Supporting Information

Phytochemical Investigation and in Vitro Cytotoxic Evaluation of Alkaloids from *Abuta rufescens*

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Materials and Methods

General experimental procedures
Melting points were determined on a Fischer Johns apparatus and are uncorrected. IR spectra were recorded on a Mattson Galaxy series FT-IR 3000 spectrophotometer. UV spectra were recorded on a Cary 100 spectrophotometer. NMR spectra were recorded on a Bruker Avance 400 spectrometer (400 MHz for $^1$H/100 MHz for $^{13}$C) or Varian Gemini 300 spectrometer (75 MHz for $^1$H MHz for $^{13}$C) in CDCl$_3$ solution at ambient room temperature. Chemical shifts are expressed relative to TMS using internal standard or the residual solvent peak as a secondary reference. Accurate mass measurement was performed on a Micromass Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer, equipped with a Z-spray interface using reserpine as the external mass calibrant. High-resolution mass spectra were acquired on Agilent ESI-TOF or JEOL LC-Mate APCI mass spectrometers. Column chromatography was conducted using silica gel 60 (230-400 mesh). Thin layer chromatography and preparative TLC were conducted using pre-coated silica gel F254 plates. Reagent grade chemicals were obtained from commercial sources and used as received. Cell images were captured using a VWR Vista Vision Inverted Microscope (Model 82026-630) equipped with a CCD DV-2B Digital Camera. Positive-control drugs [doxorubicin (purity ≥ 98% TLC), camptothecin (purity 95% HPLC), etoposide (purity 98% TLC), and vinblastine (purity ≥ 96% HPLC)] were purchased from Sigma. MTT (3-(4,5-
dimethylthiazol-2-yl)-2,5– diphenyl–tetrazolium bromide) reagent and media regents were also purchased from Sigma.

**Extraction and isolation**

Ground stems of *Abuta rufescens* (5.4 kg) were extracted four times with methanol at room temperature for two weeks each time, and the methanolic extract was evaporated to dryness to give a dark gum (263 g). This gum was partitioned in four batches. The total volume of solvents and the weight of the different fractions are given below. The methanolic extract was suspended in methanol/water (9:1, 3.6 L), and the solvent partitioned according to known procedures to provide hexane (1.5 L, 4.2 g), chloroform (3.9 L, 45.4 g), and *n*-butanol (2.0 L, 62.7 g) soluble fractions [1]. The chloroform fraction (38.0 g) was dissolved in dichloromethane (750 mL, 2 batches) and extracted with 3 x 500 mL of 5% NaOH to remove phenols. Evaporation of the organics to dryness gave 4.81 g of non-phenolic compounds. The pH of the aqueous layer was adjusted to pH 9 by 1.5 M H$_2$SO$_4$, which resulted in 12.66 g of a precipitate of phenolic alkaloids. The extraction of the aqueous filtrate with CH$_2$Cl$_2$ gave 3.1 g more of additional phenolics, which were combined with the precipitate. Column chromatography of the phenolic compounds on silica (240 g, 4.6 cm x 1.2 m column) was performed with gradient elution (500 mL fractions) using CH$_2$Cl$_2$ and then CH$_2$Cl$_2$/CH$_3$OH mixtures (progressing from 99:1 to 72:28) and finally pure methanol. Fractions eluted with 99:1 to 97:3 methanol in CH$_2$Cl$_2$ gave at least 8 spots by TLC. This mixture (0.77 g) was further chromatographed on silica (30 g, 2.5 x 30 cm, 35 mL fractions). Fractions 2-3 from this column that eluted with 98:2 CH$_2$Cl$_2$ /CH$_3$OH gave a light cherry red solution which after evaporation and subjecting to preparative plate with 97:3 CH$_2$Cl$_2$/CH$_3$OH gave wine red needles of telisatin A (9) (1.1 mg) after recrystallization from
CH₂Cl₂/CH₃OH. Fractions 8-11 eluted with 98:2 CH₂Cl₂/CH₃OH gave an orange-gold solution, which after evaporation and recrystallization from CH₂Cl₂/CH₃OH gave golden cubes of norrufescine (8) (17.0 mg).

Fractions 12-15 from the original column of the phenolic compounds eluted with 95:5 CH₂Cl₂/CH₃OH gave an orange to dark yellow solution which on evaporation (1.65 g) followed by column chromatography on silica (50 g, 2.5 x 66 cm, 75 mL fractions) gave cherry red solution in fraction 2 with 94:6 CH₂Cl₂/CH₃OH which on evaporation followed by recrystallization from CH₂Cl₂/CH₃OH gave fine yellow needless of telitoxine (10) (5.0 mg). Fractions 6-7 gave a dark red solution which on evaporation followed by silica gel preparative plate with 95:5 CH₂Cl₂/CH₃OH gave, after recrystallization from CH₂Cl₂/CH₃OH, dark red needles of subsessiline (11) (3.4 mg).

Two portions of the cytotoxic non-phenolic fraction were subjected to column chromatography on basic alumina (activity I) as well as silica gel-60 column chromatography. The results for silica (250 g) are reported here. The non-phenolic fraction (2.16 g, 5 cm x 1.2 m, 500 mL fractions) gave a complex mixture of 8-9 compounds in fractions 6-9 eluted with 99:1 CH₂Cl₂/CH₃OH including imeluteine (4), homomoschatoline (2), imenine (3), and lysicamine (5). By preparative TLC separations with CH₂Cl₂, 4 (1.0 mg) and 2 (16.0 mg) were isolated. Recrystallization from methanol was required to get pure red-orange crystals of 3 (11.0 mg).

Fraction 10, on elution with 99:1 CH₂Cl₂/CH₃OH, gave a mixture of three compounds, 5, 3, and splendidine (6), from which 3 (23.0 mg) was isolated by several recrystallizations from CH₂Cl₂/CH₃OH. Fractions 11-22 eluted with 99:1 to 98.5:1.5 CH₂Cl₂/CH₃OH on further silica chromatography (10 g, 1.2 x 31 cm, 25 mL fractions) gave the mixture of the above three alkaloids again in fractions 3-5 by elution with 97:3 CH₂Cl₂/CH₃OH. The mixture underwent
further purification by multiple preparative TLC developments with 97:3 CH₂Cl₂/CH₃OH and recrystallization several times from CH₂Cl₂/CH₃OH to give greenish-yellow needles of 5 (21.0 mg), yellow crystals of 3 (5.0 mg), and yellow crystals of 6 (0.4 mg). Imerubrine (1) next eluted from the column with 97.5:2.5 CH₂Cl₂/CH₃OH in fractions 23-24 and was further purified by chromatography on silica (10 g, 1.2 x 31 cm, 25 mL fractions) and preparative TLC to give orange-red needles of 1 (9.4 mg) recrystallized from CH₂Cl₂/CH₃OH. Isoimerubrine (7) eluted immediately after 1 in fractions 25-27 with 97:3 CH₂Cl₂/CH₃OH and only became apparent after most of 1 was removed from the column. A second preparative TLC was needed to separate it by multiple development with 3% CH₃OH/CH₂Cl₂ to give the red-orange 7 (1.1 mg). The identity of each alkaloid was confirmed by various methods including mixed m.p., co-TLC with authentic sample, NMR, IR, UV, and MS.

**Cell culture**

Cell lines HCT-116 (human colon adenocarcinoma), ACHN (human renal carcinoma), and A549 (human lung carcinoma) were purchased from American Type Culture Collection (ATCC). All cells were grown in monolayer culture. The HCT-116 line was grown using McCoy’s 5A Medium (ATCC) supplemented with 10% fetal bovine serum (Hyclone Laboratories), penicillin/streptomycin solution (100 U/mL and 100 μg/mL, respectively), 240 U/mL nystatin suspension (Sigma) or amphotericin B (2.5 μg/mL), and 1% (w/v) L-glutamine. The ACHN cells were grown in Eagle’s Minimum Essential Medium (ATCC) and supplemented as described above. A549 cells were grown as monolayers in F-12K medium (ATCC) supplemented as above. Cells were maintained in logarithmic phase of growth in a humidified incubator with 5% CO₂ atmosphere at 37°C.
MTT assay for cytotoxicity

The protocol employed for the MTT assay was similar to that reported previously by Swaffar et al. [2] with modifications. Cells were trypsinized (0.05% trypsin and 0.53 mM EDTA) and counted with a hemocytometer. Viability was determined by trypan blue exclusion (only cells having a viability >90% were used for assays). Cell stocks were made as 5X suspensions at concentrations that varied for each cell line. Prior to testing any extracts and isolated compounds, preliminary cell growth curves for each cell line were completed. Based on those preliminary experiments, cells were adjusted to the following concentrations: 1.25 x 10^5 cells/mL for HCT-116 and 3 x 10^5 cells/mL for ACHN and A549 cells. Cell suspensions in 200 μL media were plated into 96-well microtiter plates (Nunc). Wells containing only medium served as blanks. Plates were then incubated in a humidified 5% CO₂ atmosphere at 37°C for at least 4 hours to allow the cells to adhere. All extracts, drugs, and isolated compounds were dissolved in 100% sterile dimethyl sulfoxide (DMSO) at 20 mg/mL (extracts) or at 20 mM, and serial dilutions were made from this stock. Extract or drug solutions (1 μL) were added to wells in quadruplicate. Plates were incubated for 72 hours in a humidified 5% CO₂ atmosphere at 37°C and microscopically observed daily. Initial plates included wells in which alkaloids were incubated in media only, without cells. Contents of the wells were carefully aspirated and replaced with 100 μL of the appropriate unsupplemented medium. A 5 mg/mL stock solution of MTT (10 μL) in PBS was added to all wells of the plate. After incubating plates in 5% CO₂ at 37°C for four hours, 100 μL of 0.04 N HCl in isopropanol were added to all wells of the plate and thoroughly
mixed in order to solubilize formazan produced by viable cells. Well absorbances were measured at 570 nm with a BioTek Synergy plate reader. The mean absorbance of quadruplicate drug-treated wells was compared to that of control wells (treated with 0.5% DMSO alone), and results were expressed as a percentage of control absorbance. If the coefficient of variation (CV) exceeded 20% and the standard deviation exceeded 10%, the mean absorbance was re-calculated omitting the outlying absorbance value. The IC$_{50}$ values for each compound against each cell line were obtained from plots of drug concentration versus percent survival. Points on the graphs represented the mean of the results ± SD obtained from three to eight independent experiments. Doxorubicin, camptothecin, etoposide, or vinblastine were used as positive controls.

Grandirubrine (12) showed potent cytotoxic effects as evidenced in Fig. 1S. Just prior to addition of MTT to the wells of the plate after 72 h, treated HCT-116 cells were observed microscopically. Control cells treated with DMSO (A) were completely confluent, but 5 μM grandirubrine was clearly cytotoxic to the cells, as evidenced by decreased proliferation and induced cell death (B).

Fig. 1S Inverted phase contrast microscopy of human HCT-116 colon cancer cells. Cells growing on 96-well plates were exposed for 72 h to 0.5 % DMSO (A) or to 5μM grandirubrine (12) (B).
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References


2 Swaffar DS, Ireland CM, Barrows LR. A rapid mechanism-based screen to detect potential anti-cancer agents. Anti-Cancer Drugs 1994; 5: 15-23
Imenine 3
300 MHz, CDCl₃
Lysicamine 5
300 MHz, CDCl₃
Norrufescine 8 (CDCl₃, 400 MHz)
Grandinubrine 12
300 MHz, CDCl₃