonacinone $C_{35}H_{63}O_7$, MW = 594.86258
IC_{50} 64 nM, EC_{50} death 190 nM, EC_5 tau 135 nM



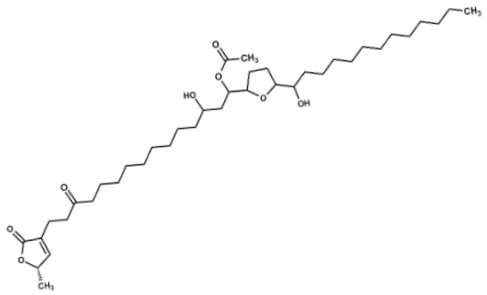
Squamocin $C_{37}H_{66}O_7$, MW = 622.9157 IC_{50} 1.4 nM, EC_{50} death 1.1 nM, EC_5 tau 0.6 nM



Bullaticin, $C_{37}H_{66}O_7$ MW = 622.9157



Sootepensin A, $C_{39}H_{70}O_8$ MW = 666.9683



Sootepensin B, $C_{39}H_{68}O_8$ MW = 664.9524

Figure 2:

are separated based on the time it takes for them to traverse the flight tube and reach the MS detector. Originally, TOF-MS was used to analyze mixtures of gases, such as N_2, O_2, CO and CO_2. However, N_2 and CO have the same molecular weights (28 atomic mass units, or amu) when measured with older TOF-MS instruments. On the other hand, modern TOF-MS instruments often have relatively high resolution and can be used to identify acetogenins [44]. However, a chemical matrix must be added to enable the production of ions when the sample is irradiated with a laser. This is called Matrix Assisted Laser Desorption and Ionization (MALDI). For example, a MALDI-TOF-TOF MS was used to produce specific patterns of fragments that unambiguous structural information on several acetogenins [44].

There are also ion trap MS instruments. For example, an LQT XL Orbitrap® was used to detect ACGs with mass accuracy of ±5 parts per million (ppm) [44]. This technology enables the determination of molecular formula. For example, the atomic mass of 12C is 12.0000,

Table 2: Suspect Acetogenins in Pawpaw Fruit Found by Direct Infusion MS.

$[M+H]^+$	Molecular Formula	$[M+Ca]^{2+}$	$[M+Mg]^{2+}$
295.1766	$C_{18}H_{30}O_3$	167.0631	159.0273
327.1414	$C_{21}H_{26}O_3$	183.0867	175.0459
381.3372	$C_{22}H_{36}O_5$	210.0870	202.1596
385.2572	$C_{25}H_{36}O_3$	212.0952	204.1028
531.4208	$C_{35}H_{62}O_3$	285.2541	277.1915
533.4326	$C_{35}H_{64}O_3$	286.2241	278.2161
535.4532	$C_{35}H_{66}O_3$	287.1956	279.2199
543.4162	$C_{37}H_{66}O_2$	291.1863	283.2520
545.4333	$C_{35}H_{60}O_4$	292.1961	284.2578
559.4492	$C_{37}H_{66}O_3$	299.1322	291.2193
561.4617	$C_{35}H_{60}O_5$	300.1966	292.2272
563.4817	$C_{35}H_{62}O_5$	301.1089	293.2359
565.4825	$C_{35}H_{64}O_5$	302.2122	294.2376
569.4402	$C_{33}H_{60}O_7$	304.2274	296.2301
573.4573	$C_{37}H_{64}O_4$	306.2313	298.228
579.4121	$C_{35}H_{62}O$	309.1774	301.2256
581.4306	$C_{35}H_{64}O_6$	310.1995	302.2223
589.4555	$C_{37}H_{64}O_5$	314.2260	306.2313
591.4020	$C_{37}H_{66}O_5$	315.2041	307.2224
593.4023	$C_{35}H_{60}O_7$	316.1911	308.2196
595.3589	$C_{35}H_{62}O_7$	317.1945	309.2162
597.4213	$C_{35}H_{64}O_7$	318.2056	310.212
605.3959	$C_{37}H_{64}O_6$	322.2197	314.1795
607.3959	$C_{37}H_{66}O_6$	323.2040	315.2045
609.4518	$C_{37}H_{68}O_6$	324.1912	316.2069
611.4704	$C_{35}H_{62}O_8$	325.1922	317.2171
613.4858	$C_{35}H_{64}O_8$	326.1939	318.2103
621.3874	$C_{37}H_{64}O_7$	330.1795	322.2137
623.3966	$C_{36}H_{62}O_8$	331.1812	323.2099
627.3835	$C_{35}H_{62}O_9$	333.1871	325.2097
629.3981	$C_{35}H_{64}O_9$	334.1963	326.1835
631.3827	$C_{35}H_{66}O_9$	335.1992	327.1882
637.3700	$C_{37}H_{64}O_8$	338.2281	330.2037
639.4128	$C_{37}H_{66}O_8$	339.2298	331.1953
645.3946	$C_{35}H_{64}O_{10}$	342.1922	334.1986
663.3932	$C_{39}H_{66}O_8$	351.1933	343.1974
665.3988	$C_{39}H_{68}O_8$	352.2048	344.1937
673.3888	$C_{37}H_{68}O_{10}$	356.1930	348.1985
681.3855	$C_{39}H_{68}O_9$	360.2811	352.2079

16O is 15.9999 and 14N is 14.0031. Thus, the compounds C_6H_{12}, C_5H_8O and $C_4H_8N_2$ have molecular weights of 84.0939, 84.0575 and 84.0688, respectively and can be distinguished by high resolution MS. So, an LQT XL Orbitrap® was used to analyze the neurotoxic ACG, annonacin. In the positive ion mode, it produced ions with Na^+ as an adduct, with sequential losses of five waters, four from the four –OH groups and one from the methyl lactone that opened in the acidic mobile phase. The Limit of Detection (LOD) was 0.3 nM in the MS mode and 0.5 μM in the MS/MS mode. In the negative ion mode, it produced a fragment that showed that C-20 was hydroxylated [44].

The same study also reported that MALDI-TOF-TOF produced more fragments at relatively high collision energies [44]. When lithium was added to the sample, it had a limit of detection of 10 nM in the MS mode and 10 μM in the MS/MS mode when Chemically Induced Ionization (CID) was used. When other ACGs were analyzed, it was found that fragment ions with an m/z=118 and/or 119 indicate the presence of a lactone of subtype-1, whereas their absence is correlated to the sub-type 2. Also, the loss of 112 amu from the lithium adducts indicates the presence of a hydroxyl group at C-4 [44].

Even though MS can identify acetogenins, pure analytical standards are needed to quantify them. Two of them, annonacin and squamocin, have been purified [43] and shared with other laboratories [1,26,50]. The concentrations of annonacin in lyophilized pawpaw (A. Triloba), graviola, atemoya, ata (A. Squamosa) and biriba (R. Mucosa) fruits were found to be 7720, 768, 3.8, 2.2 and 1.8 μg/g (dry weight), respectively [1,50]. The concentrations of squamocin in lyophilized graviola, atemoya, ata and biriba (R. mucosa) fruits were 162, 4.5, 68, 0.7 and 4.2 μg/g (dry weight), respectively [1,50]. We only had seeds from atemoya, but they had 454 and 14200 μg/g (0.454 and 14.2 mg/g) annonacin and squamocin, respectively [1]. Lyophilized graviola leaves had 306 and 17 μg/g annonacin and squamocin, respectively, but a tea made from the leaves had only 0.213% of the annonacin that was in the entire leaves and too little of the squamocin to detect [26]. That is, to analyze dried fruits, leaves or seeds for acetogenins, they must be completely extracted from the plant cells that contain them. Water (even at 100 ^{0}C) does not dissolve much of the hydrophobic (lipophilic) acetogenins. Instead, dry methanol at 100 ^{0}C and 10 MPa (100 atm) pressure in a sealed container can [1,26,50]. So, the dose of annonacin that one would consume in a tea made from dried graviola leaves would be only 0.213% of the dose that would be consumed if the entire leaves were ingested in a dietary supplement [26]. Despite the large differences in concentrations of annonacin and squamocin in graviola, atemoya, ata and biriba, they were all toxic to Lund Human Mesencephalic Neurons (LUHMES) at about the same doses [1]. So, it is quite likely that there are many other neurotoxic acetogenins in these and other fruits in the Annonaceae family.

Still, the pawpaw (Asimina Triloba) fruit might be of special concern in North America. Even though we know of no epidemiological studies on the fruit, it has been suggested that it may also be neurotoxic [49]. Not only does it contain much more annonacin than graviola, it contains many other acetogenins [52]. However, hot, pressurized methanol was not used to extract pawpaw samples when that review article was written, so there could be some that were not seen.

So, the same methanolic extract of lyophilized pawpaw fruit that was analyzed for its annonacin and squamocin content [50] was redissolved in CH_3OH containing sodium format and directly infused at a rate of 0.6 ml/min into a Bruker micro TOF-II MS in the current study. Positive ion electro spray (ESI^+) was used and a mass range of 50-3000 amu was monitored. Then, Ca^{2+} was added to a portion of the extract and mass spectra were acquired. Subsequently, Mg^{2+} was

added to a different portion of the extract and more mass spectra were acquired. The list of suspect acetogenins that was observed is shown in Table 2. This is based on the m/z values of the $[M+H]^+$ ion being within ±0.1 amu of that expected, based on the molecular formulas of known acetogenins. This includes annonacin and squamocin, which had been identified and quantified by Liquid Chromatography Coupled to High Resolution Mass Spectrometry (LC-HRMS) in a previous study [50]. They were the only two acetogenins that were available as analytical standards. At this time, the presence of the other acetogenins has not confirmed. This will require separation of the suspect acetogenins by LC.

Toxicity Studies

In addition to the epidemiological studies [4-11], several groups have investigated the *in vitro* neurotoxicity. The epidemiological studies showed that a particular variant of taupathy on the island of Guadeloupe was linked in case control studies to overconsumption of graviola fruit and tea made from the leaves [12]. As in other taupathies, there was an accumulation of the tau protein in the somatodendritic compartment of neurons. Acetogenins in graviola fruit and leaves are potent lipophilic inhibitors of mitochondrial NADH: Ubiquinone Oxidoreductase. The major acetogenin in graviola, annonacin, reduced cerebral ATP levels and causes neuronal cell loss and gliosis in the brain stem and basal ganglia [3,12]. It and several other acetogenins were found to not just reduce the ATP concentration in neurons, but also cause phosphorylated tau to be redistributed from the axon to the cell soma [12]. Annonacin has been shown to be toxic to rat primary mesencephalic [2] and striatal cells [12,13] as well as on human neuronal cells. Moreover, several acetogenins increased tau phosphorylation [12,13], changed the isoform of tau from 3R-tau to 4R-tau [54], both contributing to the pathophysiology of neurodegenerative tauopathies.

It was also shown that there is some synergy between the effects of annonacin and the expression of a mutated form of the tau protein (R406W, in which arginine 406 is mutated into tryptophan) [55]. There is much variability in the way that this mutation can affect patients. This suggests that environmental factors and diet being may also affect their health. So, it was quite important that annonacin increased the number of neurons that had phosphorylated tau in the somatodendritic compartment in several brain areas in $R406W^{+/+}$ mice as opposed to mice that had only the endogenous mouse tau ($R406W^{-/-}$). It caused degradation of the neuronal tau protein, as well as an increase in its phosphorylation and redistribution. It also activated the tau kinase Cdk5 [55].

In a more recent study, dietary supplements that contained graviola stems and leaves or pawpaw twigs were found to toxic to LUHMES cells that were derived from human mesencephalic tissue [1]. These dietary supplements were sold over the internet and advertised as being cures for cancer. As a positive control, lyophilized graviola fruit was also tested and found to be neurotoxic. In addition, pawpaw, atemoya, ata and biriba fruits as well as atemoya seeds were toxic to LUHMES cells. Despite the large differences in concentrations of annonacin and squamocin in graviola and the other four fruits, they were all toxic at about the same doses [1]. This could be due, in part, to the presence of many other neurotoxic acetogenins that were not quantified. Also, the bioavailabilities and neurotoxicities of acetogenins may be quite different when consumed by themselves or as part of the fruits. That is, acetogenins are lipophilic and may not form homogeneous doses in aqueous growth media used for the LUHMES cells. Since previous studies of *in vitro* neurotoxicities were done on ethyl acetate extracts of graviola, this solvent was used to extract the dietary supplements, the five lyophilized fruits and the atemoya seeds. However, ethyl acetate also extracts triacylglycerides, which are abundant in twigs, stems and especially seeds [51]. After the ethyl acetate was evaporated off, the oily residue that remained was made up of primarily hydrophobic triacylglycerides. It is quite likely that most of the acetogenins were trapped inside the oil and were not completely solubilized when mixed with the growth medium. So, it is hoped that hot, pressurized methanolic extracts of different parts of plants from the Annonaceae family will be evaluated for possible neurotoxicities in the future. Such extracts do not contain triacylglycerides, but do contain amphiphilic fatty acid glycosides that can form micelles that can help form a nearly homogeneous suspension of acetogenins [56]. It would be best if doses of such extracts that are dissolved in a suitable vehicle were analyzed to ensure that they are stable and the acetogenins are distributed homogeneously. This is seldom done in academic studies that have limited funding, but it is always done by pharmaceutical companies and the U.S. Environmental Toxicology Program in toxicity studies performed in accordance with Good Laboratory Practices (GLP) [57].

Another recent study showed that an aqueous extract of graviola leaves caused neuronal degeneration in the substantia nigra and neuronal vacuolization in the hippocampus of rats [58]. The neuronal damage led to an increase in glial cells in the nucleus accumbens and cerebral cortex. That is, necrotic neurons became surrounded by glia. Moreover, cytoplasmic vacuolization, clear cell foci, and eosinophilic hepatocytes emerged in the livers of the rats [58]. These effects are especially noteworthy since a tea made from 2.5 g of graviola leaves that were added to 237 ml (one cup) of water at 90 ^{0}C for 10 min had only 0.213% of the annonacin that was present in the entire leaves [26]. There is even more annonacin in graviola fruit (768 µg/g dry weight) than in the leaves (306 µg/g dry weight) [1,26]. Pawpaw fruit had 7720 µg/g dry weight [50]. Atemoya seeds had 454 and 14200 µg/g (0.454 and 14.2 mg/g) annonacin and squamocin, respectively [1]. So, if a tea made from graviola leaves can cause damage to the brain and liver of rats, the fruits and seeds of this and other plants in the Annonaceae family probably can, too, but at much lower doses. Similarly, the entire leaves that are sold as dietary supplements could be toxic, too.

Conclusion

Overconsumption of graviola has been linked to an atypical form of Parkinson's disease on the islands of Guadeloupe, Guam and New Caledonia. It does not respond to the standard treatment with L-Dihydroxyphenylalanine (L-DOPA). The neurotoxicity is caused by mitochondrial dysfunction. A class of compounds called Acetogenins (ACGs) inhibits the mitochondrial NADH: Ubiquinone Oxidoreductase (complex-I of the respiratory chain). These compounds are lipophilic polyketides that are found in plants in the Annonaceae family. Over 400 ACGs have been found in different plants in the Annonaceae family. They can be identified by NMR and LC-MS. They form adducts with Ca^{2+} and $Mg^{2+,}$ which helps to

confirm their presence in samples. Annonacin has been shown to be toxic to rat primary mesencephalic [2] and striatal cells [12,13] as well as on human neuronal cells. Moreover, several acetogenins increased tau phosphorylation [12,13] and changed the isoform of tau from 3R-tau to 4R-tau [54], both contributing to the pathophysiology. It was also shown that there is some synergy between the effects of annonacin and the expression of a mutated form of the tau protein (R406W) [55]. Also, dietary supplements that contained graviola stems and leaves or pawpaw twigs were found to toxic to LUHMES cells that were derived from human mesencephalic tissue [1]. These dietary supplements were sold over the internet and advertised as being cures for cancer. As a positive control, lyophilized graviola fruit was also tested and found to be neurotoxic. In addition, pawpaw, atemoya, ata and biriba fruits as well as atemoya seeds were toxic to LUHMES cells [1]. Still, it is hoped that hot, pressurized methanolic extracts can be tested for neurotoxicity, since they are more likely to form nearly homogeneous doses of acetogenins in micelles made from fatty acid glycosides that are in all five fruits in the Annonaceae family that were tested [1,56].

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