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# Anthelmintic acetogenin from *Annona squamosa* L. Seeds

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**ABSTRACT**

*Annona squamosa* seeds extracts showed anthelmintic activity against *Haemonchus contortus*, the main nematode of sheep and goat in Northeastern Brazil. A compound **1** was isolated from ethyl acetate extract and inhibited the egg hatching of *H. contortus* at 25 mg $ml^{-1}$. The structure of **1** was determined as a $C_{37}$ trihydroxy adjacent bis-tetrahydrofuran acetogenin based on spectroscopic analysis.

**Key words:** *Haemonchus contortus*, nematodes, Annonacea, phytotherapy.

## INTRODUCTION

In Northeastern Brazil, gastrointestinal parasitism of sheep and goats is one of the leading causes of mortality, producing high economic losses (Pinheiro et al. 2000). Among gastrointestinal nematodes, *Haemonchus contortus* is the most frequent and pathogenic, being responsible for the high mortality rate in young animals during the rainy season (Menezes et al. 1992, Arosemena et al. 1999). Trying to reduce these losses, synthetic anthelmintics are routinely used, often indiscriminately, causing reduced efficacy besides environmental pollution and food residues (Waller et al. 1995, Herd 1995). To decrease these negative impacts, the phytotherapy is an alternative tool that has been studied by many researches nowadays. The study of plants from Annonacea family has demonstrated the presence of active substances with parasiticidal effects (Duret et al. 1998). *Annona squamosa* seed powder is popularly used against insects in Northeast of Brazil (Braga 2001). In the search for new anthelmintic agents to be used in the control of goat nematodes, the anthelmintic activity of extracts and isolated compounds of *A. squamosa* seeds were evaluated on egg hatching of *H. contortus*.

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## MATERIALS AND METHODS

### PREPARATION OF *A. squamosa* EXTRACTS

The seeds of *A. squamosa* were obtained by manual extraction from fruits bought in the central market of Fortaleza. The extracts were prepared by the methodology performed by Nonfon (1990) as follows: *A. squamosa* seeds (2 kg) were triturated to a powder that was mixed with methanol/water solution (90:10, 3 l) and left in contact for seven days. This mixture was filtered and an aliquot of this initial solution (300 ml) was evaporated to dryness for bioassay analysis. The aqueous-methanol solution was evaporated under reduced pressure using a rotatory evaporator. Methanol was eliminated and the remaining aqueous solution was transferred to a separatory funnel and washed up four times (4 × 30 ml) with ethyl acetate that was evaporated to obtain the correspondent extract (15.0 g). The aqueous layer was dried in a water bath to obtain the aqueous extract (44.0 g).

### ISOLATION OF ACETOGENIN **1**

The Ethyl acetate extract (EtOAc) (15 g) was submitted to a silica gel (0.063-0.2 mm) being eluted with chloroform, ethyl acetate and methanol in mixtures of increasing polarity. Fifty-two (10 ml) fractions were obtained and compared by TLC using silica gel kieselgel 60 from Merck, visualized by exposure to $I_2$ vapor. Compound **1** (302 mg) was isolated by elution the column with 100% ethyl acetate.

### PREPARATION OF ACETYL DERIVATIVE (**1a**)

The acetyl derivative was obtained by reaction of compound **1** (100 mg) with acetic anhydride (3 ml) and pyridine (1 ml) mixture at room temperature for 24 hours. Then a hydrochloric acid solution (5%) was added (10 ml). The reactional mixture was transferred to a separatory funnel and extracted with chloroform (3 × 5 ml). The organic layer, containing the acetyl derivative was washed with water (5 × 5 ml), dried with sodium sulfate and the solvent was evaporated leaving **1a** (80 mg) (Furniss et al. 1989).

### CHEMICAL ANALYSIS OF ACETOGENIN **1**

Optical rotation was made in $CHCl_3$ (Perkin Elmer 341); IR spectra were recorded on Perkin Elmer FT-IR spectrum 100 spectrophotometer and the values are expressed in $cm^{-1}$. NMR spectra were recorded on a Brucker Avance DRX-500 spectrometer in $CDCl_3$. 70 eV EI-Mass spectra was obtained using a Hewlett-Packard 5971 GC/MS instrument employing the following conditions: column: Dimethylpolysiloxane DB-1 coated fused silica capillary column (30 m × 0.25 mm × 0.25 mm); carrier gas: He (1 ml/min); capillary injector operating at 250°C in the split mode (1:100); detector temperature: 200°C; column temperature: 35-180°C at 4°C/min then 180-250°C at 10°C/min; mass spectra: electron impact. High-resolution mass spectra were obtained in an ultrOTOF$_Q$ – ESI-TOF Mass Spectrometer, Bruker Daltonics, Billerica, MA, USA. Experiment conditions: Infusion bomb, Flux 300 $\mu$l/h, Mobile phase for solubilization: $H_2O$:MeOH (20:80). Detection mode was positive and negative for the sample. For internal calibration it was used a 10mg/ml NA-TFA solution and for external calibration a 10mM sodium formiate solution. Analysis conditions: End Plate: 4000 Volts, Capillary: 4500 Volts, Capillary Exit: 300 Volts, Skimmer 1: 50 Volts, Skimmer 2:25 Volts, Transfer: 90 $\mu$s, Collision Exit Gate: 80 $\mu$s. TLC analyses were performed on a 3-10 cm aluminum sheet precoated with silica gel 60-254 (Merck) (Solvent system: $CHCl_3$/EtOAc 3:7). $SiO_2$, 200–400 mesh (Merck) was used for column chromatography.

Compound **1** was isolated as a viscous oil: $\{\alpha\}_D^{20}$ +11.57° (c=0.001, $CHCl_3$); UV ($\lambda_{max}$, MeOH, nm): 225 (log $\varepsilon$ 3.19); IR (KBr) $\nu_{max}$ 3418, 2927, 2855, 1748, 1652, 1463, 1319, 1118, 1068, 1028, 953, 877, 756, 666 $cm^{-1}$; $^1$H NMR ($CDCl_3$, 500 MHz) and $^{13}$C NMR ($CDCl_3$, 125 MHz) are showed in Table I. EIMS 70 eV $m/z$: 481(3), 341 (5), 327 (7), 281 (45), 253 (12), 155 (5), 111 (8), 85 (32), 71 (57), 57 (72), 43 (43). A mass spectral fragmentation of compound **1** in the EI mass spectrum is shown in Figure 1.

Compound **1a** was obtained as a waxy solid: $\{\alpha\}_D^{20}$ +15.57° (c=0.001, $CHCl_3$); UV ($\lambda_{max}$, MeOH, nm): 225 (log $\varepsilon$ 2.61); IR (KBr) $\nu_{max}$ 2928, 2855, 1748, 1653, 1465, 1436, 1317, 1250, 1120, 1070, 1026, 950, 876, 759, 668 $cm^{-1}$; $^1$H NMR ($CDCl_3$, 500 MHz) and $^{13}$C NMR ($CDCl_3$, 125 MHz). EIMS 70 eV $m/z$: 481 (4), 341 (5), 281 (58), 253 (18), 155 (4), 141 (5), 137 (6), 125 (4), 111 (8), 97(17). $C_{37}H_{66}O_7$: 622.480306 (M+), 623.924945 (M+H), 645.906374 (M+Na).

### ANTHELMINTIC BIOASSAY – *in vitro* EGG HATCH TEST

*H. contortus* eggs were recovered according to Hubert and Kerboeuf (1984). Briefly, 10 g of feces were collected directly from the rectum of sheep experimentally infected with *H. contortus* mixed with tepid water and filtered through 590; 149; 101 and 30 $\mu$m aperture sieves. The eggs retained on the 30 $\mu$m sieve were collected.

The *in vitro* egg hatch test was carried out by the method of Coles et al. (1992). To perform this test, a volume of 500 $\mu$l, with 250 $\mu$l of the eggs solution (about 120 eggs) and 250 $\mu$L of extract solution were placed in 5 ml tubes. The extract solutions for the bioassay were prepared using the concentrations of the extracts tested were 25; 5; 1; 0.2; 0.04 mg $ml^{-1}$, the negative control was the diluent Tween 80 and the positive control thiabendazole (0.1 $\mu$g $ml^{-1}$). After incubation for 48 hours, a drop of Lugol was added. All larvae and eggs

**TABLE I**

**$^{1}$H (500 MHz) and $^{13}$C (100 MHz) NMR spectral data for 1 and 1a, including heteronuclear 2D shift-correlated obtained by $^{1}$H and $^{13}$C-COSY-$^{n}J_{CH}$ (n=1, HMQC, n=2 and 3, HMBC) experiments, in $CDCl_3$ as solvent, chemical shifts ($\delta$, ppm) and coupling constants ($J$, Hz, in parenthesis).[a]**

| | **1a** | | | | | |
|---|---|---|---|---|---|---|
| | $^{1}$H-$^{13}$C-COSY-$^{1}J_{CH}$ | | $^{1}$H-$^{13}$C-COSY-$^{n}J_{CH}$ | | **1** | |
| | $\delta_C$ | $\delta_H$ | $^{2}J_{CH}$ | $^{3}J_{CH}$ | $\delta_C$ | $\delta_H$ |
| C | | | | | | |
| 1 | 173.9 | – | | 2H-3; H-35 | 173.8 | – |
| 2 | 134.2 | – | 2H-3; H-35 | | 134.2 | – |
| $CH_3C$=O | 170.9; 170.8; 170.6 | – | – | – | – | – |
| CH | | | | | | |
| 5 | 74.0 | 4.91 m | | | 71.7 | 3.62 m |
| 15 | 75.0 | 4.91 m | | | 74.1 | 3.43 m |
| 16 | 80.0 | 4.04 m | | | 83.2[a] | 3.84 m |
| 19 | 81.2 | 3.93 m | | | 82.5[a] | 3.94 m |
| 20 | 81.6 | 3.93 m | | | 82.1[a] | 3.94 m |
| 23 | 80.3 | 4.04 m | | | 82.7[a] | 3.84 m |
| 24 | 75.2 | 4.91 m | | | 71.4 | 3.43 m |
| 35 | 148.8 | 7.02 s | H-36 | 2H-3; 3H-37 | 148.9 | 6.99 s |
| 36 | 77.5 | 5.05 m | H-35; 3H-37 | | 77.4 | 5.02 m |
| **$CH_2$** | | | | | | |
| 3 | 26.0 | 2.32 (t, 8.1) | | | 25.6 | 2.29 (t, 7.8) |
| 4 | 33.9 | 1.55 m | | | 37.1 | 1.56 m |
| 6 | 33.8 | 1.55 m | | | 37.3 | 1.56 m |
| 7 | 25.1 | 1.25 m | | | 24.8 | 1.28 m |
| 8-12 | 29.5-29.1 | 1.25 m | | | 28.9-29.6 | 1.28 m |
| 13 | 25.1 | 1.25 m | | | 25.1 | 1.28 m |
| 14 | 30.8 | 1.60 m | | | 33.0 | 1.56 m |
| 17 | 27.3 | 1.99 m; 1.60 m | | | 27.3 | 1.98 m; 1.65 m |
| 18 | 28.0 | 1.98 m; 1.65 m | | | 28.4 | 1.98 m; 1.65m |
| 21 | 28.2 | 1.98 m; 1.65 m | | | 28.4 | 1.98 m; 1.65 m |
| 22 | 27.3 | 1.98 m; 1.65 m | | | 27.3 | 1.98 m; 1.65 m |
| 25 | 30.8 | 1.60 m | | | 32.3 | 1.56 m |
| 26 | 25.2 | 1.25 m | | | 25.6 | 1.28 m |
| 27-31 | 29.5-29.1 | 1.25 m | | | 28.9-29.6 | 1.28 m |
| 32 | 31.7 | 1.25 m | 3H-33 | 3H-34 | 31.8 | 1.28 m |
| 33 | 22.5 | 1.25 m | 3H-34 | | 22.5 | 1.28 m |
| **$CH_3$** | | | | | | |
| 34 | 14.0 | 0.87 (t, 6.6) | | | 14.0 | 0.90 (t, 7.0) |
| 37 | 19.1 | 1.39 (d, 6.8) | | | 19.1 | 1.43 (d, 6.7) |
| **$CH_3C$**=O | 21.0, 21.1, 21.2 | 2.08, 2.05, 2.03 | | | | |

[a]Number of hydrogens bound to carbon atoms deduced by comparative analysis of DEPT-$^{13}$C NMR spectra. Chemical shifts and coupling constants (J) obtained from 1D $^{1}$H NMR spectra. $^{1}$H-$^{1}$H-COSY spectrum was also used in these assignments.

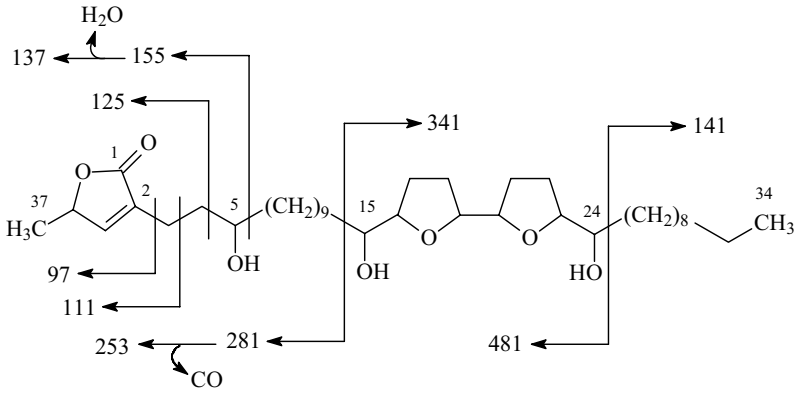


Fig. 1 – Mass spectral fragmentation of **1**.

were counted using a microscope. Five replicates were performed for each concentration. Data were expressed as percentage of egg hatching inhibition. Statistical comparisons of the results from the egg hatching inhibition by the extracts on different concentrations were performed using Kruskal-Wallis test, with significance level of 5%. The effective concentration to inhibit half eggs hatching ($CE_{50}$) for each extract was calculated by the equation which showed the tendency line with minimum determination coefficient of 70%. The values of negative and positive controls are expressed as mean results.

## RESULTS AND DISCUSSION

Table I displays the $^{1}H$ and $^{13}C$-COSY 2D NMR (HMQC and HMBC) data of compound **1** and its acetyl derivative **1a**. Compound **1** showed to be an acetogenin, a type of compound common in plants of Annonaceae family, with 37 carbons, three hydroxyl groups, an unsaturated lactone moiety ($\delta$ 6.99, H-35; $\delta$ 148.9, C-35; $\delta$ 134.2, C-2; $\delta$ 173.8, C-1) and two adjacent tetrahydrofuran (THF) rings (Figure 2). The adjacent bis-THF rings with flanking OH groups in **1** were indicated by $^{1}$HNMR resonances at $\delta$ 74.1 (C-15), 83.2 (C-16), 82.5 (C-19), 82.1 (C-20), 82.7 (C-23) and 71.4 (C-24). The relative stereochemistry of the bis-THF moiety of **1** was suggested to be *threo-trans-threo-trans-erythro* by careful comparison of the $^{1}H$ and $^{13}C$ NMR signals of **1** with stereochemically defined bis-THF acetogenins (Araya et al. 2002, Alali et al. 1999, Zhou et al. 2000, Hopp et al. 1998). The presence of three hydroxyl groups in the molecule was evidenced by an absorption band in the IR at 3418 $cm^{-1}$ and three singlets at $\delta$ 2.08, 2.05, and 2.03 in the $^{1}H$ NMR spectra of the acetyl derivative **1a**. The presence of two THF rings was suggested by signals in the $^{1}H$ NMR at $\delta$ 3.43 (H-15), 3.84 (H-16, H-19, H-24) and 3.95 (H-20, H-23), which integrated constitutes 6 protons. A third hydroxyl group was evidenced by $^{1}H$ NMR signal at $\delta$ 3.62 and a $^{13}C$ NMR signal at $\delta$ 71.7. Its position was predicted to be at C-5 based on the EIMS fragments ions at m/z 155, 111, 137, 111 and 97 (Figure 1) common in other C-5 hydroxylated acetogenins (Alali et al. 1999) and associated signals of $^{1}H$-$^{1}H$ COSY, HMQC and HMBC of the acetyl derivative **1a**. In the bidimensional homonuclear $^{1}H$-$^{1}H$ COSY spectrum of **1a**, the sequence of couplings between H-3 (t, $\delta$ 2.32) and H-4 (m, $\delta$ 1.55) and H-4 with H-5 (m, $\delta$ 4.91) assures the presence of a hydroxyl group at C-5. The IR spectra of compound **1** showed as main peaks at 3418 $cm^{-1}$ for hydroxyl groups and at 1748 and 1652 $cm^{-1}$ for the C=O and the double bond of the lactone moiety respectively, that are compatible with data of similar acetogenins from seeds of *A. squamosa* (Rupprecht et al. 1990).

The percentage of egg hatching inhibition of *H. contortus* using MeOH/$H_2O$, aqueous, EtOAc extracts and compound **1** is shown in Table II.

The highest inhibition of egg hatching was obtained with the EtOAc extract, in the concentrations of 5 and 25 mg $ml^{-1}$, which did not demonstrate significant difference from thiabendazole, at 0.1 $\mu$g $ml^{-1}$. The aqueous extract showed an inhibition percentage higher than the negative control, Tween 80, at 25 mg $ml^{-1}$, but at 5 mg $ml^{-1}$ was not statistically different from the nega-

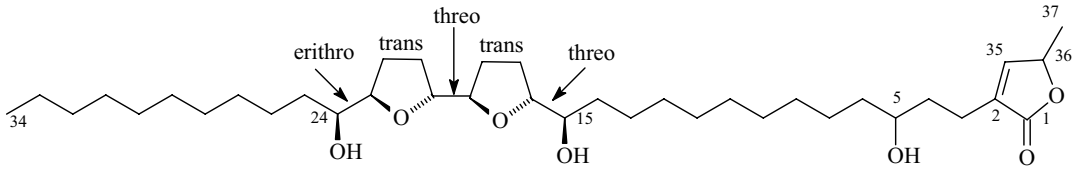


Fig. 2 – Structure of Acetogenin **1**.

**TABLE II**
**Inhibition percentage of *Annona squamosa* extracts and Compound *1* on *Haemonchus contortus* egg hatch test.**

| Concentration (mg mL$^{-1}$) | Extracts | | | |
|---|---|---|---|---|
| | Ethyl acetate | Methanol/Water | Aqueous | Compound **1** |
| 25 | $100.00^{Aab} \pm 0.0$ | $81.58^{BCbc} \pm 6.2$ | $52.64^{Bc} \pm 10.9$ | $100.00^{Aa} \pm 0.0$ |
| 5 | $99.03^{Aab} \pm 0.4$ | $81.95^{ABab} \pm 16.5$ | $14.28^{Bc} \pm 4.2$ | $99.70^{Aa} \pm 0.2$ |
| 1 | $39.47^{Bab} \pm 13.7$ | $53.10^{Ca} \pm 13.7$ | $13.74^{BCb} \pm 4.8$ | $51.60^{Ba} \pm 7.5$ |
| 0.2 | $8.97^{BCa} \pm 2.3$ | $4.81^{Dab} \pm 2.5$ | $3.02^{Db} \pm 2.5$ | $2.58^{Cb} \pm 0.8$ |
| 0.04 | $5.31^{Ca} \pm 1.3$ | $2.80^{Da} \pm 1.5$ | $6.25^{Ca} \pm 1.8$ | $3.33^{Ca} \pm 1.1$ |
| Tween 80 (3%) | $4.25^{C} \pm 0.7$ | $4.25^{D} \pm 0.7$ | $4.25^{CD} \pm 0.7$ | $4.25^{C} \pm 0.7$ |
| Thiabendazole (0.1 $\mu$g mL$^{-1}$) | $100.00^{A} \pm 0.0$ | $100.00^{A} \pm 0.0$ | $100.00^{A} \pm 0.0$ | $100.00^{A} \pm 0.0$ |

Capital letters compare means in the lines (different concentrations) and small letters in the columns (different extracts). Different letters indicate significantly different values ($P<0.05$).

tive control. The EtOAc extract was submitted to a silica gel column chromatography and the main isolated compound **1** was evaluated for its anthelmintic activity. Compound **1** inhibited the egg hatching more than 90% at 5 and 25 mg ml$^{-1}$ concentrations, similarly to the EtOAc extract. The concentration that is effective for killing half of larvae amount ($CE_{50}$) of the aqueous, methanol/water, ethyl acetate extracts and compound **1** was 10.02, 0.89, 0.76 and 0.78 mg ml$^{-1}$ respectively.

The extracts MeOH/$H_2O$, EtOAc and the acetogenin **1** at 5 mg ml$^{-1}$ concentration had similar results in the inhibition of egg hatching of *H. contortus*. This result could be explained by the fact that the extracts MeOH/$H_2O$ and EtOAc possess several chemical compounds, and many of them could display ovicidal action. The total action of the extracts is a sum of the activities of their constituents (Rates 2001). *A. squamosa* aqueous extract did not show relevant activity, similarly to the aqueous extract of *A. senegalensis* bark in the *H. contortus* egg hatch inhibition test (Alawa et al. 2003). Probably these results are due to the low solubility of acetogenins in water.

The ovicidal effect obtained with ethyl acetate extract is lower than synthetic anthelmintics nevertheless it is important when compared with other plant extracts. Assis et al. (2003) reported 20% inhibition of *H. contortus* egg hatching with ethyl acetate extract of *Spigelia anthelmia* at 12.5 mg ml$^{-1}$ and the EtOAc extract of *A. squamosa* showed 99% egg hatch inhibition at 5 mg ml$^{-1}$. The effect of eight acetogenins, isolated from *Uvaria hookeri* and *U. narum,* against *H. contortus* adult specimens showed a negative relationship between the death time in minutes and acetogenins concentration (Padmaja et al. 1993). In conclusion, the seeds of *A. squamosa* represent an alternative natural source for anthelmintic compounds.

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**RESUMO**

Extratos das sementes de *Annona squamosa* demonstraram atividade anti-helmíntica contra *Haemonchus contortus*, o principal nematódeo de ovinos e caprinos no Nordeste do Brasil. O composto **1** foi isolado do extrato acetato de etila e inibiu a eclosão dos ovos de *H. contortus* a 25 mg $ml^{-1}$. A estrutura de **1** foi determinada como uma acetogenina $C_{37}$ tri-hidroxi bis-tetrahydrofurano adjacente, baseando-se nos dados espectrais.

**Palavras-chave:** *Haemonchus contortus*, nematódeos, anonácea, fitoterapia.

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