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Antigiardial Activity of Isoflavones from *Dalbergia frutescens* Bark

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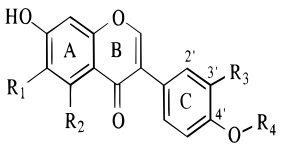
Several isoflavones [formononetin (**1**), castanin (**5**), odoratin (**6**), glycitein (**7**), pseudobaptogenin (**8**), fujikinetin (**9**), and cuneatin (**10**)] were isolated from *Dalbergia frutescens*, and their antiprotozoal activities were determined against *Giardia intestinalis*. Among these compounds, formononetin (**1**) was the most potent antigiardial agent, with an IC_{50} value of 30 ng/mL (approximately 0.1 μM), as compared to the value for metronidazole, the current drug of choice, of 100 ng/mL (approximately 0.6 μM). Three isoflavones closely related to formononetin [daidzein (**2**), biochanin A (**3**) and genistein (**4**)] were also evaluated, but they were at least 100 times less active than **1**. Formononetin (**1**) may thus be an interesting lead for development of new antigiardial agents or as a probe for a new mechanistic target.

Giardia intestinalis (also know as *Giardia lamblia*) is a flagellated protozoan parasite most frequently the cause of intestinal protozoal infections in the world [1−3] and is a common cause of waterborne diarrhea in North America.[4] In some countries, 20−30% of the population are infected.[2] Giardiasis is especially prevalent in infants and children in the developing world and can have devastating effects becauses it causes malabsorption and thus, malnutrition.[2]

Three classes of drugs are currently utilized for the treatment of giardiasis: metronidazole and derivatives; mepacrine and analogues; and nitrofurans, such as furazolidone. Metronidazole is the most widely used and is generally effective and well-tolerated. However, it has not yet received approval by the U. S. Food and Drug Administration for the indication of giardiasis. Further, treatment failures have occurred in up to 20% of patients, and reports of resistance have appeared.[2] The toxicity of metronidazole is also of concern, with gastrointestinal upset, headache, nausea, leukopenia, and an unpleasant taste commonly reported.[5] Though less frequent, neurotoxic effects, manifested as dizziness, incoordination, ataxia, and convulsions, are encountered.[6] Metronidazole has also been shown to be mutagenic,[7] though the clinical significance of this is not established. Mepacrine is no longer available in the United States, being replaced for most applications with safer and more specific drugs. Furazolidone also has serious drawbacks such as gastrointestinal disturbances, hemolytic anemia, disulfiram-like reactions to alcohol, hypersensitivity reactions, as well as evidence of tumorigenicity in rodent studies.[8] Thus, there is an evident continuing need for effective and safer agents for the chemotherapy of giardiasis.

As part of our program in the discovery of potential new pharmaceuticals from natural sources, several extracts of the bark of *Dalbergia frutescens* (Vell.) Britton (Fabaceae) showed good activity against *G. intestinalis*. This study reports on the antigiardial activity of the isoflavones (**1**, **5**−**10**) isolated from this plant, together with three related commercially available compounds (**2**−**4**). The structures of the isolated compounds were confirmed by mass spectrometry, ^1H and ^{13}C NMR spectral data analysis, and comparison with values previously reported. [9−11]



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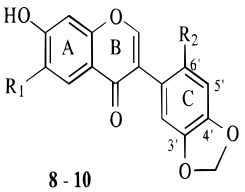

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Compound	R_1	R_2	R_3	R_4
1	H	H	H	CH_3
2	H	H	H	H
3	H	OH	H	CH_3
4	H	OH	H	H
5	OCH_3	H	H	CH_3
6	OCH_3	H	OH	CH_3
7	OCH_3	H	H	H
8	H	H		
9	OCH_3	H		
10	H	OCH_3		

The antigiardial activity of formononetin (**1**) is shown in Figure 1. The 48-h IC_{50} value of **1** was approximately 0.03 μg/mL (approximately 0.1 μM), compared to metronidazole, which had an IC_{50} value of approximately 0.1 μg/mL (approximately 0.6 μM) in our assay. In time−course studies, **1** impaired motility and reduced viability of *G. intestinalis* at 2 and 4 h of exposure (IC_{50} < 1 μg/mL), as contrasted with metronidazole, which had no effect on *G. intestinalis* cultures at these time points.

The initial extraction and screening of several hundred plants led to four extracts of *D. frutescens* with activity at <56 μg/mL. Confirmation and further evaluation showed the highest activity in the less polar hexane/ethyl acetate and ethyl acetate extracts (IC_{50} values of 8 and 11 μg/mL, respectively). Follow-up isolation permitted the activity to

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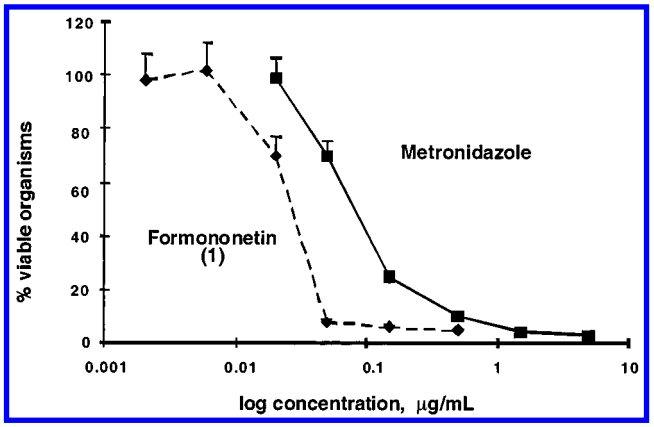

Figure 1. Concentration−response curves for formononetin (**1**) and metronidazole against *G. intestinalis*. Determinations were made at 48 h after dosing. Values are the mean ± SEM of four replicates.

Table 1. In Vitro Antigiardial Activity of Isoflavones

compound	IC_{50}
1	0.03 ± 0.01
2	3.75 ± 0.8
3	3.50 ± 0.65
4	>5.0
5	>5.0
6	NA[b]
7	NA[b]
8	0.56 ± 0.11
9	1.5 ± 0.45
10	NA[b]
Met[c]	0.1 ± 0.04

[a] Data presented are mean IC_{50} values (μg/mL) ± SEM, $n = 3$. [b] NA = no activity at 10 μg/mL. [c] Met = metronidazole.

be traced to several known isoflavones [formononetin (**1**), pseudobaptogenin (**8**), and fujikinetin (**9**)]. The active compounds were characterized, and several related isoflavones were also obtained [castanin (**5**), odoratin (**6**), glycitein (**7**), and cuneatin (**10**)]. Because **1** was the most active, three structurally related and commonly encountered isoflavones were also evaluated [daidzein (**2**), biochanin A (**3**), and genistein (**4**)]. The antigiardial assay results are shown in Table 1. Comparison of activity across this limited series suggests some structural specificity for the antigiardial activity of this class. The two most active compounds were formononetin (**1**) and pseudobaptigenin (**8**). The only difference between **1** and **8** is the presence of a methylenedioxy group attached to the C ring at C-3 and C-4. Within the methylenedioxy series (compounds **8**−**10**), the OCH_3 substitution in the A ring (**9**) results in a decrease in activity.

No cytotoxicity for formononetin (**1**) was observed in the Vero cell line at concentrations up to 50 μg/mL. Dosing of mice with formononetin (**1**) 40 mg/kg orally, once daily for 3 days, resulted in no observable adverse effects (grooming, feeding, body weight, gross behavior and appearance).

To establish whether the observed antigiardial activity could be extended to an in vivo setting, the efficacy of formononetin (**1**) was evaluated in a murine model of giardiasis.[12,13] Prior experiments failed to show any effect of **1** on cure rates or intestinal parasite burden at 1 mg/day for 4 days. In contrast, two experiments showed significantly increased cure rates, as well as decreased parasite numbers in the intestines after 4 days of treatment at 10 mg/day for 4 days (Table 2). Administration of vehicle had no effect on either cure rates or parasite burden. The findings indicate that, although **1** is active, relatively large doses are required. Further studies are needed to determine whether this may be due to poor delivery (due to stability, protein binding, etc.) of the compound to the site of action (upper small intestine).

Table 2. In Vivo Antigiardial Activity of Formononetin (**1**)[a]

group	infected/ total	trophs/hpf
control	10/10	1.8 ± 2.3
formononetin (**1**) (10 mg/day)	3/10**	0.1 ± 0.0*
control	10/10	9.7 ± 9.4
formononetin (**1**) (10 mg/day)	2/8**	0.6 ± 1.6*
control	10/10	14.1 ± 9.5
vehicle	10/10	10.9 ± 9.2

[a] All animals were infected on day 0 of the protocol, and treatment was on days 3−6. See Experimental Section for details. *$p < 0.05$, **$p < 0.005$ by two-tailed Student's *t*-test.

A previous report of antigiardial activity of plant flavonoids indicated that (−)-epicatechin was the most active of a series of 18 compounds, with an IC_{50} value of 1−2 μg/mL.[17] However, no isoflavones were evaluated in this study.

Formononetin (**1**), pseudobaptigenin (**8**), and some of their analogues have marked in vitro antigiardial activity, with the best at about five times more potent in vitro than the current drug of choice, metronidazole. In addition, the selectivity of the antigiardial effect of **1** is noteworthy, with no toxicity observed to the mammalian cell line. These isoflavones are highly effective as antigiardial agents in vitro and appear to act by a novel mechanism, as suggested by the time-course of the cytocidal activity. Also suggestive of a different mechanism is the fact that metronidazole, the current drug of choice, has marked activity against *Trichomonas* species,[18] while formononetin (**1**) was inactive at concentrations up to 25 μg/mL.[19]

Experimental Section

General Experimental Procedures. Separations were by HPLC utilizing a Waters LC module, with UV detection at 254 nm. Solvents were HPLC grade and obtained from Fisher Scientific. Mass spectra were obtained on a Bruker BioApex 3.0 system, and ^1H and ^{13}C NMR spectra on a Bruker Avance DRX-500 FT NMR spectrometer operating at 500 and 125 MHz, respectively.

Plant Material. *Dalbergia frutescens* (Fabaceae) stem bark specimens were collected in Venezuela in May 1994. Identification was determined by Dr. Charles Burandt from a voucher specimen (#13005), now maintained at the National Center for Natural Products Research at the University of Mississippi.

Extraction and Isolation. The pulverized bark (200 g) was successively extracted, after an initial hydration with 60% MeOH/water with hexane, 50% hexane/ethyl acetate, ethyl acetate, and 95% ethanol at 40 °C with overhead stirring. All four extracts of the original plant material showed activity in the original screening at a concentration of <56 μg/mL. Confirmation and further evaluation showed the highest activity in the hexane/ethyl acetate and ethyl acetate extracts (approximate IC_{50} values of 8 and 11 μg/mL of extract, respectively). These two extracts were combined for chemical investigation. The combined extract (8.5 g) was loaded on a Si gel column, which was eluted with 20% ethyl acetate/toluene to ethyl acetate and then washed with methanol. Fractions (1−640) were collected and pooled according to their TLC patterns; bioactivity in antigiardial screening showed nine active pools (30C−30K), which were rechromatographed on a Si gel column with a step gradient of $CHCl_3$ in methanol. The active fractions (56 mg) were further resolved and finally purified by HPLC over a reversed-phase C_{18} column (Ultracarb 5 ODS 30, 250 × 10 mm, Phenomenex) eluted with 50% MeOH/H_2O (0.8% TFA) to obtain compound **1** ($IC_{50} = 0.03$ μg/mL) and **5**−**10** in quantities of 2−3 mg each. Compounds **2**

(daidzein), **3** (biochanin A), and **4** (genistein) are closely related structural analogues of formononetin (**1**), differing only in the absence of the methyl group at the 4′-hydroxyl and/or the presence of the hydroxyl function at C-5. These compounds were purchased from Indofine Chemical Company, Inc. (Somerville, NJ).

Antigiardial and Cytotoxicity Assays. *Giardia intestinalis* (ATCC 30888) was grown in Keister's modified TYI-S-33 medium under nitrogen at 37 °C.[14] For each assay, 250 μL of Keister's medium containing *G. intestinalis* cells, at a concentration of 10^6/mL, were added to each well of a 96-well microplate. The plates were incubated in a modular incubator under nitrogen for 24 h at 37 °C. At 24 h, 50 μL of the serial dilutions of the crude extract were added to each well in duplicate at final concentrations of 500, 166, and 56 μg/mL. Metronidazole was used as the positive control, at concentrations of 0.5 to 5 μg/mL. Blanks and vehicle controls were included in each assay. The plates were then incubated for an additional 48 h. The viability of *G. intestinalis* was determined using a modified tetrazolium salt method with XTT.[15]

Mammalian cell cytotoxicity was evaluated using Vero cells (ATCC CCL81), using the method of Borenfreund et al.,[16] modified in regard to medium and incubation times. Cells were seeded at 5×10^4 cells per well in glutamine-supplemented Roswell Park Memorial Institute (RPMI) 1640 medium (with 60 mg/L amikacin and 10% fetal bovine serum) in 96-well microplates. After a 24-h incubation at 37 °C, test compounds were added, and plates were incubated for a further 48 h. Viability of the cells was determined with neutral red dye.[16]

Antigiardial Evaluation In Vivo. Adult C57B female mice (Taconic Farms, Germantown, NY) were utilized. Mice were inoculated by oral gavage with 500 000 trophozoites of the *G. lamblia* GS/H7 strain[13] on day 0. Treatment occurred on days 3−6, and mouse parasite burden was evaluated on day 7. In one experiment, five mice of a group of 10 were treated as described above, and the remaining five were treated for an additional 3 days and sacrificed on day 9. Because the results of both groups were comparable, they were combined and treated as one experiment. For quantitation of parasites, mice were sacrificed and the small intestines dissected out and minced in 10 mL of ice cold *G. intestinalis* medium, allowed to cool for 30 min on ice to dislodge tissue-adhering organisms, and warmed to 37 °C, which made the trophozoite motile and more easily noticeable. The number of motile trophozoites in five random fields at 200× were counted. If no organisms were noted, then a similar evaluation was performed at 25×. Cure was defined as the failure to detect any trophozoites. Results are presented as the number of animals still infected at day 7 in relation to the total number inoculated (infected/total). In those animals with evidence of persistent infection, trophozoites were enumerated; the mean ± S.D. of five fields for each animal (200×) is presented as trophs/hpf (trophozoites/high-power field).

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