A Bioactive Polyacetylene Compound Isolated from *Centella asiatica*

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Abstract

A polyacetylene compound was isolated from the aerial parts of *Centella asiatica*. The chemical structure of this new compound was identified as methyl 5-[(*E*)-9-hydroxy-1-(1-hydroxyhexyl)-2-methoxyundeca-3,10-diene-5,7-diynyloxy]pentanoate (cadiyenol). This compound induces apoptosis (63%) independent of cell cycle regimen in mouse lymphoma cells (P388D1) at 28 μM (IC_{50} = 24 ± 2 μM) in 24 hours. The compound also reduces nitric oxide production by 70 ± 2% in lipopolysacharride-activated mouse macrophages at 24 μM with no measurable cytotoxicity.

Supporting information available online at http://www.thieme-connect.de/ejournals/toc/plantamedica

Centella asiatica (L.) Urb., is an evergreen perennial of the Apiaceae family [1], [2], [3]. In the search for novel bioactive compounds, we isolated a highly functionalized polyacetylene from the aerial parts of *C. asiatica*. Here we describe the isolation, characterization and establishment of the bioactivity of this compound, cadiyenol.

HR-MS indicated the molecular formula of cadiyenol to be $C_{24}H_{36}O_6$ (M + H; calculated: 421.2590, found: 421.2213). An FT-IR spectrum showed characteristic absorptions at 3400 cm^{-1} (O-H bond), 2372 and 2344 cm^{-1} (two C ≡ C bonds), and 1738 cm^{-1} (C = O).

The ^{1}H-NMR spectrum of cadiyenol showed the presence of 5 vinyl protons: δ = 6.18 (dd, 1H), 5.9 (ddd, 1H), 5.7 (d, 16.2 Hz, 1H), 5.5 (d, 16.7 Hz, 1H) and 5.3 (d, 9 Hz, 1H), indicative of at least two double bonds; one deshielded oxygenated methine proton at δ = 4.95 (brd, 1H); a singlet at δ = 3.65, possibly due to a methoxycarbonyl group ($COOCH_3$); a multiplet at δ = 3.62; a quartet at δ = 3.6; a singlet at δ = 3.3 (OCH_3); a triplet at δ = 2.3 (possibly a CH_2 next to a carbonyl group); a triplet at δ = 0.9 (CH_3 at the end of an alkyl chain) and several other proton multiplets between δ = 1.25 and 1.9. Notably absent were methyl singlets and doublets characteristic of terpenoid compounds (See Table **1S**, Supporting Information).

A ^{13}C-NMR spectrum showed a carbonyl peak at δ = 176, four sp^2 carbons at δ = 149, 138, 117, and 111 and several other peaks between δ = 83 – 15. Further spectral analysis including HMQC, COSY and UV provided the proposed structure (Fig. **1**): methyl 5-[(*E*)-9-hydroxy-1-(1-hydroxyhexyl)-2-methoxyundeca-3,10-diene-5,7-diynyloxy]pentanoate. Further confirmation of the structure was provided by the HMBC correlations also shown in Fig. **1**. We also note that since the extraction was carried out with methanol containing hydrochloric acid, cadiyenol may not have remained in its natural form in the sample we isolated. Nevertheless, it represents a novel and interesting structure and thus investigation of its biological activity is worthwhile.

The IC_{50} of the compound against P388D1 cell lines was 24 ± 2 μM (SD n = 3) (Fig. **1S** Supporting Information). Cadiyenol was less cytotoxic than camptothecin, which had an IC_{50} of 10 nM on the same cell lines, similar to the reported value 14 nM on the same cell line [4]. The FACS assay indicated the primary mode of cell death was apoptosis, with a very minimal fraction (~1%) undergoing necrosis (Table **2S** Supporting Information). Even when the P388D1 cells were treated with 28 μM (~IC_{80}) of cadiyenol for 24 h, 63% were apoptotic with about 28% remaining normal. The treatment at the same concentration for 12 hours had no significant apoptotic effect. At all concentrations and treatment times, the compound allowed a very low incidence of necrosis and mostly induced apoptosis. Camptothecin (10 nM) was used as a positive control. In the 24 hour experiment, camptothecin caused 12% apoptotic, 20% late apoptotic/early necrotic, 67% normal and about 1% necrotic cells. Furthermore, cell cycle analysis revealed that the cell death with cadiyenol was independent of cell cycle regimen at these concentrations.

Cadiyenol inhibited nitric oxide production in a dose-dependent manner in mouse macrophage cells RAW 264.7 (Fig. **2S** Supporting Information). At 24 μM cadiyenol and 48 h incubation, the nitric oxide production was reduced by 70 ± 2% (SD n = 3). The positive control here was 100 μM *NG*-monomethyl-L-arginine (L-NMMA), to give greater than 85% inhibition at 24 h incubation. Furthermore, the cytotoxicity results (MTT assay) did not show any significant difference between viability of macrophages treated with cadiyenol and LPS, at any of the concentrations tested (0 to 24 μM) (Fig. **3S** Supporting Information).

Materials and Methods

C. asiatica grown in Malaysia was identified and the aerial parts were dried and ground by Dr. Mohd Ilham Adenan, Medicinal Plant Division, Forest Research Institute Malaysia (FRIM) (Kepong, Malaysia). A voucher specimen (FRI 50 032) of this plant was deposited at FRIM.

Approximately 60 grams of this powder were extracted with 0.3 M HCl in methanol by refluxing for 2 h. The water-insoluble compounds in the extract were precipitated by adding distilled water

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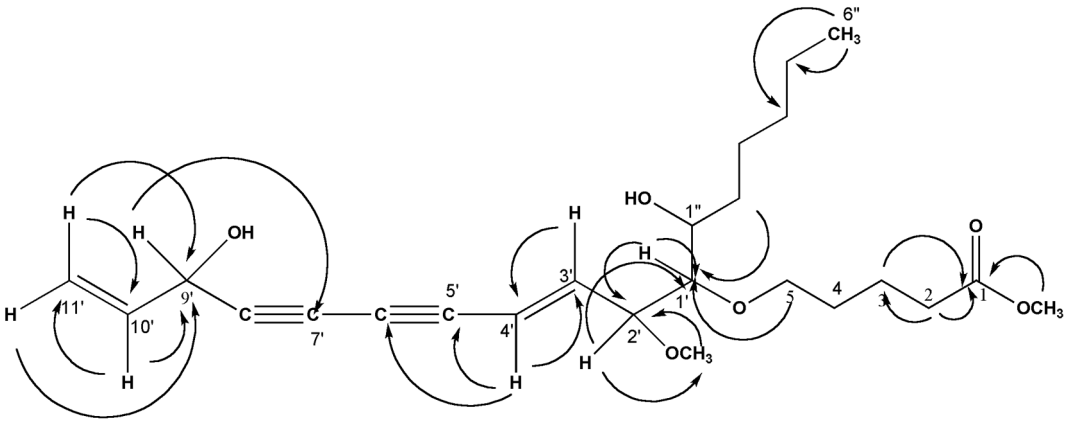

Fig. **1** Structure of cadiyenol and HMBC correlations.

(dry wt. 4.5 g, ~7.5 %). This precipitate was further fractionated by flash chromatography on a silica gel column (20 g/75 mL) starting with hexane and followed by increasing concentrations of ethyl acetate in hexane as eluent. Five fractions of 100 mL were collected. Fraction 5, eluted with hexane/ethyl acetate (80/20 v/v), contained the compound of interest, as indicated by its analytical HPLC profile. This fraction was further purified by preparative HPLC on Platinum EPS, C-18 reversed-phase column (2.5 × 10 cm; 10 μm) using gradient elution with the mobile phases: 0.05 % TFA in water and acetonitrile (Fig. **4 S** Supporting Information). This resulted in the recovery of 10 mg of cadiyenol (0.2 % of the crude extract). The peak of interest was further analyzed for purity by analytical reversed phase HPLC (Supelco Discovery HS C-18 column, 25 cm × 4.6 mm – Sigma Aldrich; St. Louis, MO, USA) using a gradient system using 0.1 % TFA and acetonitrile (R_T = 20 min), which indicated greater than 97 % purity.

Cell viability was assessed by MTT staining [5]. The control was 0.1 % DMSO in media. An appropriate amount of cadiyenol in DMSO was added to cells (cell density 5×10^4 cells per well, 96 cell plate) to give a final concentration of 0.1 % DMSO in the test well. Cell viability was determined by measuring absorbance on a microplate reader (SpectraFluor Plus – Tecan; Zurich, Switzerland) at 540 nm. Three independent experiments were carried out under this procedure, each in duplicate.

Apoptosis was determined by the FACS (fluorescence-activated cell sorting) method, using mouse lymphoma (P388D1) cells [6]. The cell pellet was incubated with 100 μL of dye according to the manufacturer's (Roche; Basel, Switzerland) procedure, and then analyzed within one hour on the flow cytometer (FACScan – Becton Dickinson; Franklin Lakes, NJ, USA).

The cell cycle analysis procedure of Leoni et al. [7] was used with slight modifications. P388D1 cells were maintained in the logarithmic phase of growth in RPMI 1640 supplemented with 10 % fetal bovine serum. The cells were synchronized into the G1/S phase by aphidicolin (3 μg/mL; Sigma), followed by treatment with cadiyenol at 0, 2 and 24 μM. As a positive control, 10 nM camptothecin (Sigma) was used. At each time point (0, 6, 12 and 24 h) cells were harvested, fixed in 70 % ethanol, stained with propidium iodide (50 μg/mL) then analyzed with FACScan (Becton Dickinson).

The ability of cadiyenol to inhibit lipopolysaccharide (LPS) stimulated nitric oxide production in mouse macrophage- RAW 264.7 cells was evaluated as described by Green et al. [8]. The macrophages were seeded at a concentration of 1.5×10^5 cells per mL in a 24-well plate. After 24 h, LPS was added (200 ng/mL) and the macrophages were treated with cadiyenol at 0 to 24 μM. The control vehicle was 1 % methanol. As a positive control L-NMMA (100 μM; Calbiochem; San Diego, CA, USA) was used [9]. Nitric oxide production after 24, 48 and 72 hours was determined by using the Griess reaction. Three experiments, each in quadruplicate, were carried out under this procedure. Cell viability was also determined using MTT assay as previously described.

Copies of the original spectra and biological activity data can be obtained from the corresponding author.

Supporting information

Additional experimental data are available as Supporting Information.

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