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Original Article

UPLC–QTOF–MS and NMR analyses of graviola (*Annona muricata*) leaves

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ABSTRACT

Graviola leaves (*Annona muricata* L., Annonaceae) are used by some people to try to treat or even cure cancer, even though over-consumption of the fruit, which contains the neurotoxins annonacin and squamocin has caused an atypical form of Parkinson's disease. In previous analyses, the fruits were extracted with methanol under ambient conditions before analyses. In the present study, UPLC–QTOF–MS and NMR were used to analyze freeze-dried graviola leaves that were extracted using dry methanol and ethanol at 100 °C and 10 MPa (100 atm) pressure in a sealed container. Methanol solubilized 33% of the metabolites in the lyophilized leaves. Ethanol solubilized 41% of metabolites in the lyophilized leaves. The concentrations of total phenolic compounds were 100.3 ± 2.8 and 93.2 ± 2.0 mg gallic acid equivalents per g of sample, for the methanolic and ethanolic extracts, respectively. Moreover, the toxicophore (unsaturated γ-lactone) that is present in neurotoxic acetogenins was found in the lipophilic portion of this extract. The concentrations of the neurotoxins annonacin and squamocin were found by UPLC–QTOF–MS to be 305.6 ± 28.3 and 17.4 ± 0.89 μg/g-dw, respectively, in the dried leaves. Pressurized methanol solubilized more annonacin and squamocin than ethanol. On the other hand, a hot, aqueous infusion solubilized only 0.213% of the annonacin and too little of the squamocin to be detected. So, graviola leaves contain significant amounts of the neurotoxins annonacin and squamocin, as well as some potentially healthy phenolic compounds. Finally, the potential neurotoxicity of whole leaves in dietary supplements could be much higher than that of a tea (hot aqueous infusion) that is made from them.

Introduction

Graviola (*Annona muricata* L.) is a tropical fruit in the Annonaceae family that is grown in Asia, South America and many tropical islands (Lannuzel and Michel, 2009; Gajalakshmi et al., 2012). The leaves can be used to make a tea (Port's et al., 2013; Hansra et al., 2014) or consumed whole as dietary supplement in capsules that may have some health effects (Torres et al., 2012). However, over-consumption of graviola and products made from it may have caused an atypical form of Parkinson's diseases on the French Caribbean island of Guadeloupe and the Pacific island of Guam (Caparros-Lefebvre and Elbaz, 1999; Champy et al., 2005; Badrie and Schauss, 2009). This is due to the presence of neurotoxic acetogenins, such as annonacin (**1**) ($C_{35}H_{64}O_7$, MW 596.88) and squamocin (**2**) ($C_{37}H_{66}O_7$, MW 622.92). Like other neurotoxic acetogenins, they contain an unsaturated γ-lactone group that is the toxicophore (Smith et al., 2014). However, the neurotoxicity could be strongly dependent on the dose. That is, the first rule of toxicology is that the dose is the poison (Smith, 2014). It was over-consumption, not consumption of graviola that caused Parkinson's disease. The dose of neurotoxic acetogenins that one consumes is very dependent on the form of graviola that is ingested. For example, one study used a tea made from 10 to 12 dry leaves that were boiled in 8 oz (about 237 ml) water for 5–7 min (Hansra et al., 2014). Since acetogenins have very low solubilities in water (Höllerhage et al., 2009), this would have been a relatively low dose. The dose

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can be estimated from a previous study that found about 56 mg of annonacin per kg of leaves when about 2.5 g of leaves were boiled for 10 min in water (Champy et al., 2005). On the other hand, much higher doses would be consumed if one were to ingest the entire leaves in a dietary supplement. That is, one group reported that the "therapeutic dose" of graviola leaves is 2–3 g, taken 3 or 4 times daily (Sun et al., 2014). If whole leaves are consumed, the expected dose would be higher than that in a tea. To estimate the concentration, 100 g of dried leaves were extracted with 1 l of methanol (Champy et al., 2005). This recovered almost six times as much annonacin (about 300 mg/kg) as did the boiling water. However, this analysis was done with matrix assisted laser desorption and ionization coupled to time of flight mass spectrometry or MALDI–TOF MS (Champy et al., 2005), which separates analytes based on their molecular weights. So, annonacin will not be separated from its isomers. This could cause an over-estimation of the concentration of annonacin, since its isomers will also be detected by the MALDI–TOF MS. It was observed, boiling methanol was not able to solubilize all the annonacin (**1**) or squamocin (**2**) from another fruit in the Annonaceae family, the North American pawpaw (*Asimina triloba*; Potts et al., 2012). That is, the method used to extract annonacin and squamocin can affect the results of the analysis. Boiling water will not solubilize all the annonacin or squamocin. Instead, pressurized liquid extraction using dry methanol at 100 °C and 10 MPa (100 atm) pressure can solubilize over seven times as much annonacin and even some squamocin from the fruits (Potts et al., 2012). So, it is important to use hot, dry pressurized methanol to extract all the annonacin and squamocin. It is also important to use liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS) to separate and detect isomers of annonacin and squamocin (Levine et al., 2015).

LC-HRMS is especially useful when trying to quantify trace components, like acetogenins. The MS can be tuned so that it is as sensitive as possible for the analytes of interest (Smith, 2014). However, the sensitivities for other analytes that are present in a sample are quite different. So, standards are required when quantifying analytes by MS. On the other hand, ^{1}H NMR has the same sensitivity for all the hydrogens (^{1}H isotope) in the sample (Smith, 2014). So, it can be used without standards to determine the relative concentrations of different types of hydrogens in a sample (Smith, 2014). Moreover, ^{13}C NMR can be used to determine how many different kinds of carbons are in a sample and to identify the types of compounds that are present (Smith, 2014). Therefore, NMR can be very useful in analyzing relatively unknown samples. NMR was used to analyze extracts of *A. muricata* for the presence of the α,β-unsaturated-γ-lactone toxicophore that is present in neurotoxic acetogenins (Machado et al., 2015).

Even though graviola leaves have been reported to contain about 300 mg/kg annonacin (Höllerhage et al., 2009), 32 mg/kg total phenolics and 5.6 mg/kg total flavonoids (Port's et al., 2013), little is known about its major chemical constituents. Even the concentration of total soluble substances is not well known. That is, the concentration depends on the method used to extract the soluble substances. For example, when dried leaves were soaked in distilled water for 48 h, about 5% of the solids dissolved (Florence et al., 2014). Another obtained only a 3.62% yield when leaves were extracted twice with distilled water at room temperature (Adewole and Ajewole, 2009). When soaked for 48 h in 80% ethanol, about 10.55% of the compounds in the leaves dissolved (Foong and Hamid, 2012; Hamizah et al., 2012). A different study percolated dry leaves with 95% ethanol to get a 19.3% yield of soluble substances (Singleton et al., 1999).

So, the aims of this work were characterizing the extracts obtained by this technique and see if methanol and ethanol present different results, as well as analyze annonacin and squamocin by UPLC–QTOF–MS.

Materials and methods

Chemicals and graviola leaves

Methanol (CH_3OH), chloroform ($CHCl_3$), acetonitrile (CH_3CN) and ethanol were from Honeywell Burdick & Jackson (Muskegon, MI, USA). Deuterated chloroform ($CDCl_3$), deuterated methanol (CD_3OD) and deuterated water (D_2O) were from SigmaAldrich (St. Louis, MO). Leucine-enkephalin was purchased from Waters (North Kingstown, RI, USA). Lyophilized graviola leaves were obtained by Ingrid de Moraes from a commercial cultivation located in the city of Trairi (Ceará, Brazil), latitude 3°22′15.98″S and longitude 39°17′34.46″W. Voucher specimens are kept at Embrapa in Fortaleza. The voucher number is 49002. After being harvested, the leaves were washed, sanitized with a sodium hypochlorite solution (100 μl l^{-1} or 100 ppm) and then lyophilized in a model LP 510 lyophilizer (Liobrás, São Carlos, SP - Brazil), at Embrapa Tropical Agroindustry located in Fortaleza, Ceará, Brazil.

Extractions

About 10 g of lyophilized leaves were mixed with enough of HydroMatrix™ (SigmaAldrich, St. Louis, MO) to fill the 100 ml stainless steel sample cell used in an Accelerated Solvent Extractor (ASE, ThermoFisher Scientific, Sunnyvale, CA). Then, CH_3OH or ethanol was added while the temperature and pressure were increased to 100 °C and 10.3 MPa (100 atm) over a 3 min time (static time). Next, the solvent was purged into a collection vessel. A total of four cycles were run to statically extract the sample, resulting in a total volume of about 160 ml. The solvent was evaporated off and the oily residues remaining after extraction with each solvent were weighed. A portion of the residue obtained from the methanol extraction was dissolved in 99.99% CD_3OD and analyzed by NMR. Portions of the methanolic and ethanolic residues were analyzed for total phenolics, as described below. Then, portions of the residues obtained from the methanolic and ethanolic extraction were partitioned between $CHCl_3$ and water. The $CHCl_3$ phase was collected, the solvent evaporated off, the residue redissolved in $CDCl_3$ and NMR spectra acquired. Finally, a 10 g portion of dried leaves were extracted by ultrasonication. It was done with 200 ml of 50% ethanol plus 50% water at 40 °C for 10 min, using an Ultracelaner 1450 ultrasonicator from Unique (Indaia, SP, Brazil). It was done at a frequency of 20 kHz and with 800 W power. This extract was also analyzed for total phenolics.

Also, three portions of about 2.5 g and one portion of about 5.0 g of lyophilized leaves were added to 237 ml (one cup) of water at 90 °C for 10 min, after which the solutions were filtered and analyzed by LC–MS/MS.

NMR analyses

^{1}H and ^{13}C{^{1}H}-NMR spectra were obtained using an Agilent DD2 600 MHz NMR (Santa Clara, CA). A 30° pulse width and 1 s pulse delay were used for the ^{1}H NMR, while a 30° pulse width and 2 s pulse delay were used for the ^{13}C NMR spectra. Chemical shifts were referenced to either the CD_3OD peaks at 3.35 and 4.78 (for ^{1}H) and 49.3 ppm (for ^{13}C) or the $CDCl_3$ peaks at 7.27 and 77.23 ppm for ^{1}H and ^{13}C, respectively.

Analysis for total phenolic compounds

The concentrations of total phenolics in the methanolic and ethanolic extracts were determined using the Folin–Ciocalteu reagent and a gallic acid reference standard, as described

previously (Singleton et al., 1999). Results were expressed as mg gallic acid equivalents per g of sample, or mg GAE/g-dw.

UPLC–QTOF–MS analysis

First, about 10 mg of each of the residues remaining after evaporating the solvent off of the hot, pressurized extracts was dissolved in 5 ml of ethanol:H_2O (1:1, v/v) and passed through a LC-18 Supelcoclean 6 ml (0.5 g) solid phase extraction (SPE) cartridge (Supelco, Bellefonte, PA, USA) that was preconditioned by washing it three times with CH_3OH and then equilibrated with 3 ml of deionized water. The acetogenins were eluted with 6 ml of CH_3CN. The CH_3CN was evaporated off and the residue redissolved in 1 ml of CH_3CN:H_2O (15:7, v/v). The residues from each sample were analyzed by positive ion UPLC–MS analysis using a Xevo UPLC-QTOF from Waters (Milford, MA) and an Acquity UPLC BEH C18 column, 1.7 μm, 2.1 mm × 100 mm. A gradient elution was used with a mixture of H_2O and CH_3CN and 0.1% formic acid, flowing at 400 μl/min. That is, even though the concentrations of H_2O and CH_3CN varied, the concentration of formic acid did not. It was held constant at 0.1%. It started with 35:65 H_2O/CH_3CN (v/v) for the first 25 min, then increased linearly to 1:9 H_2O/CH_3CN (v/v) from 25 to 30 min. It was held at 1:9 H_2O/CH_3CN (v/v) from 30 to 35 min, then decreased linearly to 35:65 H_2O/CH_3CN (v/v) from 35 to 36 min and held at this from 36 to 40 min. The MS spectra were acquired in positive ion mode with an electrospray ionization (ESI) source. The MS parameters were as follows: desolvation temperature 350 °C, capillary voltage 3.2 kV, sampling cone voltage 32 V, extraction cone voltage 1.0 V, source temperature 120 °C, cone gas 2 l/h, desolvation gas flow 350 l/h, purge gas flow 350 l/h, collision energy 5.0 V. Centroid data were collected for each sample in a range 110–800 Da, and the *m/z* value of all acquired spectra was automatically corrected during acquisition based on lockmass by infusing 200 pg/l of leucine-enkephalin thorough Lockspray at a flow rate of 20 μl/min. The calibration was performed to achieve an acceptable accuracy error of ±5 ppm. The accurate mass and molecular formula denomination were acquired with the MassLynx 4.1 software (Waters MS Technologies).

Annonacin and squamocin standards from Pierre Champy at the University of Paris were used to identify and quantify the peaks due to annonacin (**1**) and squamocin (**2**). They eluted at 14.1 and 15.6 min, respectively. Ions with *m/z* = 597.47 and 623.48 Da were used to quantify annonacin and squamocin, respectively. Calibration curves were obtained using 10–200 ng/ml annonacin and squamocin, assuming that the standards were 100% pure. Samples were injected in triplicate with results reported as the average ± standard deviation.

Results and discussion

Extraction

Dry methanol at 100 °C and 10 MPa (100 atm) pressure solubilized 33% of the leaves, while ethanol under the same conditions solubilized 41%. So, hot, pressurized methanol extracted much more material than boiling (or percolating) under ambient pressure, which solubilized 19.3% of the leaves in a previous study (Port's et al., 2013). The concentration of total phenolic compounds that were found using hot, pressurized methanol extraction was 100.3 ± 2.8 mg GAE/g. This compares to 93.2 ± 2.0 mg GAE/g in the hot, pressurized ethanol extract, 54.6 mg GAE/g in the ultrasonic extract using 50% ethanol and the value of 33 mg GAE/g that was found by others when leaves were extracted with boiling water. So, extraction with dry methanol at 100 °C and 10 MPa (100 atm) pressure can extract more phenolic compounds than boiling water under ambient pressure.

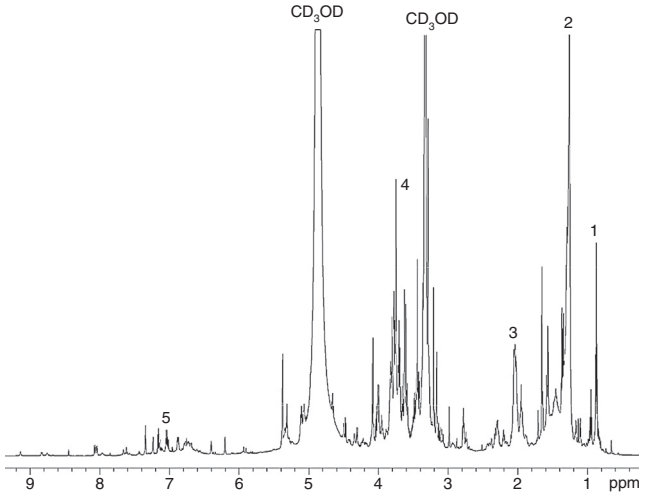


Fig. 1. ^{1}H-NMR spectrum of methanolic extract (100 °C, 100 MPascal) of lyophilized graviola leaves, in CD_3OD. Peaks: 1: –C**H**$_3$; 2: –(C**H**$_2$)$_n$; 3: **H**$_2$C–CH=CH–; 4: **H**CO of sugars 5: phenyls and/or phenolics.

NMR analysis

The ^{1}H and ^{13}C{^{1}H}-NMR spectra of the methanolic extract of lyophilized graviola leaves are shown in Figs. 1 and 2. They both contain signals (chemical shifts) due to the CH_3–$(CH_2)_n$$CH_2$COOR of esters (^{1}H: 0.66–1.0 and 1.1–1.4 ppm; ^{13}C: 14.6, 16.3–39.3 and 175–177 ppm), the –HCOH and –H_2COH of carbohydrates (^{1}H: 3.0–5.4 ppm; ^{13}C: 62.2–108.8 ppm) and the HC=C of aromatic hydrogens in phenolic compounds (^{1}H: 6.4–8.1 ppm; ^{13}C: 125.5–166.2 ppm). There are also signals due to –CH_2–HC=CH– portion of unsaturated fatty acyls (^{1}H: 6.4 ppm; ^{13}C: 108.9–119.5 ppm). The relative contributions to the total peak area in the ^{1}H spectrum were 24% alkyl (CH_3–$(CH_2)_n$), 46% carbohydrate, and 4.2% phenyl. There were no peaks due to di- or triglycerides. The alkyl, carbohydrate and carbonyl carbons all produced chemical shifts that were consistent with the presence of esters of fatty acyls and sugars. These are more commonly called fatty acid glycosides. They have been found in the tropical fruit called noni (*Morinda citrifolia*) and have anticancer properties

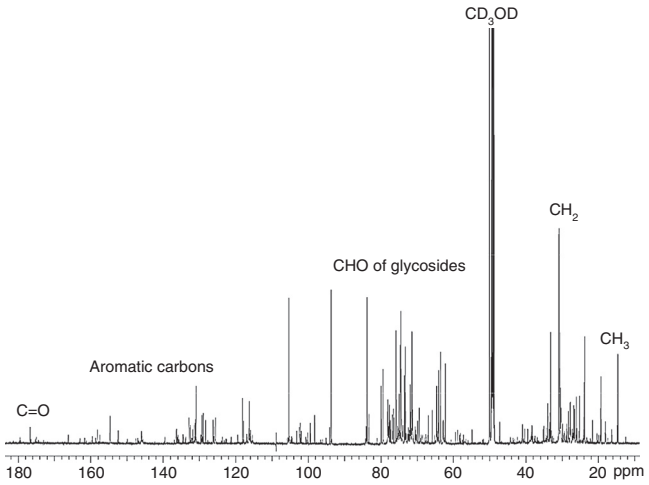


Fig. 2. ^{13}C{^{1}H}-NMR spectrum of methanolic extract (100 °C, 100 MPascal) of lyophilized graviola leaves, in CD_3OD.

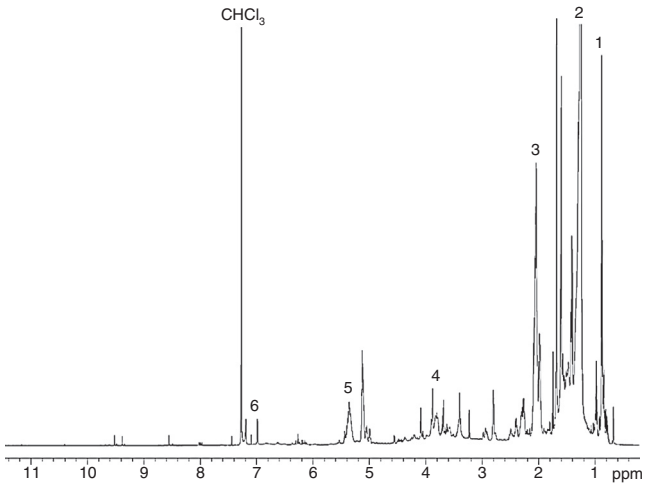


Fig. 3. ^{1}H NMR spectrum of the portion of the methanolic extract (100 °C, 10 MPa) of oven dried graviola leaves that partitioned into $CHCl_3$, redissolved in $CDCl_3$. Peaks: 1: –C**H**$_3$; 2: –(C**H**$_2$)$_n$; 3: **H**$_2$C–CH=CH–; 4: **H**CO of sugars; 5: **H**C=C**H**; 6: HC=C in the unsaturated γ-lactone toxicophore in acetogenins; 0.1% $CHCl_3$ in 99.9% $CDCl_3$ solvent.

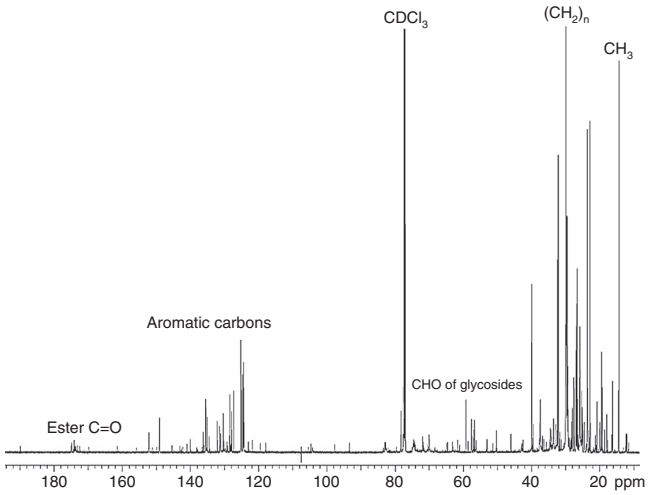
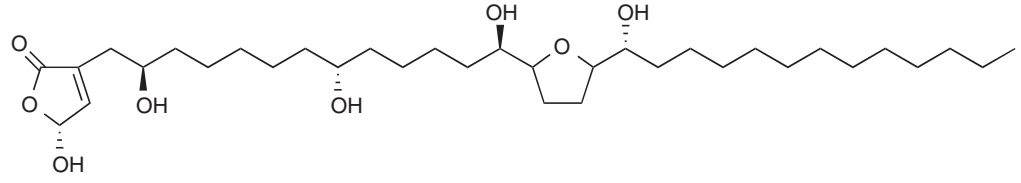
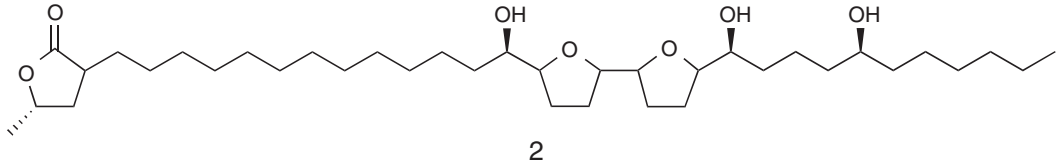


Fig. 4. ^{13}C{^{1}H}-NMR of the portion of the methanolic extract (100 °C, 10 MPa) of oven dried graviola leaves that partitioned into $CHCl_3$, redissolved in $CDCl_3$.

(Smith et al., 2014; Akihisa et al., 2007; Kim, 2010). It should also be noted that even though the methanolic extract was green due to the presence of chlorophyll, the chlorophyll was present at too low of a concentration to produce peaks in the NMR spectra. There may also be some signals due to terpenes that may be slightly soluble in methanol, even though they are usually extracted using hexane.



1



2

About 37.5% of the methanolic extract partitioned into $CHCl_3$. The ^{1}H and ^{13}C{^{1}H}-NMR spectra of this lipophilic portion of the methanolic extract are shown in Figs. 3 and 4. There are chemical shifts at or about 7.0 and 7.2 ppm due to the unsaturated γ-lactone toxicophore that is in neurotoxic acetogenins (Luo et al., 2013), and very small signals due to four different HC=C hydrogens in chlorophyll at 8.00, 8.55, 9.29 and 9.52 ppm.

In comparison, the ^{1}H and ^{13}C{^{1}H}-NMR spectra of the portion of the ethanolic extract that partitioned into $CDCl_3$ are shown in the Supplementary Material. The chemical shifts due to the unsaturated γ-lactone toxicophore are also present.

LC–MS analysis

Seven point calibration curves each for annonacin (**1**) and squamocin (**2**) showed adequate linear correlation between concentration and area ($R^2 > 0.99$), the linear range of standard compounds was between 10 and 200 ng/ml for standard compounds. Standards and samples were injected in triplicate. The injections showed that the results are highly reproducible and repetitive. The LC–MS chromatograms of 200 ng/ml annonacin and squamocin standards and the methanolic extract of graviola leaves are shown in Fig. 5. The concentrations of the neurotoxins annonacin and squamocin were found to be 1.68 ± 0.08 and 0.023 ± 0.001 mg/g-dw, respectively in the dried leaves. Pressurized methanol solubilized more annonacin and squamocin than ethanol. That is, 305.6 ± 28.3 μg/g-dw annonacin was found in the methanolic extract and 226.0 ± 0.07 μg/g-dw was found in the ethanolic extract. On the other hand, 17.4 ± 0.89 μg/g-dw squamocin was found in the methanolic extract, and 4.75 ± 0.41 mg/g-dw squamocin was found in the ethanolic extract. On the other hand, a hot, aqueous infusion (tea) solubilized only 0.213% (0.65 ± 0.07 μg/g-dw) of the annonacin from 2.5 g of dry leaves and too little of the squamocin to be detected. When 5.0 g of leaves were used to prepare a tea, only 0.108% (0.33 μg/g-dw) of the leaves. So, tea bags containing about 5 g of dry graviola leaves will not solubilize much more annonacin than tea bags containing 2.5 g of leaves. Thus, whole graviola leaves contain significant amounts of the neurotoxins annonacin and squamocin, as well as phenolic compounds. Moreover, the potential neurotoxicities of whole leaves in dietary supplements could be much higher than that of a tea that is made from them.

In conclusion much more material in dried graviola leaves can be solubilized using pressurized liquid extraction and either dry methanol or ethanol, with ethanol solubilizing a little bit more.

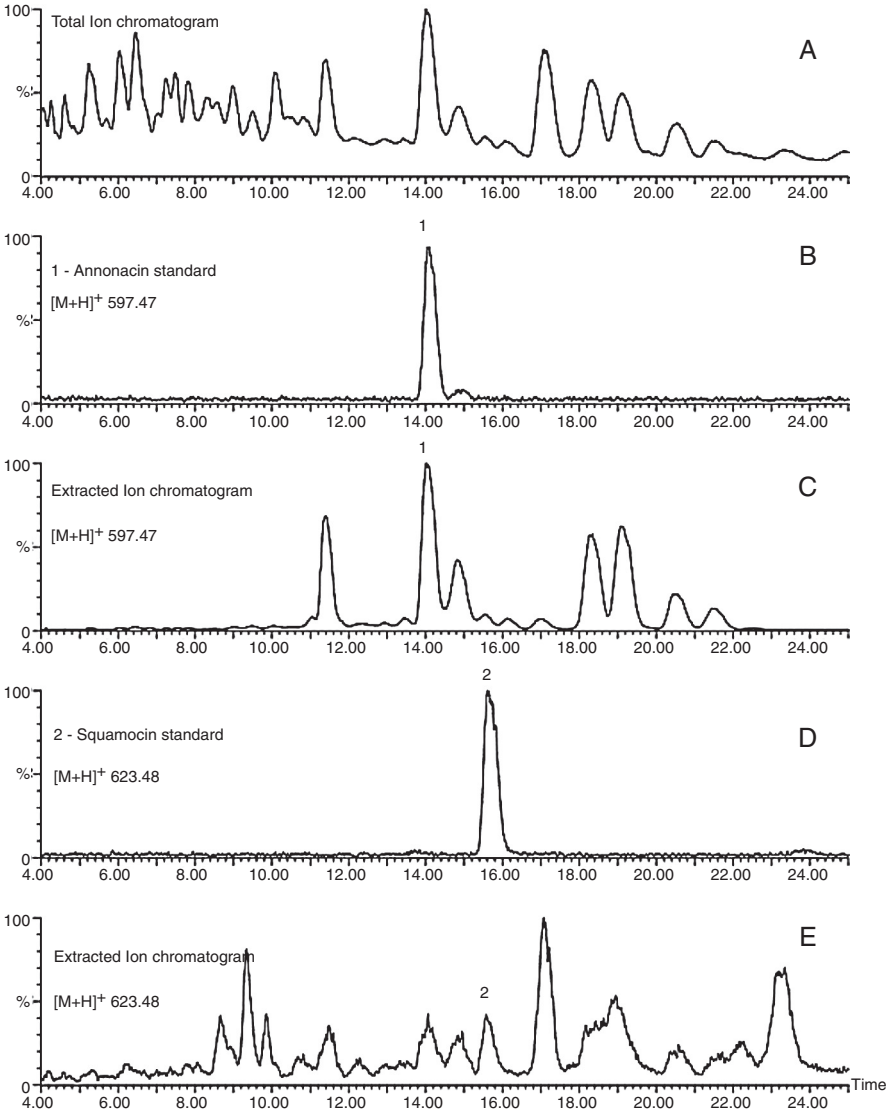


Fig. 5. UPLC–QTOF–MS chromatogram of the hot, pressurized methanolic extract (100 °C, 10 MPa) of lyophilized graviola leaves – total ion chromatogram of the sample (A); annonacin standard – 200 ng/ml (Rt = 14.1 min) (B); all peaks of ions $[M+H]^+ = 597.47$ extracted ion chromatogram of the sample (C); squamocin standard – 200 ng/ml (Rt = 15.6 min) (D); all peaks of ions $[M+H]^+ = 623.48$ extracted of the sample (E).

The NMR spectra were dominated by peaks due to fatty acid glycosides, which may have anticancer and other health benefits (Kim et al., 1998; Akihisa et al., 2007; Smith, 2014). They are also surfactants, so they will probably increase the solubilities of acetogenins in hot water. The NMR spectra also contained chemical shifts due to the unsaturated γ-lactone toxicophore that is in annonacin and squamocin (Machado et al., 2015). They are also surfactants, so they will probably increase the solubilities of acetogenins in hot water. Finally, the total phenolics in the hot, pressurized methanolic and ethanolic extracts were 83 and 64 mf GAE/g, respectively.

Moreover, methanol at 100 °C and 10 MPa (100 atm) pressure solubilized 33% of the lyophilized graviola leaves. Ethanol solubilized 41% of the lyophilized leaves. The concentrations of total phenolic compounds were 83 and 64 mg GAE/g for the methanolic and ethanolic extracts, respectively. Moreover, the toxicophore (unsaturated γ-lactone) that is present in neurotoxic acetogenins was found in the lipophilic portion of these extracts. The concentrations of the neurotoxins annonacin (**1**) and squamocin (**2**) were found by UPLC–TOF–MS to be 305.6 ± 28.3 and 17.4 ± 0.89 mg/g, respectively in the dried leaves. Pressurized methanol solubilized more annonacin than ethanol, while pressurized ethanol solubilized more squamocin than methanol. On the other hand, a hot, aqueous infusion (tea) made from 2.5 g of dried leaves in 237 ml (one cup) of water solubilized only 0.213% (0.65 ± 0.07 μg/g-dw) of the annonacin and too little of the squamocin to be detected.

In summary, we conclude that hot, pressurized ethanol can solubilize more material than ethanol, the concentrations of total phenolic compounds were 100.3 ± 2.8 and 93.2 ± 2.0 mg gallic acid equivalents per g of sample for the methanolic and ethanolic extracts, respectively, the toxicophore (unsaturated γ-lactone) that is present in neurotoxic acetogenins was found in the lipophilic portion of these extracts, the concentrations of the neurotoxins annonacin and squamocin were found by UPLC–TOF–MS to be 305.6 ± 28.3 and 17.4 ± 0.89 mg/g, respectively in the dried leaves, pressurized methanol solubilized more annonacin and squamocin than ethanol, and a hot, aqueous infusion (tea) made from 2.5 g of dried leaves in 237 ml (one cup) of water solubilized only 0.213% (0.65 ± 0.07 μg/g-dw) of the annonacin and too little of the squamocin to be detected.

This work should not be taken as reflecting FDA policy or regulations.

Authors' contributions

IVMM, PRVR, FLS, KMC, GJZ, ESB and RES conceived and designed the experiments. IVMM, PRVR, FLS, KMC, GJZ and ESB did the LC-MS and total phenolics analyses as well as data interpretation. RL did the NMR analyses. IVMM, KMR and KT did the pressurized liquid extractions. RES wrote the article. Everyone read the article and suggested some changes.

Conflicts of interest

The authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjp.2015.12.001.

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