Induction of quinone reductase (QR) by withanolides isolated from Physalis angulata L. var. villosa Bonati (Solanaceae)

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A B S T R A C T

In the present study, the EtOAc extract of the persistent calyx of Physalis angulata L. var. villosa Bonati (PA) was tested for its potential quinone reductase (QR) inducing activity with glutathione (GSH) as the substrate using an UPLC–ESI-MS method. The result revealed that the PA had electrophiles that could induce quinone reductase (QR) activity, which might be attributed to the modification of the highly reactive cysteine residues in Keap1. Herein, three new withanolides, compounds 3, 6 and 7, together with four known withanolides, compounds 1, 2, 4 and 5 were isolated from PA extract. Their structures were determined by spectroscopic techniques, including 1H-, 13C-NMR (DEPT), and 2D-NMR (HMBC, HMQC, 1H, 1H-COSY, NOESY) experiments, as well as by HR-MS. All the seven compounds were tested for their QR induction activities towards mouse hepa 1c1c7 cells.

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1. Introduction

Withanolides are a group of C28 steroidal lactones that occur primarily in 12 genera of the plant family Solanaceae [1], which have been studied previously for their antioxidant, anti-inflammatory, antitumor, cytotoxic and immunomodulating activities [2–3]. Physalis angulata L. var. villosa Bonati (PA) is a plant distributed mainly in China and Vietnam. It is the variety of PA with potential pharmaceutical value which usually appears in grass but not currently covered by the artificial cultivation. Ergostane-type steroidal compounds, including physagulins A–G, withangulatin A, and physalins have been isolated from P. angulata [4], but for the natural products in the variety of PA, there has been little reports. Phase II enzymes such as quinone reductase (QR) are primarily responsible for the metabolic detoxification of chemical carcinogens and other harmful oxidants. Therefore, induction of QR is suggestive of cancer prevention at the tumor initiation stage [5]. Research has shown that Michael addition acceptors which have the ability to conjugate with glutathione (GSH) have phase II detoxifying enzyme-inducing and nuclear factor-κB (NF-κB)-inhibiting activities [6]. Therefore, an UPLC–ESI-MS method with GSH as the substrate was used to detect the electrophiles in PA. Finally, seven compounds were isolated from the PA extract and among them five compounds could conjugate well with GSH. QR induction activity was measured for these compounds to evaluate their cancer chemopreventive properties.

2. Experimental

2.1. Materials and methods

An UPLC mass system (Ultra Performance Liquid Chromatography, Waters, Milford, MS, USA) and a BEH (Bridge Ethylidene Hybrid Particles) C18 column (2.1 mm × 100 mm, 1.7 μm) were used to perform the chromatographic analyze with an electrospray (ESI) source in positive mode. 1D- and 2D-NMR spectra were recorded on Bruker UltraShield 400 and 500 MHz spectrometer using DMSO-d6 as solvents with TMS as the internal standard. HR-ESI-MS spectra were obtained using Bruker MicrOTOF mass spectrometer. Preparative HPLC using a Agilent-1200 system with photodiode array detector and Zorbax-C18 column (21.2 mm × 250 mm, 7 μm) were used to purify compounds isolated from preparative MPLC. Preparative MPLC was carried out on a ODS C18 column (25 mm × 500 mm) and photodiode array detector. Jasco P-1030 polarimeter was used to measure the optical rotations of the...
compounds. The IR spectra were recorded on a Bruker Vector 22 spectrometer. Silica gel (100–200 mesh) was purchased from Qingdao Puke Silica Gel Co., Ltd. (Qingdao, China). Ethyl acetate and petroleum ether were purchased from Tianjin Damao Chemical Reagents Co., Ltd (Tianjin, China). GSH was purchased from Sigma–Aldrich (St. Louis, MO, USA). Tris was purchased by Beyotime Biotechnology Co. (Jiangsu, China). Acetonitrile of UPLC grade was purchased from Merck (Darmstadt, Germany). Deionized water was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Plant material

The persistent calyx (1 kg dried weight) was collected from the suburb of Hangzhou city, Zhejiang Province of China in August, 2012. The plant material was identified by Dr. Bo Yang (Zhejiang Chinese Medical University) and deposited at Ocean College, Zhejiang University, China, under the Acquisition Number A20120820.

2.3. UPLC–ESI-MS analysis of the PA reacted with GSH

To analyze, we used UPLC–ESI–MS with GSH as the substrate and we detected electrophiles in PA. The 20 mg ethyl acetate part of PA extract was extracted in methanol (1 mL). After centrifugal separation, 40 μL of supernatant was added to 360 μL of 5 mM GSH dissolved by 25 mM Tris–HCl buffer (pH 8.0). For another 40 μL, GSH was taken out as a control. Then they were both incubated at 37°C for 2 h. The UPLC ionization source parameters were set up as follows: the capillary voltage and extractor voltage were set up at 3.5 kV and 3 V, respectively. The source temperature and desolvation temperature were maintained at 120°C and 350°C, respectively. The velocity of cone gas and desolvation gas were 50 L h⁻¹ and 600 L h⁻¹, respectively and both of them were using nitrogen. Collision-induced dissociation was performed at a pressure of 4 × 10⁻⁹ mbar using argon as collision gas in the collision cell. In the whole UPLC system, A (H₂O) and B (CH₃CN) were used as the mobile phase. For PA, a gradient program was applied according to the following method: 0–1.5 min, 30% B, 1.7–6.5 min, 40% B, 7–9.2 min 50% B and maintained 30% B during the next 0.8 min. The injection volume was 1 μL with the flow rate of 0.3 mL/min, and the column and sample temperature were maintained at 35°C and 25°C, respectively.

2.4. Extraction and isolation

Dried persistent calyx (1 kg) was extracted with 95% EtOH for four times (5 L × 4) under reflux, and 130 g of extract was obtained. The extract was suspended in water (1 L) and then extracted successively with petroleum ether (1 L × 3) and ethyl acetate (1 L × 3). The ethyl acetate part (20 g) was subjected to silica gel column chromatography (5 cm × 100 cm, 300 g silica gel) and eluted with a gradient of petroleum ether/ethyl acetate (10:1; 5:1; 2:1; 1:1; 1:2; 1:3; 0:1, v/v) to give 13 fractions (Fr. A–M). The volumes of eluting solvents for each gradient were 4.8 L. Fr. M (1.4 g of 20 g) was separated by preparative MPLC directly using CH₃OH–H₂O as the mobile phase (65% maintained for 40 min, flow rate 20 mL/min) to obtain compounds 1 (6 mg, tₑ= 25.0 min), 2 (6 mg, tₑ= 36.0 min). Fr. K (1.8 g of 20 g) was also subjected to preparative MPLC using the same method as Fr. M to obtain compounds 3 (15 mg, tₑ= 13.0 min) [7], 4 (8 mg, tₑ= 22.0 min), 5 (20 mg, tₑ= 27.0 min). Fr. J (1.6 g of 20 g) was separated by preparative MPLC (70% CH₃OH–H₂O maintained for 20 min, flow rate 20 mL/min) to afford two fractions H₁ (500 mg, tₑ= 8.0 min) and H₂ (800 mg, tₑ= 16.0 min). Then Fr. H₁ was subjected to preparative HPLC (65% CH₃OH–H₂O maintained for 30 min, flow rate 10 mL/min) to obtain compound 6 (14 mg, tₑ= 27.0 min) [8]. Fr. I (2.0 g of 20 g) was first separated by preparative MPLC using a 50 min gradient from 35% methanol to 75% methanol (flow rate 10 mL/min) to afford five fractions (I₁–I₅). Then Fr. I₅ was subjected to preparative HPLC (75% CH₃OH–H₂O maintained for 30 min, flow rate 10 mL/min) to obtain compound 7 (20 mg, tₑ= 17.0 min) [7].

2.4.1. Physagulin P (3)

Light yellow, amorphous powder (CH₃OH): [α]D +33.6° (c = 0.05, CH₃OH); IR (neat) νmax 3495, 1686, 1522 cm⁻¹; HR-ESIMS [M–H₂O + Na]⁺ at m/z 567.2579 (calcld. for C₃₀H₄₂NaO₁₀, 567.2570). ¹H and ¹³C NMR see Table 1.

2.4.2. Physagulin Q (6)

White, amorphous powder (CH₃OH): [α]D +52.3° (c = 0.05, CH₃OH); IR (neat) νmax 3492, 1741, 1689 cm⁻¹; HR-ESIMS [M+N]+ at m/z 569.2292 (calcld. for C₃₀H₃₉ClNaO₇, 569.2282). ¹H and ¹³C NMR see Table 1.

2.4.3. 14-Epi-physagulin P (7)

White, amorphous powder (CH₃OH): [α]D +52.3° (c = 0.05, CH₃OH); IR (neat) νmax 3492, 1741, 1689 cm⁻¹; HR-ESIMS [M+N]+ at m/z 585.2234 (calcld. for C₃₀H₄₂NaO₁₀, 585.2276). ¹H and ¹³C NMR see Table 1.

2.5. Cell culture and assay of QR induction

For the evaluation of pure isolates as inducers of QR, cultured mouse hepa 1c1c7 cells (obtained from ATCC) were used as described previously [9,10]. The cells were maintained in α-minimum essential medium supplemented with 0.1% penicillin–streptomycin and 10% fetal bovine serum (GIBCO, NY, USA). Then 96-well plates were seeded at a density of 2 × 10⁴ cells/mL (100 μL/well) and incubated for 24 h at 37°C in a 5% CO₂ incubator. After that, the test compounds were added, and the cells were incubated for an additional 48 h. After the medium was decanted, the cells were incubated with 50 μL of 0.8% digitonin and 2 mM ethylene diamine tetraacetic acid (EDTA) solution (pH 7.8) for 10 min at 37°C. Then, 200 μL of a mixed solution containing bovine serum albumin (0.67 mg/mL), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.72 mM), 1.5% Tween 20, 0.5 M Tris–HCl, 5 mM FAD, 150 mM glucose-6-phosphate, 2 units/mL glucose-6-phosphate dehydrogenase, 50 mM NADP, and 50 mM menadione were added into each well. After incubation for 3 min, the plates were scanned at 550 nm. Stock solutions of the compounds and PA were dissolved in DMSO, and the final concentration of DMSO was kept at 0.5% (v/v). 4′-Bromoflavone in DMSO and DMSO were used as the positive and negative control respectively, both with the final concentrations of 0.5% (v/v). The cytotoxicity of hepa 1c1c7 cells and the isolated compounds were determined by a crystal violet assay as previously described [6]. Experiments were carried out three times on separate occasions.

3. Results and discussion

3.1. UPLC–ESI-MS analysis of PA conjugated with GSH

From Fig. 1 we can see that 11 peaks were weakened and two peaks disappeared by comparing UPLC-DAD chromatograms of PA before and after incubating with GSH, which suggested that these compounds might represent electrophiles with QR inducing activity. Among the seven compounds isolated from PA extract, two new compounds 6 and 7 and three known compounds 2, 4 and 5 conjugated well with GSH (Fig. 2). The GSH conjugates were identified clearly from the total ion current chromatograms in positive ion mode, with the fragments 6*: [546 + GSH + H]+, 7*
Table 1

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\( ^{1}H \) and \( ^{13}C \) NMR spectra of compound 3 were obtained at 400 and 100 MHz, compounds 6 and 7 were obtained at 500 and 125 MHz, all assignments were based on DEPT, \( ^{1}H–^{1}H \) COSY, HSQC, HMBC and NOESY spectra in DMSO-d6.

Fig. 1. UPLC-DAD chromatograms of the PA before (green) and after (red) incubating with GSH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Three new compounds 3, 6 and 7 together with four known compounds 1, 2, 4 and 5 were isolated from the persistent calyx of PA (Fig. 3). The structures were determined by analysis of their spectroscopic data, including DEPT, $^1$H–$^1$H COSY, HSQC, HMBC and NOESY NMR spectra, and comparison with the literature data (Fig. 4).

Compound 1, obtained as colorless needles, was named as physagulin K. It had the molecular formula C$_{30}$H$_{42}$O$_9$, with mp 190–193 °C and $[a]_D^27 + 92.8$ (c = 2.00, MeOH) [11].

Compound 2, obtained as a solid, was named as physagulin J. It had the molecular formula C$_{30}$H$_{42}$O$_8$, with $[a]_D^27 + 30.9$ (c = 1.15, MeOH) [11].

Compound 4, obtained as a colorless powder, was named as physagulin N. It had the molecular formula C$_{31}$H$_{44}$O$_9$, with $[a]_D^27 + 100.5$ (c = 0.20, MeOH) [12].

Compound 5, obtained as a homogeneous amorphous powder, was named as withamininin. It had the molecular formula C$_{30}$H$_{40}$O$_8$, with mp 208 °C and CD (MeOH): $\Delta e_{332} = 0.08$, $\Delta e_{252} = 0.22$ [13].

All of their NMR spectra are shown in supporting information (Table S1).

Compound 3 was obtained as a light yellow, amorphous powder (CH$_3$OH) with a molecular formula of C$_{30}$H$_{42}$O$_{10}$ by HR-ESIMS. The IR absorption peaks were indicative of hydroxyl (3495 cm$^{-1}$), ester (1686 cm$^{-1}$) and $\alpha,\beta$-unsaturated ketone group (1522 cm$^{-1}$).

Inspection of the 1D and 2D NMR data revealed that compound 3 possessed the structure of a withanolide [14]. Furthermore, compound 3 was found to have different functional groups at ring D from the known compound 1. One more hydroxyl group was

[562–2H$_2$O + GSH + H]$^+$, 2'[530 + GSH + H]$^+$, 4'[544–2H$_2$O + GSH + H]$^+$, as shown in Fig. 2.

3.2. Phytochemical investigation

Three new compounds 3, 6 and 7 before (green) and after (red) incubating with GSH. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
observed in addition to the hydroxyl groups at C-5, C-6, C-14 and C-17 ($\delta$ 75.6, $\delta$ 72.9, $\delta$ 75.8 and $\delta$ 81.2 ppm, respectively) when the NMR data were compared with those of compound 1. The HMBC correlations from $\delta$ 3.50 (s) attributed to the carbon signal at $\delta$ 58.4 to $\delta$ 46.1 (C-13), 76.3 (C-15), and 81.2 (C-17) indicated the hydroxyl group to be at C-16. The orientation of the hydroxyl group was deduced to be $\alpha$ based on an NOE between H-16 signal at $\delta$ 3.50 (s) and H-18 signal at $\delta$ 1.06 (s). Structure is shown in Fig. 3 and the compound was named physagulin P.

Compound 6 was obtained as a white, amorphous powder (CH$_3$OH) with a molecular formula of C$_{30}$H$_{39}$ClO$_7$ by HR-ESIMS. The IR absorption peaks were indicative of hydroxyl (3484 cm$^{-1}$), ester (1735 cm$^{-1}$) and $\alpha$, $\beta$-unsaturated ketone group (1677 cm$^{-1}$). The 3:1 isotopic peak observed in HR-ESIMS spectrum confirmed that there was a chlorine atom in the structure. Inspection of the 1D and 2D NMR data revealed that the functional groups of compound 6 at ring B were different from those of compound 5. More concretely, the substituent group at C-6 changed from hydroxyl to chloro compared with that of compound 5 and the $^{13}$C signal at C-6 shifted from $\delta$ 73.0 to $\delta$ 66.8 as a consequence [15]. In the NOESY spectrum, cross peak of $\delta$ 4.30 (m, H-6) with $\delta$ 1.07 (s, H-19) indicated that the orientation of the chlorine group to be $\alpha$. Furthermore, the orientation of the hydroxyl group at C-5 was deduced to be $\beta$ which was also different from that of compound 5, because the $^{13}$C signal of C-19 methyl group at $\delta$ 7.4 explained the cis relationship between rings A and B. Structure was shown in Fig. 3 and the compound was named physagulin Q.

Compound 7 was obtained as a white, amorphous powder (CH$_3$OH) with a molecular formula of C$_{30}$H$_{42}$O$_{10}$ by HR-ESIMS. In the IR spectrum of 7, absorption bands appeared at hydroxyl (3492 cm$^{-1}$), ester (1741 cm$^{-1}$) and $\alpha$, $\beta$-unsaturated ketone group (1689 cm$^{-1}$). All the spectra data revealed that the structure of compound 7 was very similar to that of compound 3 except the carbon signals at C-14. For compound 3, the $^{13}$C signal at C-14 was $\delta$ 75.8 while it remained $\delta$ 82.9 in compound 7, which indicated that these two compounds were epimers at C-14. According to the literature, 15$\alpha$-AcO/14$\beta$-OH substitution generally gives rise to $^{13}$C NMR chemical shifts for C-14 of 76–80 ppm, whereas the 15$\alpha$-AcO/14$\alpha$-OH form exhibits $\delta$ (C) values of 83–84 ppm [16]. So the orientations of the hydroxyl groups at C-14 in compounds 3 and 7 were possibly deduced to be $\beta$ ($\delta$ 75.8) and $\alpha$ ($\delta$ 82.9) respectively, but they still couldn’t be confirmed only by the description of the reference. Meanwhile, the HMBC correlations from $\delta$ 3.52 (s) attributed to the carbon signal at $\delta$ 58.4–$\delta$ 46.1 (C-13), $\delta$ 76.5 (C-15), and $\delta$ 80.7 (C-17) indicated that there was also a hydroxyl group at C-16. The orientation of the hydroxyl group was deduced to be $\alpha$ based on an NOE between H-16 signal at $\delta$ 3.52 (s) and H-18 signal at $\delta$ 1.07 (s). Structure was shown in Fig. 3 and the compound was named 14-epi-physagulin P.

**Fig. 3.** Structures of compounds 1–7.

1 R= $\beta$-OH  
2 R= $\alpha$-H  
3 physagulin P  
4  
5 $R_1=$ $\alpha$-OH $\beta$-OH  
6 $R_2=$ $\beta$-OH $\alpha$-Cl  
7 14-epi-physagulin P
3.3. QR induction activities of isolated withanolides

Since the chemical composition in PA extract has the ability to conjugate with GSH, we measured the QR induction activity of withanolides from PA towards mouse hepa 1c1c7 cells. Compounds 2 and 7 showed potent QR induction activities with IR value of 2.74 ± 0.01 and 2.85 ± 0.02, respectively. Compounds 1, 3 and 5 exhibited moderate QR induction activities in this assay. Although compounds 4 and 6 conjugated well with GSH, their QR induction activities were very weak (Table 2). A possible reason was that the solubility of both compounds were very weak in the cell culture medium. Compound 4 had nearly no hydrophilic groups in the structure so that the nonpolar compound could not dissolve well in the cell culture medium. For compound 6, a chlorine atom was observed in the structure so that its solubility in the cell culture medium was also very weak.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.04.015.

References


