JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

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Isolation and Identification of *Flavobacterium columnare* and *Streptococcus iniae* Antibacterial Compounds from the Terrestrial Plant *Atraphaxis laetevirens*

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ABSTRACT: Columnaris disease, enteric septicemia of catfish, and streptococcosis are common bacterial diseases of certain freshwater fish and are caused by *Flavobacterium columnare*, *Edwardsiella ictaluri*, and *Streptococcus iniae*, respectively. During the process of evaluating several species of plants to isolate and identify compounds with toxicity against these bacteria, a promising extract from the aerial parts of the terrestrial plant *Atraphaxis laetevirens* (Ledeb.) Jaub. et Spach (Polygonaceae Juss.) was selected for bioassay-guided fractionation using a rapid microplate bioassay. The active dichloromethane extract was subjected to liquid−liquid partitioning, and active fractions were further separated by normal-phase column chromatography and normal-phase high-performance liquid chromatography (HPLC). Nepodin (**3**) and emodin (**4**) were isolated from two fractions with strong toxicities against *S. iniae*. A chloroform fraction was further separated by normal-phase column chromatography to yield two active fractions against *F. columnare*, and these fractions contained chrysophanol (**1**), physcion (**2**), and nepodin (**3**). Compound **1** had strong activity, and compound **3** had moderate activity against *F. columnare*, while compounds **2** and **4** were not toxic at the concentrations tested.

KEYWORDS: *Anthraquinones, antibacterial activity, Atraphaxis laetevirens, Edwardsiella ictaluri, Flavobacterium columnare, Streptococcus iniae*

INTRODUCTION

The two most common bacterial diseases of pond-raised channel catfish (*Ictalurus punctatus*) in the southeastern United States are columnaris disease and enteric septicemia of catfish (ESC). Columnaris-disease-related problems are not only common to the channel catfish aquaculture industry but also occur worldwide in many other species of freshwater fish (e.g., rainbow trout, salmon, and tilapia) that can result in heavy economic losses to these aquaculture industries.[1,2] The etiological agent for columnaris disease is the bacterium *Flavobacterium columnare*, and this Gram-negative motile rod (2−10 μm in length) is in the family Flavobacteriaceae.[3] Columnaris diseases can result in severe necrosis of gill tissue, skin ulceration from systemic infection, and high mortalities in fish. The etiological agent for ESC is the bacterium *Edwardsiella ictaluri*, and this Gram-negative weakly motile rod (1−3 μm in length) is in the family Enterobacteriaceae.[2] The gross lesions of ESC in channel catfish include pale gills, small depigmented lesions and/or ulcers (1−3 mm) on the backs of infected fish, open lesion along the central skull line between the eyes, and hemorrhage at the base of the fins, under the jaw, and on the belly. In addition to high mortality rates of channel catfish, prevention and treatment approaches can cost producers millions of dollars annually.[2]

Another bacterial disease of significance in farmed freshwater fish, although less so in pond-raised channel catfish, is streptococcosis, which is a prevalent problem in fish species such as tilapias (*Oreochromis* spp.) and hybrid striped bass [*Morone chrysops* female × *Morone saxatilis* male (Percichthyidae)].[4] One of the several streptococcal species of bacteria that have been attributed as the cause streptococcosis in freshwater fish is *Streptococcus iniae*, a Gram-positive spherical-shaped cell (0.5−2.0 μm in diameter). In tilapia, the gross lesions of streptococcosis include hemorrhage in the skin and at the base of fins, epidermal lesions and/or bloody ulcers, and opaque corneas.[2] Streptococcosis can result in very high mortality rates, and therefore management approaches include prevention as well as treatment once the disease is determined to be present within a population of fish.

Catfish producers currently have several available management approaches for columnaris disease and ESC, including the application of medicated feeds, live attenuated vaccines,[5] and non-antibiotic therapeutants, such as 35% Perox-Aid, for external columnaris.[2] Perox-Aid is not recommended for use in earthen ponds without water exchange. Additional inorganic agents, such as copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$) and

Received: July 25, 2012
Revised: October 1, 2012
Accepted: October 3, 2012
Published: October 3, 2012

potassium permanganate ($KMnO_4$), have been mentioned as potential treatments for columnaris disease,[6] but the efficacy of these compounds can be adversely impacted by certain water-quality variables. In addition, extra care must be taken when using these therapeutants because of their broad-spectrum toxicity toward nontarget organisms (e.g., channel catfish).[7]

Preventive management approaches for streptococcosis should include maintaining high water quality, application of high-quality diets, adequate water exchange and disinfection, and removal of fecal waste from recirculating water systems.[2] Currently, only florfenicol (Aquaflor) is approved in the United States for treatment of streptococcal septicemia caused by *S. iniae* in warmwater fish. Vaccination appears to be a very promising approach for protection against *S. iniae* infection in Nile tilapia.[8]

The discovery of novel, environmentally safe, natural, and natural-based antibacterial compounds would greatly benefit aquaculturists because of the limitations or the absence of current management approaches available to producers for controlling the bacterial species responsible for columnaris disease, ESC, and streptococcosis. As part of our discovery process to identify active compounds against isolates of *F. columnare*, *E. ictaluri*, and *S. iniae*, crude extracts from various plants collected in Kazakhstan were selected for evaluation using a rapid bioassay. Active crude extracts from the aerial parts of the terrestrial plant *Atraphaxis laetevirens* (Ledeb.) Jaub. et Spach (Polygonaceae Juss.) were found to be the most promising and were subsequently chosen for bioassay-guided investigation studies. Bioassay evaluations of isolated compounds from these crude extracts were also performed.

MATERIALS AND METHODS

General Procedures. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity Inova AS600 spectrometer (Varian, Palo Alto, CA). Electron impact mass spectrometry (EIMS) spectra were recorded on a Varian CP-3800 gas chromatograph coupled to a Varian Saturn 2000 mass spectrometer (Varian, Inc., Palo Alto, CA). Column chromatography was performed using a Biotage Isolera One flash purification system (Biotage, Uppsala, Sweden). High-performance liquid chromatography (HPLC) was performed using an Agilent 1100 HPLC system (Agilent, Palo Alto, CA).

Plant Materials. The terrestrial plant *A. laetevirens* (Ledeb.) Jaub. Et Spach was collected for evaluation. The aerial parts of *A. laetevirens* were collected at the end of the flowering stage on May 19, 2006, in the Syugaty Mountains in Kazakhstan. A voucher specimen number 2198/25 has been deposited in the Institute of Botany and Phytointroduction Herbarium, Almaty, Kazakhstan.

Extraction and Isolation. The aerial parts of *A. laetevirens* were extracted by sequential soaking at room temperature with CH_2Cl_2, EtOH, and H_2O. The aerial parts (0.3 kg) of *A. laetevirens* extracted with 1.8 L of CH_2Cl_2 for 24 h at room temperature provided 6.1 g of CH_2Cl_2 extractables. For isolation of compounds for toxicity against the bacteria from the active CH_2Cl_2 crude extract, a portion of the CH_2Cl_2 extract (2.9 g) was partitioned between 500 mL (first) plus 200 mL (2×, second and third) of *n*-hexane and 400 mL of 90% MeOH [9:1 (v/v) MeOH/H_2O]. A total of 114 mL of water was added to the 90% MeOH fraction to adjust its concentration to 70% MeOH [7:3 (v/v) MeOH/H_2O], and then the 70% MeOH fraction was partitioned with 300 mL (3×) of $CHCl_3$. The MeOH involved in the 70% MeOH fraction was removed by rotary evaporation, and the resulting H_2O fraction was partitioned with 200 mL (3×) of EtOAc. The *n*-hexane fraction (169.8 mg) was subjected to step-gradient normal-phase column chromatography on a 100 g, 157 × 39 mm, 40−63 μm SNAP KP-Sil cartridge (Biotage, Charlotte, NC) eluted with *n*-hexane/acetone [step 1, from 100:0 to 30:70 (v/v), 1800 mL; step 2, from 30:70 to 0:100 (v/v), 600 mL] to afford fractions H1 (0.8 mg), H2 (0.2 mg), H3 (59.9 mg), H4 (47.2 mg), H5 (13.1 mg), H6 [emodin (**4**)] (10.8 mg, 0.0076% yield), H7 (93.5 mg), and H8 (41.8 mg) (MeOH-washed fraction). A portion of fraction H3 (5.0 mg) was further separated by normal-phase HPLC [Luna 5 μm Silica (2) 100 Å, Phenomenex, 250 × 10 mm; eluent, 99.3:0.7 (v/v) *n*-hexane/isopropyl alcohol; flow rate, 4.7 mL/min; ultraviolet (UV) detection, 210 nm] to yield chrysophanol (**1**) (2.1 mg, 0.0176% yield, t_R of 5.1 min) and physcion (**2**) (1.4 mg, 0.0118% yield, t_R of 6.1 min). A portion of fraction H4 (18.9 mg) was further separated by semi-preparative normal-phase HPLC [Luna 5 μm Silica (2) 100 Å, Phenomenex, 250 × 10 mm; eluent, 80:20, (v/v) *n*-hexane/isopropyl alcohol; flow rate, 4.7 mL/min; UV detection, 210 nm] to yield nepodin (**3**) (6.9 mg, 0.0121% yield, t_R of 6.1 min) (H4a). A portion of the $CHCl_3$ fraction (605.0 mg) was subjected to step-gradient normal-phase column chromatography on a 100 g, 157 × 39 mm, 40−63 μm SNAP KP-Sil cartridge (Biotage, Charlotte, NC) eluted with $CHCl_3$/MeOH [step 1, 95:5 (v/v), 1500 mL; step 2, from 95:5 to 29:71 (v/v), 600 mL; from 29:71 to 0:100 (v/v), 600 mL] to afford fractions C1 (0.4 mg), C2 (85.9 mg), C3 (83.5 mg), C4 (25.3 mg), C5 (46.8 mg), C6 (70.1 mg), C7 (39.7 mg), C8 (115.4 mg), C9 (5.1 mg), C10 (1.7 mg), and C11 (3.6 mg).

*Chrysophanol (**1**).* Yellow solid. ^1H NMR ($CDCl_3$) δ: 12.09 (1H, s, OH-8), 11.98 (1H, s, OH-1), 7.79 (1H, dd, *J* = 1.0, 7.5 Hz, H-5), 7.64 (1H, dd, *J* = 7.5, 8.4 Hz, H-6), 7.62 (1H, brs, H-4), 7.26 (1H, d, *J* = 1.0, 8.4 Hz, H-7), 7.07 (1H, brs, H-2), 2.44 (3H, s, CH_3). EIMS *m*/*z*: 254.0.

*Physcion (**2**).* Yellow solid. ^1H NMR ($CDCl_3$) δ: 12.28 (1H, s, OH-1), 12.09 (1H, s, OH-8), 7.60 (1H, d, *J* = 1.5 Hz, H-5), 7.34 (1H, d, *J* = 2.5 Hz, H-4), 7.05 (1H, d, *J* = 1.5 Hz, H-7), 6.66 (1H, d, *J* = 2.5 Hz, H-2), 3.92 (3H, s, OCH_3), 2.43 (3H, s, CH_3). EIMS *m*/*z*: 284.0.

*Nepodin (**3**).* Yellow solid. ^1H NMR ($CDCl_3$) δ: 10.21 (1H, s, OH-8), 7.44 (1H, dd, *J* = 7.8 and 7.8 Hz, H-6), 7.05 (1H, d, *J* = 7.8 Hz, H-5), 6.95 (1H, brs, H-4), 6.81 (1H, d, *J* = 7.8 Hz, H-7), 2.73 (3H, s, acetyl CH_3), 2.62 (3H, s, CH_3). EIMS *m*/*z*: 216.2.

*Emodin (**4**).* Yellow solid. ^1H NMR (CD_3COCD_3) δ: 12.14 (1H, s, OH-1), 12.02 (1H, s, OH-8), 7.51 (1H, brs, H-5), 7.21 (1H, d, *J* = 2.4 Hz, H-4), 7.09 (1H, brs, H-7), 6.63 (1H, d, *J* = 2.4 Hz, H-2), 2.44 (3H, s, CH_3). ^{13}C NMR ($CDCl_3$) δ: 191.7 (C-9), 182.2 (C-10), 166.5 (C-3), 166.3 (C-1), 163.3 (C-8), 149.6 (C-6), 136.6 (C-14), 134.2 (C-11), 125.0 (C-7), 121.5 (C-5), 114.5 (C-12), 110.5 (C-13), 109.8 (C-4), 108.9 (C-2), 22.1 (CH_3). EIMS *m*/*z*: 270.2.

Antibacterial Bioassay. An isolate of *E. ictaluri* (isolate S02-1039) was obtained from Tim Santucci (College of Veterinary Medicine, Mississippi State University, Stoneville, MS). To ensure purity, cultures of *E. ictaluri* were maintained on 3.8% Mueller−Hinton (MH) agar plates (pH 7.3) (Becton, Dickinson and Company, Sparks, MD). Prior to conducting the bioassay, single colonies of *E. ictaluri* were used to prepare the assay culture material by aseptically transferring bacterial cells from colonies to 45 mL of 3.8% MH broth to form a bacterial cell density of 0.5 McFarland standard.[9] An isolate of *F. columnare* [isolate ALM-00-173 (genomovar II)] was obtained from Dr. Covadonga Arias (Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL). The purity of cultures of the *F. columnare* isolate was assured by streaking the bacteria for isolation onto modified Shieh (MS) agar plates (pH 7.2−7.4)[10] and then checking after incubation at 29 ± 1 °C for 3−5 days that only one bacterial colony type was present. Prior to conducting the bioassay, single colonies of *F. columnare* were used to prepare the assay culture material by culturing in 75 mL of MS broth (24 h) at 29 ± 1 °C at 150 rpm on a rotary shaker. After overnight incubation, a 0.5 McFarland standard of *F. columnare* assay material was prepared by transferring cells from the broth culture to fresh MS broth. A culture of *S. iniae* (isolate LA94-426) was provided by Dr. Ahmed Darwish (formerly at Harry K. Dupree Stuttgart National Aquaculture Research Center, USDA−ARS, Stuttgart, AR). The purity of cultures of *S. iniae* was assured by streaking the bacteria for isolation onto Columbia CNA agar plates containing 5% sheep blood (Remel, Inc., Lenexa, KS) and then checking after incubation at 29 ± 1 °C for 3−5 days that only one bacterial colony type was present. Bioassay culture material of *S.*

Table 1. Results of the Bioassay Evaluation of the Hexane Partition and Its Fractions from Aerial Parts of *A. laetevirens* for Toxicity against *S. iniae*

fraction or compound	IC_{50}[a]	RDCF[b]	RDCO[c]	MIC[d]	RDCF	RDCO
florfenicol	0.91 (0.16)[e]			0.36 (0)		
oxytetracycline HCl	0.12 (0.02)			0.03 (0.02)		
hexane fraction	2.0 (0)	2.2	16.7	10.0 (0)	27.8	333.3
H3	>100.0	>109.9	>833.3	>100.0	>277.8	>3333.3
H3a[f]	>25.4	>27.9	>211.8	>25.4	>70.6	>847.3
H3b[g]	>28.4	>31.2	>236.9	>28.4	>79.0	>947.7
H4	>100.0	>109.9	>833.3	>100.0	>277.8	>3333.3
H4a[h]	6.4 (1.1)	7.0 (0.1)	53.2 (0.2)	2.32 (0)	6.4 (0)	77.3 (0)
H5	57.0	62.6	475.0	100.0	277.8	3333.3
H6[i]	9.1 (1.5)	10.0 (0.1)	75.5 (0.2)	2.70 (0)	7.5 (0)	90.0 (0)
H7	79.0	86.8	658.3	100.0	277.8	3333.3
H8	32.0	35.2	266.7	10.0	27.8	333.3

[a]IC_{50}, 50% inhibition concentration (mg/L). [b]RDCF = relative-to-drug-control florfenicol. [c]RDCO = relative-to-drug-control oxytetracycline HCl. [d]MIC = minimum inhibition concentration (mg/L). [e]Numbers in parentheses are the standard error of the mean. [f]H3a = chrysophanol (**1**). [g]H3b = physcion (**2**). [h]H4a = nepodin (**3**). [i]H6 = emodin (**4**).

iniae was prepared in the same manner and conditions as used for the *F. columnare* isolate, except 3.8% MH broth was used and the broth cultures were incubated for 18 h instead of 24 h prior to preparing the 0.5 MacFarland standard.

The crude extract from *A. laetevirens*, extract fractions, and isolated pure compounds were evaluated for antibacterial activity using a rapid 96-well microplate bioassay and following the procedures of a previous report.[9] Florfenicol and oxytetracycline HCl, antibiotics that can be used in medicated feed, were included as positive drug controls. Also, control wells (no test material added) were included in each assay. The initial crude extract samples were dissolved in either acetone or water. Extract fractions obtained later were dissolved in either acetone, hexane, methanol, or dichloromethane (also used for chloroform fractions) depending upon the eluant used to obtain the extract fraction. All isolated pure compounds were dissolved separately in dichloromethane. Drug controls were dissolved in ethanol. Technical-grade solvents were used in this study. Final test concentrations of the crude extracts and extract fractions in the microplate wells were 0.1, 1.0, 10.0, and 100.0 mg/L. Final concentrations of test compounds and drug controls were 0.01, 0.1, 1.0, 10.0, and 100.0 μM. Three replications were used for each dilution of each crude extract, extract fraction, test compound, and controls. Final results were converted to units of mg/L to allow for comparison to previous studies.

To determine the 24 h 50% inhibition concentration (IC_{50}) and minimum inhibition concentration (MIC), sterile 96-well polystyrene microplates (Corning Costar Corp., Acton, MS) with flat-bottom wells were used for extracts, fractions, and test compounds dissolved in either water or methanol, while sterile quartz 96-well microplates (Hellma Cells, Inc., Forest Hills, NY) were used for extracts, fractions, or test compounds dissolved in either acetone, hexane, or dichloromethane. Initially, dissolved test material or drug controls were micropipetted separately into individual microplate wells (10 μL/well), and solvent was allowed to completely evaporate before 0.5 MacFarland bacterial culture (prepared as described previously[9]) was added to the microplate wells (200 μL/well). For the initial crude extract dissolved in water, 10 μL/well was added to microplate wells, while sterile water was added to drug control wells (10 μL/well) before the addition of 190 μL/well of culture material. Microplates were incubated at 29 ± 1 °C. A SpectraCount microplate photometer (Packard Instrument Company, Meriden, CT) was used to measure the absorbance (630 nm) of the microplate wells at times 0 and 24 h.

The means and standard deviations of absorbance measurements were calculated, graphed, and compared to controls to help determine the 24 h IC_{50} and MIC for each crude extract, extract fraction, and test compound.[9] The 24 h IC_{50} and MIC results for each test fraction and compound were divided by the respective 24 h IC_{50} and MIC results obtained for the positive controls florfenicol and oxytetracycline to determine the relative-to-drug-control florfenicol (RDCF) and relative-to-drug-control oxytetracycline (RDCO) values.

RESULTS AND DISCUSSION

Previous studies have isolated many bioactive compounds from plants collected in Kazakhstan, thereby demonstrating the benefits of sourcing plants from this region for the discovery of pesticide candidates.[11−15] In the present study, 18 crude extracts of plants collected in Kazakhstan were screened for toxicity against the Gram-negative bacteria *F. columnare* (isolate ALM-00-173) and *E. ictaluri* (isolate S02-1039) and the Gram-positive bacterium *S. iniae* (isolate LA94-426). The 24 h IC_{50} and MIC values of all tested extracts for toxicity against *E. ictaluri* (>100.0 and ≥100.0 mg/L, respectively) were much higher than that of the drug control florfenicol (0.2 and 0.1 mg/L, respectively). In contrast, the 24 h IC_{50} and MIC values of the CH_2Cl_2 extract from the aerial parts of *A. laetevirens* for toxicity against *F. columnare* were the lowest (18.0 and 10.0 mg/L) compared to the other tested extracts (>100.0 and ≥100.0 mg/L, respectively). Furthermore, the 24 h IC_{50} results for the CH_2Cl_2 and EtOH extracts from the aerial parts of *A. laetevirens* for toxicity against *S. iniae* were lower (40.0 and 95.0 mg/L, respectively) than all but two of the other tested extracts (>100.0 mg/L). The MIC results for the EtOH extract from the aerial parts of *A. laetevirens* for toxicity against *S. iniae* were lower (0.1 mg/L) than all but two of the other tested extracts (≥100.0 mg/L). Thus, the conclusion was made based on the collective results that the CH_2Cl_2 extract from the aerial parts of *A. laetevirens* contained an active compound(s) possessing strong toxicities against both *F. columnare* and *S. iniae*. Subsequently, this extract was chosen for bioassay-guided fractionation to discover compounds with activities against *F. columnare* and *S. iniae*.

To isolate the active compounds in the extract from *A. laetevirens*, the CH_2Cl_2 extract was separated into hexane, $CHCl_3$, EtOAc, and H_2O fractions by liquid−liquid partitioning. The 24 h IC_{50}, MIC, and respective RDCF values of all tested fractions for toxicity against *E. ictaluri* were higher compared to results for florfenicol, especially the RDCF values. In contrast, the RDCF value of the 24 h IC_{50} of the hexane fraction for toxicity against *F. columnare* was the lowest, followed by those of the $CHCl_3$, EtOAc, and H_2O fractions. The RDCF value of the MIC of the $CHCl_3$ fraction was the

lowest, followed by those of the EtOAc, H_2O, and hexane fractions. Furthermore, the 24 h IC_{50}, MIC, and respective RDCF values of the hexane fraction for toxicity against *S. iniae* were the lowest, followed by those of the $CHCl_3$, EtOAc, and H_2O fractions. Thus, the hexane and $CHCl_3$ fractions of the CH_2Cl_2 plant extract were determined to likely possess a compound(s) with strong toxicities against *S. iniae* and *F. columnare*, respectively.

The hexane fraction was further separated by normal-phase column chromatography and normal-phase HPLC. The 24 h IC_{50}, MIC, and respective RDCF and RDCO values of H4a and H6 fractions for toxicity against *S. iniae* were lower than those of the other fractions (Table 1). These results suggested that H4a and H6 fractions contained compounds with strong toxicities against *S. iniae*. The compounds **1**−**4** were isolated from H3a, H3b, H4a, and H6 fractions, respectively. The gross structures of compounds **1**, **2**, and **3** (Figure 1) were elucidated

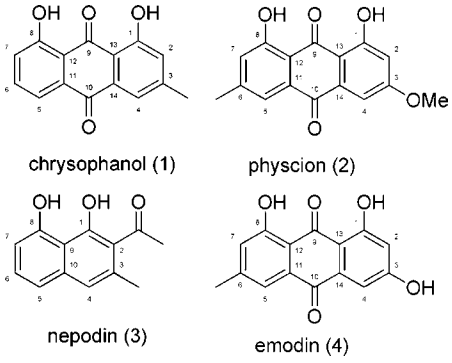

Figure 1. Structures of chrysophanol (**1**), physcion (**2**), nepodin (**3**), and emodin (**4**).

by analyses of EIMS and ^{1}H NMR spectra, which were in agreement with literature data of chrysophanol, physcion, and nepodin, respectively.[16,17] The gross structure of compound **4** (Figure 1) was elucidated by analyses of EIMS and ^{1}H and ^{13}C NMR spectra, which were in agreement with the literature data of emodin.[16] This is the first report on the presence of compounds **1**−**4** in *A. laetevirens*. Chrysophanol (**1**), physcion (**2**), and emodin (**4**) belong to the class of compounds designated as anthraquinones, and their structures are very similar to each other, except for the group attached to C6 for compound **1** or C3 for compounds **2** and **4**. Emodin (**4**) showed the strongest toxicity against *F. columnare* among compounds **1**, **2**, and **4**. Thus, the active site in the structure of the anthraquinones from *A. laetevirens* appears to be influenced by the hydroxyl group attached at C3 (or C6). Also, compounds **3** and **4** might play important roles in the extracts from *A. laetevirens* for antibacterial activities against *S. iniae*.

The $CHCl_3$ fraction was further separated by normal-phase column chromatography. The 24 h IC_{50} and respective RDCF values of C2 and C3 fractions for toxicity against *F. columnare* were lower than those of other fractions (Table 2). The MIC and respective RDCF values of C2, C3, and C6 fractions for toxicity against *F. columnare* were lower than those of other fractions. These results suggested that C2 and C3 fractions contained compounds with strong toxicities against *F. columnare*. Analyses of ^{1}H NMR revealed that compounds **1**, **2**, and **3** were constituents in the C2 fraction in the ratio of 2:2:3, respectively, and in the C3 fraction at 1:1:6, respectively. Furthermore, pure compounds **1**−**3** were evaluated against *F. columnare*. Emodin (**4**) was also evaluated against *F. columnare* because of its known presence in a *n*-hexane fraction that was evaluated against *S. iniae* (Table 1). Results determined that compound **1** had the strongest activity against *F. columnare* based on MIC results (MIC = 1.4 mg/L), while compound **3** had moderate activity, with a 24 h IC_{50} of 13.8 mg/L and MIC of 23.2 mg/L (Table 3). Thus, the methoxyl and hydroxyl groups attached to C3 (or C6) appear to decrease the activity of anthraquinones from *A. laetevirens*. Also, compounds **1** and **3** might play important roles in the extracts from *A. laetevirens* for antibacterial activities against *F. columnare*. Emodin (**4**), which had strong activity against *S. iniae* (Table 1), was not toxic against *F. columnare* at any of the concentrations evaluated (Table 2). The MIC results indicate that compounds **1** and **4** had selective toxicity against the two different bacterial species used in this study. Conversely, compound **3** was toxic against both *S. iniae* and *F. columnare*, although antibacterial activity was stronger against *S. iniae*.

Table 2. Results of the Bioassay Evaluation of the $CHCl_3$ Partition and Its Fractions from Aerial Parts of *A. laetevirens* for Toxicity against *F. columnare*

fraction or compound	IC_{50}[a]	IC_{50} RDCF[b]	MIC[c]	MIC RDCF
Bioassay 1				
florfenicol	0.31		1.0	
C2[d]	7.9	25.5	10.0	10.0
C3[e]	7.0	22.6	10.0	10.0
C4	>100.0	>322.6	100.0	100.0
C5	>100.0	>322.6	100.0	100.0
C6	31.0	100.0	10.0	10.0
C7	>100.0	>322.6	100.0	100.0
C8	47.0	151.6	100.0	100.0
Bioassay 2				
florfenicol	0.26		1.0	
$CHCl_3$	28.0	107.7	10.0	10.0
C9	>100.0	>384.6	100.0	100.0
C11	100.0	384.6	100.0	100.0

[a]IC_{50} = 50% inhibition concentration (mg/L). [b]RDCF = relative-to-drug-control florfenicol. [c]MIC = minimum inhibition concentration (mg/L). [d]C2 = composed of chrysophanol (**1**), physcion (**2**), and nepodin (**3**) at 2:2:3. [e]C3 = composed of chrysophanol (**1**), physcion (**2**), and nepodin (**3**) at 1:1:6.

This study is the first report on the antibacterial activities on compounds **3** and **4** against *S. iniae* and compounds **1** and **3** against *F. columnare*. Nepodin (**3**) and emodin (**4**) appear to be the most promising against *S. iniae*. While compound **1** had strong activity against *F. columnare* based on MIC results, the lack of toxicity as indicated by the 24 h IC_{50} of >25.42 mg/L (Table 3) indicates that *F. columnare* may be able to overcome the initial toxicity of this compound, and therefore, it is not as promising for additional studies. Future studies (e.g., challenge studies) need to be performed to determine the potential of compounds **3** and **4** for use in managing streptococcosis in certain types of freshwater fish, such as channel fish. Such studies would include the evaluation of the potential enhancement of the antibacterial activities of compounds **3** and **4** against *S. iniae* when both compounds are applied together. In addition, efficacy studies might include the use of compound **3** or **4** with florfenicol, so that reduced doses of florfenicol would be required to effectively manage *S. iniae* infections.

Table 3. Results of the Bioassay Evaluation of Antibacterial Compounds Present in Aerial Parts of *A. laetevirens* for Toxicity against *F. columnare*

compound	IC_{50}[a]	IC_{50} RDCF[c]	IC_{50} RDCO[d]	MIC[b]	MIC RDCF	MIC RDCO
florfenicol	0.84 (0.1)[e]			0.36 (0)		
oxytetracycline HCl	0.56 (0.2)			0.46 (0)		
chrysophanol (**1**)	>25.4	>30.3	>45.4	1.4 (1.1)	3.9 (3.2)	3.0 (2.5)
physcion (**2**)	>28.4	>33.9	>50.8	>28.4	>79.0	>61.8
nepodin (**3**)	13.8 (4.8)	17.3 (7.5)	31.2 (19.0)	23.2 (0)	64.5 (0)	50.5 (0)
emodin (**4**)	>27.0	>32.2	>48.3	>27.0	>75.1	>58.7

[a]IC_{50} = 50% inhibition concentration (mg/L). [b]MIC = minimum inhibition concentration (mg/L). [c]RDCF = relative-to-drug-control florfenicol. [d]RDCO = relative-to-drug-control oxytetracycline HCl. [e]Numbers in parentheses are the standard error of the mean.

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Funding

Financial support in part from the International Science and Technology Center (ISTC) Project K1896 is greatly appreciated.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Phaedra Page, Solomon Green III, Marcuslene Harries, and Amber Reichley, from the Natural Products Utilization Research Unit, USDA–ARS, for technical assistance.

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