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Flavones from *Struthiola argentea* with anthelmintic activity *in vitro*

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Abstract

Parasitic diseases caused by helminthes lead to significant health hazards to animals resulting in enormous economic impact. While a number of anthelmintics are currently available, all are encountering resistance and ones with a mode of action are needed. We report herein bioassay-guided isolation of three anthelmintic flavones **1**–**3**, including the flavone, 5,6,2′,5′,6′-pentamethoxy-3′,4′-methylenedioxyflavone (**3**) from the methanol extract of *Struthiola argentea* (Thymelaeaceae). The structure of **3** was elucidated by analysis of its 1D and 2D NMR and MS data. The two major flavones produced by this plant were also isolated and identified as yuankanin (**4**) and amentoflavone (**5**). A number of flavones related to the compounds isolated from *S. argentea* were acquired and tested to ascertain structure activity relationships. The isolation, structure, anthelmintic activity and structure activity relationships of the flavones are described. Compound **3** exhibited the most potent *in vitro* activity with 90% inhibition of larval motility at 3.1 μg/mL and compound **15** showed modest *in vivo* activity.

Keywords: Flavones; *Struthiola argentea*; Anthelmintics; *Haemonchus contortus*; *Heligmosomoides polygyrus*; Thymelaeaceae

1. Introduction

One of the greatest impediments to productivity in the sheep and goat industry is infection by internal parasites such as *Haemonchus contortus*. In the past 50 years, significant progress has been made in the development of anthelmintic drugs, including the development of the current classes of synthetic drugs, the most important being the benzimidazoles and imidazothiazoles (tetramisole/levamisole). However, the biggest achievement was introduction of the avermectin class of macrolactones in the early 1980s. The discovery of this class of polyketide natural products led to anthelmintic drugs such as ivermectin and doramectin with excellent broad-spectrum activity and superior potency. Unfortunately, resistance to all of these classes of drugs has been observed, leading to the need for continuous discovery and development of new classes of anthelmintics, particularly those with novel modes of action (McKellar and Jackson, 2004).

We have had a long standing interest in the discovery of anthelmintics going back to avermectin using various screening strategies. Our current strategy involves the screening of extracts of plants, bacterial and fungal fermentations employing *in vitro* measurement of *H. contortus* inhibition of motility in a high throughput assay (Michael et al., 2001). This species is one of the most prevalent parasitic worms that infect small ruminants. The *in vitro* activity is followed up by measurement of the activity in an *in vivo* model using systemic infection by *Heligmosomoides polygyrus* in mice (Fonseca-Salamanca et al., 2003). The

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methanol extract of *Struthiola argentea* (Thymelaeaceae) whole plant exhibited *in vitro* activity and was therefore selected for further study. Bioassay-guided fractionation using the *in vitro H. contortus* assay led to isolation of three active flavones **1**–**3**, including the new flavone **3**. The isolation, structure elucidation and evaluation of *in vitro* and *in vivo* activities of these compounds along with a series of over 20 other flavones are described.

2. Results and discussion

The whole plant of *S. argentea* was extracted with methanol and was partitioned between hexanes and MeOH/H_2O, then CH_2Cl_2 and MeOH/H_2O. White insoluble material at the interface of the CH_2Cl_2 partitions was filtered off and identified as nearly pure yuankanin (**4**) from HRMS and ^{1}H NMR data. The ^{1}H NMR spectroscopic data for **4** is given in Section 4 since the only data in the literature appears to be from a 1973 report of the TMS derivative (Núnez-Alarcón et al., 1973). To ensure the correct assignments for **4**, a ROESY experiment was conducted. Key correlations observed were from the methoxy protons to H-6 and H-8 of the flavone, and from H-1″ (anomeric) of the glucose moiety to H-6 of the flavone.

The hexanes and methylene chloride extracts were combined and chromatographed on reversed-phase (C_8) HPLC in acetonitrile/water followed by reversed-phase (C_{18}) HPLC in methanol/water to give flavones **1**–**3** and **5**. Flavone **1** was identified as 5,6,2′,6′-tetramethoxyflavone (zapotin), and **2** was identified as 5,6,2′-trimethoxyflavone by comparison of the UV, MS, and NMR spectroscopic data of these compounds to the corresponding literature data (Dreyer and Bertelli, 1967; Meyer et al., 1985; Ito et al., 1998; Budzianowski et al., 2005). Amentoflavone (**5**) was also identified by comparison of ^{1}H NMR spectroscopic data to the literature (Terashima et al., 1999). Compounds **1** and **2** have previously been reported to have antitumor (Meyer et al., 1985) and antimutagenic properties (Ito et al., 1998).

Compound **3** was obtained as a pale yellow solid. Its molecular formula, $C_{21}H_{20}O_9$, was deduced from the molecular ion of 417.1178 $[M+H]^+$ in the HRESIFTMS. The carbon count was confirmed by analysis of the ^{13}C NMR spectrum, which showed 21 signals. The ^{1}H NMR spectrum showed 20 protons, including five three-proton singlets at δ_H 3.99, 3.97, 3.92, 3.90, and 3.79 ppm, which indicated the presence of five methoxy groups. The two-proton singlet at δ_H 6.00 ppm was consistent with the presence of a methylenedioxy group. The remaining protons present in the ^{1}H NMR spectrum were a one-proton singlet at δ_H 6.26 ppm, as well as two one-proton doublets each with $J = 9$ Hz resonating at δ_H 7.28 and 7.19 ppm (see Fig. 1).

The UV, NMR, and MS data for **3** suggested it to be a flavone that consisted of five methoxy groups and a methylenedioxy group. The doublets at δ_H 7.28 and 7.19 ppm correlated to one another in the ^{1}H,^{1}H-COSY spectrum,

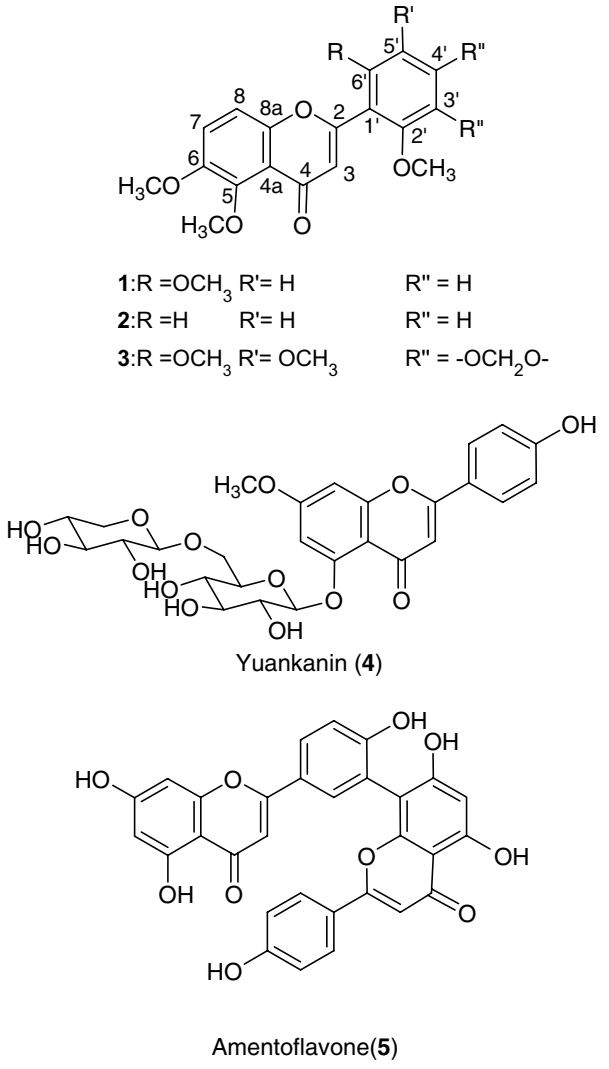


Fig. 1. Structures of compounds **1**–**5**.

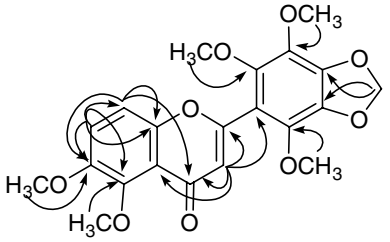


Fig. 2. HMBC correlations of compound **3**.

and were assigned to H-7 and H-8, respectively, by HMBC (Fig. 2). The ^{1}H NMR signal appearing at δ_H 6.26 ppm showed an HMBC correlation to the carbonyl carbon (C-4) at δ_C 177.9 ppm leading to its assignment as H-3. The HMBC correlation of H-3 to C-4a at δ_C 119.0 ppm and its HSQC correlation to δ_C 114.8 (C-3) ruled out the possibility of an isoflavone. All remaining positions were, therefore, substituted. Lack of symmetry in the B ring precluded the substitution of the methylenedioxy group at C5–C6. Furthermore, HMBC correlations of H-7 to two oxygen-

ated carbons C-5 (δ_C 148.0) and C-6 (δ_C 149.8) that also exhibited HMBC correlations to two methoxy group protons resonating at δ_H 3.99 and δ_H 3.92 suggested that C-5 and C-6 possessed methoxy substitutions. The remaining three methoxy groups and the methylenedioxy group substituted the five positions of the B ring. The methylenedioxy could occupy C-2′ and C-3′, or C-3′ and C-4′. 1D NOE-difference NMR spectroscopy was used to ascertain the substitution pattern of ring B (Fig. 3). Irradiation of the singlet at δ_H 6.26 ppm (H-3) produced nOe enhancements of the methoxy groups δ_H 3.90 (2′-OCH_3) and δ_H 3.79 (6′-OCH_3) indicating that C-2′ and C-6′ were substituted with methoxy groups (Fig. 3) leading to the substitution of the methylenedioxy group at C-3′ and C-4′. Therefore, the remaining methoxy group was located at C-5′ (δ_H 3.97), which was confirmed by the nOe enhancement observed upon irradiation of the methoxy signal at δ_H 3.79 (Fig. 3).

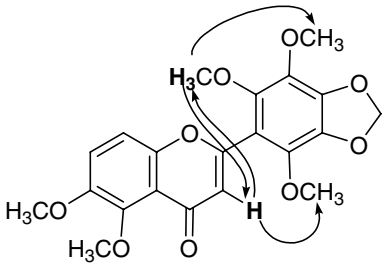
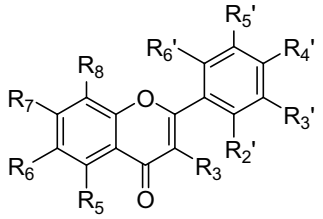


Fig. 3. Nuclear Overhauser enhancements of compound **3**.

These compounds were tested in the *in vitro* assay and data is summarized in Table 1. To study structure activity relationships of methoxylated flavones for the anthelmintic properties, we acquired a series of commercially available methoxylated flavones (**6**–**25**). The *in vitro* data are presented as a measure of effective concentration (EC_{90}) in which a particular compound concentration caused 90% inhibition of larval motility compared to the control. The new flavone **3** exhibited the most potent activity with an EC_{90} of 3.1 μg/mL which is significantly (>17-fold) less

Table 1
Anthelmintic activities of flavones



	R_3	R_5	R_6	R_7	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$	$R_{6'}$	*In vitro*[a] (EC_{90})	*In vivo*[b]
1	H	OMe	OMe	H	OMe	H	H	H	OMe	22	NT
2	H	OMe	OMe	H	OMe	H	H	H	H	19	NA
3	H	OMe	OMe	H	OMe	–OCH_2O–		OMe	OMe	3.1	NT
4	H	O-disacc	H	OMe	H	H	OH	H	H	>100	NT
5	H	OH	H	OH	H	8-Apigenin	OH	H	H	>100	NT
6	H	OMe	OMe	OMe	H	OMe	OMe	OMe	H	>100	NT
7	H	H	H	OMe	H	OMe	OMe	OMe	H	>100	NT
8	OMe	H	OMe	H	OMe	OMe	H	H	H	53	NT
9	H	OMe	H	OMe	H	OMe	OMe	H	H	>100	NT
10	OMe	H	OMe	H	H	OMe	OMe	H	H	>100	NT
11	H	OMe	OMe	OMe	H	H	H	H	H	32	NT
12	H	H	OMe	H	OMe	H	OMe	H	H	>100	NT
13	OMe	H	H	OMe	H	OMe	H	H	H	>100	NT
14	H	OMe	H	OMe	H	H	OMe	H	H	18	13
15	H	H	OMe	H	OMe	OMe	H	H	H	10	49
16	H	H	H	OMe	H	OMe	OMe	H	H	>100	NT
17	OMe	H	OMe	H	H	OMe	H	H	H	7.9	NA
18	H	H	H	OMe	OMe	H	OMe	H	H	>100	NT
19	H	H	H	OMe	OMe	OMe	H	H	H	10	NA
20	OMe	H	OMe	H	OMe	H	H	H	H	61	NT
21	H	OMe	H	OMe	OMe	H	H	H	H	>100	NT
22	H	OMe	H	OMe	H	OMe	H	H	H	>100	NT
23	H	OH	OMe	OMe	H	OH	OMe	H	H	>100	NT
24	H	OMe	H	H	OMe	H	H	H	H	>100	NT
25	H	H	OMe	H	OMe	H	H	H	H	>100	NT
Ivermectin	–	–	–	–	–	–	–	–	–	0.18	98

[a] *In vitro* assay against *H. contortus*. EC_{90} indicates effective concentration (in μg/mL) at which 90% loss of motility of the larvae was observed. The EC_{90} data are an average of two experiments.

[b] *In vivo* assay against *H. polygyrus* in mice at a dose of 25 mg/kg intramuscular. Number shown is % reduction in worm counts compared to control. Ivermectin is positive control, tested at 10 mg/kg. NT, not tested; NA, not active at 25 mg/kg.

active than the ivermectin control ($EC_{90} = 0.18$ μg/mL). All other flavones exhibited EC_{90} ranging from 7.9 to >100 μg/mL. The most active of the commercial flavones *in vitro* were the trimethoxyflavones **14**, **15**, **17**, and **19**. These four flavones were selected for *in vivo* evaluation using the *H. polygyrus* mouse model (Fonseca-Salamanca et al., 2003). Three mice were used for each study dosed intramuscularly at 25 mg/kg and the results are also presented in Table 1. Only compound **15** (6,2′,3′-trimethoxyflavone) exhibited modest *in vivo* activity with a $49 \pm 10\%$ reduction of worm counts compared to the control group. Compound **3** did not show any *in vivo* activity at 25 mg/kg. Lack of enough quantities prevented *in vivo* evaluations of compounds **1** and **2**. No clear structure activity pattern emerged from this study.

3. Conclusion

We have isolated three anthelmintic flavones from the whole plant extract of *Struthiola argentea*, including a highly substituted new flavone 5,6,2′,5′,6′-pentamethoxy-3′,4′-methylenedioxyflavone (**3**). This is the first report of secondary metabolites from the genus *Struthiola*, as well as the first report of the anthelmintic activity of flavones. An attempt was made to determine anthelmintic structure activity relationships of flavones; however, no clear pattern emerged.

4. Experimental

4.1. General

All reagents were obtained from Sigma–Aldrich and were used without further purification. NMR spectra were obtained on a Varian Inova 500 MHz spectrometer operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei. IR spectral data were obtained on a Perkin–Elmer Spectrum One spectrometer. UV/VIS spectra were collected on a Perkin–Elmer Lambda 35 UV/VIS spectrometer. Low-resolution mass spectra were obtained on an Agilent MSD, and high-resolution mass spectra were obtained on a Thermo Finnigan LTQ-FT with the standard Ion Max API source (without the sweep cone) and ESI probe. Flavones **6**–**25** were purchased from Indofine Chemical Company (Hillsborough, NJ). Ivermectin was obtained as the commercial product Ivomec Injection (1%) for Cattle and Swine (Merial, Duluth, GA). Swiss Webster mice were obtained from Taconic Labs (Germantown, NY).

4.2. Plant material

Whole plants of *S. argentea* were collected in South Africa, Eastern Cape, Willowmore, in October 2000. Voucher specimens are stored at the New York Botanical Garden, Bronx, NY (RB291A).

4.3. Extraction and isolation

The whole plant of *S. argentea* (1.25 kg) was extracted with methanol (5 L) at room temperature for 5 days. The solvent was removed *in vacuo* yielding 45.2 g of extract. A 12.7 g portion of the methanol extract was dissolved in 170 mL of 9:1 MeOH/H_2O and extracted with hexanes (3×170 mL). The 9:1 MeOH/H_2O layer was diluted with water to 3:2 MeOH/H_2O and extracted with CH_2Cl_2 (3×250 mL). Insoluble yuankanin (**4**) (625 mg) at the interface was removed by filtration. The CH_2Cl_2 and hexane layers were dried over Na_2SO_4, combined, and evaporated to give a dark green material (1.56 g). This extract was dissolved in a minimum volume of a mixture of 1:1 acetone/MeOH and further fractionated by preparative HPLC (Zorbax RX-C_8, 250×21.2 mm, 25–100% MeCN in H_2O, both w/0.1% TFA, over 40 min, 10 mL/min) in multiple injections. Fractions were collected every 0.5 min, 80 fractions total. Fractions 36–40 contained pure **5** (104.9 mg), compound **1** was present in fractions 48–49, **2** was present in fractions 50 and 51, and **3** was present in fractions 51 and 52. Semi-preparative HPLC was used for final purification of **1**–**3** (Zorbax RX-C_{18}, 250×9.4 mm, 25–100% MeOH in H_2O, both w/0.1% TFA over 20 min, 3 mL/min) in multiple injections yielding **1**: 3.0 mg, **2**: 3.1 mg, **3**: 16.0 mg.

4.4. Characterization data

4.4.1. 5,6,2′,5′,6′-Pentamethoxy-3′,4′-methylenedioxyflavone (*3*)

Pale yellow solid: IR ν_{max} (ZnSe film): 2940, 2842, 1641, 1479, 1418, 1355, 1283, 1047 cm^{-1}; UV λ_{max} (MeOH) nm (log ε): 203 (4.48), 230 (4.35), 329 (3.82); ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.28 (1H, *d*, $J = 9$ Hz, H-7), 7.19 (1H, *d*, $J = 9$ Hz, H-8), 6.26 (1H, *s*, H-3), 6.00 (2H, *s*, –OCH_2O–), 3.99 (3H, *s*, C-5 –OCH_3), 3.97 (3H, *s*, C-5′ –OCH_3), 3.92 (3H, *s*, C-6 –OCH_3), 3.90 (3H, *s*, C-2′ –OCH_3), 3.79 (3H, *s*, C-6′ –OCH_3); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 177.9 (C-4), 158.22 (C-2), 152.4 (C-8a), 149.8 (C-6), 148.0 (C-5), 146.3 (C-6′), 141.6 (C-3′ or C-4′), 136.4 (C-2′), 134.4 (C-3′ or C-4′), 133.2 (C-5′), 119.1 (C-7), 119.0 (C-4a), 114.8 (C-3), 113.6 (C-1′), 113.5 (C-8), 101.9 (–O*C*H_2O–) 62.1 (C-6′ –O*C*H_3), 61.9 (C-5 –O*C*H_3), 60.6 (C-5′ –O*C*H_3), 60.4 (C-2′ –O*C*H_3), 57.3 (C-6 –O*C*H_3); HRESIFTMS *m/z* 417.1178 (calcd for $C_{21}H_{20}O_9 + H$, 417.1185).

4.4.2. Yuankanin (*4*)

White amorphous powder: ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 7.90 (2H, *d*, $J = 8.5$ Hz, H-2′, H-6′), 7.01 (1H, *d*, $J = 2.5$ Hz, H-8), 6.91 (2H, *d*, $J = 8.5$ Hz, H-3′, H-5′), 6.86 (1H, *d*, $J = 2.5$ Hz, H-6), 6.68 (1H, *s*, H-3), 4.78 (1H, *d*, $J = 7.5$ Hz, H-1″), 4.19 (1H, *d*, $J = 7.5$ Hz, H-1‴), 3.97 (1H, *d*, $J = 10.5$ Hz, H-6″a), 3.88 (3H, *s*, –OCH_3), 3.69 (1H, *dd*, $J = 10.5$, 5.0, H-5‴eq), 3.65 (1H, *dd*, $J = 10.5$, 6.5, H-6″b), 3.57 (1H, *dd*, $J = 9.0$, 6.5, H-5″), 3.37 (1H, *t*, $J = 8.0$, H-2″), 3.29–3.30 (2H, *m*, H-3″,

H-4‴), 3.21 (1H, *t*, $J = 9.0$, H-4″), 3.11 (1H, *t*, $J = 9.0$ Hz, H-3‴), 3.01 (1H, *t*, $J = 11.0$ Hz, H-5‴ax), 2.98 (1H, *t*, $J = 9.0$ Hz, H-2‴). HRESIFTMS *m/z* 579.1705 (calcd for $C_{27}H_{30}O_{14} + H$, 579.1713).

4.5. Biological assays

The *in vitro* assay against *H. contortus* was used as described previously (Michael et al., 2001). The *in vivo* mouse assay was modified from Fonseca-Salamanca et al. (2003) and performed as follows: Swiss Webster mice (≈30 g) were inoculated with 200–400 L3 *H. polygyrus* larvae. The mice were checked for infection around day 12 post inoculation then dosed IM (intramuscularly) with test compound in triplicate. On day three post-treatment, mice were euthanized and the intestine was collected (from below stomach and above cecum), opened, and placed in sterile H_2O (5 ml). Mucosa was scraped and rinsed through a 200-mesh screen. The rinseate was then examined for the presence of worms and worms were counted. Worm counts for treated mice were then compared to infected, untreated mice as negative controls, and infected mice treated with ivermectin (10 mg/kg) as positive controls.

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