Pharmacognosy Research [Phcog Res.]
Vol 1, Issue 3, May-Jun, 2009 Page 130-135
[Rapid Publication]
A Supplement to Pharmacognosy Magazine – [Phcog Mag.]
Rapid publication process initiated: 10 Mar, 2009
Accepted: 9 April, 2009

PHCOG RES.: Research Article

Cytotoxic acetogenins from *Annona glabra* cultivated in Egypt

Ahmed Abdel-lateff,[a]* Bassem S. El-Menshawi,[b] Mohamed Y. Haggag[c] and Mahmoud A. Nawwar[d]

[a]*Pharmacognosy Department, Faculty of Pharmacy, Minia University, 61519 Minia, Egypt.*
.[b]*Pharmacognosy Department, National Research Centre Dokki, 12622 Giza, Egypt.*
[c]*Pharmacognosy Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.*
[d]*Tanning and Protein Department, National Research Centre Dokki, Giza Egypt*
***Telephone & fax** 002-086-2369075**; e-mail** aabelazim@yahoo.co.uk*

ABSTRACT
Bio-assay guided fraction of the methanolic extract of *Annona glabra* seeds *(*Annonaceae), cultivated in Egypt, revealed to the isolation of three bis-tetrahydrofuran acetogenins; squamocin-C (**1**), squamocin-D (**2**), and annonin I (**3**). Compounds **1** and **2** were obtained as stereoisomeric mixture. All isolates were assayed for their cytotoxicity twards brine shrimp and five in vitro cancer cell lines (A549, HT29, MCF 7, RPMI, and U251), and showed significant activity The structures of all compounds were determined by interpretation of their NMR and MS analyses.
Keywords: *Annona glabra*, Annonaceae, Acetogenin, Cytotoxic, Brine shrimp

INTRODUCTION

Annonaceae is a family of aromatic trees, shrubs or climbers, which grow in tropical and subtropical regions (1). Annonaceae comprises *ca* 120 genera and more than 2000 species (2). The plants exhibit a broad range of potent biological activities, [*viz*. cytotoxicity, antitumor, immunosuppressant antimalarial, antimicrobial, antiparasitic, and insecticidal] (3-5). Accordingly, several members of Annonaceae have attracted more and more attention (3, 4). Activity-directed fractionation of *Uvaria acuminata* examined by Jolad *et al*. (6), using 3ps (*in-vivo* murine leukemia), led to the isolation and structure elucidation of uvaricin an unusual antitumor compound. This is the first example of a new class of extremely bioactive compounds that are now referred to annonaceous acetogenins (5). The number of these acetogenins have reached now more than 230 compounds. Annonaceous acetogenins were found in the following genera *Annona, Goniothalamus, Rollinia, Uvaria, Asimina* and *Xylopia* (1).

Previous published studies of the cytotoxic principles isolated from *A. glabra* indicated that it is a productive plant. For instance; squamocin, asimicin, and desacetyl uvarcin; annonacin, corossolon, and solamin; glacins A and B. (7) and annoglaxin and 27-hydroxybullatacin (8); anoglabayin, a novel dimeric kaurane diterpenoid, and an acetogenin annomontacin (9); and an ent-kauran-19-al-17-oic acid derivatives (10).

In the current paper, the isolation and structure elucidation of three acetogenins are reported. Successive fractionation of the MeOH extract by vacuum liquid chromatography (VLC) over silica followed by normal-(NP) and preparative thin layer chromatography yielded compounds **1**-**3**. The potency against human tumor cell lines has reported for many acetogenin compounds, this study reports for the first time the range of cytotoxicity of squamocin-C, squamocin-D and annonin I against human tumors *in-vitro*.

MATERIAL AND METHODS

General procedures

^{1}H and ^{13}C-NMR spectra were recorded on Varian 500 MHz spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively). FAB, EI and CI mass spectra were measure by VG-250 S mass spectrometer, Pre-coated silica gel 60 GF_{254} plates (E. Merck) were used for TLC. Sephadex LH20 (Pharmacia Fine Chemicals, Sweden). The compounds were detected by UV absorption at

λ_{max} 254 and 366 nm followed by spraying with anisaldehyde/H_2SO_4 reagent and heating at 110 ^{0}C for 1-2 min.

Plant material

The seeds of Annona glabra were collected from Zoo garden, Giza, Egypt. The plant material was kindly identified by Agr. Eng. Badia Diwan, Herbarium of Orman Botanical Garden, A voucher specimen (A-3) was deposited in herbarium of the Pharmacognosy Department, National Research Centre Dokki, Giza, Egypt.

Extraction and isolation

The air-dried seeds of *Annona glabra* L. (620 g), collected from Zoo Garden Giza, Egypt, were extracted and fractionated according to scheme published by Cassady *et al*. (12). The defatted fraction (F000) and (F005) showed the highest toxicity towards brine shrimp (LC_{50} 2.75 and ≤ 0.5 ppm, respectively). Further methanolic extraction of F000 led to more active fraction with LC_{50} against brine shrimp (1.5 ppm), led to two fractions (F00A and F00B). The F00A (3 g) subjected to flash column on silica gel 60H (5-40 μm), eluted with n-hexane: EtOAC (7:3), 5 ml each, were collected. According to TLC pattern, were combined into 18 pools, monitored by brine shrimp lethality proved that the F00A-16, had significant cytotoxicity. F00A-16 (140 mg) fractionated on silica gel 60H (5-40 μm), employing flash column and eluted with n-hexane:CH_2Cl_2:Acetone (1.0:4.0:5.0), led to 14 fractions (5 ml each), pooled together into six pools, based on the TLC pattern. The purification was achieved by Sephadex LH20 column using MeOH as eluting solvent lead to mixture of compounds **1** and **2** (4 mg). F005 was fractionated by using flash column on silica gel 60H (5-40 μm, 40 x 3 cm), gradient elution from CH_2Cl_2 to MeOH, 80 ml each, were collected and combined into seven pools according to their TLC pattern. F0053 possessed the highest cytotoxicity (% lethality 40 % at 40 ppm). Further fractionation of F0053 on NP-silica gel employing CH_2Cl_2:MeOH: Acetone:n-Hexane (9.5:0.5) yielded compound **3**, which purified by Sephadex LH20, eluted by MeOH.

BIOLOGICAL ACTIVITY

Brine shrimp

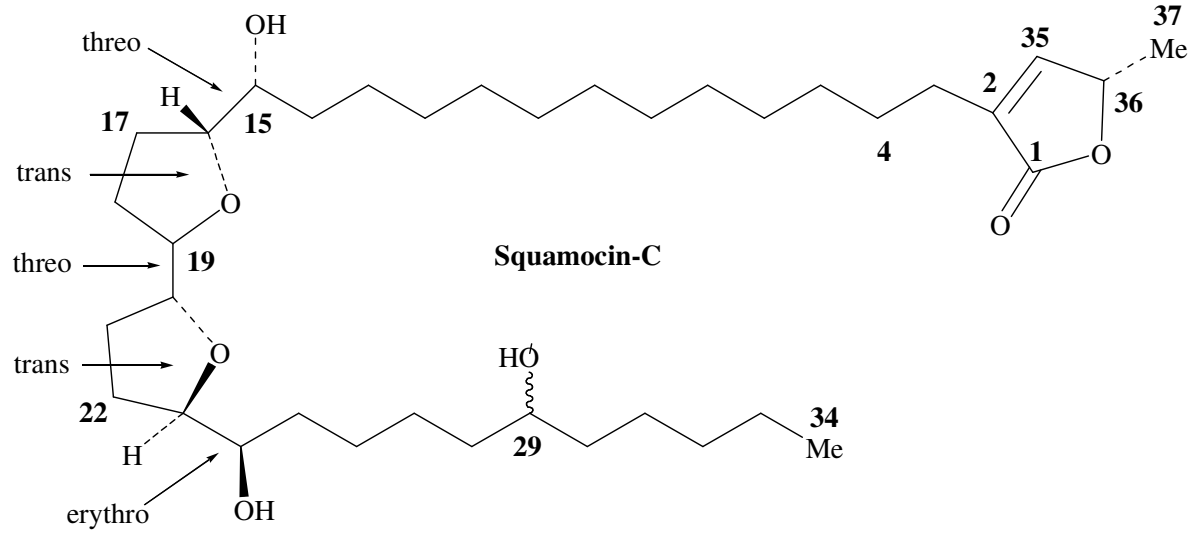
A solution of sea water was made by dissolving 32.5g [A natural blend of salts and trace element, for sea water fish (Sera Company, Germany, Aquaristik Gmbh, D5138 Henisberg)] in distilled water (1L). *ca* 1 mg of brine shrimp, *Artemia salina* (Leach), eggs was added in a hatching chamber (22 × 32cm). The hatching chamber was kept under an inflorescent bulb for 48 h for the eggs to hatch into shrimp larvae (nauplii). 50 mg of tested extracts / fractions; or 1 mg of pure compounds, dissolved in 5 ml of solvent in which they were soluble and from this, 5, 50 and 500 uL of each solution was transferred into vials corresponding to 10, 100 and 1000 μg/ml, respectively. Each dosage was tested in triplicate. The test vials and one control containing 500 uL of solvent were allowed to evaporate to dryness under nitrogen. 10 larvae (nauplii) of *Artemia salina* were transferred into each vial and the volume completed into 5 ml with sea salt solution (± DMSO) immediately after adding the nauplii, 24 h later, the number of surviving shrimp at each dosage was counted and recorded. LC_{50} values were determined statistically (11).

Human tumor cell cytotoxicity assay (HTCC)

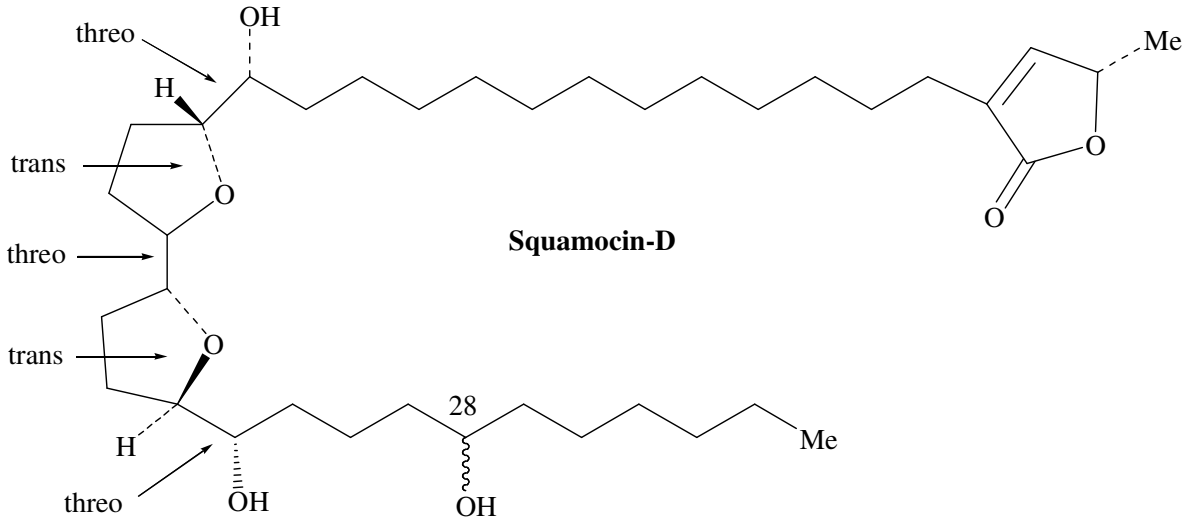
This cytotoxicity was carried out at the Comprehensive Cancer Centre, Ohio State University, Columbus, OH. From growing stock cultures, cells are inoculated into 96 well tissue culture plates on day one (D1) at appropriate concentrations (1000-2000 cells depending on the cell line), then incubated for 24 hours. Test compounds are then added on day two (D2) in five log dilutions beginning with the highest soluble concentration, (four wells for each concentration). Simultaneously, negative controls (no treatment) and positive controls (Adriamycin, 5 log dilutions) are included then ED_{50} according to standard methods was calculated (12).

RESULTS AND DISCUSSION

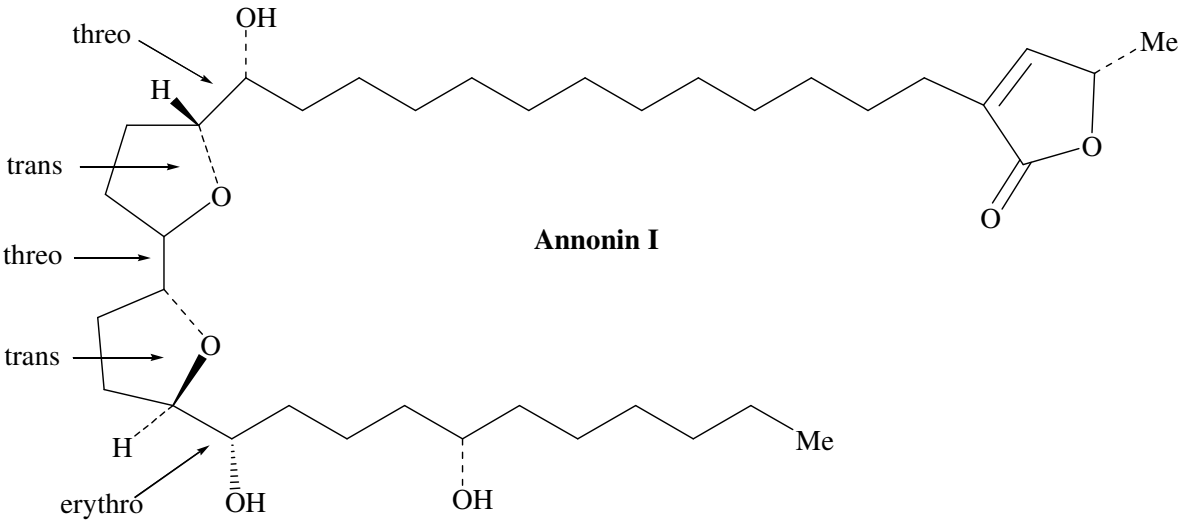
The FAB-MS exhibited molecular weight of 622 and indicated a molecular ion peak m/z 623.6 $[M+H]^+$. The spectrum also showed an ion peak at m/z 646 attributable to an adduct with sodium. High Resolution EI-M Spectrum showed two major patterns of ion peaks assignable to two stereo-isomeric acetogenins. They were found to be identical to those reported for squamocin-C (**1**) and squamoicn-D (**2**), C_{37} H_{66} O_7. ^{1}H, ^{13}C and 2D-NMR spectral analysis proved the previous achieved structures. However, the NMR data failed to differentiate between each of the two isomer structures (squamocin-C and squamocin-D). ^{1}H-NMR spectrum revealed the presence of a pattern of proton signals assignable to α, β-unsaturated, methyl, γ-lactone moiety as evidenced by the proton resonance, appearing in the form of a quartet at δppm 6.98 assignable to the olefenic proton H-35 and also by the proton



Squamocin-C



Squamocin-D



Annonin I

Table 1: ^{13}C NMR [$CDCl_3$, 125 MHz], spectral data for compounds 1 and 2

Carbon Number	δc[a]		Carbon Number	δc	
	1	2		1	2
1	173.9 (s)	173.9 (s)	20	82.5 (d)	82.5 (d)
2	134.5 (s)	134.5 (s)	21	28.9 (t)	28.9 (t)
3	25.2 (t)	25.2 (t)	22	25.2 (t)	25.2 (t)
4	27.4 (t)	27.4 (t)	23	82.9 (d)	82.9 (d)
5	29.2 (t)	29.2 (t)	24	71.5 (d)	74.2 (d)
6	29.4 (t)	29.4 (t)	25	32.6 (t)	32.6 (t)
7	29.7 (t)	29.6 (t)	26	22.0 (t)	22.0 (t)
8	29.6 (t)	29.5 (t)	27	25.6 (t)	37.3 (t)
9	29.4 (t)	29.4 (t)	28	37.5 (t)	71.8 (d)
10	28.4 (t)	28.4 (t)	29	71.8 (d)	37.5 (t)
11	29.9 (t)	29.9 (t)	30	37.5 (t)	25.6 (t)
12	28.4 (t)	28.4 (t)	31	29.7 (t)	29.7 (t)
13	25.7 (t)	25.7 (t)	32	31.9 (t)	31.9 (t)
14	33.4 (t)	33.4 (t)	33	22.6 (t)	22.6 (t)
15	74.2 (d)	74.2 (d)	34	14.1 (q)	14.1 (q)
16	83.4 (d)	83.3 (d)	35	148.9 (d)	148.9 (d)
17	28.4 (t)	28.4 (t)	36	77.4 (d)	77.4 (d)
18	27.4 (t)	27.4 (t)	37	19.2 (q)	19.2 (q)
19	82.3 (d)	82.3 (d)			

[a]*Implied multiplicities as determined by DEPT (C = s, CH = d, CH_2 = t, CH_3 = q).*

Table 2: Cytotoxic activity of the fractions and compounds 1-3 on HTCL

Items	ED_{50} (ppm)				
	A-549	HT-29	MCF-7	RPMI	U251
F001	0.00130	$5 \times 10^{\circ}$	0.0013	0.0013	0.0013
F003	0.00060	$2 \times 10^{\circ}$	0.0001	6×10^{-4}	2×10^{-2}
F005	2×10^{-3}	2×10^{-1}	0.001	0.0010	0.001
1 & 2	7×10^{-4}	5×10^{-1}	7×10^{-4}	5×10^{-4}	6×10^{-4}
3	4×10^{-3}	No	5×10^{-3}	No	3×10^{-3}

A-549 Lung carcinoma; HT-29 Colon adenocarcinoma; U251-MG Glioblastoma multiforme; No = not active; RPMI-7951 Malignant melanoma; MCF-7 Breast adenocarcinoma ; F000 was not tested; HTCL is human tumor cell lines.

resonances at δppm 4.99 (dq, H-36) and δ 2.26 (t, 2H-3) as well. The equivalent three-methyl protons (3H-37) in this moiety revealed their resonance as a doublet at δ 1.41. 2D-Chemical Shift Correlation Spectrum (COSY) clearly showed the correlation of H-37 to H-36, H-36 to H-35, of H-35 to H-3 and of H-36 to H-3. In the same spectrum a cross peak correlating the signal of H-3 methylene protons (δ 2.21, t, *J* = 7.7 Hz) with the signal of H-4 methylene protons (δ 1.5, m) was recognized. The spectrum also revealed the presence of seven oxymethine proton resonances appearing as complicated multiplets at δppm 3.39 (1H), 3.52 (1H) and 3.80-3.90 (5H). The spectrum also showed the presence of a terminal methyl proton resonance appearing as a triplet at δppm 0.88 (Me-34). The COSY spectrum also showed the correlation of 2 H-32 to 2H -33 and of 2H -33 to 3H -34.Detailed analysis of this spectrum proved the correlation of H-15 (δ 3.39 m) to H-16 (δ3.86, m) and of H-23 (δ 3.86 m) to H-24 (δ 3.39, m). ^{13}C-NMR spectrum, on the other hand, confirmed the presence of α, β-unsaturated γ-lactone moiety. It proved the presence of seven oxygenated carbons exhibiting their resonances at δ ppm 83.325, 82.25, 82.530 and 82.85, assignable to C-16, C-19, C-20 and C-23, respectively. The remaining three hydroxylated carbons (C-15, C-24 and C-28/C-29) received their resonances at δppm 74, 71 and 71.8 respectively (Table 1). The site of attachment of the

hydroxyl groups was further confirmed through the fragmentation pattern recognized in the EI-M spectrum. ^{1}H-^{13}C Chemical Shift Correlation HETCOR, finally confirmed the achieved structures. These data was consistence with the previous published data (13).

CI-MS spectrum of **3**, exhibited molecular weight of 622 and revealed an adduct with $[CH_3CH_2]^+$ at m/z = 651.6. The received EI-MS data was found to be identical to those reported for Annonin I (**3**), C_{37} $H_{66}O_7$. ^{1}H-NMR spectral analysis proved the previous achieved structure as follows. It revealed the presence of a pattern of signals assignable to α-β unsaturated γ-lactone moiety as evidented by proton resonance, appearing in the form of quartet at δ ppm 6.95, assignable to olefenic proton H-35 and also by the proton resonances at δ ppm 4.96 (dq H-36) and δppm 2.26 (t, 2H-3) as well. The non equivalent three methyl protons (3H-37) in this moiety revealed their resonances as doublet at δppm 1.40. The spectrum also revealed the presence of seven oxymethine proton resonances appearing as multiplets at δppm 3.37 (1H), 3.58 (1H) and 3.78 $\rightarrow$ 3.95 (5H).The spectrum also showed the presence of a terminal methyl proton resonance appearing as a triplet at δppm 0.87 (3H-34). The obtained data was consistence with those reported on the annonin I [14].

Compounds **1**-**3** showed were isomeric acetogenins in *Annona glabra*, *viz* Squamocin-C, Squamocin-D, and Annonin I. These compounds were previously reported only from *Annona squamosa* (13). It could be safely concluded through this study, that *A. glabra* represents their second natural source. These compounds isolated for the first time from *Annona glabra*. Previous studies on *A. glabra* have reported on the isolation of squamocin, asimicin, and desacetyl uvarcin from its seeds in Japan (1); and annonacin, corossolon, and solamin from the bark in China (3). Only one out of these 6 previously reported compounds, *viz* squamocin possesses isomeric relationship with the three compounds isolated in this work. While the potency against human tumor cell lines has been reported for many acetogenin compounds, this study reports for the first time the range of cytotoxicity of squamocin-C and squamocin-D against human tumors *in-vitro*. It is also of importance to found that annonin I possesses higher activity against lung carcinoma A-549, breast adenocarcinoma and glioblastoma multiforme.

Squamacin C (1)

Amorphous white powder ^{1}H NMR (500 MHz, $CDCl_3$): 0.88 (3H, t, *J*= 6.8, 34), 1.41 (3H, d, *J* = 6.8, 37), 2.26 (2H,ddt, *J* = 7.7 Hz, H-3), 1.5 (2H, m, H-4), 1.2-1.7 (18H, m, H 5-13), 1.30 (2H, m, H-14), 3.39 (H, m, H-15 and H-24), 3.80 (1H, m, H-16), 1.50-1.90 (H, m, H-17, H-18, H-21, H-22), 3.80 - 3.90 (4H, m, H-16, H-19, H-20, H-23), 1.76-1.87 (2H, m, 21), 1.30-1.40 (8H, m, 25-28), 3.58-3.60 (1H, m, 29), 1.30 (8H, m, 30-33), 6.98 (1H, d, *J* = 1.4, 35), 4.99 (1H, dq, *J* = 6.8, 0.4, 36), ^{13}C NMR: Table 1

Squamacin D (2)

^{1}H NMR (500 MHz, $CDCl_3$): 0.88 (3H, t, *J*= 6.8, 34), 1.41 (3H, d, *J* = 6.8, 37), 2.26 (2H,ddt, *J* = 7.7 Hz, H-3), 1.5 (2H, m, H-4), 1.2-1.7 (18H, m, H 5-13), 1.30 (2H, m, H-14), 3.39 (H, m, H-15), 3.80 (1H, m, H-16), 1.50-1.90 (H, m, H-17, H-18, H-21, H-22), 3.80 - 3.90 (4H, m, H-16, H-19, H-20, H-23), 1.76-1.87 (2H, m, 21), 1.30-1.40 (6H, m, 25-27), 1.35-1.37 (2H, m, 29), 3.59-3.61 (1H, m, 28), 1.30 (8H, m, 30-33), 6.98 (1H, d, *J* = 1.4, 35), 4.99 (1H, dq, *J* = 6.8, 0.4, 36), ^{13}C NMR: Table 1

Annonin 1 (3)

^{1}H NMR (500 MHz, $CDCl_3$): 0.88 (3H, t, *J* = 6.8, 34), 1.41 (3H, d, *J* = 6.8, 37), 2.26 (2H,ddt, *J* = 7.7 Hz, H-3), 1.5 (2H, m, H-4), 1.2-1.7 (18H, m, H 5-13), 1.30 (2H, m, H-14), 3.39 (H, m, H-15), 3.39 (H, m, H-24), 3.80 (1H, m, H-16), 1.50-1.90 (H, m, H-17, H-18, H-21, H-22), 3.80 - 3.90 (4H, m, H-16, H-19, H-20), 3.78-3.85 (H, m, H-23), 1.76-1.87 (2H, m, 21), 1.30-1.40 (8H, m, 25-28), 3.58-3.60 (1H, m, 29), 1.30 (8H, m, 30-33), 6.98 (1H, d, *J* = 1.4, 35), 4.99 (1H, dq, *J* = 6.8, 0.4, 36).

ACKNOWLEDGMENT

Thanks also go to Prof. Dr. David L. Smith, Head of Mass Spectrometry Center, University of Nebraska Lincoln, USA. for kindly analyzing some of the samples by FAB-MS. Sincere thanks to Prof. John M. Cassady for performing the HTCL cytotoxicity screening, Chemistry department, and the Ohio Cancer Centre, respectively, of Ohio State University.

REFERENCES

1. K. J. Rupprecht, Y. H. Hui, J. L. Mclaughlin. Annonaceous acetogenins. *J. Nat. Prod.* **53**: 237-278, (1990).
2. M. Leboeuf, A. Cavé, P. K. Bhaumik, B. Mukherjee, R. Mukherjee. The Phytochemistry of the Annonaceae. *Phytochemistry*. **21**:2783-2813, (1982).
3. X. P. Fang, M. J. Rieser, Z. M. Gu, G. X. Zhao, J. L. Mclaughlin. Annonaceous acetogenins: An updated review. *Phytochemical Analysis.* **4**: 49-67, (1993).
4. X. P. Fang, M. J. Rieser, Z. M. Gu, G. X. Zhao, L. J. Mclaughlin. Annonaceous acetogenins: An updated review. *Phytochemical Analysis.* **4**: 27-48, (1993).

5. A. Cavè, D. Cortes, B. Figader, R. Hocquemiller, O. Laprevote, A. Laurens, M. Leboeuf M. (*Phytochemical Potential of Tropical Plants*" Plenum Press, New York, P.167, 1993).
6. S. D. Jolad, J. J. Hoffmann, K. H. Schram, J. R. Cole, M. S. Tempesta, G. R. Kriek, R. B. Bates. Uvaricin, a new antitumor agent from *Uvaria accuminata* (Annonaceae); *J* Org. Chem. **47**: 3151–3153, (1982).
7. X. X. Liu, F. Q. Alali, D. C. Hopp, L. L. Rogers, E. Pilarinou, J. L McLaughlin. A. Glabracins. Two new acetogenins from Annona glabra *Bioorg Med.Chem.* **6**:959-65, (1998).
8. X. X. Liu, E. Pilarinou, J. L McLaughlin. Two novel acetogenins, annoglaxin and 27 hydroxy bullatacin, from *Annona glabra. J. Nat. Prod.*, **62**, 848-852, (1999).
9. C. H. Chen, T. J. Hsieh, T. Z. Liu, C. L. Chern, P. Y. Hsieh, C. Y. Chen. Annoglabayin, a novel dimeric kaurane diterpenoid, and apoptosis in Hep G2 cells of annomontacin from the fruits of Annona glabra. *J. Nal. Prod*, **67**: 1942-1946, (2004).
10. Y. H. Zhang, H. Y. Peng, G. H. Xia, M. Y. Wang, Y. Han Anticancer effect of two diterpenoidcompounds isolated from Annona glabra Linn. *Acta Pharmacological Sin*, **25**: 937-42, (2004).
11. B. N. Meyer, N. R. Ferrigni, J. E. Putnam, L. B. Jacobsen, D. E. Nichols, J. L. McLaughlin. Brine Shrimp: A convenient general bioassay for active plant constituents. *Planta Medica*, **45**:31–34, (1982).
12. J. M. Cassady, W. M. Baird, C. J. Chang. Natural products as a source of potential cancer chemotherapeutic and chemopreventive agents. *J. Nat. Prod.* **53**: 23- 41, (**1990**).
13. M. Sahai, S. Singh, M. Singh, K. Y. Gupta, S. Akashi, R. Yuji, K. Hirayama, H. Asaki, H. Araya, K. Kakinuma, H. Hara, T. Eguchi, K. Kakinuma, Y. Fujimoto. Annonaceous Acetogenins from the seeds of *Annona squamousa*; adjacent bis-tetrahydrofuranic acetogenins *Chem. Pharm. Bult.* **42**: 1163-1174, (1994).
14. L. Born, F. Lieb, P.J. Lorentzen, H. Moeschler, M. Nonfon, R. Soellner, D. Webdisch. The relative configuration of Acetogenins isolated from *Annona squamosa* Annonin I and Annonin IV. *Planta Medica*, **45**: 312-316, (1990).