Supporting Information to:

Piper cubeba Demonstrates Anti-Estrogenic and Anti-Inflammatory Properties

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Cell culture
The human cell lines LNCaP-FGC, MCF-7, MDA and THP-1 were obtained from the American Type Culture Collection. LNCaP cells were cultured with RPMI 1640 medium containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate and 10% FBS. Phenol red free Minimum Essential Medium Eagle (MEM) containing 2 mM L-glutamine, 2.2 g/L sodium bicarbonate and 10% FBS was used to culture the MCF-7 and MDA cells. THP-1 cells were kept in RPMI 1640 medium containing 2 g/L NaHCO₃, 10 mM HEPES, 1 mM Na-Pyruvate, 30 µM mercaptoethanol and 10% FBS. Normal human prostate epithelial cells (PrEC) were purchased from CAMBREX Bio Science Walkersville, Inc. PrEC are primary cells isolated from a patient. They were cultured according to the supplier’s instructions. HL-60 cells (DSMZ) were cultured in complete RPMI 1640 medium supplemented with 10% FBS. 1% (v/v) penicillin/streptomycin solution was added to all cell cultures. The cell lines were kept in a humidified incubator at 37°C and 5% CO₂ and passaged at 70-80% confluency. Cultures used in subsequent experiments were passaged less than 25 times.

Details of the chromatographic analysis of extracts
An aliquot of the fluid extract was analyzed by a Waters HPLC system with UV-VIS detection (280 - 600 nm) using a Nucleosil 120 – 3, C18 (250 × 4.6 mm) column with a pre-column of the same material (both Macherey Nagel) as stationary phase. The mobile phase consisted of two solvent systems {A: 0.1% trifluoroacetic acid in water (v/v) and B: 100% acetonitrile in a gradient (0-30 min 90% A, 30-45 min 50% A, 45-46 min 10% A, 46-50 min 10% A)}. The column temperature was at 40°C and the flow rate was set to 1.0 mL/min. UV detection was carried out at 280 nm and quantification of cubebin in the extract was performed using synthetic cubebin as reference substance. The calculated extract yield of cubebin based on the weight of the de-fatted residue was approximately 8%.

The chromatogram of the extract showed 4 peaks, which were identified to be lignans based on their distinctive UV spectra. One of them was identified as a cubebin peak by comparing its retention time and UV spectra with synthetic cubebin. By quantifying the peak areas, cubebin appears to be the dominant lignan present (roughly 50%). A typical chromatogram is given in the supporting material (Fig. 1S).
Fig. 1S HLPC chromatogram of the lignan peaks present in P9605 extract. The cubebin peak was identified and quantified by comparing it with an external cubebin standard. The other lignans were recognised based on their typical UV spectra (indicated in the boxes).

DNA detection assay

DNA amount was quantified using CyQuant cell proliferation assay kit (Molecular Probes). Cells (5000 cells/well in 96-well plates) were treated with P9605 (3, 10, 30 µg/mL) for 4 days. After incubation with the samples, the medium was discarded and the plates were frozen at -80°C. The CyQuant GR dye used in this assay exhibits strong fluorescence enhancement when bound to DNA or RNA. After thawing the plate, the cells were incubated with 195 µL of CyQuant lysis buffer containing DNA-free RNase (1.35 Kunitz units/mL) for 1 h at room temperature to eliminate the RNA. 5 µL of CyQuant GR dye reagent was then added to every well and incubated for 5 min in the dark. TECAN infinite 200 multifunctional microplate reader (Tecan) was used to measure fluorescence with the excitation wavelength set at 485 nm and the emission wavelength at 530 nm.

The assay was linear over a range of 50 to 50,000 cells under these conditions. The DNA quantities were calculated using a DNA standard curve.

Aromatase activity

P9605 samples (3, 10, 30 µg/mL) and formestane, a synthetic aromatase inhibitor, were incubated with an enzyme/substrate mixture of aromatase, CYP19, (BD Bioscience) and 3H-androstenedione in the presence of a NADPH regenerating system (BD Bioscience). Aromatase catalyses the conversion of androstenedione into estrone, H2O and formaldehyde. The H2O by-product is radioactive. After 15 min incubation at 37°C, the non-metabolised 3H-
androstenedione was extracted with dichloromethane for 5 min. After centrifugation (3000 g, 5 min), the water phase was removed and treated with 2% dextrane coated charcoal for 30 min before centrifugation (4000 g, 10 min). The supernatant was then measured with the scintillating counter. The activity of aromatase per sample was determined by measuring the quantity of radioactive by-product generated and expressed as a percentage of the solvent control.

Fig. 2S Inhibitory action on aromatase by P9605. Formestane, a well-known synthetic inhibitor, acts as a control for the enzymatic assay. Data represent means ± SD of 3 experiments. * p < 0.05 vs. control, ** p < 0.01 vs. control, *** p < 0.001 vs. control.
Fig. 3S Dose-dependent inhibitory effect of P9605 on the activities of COX 1 and 2 (A) *** indicates the statistical significant for both cyclo-oxygenases, 5-LOX (B) and IL-6 production (C). Data represent means ± SD of at least 3 experiments. *** p < 0.001 vs. control.