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# Selective Polarity- and Adsorption-Guided Extraction/Purification of *Annona* sp. Polar Acetogenins and Biological Assay Against Agricultural Pests

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## ABSTRACT

*Annonaceae* acetogenins (AG) comprise a family of natural chemical modifications of long-chain fatty acids ($C_{35-37}$) bearing one to several hydroxyls (less often oxo), middle-chain tetrahydrofuran rings, and a γ-lactonized, α/β-unsaturated carboxyl group. Acetogenins' strong biological activity as larvicides, pesticides, and antitumorals is dependent on these structural variations. The hydroxylation degree is particularly important for these etfects. Seeds, albeit rich in fats (mostly triacylglycerols, [TAG]), are a nonpredatory source of these drugs as compared to other botanical parts such as roots and stems. Conventional lipid extractions lead to quantitative lipid recovery and then the unfavorable natural ratio of TAG:AG in the range >90:<0.1 These extracts thus require, for instance, partitions and extensive sílica gel column chromatographic steps, in order to enrich or purify the AG fraction(s). Great operational difficulties result from the similar polarity and mol. wt. range of TAG and AG when carrying out these purification steps. An alternative fast two-step procedure to obtain polar acetogenin (pAG)-enriched preparations was developed. The extraction procedure for *Annona* spp. seeds pAG was carried out with acetonitrile ($E^\circ = 0.65$; $\log P = -0.33$) as a polar organosolvent, followed by the adsorption of the solvent-free extract on activated charcoal, then washed with hexane and/or chloroform ($E^\circ = 0.0$

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and 0.40: log P = 3.5 and 2.0) for most of the contaminating TAG removal, and then with acetone ($E^\circ$ = 0.56; log P = −0.23) to the desorption of an enriched **pAG** fraction. An alternative procedure for pAG extraction was supercritical fluid extraction (SFE) at moderate thermopressurization conditions (65–82°C; 120–130 atm) using $CO_2$, with 10% acetonitrile as the polarity modifier. The pAG fractions' bioactivity was evaluated with the brine-shrimp test (BST), and for feed deterrance, growth inhibition, and lethality against the high-impact agricultural pests *Anticarsia gemmatalis* and *Pseudaletia sequax* caterpillars feeding on soya or grass leaves sprayed with a 10% alcohol-stabilized emulsion of pAG.

**Index Entries:** *Annonaceae;* polar acetogenins; acetonitrile; downstream; caterpillars.

## INTRODUCTION

Acetogenins from *Annonaceae,* and particularly from the richest genera, *Annona, Rollinia, Asimina, Uvaria,* and *Goniothalamus,* display strong biological activity against several organisms because of their particular mode of action, that is, inhibition of the mitochondrial NADH: ubiquinone reductase. Effective doses as low as $ED_{50} = 10^{-2}$ μg/mL were found using VERO as a tumoral cell line model. Sixty-one new structural acetogenin variations were recently described, comprised of three main groups according to the number of tetrahydrofuran (THF) rings or their precursors (epoxides) in the biosynthetic route *(1).* lmportant potential applications of acetogenins in the oncology field are being reported (one example is in human prostate tumor *[2]).*

*Annona muricata* (soursop; "guanabana," "graviola") is one of the most-studied models as a mono-THF acetogenin source, and as many as 16 bioactive fatty acid lactones were found in its leaves *(3–6).* Among them, gigantetrocin A, muricatetrocins A and B, annonacin A, goniothalamicin, and annonacion-10-one were also found in the seeds, along with the lesser hydroxylated forms like murisolin, solamin, corossolone, and diepomuricanin. The seed acetogenins bearing 4 hydroxyls (gigantetrocins and muricatetroxins) are particularly bioactive, since increased polarity correlates with bioactivity *(1).* The chemical structure for one of these compounds is shown in Fig. 1.

Downstream processing of two di-THF acetogenins (bullatacin and bullatacinone) from the bark of *Annona bullata* was based on sequential partitions of a crude ethanol extract using biphasic systems, namely chloroform/methanol and hexane/90% methanol. Final purification steps were chromatography and rechromatography on silica gel columns eluted with hexane/chloroform/methanol and chloroform/ethyl acetate/methanol gradients. About 195 mg of pure bullatacin, a tetrahydroxylated $C_{37}$-(THF)

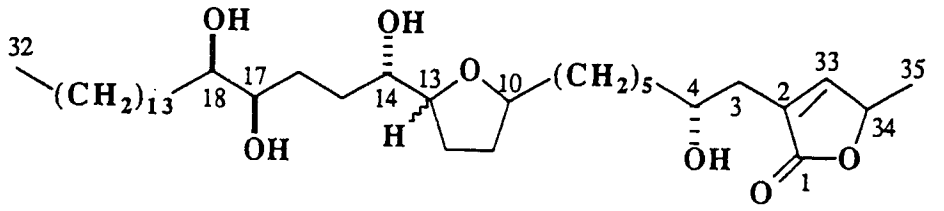

Fig. 1. Gigantetrocin A from *A. muricata* seeds (example of a pAS structure used with permision from ref. *1*).

2 acetogenin, was thus obtained from approx. 4 kg of *A. bullata* bark, as well as 13 mg of bullatacinone, the related oxo-trihydroxylated form *(7)*.

Interested in the highly hydroxylated and more bioactive acetogenins (polar acetogenins [pAG]), the authors adopted *A. muricata* seeds as a non-destructive working source for an alternative and faster downstream process. The proposed methodology took in account the high neutral lipid (TAG) content (~ 30%) previously reported for these particular seeds *(8)*.

## MATERIALS AND METHODS

### Seeds and Lipid Material Extraction by Organosolvents or Supercritical Fluid Extraction (SFE)

Ripened fruits from *A. muricata* ("graviola"), *Annona* sp. (probably *squamosa;* "fruta-do-conde"), and *Annona coriacea* ("pinha-do-cerrado" [PR]) were respectively collected at Feira de Santana (Brasfrut, Bahia State, Brazil), Porecatu, and Jaguariaiva (Parana State), Brazil. Washed and sun-dried seeds were powdered in a Waring blender (Dynamics, New Hartford, CT; 30-mesh sieve), and stored in plastic bags with an average 10% moisture content. Lipid material extraction was carried out with 3 vol of each organosolvent overnight at 28°C in a rotatory shaker. Acetonitrile (polarity, $E° = 0.65$, Riedel scale; hydrophobicity, $\log P = -0.33$) was the preferable solvent. Following filtration by a frit glass screen, extract solvent was removed in a rotatory vacuum evaporator. Acetonitrile extracts were designated as AE. Guidelines on solvent polarity were taken from a published log-P hydrophobicity-ordered list *(9)*, and from a Riedel-de-Haen table (Seelze, Germany).

Supercritical fluid extraction (SFE) was carried out in a steel apparatus (reactor working vol = 10 mL) specially designed by one of the authors (F. M. Lanças) from the Chromatography Laboratory at the University of São Paulo (USP) at São Carlos-SP *(10)*. The SFE apparatus consists of a crushed-ice bath involving a 500-mL high-pressure gas-mix chamber (carbonic anhydride and nitrogen), with or without addition of 0.1 vol of modifier solvent (acetonitrile or hexane), an extraction vessel (sample-containing reactor) in a thermostat-equipped water bath, a restrictor

outlet, and a collection vessel containing methanol in a cryogenic bath. Moderated ranges of temperature (maximum 82°C) and pressure (120–130 atm) were employed. Gas flow was set at 45 mL/min for dynamic SFE of solutes, after an equilibration time of 10 min at the lowest temperature (65°C; static SFE; no gas flow). During the average time of 90 min for each run, temperature increased from 65 to 82°C, and fractions were collected at 10 min intervals. Sample solvent of the SFE fractions from powdered seeds was evaporated in a vacuum oven, and each residue was dissolved in chloroform:methanol (2:1) or acetonitrile:water (85:15) for analyses by thin layer chromatography (TLC) or high-presssure liquid chromatography (HPLC).

## Acetogenins Enrichment and Purification

Acetogenin extract (light brown oil) was suspended in petroleum ether or hexane, thoroughly mixed with 4–5 parts activated charcoal (Sigma C-3585; St. Louis, MO), and left in contact for 1 h. Filtration was carried out in a frited glass filter/Buchner flask assembly using a double layer of filter paper and applying 2 column vol of each solvent from an eluotropic series: hexane ($E^\circ$ = 0.0; glycerides and less polar acetogenins); chloroform ($E^\circ$ = 0.40; remanent glycerides and midpolar acetogenins); acetone ($E^\circ$ = 0.56; polar acetogenins, pAG), and finally, methanol ($E^\circ$ = 0.95). A triglyceride-free pAG standard was further obtained on neutral alumina or silica gel 60 columns (gradient elution with hexane:chloroform up to 70:30, and then acetone).

### *Analytical Procedures*

Thin-layer chromatography was carried out in Merck (Darmstadt, Germany) silica gel chromatoplates G 60 (art. 105553) with hexane:chloroform:nitroethane:ethyl acetate:acetone:methanol:acetonitrile:water, 120:20:40:40:10:20:16:1 as mobile phase. Lipids were detected with four different reagents *(11):* iodine vapor (unsaturated lipids); phosphomolibdic acid, saturated solution in ethanol (general lipids) at 120°C, 10 min; 1% anisaldehyde in sulfuric acid:methanol (5:95) for the differentiation between TAG and phytosterols (deep lilac to wine color) and acetogenins (yellow to olive color) at 105°C for 5 min; and the Kedde reagent, 3–5-dinitrobenzoic acid, followed by KOH, both as 5% methanolic solutions, as a selective reagent sequence for acetogenins (lactones; pink to wine color, fast-fading).

HPLC was run on a Spectra Physics C-18 reverse-phase column assembled in a Waters multimodule (SC 600E/WISP 712) with double monitoring with a 410 DRI or 484 UV detector at 215 nm, using acetonitrile:water (85:15) as mobile phase at a 0.75 mL/min flow rate. Sample solvent mix (chloroform:methanol:water or aqueous acetonitrile, detected as the initial chromatographic peaks) was adjusted to ensure complete lipid solubilization.

## Biological Assays

The brine shrimp test (BST) *(12)*, affording the $LD_{50}$ for *Artemia salina* nauplii (24 h after the cyst hatching in artificial sea water), was performed as a routine cytotoxic activity assay. Feed deterrance, growth inhibition, and lethality were assayed against *Pseudaletia sequax* larvae (V instar) feeding on fresh leaves of *Penisetum clandestinum* ("kikuyo grass," 7-cm length and 0.8-cm width leaf pieces), or against *Anticarsia gemmatalis* caterpillars feeding on fresh leaves of *Glycine max* (soya; 2.5- to 3.5-cm length young leaves). Plant leaves were sprayed (10 s) with a fine mist of a milky, homogeneous, and stable suspension of pAG (fraction eluted from active charcoal with acetone) in 10% ethanol, in order to ensure an 18 (low dose), 36 (medium dose), and 54 μg (high dose)/leaf as determined in grass leaf pieces. Control leaves were sprayed with the same solvent or water. Following solvent evaporation, leaves were offered *ad libitum* for the growing caterpillars for 2 d. From d 3 untreated leaves were the exclusive food in all experiments.

## RESULTS AND DISCUSSION

The current fractionation flow sheet is depicted in Fig. 2. Progressive enrichment of pAG was obtained in the sequence from acetonitrile extraction to the activated charcoal fractionation step. Compared to an ethanol extract Fig. 3A, B; (lanes EE-1–3) from *A. muricata* seeds, whose AG content is barely visible with both the chromogenic anisaldehyde and Kedde reagent sprays, because of the dominance of TAG (triglycerides), the alternative acetonitrile extract (same Fig., lane EA) displayed an approx 1:2–1:3 AG:TAG ratio (color profiles in Fig. 3A, B). Higher TAG content in the ethanolic extracts, compared to the acetonitrile extract, was qualitatively confirmed using the lipase/glycerokinase/glycerophosphate oxidase/peroxidase/benzoquinone-monoimido-phenazone kit (Diagnostic Systems, Holzheim, Germany). Gravimetric measurement of total fat content of *A. muricata* seeds, after exhaustive ethanol or acetonitrile extractions, corresponded to 33 and 26%, respectively. The corresponding figures for *A. coriacea* were 20 and 13%. The acetone eluate, when processing the acetonitrile extract on an activated charcoal bed (lane AC-A), was progressively enriched in pAG because of the efficient and previous elution of TAG (and also most of the less polar acetogenins) with hexane, followed by chloroform. In fact, considering the TLC fast-moving spot as TAG, since a triolein standard cochromatographed with the same Rf, the AC-A fraction is purer than the equivalent step of the previously patented procedure *(7)*, namely, the aqueous methanolic phase from the hexane/90% methanol partition (lane M[H]; Fig. 3A, B). For the sake of illustration, the complete purification of the dominant and most polar acetogenin(s), attained on an alumina pad washed with a chloroform:acetone gradient (up to 70:30,

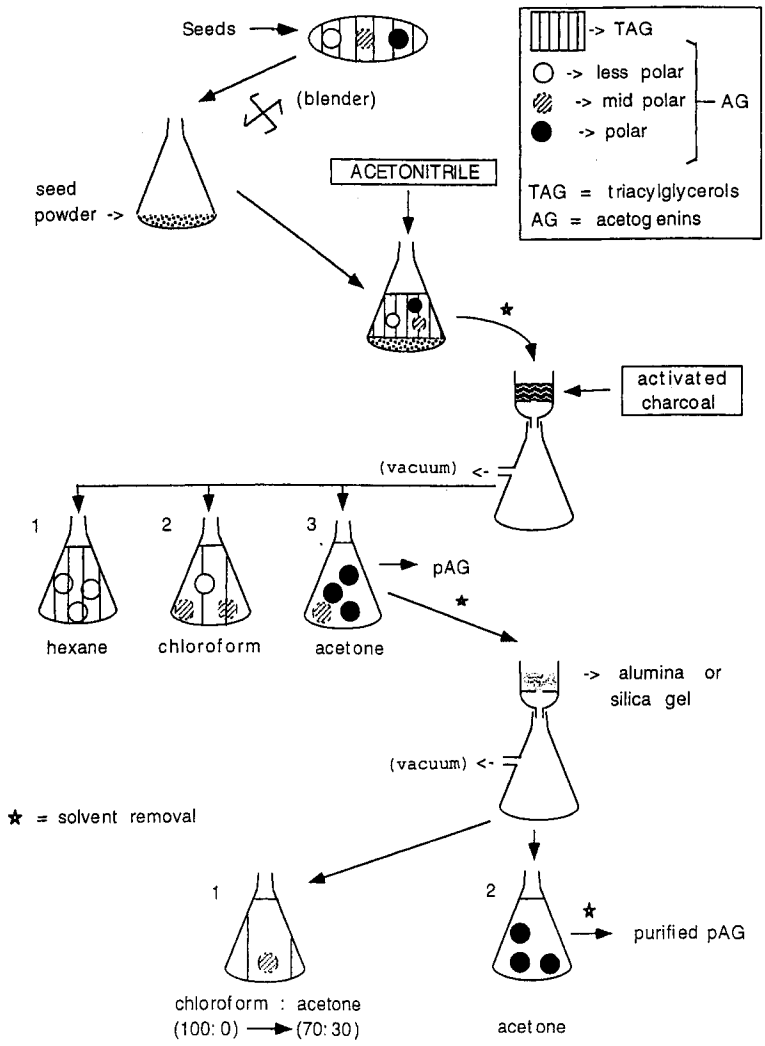


Fig. 2. Downstreaming for pAGs using acetonitrile as extractant and activated charcoal for fractionation and purification.

v/v, and then pure acetone), is also shown (pure pAG fraction in Fig. 2, and lane AL-A in Fig. 3A, B). The relative profiles for polar acetogenins (pAG; peaks with Rt = 9.7 and 11.0 min as monitored at 215 nm) were also verified in the same samples by HPLC (Fig. 4A–C, referring to initial alternative acetonitrile extract, acetone eluate from charcoal, and acetone eluate from alumina, respectively Fig. 4D refers to the initial classic ethanol extract). Quantitation indicated that the sum of the two main peaks (a resolution not attained in TLC) corresponded to 43% of all 215 nm-detectable material. Scanning the TLC plates (or their derived color pictures) on a Shimadzu CS-9301-PC Dual Wavelength Flying Spot Densitometer (results not shown), using the most suitable wavelength (545 nm) for both anisalde-

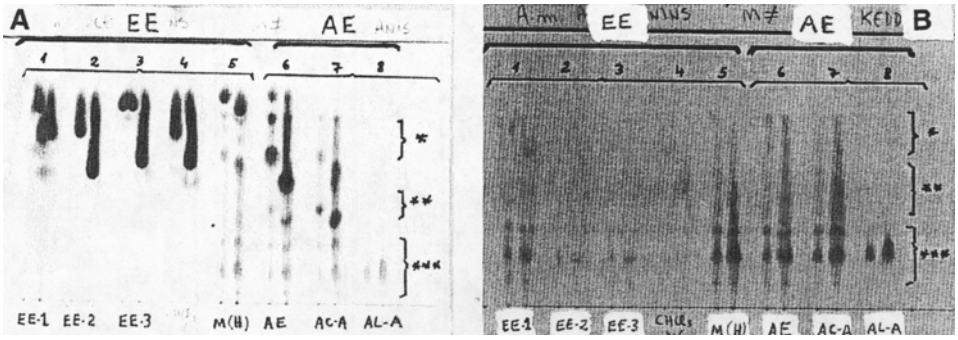


Fig. 3. TLC monitoring for *A. muricata* seed acetogenins extraction and purification. **(A)** p-anisaldehyde spray. **(B)** Kedde's reagent spray. EE, ethanol extraction and derived fractions; EE-1, EE-2, EE-3, sequential initial ethanol extracts; M(H), aqueous methanolic phase from the second partition of the EE mix; AE, acetonitrile extract; AC-A, acetone eluate from the activated charcoal bed; AL-A, acetone fraction from the alumina bed.*, **, and ***, less polar, midpolar, and pAG, respectively.

hyde and Kedde reagent chromogenic sprays, indicated that pAG contributions were 11 and 42% of the colored components, respectively. It may be recalled, as shown in Fig. 3A, B, that anisaldehyde is an efficient chromogen for both TAG (wine to violet) and AG (yellow to olive), compared to the Kedde reagent, specific for AG (light to deep pink; no color for TAG and other related or unrelated lipids). Efficiency of acetonitrile as a selective extractant for all acetogenins, irrespective of their polarity, was also successfully assessed using other species of *Annona*. The TLC results for the sp. *coriacea*, for instance, indicated an even better ratio of AG:TAG, but this may be attributed to the lesser relative content of TAG in this seed type (J. D. Fontana, unpublished data).

Pursuing the same goal, that is, the preferential extraction of polar acetogenins, SFE was carried on *A. muricata* powdered seeds using either 10% hexane or 10% acetonitrile as polarity modifiers for the carbonic anhydride thermopressurized flowing stream. TLC analysis indicated best results for the $CO_2$ acetonitrile mix, whose earlier fractions, 1–4 among 10, were also particularly enriched on pAG, most of TAG being collected in fractions 5–9 in the higher temperature range. The $CO_2$–hexane mix revealed a preferential extractant for TAG, thus concentrated in the initial five fractions, no significant lipid amounts being detectable in fractions 6–10. The encouraging results obtained with the inclusion of acetonitrile in SFE is being better explored and the complete results will be published elsewhere.

An emulsifier agent is needed for the application of pAG in agricultural plague control. The choice for this purpose was 10% ethanol. Fig. 5A–C show the comparative effectiveness of this low-cost emulsifying mixture on whole oil (WO), acetonitrile extract (AE), and semipurified polar

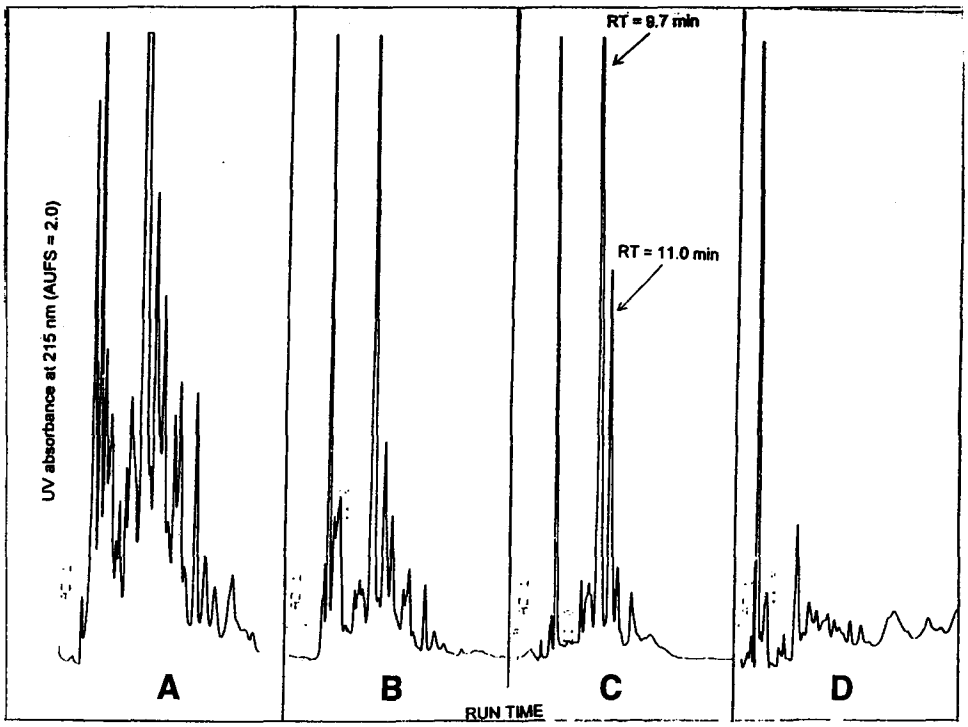


Fig. 4. Comparative HPLC analyses for the extracts or purified fractions of *A. muricata* seed polar acetogenins (pAG). **(A)** Initial acetonitrile extract (AE); **(B)** acetone eluate from AE purification on an activated charcoal bed (AC-A); **(C)** acetone eluate from AC-A final purification on an alumina bed (AL-A); **(D)** initial ethanol extract. (UV monitoring at 215 nm with an AUFS = 2.0. Earlier peaks correspond to different solvent sample mix. Samples at 50 mg/mL; injection volume of 15 μL for samples A and D, and 5 μL for samples B and C. A sample of a triolein standard presented no detection in the 25 min run).

acetogenins (AC-A) samples at a O.25% concentration (w/v). Pictures were taken just after mixing (A), 3 h (B), and 24 h (C). As is clearly visible, the progressive enrichment of *A. muricata* seed lipomaterial in polar acetogenins leads to partial (Fig. 5B; sample AE), or even complete, emulsion stabilization (Fig. 5C, sample AC-A), when 10% aqueous ethanol replaced pure water or other emulsifying agents. This latter preparation of pAG was then used as a fine mist for spraying of legume (soya) or grass (wheat and "kykuio") leaves (18, 36, and 54 μg of pAG/leaf) in order to check acetogenin bioactivity against the caterpillar pests *Anticarsia gemmatalis* and *Pseudaletia sequax*. The use of low and medium doses led to 10 and 30% mortality, respectively, reduced food consumption (feed deterrance), enlargement of the larval period, and generation of a surplus instar (designed as the 7th, not existing in the controls). Results of this triplicate experience are detailed in Table 1. Wheat caterpillar (*P. sequax*) proved to be more resistant to pAG inhibitory action, since the highest dose (54 μg/leaf cut)

Table 1
Food Consumption by *Anticarsia gemmatalis* Caterpillars for the Three Final Instar Periods, Larval Period Delay, and Mortality Rate Following a 2 d-Period Feeding on *Glycine max* Leaves Treated or Untreated with Polar Acetogenins (pAG).

| Treatment | Average food consumption (%) | | | Larval Period (d) | Mortality (%) |
|---|---|---|---|---|---|
| | 5th instar | 6th instar | 7th instar[a] | | |
| Control; water | 51.3 ± 8.7 | 77.5 ± 7.0 | – | 19.9 ± 1.3 | 0% |
| Control; 10% ethanol | 55.8 ± 13.2 | 75.2 ± 9.4 | – | 19.3 ± 1.8 | 0% |
| pAG[b]; low dose | 18.3 ± 3.4 | 35.3 ± 10.4 | 74.7 ± 13.7 | 25.0 ± 2.5 | 10% |
| pAG[b]; medium dose | 20.9 ± 7.6 | 49.2 ± 22.9 | 72.8 ± 15.5 | 29.3 ± 3.0 | 30% |

[a] Surplus instar resulting from the cytotoxic action of acetogenins actually seen.
[b] pAG, polar acetogenins (acetone eluate from activated charcoal; doses detailed in Materials and Methods).

resulted only in delayed larval growth because of the feed deterrance, which persisted until the fifth d, fresh untreated food allowing further recovery for the initial effect (results not shown).

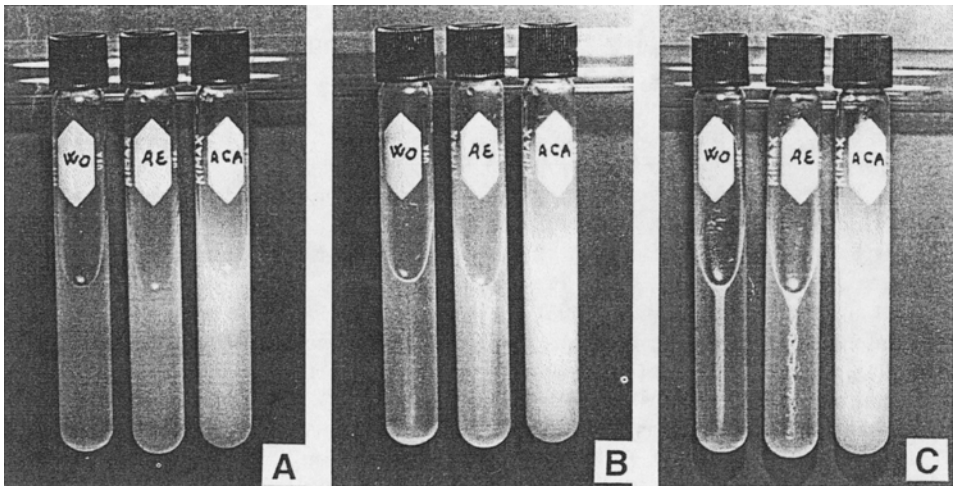


Fig. 5. *A. muricata* crude and semipurified acetogenins forrnulation in 10% ethanol as emulsifier. WO, seed whole oil; AE, seed acetonitrile extract; AC-A, acetone eluate from the activated charcoal bed. (All samples at 0.25 g% were vigorously mixed in a Vortex for 1 min and pictures from the laid test tubes were taken just after mix **(A)**, after 3 h **(B)**, and after 24 h **(C)**.

## CONCLUSIONS

As a nonpredatory source for polar and more bioactive pesticidal and antitumoral acetogenins, *Annona muricata* seeds were conveniently and quickly processed using acetonitrile as a more selective extractant, compared to ethanol. The time-consuming steps of partitions were replaced by adsorption and desorption of the polar acetogenins on activated charcoal. A second promising extraction method was supercritical fluid extraction, provided 10% acetonitrile was used as polarity modifier for the moderately thermopressurized carbonic anhydride supercritical stream. Polar acetogenins semipurified fraction produced stable emulsions in 10% ethanol, even at a 0.25% concentration, thus facilitating their uniform application for agricultural pests control. The soya caterpillar was significantly affected.

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