HHS Public Access

Author manuscript

Phytochem Lett. Author manuscript; available in PMC 2018 June 01.

Published in final edited form as:

Phytochem Lett. 2017 June ; 20: 54–60. doi:10.1016/j.phytol.2017.03.012.

Secondary Metabolites from the Leaves of the Medicinal Plant Goldenseal (*Hydrastis canadensis*)

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Abstract

The study presented herein constitutes an extensive investigation of constituents in *Hydrastis canadensis* L. (Ranunculaceae) leaves. It describes the isolation and identification of two previously unknown compounds, 3,4-dimethoxy-2-(methoxycarbonyl)benzoic acid (**1**) and 3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-*C*-dimethyl-flavone (**2**), along with the known compounds (±)-chilenine (**3**), (2*R*)-5,4′-dihydroxy-6-*C*-methyl-7-methoxy-flavanone (**4**), 5,4′-dihydroxy-6,8-di-*C*-methyl-7-methoxy-flavanone (**5**), noroxyhydrastinine (**6**), oxyhydrastinine (**7**) and 4′,5′-dimethoxy-4-methyl-3′-oxo-(1,2,5,6-tetrahydro-4*H*-1,3-dioxolo-[4′,5′:4,5]-benzo[1,2-*e*]-1,2-oxazocin)-2-spiro-1′-phtalan (**8**). Compounds **3**-**8** have been reported from other sources, but this is the first report of their presence in *H. canadensis* extracts. A mass spectrometry based assay was employed to demonstrate bacterial efflux pump inhibitory activity against *Staphylococcus aureus* for **2**, with an IC_{50} value of 180 ± 6 μM. This activity in addition to that of other bioactive compounds such as flavonoids and alkaloids, may explain the purported efficacy of *H. canadensis* for treatment of bacterial infections. Finally, this report includes high mass accuracy fragmentation spectra for all compounds investigated herein which were uploaded into the Global Natural Products Social molecular networking library and can be used to facilitate their future identification in *H. canadensis* or other botanicals.

Keywords

Hydrastis canadensis; goldenseal; alkaloids; flavonoids; efflux pump inhibitors

1. Introduction

The medicinal plant *Hydrastis canadensis* L. (Ranunculaceae) has a long history of use for the treatment of infections. Native Americans, particularly the Cherokee, used goldenseal roots to treat skin and eye infections, while other populations have used goldenseal tonics to treat gastrointestinal irritation (Foster et al., 2000). *H. canadensis* roots have been extensively profiled (Le et al., 2013; Bharathi et al., 2012; McNamara et al., 2004), although only a few reports have described the composition of *H. canadensis* leaves (Junio et al.,

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2011; Douglas et al., 2010). *H. canadensis* has been of recent interest due to its ability to inhibit the growth of pathogenic bacteria, including *Staphylococcus aureus* (Cech et al., 2012). This activity was originally attributed to the antimicrobial alkaloid berberine and to other alkaloids that the plant contains (Knight, 1999; Scazzocchino et al., 2001; Hwang et al., 2003). Recently, it has been shown that the activity of *H. canadensis* leaves is more complex. Three flavonoids, sideroxylin, 6-desmethyl sideroxylin and 8-desmethyl sideroxylin (Junio et al., 2011) were shown to synergistically enhance the antimicrobial activity of goldenseal alkaloids. These flavonoids act as bacterial efflux pump inhibitors, facilitating accumulation of berberine within bacterial cells and thereby reducing the necessary quantity of berberine (or other alkaloids) to achieve antimicrobial activity (Junio et al., 2011).

Botanicals are chemically complex and contain many compounds that may possess diverse structures and biological activities. On the basis of the previously reported interesting biological activity of *H. canadensis* leaves, we endeavored to conduct more in-depth chemical profiling of this botanical. With these studies, we sought to identify efflux pump inhibitors from *H. canadensis*, and to generate a more comprehensive profile of chemical compounds in this botanical than has previously been published.

2. Results and Discussion

2.1. Structures of isolated compounds

Investigation of *Hydrastis canadensis* leaves led to isolation of two new compounds, 3,4-dimethoxy-2-(methoxycarbonyl)benzoic acid (**1**) and 3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-*C*-dimethyl-flavone (**2**), together with six additional compounds (**3-8**) that are known but new to *Hydrastis canadensis* (Fig. 1). (±)-Chilenine (**3**), an isoindolobenzazepine alkaloid, was previously reported from *Berberis enpetrifolia* (Fajardo et al., 1982); flavonones (2*R*)-5,4′-dihydroxy-6-*C*-methyl-7-methoxy-flavanone (**4**) and 5,4′-dihydroxy-6,8-di-*C*-methyl-7-methoxy-flavanone (**5**) were isolated from leaf wax of *Callistemon coccineus* (Wollenweber et al., 2000); and the isoquinolone derivatives noroxyhydrastinine (**6**) and oxyhydrastinine (**7**) were obtained from *Thalictrum minus* and *Hypecoum erectum*, respectively (Doskotch et al., 1969; Zhang et al., 1995). Compound **8**, 4′,5′-dimethoxy-4-methyl-3′-oxo-(1,2,5,6-tetrahydro-4*H*-1,3-dioxolo-[4′,5′:4,5]-benzo[1,2-*e*]-1,2-oxazocin)-2-spiro-1′-phtalan, was reported previously as a product of β-hydrastine-N-oxide under reflux conditions (Klötzer and Oberhänsli, 1973). Given that β-hydrastine is an abundant constituent of *H. canadensis* (Le et al., 2014), it is possible that compound **8** is an isolation artifact and not a constituent of *H. canadensis*. Additionally, nine compounds known to be constituents of *H. canadensis* were also isolated. These include berberine (**9**) (Qiu et al., 2008), (−)-canadine (**10**) (Malhotra et al., 1989), sideroxylin (**11**), 6-desmethyl-sideroxylin (**12**), 8-desmethyl-sideroxylin (**13**) (Junio et al., 2011), β-hydrastine (**14**) (Seger et al., 2004), (−)-8-oxocanadine (**15**), 8-oxotetrahydrothalifendine (**16**) (Pinho et al., 1992), and oxyberberine (**17**) (Singh et al., 2010). The structures of these known compounds were determined by comparing their spectroscopic data with those reported in the literature.

Compound **1** was obtained as white amorphous powder. High resolving power electrospray ionization mass spectrometry (HRESIMS) analysis indicated an ion at *m/z* 241.0702 $[M+H]^+$ (calcd for $C_{11}H_{13}O_6^+$, 241.0707), suggesting six degrees of unsaturation. The NMR spectral data (Table 1) allowed the assignment of two aromatic protons (δ_H = 6.98 and 7.87) and three methoxy groups (δ_H = 3.87, 3.94 and 3.95). The HMBC analysis allowed correlation of two of the methoxy groups, (δ_H = 3.87 and 3.95) with carbon C-3 and C-4 (δ_C = 145.9 and 157.4), respectively. Additionally, the correlation between H-5 (δ_H = 6.98) and the resonance at δ_C = 145.9 (C-3), and between H-6 (δ_H = 7.87) with δ_C = 157.4 (C-4) supported the placement of the methoxy groups. The HMBC correlations between H-5 (δ_H = 6.98) and δ_C = 118.7, and H-6 (δ_H = 7.87) with δ_C = 131.8 supported the assignment of the carboxylic group at C-1 and the methoxycarbonyl group at C-2 (Fig. 2). Therefore, the structure of **1** was established as 3,4-dimethoxy-2-(methoxycarbonyl)benzoic acid.

Compound **2**, obtained as a yellow amorphous powder, showed in HRESIMS an ion at *m/z* 359.1129 $[M+H]^+$ (calcd for $C_{19}H_{19}O_7$, 359.1125). The UV maxima absorption bands at λ_{max} 377, 346 and 258 nm were suggestive of a flavone skeleton, given that the absorbance maxima of flavonols are generally at longer wavelengths (350–385 nm) (Tsimogiannis et al., 2007). The ^{1}H-NMR data (Table 2) indicated the presence of two aromatic methoxy groups (δ_H = 3.80 and 4.00), two C-methyls (δ_H = 2.41 and 2.23), and three aromatic protons (δ_H = 7.87, 7.82 and 7.00). Analysis of the ^{13}C-NMR spectrum (Table 2) showed the presence of a α,β-unsaturated carbonyl (δ_C = 175.9) and a signal at δ_C = 136.2, which together with the proton signals of the aromatic rings corresponding to a flavonol skeleton. HMBC correlations of 7-OCH_3 (δ_H = 3.80), 6-CH_3 (δ_H = 2.23) and 8-CH_3 (δ_H = 2.41) methyl protons with C-7 (δ_C = 163.0) support the position of the substituents in ring A (Fig. 3). In addition, the methoxy and hydroxy group in the B ring were assigned based on the correlation between 4′-OCH_3 (δ_H = 4.00) with the C-4′ (δ_C = 148.4), and the correlations between 5′-H (δ_H = 7.00) with C-1′ (δ_C = 124.6) and C-3′ (δ_C = 145.8) in the HMBC spectrum. Additionally, the HMBC correlations between 2′-H (δ_H = 7.82) and 6′-H (δ_H = 7.87) with C-2 (δ_C = 145.8) supported the connectivity of ring B to C-2 (ring C) (Fig. S10). On the basis of this evidence, the compound was determined to be 3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-*C*-dimethyl-flavone.

Reisolation of known compounds from botanical extracts is a common problem when seeking to identify novel compounds from botanicals such as *H. canadensis*. To facilitate future identification of compounds **1**–**17** in botanical mixtures, tandem high resolving power electrospray ionization mass spectrometry (HRESIMS-MS) was employed to collect fragmentation spectra of all seventeen compounds in both the positive ion mode (Table S1) and the negative ion mode (Table S2). Notably, these fragmentation spectra were collected with high mass accuracy (<10 ppm), enabling the confirmation of molecular formulae of many of the fragments. An example of such a high resolution fragment spectrum is provided in Fig. 4 for **2**. High mass accuracy measurements of fragmentation data enable assignment of molecular formulae not just for the intact molecule, but also for its fragments. The fragments with *m/z* of 344.0890, 329.0654, and 316.0941 represent rearrangements and losses from the C ring, and those with *m/z* of 301.0708 and 259.0965 represent a partial loss of the C ring with bonds formed with the hydroxyl group at carbon 3. The fragments with

m/z of 195.0653 and 179.0347 represent the remaining A ring along with the ketone at carbon 4. An additional fragmentation spectrum of berberine (**9**), the most abundant alkaloid present in goldenseal (Le et al., 2014) can be found as Supporting Information (Fig. S26). Spectra for **2,** berberine, and the remaining *H. canadensis* compounds identified herein were uploaded into the Global Natural Product Social molecular networking library to facilitate identification of these compounds or their structural analogues by other researchers (Wang et al., 2016). Fragment masses can also be found in Tables 1S and 2S.

2.2. Efflux pump inhibitory activity of isolated compounds

The two new compounds isolated as part of this study [3,4-dimethoxy-2-(methoxycarbonyl)benzoic acid (**1**) and 3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-*C*-dimethyl-flavone (**2**)], as well as the compounds **4** and **16** were tested for biological activity. Specifically, a mass spectrometry based assay was employed to evaluate the ability of these compounds to inhibit efflux of an efflux pump substrate (ethidium bromide) from *Staphylococcus aureus* cells (Brown et al., 2015). The rationale for evaluating efflux pump inhibitory activity is that drug efflux constitutes a major form of antibiotic resistance in bacteria (Kaatz, 2005). Thus, compounds that prevent efflux of toxins from cells have the potential of contributing to antimicrobial activity. Efflux inhibitory activity against *Staphylococcus aureus* is particularly relevant given that this pathogen is responsible for approximately 50% of all skin infections, and *Hydrastis canadensis* is traditionally used in the treatment of such infections (McCaig et al., 2006).

Compound **2** demonstrated moderate inhibitory activity of efflux from *Staphylococcus aureus* with an IC_{50} value of 180 ± 6 μMCompounds **1**, **4** and **16** were inactive. Fig. 5 shows the raw experimental data evaluating the efflux pump inhibitory activities of compounds **1**, **2**, and the positive control, carbonyl cyanide *m*-chloro phenylhydrazone (CCCP), which had an IC_{50} of 270 ± 50 μM. The data shown are relative quantities (as measured by mass spectrometric peak area) of ethidium ion in the spent bacterial media after exposure to an increasing amount of the test compound or control. As demonstrated for both CCCP and **2**, when efflux is blocked, the quantity of ethidium present in the media decreases (Fig. 5). Ethidium concentration remains high regardless of concentration for the compound that does not possess efflux inhibitory activity (Compounds **1**, **4** & **16**).

4. Experimental

4.1. General experimental procedures

Optical rotations at the sodium D-line wavelength of pure compounds were measured with a Rudolph Research Autopol (II) Polarimeter. 1D and 2D NMR spectra were recorded using a JEOL ECS-400 NMR spectrometer equipped with a high sensitivity JEOL Royal probe operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C, or an Agilent 700 MHz NMR spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a cryoprope, operating at 700 MHz for ^{1}H and 175 MHz for ^{13}C. Chemical shifts are reported as δ values (ppm), and coupling constants (*J*) were measured in Hz. HRESIMS was performed on a Thermo LTQ Orbitrap XL mass spectrometer equipped with an electrospray ionization source. HPLC was carried out using a Varian ProStar HPLC system equipped

with ProStar 210 pumps and a ProStar 335 photodiode array detector (PDA), with data collected and analyzed using Galaxie Chromatography Workstation software (version 1.9.3.2). For preparative HPLC, a Phenomenex Gemini-NX C_{18} Column (5 μm; 250 × 21.2 mm) was used at a 21 mL/min flow rate. Flash chromatography was performed on a Teledyne ISCO CombiFlash® R_f using 80 g or 120 g RediSep® RF Silica Column (35 – 70 μm particle size) and 12 g RediSep® R_f Gold HP Silica Columns (20 – 40 μm particle size, Teledyne ISCO, Lincoln, NE, USA); and monitored by UV and evaporative light-scattering detectors. UV spectra were measured with a ProStar 335 photodiode array UV detector (PDA) and the reported λmax values were collected from the spectra for relevant compounds eluting from the HPLC. All other reagents and solvents were obtained from Fisher Scientific and were used without further purification.

4.2. Plant material

Hydrastis canadensis L. (Ranunculaceae) was collected in Hendersonville, North Carolina (NC, N 3524.2770, W 08220.9930, 702.4 m elevation), in July 2013. The plants were cultivated in their native environment, a hardwood forest understory. A voucher specimen (NCU583414) was deposited in the University of North Carolina Herbarium, Chapel Hill, NC and the identity was verified by herbarium director Dr. Alan S. Weakley.

4.3. Extraction and isolation

The isolation scheme is provided as Supporting Information (Fig. S1). Batches of dried *H. canadensis* plant were pulverized into fine powder using a commercial coffee grinder (Kitchen Aid). *H. canadensis* powder was percolated in MeOH overnight, and the MeOH extract was concentrated *in vacuo* and subjected to liquid-liquid partition, as described previously (Junio et al., 2011). This concentrated extract was defatted by partitioning between 10% aqueous MeOH and hexane (1:1), and the aqueous MeOH fraction was partitioned further between EtOAc:MeOH:H_2O (4:1:5). The organic layer was washed with 1% saline solution to remove tannins.

The first stage of normal-phase flash chromatography (120 g silica gel column) was conducted with a Hex/$CHCl_3$/MeOH gradient, yielding 8 primary fractions (FI-FVIII). Fraction FII, FIII and FIV were subjected to a second stage of normal-phase flash chromatography (80 g silica gel column) with a Hex/EtOAc/MeOH gradient to give 5 (FII1-FII5), 3 (FIII1-FIII3) and 6 (FIV1-FIV6) subfractions, respectively.

The compounds were purified using reversed-phase preparative HPLC with a Phenomenex Gemini-NX C_{18} column at a 21 mL/min flow rate. Fraction FII2 (32.5 mg) (eluents A: H_2O 0.1% formic acid, B: CH_3CN, gradient: B 45% at time 0, B 75% at time 20 min, B 100% at time 25 min) yielded the compounds (2*R*)-5,4′-dihydroxy-6-*C*-methyl-7-methoxy-flavanone (**4**; 1.5 mg), 5,4′-dihydroxy-6,8-di-*C*-methyl-7-methoxy-flavanone (**5**; 0.7 mg), and *3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-C-dimethyl-flavone* (**2**; 0.6 mg).

Fractions FII3 (30.1 mg), FII4 (7.3 mg) and FIII1 (26.9 mg) were purified (eluents A: H_2O 0.1% formic acid, B: CH_3CN, gradient: B 35% at time 0, B 90% at time 27 min, B 100% at time 30 min) to obtain oxyhydrastinine (**7**; 2.0 mg), chilenine (**3**; 0.8 mg) for the first

fraction, noroxyhydrastinine (**6**; 0.9 mg) for the second fraction, and (2*R*)-5,4′-dihydroxy-6-*C*-methyl-7-methoxy-flavanone (**4**; 0.5 mg) for the last fraction.

Fraction FIV3 (96.3 mg) (eluents A: H_2O 0.1% formic acid, B: CH_3CN, gradient: B 30% at time 0, B 90% at time 20 min, B 100% at time 22 min) and fraction FIV5 (42.3 mg) (eluents A: H_2O 0.1% formic acid, B: CH_3CN, gradient: B 30% at time 0, B 65% at time 20 min, B 100% at time 22 min) yielded chilenine (**3**; 1.0 mg) and 3,4-dimethoxy-2-(methoxycarbonyl) benzoic acid (**1**; 1.2 mg), respectively.

3,4-dimethoxy-2-(methoxycarbonyl)benzoic acid (**1**): white powder; UV λmax 208 and 259 nm; ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) data see Table 1. HRESIMS *m*/*z* 241.07021 $[M+H]^+$ (calcd for $C_{11}H_{13}O_6$ 241.0707).

3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-C-dimethyl-flavone (**2**): yellow powder; UV λmax 217, 258, 346 and 377 nm; ^{1}H (700 MHz) and ^{13}C NMR (175 MHz) data see Table 2. HRESIMS *m*/*z* 359.11287 $[M+H]^+$ (calcd for $C_{19}H_{19}O_7$ 359.1125).

(±)-chilenine (**3**): white powder; $[\alpha]_D^{25} = 0.0$ (c 0.22, MeOH); UV λmax 215 and 318 nm; ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 7.36 (1H, d, *J* = 8.4 Hz, H-11), 7.05 (1H, d, *J* = 8.4 Hz, H-12), 6.71 (1H, s, H-1), 6.66 (1H, s, H-4), 5.95 (2H, dd, *J* = 7.2, 1.2 Hz, OCH_2O), 4.26 (1H, m, H-6), 3.99 (3H, s, OCH_3-9), 3.87 (3H, s, OCH_3-10), 3.56 (1H, m, H-5), 3.30 (1H, m, H-6), 3.11 (1H, m, H-5); ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 199.8 (C-14), 166.7 (C-8), 154.5 (C-10), 151.6 (C-3), 147.1 (C-2), 146.7 (C-9), 133.7 (C-4a), 131.7 (C-12a), 130.7 (C-14a), 124.1 (C-9a), 120.6 (C-12), 116.3 (C-11), 109.4 (C-4), 109.2 (C-1), 101.9 (OCH_2O), 94.9 (C-13), 62.6 (OCH_3-9), 56.6 (OCH_3-10), 38.9 (C-6), 30.7 (C-5); HRESIMS *m*/*z* 384.10751 $[M+H]^+$ (calcd for $C_{20}H_{18}NO_7$ 384.1078).

(2R)-5,4′-dihydroxy-6-C-methyl-7-methoxy-flavanone (**4**): yellow oil; $[\alpha]_D^{25} = -7.33$ (c 0.3, MeOH); UV λmax 216, 291 and 338 nm; ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 12.1 (OH-5), 7.35 (2H, d, *J* = 8.4 Hz, H-2′ and H-6′), 6.89 (1H, d, *J* = 8.4 Hz, H-3′ and H-5′), 6.07 (1H, s, H-8), 5.35 (1H, dd, *J* = 13.2, 2.8, H-2), 3.83 (3H, s, OCH_3-7), 3.09 (1H, dd, *J* = 17.2, 13.2 Hz, H-3), 2.77 (1H, dd, *J* = 17.2, 2.8 Hz, H-3), 2.01 (1H, s, CH_3-6); ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 196.2 (C-4), 165.8 (C-7), 161.3 (C-9), 160.5 (C-5), 156.2 (C-4′), 130.9 (C-1′), 128.1 (C-2′ and C-6′), 115.8 (C-3′ and C-5′), 106.1 (C-6), 102.9 (C-10), 90.9 (C-8), 79.2 (C-2), 55.9 (OCH_3-7), 43.5 (C-3), 7.0 (CH_3-6); HRESIMS *m*/*z* 301.10709 $[M+H]^+$ (calcd for $C_{17}H_{17}O_5$ 301.10780).

5,4′-dihydroxy-6,8-di-C-methyl-7-methoxy-flavanone (**5**): yellow oil; $[\alpha]_D^{25} = -6.86$ (c 0.12, MeOH); UVλmax 192, 222, 282 and 361 nm; ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 12.0 (OH-5), 7.35 (2H, d, *J* = 8.4 Hz, H-2′ and H-6′), 6.89 (1H, d, *J* = 8.4 Hz, H-3′ and H-5′), 5.34 (1H, dd, *J* = 12.8, 2.8 Hz, H-2), 3.74 (3H, s, OCH_3-7), 3.06 (1H, dd, *J* = 17.2, 12.8 Hz, H-3), 2.83 (1H, dd, *J* = 17.2, 2.8 Hz, H-3), 2.10 (3H, s, CH_3-6), 2.07 (3H, s, CH_3-8); ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 197.7 (C-4), 162.5 (C-7), 160.2 (C-5), 158.1 (C-4′), 156.4 (C-9), 131.5 (C-1′), 127.8 (C-2′ and C-6′), 119.5 (C-8),116.2 (C-3′ and C-5′), 108.2 (C-6), 104.0

(C-10), 79.7 (C-2), 63.5 (OCH_3-7), 40.7 (C-3), 9.9 (CH_3-8), 9.3 (CH_3-6); HRESIMS *m*/*z* 315.12292 $[M+H]^+$ (calcd for $C_{18}H_{19}O_5$ 315.1227).

Noroxyhydrastinine (**6**): yellow powder; UV λmax 222, 261 and 306 nm; ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 7.50 (1H, s, H-8), 6.66 (1H, s, H-5), 6.01 (2H, s, OCH_2O), 3.53 (2H, t, *J* = 6.8, 6.4 Hz, H-3), 2.91 (1H, t, *J* = 6.8, 6.4 Hz, H-4); ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 166.5 (C-1), 151.5 (C-6), 147.2 (C-7), 135.0 (C-5a), 121.5 (C-8a), 108.0 (C-8), 107.5 (C-5), 101.8 (OCH_2O), 40.4 (C-3), 28.3 (C-4); HRESIMS *m*/*z* 192.06526 $[M+H]^+$ (calcd for $C_{10}H_{10}NO_3$ 192.0655).

Oxyhydrastinine (**7**): yellow pale powder; UV λmax 222, 264 and 304 nm; ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 7.54 (1H, s, H-8), 6.61 (1H, s, H-5), 5.99 (2H, s, OCH_2O), 3.51 (2H, t, *J* = 6.8, 6.8 Hz, H-3), 2.90 (1H, t, *J* = 6.8, 6.8 Hz, H-4), 3.13 (3H, s, N-CH_3); ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 164.7 (C-1), 150.4 (C-6), 146.9 (C-7), 133.6 (C-5a), 123.5 (C-8a), 108.3 (C-8), 107.0 (C-5), 101.6 (OCH_2O), 48.3 (C-3), 35.3 (N-CH_3), 28.1 (C-4); HRESIMS *m*/*z* 206.08127 $[M+H]^+$ (calcd for $C_{11}H_{12}NO_3$ 206.08117).

4′,5′-dimethoxy-4-methyl-3′-oxo-(1,2,5,6-tetrahydro-4H-1,3-dioxolo-[4′,5′:4,5]-benzo[1,2-e]-1,2-oxazocin)-2-spiro-1′-phtalan (**8**): yellow pale powder; $[\alpha]_D^{25} = +4.8$ (c 0.17, MeOH); UV λmax 218, 245 and 300 nm; ^{1}H-NMR (400 MHz, $CDCl_3$) *δ*: 7.04 (2H, d, *J* = 8.4 Hz, H-6′), 6.75 (1H, s, H-7), 6.36 (1H, s, H-11), 6.33 (1H, d, *J* = 8.4 Hz, H-7′), 6.00 and 5.94 (2H, s, OCH_2O), 4.28 (1H, m, H-1), 4.11 (3H, s, OCH_3-4′), 3.88 (3H, s, OCH_3-5′), 3.35 (1H, m, H-6), 3.07 (2H, m, H-5), 2.76 (3H, s, N-CH_3), 2.70 (2H, m, H-1 and H-6); ^{13}C-NMR (100 MHz, $CDCl_3$) *δ*: 165.8 (C-3′), 154.0 (C-5′), 148.1 (C-4′), 146.9 (C-8), 145.4 (C-10), 138.9 (C-7′a), 135.1 (C-7a), 126.9 (C-11a), 119.7 (C-7′), 119.1 (C-4′a), 118.0 (C-6′), 112.6 (C-11), 111.1 (C-7), 109.5 (C-2), 101.2 (C-9), 62.5 (OCH_3-4′), 62.1 (C-5), 56.8 (OCH_3-5′), 49.2 (N-CH_3), 40.6 (C-1), 36.6 (C-6); HRESIMS *m*/*z* 400.1375 $[M+H]^+$ (calcd for $C_{21}H_{22}NO_7$ 400.1391).

4.4. Collection of HRESIMS fragmentation data

Each of the 17 isolated compounds were suspended in MeOH at either 1 mg/mL or 0.1mg/mL and subjected to ultraperformance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis via a Waters Acquity UPLC with an Acquity UPLC column (BEH C_{18}, 1.7 μm, 2.1 × 50 mm), Waters Corporation, Milford, MA) coupled to a Thermo Q Exactive Plus orbitrap mass spectrometer with heated electrospray ionization (Thermo Fisher Scientific, MA, USA). The compounds were eluted from the column at a flow rate of 0.3 mL/min using a binary solvent system with A consisting of water with 0.1% formic acid additive and solvent B consisting of acetonitrile with 0.1% formic acid additive. The gradient was as follows: 95:5 (A:B) from 0 to 1 min, increasing to 90:10 (A:B) from 1 to 2 min, 80:20 (A:B) from 2 to 3 min, 60:40 (A:B) from 3 to 4 min, 70:30 (A:B) from 4 to 5 min, 0:100 (A:B) from 5 to 6 min and held from 6 to 7 min, 95:5 (A:B) from 7 to 8 min and held from 8 to 9 min. Duplicate analyses of each sample were conducted in both positive and negative polarities using the following settings: spray voltage, 3.7 kV; capillary temperature, 350 °C; sheath gas, 25; auxiliary gas, 5; S-lens RF level, 50. Each compound was chosen for fragmentation via high energy collision-induced dissociation (HCD,

normalized collision energy set to 50) from an inclusion list for both polarities. To determine the average mass accuracy of the product ions, the fragmentation spectra of **2** was compared to theoretical fragments produced by ACD MS fragmenter (Advanced Chemistry Development, Inc. Toronto, Canada). The resulting accurate mass of the predicted chemical formulas and hypothetical structures were matched with experimental data and were within 10 ppm mass error (Fig. 4).

4.5. Efflux pump inhibition assay

Four of the isolated compounds (**1, 2, 4** and **16**) were of sufficient purity (91%, 98%, 95% and 100%, respectively, by LC-UV) and quantity for evaluation via an efflux pump inhibition assay, as previously described (Brown et al., 2015). The assay was modified from the previously reported method in the chromatographic gradient, in some of the mass spectrometric conditions, and in the use *Staphyloccocus aureus* strain SA1199 (Kaatz and Seo, 1995).

The gradient was as follows: 95:5 (A:B) from 0 to 1 min, increasing to 0:100 (A:B) from 1 to 3.5 min, held from 3.5 to 9.5 min, 95:5 (A:B) from 9.5 to 10 min. A divert valve was utilized, with the valve set to waste from 0 to 1.5 min and to inject from 1.5 to 10 min. The mass spectrometric analyses were conducted under the following conditions: spray voltage, 3 kV; capillary temperature, 250°C; vaporizer temperature, 40°C; sheath gas, 40; aux gas, 30; tube lens offset, -112. Mass spectral dose-response data were analyzed with SigmaPlot (Systat Software, San Jose, CA) to calculate IC_{50} values for each of the active compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by the National Center for Complementary and Integrative Health (NCCIH), a component of the National Institute of Health (NIH), by grant numbers 1 R01 AT006860 (to NBC) and F31 AT009164 (to ERB). We thank Bill Burch for supplying plant material and Noemi Paguigan, Vincent Sica, and Diana Kao for helpful suggestions related to the manuscript. Mass spectrometry data were collected in the Triad Mass Spectrometry facility.

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Appendix A. Supplementary data

The isolation scheme and NMR spectra are available as Supporting Information.

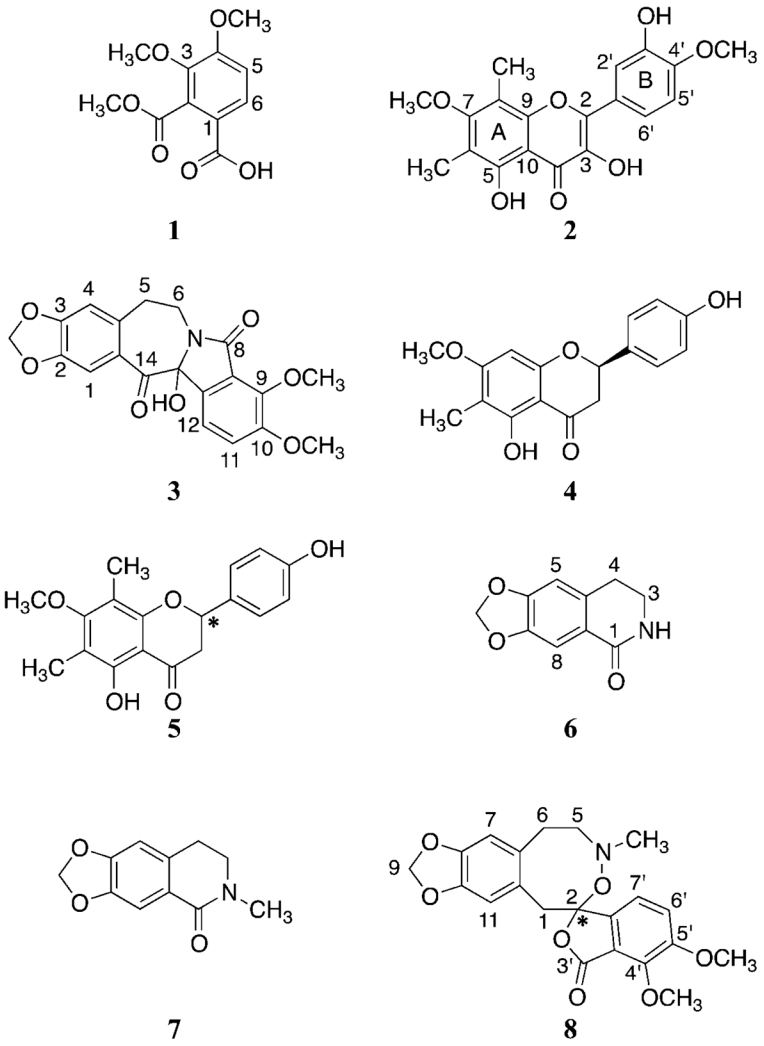

Fig. 1.

Structures of compounds **1-8**, which are reported for this first time in this report as constituents of goldenseal *(Hydrastis canadensis*). The configuration at locations with asterisks are unknown.

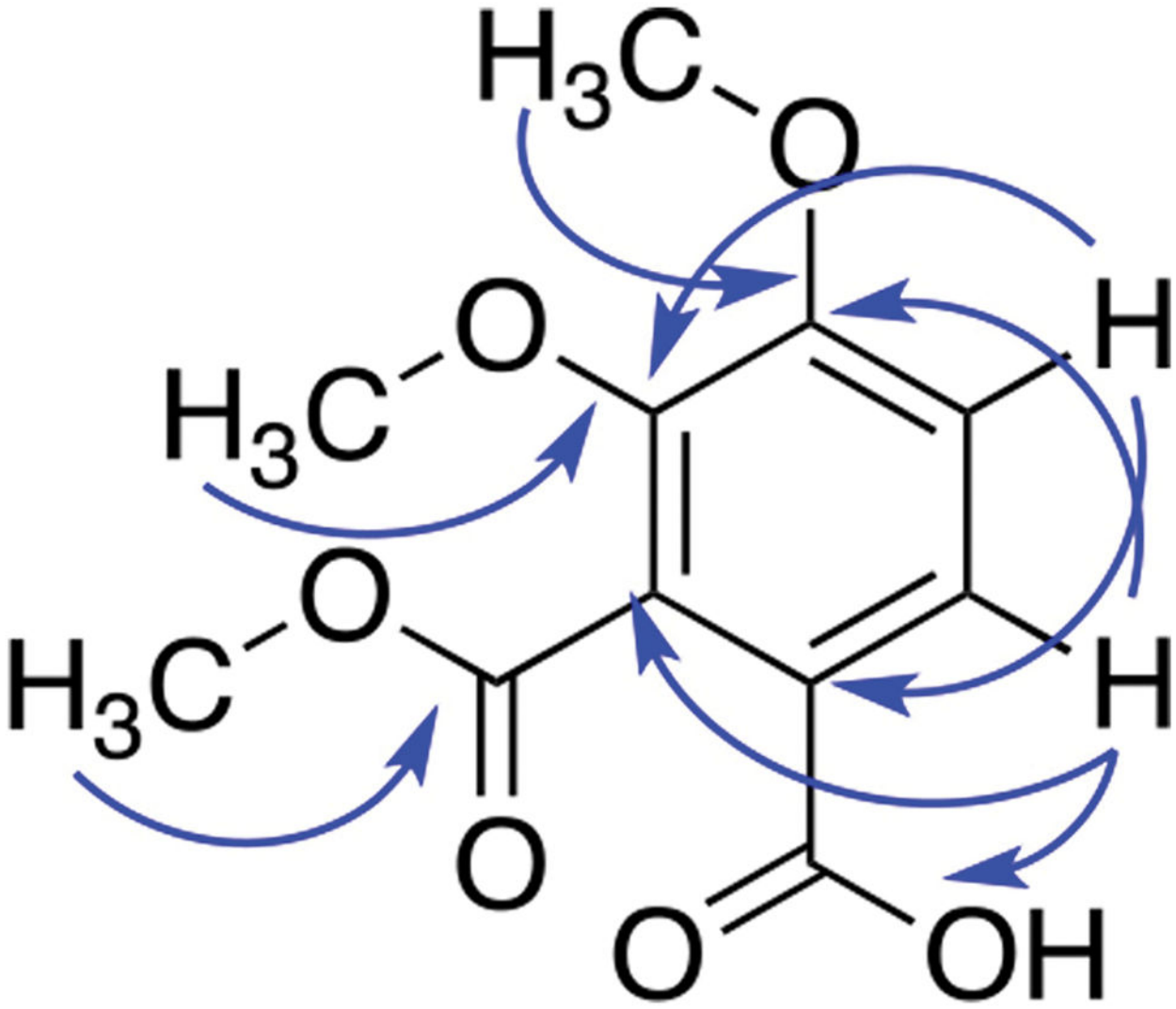

Fig. 2.
HMBC correlations of **1**.

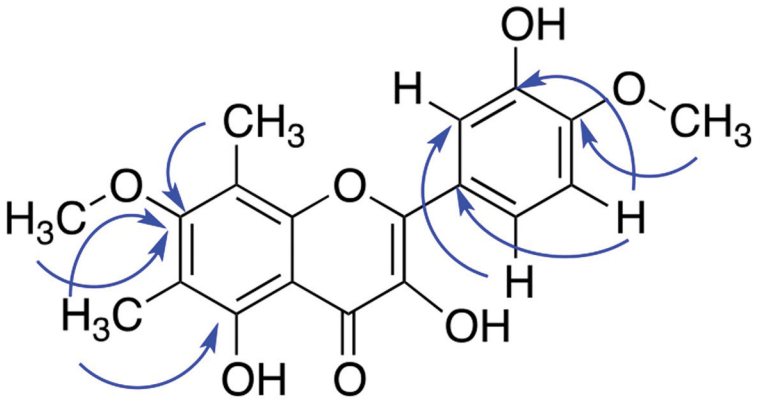

Fig. 3.
HMBC correlations of **2**.

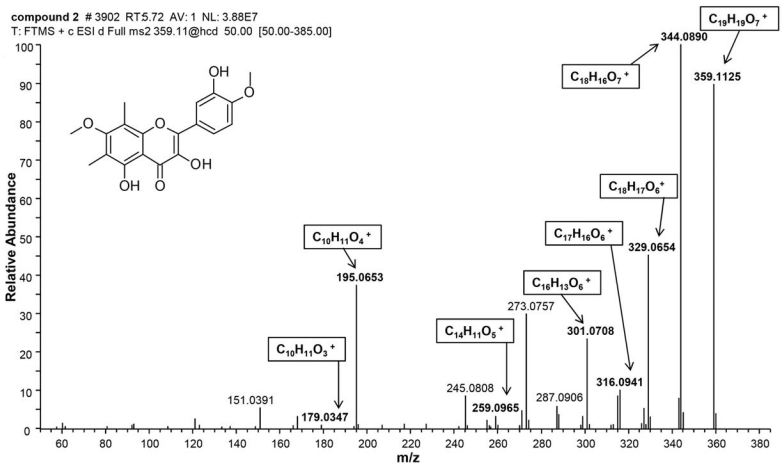

Fig. 4.

Accurate mass fragmentation spectrum of 3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-*C*-dimethyl-flavone (**2**) in the positive polarity. Fragments and molecular formulas were predicted using ACD MS fragmenter (Advanced Chemistry Development, Inc. Toronto, Canada) and compared to experimental data. Bold ions had a mass error within 10 ppm of the associated molecular formula.

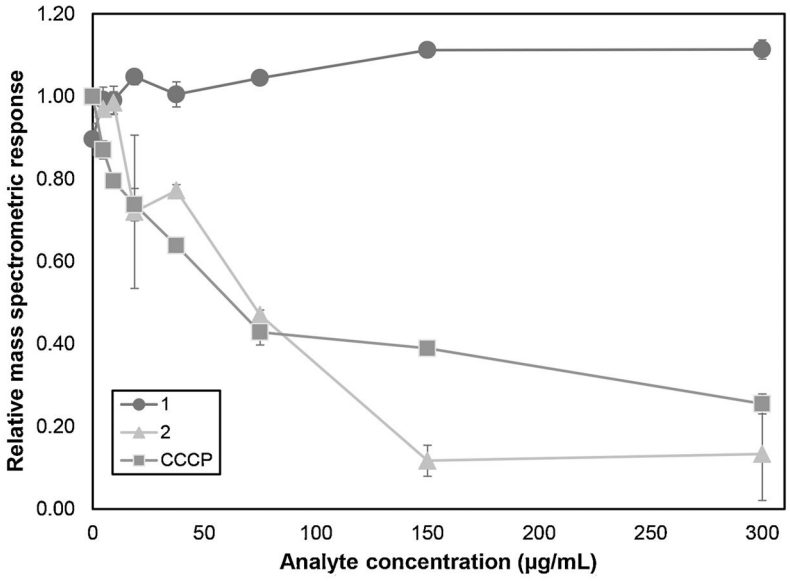

Fig. 5.

Efflux pump inhibition assay data for 3,4-dimethoxy-2-(methoxycarbonyl)benzoic acid (**1**) and 3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-*C*-dimethyl-flavone (**2**). The positive control for this assay is carbonyl cyanide *m*-chloro-phenylhydrazone (CCCP), a compound that inhibits efflux by collapsing the proton motive force across the cell membrane (Hopfer et al., 1967). Each data point is the mean of triplicate measurements from separate bacterial cultures (biological replicates) and error bars represent standard error of the mean.

Table 1

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectroscopic data[a] for *3,4-dimethoxy-2-(methoxycarbonyl)benzoic acid* (**1**).

Position	δ_C	δ_H, m (*J* in Hz)	HMBC[b]
1	118.7		
2	131.8		
3	145.9		
4	157.4		
5	112.2	6.98, d (8.8)	C-1, C-3
6	128.4	7.87, d (8.8)	C-2, C-4, C-1a
1a	168.6		
2a	167.5		
3-OCH_3	61.9	3.87, s	C-3
4-OCH_3	56.3	3.95, s	C-4
2a-OCH_3	53.0	3.94, s	C-2a

[a]^{1}H and C chemical shifts with reference to $CDCl_3$ (δ_H = 7.26 ppm) and $CDCl_3$ (δ_C = 77.16 ppm), respectively.

[b]HMBC correlations are from the proton stated to the indicated carbon.

Table 2

^{1}H (700 MHz) and ^{13}C (175 MHz) NMR spectroscopic data[a] for *3,5,3′-trihydroxy-7,4′-dimethoxy-6,8-C-dimethyl-flavone* (**2**).

Position	δ_C	δ_H, m (*J* in Hz)	HMBC[b]
2	145.8		
3	136.2		
4	175.9		
5	156.0		
6	113.4		
7	163.0[c]		
8	109.4		
9	152.4		
10	105.8		
1′	124.6		
2′	113.7	7.82, d (2.1)	C-2, C-6′
3′	145.8		
4′	148.4		
5′	110.7	7.00, d (9.1)	C-1′, C-3′
6′	121.1	7.87, dd (9.1, 2.1)	C-2, C-2′
6-CH_3	8.4	2.23, s	C-5, C-6, C-7
8-CH_3	8.7	2.41, s	C-7, C-8, C-9
7-OCH_3	60.8	3.80, s	C-7
4′-OCH_3	56.2	4.00, s	C-4′
3-OH		6.64	C-3, C-4
3′-OH		5.71	C-2′, C-3′
5-OH		11.80	C-5, C-10

[a] ^{1}H and ^{13}C chemical shifts with reference to $CDCl_3$ (δ_H = 7.26 ppm) and $CDCl_3$ (δ_C = 77.16 ppm), respectively.

[b] HMBC correlations are from the proton stated to the indicated carbon.

[c] obtained from HMBC