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Kaurenoic Acid from Pulp of *Annona cherimolia* in regard to Annonaceae-induced Parkinsonism

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Guadeloupean Parkinsonism has been linked epidemiologically to the consumption of Annonaceae fruits. These were proposed to be etiological agents for sporadic atypical Parkinsonism worldwide, because of their content of neurotoxins such as isoquinolinic alkaloids and Annonaceous acetogenins. The pulp of *Annona cherimolia* Mill. from Spain was screened for these toxic molecules using Matrix-Assisted Laser Desorption Ionisation – Time of Flight mass spectrometry (MALDI-TOF MS) and it was found not to be a source of exposure. However, kaurenoic acid, a diterpene considered to be cytotoxic, was detected in high amounts (66 mg/fresh fruit). Treatment of rat embryonic striatal primary cultures, up to a high concentration (50 µM), did not cause neuronal death nor astrogliosis, suggesting that this molecule is not at risk of implication in human neurodegenerative diseases.

Keywords: *Annona cherimolia*; Annonaceae; Annonaceous acetogenins; Guadeloupean parkinsonism; isoquinolinic alkaloids; kaurenoic acid.

INTRODUCTION

Guadeloupean parkinsonism (Guad-Park) is a sporadic levodopa-unresponsive form of atypical Parkinsonism endemic in the Caribbean island of Guadeloupe. Patients present motor symptoms similar to those of progressive supranuclear palsy (PSP) and dementia. Degenerative features in magnetic resonance imaging, electrophysiological abnormalities and sleep disorders were characterized (Lehéricy *et al.*, 2010, and see ref. cit.). Autopsy of three patients showed marked neurodegeneration in the *substantia nigra*, basal ganglia and thalamus, and revealed that Guad-Park is a tauopathy (see Camuzat *et al.*, 2008). Case–control studies demonstrated a significant association between Guad-Park and the regular consumption of *Annona* spp. (Annonaceae) as herbal teas and fruits (Lehéricy *et al.*, 2010). Atypical Parkinsonian syndromes in populations consuming Annonaceae are also reported in New-Caledonia and in some communities in London. Implication of *Annona* spp. in the Parkinsonism-Dementia Complex of the island of Guam, concomitantly with that of *Cycas circinalis* L., is suggested (Steele *et al.*, 2002, and see ref. cit.).

Two classes of compounds commonly found in Annonaceae are proposed as potential neurotoxins: (a) isoquinolinic alkaloids (Fig. 1a) are a class of candidate toxins for idiopathic Parkinson's disease (Nagatsu, 2002) and, possibly, tauopathies (Höllerhage *et al.*, 2009); alkaloids from *Annona muricata* L. (soursop) exert weak neurotoxicity *in vitro* (Lannuzel *et al.*, 2002). Structurally related to dopamine, they may account for symptomatic effects (Banning *et al.*, 1980; Watanabe *et al.*, 1983). Leaves, twigs and roots of *A. cherimolia* Mill. (cherimoya) yielded several benzylisoquinolines and aporphines (Chulia *et al.*, 1995; Chen *et al.*, 1997, 2001; Fresno and Cañavate, 1983). Fruits of *A. muricata* and *A. squamosa* L. (custard-apple) were shown to contain these compounds (Champy *et al.*, 2005; Hasrat *et al.*, 1997; Kotake *et al.*, 2004); (b) Annonaceous acetogenins, a large family of lipophilic complex I inhibitors specific to Annonaceae (Bermejo *et al.*, 2005), appear as prime candidate toxins. They induce Tau hyperphosphorylation and redistribution, disruption of microtubule network and cell death in primary neuronal cultures. Annonacin, a prototypical acetogenin (Fig. 1b), was shown to induce neurodegeneration after subchronic systemic intoxication in rats (Höllerhage *et al.*, 2009, and see ref. cit.). Acetogenins appear to be abundant in *A. muricata* pulp from various origins, and were also identified in the fruits of *A. squamosa* (Champy *et al.*, 2009, and see ref. cit.) and *Asimina triloba* Dunal. (paw-paw; Pomper *et al.*, 2009). The French food security agency (AFSSA) expressed safety concerns about the consumption of *Annona* spp. (AFSSA, 2010).

Ent-kauranes diterpenoids are another class of small molecules identified in several Annonaceae, including the stems of *A. cherimolia* (Chen *et al.*, 1998). *Ent*-kaur-16-en-19-oic acid (kaurenoic acid, KA, Fig. 2) was shown

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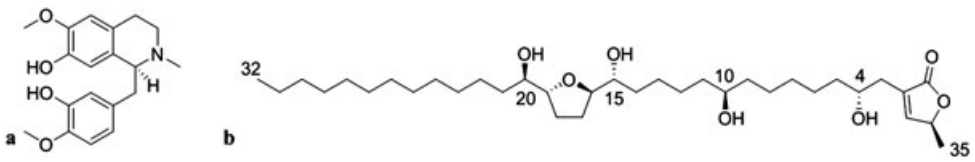

Figure 1. Prototypical benzyl-isoquinolinic alkaloid – (*S*)-Reticuline (a), and Annonaceous acetogenin – Annonacin (b).

to be abundant in the pulp of *A. squamosa* (0.9 g/kg; Andrade *et al.*, 2001). Isolation from the fruit of *A. cherimolia* was published during preparation of this manuscript (Miyashita *et al.*, 2010). This molecule exerts various biological activities (Ghisalberti, 1997) and raised safety concerns because of its cytotoxicity and genotoxicity in various cell lines *in vitro* (see Garcia *et al.*, 2007). It is noteworthy that safety assessments for steviosides and their metabolite steviol (*ent*-13-hydroxy-kaur-16-en-18-oic acid) were recently reviewed (Brusick, 2008). However, KA was shown to cross the blood–brain barrier (Dalo *et al.*, 2007), and its possible implication in the aetiology of Guad-Park was never questioned.

The remarkable chemical homogeneity amongst Annonaceae suggests that all edible species of this family might constitute dietary sources of presumably toxic compounds. *Annona cherimolia* Mill. is a fruit tree from South-America, cultivated in tropical and sub-tropical regions. Spain leads the world in cherimoya production, with some 3600 ha cultivated in the southern part of the country, which yielded 20000 tonnes of fruits in 1991 (VanDamme and Scheldeman, 1999). Therefore the pulp of fruits ('fino de jete' cultivar) harvested in autumn in Spain were investigated.

MATERIAL AND METHODS

General experimental procedures. Optical rotation was measured with a Schmidt-Haensch Polartronic I polarimeter. IR spectra were recorded on a Bruker Vector 22 spectrometer. Electrospray mass spectrometry (ESIMS) was performed with a Navigator mass spectrometer (Thermofinnigan, France). 1D and 2D NMR spectra were obtained with a Bruker AC-200 (200 MHz) and Bruker AM-400 (400 MHz). Matrix-Assisted Laser Desorption Ionisation – Time of Flight mass spectrometry (MALDI-TOF MS) was performed with a Perseptive Voyager DE STR MALDI time-of-flight mass spectrometer (Perseptive Biosystems, Framingham, MA, USA), equipped with a Tektronix TDS 540 C digital oscilloscope (500 MHz, digitization rate 2 Gigasamples.s^{-1}) and with a N_2 laser (337 nm wavelength). 2,4,6-trihydroxy-acetophenone in MeOH/H_2O (1/1) was used as the matrix (10 mg/mL; Aldrich Chemical Co., Milwaukee, WI, USA); Pepmix 5 was used as an internal standard (bradykinin [1–5]: 573.3150 Da and bradykinin [1–7]: 757.3998 Da; 1/20; LaserBio Labs, Sofia-Antipolis, France). HPLC analyses were performed with a Beckman 112 pump, a Varian 9050 spectrophotometer (214 nm) and a U6K Waters injector on a Prepak μBondapak C_{18} column (10 μm, 8 × 100 mm). Preparative HPLC system was composed of a Waters 590 pump, a Waters 484 spectrophotometer (210 nm), a Waters SSV injector and a ABB SE120 recorder on a Prepak μBondapak C_{18} column (10 μm, 25 × 100 mm). For thin layer chromatography (TLC), the following material and reagents were used: Merck 60F_{254} silica plates; Kedde reagent (dinitrobenzoic acid 10% in MeOH, then KOH 2 N in EtOH) for detection of unsaturated butyrolactones; Dragendorff's reagent for detection of alkaloids. Open column chromatographies (CC) were performed with Sephadex® LH-20 (Pharmacia) and silica gel Merck 60 H (Merck 7736; VWR, Fontenay sous Bois, France). Solvents were purchased from Carlo-Erba (VWR) and HPLC and MS solvents from Prolabo (VWR). Labeled cells were visualized under an epifluorescence microscope (DM IRB, Leica, Wetzlar, Germany) equipped with an ORCA-ER digital camera (C4742-95, Hamamatsu, Hersching, Germany) and a computer-based image analysis system (Simple-PCI 6.1, Cimaging Systems, Hersching, Germany).

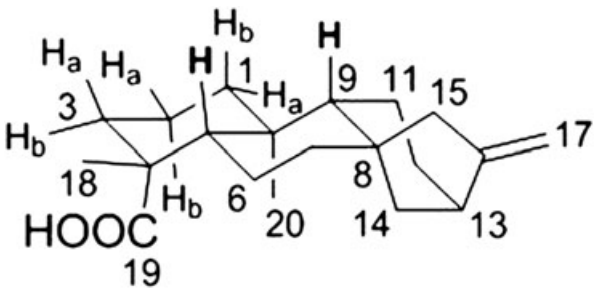

Figure 2. Kaurenoic acid.

Plant material. Forty six fruits (9.6 kg) originating from Spain were purchased in Rungis market (France). Seeds and pericarps were discarded. The pulp was dried at a moderate temperature.

Extraction procedure. The dried pulp (~ 2.5 kg) was extracted with MeOH (6 × 5 L) to give extract **A** (847 g), which was partitioned between MeOH, water and CH_2Cl_2 (3:7:10). After concentration, the hydromethanol layer was subjected to a classical procedure for purification of alkaloids to yield fraction **B** (190 mg). The chloromethylenic counter-extract was named **C** (23 g).

MALDI-TOF MS. Acetogenins were searched in **A**, **C** and subsequent fractions (10 mg/mL) in positive mode, as $[M+Na]^+$ and $[M+K]^+$ adducts; alkaloids were searched in **A** and **B** (10 mg/mL) as $[M+H]^+$ ions, under previously described conditions (Champy *et al.*, 2005, 2009).

Purification and determination of kaurenoic acid. Fraction **C** was subjected to exclusion CC (Sephadex® LH-20, CH_2Cl_2/MeOH 2:1) to yield fractions **F-1** to **F-8**, followed by CC on silica gel of fraction **F-4** (60 H, C_6H_{12}/AcOEt/iPrOH 70/8/4) and Reverse-phase high performance liquid chromatography (RP-HPLC) (CH_3CN/H_2O 70:30, flow rate: 9 mL/min) to give KA (164 mg; TLC: CH_2Cl_2/AcOEt/MeOH 8.5:1:0.5, R_f 0.78; RP-HPLC: MeOH/H_2O 9:1, R_T 9.6 min).

Ent-kaur-16-en-19-oic acid: White crystals; mp: 179–180 °C; $[\alpha]_D$= −13.4° (c 0.015, CH_2Cl_2); IR: 3001, 2928, 2853, 2361, 1692, 1656, 1470, 1446, 1367, 1325, 1263, 1209, 1194, 1180, 1155, 1089, 1023, 955, 873, 798, 741 cm^{-1}; UV (MeOH) λ_{max}: 209.6 nm. ESIMS *m/z* = 325: $[M+Na]^+$; HR-ESIMS: *m/z* = 301.2174, calcd. for $[C_{20}H_{30}O_2\text{-}H]^-$: 301.2158. ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.81 *m* (1 H): H-1a; 0.95 *s* (3 H): H-20; 1.01 *m* (1 H): H-3a; 1.05* *m* (1 H): H-5; 1.07* *m* (1 H): H-9; 1.14 *m* (1 H): H-14a; 1.24 *s* (3 H): H-18; 1.41 *m* (1 H) H-2b; 1.44 *m* (1 H): H-7a; 1.47 *m* (1 H): H-12a; 1.51 *m* (1 H): H-7b; 1.57 *m* (2 H): H-11; 1.62 *m* (1 H): H-12b; 1.83 *m* (2 H): H-6; 1.87 *m* (1 H): H-2a; 1.90 *m* (1 H): H-1b; 1.99 *m* (1 H): H-14b; 2.05 *m* (2 H): H-15; 2.31 *m* (1 H): H-3b; 2.64 *m* (1 H): H-13; 4.74 *s* (1 H): H-17a; 4.80 *s* (1 H): H-17b. *: interchangeable. ^{13}C NMR ($CDCl_3$, 400 MHz, JMOD): 15.6: C-20 (CH_3); 18.4: C-11 (CH_2); 19.1: C-2 (CH_2); 21.8: C-6 (CH_2); 29.0: C-18 (CH_3); 33.1: C-12 (CH_2); 37.8: C-3 (CH_2); 39.7: C-10 (C_{IV}); 39.7: C-14 (CH_2); 40.7: C-1 (CH_2); 41.3: C-7 (CH_2); 43.7: C-4 (C_{IV}); 43.8: C-13 (CH); 44.2: C-8 (C_{IV}); 48.9: C-15 (CH_2); 55.1: C-9 (CH); 57.0: C-5 (CH); 103.0: C-17 (C = $\underline{C}H_2$); 155.9: C-16 (C_{IV}); 184.5: C-19 (COOH). Key NOESY correlations: H-5 → H-1b, H-9; H-9 → H-11, H-15; H-15 → H-17a; H-12 → H17b.

The total amount of KA was determined by Reverse-phase high performance liquid chromatography with photodiode array-detection (RP-HPLC-DAD) (CH_3CN/H_2O 70/30, flow rate: 1 mL/min) in fractions containing the molecule, according to their HPLC profile (co-injections wih KA; fractions **F-3-3** to **F-3-8**, obtained from **F-3** of CC Sephadex® LH-20 by fractionation on CC silica gel 60 H (C_6H_{12}/AcOEt 1:0→7:3): 639 mg, 565 mg, 583 mg, 693 mg, 69 mg, 33 mg; Fraction **F-4**: 418 mg). For quantification, KA and fractions were diluted extemporaneously in MeOH, and 100 µg of each fraction was injected. The study was conducted at 210 nm, with a limit of quantification of 5×10^{-2} µg KA injected. The straight-line regression equation was obtained with six dilutions of KA, injections ranging from 1 to 10^3 µg, by plotting peak area against concentration, with excellent correlation ($R^2 > 0.9997$). Precision was tested for three concentrations of the standard corresponding to the low, medium and high ranges of the calibration curve, and was comprised between 2% and 4% (relative standard deviation).

***In vitro* study.** Striatal primary cultures were prepared from Wistar rat (Janvier Breading Centre, Le Genest St Isle, France) embryos (day 16.5) and cultured as described previously (Höllerhage *et al.*, 2009). Fractions **A**, **C** and KA were dissolved in DMSO and stored at −20 °C. On day 5–6 *in vitro*, the cultures were treated for 48 h with fractions or KA (10 mg/mL and 1–50 µM respectively, DMSO < 0.01%). Immunohistological fluorescent labeling of neurons and astrocytes was performed with NeuN and anti-GFAP primary antibodies (Glostrup, Denmark), and after washing, the cells were incubated with the appropriate secondary antibody: Cyanide 3-conjugated goat anti-mouse IgG (1:500, Jackson ImmunoResearch, West Grove, PA, USA) and Alexa 488 goat anti-rabbit (1:500, Molecular probes, France). Nuclei were visualized with the fluorescent DNA stain Hoechst 33342, as described (Höglinger *et al.*, 2003). All cells in five randomly distributed visual fields were analysed and quantified at a 40× magnification. For each cell, the neuronal integrity (loss of dendrites) was determined by inspection by phase contrast microscopy, nuclear integrity (DNA condensation) and morphological changes in astrocytes, by fluorescence microscopy, in three wells from three independent experiments.

RESULTS

The seedless pulp of about 50 fruits of *A. cherimolia* was dried and extracted with MeOH to give extract **A**, which was partitioned between MeOH, water and CH_2Cl_2. From the hydromethanol layer, an alkaloidic extract **B** of very low mass was obtained. **A**, **B** and counter-extracts were negatively searched for alkaloids by TLC using Dragendorff's reagent. A more sensitive method, MALDI-TOF MS allowing detection of trace elements in complex matrixes and used previously for the analysis of alkaloids in *A. muricata* (Champy *et al.*, 2005), confirmed the absence of isoquinolines. Searches for acetogenins in extract **A**, chloromethylenic counter-extract **C** and subsequent fractions, conducted using MALDI-TOF MS (l.o.d. ~150 fmol/spot; Champy *et al.*, 2009), also proved negative. In parallel, *in vitro* neurotoxicity of **A** and **C** was evaluated in rat embryos primary striatal cultures – a model chosen because neuropathological examination of guadeloupean patients showed particular degeneration and astrogliosis in the *Caudate putamen*. Due to poor yield, extract **B** was not tested. No impairment of neuronal and astrocytic survival was observed (10 mg/mL, for 48 h). It is noteworthy that during previous investigation of *A. muricata* leaves, total extracts containing acetogenins in low amounts did not show significant toxicity, while purified fractions did (unpublished data). Nevertheless, in fraction **C**, a compound of remarkable abundance was noticed. It was thus isolated, using classical chromatographic techniques, and determined to be *ent*-kaur-16-en-19-oic acid (KA) on the basis of its spectral data (UV, IR, 1D and 2D ^{1}H and ^{13}C NMR, ESIMS), and by co-injection in RP-HPLC with an authentic sample. Presence of KA in other fractions was challenged and quantification was performed using RP-HPLC-DAD, a method previously used for the same mean (Andrade *et al.*, 2001; Oliveira *et al.*, 2002). One fresh fruit (200 g) was thus estimated to contain 66.3 ± 2.7 mg of the molecule. Putative neurotoxicity of KA was challenged in rat embryos primary striatal cultures, at concentrations up to 50 µM, for 48 h. Neuronal morphology and survival were unaltered in treated cells, as evaluated by NeuN-staining. Hoechst staining did not show nuclear alterations. Anti-GFAP immunofluorescence did not allow the observation of astrocytic alterations or astrogliosis.

DISCUSSION

KA appears to be abundant in the pulp of *A. cherimolia*, as was shown for *A. squamosa* (Andrade *et al.*, 2001). The molecule does not exert toxicity in a sensitive and accurate *in vitro* model for assessment of neurodegeneration. In comparable paradigms, alkaloidic totum of the leaves of *A. muricata* and purified acetogenins, respectively, induced moderate (IC_{50} ~20–50 µM;

Lannuzel *et al.*, 2002) and severe (IC_{50} ~10 nM; Höllerhage *et al.*, 2009, and see ref. cit.) neuronal death. After treatment with KA, the nuclei showed no abnormalities in proliferating and non-proliferating cellular types in the cultures, in contrast with the pro-apoptotic and genotoxic potential reported previously for KA (see above). These data indicate that, despite possible exposure, implication of KA in the pathogenesis of Guad-Park or other forms of sporadic Parkinsonism appears to be unlikely. This observation is of interest in regard to the relatively widespread occurrence of KA in various food sources and medicinal products (Chang *et al.*, 1998; Garcia *et al.*, 2007), and to the environmental etiological hypothesis for worldwide sporadic Parkinsonian syndromes.

Nevertheless, the plant material we used did not to contain isoquinolinic alkaloids, contrary to observations made for other *Annona* pulps (Kotake *et al.*, 2004) and other parts of *A. cherimolia* (Chulia *et al.*, 1995; Chen *et al.*, 2001; Fresno and Cañavate, 1983), nor Annonaceous acetogenins (Bermejo *et al.*, 2005). However, their absence in our batch does not rule out the implication of the species in Guad-Park and sporadic atypical Parkinsonism in tropical areas, as their content of secondary metabolites can be highly variable between varieties and for environmental reasons. Aside from clinical and epidemiological evaluation in heavy Annonaceae consumers, a chemical study of sources of intoxication, including *A. cherimolia* from diverse geographical origins, should be pursued.

Other Abbreviations Used

GFAP: glial acidic fibrillary protein; HR: high resolution; l.o.d.: limit of detection.

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Conflict of Interest

The authors have no conflict of interest.

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