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Screening of Acetogenin-Producing Plants in Brazilian Flora

Annona and *Rollinia* Seed Oil as a Source of the Bioactive Compounds and Subproducts

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ABSTRACT

The acetogenins are strongly bioactive natural compounds present in the bark, roots, leaves, and seeds of many *Annonaceae* plants. They are modified fatty acids and their cytotoxicities have been determined for different biological models including the in vitro growth inhibition of several human cancer cell lines.

Very low acetogenin yield (<0.1 g%) has been found previously in native phytobiomass, and we have now investigated the nonpredatory exploitation of the seeds as acetogenin sources characterizing the seed triacylglycerols (dominant fraction; >90% of the whole lipid extracts) as potential valuable by-products.

Supercritical fluid extraction (SFE) was utilized as an alternative for both kinds of lipid biomass, acetogenins and fats, and manipulation of *Annona* explants for obtaining in vitro callus and/or new plants.

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Index Entries: Acetogenins; *Annonaceae* seed oil; tetrahydrofuranoid fatty acid lactones; *Annona; Rollinia;* polyketides.

INTRODUCTION

A group of natural compounds collectively designated as "acetogenins" is stimulating interest because of its strong biological activity. Acetogenins may be briefly defined as modified long-chain fatty acids presenting a middle chain-insertion of tetrahydrofuran (THF) ring(s). The long C_{35} or C_{37} aliphatic chain presents two other additional features in the structure: one to four hydroxyl substituents (less often, part of them as the isomeric keto form) at variable relative positions and, as a result of the loss of water, a lactone function instead of the free carboxyl group. A representative but not universal formula for this class of bioactive substances is that of Fig. 1. It corresponds to the first isolate, "uvaricin" (*1*), obtained from the roots of *Uvaria accuminata*, and its C_{39} chain bears one unusual *O*-acetyl substituent at the C(24)-OH position. Its structure displays two of the usual aspects: The lactone group belongs to the α, β unsaturated type and the double THF ring arrangement is adjacent.

A comparative view of the natural distribution of acetogenins within the *Annonaceae* family plants from several regions of the world is shown in Table 1. The list is not exhaustive but some pertinent comments are:

1. Except for uvaricin, all them display either either a C_{35} or a C_{37} chain length;
2. Except for bullatacinone, isoannonacin, and isoannonacinone, the γ-lactone ring is of the α, β unsaturated type;
3. The alkyl chain may present, depending on each particular acetogenin, hydroxyl (less often oxo) substituents in 16 different possibilities (see the labeled C* in Fig. 1); and
4. Tetrahydrofuran modification of the fatty acid chain length may involve one or two rings, and in the latter case, they may occur adjacent or not.

Considering any whole plant source, acetogenins are more often found, or at least they occur more concentrated, in the bark, roots, and seeds. Leaves are not to be excluded but because of their richest content on lipophylic pigments, undoubtedly, they present as the more tedious feedstuff to be worked up. Most of the cumulative research was performed in the genera *Annona* and *Rollinia* but the pioneering report corresponding to the chemical description of the antitumoral drug uvaricin resulted from the genus *Uvaria* (*1*).

Once purified, acetogenins generate crystalline or waxy compounds whose melting points seldom exceed 100°C. The optical activity is invariably dextrorotatory and within the range 5–30°C.

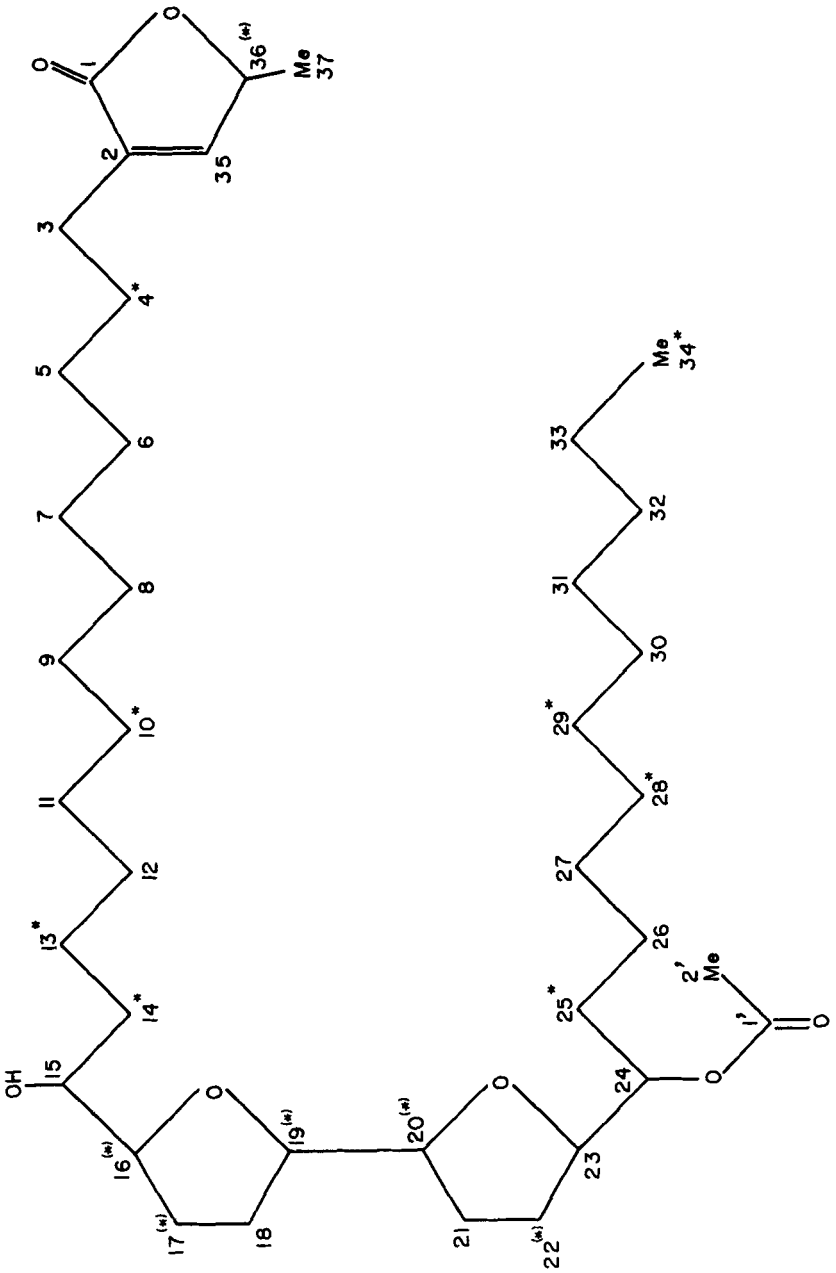

Fig. 1. Uvaricin structure, the first reported natural acetogenin obtained from roots of *Uvaria accuminata* (*1*). * Potential positions of hydroxyl or keto substituents often found in other adjacent *bis*(THF)-bearing acetogenins. (*) Additional positions of hydroxyl or keto substituents found in non-adjacent *bis*(THF)-bearing acetogenins.

Table 1
Cumulative Examples on Acetogenin Research

Year (ref.)	*Annonaceae source* (botanical part)	Structural features (trivial name)
1982 (*1*)	*Uvaria accuminata* (roots)	$C_{39}H_{68}O_7$; mw = 648; $(THF)_2$[a] C-15 = -OH; C-24 = *O*-acetyl (uvaricin)[d]
1984 (*24*)	*Rollinia papilionella* (roots)	$C_{37}H_{66}O_7$; mw = 622; $(THF)_2$[a] C-15, 24, 25 = -OH (rollinicin and isorollicinin)[d]
1984 (*2*)	idem	$C_{37}H_{66}O_7$; mw = 622; $(THF)_2$[a] C-14 = =O; C-15, 24 = -OH (rollinone)
1985 (*25*)	*Uvaria accuminata* (roots)	$C_{37}H_{66}O_6$; mw = 606; $(THF)_2$[a] C-15, 24 = -OH (desacetyluvaricin)[d]
1986 (*26*)	*Asimini triloba* (bark; seeds)	$C_{37}H_{66}O_7$; mw = 622; $(THF)_2$[a] C-4, 15, 24 = -OH (asimicin)[d]
1987 (*27*)	*Rollinia muscosa* (seeds)	$C_{37}H_{66}O_7$; mw = 662; $(THF)_2$[a] C-4, 15, 24 = -OH (rolliniastin I)[d]
1989 (*3*)	*Annona bullata* (bark)	$C_{37}H_{66}O_7$; mw = 622; $(THF)_2$[a] C-4, 15, 24 = -OH (bullatacin)[d]
		$C_{37}H_{66}O_7$; C-15, 24 = -OH; C-36 = =O (bullatacinone)
1989 (*28*)	*Rollinia mucosa* (seeds)	$C_{37}H_{66}0_7$; mw = 622; $(THF)_2$[a] C-4, 15, 24 = -OH (rolliniastatin 2)[d]
1989 (*29*)	*Annona bullata* (bark)	$C_{37}H_{66}O_8$; mw = 638; THF----THF[b] C-4, 16, 19, 24 = -OH (bullatalicin)[d]
1989 (*6*)	*Annona densicoma* (bark)	$C_{35}H_{64}O_7$; mw = 596; $(THF)_1$[c] C-4, 10, 15, 20 = -OH (annonacin)[d]
		$C_{35}H_{62}O_7$; mw = 594; $(THF)_1$[c] C-4, 15, 20 = -OH; C10 = =O (annonacin-10-one)
		$C_{35}H_{64}O_7$; mw = 596; $(THF)_1$[c] C-10, 15, 20 = -OH; C34 = =O (isoannonacin)
		$C_{35}H_{62}O_7$; mw = 594; $(THF)_1$[c] C-15, 20 = -OH; C-10, 34 = =O (isoannonacin-10-one)

Table 1 (Continued)

Year (ref.)	Annonaceae source (botanical part)	Structural features (trivial name)
1990 (*19*)	*Rollinia sylvatyca* (fruits)	$C_{37}H_{66}O_8$; mw = 638; THF----THF[b] C-4, 16, 19, 24 = -OH (sylvaticin)[d]
1990 (*30*)	*Goniothalamus giganteus* (bark)	$C_{37}H_{66}O_8$; mw = 638; THF----THF[b] C-4, 14, 17, 22 = -OH (gigantecin)[d]
1991 (*31*)	*Annona reticulata* (bark)	$C_{35}H_{68}O_5$; mw = 592; $(THF)_1$[c] C-17, 22 = -OH (reticulatacin)[d]
1991 (*4*)	*Annona muricata* (seeds)	$C_{35}H_{64}O_5$; mw = 564; $(THF)_1$[c] C-15, 20 = -OH (solamin)[d]
1991 (*5*)	*Annona cherimolia* (seeds)	$C_{35}H_{62}O_7$; mw = 594; $(THF)_2$[a] C-4, 13, 22 = -OH (molvizarin)[d]
		$C_{37}H_{66}O_7$; mw = 622; $(THF)_2$[a] C-15, 24, 29 = -OH (motrilin)[d]
		$C_{37}H_{66}O_7$; mw = 622; $(THF)_2$[a] C-15, 24, 28 = -OH (squamocin)[d]
1992 (32)	*Asimini triloba* (bark)	$C_{37}H_{66}O_7$; mw = 622; $(THF)_2$[a] C-4, 15, 24 = -OH (trilobacin)[d]

[a]Indicates an adjacent *bis*-tetrahydrofuran ring.
[b]Indicates that the *bis*-tetrahydrofuran has no adjacent rings.
[c]Indicates a single tetrahydrofuran ring.
[d]Indicates that the particular structure bears an insaturation i the γ-lactone.

As a result of the dominance of the apolar portion of the molecule, acetogenins display solubility and physical properties closer to the common lipids. This circumstance, allied to their occurrence in reduced amounts in the plant kingdom, causes difficulty when working a purification schedule. Relatively fat-free issues like bark unfortunately have tenths of organo-solvent-soluble (phenolic) contaminants; a seed, in turn, has most of its mass corresponding to triacylglycerols, an ideal mixing-phase to aceto-genins, thus creating additional obstacles to partition chromatography during the isolation procedure. Furthermore, acetogenins' mol-wt range (596–638 daltons) is just below that of their dragging natural partners, the

triglycerides. Also concerning the main intramolecular chemical linkages for both groups (lactone and ester, respectively), no large difference on polarity is to be expected. These considerations may help to explain the tedious sequence of steps when performing plant extraction with large volumes of solvent and the following column chromatography (e.g., silica gel eluted with increasing polarity gradient from hexane to methanol) to achieve a pure bioactive compound. Examples for pure acetogenin yields from different botanical parts are given as projections from the published partial schedules of fractionation. It was possible to isolate only about 96 mg of pure rollinone from 7.1 kg of dried roots of *R. papilionella* (*2*), a net yield of <0.002 g%. The powder from 3.9 kg of *Annona bullata* bark resulted in about 195 mg of pure bullatacin (*3*), a 0.005% yield. Seeds from *A. muricata* (860 g) furnished about 14 mg of pure solamin (*4*), a partial yield about 0.002 g%. Another seed brand (1 kg amount of *A. cherimolia*) led to sequential isolation of 100 mg of molvizarin, 35 mg of motrilin, 300 mg of rolliniastatin-2, and 450 mg of squamocin, the respective yields in the 0.0035–0.045 g% range (*5*).

Acetogenins' biological activity has been measured in several models in order to establish their antimitotic, antitumor, cytotoxic, antimicrobial, and pesticidal actions. The (in vitro) antitumorogenic effect of acetogenins, owing to the extremely low concentrations required to attain the ED_{50} for several murine and human cancer cell lines in the pg to 1/10 μg/mL range (*3,6*), is a judicious finding to encourage all research efforts chaneling these compounds to animal and human trials that, most certainly, will give the favorable results to support the application of acetogenins as new marketable drugs. One patent on the pesticidal uses of acetogenins is already issued in the United States (*7*).

Regarding the utilization of *Annonaceae* for the isolation of acetogenins, it would advisable to keep in mind the destructive run to yew tree (*Taxus*, spp *brevifolia* and *baccata*) launched just after the reports on taxol, the natural antileukemic and antitumoral active principle found in the bark (*8*) and needles of such a plant. In a coarse comparison, *Annonaceae* are by far more common in the world than *Taxus*, but any intensive exploration of botanical parts such as roots and bark is to be seen as deeply predatory. The genera *Annona* and *Rollinia* usually provide, in several countries, edible fruits in a practice similar to that in the United States concerning *Asimina triloba*. Seeds, following the tasteful fruit pulp ingestion or the industrial preparation of canned juice, are the most attractive source for acetogenin isolation.

Following our interest in seed xyloglucans as substrates to the characterization of the less studied group of xyloglucanase enzymes, we decided to extend the research to the characterization of *Annonaceae* from the Brazilian flora as source of bioactive acetogenins. The huge availability of native and cultivated phytobiomass in the country may allow researchers to add valuable information to the high quality research on acetogenins initiated

by Arizona University about a decade ago and further enriched, among others, by the elegant series of publications from Purdue University. To the best of our knowledge, the first initiative toward acetogenins in Brazil corresponded to our communication (*9*). Our midterm prospects will be concentrated on whole seed crude oil characterization and its uses as a nonpredatory procedure. Because of the very high ratio of triglycerides: acetogenins (>1000:1), this focus claims for a nonwaste-generating procedure saving the prominent subproduct.

As an innovation on the subject, we introduced the application of Supercritical Fluid Extraction (SFE) as an alternative for the routinely described organosolvent extractions and a micropropagation schedule from *Annona* seedlings.

MATERIALS AND METHODS

Collection and Processing of Seeds and Other Botanical Parts

Seeds of an acetogenin-producing referential plant, *Asimini triloba,* were purchased from Mellinger's (N. Lima, OH). Seeds and limited amounts of roots, stem, or twig bark, and leaves of Brazilian *Annonaceae* were obtained from native trees in Porecatu, Curitiba, Guaraqueçaba, and Quatro Barras (State of Parana) and also from Itajai (State of Santa Catarina). The taxonomy of the presently studied biological material is merely tentative since the floral apparatus was available only for a few specimens. Except for seeds, moisture was removed by freeze-drying in order to avoid microbial infection. Seeds were air-dried (average water content in the range 10–20%). Prior to the organosolvent extractions, all materials were finely powdered in a knife blendor (Cole-Parmer, Chicago, IL; maximal speed, 2 × 30 s). The conventional lipid extractants were: methylene chloride:methanol (1:1) or heptane in a solvent:phytobiomass ratio of 6–8:1 (v/v). Following contact for at least 8 h in a rotatory shaker, the solvent was brought to incipient boiling in a water bath and quickly filtered through fritted glass covered by a double layer of filter paper. The residues were twice washed with warm ethanol, and the extracts combined. Solvent removal was carried using a Büchi evaporator. Gravimetric measurements were carried out in an Ainsworth model A-2400DR balance (gram to kilogram range) or in a Sartorius H-30 instrument (New York) (0.1 mg sensitivity). The SFE was performed with hexane using "homemade" equipment consisting of a gas tank, high pressure vessel, extraction vessel inserted in an oven, restrictor outlet, collection vessel, and cryogenic trap, all adapted with the necessary valves and pressure/temperature meters (*10*). The applied pressure was 105 atm at 80°C (seeds) or 175°C (bark).

Lipophilic Material Analyses

Thin layer chromatography (TLC) was carried out in silica gel plates (Merck's chromatoplates 5553) with the following stationary phases: chloroform:methanol (9:1); chloroform:methanol:water (80:19:1); and petrol ether (b.p. 30–65°C):ethyl ether:acetic acid (90:10:1), the latter for resolution of triglycerides (*11*). Plates were examined under short (254 nm) and long (365 nm) UV wavelengths in a C-70G Chromatovue cabinet (San Gabriel, CA) and the lipids detected with iodine vapor followed by 5% ethanolic phosphomolibdic acid at 110°C for 5–10 min. Seed triglyceride profiles were obtained by high-temperature–high-resolution gas chromatography (HT-HRCG) on an L.M.S. HT column (6 m × 0.25 mm id; film thickness 0.08 μm) operated at 350°C with hydrogen as carrier gas (1.73 mL/min; linear velocity 58.8 cm/s) and sample volume of 1.0 μL from solutions of 2 mg/mL of heptane. The gas chromatograph was the HP-5890 series II coupled to a FID detector and an HP-3393A integrator. Crude lipophilic extracts from seeds and other botanical parts were partially fractionated using adsorption column chromatography with alumina (neutral aluminum oxide) or silicic acid as stationary phase, and by molecular sieving using Sephadex LH-20. Prior to HT-HRCG, triglycerides from crude oils were purified by absorption on neutral alumina and selective elution with ethyl ether.

Bioactivity Monitoring

Cytotoxicity of the whole extracts and resulting fractions was evaluated by the brine shrimp lethality test (BSLT) (*12*), and during the routine fractionation procedures the sample amount was increased to 0.5 mg to shorten the dead time of the nauplii to a 1–3 h incubation period. Taking the cationic and anionic composition of sea water as a guide (*13*), an artificial incubation medium for BSLT was prepared, expressed in g/L: sodium chloride 26.3; potassium chloride 0.75; calcium chloride 1.11; magnesium chloride (hexahydrate) 5.1; magnesium sulfate (heptahydrate) 6.2 g; hydrogen sodium carbonate 0.21 g; and sodium bromide 0.08, the final pH being adjusted to 8.1 with a few drops of 2*M* NaOH.

In Vitro Culture of Acetogenin-Producing Plants

Callus production and in vitro organogenesis of *Annona muricata* was carried out using the procedures previously established for *Araucaria angustifolia* micropropagation (*14,15*) with modifications in terms of thiamine and 2,4-D components.

RESULTS AND DISCUSSION

Since bullatacin extracted from branches and twigs of *Annona bullata* (3) was recently noted as being 300 times more efficient in inhibiting tumor growth than taxol extracted from *Taxus* (*16*), the research on acetogenins is experiencing an exponential increase. Furthermore, in contrast with the few species of *Taxus*, the acetogenin sources of the ubiquitous family *Annonaceae*, encompasses about 130 genera and 2300 species in the world flora (*17*). There are at least 29 genera and 260 species reported in Brazil (*18*) and the epicuticular wax of 24 spp. has been studied for taxonomic purposes (*19*). Almost all of the research on *Annonaceae* acetogenins (Table 1) was carried out on the *Annona* and *Rollinia* genera, whose edible and tasty fruits (pulp) are popularly known, in Brazil, as "ata," "pinha," "fruta-do-conde," "condessa," and "araticum." One of them, *A. muricata*, is presently explored on a commercial scale, for the production of pulp juice and ice cream. The botanical equivalent of these plants within the US flora is almost certainly custard-apple and not paw-paw (Indiana banana).

The *Annonaceae*, however, present two complications. On one hand, the huge number of species of flora recently collected in the Amazon basin led to the taxonomical attribution of 12 new species of *Rollinia* (*20*) by the Institute of Systematic Botany of Utrecht. On the other hand, each defined genus and species may furnish a lot of different bioactive compounds (e.g., 11 as detected in *A. bullata* [*16*]). Chirality is an additional complication. Sylvatacin, obtained from *R. sylvatica*, is a tetra-hydroxylated C_{37} acetogenin and it possesses nine independent asymmetric centers leading to 512 possible stereoisomers (*21*).

We have now directed our focus to the exploitation of the whole seed oil of a few but widespread species as sources for both acetogenin (bioactive main product) and glyceride fraction (dominant seed mass byproduct), since the latter could act as a natural and ideal solvent for the former. Two opposite views might be considered when looking at low contents of acetogenins. First, there is great difficulty in carrying out the final purification step, ideally crystallization, for the isolation of valuable acetogenins. And second, a much more simplified procedure might be used if a mixture of the closer related lipophilic classes, acetogenins + triglycerides, were intended to be saved together. As an example, 1 kg of seeds is required to obtain only 16 mg of solamin (*4*). Since the crude oil content of this acetogenin source is about ~20%, there is native solution of 8 mg solamin/100 mL triglyceride. In this case, it is noteworthy that the natural (lipo)solution of acetogenin, namely the seed crude extract, had a nominal drug concentration 80–266 times higher than those respectively observed in the ED_{50} for KB (human epidermoid carcinoma) and VERO (Africa

Table 2
Comparative Features of Seeds from Brazilian *Annonaceae*

Plant or fruit characterization	Mean[a] weight, g	% Oil[b] content, %
1. Paw-paw (*Asimina triloba*) (US reference)	0.93	46%
2. Sweet yelow pulp; brown seeds, "count fruit" (FCg-ANGLN) (*A. squamosa*?)	0.28	33%[c]
3. Deeply sweet pulp; black shiny seeds, fruit shape related to 2., but with easily detachable carpids (FCp-MMun)	0.33	28%
4. Giant conical fruits; sharp pointed (fruit) carpids; ellipsoidal seeds (*Annona muricata*)	0.36	30%
5. A sp. related to 6, but seeds arising from unripened fruits	0.08	10%
6. >12 m tall tree; ox heart-shaped fruit; ripened pulp diarrheic to monkeys; very small seeds (*Annona cacans*)	0.20	32%
7. Corky roots, smooth fruit epicarp, waxy leaves; flat, wide seeds (a typical mangrove custard apple or sweetsop) (ARTCM-GçBA-*Rollinia sericea*)	0.26	35%[d]
8. Rough pericarp; drop-shaped brown seeds (ARTCM-LTR) (*Rollinia rugulosa*)	0.32	35%

[a]Air-dried seeds (moisture content extremes 11 and 23%).
[b]Methylene chloride: methanol extracts.
[c]The duplicate hot heptane extract afforded 31% yield.
[d]The plant bark yield was ca. 6%.

green monkey kidney) cell lines. If the same argument is extrapolated to annonacin, one of the five other bioactive acetogenins extractable from the same seed source, the idea of the use of a triglyceride:acetogenin mix is further reinforced, as annonacin proved to be 100–300 times more cytotoxic than solamin against the same cell lines (4).

We have also found cytotoxicity for brine shrimp in roots, bark, and leaves, besides that always detected in the corresponding seeds, but the exploitation of the first named botanical parts is clearly predatory, until a large plantation of the most productive species is available.

When assaying 1-mg amounts of each crude extract obtained from the different parts from the same plant source n. 2 (Table 2), we found the following order of decreasing cytotoxicity for brine shrimp (fast BSLT): whole seeds > kernel seed > husk seeds > cortex-free roots > leaves >fruit epicarp > root cortex. The same comparative test on whole seeds from different plants was: n. 2 (*Annona squamosa*?) > n. 7 *Rollinia sericea* > n. 3 (*A. reticulata*? > n. 8 *R. rugulosa* > n. 1 *Asimina triloba* > n. 4 *Annona muricata* >> n. 6 *A. cacans* (seed numbers as in Table 2). In comparison

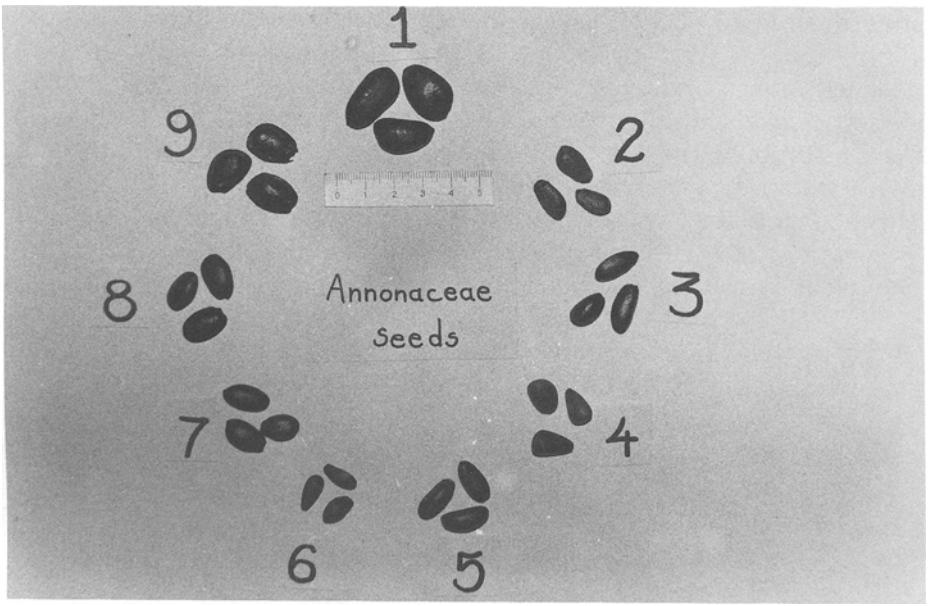

Fig. 2. Visual comparison of *Annonaceae* seeds. 1 = ex- the US paw-paw tree, *Asimini triloba;* 2–9 = ex-Brazilian sources; details about the plant sources in Table 2.

with the above crude extracts, the partial purification of the plant n. 2 crude seed oil by column chromatography allowed enrichment of the cytotoxic compounds, as detected in the fraction eluted of the cytotoxic compounds, as detected in the fraction eluted in the maximal inclusion volume of Sephadex LH-20, in the most polar eluant (methanol) for neutral alumina (following petrol and ethyl ether), and in the medium polarity eluant (ethyl acetate) for the silicic acid column (after methylene chloride but before methanol). The resulting fractions did not have the sufficient purity and amounts for physical or chemical analyses, since only 1–2 g of crude oil could be loaded in the columns.

In Fig. 2 some of the *Annonaceae* Brazilian seeds are compared to a US reference, paw-paw (*A. triloba*) giant seeds. The greatest variations concerned seed size (2.5 × 1.2 cm length to width for paw-paw and 1.3–1.7 × 0.6–1.2 cm for Brazilian spp.), shape, external color, brightness, and weight. Two reliable and measurable parameters, weight and crude oil content, are recorded in Table 2. The smallest seed number found per fruit was 17 and the largest 78, meaning a reasonable supply of crude oil (triglycerides) in view of the fat yields, which range on the average from one-quarter to one-third of the seed weight. Since the seeds were not freeze-dried, the actual moisture content from 11–20% would further improve the net oil yields. A close examination of two other aspects, namely the dimensions of the "micropylar plug" (22) and the type of ruminations, a rich internal architecture connecting the kernel to the tegmen, the

innermost layer of the husk, also proved to be useful in terms of species differentiation. Most of the seeds can be separated into its two main integral anatomical parts, the husk (testa + tegmen) and the kernel. On seed dissection the splitting of the ruminations to these two halves was variable. As a result of the separation of anatomical parts, oil yields of seed sample 2, for instance, were, respectively, 4% (husk) and 42% (kernel), but brine shrimp cytotoxicity was detected in both portions even when the extraction was carried out with pure methylene chloride or ethyl acetate instead of the usual more polar methylene chloride:methanol (1:1 v/v) mixture.

The TLC profiles of most of the seed extracts using the organosolvents, as well as some extracts from other botanical parts, were compared as shown in Fig. 3. The middle plate standards were oleic acid, corn oil, and hydroxystearic acid (R_f = 0.71, 0.89, and 0.62, respectively). Except for the minor polar components in sample 6 and an apparent larger free fatty acid content of sample 7, all seed samples (lanes 1–8) showed triglycerides as the main component, the strongest spot being close to the solvent front with $R_f \sim 0.90$). Prior to the chemical spray of phosphomolybdic acid, these spots were deeply stained in an iodine atmosphere, indicating the unsaturated nature of the their fatty acids. Leaves (lane 13), bark (lane 14), and cortex-free roots (lane 15), all samples being from plant source 2 (Table 2), had fairly distinctive TLC profiles as shown by the richly fluorescent fingerprint, observed under UV light (result not shown). In the latter sample, the alternative extraction procedure with hot heptane (lane 15a) made a large difference, resulting in a cleaner but similarly active extract as in lane 15, which did not occur with the seed material (compare lanes 2 and 2b, obtained with polar and apolar extractants, respectively).

The TLC behavior was further studied in order to confirm the triglycerides as being the almost exclusive component of the extracted lipidic biomass (Fig. 4A,B). They gave the most intense line at R_f = 0.39 (Fig. 4A), as expected in the usual solvent system proposed by Randerath (*11*), and the composition was not affected by the extraction schedule (2b = hot heptane; 2 and 3 = room temperature methylene:methanol mixture; 3s = SFE with hexane). An indication that SFE could be equally applied to other botanical parts was demonstrated with another source of bioactive compounds, namely the stem bark of the mangrove *Rollinia* (sample 7; Table 2) (TLC lanes 17 and 17s, respectively, for the conventional and SFE extractions). The lipid yields of the preliminary SFE from seed n. 3 (11%) and plant n. 7 bark (17%) were, respectively, less and greater than those obtained by conventional extractions, and reflected the contribution of the temperature range in the SFE procedure. Carbon dioxide, with relatively low critical temperature and pressure (31°C and 73 atm) and already approved for fat extraction (*23*), is then an ideal nonpolluting alternative for SFE on *Annona* and *Rollinia* acetogenins. Whatever the botanical source, because of the extremely low content on acetogenin(s), a huge residual matter is generated consisting of lignocellulosics, reserve

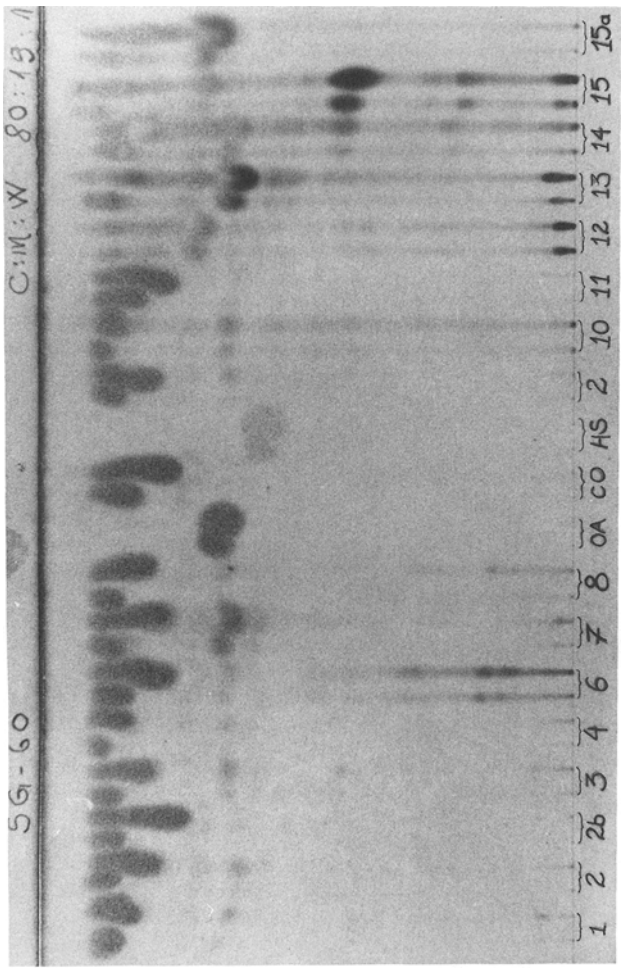

Fig. 3. TLC profile of the lipophilic extracts from seeds and other botanical parts of *Annonaceae* (solvent B, phosphomolybdic acid spray). 1–8 = seed extracts with methylene chloride:methanol; seed numbering as in Table 2. 10–15 = similar extracts from seed husk, seed kernel, fruit pericarp, leaves, bark of twigs, and cortex-free roots of plant source n. 2 (Table 2). 2b and 15a = duplicate extracts from 2 and 15, using hot heptane. OA, CO, and HS = lipid standard of oleic acid, corn oil, and hydroxystearic acid.

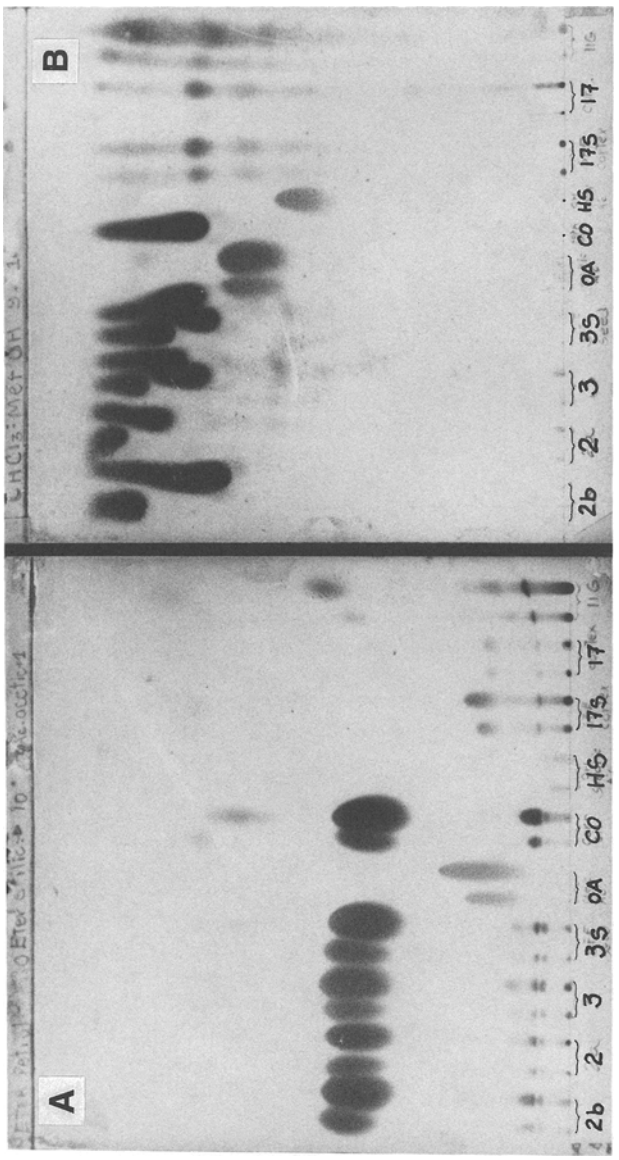

Fig. 4. Comparative TLC profiles of *Annonaceae* seed and bark triglycerides as function of the extraction procedures. (**A**) solvent (C); (**B**) solvent (A). 2, 3, and 17 = methylene chloride: methanol routine extractions of seed n . 2, seed n. 3, and bark from plant source n. 7 (Table 2), respectively. 2b = the duplicate hot heptane extract for 2. 3s and 17s = the duplicate SFE extracts for 3 and 17. OA, CO, and HS = lipid standard of oleic acid, corn oil, and hydroxystearic acid.

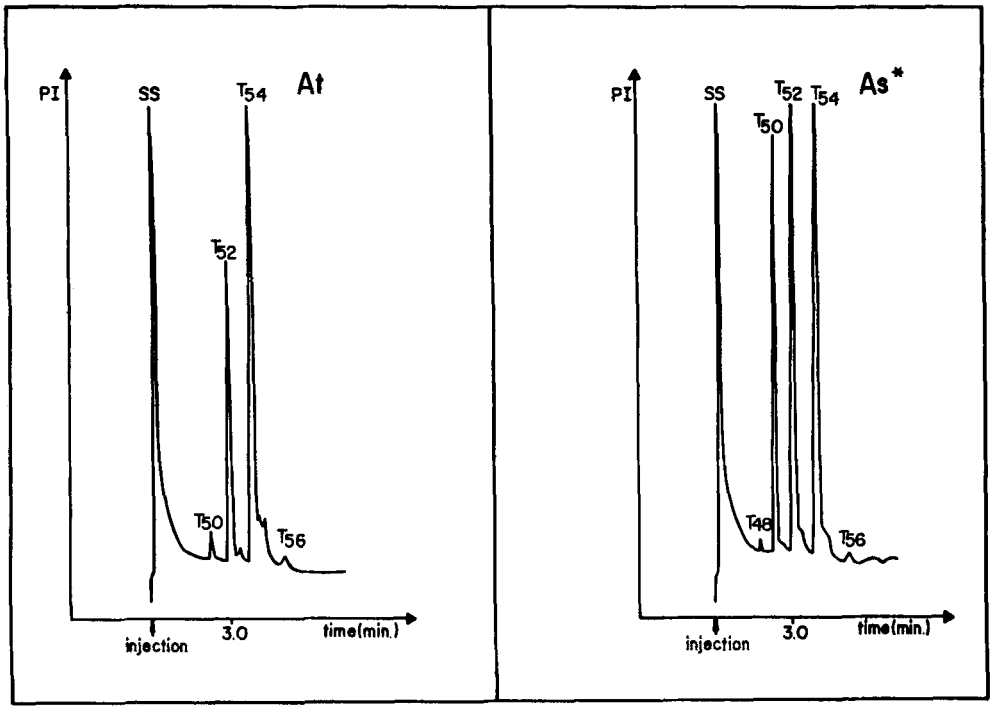

Fig. 5. HT-HRCG of the triglyceride fraction of two *Annonaceae* seed oil. At = *Asimina triloba;* As* = plant source 2 (Table 2). (Details as described in Materials and Methods).

polysaccharides, and protein, a situation that is further aggravated by the correspondingly large volumes of organic solvents used and to be recovered. This may be the best contribution of a CO_2-based SFE.

Following the partial purification of the crude seed extracts from paw-paw (reference; *Asimina triloba; At*) and from the most active source studied herein (Table 2; seed n. 2 = FCg-ANGLN or As*) on twin alumina columns, the respective fractions eluted with petrol ether (b.p. 30–65°C), and which contained triglycerides, were submitted to HT-HRCG. The results were those of Fig. 5. Triglyceride peak elution according to the increase in the total C number (i.e., the sum of the three fatty acid chains) showed that the dominant group (75.4%) for *Asimina* (T_{54}; retention time = 3.8 min) also accounted for 41.4% of As* triglycerides. T_{54} may correspond to the insaturated tripalmitolein as one of the hypothetical structures if the alcoholic positions of glycerol were homogeneously substituted, that is $C_{18} \times 3$. The only other significant group in paw-paw (T_{52}; 20.7%) was also present in our seed extract and in this case occurred as the major peak (44.7%). The different feature was the occurrence of a third group (T_{50}) in a significative proportion only as As* sample (12.7%; <2% in the At reference). The identification of the exact nature of the respective fatty

acids is in progress as well as other pertinent oil properties, such as iodine number and saponification index. It may be recalled that the determination of the fatty acid profile is a classic methodology for classification of pathogenic bacteria according to the guidelines from the Centers for Disease Control (Atlanta, GA), and then the application of this technique to our *Annonaceae* seeds might prove to be an additional criterion for plant chemotaxonomy at the genus and/or species level.

A final valuable result was obtained with seedlings when looking for micropropagation and tissue culture procedures, and the most intensively cultivated species, *Annona muricata,* was then used. Hypocotyls from the recently germinated seeds were cut and aseptically transferred to the complex culture medium. As shown in Fig. 6A, within the period of 2–3 wk the uniform stem cylinder evolved to the double-ended thickening. The middle segment of this dumbbell-shaped callus further emitted profuse sprouts, that is, an incipient organogenesis took place. The sequence of this experiment, following the transfer of the callus to the fresh medium thus completing 4–5 wk, resulted in sprout evolution to leaflet (Fig. 6B), and then, into a complete plantula (Fig. 6C). Therefore, both callogenesis and micropropagation could turn into practices that compensate for predatory procedures (i.e., use of roots and bark). For this purpose, callus and in vitro plants should ensure a satisfactory productivity for the bioactive compounds of interest.

CONCLUSIONS

Seeds of *Annonaceae* can be considered a true nonpredatory source of bioactive acetogenins. Furthermore, a technological fate is being devised for a dominant seed biomass, that of triglycerides through its use as a natural solvent for the acetogenins.

Also both the SFE and callus/organogenesis through in vitro manipulations were attempted with promising results.

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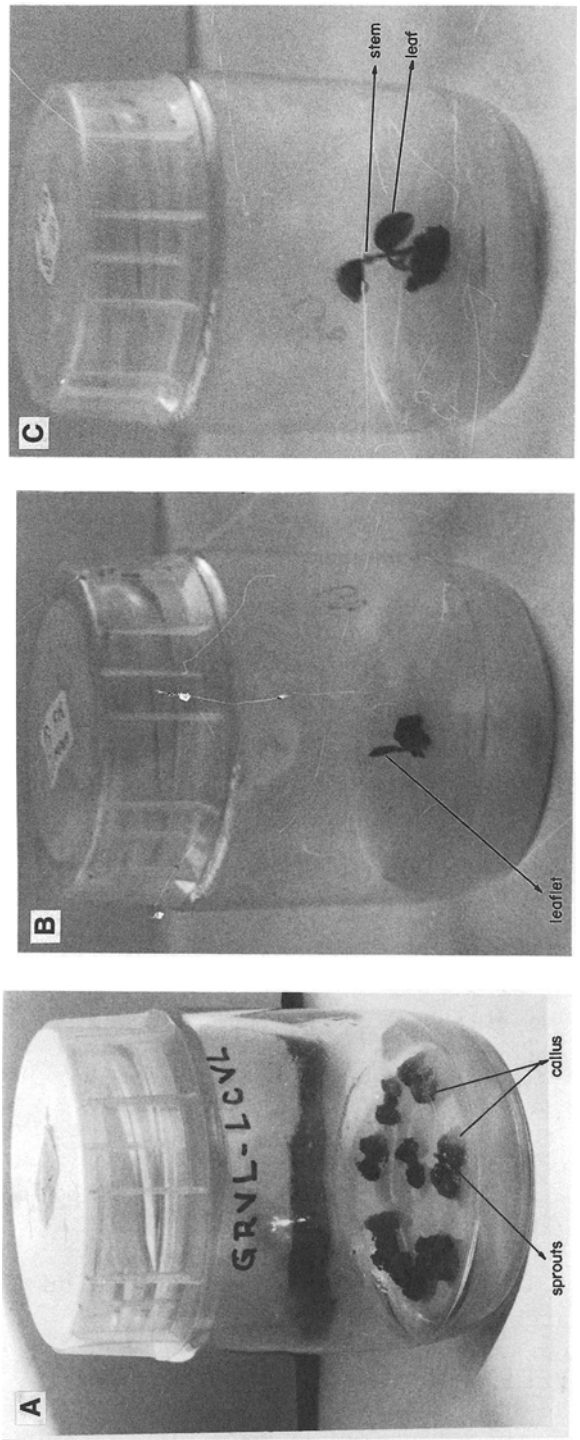

Fig. 6. In vitro culture of *Annona muricata* hypocotyls. (Note the callus protuberances at the botanical piece endings and the onset of the organogenesis in the middle piece in **A**; note the formation of the complete plantula in **C**.)

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