



Annonacin in *Asimina triloba* fruit: Implication for neurotoxicity

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ABSTRACT

Introduction: The acetogenin, annonacin, from the tropical annonaceous plant *Annona muricata*, is a lipophilic, mitochondrial complex I inhibitor reported to be more toxic than rotenone to mesencephalic neurons. The temperate annonaceous plant *Asimina triloba* (pawpaw) is native to the Eastern United States and products are available online. This study determined whether annonacin is in the pawpaw fruit pulp and whether it or the crude ethyl acetate extract is toxic to cortical neurons.

Methods: Pawpaw extract was prepared by pulp extraction with methanol and liquid–liquid partitioning with ethyl acetate (EtOAc). Annonacin was isolated from the crude EtOAc extract via column chromatography using a gradient solvent system of increasing polarity. Mass spectroscopy, nuclear magnetic resonance and infrared spectroscopy were used to compare isolated material with synthetic annonacin data and a natural annonacin sample. Toxicity of isolated annonacin and the total EtOAc extract was determined in primary rat cortical neurons using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay.

Results: The average concentration of annonacin in the fruit pulp was 0.0701 ± 0.0305 mg/g. Purified annonacin (30.07 μ g/ml) and crude EtOAc extract (47.96 μ g/ml) induced 50% death of cortical neurons 48 h post treatment. Annonacin toxicity was enhanced in the presence of crude extract.

Discussion: Pawpaw fruit contains a high concentration of annonacin, which is toxic to cortical neurons. Crude fruit extract also induced neurotoxicity, highlighting the need for additional studies to determine the potential risks of neurodegeneration associated with chronic exposure to pawpaw products.

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1. Introduction

Annonaceous acetogenins, specifically annonacin, isolated from *Annona muricata* (soursop, graviola, guanabana, Brazilian pawpaw, prickly custard apple) fruit, bark and leaves are naturally occurring compounds exhibiting a range of biological activities related to inhibition of mitochondrial complex I of the electron transport chain (Degli Esposti et al., 1994; Bermejo et al., 2005). The annonaceous plants have been investigated for their potential as cancer treatments (McLaughlin, 2008; Liaw et al., 2010), but also for neurotoxicity (Champy et al., 2004; Lannuzel et al., 2006) due to their high content of acetogenins (Champy et al., 2005, 2009). Annonacin has been reported to cause cell death and tau pathology in mesencephalic cultures (Lannuzel et al., 2003; Escobar-

Khondiker et al., 2007; Hollerhage et al., 2009), and neurodegeneration of the basal ganglia and brainstem after its chronic, intravenous administration to rats (Champy et al., 2004). Nigrostriatal pathology, mitochondrial impairment and/or tau pathology are seen in numerous neurodegenerative disorders including Parkinson's disease, Alzheimer's disease (AD) and progressive supranuclear palsy (PSP) (Albers and Beal, 2002; Robert and Mathuranath, 2007; Di Filippo et al., 2010). Moreover, regular consumption of fruit and tea from annonaceous plants was reported to be higher in patients with PSP and with an unclassifiable atypical parkinsonian disorder than controls in the French West Indies (Caparros-Lefebvre and Elbaz, 1999; Caparros-Lefebvre et al., 2002; Lannuzel et al., 2007).

Annonacin has been reported to be present in the bark and seeds of *Asimina triloba* (pawpaw, prairie banana, poor man's banana, Ozark banana, Banango, also commonly referred to as its native states "banana", e.g. Indiana/Hoosier banana, Kentucky banana, etc.) (McLaughlin, 2008), which grows throughout the Eastern United States and is touted as a potential alternative cash crop to tobacco. Furthermore, there is an annonacin-containing

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commercial supplement made from pawpaw twig extracts, Paw-Paw Cell-Reg™, that is marketed as beneficial for overall health (www.naturessunshine.com) and as a safe complement to cancer therapy (Wang et al., 2001; McLaughlin, 2008; Cuendet et al., 2008; Pomper et al., 2009; Coothankandaswamy et al., 2010). We hypothesized that annonacin is also present in the pawpaw fruit pulp, which is the most commonly consumed tree product. This study determined the presence and concentration of annonacin in the pawpaw fruit and its toxicity to cortical neurons, which are affected in several neurodegenerative disorders such as AD and fronto-temporal dementia (FTD). We also determined the toxicity of the crude organic extract.

2. Materials and methods

2.1. Isolation

Annonacin was isolated from *A. triloba* using frozen (<http://www.integrationacres.com/products.html>, May 18, 2011) and fresh (A Little Piece of Paradise Farm, Lincoln County, Kentucky) fruit pulp following standard extraction and chromatography techniques (Liaw et al., 2005) using an optimized solvent system. Fruit pulp (~1000 g) was homogenized with methanol (800 ml) using a commercial blender and vacuum filtered at room temperature overnight or until nearly dry. Liquid–liquid partitioning of the filtrate into ethyl acetate (EtOAc, 3 × 200 ml) yielded the crude organic extract (cEX), which was dried and concentrated for weight determination. Separation of extracts and isolation of annonacin from a known amount of crude extracts was obtained by repeated open column chromatographies (25 mm × 250 mm glass column) on silica gel 60 (Merck 9385, 235–400 mesh) or 62 (Mallinckrodt 6551, 60–200 mesh) using a gradient solvent system of hexane with increasing concentrations of EtOAc and finally 5% methanol in EtOAc. Annonacin and acetogenin containing fractions were identified by thin layer chromatography (TLC) with chloroform/methanol (9:1) using standard visualization techniques (i.e. anisaldehyde/ethanol/sulfuric acid stain) as well as the Kedde reagent (dipping of TLC plate in 3,5-dinitrobenzoic acid/methanol (2%, w/v) followed by dipping in potassium hydroxide/methanol (2 M) and weight determined following solvent removal *in vacuo*). The entire extraction and isolation process was repeated on 5 separate batches of frozen fruit and one batch of fresh fruit. High resolution mass spectroscopy (HRMS, specifically FTMS using ESI) and nuclear magnetic resonance (NMR, 500 MHz ¹H and ¹³C) were used to compare the isolated annonacin to an annonacin sample from *A. muricata* and synthetic annonacin data (Hu et al., 2001). An annonacin tetraacetate derivative was prepared by dissolving crude annonacin (79 mg, 0.13 mmol) in pyridine (200 μl, 2.5 mmol) and acetic anhydride (100 μl, 1.0 mmol). TLC confirmed full consumption of the starting material during the reaction. Chromatographic purification of the acetylated product (hexane/EtOAc 2:1) yielded the tetraacetate derivative and spectral data were compared to that of isolated annonacin. Optical rotations and infrared spectra (Fourier transform infrared spectroscopy, FTIR) were also recorded for isolated samples of annonacin and annonacin tetraacetate.

2.2. Chemicals

Solvents and reagents used for extractions were American Chemical Society grade and used as commercially supplied. Stock solutions of 1 mM rotenone (Sigma R8875) were prepared in dimethyl sulfoxide (DMSO) or EtOAc prior to each experiment. Purified annonacin and cEX were dissolved in DMSO or EtOAc to make 50 mM and 75 mg/ml stock solutions, respectively, which were stored at –80 °C. Acetogenin-free fractions (ACG-free) isolated from cEX alongside annonacin were diluted in DMSO to

22 mg/ml and stored at –80 °C. Serial dilutions of drugs were prepared in basal medium Eagle (BME, Sigma) immediately before each experiment.

2.3. Cell cultures

Cortical neurons were cultured from postnatal day 0 (P0) Sprague–Dawley rat pups according to previously published methods (Hetman et al., 1999). The handling of animals followed Institutional Animal Care and Use Committee approved procedures. For viability assays, cultured neurons were plated in 96-well culture plates coated with poly-D-lysine (PDL) at a density of 1500–2000 cells/mm². 1-(β-D-Arabinofuranosyl) cytosine (2.5 μM) was added to all wells on day 2 *in vitro* (DIV 2) to inhibit proliferation of non-neuronal cells. Cells were treated with annonacin, cEX, or vehicle (DMSO or EtOAc) on DIV 5. Rotenone is a model neurotoxin and was therefore used as a positive control while the ACG-free extract served as a negative control for the extraction process. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is converted to a formazan product by mitochondrial dehydrogenase in living cells. As the rate of conversion is proportional to the number of surviving cells, it provides a convenient measure of cell viability (Mosmann, 1983). Therefore, the MTT assay was used as previously described (Hansen et al., 1989) to assess the toxicity of each treatment relative to vehicle-treated controls.

2.4. Statistical analysis

One-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to determine significance and non-linear regression analysis to calculate the LD₅₀ values for annonacin and crude extracts.

3. Results

3.1. Isolation

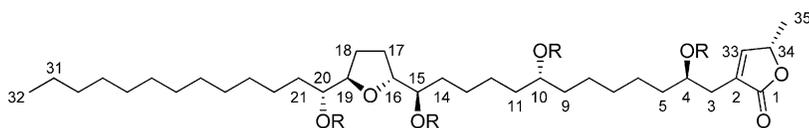
Data from TLC, FTMS, ¹H- and ¹³C-NMR, FTIR spectroscopic analyses and optical rotations revealed the presence of annonacin (HRMS calculated for C₃₅H₆₄O₇ *m/z* [m+Na]⁺: 619.4543; Found: 619.4546 ± 0.6 ppm. [α]_D²⁰: +20.4° (c = 0.51, CHCl₃)) in extracts isolated from pawpaw fruit pulp (Table 1, Fig. 1A). Annonacin tetraacetate was successfully prepared (HRMS calculated for C₄₃H₇₂O₁₁ *m/z* [m+Na]⁺: 787.4966; Found: 787.4963 ± 0.4 ppm. [α]_D²⁰: +7.45° (c = 0.51, CHCl₃)) and isolated as determined by TLC, NMR, FTMS and FTIR (Table 1, Fig. 1B).

3.2. Quantification

The amount of annonacin isolated per gram of frozen fruit pulp was 0.0701 ± 0.0305 mg as determined by average weight from extractions on 5 separate batches (Table 2). On average, annonacin made up 10% of the total crude EtOAc extract. One additional extraction was done on locally harvested fresh fruit, which gave an annonacin yield of 0.1226 mg/g of pulp.

3.3. Neurotoxicity

Annonacin treatment reduced the number of viable cells remaining in the culture 48 h after treatment at concentrations as low as 10 μM (*p* < 0.001, Fig. 2A) as determined by MTT assay. ACG-free treatment did not result in cell death indicating that the processing of extract components *per se* is not sufficient for procuring toxic material (Fig. 2B). Pure annonacin 30.07 μg/ml (i.e. 50.45 μM) and cEX (47.96 μg/ml) reduced the number of viable cells compared to controls by 50% 48 h after treatment. Compari-

Table 1Chemical structures and ^1H and ^{13}C NMR data for annonacin (R=H) and annonacin tetraacetate (R=Ac).

R=H, ^1H assignment	R=H, ^{13}C assignment	R=Ac, ^1H assignment	R=Ac, ^{13}C assignment
–	C-1: 174.7	–	C-1: 173.4
–	C-2: 131.2	–	C-2: 130.1
H-3a: <i>dd</i> , 2.40 (15.2, 8.0)	C-3: 33.3	H-3a: <i>dd</i> , 2.52 (14.4, 4.0)	C-3: 30.8
H-3b: <i>dd</i> , 2.52 (15.2, 8.0)		H-3b: <i>dd</i> , 2.52 (14.4, 4.0)	
H-4: <i>m</i> , 3.85	C-4: 69.9	H-4: <i>m</i> , 4.80–5.02	C-4: 71.8
H-5–H-9: <i>m</i> , 1.25–1.62	C-5–C-9: 22.7–37.3	H-5–H-9: <i>m</i> , 1.25–1.40	C-5–C-9: 22.6–34.0
H-10: <i>m</i> , 3.59	C-10: 71.7	H-10: <i>m</i> , 4.80–5.02	C-10: 74.0
H-11–H-14: <i>m</i> , 1.25–1.62	C-11–C-14: 22.7–37.3	H-11–H-14: <i>m</i> , 1.25–1.40	C-11–C-14: 22.6–34.0
H-15: <i>dt</i> , 3.41 (12.0, 6.8)	C-15: 74.0	H-15: <i>m</i> , 4.80–5.02	C-15: 74.7
H-16: <i>m</i> , 3.81	C-16: 82.7	H-16: <i>m</i> , 3.97	C-16: 79.4
H-17: <i>m</i> , 1.65/ <i>m</i> , 1.98	C-17: 28.8/29.7	H-17: <i>m</i> , 1.73/ <i>m</i> , 1.94	C-17: 28.0
H-18: <i>m</i> , 1.65/ <i>m</i> , 1.98	C-18: 28.8/29.7	H-18: <i>m</i> , 1.73/ <i>m</i> , 1.94	C-18: 28.0
H-19: <i>m</i> , 3.81	C-19: 82.7	H-19: <i>m</i> , 3.97	C-19: 79.5
H-20: <i>dt</i> , 3.41 (12.0, 6.8)	C-20: 74.1	H-20: <i>m</i> , 4.80–5.02	C-20: 74.9
H-21–H-31: <i>m</i> , 1.25–1.62	C-21–C-31: 22.7–37.3	H-21–H-31: <i>m</i> , 1.25–1.40	C-21–C-31: 22.6–34.0
H-32: <i>t</i> , 0.88 (6.8)	C-32: 14.1	H-32: <i>t</i> , 0.88 (7.0)	C-32: 14.1
H-33: <i>d</i> , 7.18 (1.4)	C-33: 151.9	H-33: <i>d</i> , 7.09 (1.5)	C-33: 151.9
H-34: <i>qd</i> , 5.06 (6.8, 1.4)	C-34: 78.0	H-34: <i>qd</i> , 5.09 (7.0, 1.5)	C-34: 77.5
H-35: <i>d</i> , 1.40 (6.8)	C-35: 19.1	H-35: <i>d</i> , 1.40 (7.0)	C-35: 18.9
		–	C=O (Ac): 170.6; 170.8; 170.9
		CH ₃ (Ac): <i>s</i> , 2.03; <i>s</i> , 2.04; <i>s</i> , 2.07	CH ₃ (Ac): 21.1–21.2

Isolated and purified materials were dried and dissolved in CDCl_3 (chemical shifts δ in ppm; coupling constants J in Hz; 100 and 400 MHz).

son of LD_{50} values between pure annonacin and the amount of annonacin present in cEX ($\text{LD}_{50} = 4.80 \mu\text{g}/\text{ml}$) confirmed that cEX has more toxic potential than annonacin by itself ($p < 0.0001$, Fig. 2C).

4. Discussion

Herein we have: (1) found annonacin in fruit pulp of the annonaceous pawpaw tree, *A. triloba*, (2) determined the amount of annonacin per gram of fruit pulp, and (3) shown that annonacin and crude acetogenin-containing extracts are toxic to primary cortical neurons. These findings are important particularly in view of previous reports of neurotoxicity associated with *in vivo* and *in vitro* exposure to annonacin extracted from fruit or teas of *A. muricata*, a tropical annonaceous relative of pawpaw.

4.1. Isolation and quantification

We optimized a method for reliable extraction and isolation of annonacin from pawpaw fruit pulp. The presence of annonacin was determined by TLC and mass spectroscopy (FTMS) using high-field NMR and infrared spectroscopy (FTIR) to confirm its identity by comparison of spectral data with data from a natural annonacin sample as well as that reported for synthetic annonacin. When examined by TLC in multiple solvent systems, the isolated annonacin co-spotted with the *A. muricata* sample. NMR shifts were consistent with those previously reported for synthetic annonacin (Hu et al., 2001) and FTMS and NMR spectra corresponded with that of the annonacin sample. The successful formation of annonacin tetraacetate provided additional confirmation that our isolated material was annonacin as noted by shifts in FTMS, NMR and FTIR absorptions where expected. Specifically the loss of OH bending in the FTIR tetraacetate spectra indicated disappearance of the 4 hydroxyl groups that were evidenced by a broad absorption band at 3416.74 cm^{-1} in the annonacin spectra. Furthermore, the optical rotation of our purified material

corresponds to that of annonacin prepared by stereoselective synthesis, (Hu et al., 2001) which confirms that it possess the same stereochemistry as annonacin. Based on repeated extractions we determined that annonacin represents 0.007% of pawpaw pulp by weight determination. This is higher than what is reported in *Annona cherimola* seeds (0.0004%) (Kim et al., 2001) and *A. muricata* (sour sop) pulp 0.002% (Champy et al., 2005). The fruit pulp of *A. triloba* is a new, easily accessible source of biomass and our extraction and isolation process procures a higher yield of annonacin than the current, multistep synthetic route (Hu et al., 2001), making it useful in exploring its potential as a novel model neurotoxin.

4.2. Neurotoxicity

Acute annonacin treatment was toxic to cortical neurons (P0) at low micromolar concentrations while treatment with the ACG-free extract did not affect neuronal viability, indicating that the neurotoxicity of annonacin was not an artifact of the extraction process. Furthermore, annonacin was more toxic in the presence of cEX than when purified in terms of the amount of annonacin present in each treatment, suggesting that multiple acetogenins synergistically affect neuronal viability. The presence of bis-tetrahydrofuranic acetogenins such as bullatacin is likely contributing to the neurotoxicity of cEX (Bermejo et al., 2005; Hollerhage et al., 2009). We found annonacin to be less potent in cortical neurons than previously reported in mesencephalic and striatal neurons (Escobar-Khondiker et al., 2007; Hollerhage et al., 2009), which are more sensitive to such treatments. As annonacin was less potent than rotenone in our experimental model, one can speculate that the greater toxicity of the latter may be due to rotenone's interactions with additional cellular targets other than the mitochondrial complex I (Klintworth et al., 2007; Choi et al., 2008; Belcastro et al., 2009; Gill and Perez-Polo, 2009). Prior work from Dr. Hetman's lab has demonstrated that 80% or more of cells in the culture are neurons. Indeed in annonacin treated cells, there

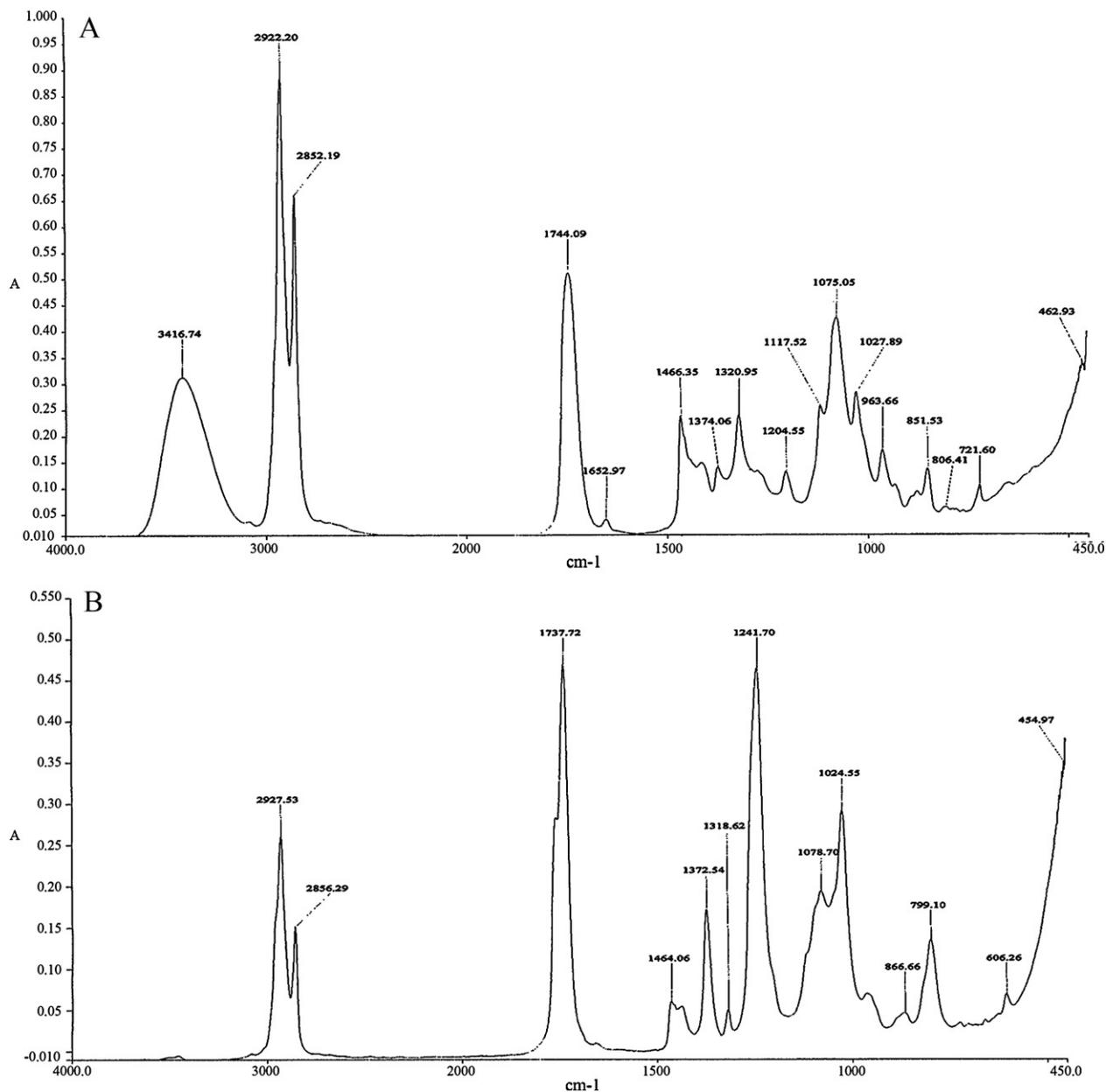


Fig. 1. Fourier transform infrared (FTIR) spectra of annonacin isolated from *Asimina triloba*. Broad peak at 3416.74 cm^{-1} = OH groups, 2922.2 cm^{-1} = CH_2s , 2852.19 cm^{-1} = THF ring, 1744.09 cm^{-1} = butenolide (A) compared to spectra of the tetraacetate derivative 2927.53 cm^{-1} = CH_2s , 2856.29 cm^{-1} = THF ring, 1737.72 cm^{-1} = acetates with butenolide shoulder (B). Note, as expected, in (B) there is no longer a peak corresponding to the OH groups confirming complete consumption of starting material and tetraacetate formation. Purified samples dissolved in CHCl_3 and spotted on salt plates for spectral acquisition using Perkin-Elmer Spectrum 100 instrument.

Table 2

Annonacin content of *Asimina triloba* vs. *Annona muricata*.

	Average total fruit weight ^a (g)	Amount annonacin per fruit (mg)	Amount annonacin per gram of fruit pulp ^b (mg/g)
<i>Asimina triloba</i>	~300		
Frozen fruit pulp		7.0	0.070
Fresh fruit pulp ^c		12.3	0.123
<i>Annona muricata</i>	~800	15.0	0.023

^a Note these values reflect weight of the whole fruit (i.e. including skin, seeds and pulp).

^b The amount of annonacin per gram of fruit pulp is based on the amount of pulp used in the extraction, not the weight of the whole fruit.

^c Quantities in fresh fruit are based on data from one extraction; one pawpaw fruit yielded 100 g of pulp on average.

were no MAP2-positive cells remaining in the culture (data not shown) after 48 h, indicating toxicity to neuronal cells as MAP2 is a neuron-specific marker. To date, *A. triloba* toxicity has only been documented in cancer cell lines, and in cancerous murine models (McLaughlin, 2008). Our finding of *in vitro* cortical neurotoxicity corroborates previous reports of annonacin neurotoxicity to nigral neurons (Lannuzel et al., 2002, 2003; Champy et al., 2004; Escobar-Khondiker et al., 2007) broadening the significance of annonacin-induced toxicity as it relates to neurodegeneration.

Previous reports assumed human exposure levels based on phytochemical and epidemiological studies and comparison of *in vivo* findings. These estimations may be insightful for preliminarily assessing the risk of consuming pawpaw products; however, human exposure levels (i.e. levels of annonacin in blood or brain parenchyma) may not be extrapolated from our results, which

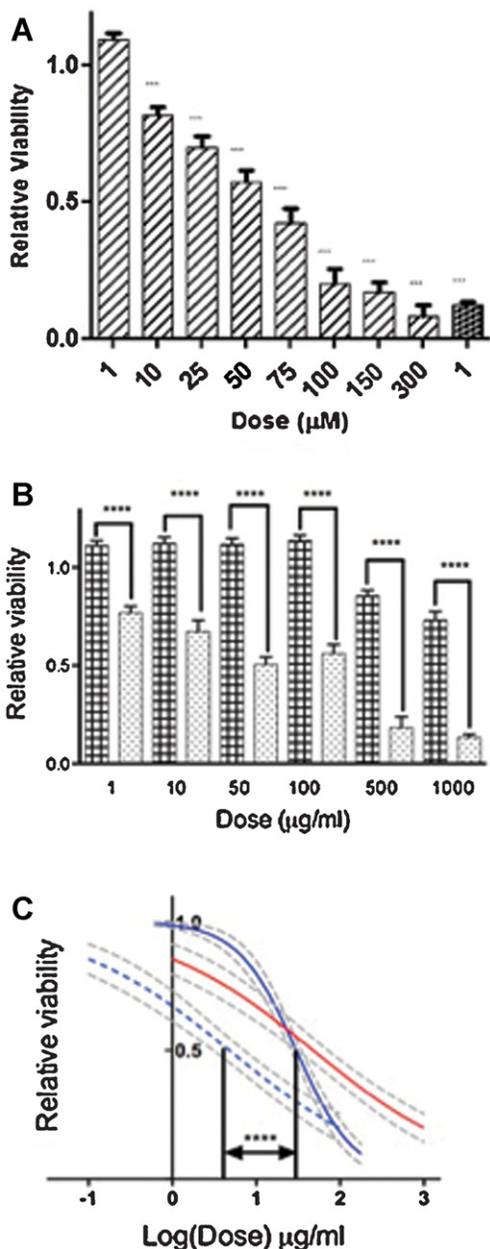


Fig. 2. Cortical neurotoxicity of *Asimina triloba* fruit extracts 48 h after treatment. Panel A: MTT assay of cortical neurons 48 h after treatment with annonacin isolated from *Asimina triloba* fruit pulp. LD₅₀ for annonacin = 50.45 µM, (i.e.) ±1.07. Diagonal lines = annonacin (µM), checked line = rotenone (µM) p -value < 0.001 (***). Panel B: crude extract (cEX, light checked bars) relative viability was significantly lower at each dose than ACG-free (plaid bars) treatment p -value < 0.0001 (****). All doses of cEX significantly reduced cell viability compared to controls (p < 0.001). ACG-free treatment only significantly reduced viability at high concentrations (≥ 500 µg/ml, p < 0.01). Bars represent relative viability, normalized to controls (mean ± SEM). p -Values determined by one-way ANOVA with Dunnett's post hoc comparison. Panel C: dose-response curves for cEX (LD₅₀ = 47.96 µg/ml) (solid red line) and for pure annonacin (µg/ml) (solid blue line) compared to amount of annonacin (µg/ml) in the crude extracts (dashed blue line) 48 h post-treatment. LD₅₀ of pure annonacin alone (30.07 µg/ml, i.e. 50.45 µM) is significantly higher than that of annonacin in the presence of crude extracts (4.80 µg/ml, p < 0.0001 (****)). LD₅₀ values determined by nonlinear regression of log (dose) vs. relative viability. Gray dashed lines represent 95% confidence intervals for dose-response data.

cannot be directly compared to studies done *in vivo*. Although it has been demonstrated that annonacin crosses the blood brain barrier (Champy et al., 2004), additional studies should determine the bioavailability of annonacin and further elucidate the neurotoxicity of other compounds in the fruit. Likewise, as we

used an acute treatment method, the effects of chronic, *in vivo* exposure to the total extract should be determined before firm conclusions can be made regarding the risk associated with regular consumption of pawpaw products.

5. Conclusions

Identification and quantification of annonacin in pawpaw fruit is of interest considering the neurotoxicity reported here and in previous studies (Champy et al., 2004; Escobar-Khondiker et al., 2007; Hollerhage et al., 2009). The high concentration of annonacin in pawpaw fruit is particularly relevant because consumption of pawpaw in the United States could be much more widespread than that of other annonaceous fruits considering (1) it is the only edible annonaceous fruit that is not confined to tropical areas, (2) it grows throughout the Eastern United States, (3) is marketed on the worldwide web therefore available to consumers all year round (e.g. frozen pulp, jam), and (4) is currently marketed for its potential use in alternative medicine (Zhao et al., 1992; McLaughlin, 2008; Cuendet et al., 2008; Coothankandaswamy et al., 2010). This growing in popularity of pawpaw fruit highlights the importance of ours and other's findings of annonacin neurotoxicity with regard to public health and supports the inclusion of pawpaw exposure/consumption in studies investigating potential risk factors for neurodegeneration.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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References

- Albers DS, Beal MF. Mitochondrial dysfunction in progressive supranuclear palsy. *Neurochem Int* 2002;40:559–64.
- Belcastro V, Tozzi A, Tantucci M, Costa C, Di Filippo M, Autuori A, et al. A2A adenosine receptor antagonists protect the striatum against rotenone-induced neurotoxicity. *Exp Neurol* 2009;217:231–4.
- Bermejo A, Figadere B, Zafra-Polo MC, Barrachina I, Estornell E, Cortes D. Acetogenins from Annonaceae: recent progress in isolation, synthesis and mechanisms of action. *Nat Prod Rep* 2005;22:269–303.
- Caparros-Lefebvre D, Elbaz A. Possible relation of atypical parkinsonism in the French West Indies with consumption of tropical plants: a case-control study. *Caribbean Parkinsonism Study Group. Lancet* 1999;354:281–6.
- Caparros-Lefebvre D, Sergeant N, Lees A, Camuzat A, Daniel S, Lannuzel A, et al. Guadeloupean parkinsonism: a cluster of progressive supranuclear palsy-like tauopathy. *Brain* 2002;125:801–11.
- Champy P, Guerin V, Laprevote O. MALDI-TOF MS, profiling of annonaceous acetogenins in *Annona muricata* products for human consumption. *Molecules* 2009;14:5235–46.
- Champy P, Hoglinger GU, Feger J, Gleye C, Hocquemiller R, Laurens A, et al. Annonacin, a lipophilic inhibitor of mitochondrial complex I, induces nigral and striatal neurodegeneration in rats: possible relevance for atypical parkinsonism in Guadeloupe. *J Neurochem* 2004;88:63–9.

- Champy P, Melot A, Guerineau Eng V, Gleye C, Fall D, Hoglinger GU, et al. Quantification of acetogenins in *Annona muricata* linked to atypical parkinsonism in guadeloupe. *Mov Disord* 2005;20:1629–33.
- Choi WS, Kruse SE, Palmiter RD, Xia Z. Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. *Proc Natl Acad Sci USA* 2008;105:15136–41.
- Coothankandaswamy V, Liu Y, Mao SC, Morgan JB, Mahdi F, Jekabsons MB, et al. The alternative medicine pawpaw and its acetogenin constituents suppress tumor angiogenesis via the HIF-1/VEGF pathway. *J Nat Prod* 2010;73:956–61.
- Cuendet M, Oteham CP, Moon RC, Keller WJ, Peardon PA, Pezzuto JM. Dietary administration of *Asimina triloba* (Paw Paw) extract increases tumor latency in *N*-methyl-*N*-nitrosourea-treated rats. *Pharm Biol* 2008;46:3–7.
- Degli Esposti M, Ghelli A, Ratta M, Cortes D, Estornell E. Natural substances (acetogenins) from the family Annonaceae are powerful inhibitors of mitochondrial NADH dehydrogenase (complex I). *Biochem J* 1994;301(Pt 1):161–7.
- Di Filippo M, Chiasserini D, Tozzi A, Picconi B, Calabresi P. Mitochondria and the link between neuroinflammation and neurodegeneration. *J Alzheimers Dis* 2010;20 (Suppl 2): S369–79.
- Escobar-Khondiker M, Hollerhage M, Muriel MP, Champy P, Bach A, Depienne C, et al. Annonacin, a natural mitochondrial complex I inhibitor, causes tau pathology in cultured neurons. *J Neurosci* 2007;27:7827–37.
- Gill MB, Perez-Polo JR. Bax shuttling after rotenone treatment of neuronal primary cultures: effects on cell death phenotypes. *J Neurosci Res* 2009;87:2047–65.
- Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* 1989;119:203–10.
- Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by brain-derived neurotrophic factor is mediated by extracellular signal-regulated kinase and phosphatidylinositol 3-kinase. *J Biol Chem* 1999;274:22569–80.
- Hollerhage M, Matusch A, Champy P, Lombes A, Ruberg M, Oertel WH, et al. Natural lipophilic inhibitors of mitochondrial complex I are candidate toxins for sporadic neurodegenerative tau pathologies. *Exp Neurol* 2009;220:133–42.
- Hu TS, Yu Q, Wu YL, Wu Y. Enantioselective syntheses of monotetrahydrofuran annonaceous acetogenins tonkinecin and annonacin starting from carbohydrates. *J Org Chem* 2001;66:853–61.
- Kim DH, Son JK, Woo MH. Annonocheerin, annonacin and annomontacin: a novel and two known bioactive mono-tetrahydrofuran annonaceous acetogenins from *Annona cherimolia* seeds. *Arch Pharm Res* 2001;24:300–6.
- Klintonworth H, Newhouse K, Li T, Choi WS, Faigle R, Xia Z. Activation of c-Jun N-terminal protein kinase is a common mechanism underlying paraquat- and rotenone-induced dopaminergic cell apoptosis. *Toxicol Sci* 2007;97:149–62.
- Lannuzel A, Hoglinger GU, Champy P, Michel PP, Hirsch EC, Ruberg M. Is atypical parkinsonism in the Caribbean caused by the consumption of Annonaceae? *J Neural Transm Suppl* 2006;153–7.
- Lannuzel A, Hoglinger GU, Verhaeghe S, Gire L, Belson S, Escobar-Khondiker M, et al. Atypical parkinsonism in Guadeloupe: a common risk factor for two closely related phenotypes? *Brain* 2007;130:816–27.
- Lannuzel A, Michel PP, Caparros-Lefebvre D, Abaul J, Hocquemiller R, Ruberg M. Toxicity of Annonaceae for dopaminergic neurons: potential role in atypical parkinsonism in Guadeloupe. *Mov Disord* 2002;17:84–90.
- Lannuzel A, Michel PP, Hoglinger GU, Champy P, Jousset A, Medja F, et al. The mitochondrial complex I inhibitor annonacin is toxic to mesencephalic dopaminergic neurons by impairment of energy metabolism. *Neuroscience* 2003;121:287–96.
- Liaw CC, Chang FR, Chen SL, Wu CC, Lee KH, Wu YC. Novel cytotoxic monotetrahydrofuranic annonaceous acetogenins from *Annona montana*. *Bioorg Med Chem* 2005;13:4767–76.
- Liaw CC, Wu TY, Chang FR, Wu YC. Historic perspectives on annonaceous acetogenins from the chemical bench to preclinical trials. *Planta Med* 2010;76:1390–404.
- McLaughlin JL. Paw paw and cancer: annonaceous acetogenins from discovery to commercial products. *J Nat Prod* 2008;71:1311–21.
- Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55–63.
- Pomper KW, Lowe JD, Crabtree SB, Keller W. Identification of annonaceous acetogenins in the ripe fruit of the North American pawpaw (*Asimina triloba*). *J Agric Food Chem* 2009;57:8339–43.
- Robert M, Mathuranath PS. Tau and tauopathies. *Neurol India* 2007;55:11–6.
- Wang LQ, Li Y, Min BS, Nakamura N, Qin GW, Li CJ, et al. Cytotoxic mono-tetrahydrofuran ring acetogenins from leaves of *Annona montana*. *Planta Med* 2001;67:847–52.
- Zhao G, Hui Y, Rupprecht JK, McLaughlin JL, Wood KV. Additional bioactive compounds and trilobacin, a novel highly cytotoxic acetogenin, from the bark of *Asimina triloba*. *J Nat Prod* 1992;55:347–56.