Supporting Information to:

Triterpenoidal Saponins: Bioactive Secondary Metabolites from
Zygophyllum coccineum

Elham Amin1,5, Seham S. El-Haway2, Magda M. Fathy2, Rabab Mohammed1, Zulfiqar Ali5,
Nurhayat Tabanca3, David E. Wedge3, James J. Becnel4, Ikhlas Khan5

Affiliation
1 Faculty of Pharmacy, Beni Suef University, Egypt
2 Faculty of Pharmacy, Cairo University, Egypt
3 U.S. Department of Agriculture, Agricultural Research Service, Natural Products Utilization
Research Unit, University of Mississippi, University, MS, USA
4 USDA, ARS, Center for Medical, Agricultural, and Veterinary Entomology Gainesville, FL,
USA
5 National Center for Natural Product Research, University of Mississippi, University, MS, USA

Correspondence
Ikhlas A. Khan, Ph.D.
National Center for Natural Products Research,
Research Institute of Pharmaceutical Sciences and
Department of Pharmacognosy, School of Pharmacy
The University of Mississippi
University
MS 38677
USA
Tel.: +1-662-915-7821
Fax: +1-662-915-7062
ikhan@olemiss.edu
**General experimental procedures**

HR-ESI-MS data were obtained on an Agilent Series 1100 SL mass spectrometer. NMR spectra were recorded on Varian AS 400 NMR spectrometers. IR spectra were recorded on a Bruker Tensor 27 spectrophotometer. Specific rotations were measured at ambient temperature using a Rudolph Research Analytical Autopol IV automatic polarimeter. Flash column chromatography was performed on a Biotage SP-1 system.

**Extraction and isolation**

The dried, powdered aerial parts of *Z. coccineum* (2 kg) were extracted with 80% methanol (10 × 4 L) and evaporated under reduced pressure to give a residue (300 g). A part (150 g) was dissolved in water and sequentially fractionated with petroleum ether, chloroform, ethyl acetate, and *n*-butanol. The *n*-butanol extract (20 g) was chromatographed over a silica gel column (600 g, 60 × 8 cm) by using EtOAc/CHCl₃/MeOH/H₂O 15:8:4:1 (10 L) and 6:4:4:1 (6.5 L) to yield seven main fractions (A–G). Fraction A (99 mg) was subjected to silica gel (15 g, 1 × 35 cm) column chromatography (CC) and eluted with CHCl₃/MeOH 9:1 (200 mL) to afford 3 (33 mg).

Fraction B (162 mg) was fractioned by using a Sep-Pak cartridge (C18, 10 g) with an MeOH/H₂O gradient [1:9 (50 mL), 3:7 (50 mL), and 9:1 (200 mL)] to yield three fractions (B₁, B₂, and B₃). Fraction B₃ (80 mg, MeOH/H₂O, 9:1) was chromatographed by using silica gel (50 g, 2.5 × 50 cm) CC and eluted with CHCl₃/MeOH/H₂O 4:1:0.1 (250 mL) to obtain 6 (28 mg).

Fraction C (150 mg) was applied to silica gel CC (80 g, 2.5 × 50 cm) using CHCl₃/MeOH/H₂O 4:1:0.1 (2 L) to give 5 (16 mg). Fraction D (300 mg) was chromatographed on a Biotage system [SP-1 (40 + M), C18 column, MeOH/H₂O 3:2 (2 L)] followed by silica gel CC [20 g, 1 × 35 cm, CHCl₃/MeOH/H₂O 4:1:0.1 (150 mL)] to afford 1 (9 mg). Fraction E (2.5 g) was chromatographed on a Biotage system [SP-1 (40 + M), C18 column, MeOH/H₂O 3:7 (1 L), 1:1 (3.5 L)] to obtain E₁ and E₂. Fraction E₁ (370 mg) was further separated by Sephadex LH-20 using MeOH/H₂O 9:1 (700 mL) to give 10 (100 mg). Fraction E₂ (560 mg) was purified on a Biotage system [SP-1 (25 + M), silica gel column, EtOAc/CHCl₃/MeOH/H₂O 15:8:4:1 (1.1 L)] to afford 2 (250 mg) and 4 (35 mg).

Fraction F (900 mg) was submitted to a Biotage system [SP-1 (40 + M), C18 column, MeOH/H₂O/acetic acid 60:40:0.1(2.1 L)] to give F₁ and F₂. Fraction F₁ (100 mg) was applied to silica gel CC [50 g, 2.5 × 50 cm, CHCl₃/MeOH/H₂O 4:1:0.1 (1 L)] to give 9 (33 mg). Fraction F₂ (35 mg) was applied to silica gel CC [20 g, 1 × 35 cm,
CHCl₃/MeOH/H₂O 4:1:0.1 (0.6 L) to afford 7 (18 mg). Fraction G (190 mg) was purified on a Biotage system [SP-1 (40 + M), C18 column, MeOH/H₂O 2:3 (3 L)] to afford 8 (25 mg). The chloroform fraction (500 mg) was chromatographed by using silica gel CC (50 g, 2.5 × 50 cm) and CHCl₃/actone [20:1 (1 L)] to give 11 (10 mg).

**Antifungal assay**

A standardized 96-well microdilution broth assay developed by Wedge and Kuhajek [1] for the discovery of natural fungicidal agents was used to evaluate the antifungal activity of test compounds towards *Botrytis cinerea*, *Colletotrichum acutatum*, *C. fragariae*, *C. gloeosporioides*, *Phomopsis viticola*, *P. obscurans*, and *Fusarium oxysporum*. Each microtiter test well received 80 μL of RPMI 1640 (Roswell Park Memorial Institute mycological broth 1640; Life Technologies) and 3-((N-morpholino)propanesulfonic acid (Sigma Chemical Co.) buffered broth, 100 μL of conidial suspension at 1.0 × 10⁴ conidia/mL, and 20 μL of test compound solution. The commercial fungicide captan was used as an internal fungicide standard in all assays. Each fungus was challenged in a dose–response format using test compounds, with final treatment concentrations of 0.3, 3.0, and 30.0 μM. Microtiter plates (Nunc MicroWell, untreated) were covered with a plastic lid and incubated in a growth chamber at 24 ± 1 °C during a 12-h photoperiod under a light intensity of 60 ± 5 μmol/m²/s. Growth was then evaluated by measuring the absorbance (620 nm) of each well using a microplate reader (SpectraCount; Packard Instrument Company).

Using the 96-well plate micro-bioassay format, each chemical was evaluated in duplicate at three concentrations. Sixteen wells containing broth and inoculum served as positive controls, and eight wells containing solvent at the appropriate concentration and broth without inoculum were used as negative controls. The experiments were repeated three times. Mean absorbance values and standard errors were used to evaluate fungal growth at 48 h and 72 h, except for *P. obscurans* and *P. viticola*, whose data were recorded at 144 h. Analysis of variance of means for percentage inhibition/stimulation of each fungal species at each dose of test compound relative to the untreated positive growth controls was used to evaluate fungal growth. Treatments were arranged as a split-plot design repeated four times. Whole plots were fungal isolates and subplots were chemicals. Each dose level and response time was analyzed separately. The SAS (Statistical
Analysis System) analysis of variance procedure was used to identify significant factors, and Fisher’s protected LSD was used to separate means.

Mosquitoes
The Gainesville strains of *Aedes aegypti* (established in 1952) and *Culex quinquefasciatus* (established in 1995) were reared in the insectary of the Mosquito and Fly Research Unit at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), USDA-ARS, Gainesville, Florida. Standard rearing protocols that have been previously described were followed [2, 3].

Mosquito larvicidal assay
Test samples were diluted in methanol (MeOH), and serial dilutions were performed for each test compound (six concentrations between 8 and 500 ppm). Larvae assays were performed in 24-well plates using five first-instar larvae of *Aedes aegypti* in each well. Each well contained 950 µL of water, 40 µL of larvae food solution, and 10 µL of MeOH (control) or 10 µL of serially diluted test compound. Mortality data were recorded 24 h post-exposure.

Mosquito adult topical assay
To determine the toxicity of each chemical against female *Aedes aegypti* and *Culex quinquefasciatus*, the compound was serially diluted as above and topically applied to individual mosquitoes. Prior to topical application, 5- to 7-day-old females were briefly anaesthetized for 30 seconds with carbon dioxide and placed on a 4 °C chill table. A droplet of 0.5 µL of chemical solution was applied to the dorsal thorax using a 700 series syringe and a PB600 repeating dispenser. A screening dose of 3.125 ppm per female was used on 25–30 females. Tests were replicated three times. Control treatments with 0.5 µL of acetone alone resulted in mortality rates of less than 10%. After treatment, mosquitoes were kept in plastic cups and supplied with 10% sucrose solution for 24 h before mortality was recorded. Temperature and humidity were maintained at 26 °C and 80% RH, respectively.

References
