SUPPLEMENTARY MATERIAL

Alkaloids from the root of Indonesian *Annona muricata* L.

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Abstract

Annona muricata L. has been used traditionally in Indonesia to treat disease. Phytochemical studies on the alkaloid fractions from the root of *Annona muricata* L. from Malang-Indonesia resulted in the isolation of an unreported benzylisoquinoline alkaloid (+)-xylopine **5** as well as four known alkaloids (**1**-**4**). The crude methanol extract and alkaloid fractions were tested against *Plasmodium falciparum* K1 and against bacteria (*Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter buamanii*, *Pseudomonas aeruginosa*, Methicillin-resistant *Staphylococcus aureus*) with insignificant activities (MIC > 32 µg/mL). Individual alkaloids were tested against a human suspension cancer cell line (HL-60 leukemia cells) and two human fibroblastic cancer cell lines (A549 lung cancer cells and HepG2 liver cancer cells) in which compound **5 w**as the most toxic alkaloid with IC_{50} values ranging from 20-80 µM.

Keywords
Indonesian medicinal plant; *Annona muricata*; alkaloid; anti-cancer; anti-malarial; anti-bacteria

Cytotoxicity Method

Chemicals and materials

Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle Medium (DMEM), trypsin (2.5% w/v in phosphate buffered saline (PBS)), and penicillin/streptomycin (10,000 U/mL, 10,000 μg/mL) were purchased from Life Technologies (New York, USA). Certified foetal bovine serum (FBS) was obtained from Bovogen (Waltham, USA) and was heat-deactivated before use (60 ˚C, 1 h). CELLSTAR® 96-well cell culture plates, and CELLSTAR® filter cap cell culture flasks (75 cm^2) were purchased from Greiner Bio-One (Kremsmünster, Austria). MTT (98%) and trypan blue (0.5% in PBS) were obtained from Sigma Aldrich (St Louis, USA). PBS tablets (1 tablet per 100 mL of MilliQ water contains 0.8 g sodium chloride; 0.02 g potassium chloride; 0.115 g disodium hydrogen phosphate; 0.02g potassium dihydrogen phosphate, Oxoid) and sodium dodecyl sulfate (SDS, >99%) were purchased from Thermo Fisher Scientific (Australia). Hydrochloric acid (HCl, 32%) was obtained from Ajax Finechem (Australia).

HL-60 cell line

HL-60 cells are human acute promyelocytic leukemia cells derived from a 36-year-old Caucasian female with acute promyelocytic leukemia. HL-60 cells were grown from semi-permanents (obtained from Associate Professor Ronald Sluyter (UOW), originally purchased from ATCC). The cells were grown in growth medium containing IMDM, FBS (20% v/v) and penicillin/streptomycin (200 units/mL and 200 μg/mL, respectively). The cells were grown in cell culture flasks, at 37 ˚C.

HepG2 cell line

HepG2 cells are human liver cancer cells derived from a 15-year-old Caucasian male with a well-differentiated hepatocellular carcinoma. HepG2 cells were grown from semi-permanents (obtained from Professor Mark Wilson (UOW), originally purchased from ATCC). The cells were grown in growth medium containing DMEM, FBS (10% v/v) and penicillin/streptomycin (200 units/mL and 200 μg/mL, respectively). The cells were grown in cell culture flasks, at 37 ˚C. HepG2 cells were harvested with trypsin (0.25% in PBS, 5 min incubation).

A549 cell line

A549 cells are human lung cancer cells derived from a 58-year-old Caucasian male with a lung carcinoma. A549 cells were grown from semi-permanents (purchased from ATCC). The cells were grown in growth medium containing DMEM, FBS (10% v/v) and penicillin/streptomycin (200 units/mL and 200 μg/mL, respectively). The cells were grown in cell culture flasks, at 37 ˚C. HepG2 cells were harvested with trypsin (0.25% in PBS, 5 min incubation).

Protocol for MTT cytotoxicity assays

The suspension cells (HL-60, 2×10^5 cells/50 μL in IMDM) were added to the specified wells (rows 2-7, columns B-I) of a 96 well flat bottom plate and treated immediately. An alternative method was used for the adherent cells, whereby the HepG2 cells (1×10^4 cells in 100 μL/well) or A549 cells (4×10^4 cells in 100 μL/well) were seeded in growth medium and incubated (37 ˚C) for 24 h prior to treatment to allow the cells to adhere to the bottom of the wells. The growth medium was then removed and fresh DMEM (50 μL/well) was added.

Stock solutions of each treatment solution were prepared (10,000 μg/mL, DMSO). The stock solutions were serially diluted in DMSO to achieve a range of concentrations that were 50

times more concentrated than the desired concentrations used for analysis. Each concentration of compound was then diluted 1 in 50 with treatment medium (HL-60, IMDM; HepG2 and A549, DMEM) immediately prior to treatment to give the range of concentrations to be analysed (2% DMSO). The treatment solutions (50 μL) were added to each of the wells in columns C-I (most dilute in column C, most concentrated in column I), while IMDM or DMEM (50 μL) was added to the control well cells (column B). The plates were incubated (37 ˚C, 5% CO_2) for 24 h, after which MTT solution (20 μL, 5 mg/mL, PBS) was added to the wells and the plates were incubated (37 ˚C, 5% CO_2) for 4 h to allow for formazan development. The solubilising solution (100 μL, 10% SDS in 0.01 M HCl) was added to the specified wells and the plates were incubated (37 ˚C, 5% CO_2) overnight.

Absorbance readings were recorded at 570 nm and 690 nm (BMG LabTech Polarstar Omega microplate reader) and concentration-response curves (using equation 1) were produced in order to calculate the IC_{50} value for each compound.

$$\text{Cell survival (\%)} = \frac{(A_{570} - A_{690})\ \text{treated cells}}{(A_{570} - A_{690})\ \text{control cells}} \times 100 \qquad \text{Equation 1}$$

The calculated cell viability was plotted against the treatment concentrations using Microsoft EXCEL™ [1] to determine the IC_{50} value of each compound. All MTT assays were performed at least three times to obtain reproduceable IC_{50} values. Statistical analysis was performed with one-way ANOVA and Tukey's multiple comparison test using GraphPad Prism.[2]

References

1. *Microsoft EXCEL™ 2010*, Microsoft Corporation: Redmond Washington USA, 2010.
2. *GraphPad Prism*, version 5 for Windows; GraphPad Software: San Diego California USA, 2011.

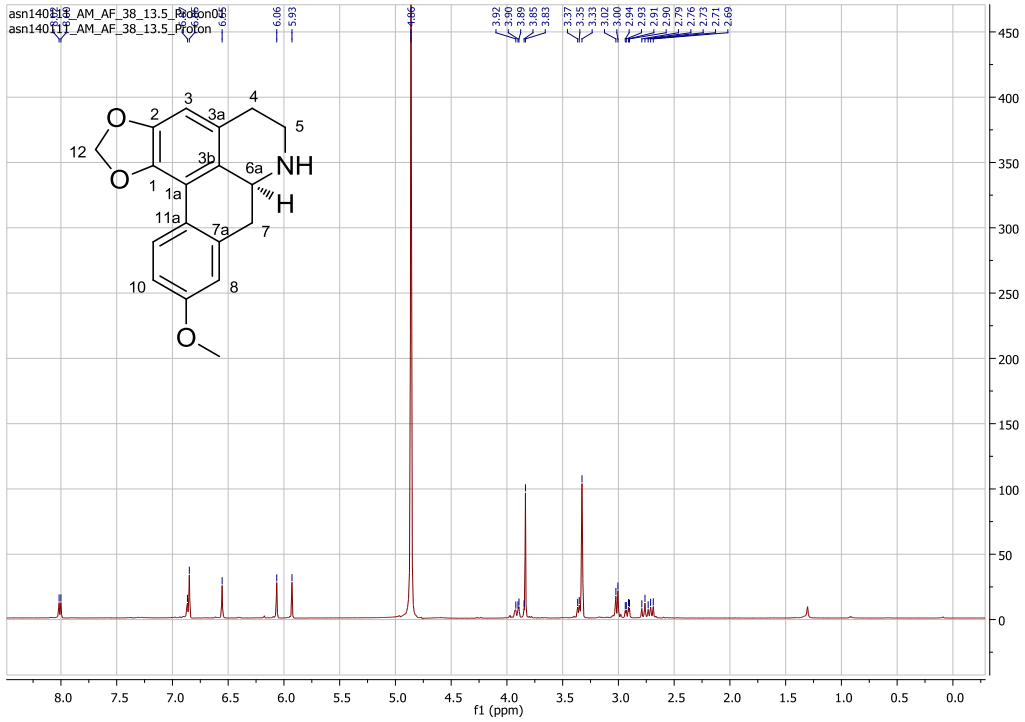


Figure S1. ^{1}H-NMR spectrum of (+) Xylopine 5 (CD_3OD)

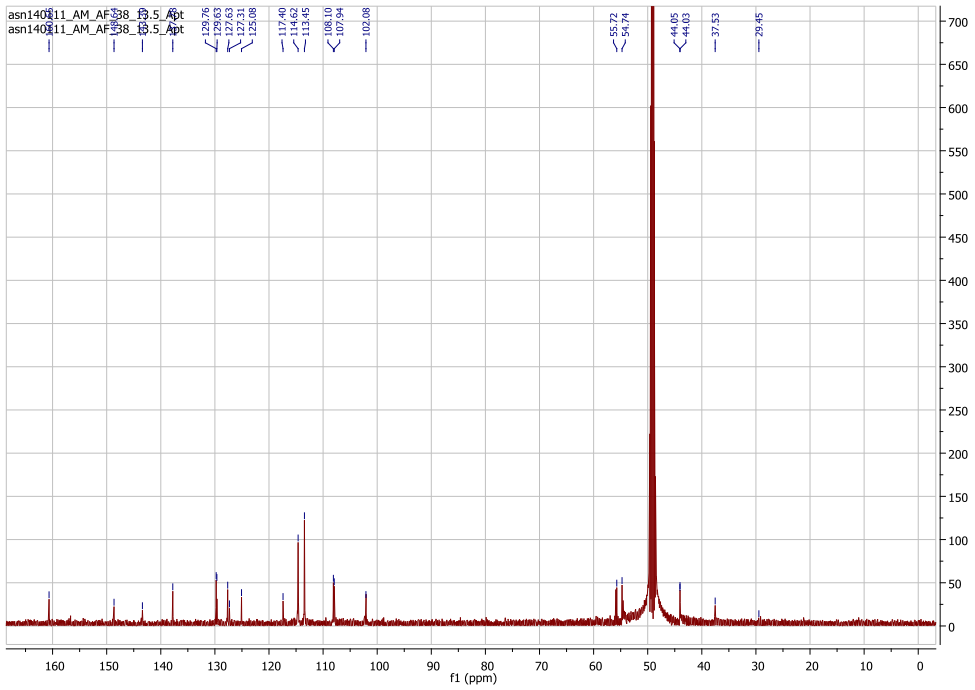


Figure S2. ^{13}C-NMR spectrum of (+) Xylopine 5 (CD_3OD)

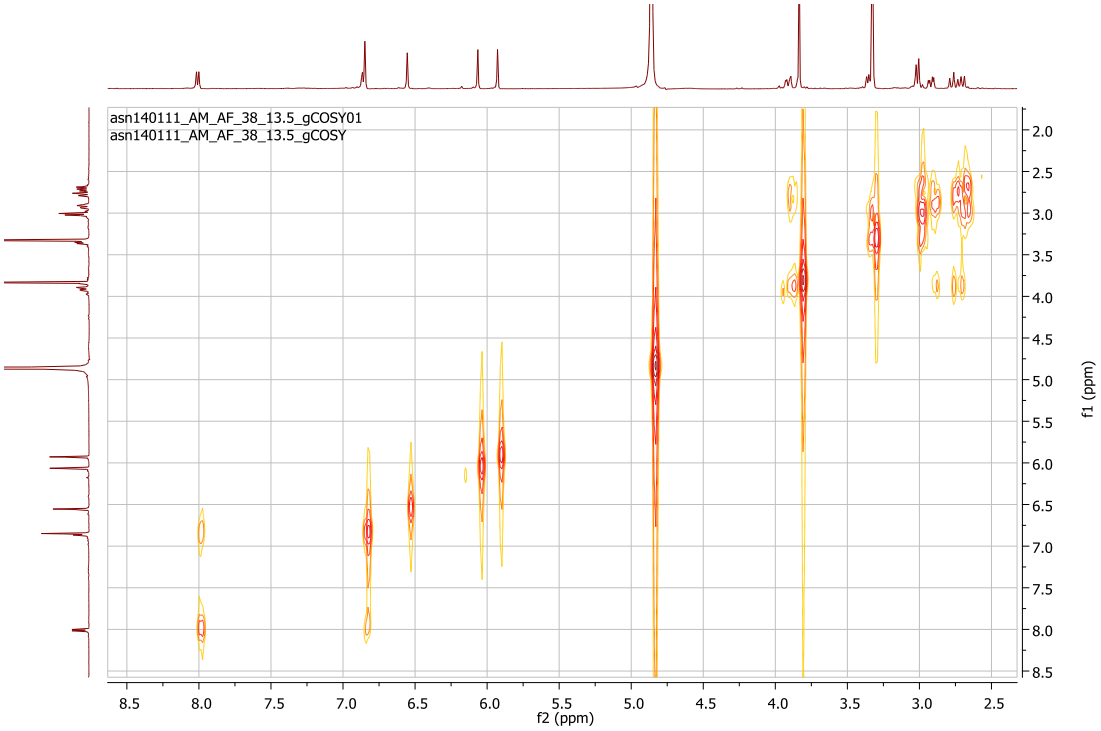


Figure S3. gCOSY spectrum of (+) Xylopine 5 (CD_3OD)

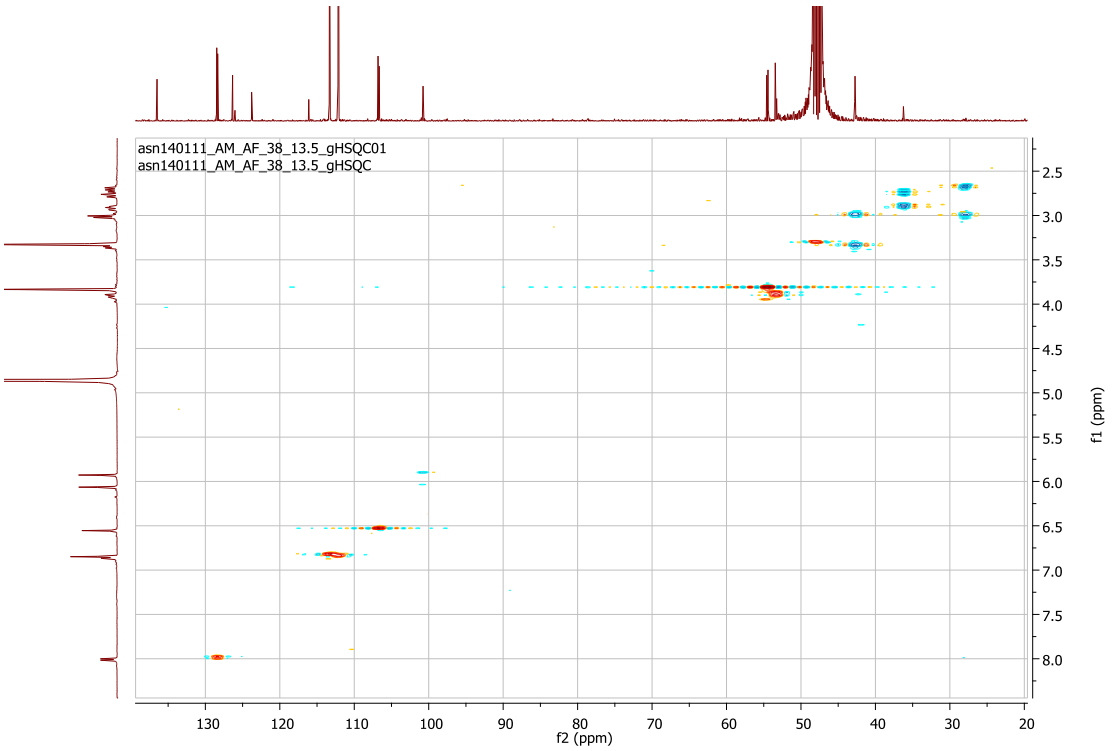
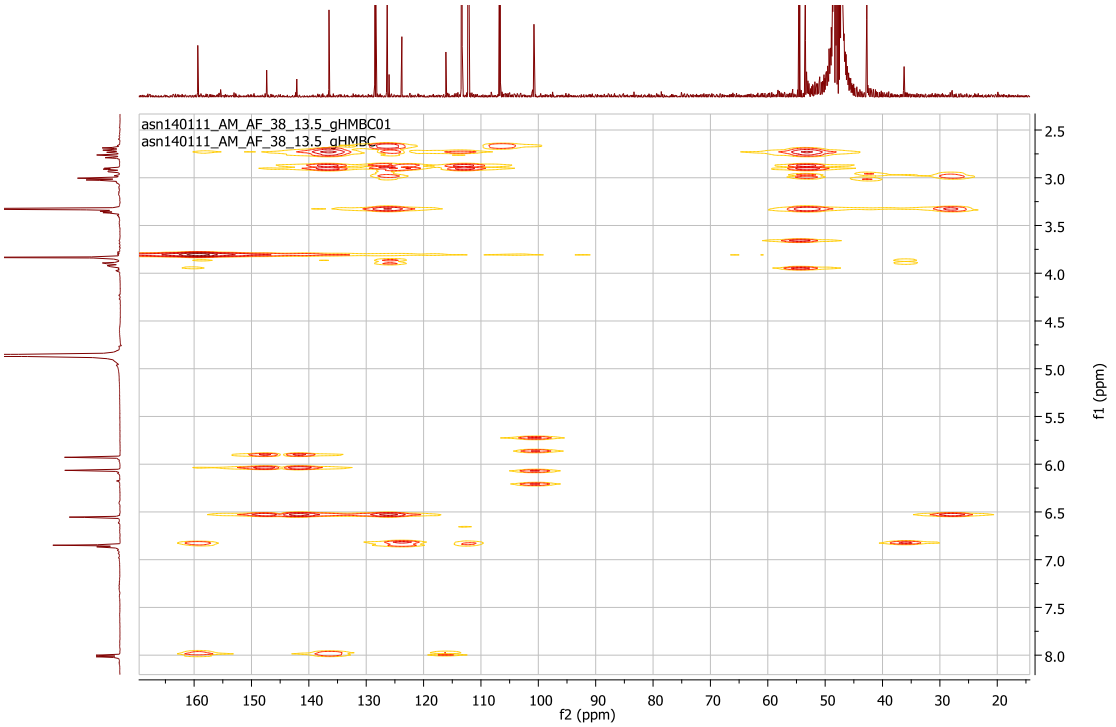


Figure S4. gHSQC spectrum of (+) Xylopine 5 (CD_3OD)



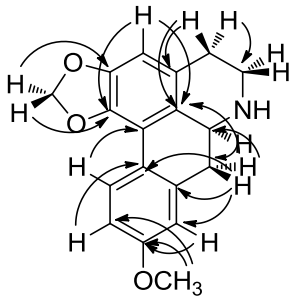


Figure S5. gHMBC spectrum of (+) Xylopine 5 (CD_3OD) and long range proton-carbon correlation

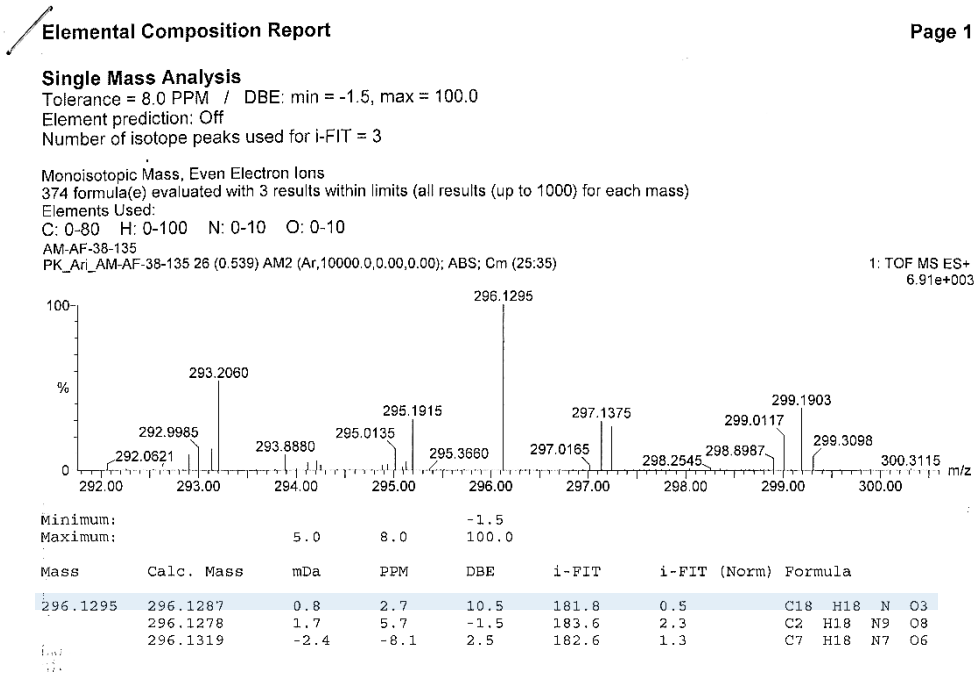
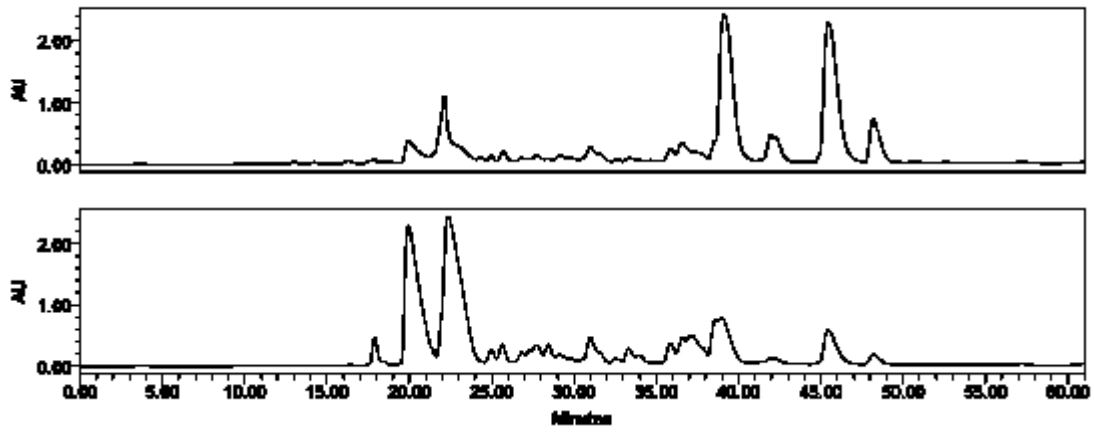
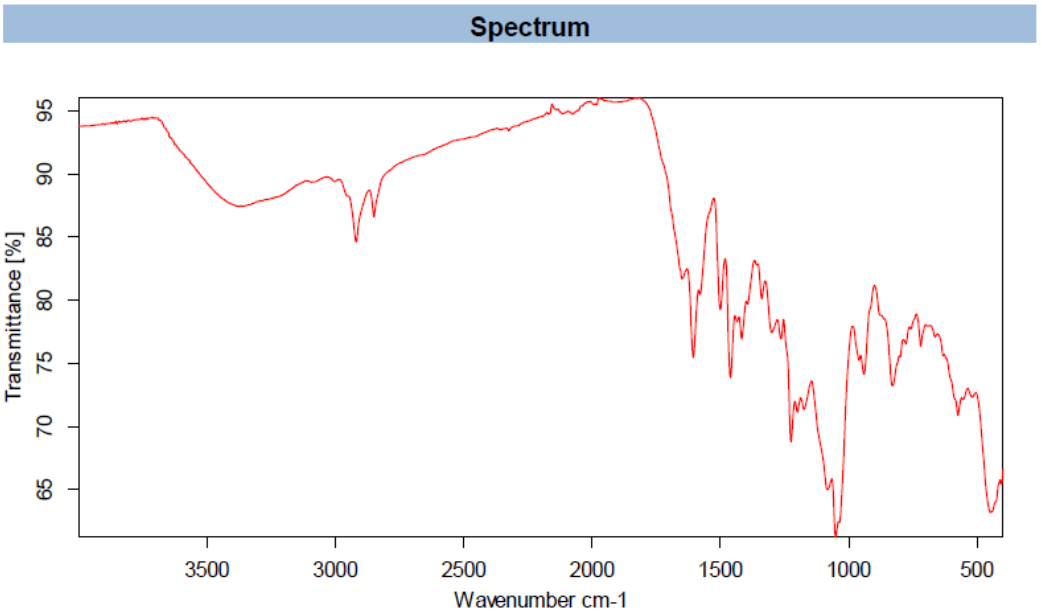


Figure S6. HRESI mass spectrum of (+)-xylopine 5



Note: Chromatogram of alkaloid fraction on optimized method at 254 nm (top) and 280 nm (bottom).

Figure S7. Semi Prep-HPLC profile of isolation of alkaloid from *Annona muricata*



Wave number	Abs. intensity	Rel. intensity	Width	Threshold	Shoulder
2919.355	0.846	0.105	731.247	28.42	0
1606.014	0.755	0.148	64.073	36.24	0
1500.286	0.792	0.061	1805.231	11.65	0
1461.379	0.739	0.120	96.048	26.66	0
1225.240	0.688	0.075	957.502	13.87	0
1051.109	0.612	0.224	120.073	57.27	0
940.903	0.741	0.055	376.544	10.93	0
830.276	0.732	0.070	75.827	16.39	0
447.391	0.632	0.038	63.369	7.06	0
2850.304	0.866	0.022	14.818	35.57	0
1417.377	0.769	0.034	65.785	19.97	0
1174.338	0.713	0.013	67.785	8.78	0
1200.084	0.711	0.011	12.006	8.86	0
574.838	0.709	0.030	221.269	33.63	0
1650.573	0.817	0.023	1719.275	5.36	0

Figure S8. IR spectrum of (+)-xylopine 5

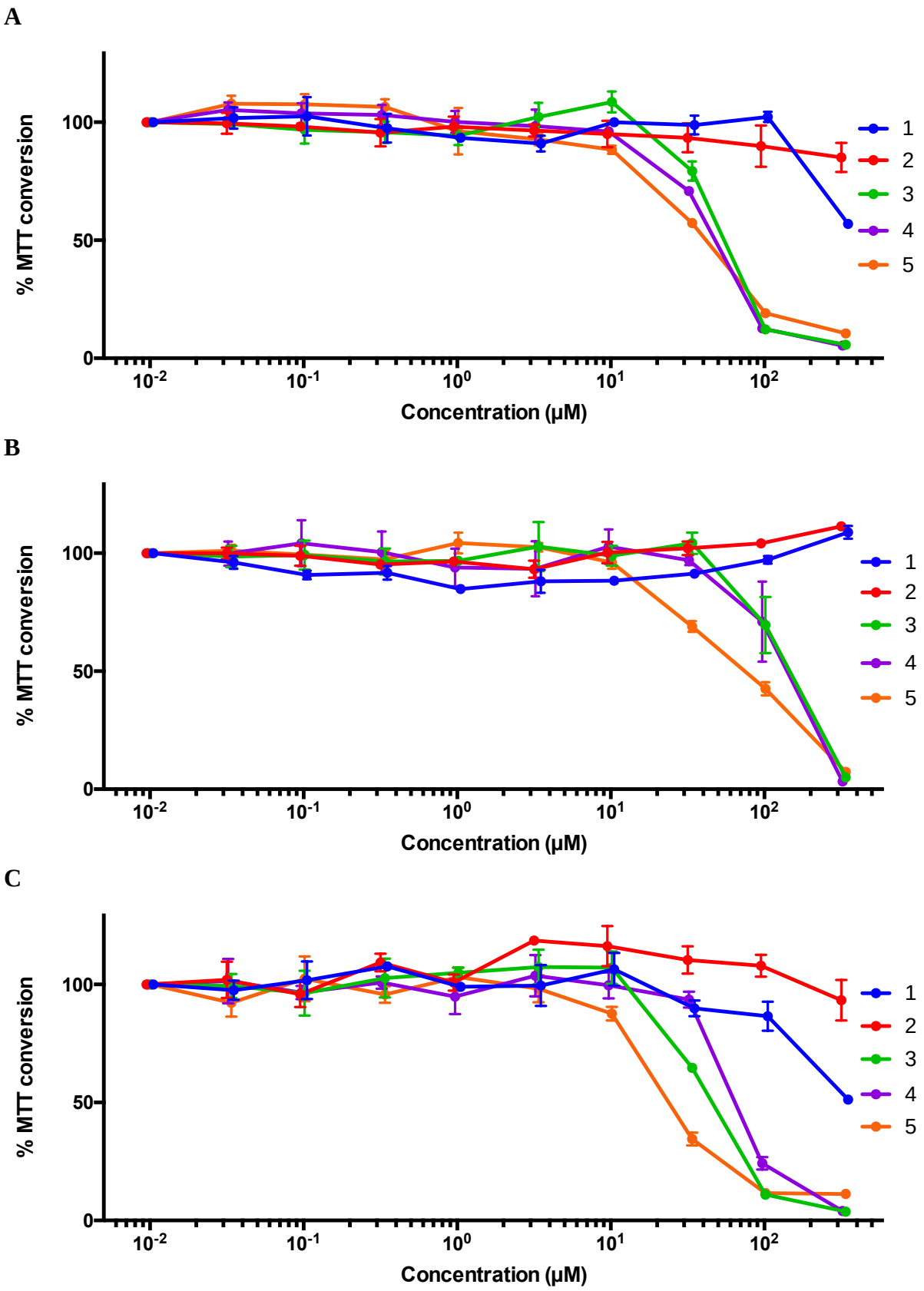


Figure S9. The concentration-response curves for the specified compounds in the human suspension cancer cell line (HL-60 leukemia cells, (A)) and the human fibroblastic cancer cell lines (A549 lung cancer cells (B) and HepG2 liver cancer cells (C)).

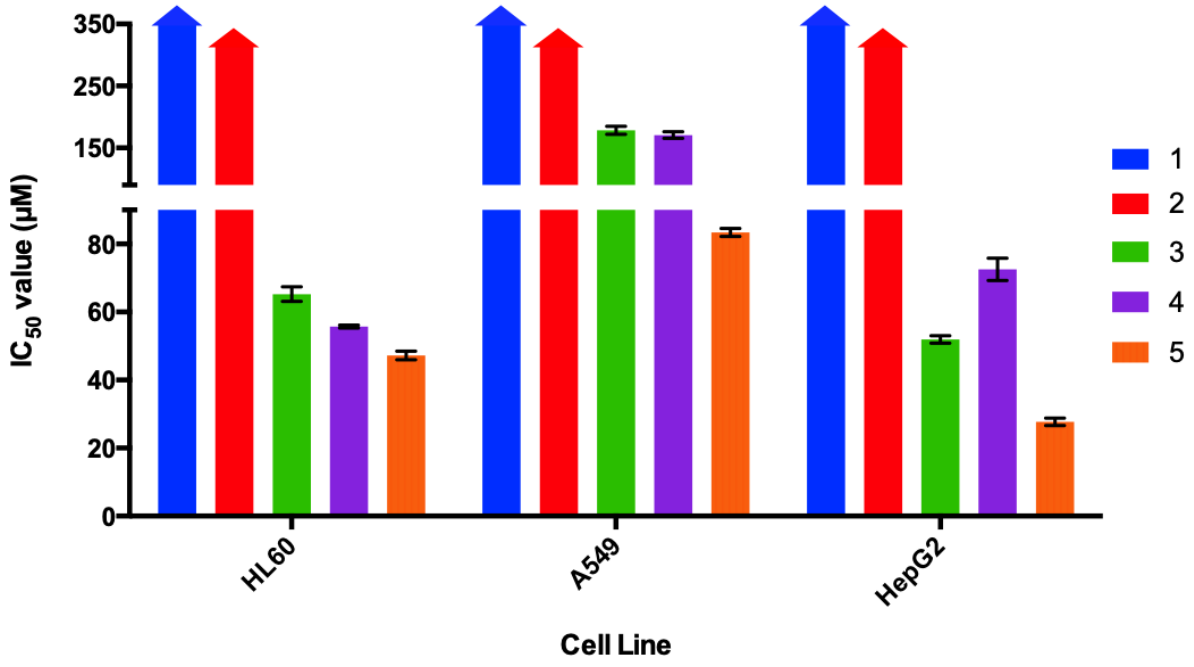


Figure S10. Summary of the IC_{50} values obtained for the specified compounds in the human cancer cell lines: HL-60, A549 and HepG2 cells. IC_{50} values for compounds **1** and **2** were not determined due to solubility limitations, but were greater than 300 μM (as indicated by column arrow). Each column represents the average IC_{50} value determined from triplicate MTT assays.