Notes

Antimycobacterial Flavonoids from the Leaf Extract of *Galenia africana*

Sannah P. N. Mativandlela,[†] Tshilidzi Muthivhi,[‡] Haruhisa Kikuchi,[§] Yoshiteru Oshima,[§] Chris Hamilton,[⊥] Ahmed A. Hussein,[II] Martha L. van der Walt,[†] Peter J. Houghton,[○] and Namrita Lall*[,▽]

South African Medical Research Council, TB Epidemiology and Intervention Research Unit, South African National Blood Service, Pretoria, South Africa, Graduate School of Pharmaceutical Sciences, Tohuku University, Sendai, Japan, School of Chemistry Science and Pharmacy, University of East Anglia, Norwich, NR4-7TJ, U.K., Chemistry of Medicinal Plants Department, Pharmaceutical Sciences Division, National Research Centre, Dokki, Cairo, Egypt, Department of Pharmacy, Franklin-Wilkins Building, 150 Stamford Street, King's College London, London, SE1 9NN, U.K., and Department of Plant Science, University of Pretoria, Pretoria, South Africa

Received December 8, 2008

The bioassay-guided fractionation of the EtOH extract of the leaves of *Galenia africana* led to the isolation of three known flavonoids, (2*S*)-5,7,2′-trihydroxyflavanone (**1**), (*E*)-3,2′,4′-trihydroxychalcone (**2**), and (*E*)-2′,4′-dihydroxychalcone (**3**), and the new (*E*)-3,2′,4′-trihydroxy-3′-methoxychalcone (**4**). Compounds **1** and **3** exhibited moderate antituberculosis activity. During synergistic studies, a combination of compound **4** and an existing antituberculosis drug, isoniazid, reduced their original MICs 4-fold, resulting in a fractional inhibitory concentration of 0.50. The most pronounced effect was demonstrated by compound **1** and isoniazid reducing their MICs 16-fold and resulting in an FIC of 0.12. Both EtOH extract and isolated compounds failed to exhibit any NADPH oxidase activity at 800.0 μM concentrations, indicating that mycothiol disulfide reductase is not the target for their antituberculosis activity.

The occurrence of multidrug resistance among *Mycobacterium tuberculosis* needs surveillance and control. The entry of *M. tuberculosis* into the host macrophages is the key component of TB pathogenesis. How this process occurs remains poorly understood, but one mechanism may involve the migration of macrophages containing *M. tuberculosis* across the alveoli to lymph nodes.

First-line drugs used for the treatment of tuberculosis (TB) include isoniazid (INH), rifampicin (RIF), ethambutol (EMB), streptomycin (STR), and pyrazinamide (PZA).[1] Although the existing standard regimen is very effective against TB, the long treatment duration (6 months), the toxicity, and the potential for drug−drug interactions, particularly in the setting of antiretroviral treatment, are all factors underlining the need for new antituberculosis drugs.[2]

Aerial parts of *Galenia africana* L. var. *africana* are being used in South Africa to treat venereal sores, asthma, coughs, wounds, eye infections, TB, and skin diseases. Indigenous tribes chew the leaves to relieve toothache.[3,4] No phytochemical study has been conducted previously on this plant. As part of our search for finding new TB agents from native South African medicinal plants, we report herein the isolation and identification of three known flavonoids, (2*S*)-5,7,2′-trihydroxyflavanone (**1**), (*E*)-3,2′,4′-trihydroxychalcone (**2**), and (*E*)-2′,4′-dihydroxychalcone (**3**), and the new (*E*)-3,2′,4′-trihydroxy-3′-methoxychalcone (**4**). The identities of the known compounds **1**, **2**, and **3** were established by comparing their observed and reported physical data.[5−7] The structure of the new compound was elucidated particularly on the basis of its NMR and MS data.

The EtOH extract of the leaves of *G. africana* was fractionated using silica gel column chromatography as well as gel permeation chromatography−high-pressure liquid chromatography (GPC-HPLC) to afford compounds **1**, **2**, **3**, and **4**. Compounds **1**, **2**, and **3** have been isolated from other plants (*Scutellaria amabilis*, *Muntingia calabura*, and *Zuccagnia punctata*); however, compound **4** has not been isolated from natural sources.[8−10] This is the first report of all four compounds from *G. africana*.

Compound **4** was obtained as an amorphous solid, and its molecular formula, $C_{16}H_{14}O_5$, was established by the HREIMS data observed at *m/z* 286.0829 (calcd for $C_{16}H_{14}O_5$, $[M]^+$, 286.0841, Δ −1.2 mmu). The ^{13}C NMR spectrum showed signals due to one carbonyl carbon (δ_C 193.5), six sp^2 quaternary carbons (δ_C 159.8, 158.8, 157.9, 137.2, 135.8, 115.2), eight sp^2 methine carbons (δ_C 145.1, 130.8, 127.7, 121.6, 121.2, 118.7, 116.1, 108.4), and one methoxy carbon (δ_C 60.5). The ^1H NMR and COSY data of **4** indicated the presence of a 1,3-disubstituted benzene ring (δ_H 7.33 (1H, dt, J = 7.7, 1.0 Hz, H-6), δ_H 7.26−7.30 (2H, m, H-2 and 5), and δ_H 6.94 (1H, ddd, J = 8.0, 2.5, 1.0 Hz, H-4)), a 1,2,3,4-tetrasubstituted benzene ring (δ_H 7.94 (1H, d, J = 9.1 Hz, H-6′) and δ_H 6.52 (1H, d, J = 9.1 Hz, H-5′)), two *E*-olefinic protons (δ_H 7.87 (1H, d, J = 15.4 Hz, H-α) and δ_H 7.80 (1H, d, J = 15.4 Hz, H-β)), and a methoxy group (δ_H 3.85 (3H, s)) (Figure 2). In the HMBC spectrum of **4**, the correlations of H-5 to C-1 and C-3 revealed the connection between C-1/C-6 and C-3/C-4 in a 1,3-disubstituted benzene ring. The correlations of H-5′ to C-1′ and C-3′; H-6′ to C-2′ and C-4′; and *O*-methyl protons to C-3′ indicated that C-6′−C-1′−C-2′−C-3′−C-4′−C-5′ were connected and a methoxy group was attached to C-3′ in a 1,2,3,4-tetrasubstituted benzene ring. HMBC correlations were observed for H-α, H-β, and H-6′ to a carbonyl carbon; H-α to C-1; and H-β to C-6, suggesting that two benzene rings were linked by an α,β-unsaturated carbonyl moiety (Figure 2). Therefore, the structure of **4** was deduced to be the new (*E*)-3,2′,4′-trihydroxy-3′-methoxychalcone.

The EtOH extract of *G. africana* and the isolated compounds were investigated for their antituberculosis activity. The EtOH extract was found to exhibit an MIC of 0.78 and 1.20 mg/mL against

* To whom correspondence should be addressed. Tel: +27 12 420 2524. Fax: +27 12 325 5099. E-mail: namrita.lall@up.ac.za.
[†] SAMRC Pretoria.
[‡] SANBS South Africa.
[§] Tohuku University.
[⊥] East Anglia University.
[II] National Research Centre, Egypt.
[○] King's College London.
[▽] Pretoria University.

10.1021/np800778b CCC: $40.75
Published on Web 11/17/2009

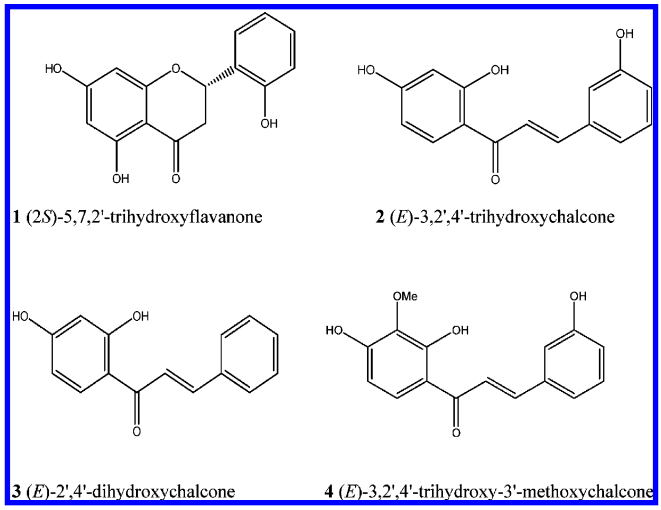

Figure 1. Structures of isolated compounds from *G. africana*.

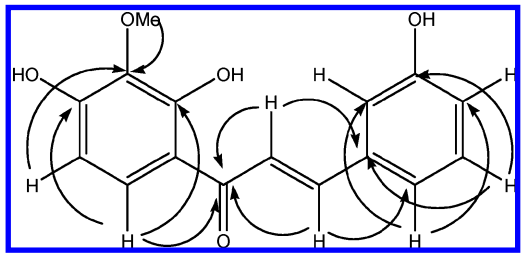

Figure 2. HMBC correlations of compound **4**.

Mycobacterium smegmatis and *M. tuberculosis*, respectively. The MIC of **1** was found to be 110.2 and 367.6 μM against *M. smegmatis* and *M. tuberculosis*, respectively. Compound **3** exhibited an MIC of 195.3 μM against *M. tuberculosis* (Table 1). The isolated compounds were found to be more bactericidal than the EtOH extract, which resulted in 99.5% killing of *M. tuberculosis*. During synergistic studies, a combination of compound **4** and INH reduced their original MICs 4-fold, resulting in an FIC of 0.50. The most pronounced effect was demonstrated by compound **1** and INH, reducing their MICs 16-fold and resulting in an FIC of 0.12.

The EtOH extracts of *G. africana* and purified compounds were tested for cytotoxicity in human macrophage U937 cell lines. IC_{50} values of samples are presented in Table 1. Effective doses of antimycobacterial drugs were evaluated in a macrophage model to ensure intracellular drug effectiveness of compounds. The antituberculosis activity of isolated compounds against *M. tuberculosis* residing within U937 macrophage cells showed good inhibitory activity (Table 1). The low MIC of 0.05 mg/mL shown by the EtOH extract of *G. africana* in TB-infected macrophages as compared to the MIC of 1.20 mg/mL of the extract against the sole organism indicated that the EtOH extract could be absorbed well by macrophages, leading to the increased interaction with the bacteria.

The EtOH extract and the purified compounds were also screened for their inhibitory activity against mycothiol disulfide reductase (Mtr). Mtr is an NADPH-dependent oxidoreductase responsible for maintaining the high thiol:disulfide ratio of mycothiol within the mycobacteria.[11] Inhibition of Mtr increases susceptibility to oxidative stress. Molecules bearing Michael-acceptor motifs sometimes operate as covalent (time-dependent) inhibitors of similar disulfide reductases (e.g., trypanothione reductases) via conjugate addition with an active site cysteine thiol.[12] Both EtOH extract and isolated compounds failed to exhibit any NADPH oxidase activity at 800.0 μM concentrations, indicating that Mtr is not the target for their antituberculosis activity.

Thus, the EtOH extract of *G. africana* and its purified compounds showed moderate antituberculosis activity. However, the synergistic activity of purified compounds with the antituberculosis drug INH was significant. It will be worthwhile evaluating the efficacy of purified compounds in combination with TB drugs in preclinical studies.

Experimental Section

General Experimental Procedures. Optical rotations were obtained with a P-1030 polarimeter (JASCO). NMR spectra were recorded on a JNM ECA-600 (JEOL). Chemical shifts for ^{1}H and ^{13}C NMR are given in parts per million (δ) relative to TMS (δH 0.00) and residual solvent signals (δ_C 49.0 and 29.8 for methanol-d_4 and acetone-d_6, respectively) as internal standards. Mass spectra were measured on JMS AX-500 and AX-700 (JEOL) instruments. Analytical TLC was performed on silica gel 60 F_{254} (Merck). Column chromatography was carried out on silica gel 60 (70−230 mesh, Merck). The GPC-HPLC (20.0 mm × 500 mm) was carried out on an LC-908W instrument (Japan Analytical Industry). For antituberculosis activity tests, *M. tuberculosis* H37Rv (ATCC 27264) was obtained from American Type Culture Collection and INH was obtained from Sigma Chemical Co. (Sigma-Aldrich, South Africa). For cytotoxicity tests, U937 cell lines and RPMI 1640 (developed at Roswell Park Memorial Institute) were obtained from Highveld Biologicals Co. (South Africa). Recombinant *M. tuberculosis* mycothione reductase was overexpressed and purified from *M. smegmatis* MC^2 155. Other chemicals and reagents used were of analytical grade.

Plant Material. The leaves of *G. africana* were collected in the Northern Cape, South Africa. A voucher specimen (93723) was deposited and identified by Ms. Magda Nel, an ecologist at the H.G.W.J. Schweickerdt Herbarium (PRU), University of Pretoria, South Africa.

Extraction and Isolation. Extraction of *G. africana* was the same as previously reported.[13] The total concentrated EtOH extract (20.0 g) was subjected to silica gel column chromatography (2.5 kg), eluting with *n*-hexane− EtOAc mixtures of increasing polarity (0 to 100%). Six major fractions were obtained, of which only three showed antimycobacterial activity against *M. tuberculosis*. The three fractions were further subjected to column chromatography as above. Fraction 3 (112.0 mg) was subjected to a GPC HPLC column eluted with $CHCl_3$−MeOH (4:1) to give pure (2*S*)-5,7,2′-trihydroxyflavanone (**1**)

Table 1. Antimycobacterial, Intracellular, and Cytotoxicity Activity of Compounds from *G. africana* Leaves

	M. smegmatis		*M. tuberculosis*		intracellular activity				cytotoxicity
tested sample	MIC[a] (μM)	MBC[b] (μM)	MIC (μM)	ΔGI[c]	% inhibition, MBC	MIC (μM)	ΔGI	MBC (μM)	IC_{50}[d] (μM ± SD)
G. africana (EtOH extract in mg/mL)	0.78	1.56	1.20	0.0 ± 0.0	90.0	0.05	23.0 ± 16.2	12.50	120.0 ± 2.31
(2*S*)-5,7,2′-trihydroxyflavanone (**1**)	110.20	441.10	367.60 (S[e])	8.0 ± 2.8	99.5	367.60 (S)	9.0 ± 6.3	735.10	110.3 ± 2.16
(*E*)-3,2′,4′-trihydroxychalcone (**2**)	na[f]	nt[g]	416.60 (S)	2.0 ± 1.4	99.5	183.80 (S)	18.5 ± 4.9	367.50	415.3 ± 2.16
(*E*)-2′,4′-dihydroxychalcone (**3**)	468.70	234.30	195.30 (S)	2.0 ± 1.4	99.5	195.30 (S)	2.0 ± 1.4	416.50	080.2 ± 1.15
(*E*)-3,2′,4′-trihydroxy-3′-methoxychalcone (**4**)	na	nt	174.80 (S)	19.0 ± 2.6	99.5	104.80 (S)	13.0 ± 0.7	183.70	333.2 ± 1.15
ciprofloxacin (positive drug control for *M. smegmatis*)	1.50	2.20	nd[h]	nd	nd	nd	nd	nd	nd
isoniazid (positive drug control for *M. tuberculosis*)	nd	nd	1.40 (S)	2.0 ± 1.4	nd	2.90 (S)	1.0 ± 0.4	nd	nd
doxorubin (positive for U937 cell lines)	nd	nd	nd	nd	nd	nd	nd	nd	1.1 ± 0.15

[a] MIC. [b] MBC. [c] ΔGI value (mean ± SD) of the control vial (10^{-2}) was 38.0 ± 3.8 for the sensitive strain. [d] IC_{50} concentration of samples on U937 cell line. [e] Susceptible. [f] Not active at the highest concentration tested (620.0 μg/mL). [g] Not tested for MBC determination. [h] Not determined.

Table 2. Synergistic Activity of Compounds **1**−**4** in Combination with Isoniazid against *M. tuberculosis* Using the BACTEC Method

	M. tuberculosis synergistic activity		
compound or combination	MIC[a] (μM)	ΔGI[b]	FIC[c]
(2*S*)-5,7,2′-trihydroxyflavanone (**1**)	367.6 (S[d])	5.0 ± 3.5	nd[e]
(*E*)-3,2′,4′-trihydroxychalcone (**2**)	416.6 (S)	2.0 ± 1.4	nd
(*E*)-2′,4′-dihydroxychalcone (**3**)	195.3 (S)	6.0 ± 4.2	nd
(*E*)-3,2′,4′-trihydroxy-3′-methoxychalcone (**4**)	174.8 (S)	1.0 ± 2.6	nd
INH	1.4 (S)	0.0 ± 0.0	nd
1 + INH	1/16 +1/16 (S)	0.0 ± 0.0	0.12
2 + INH	1/2 +1/2 (S)	2.5 ± 2.1	1.00
3 + INH	1/8 + 1/8 (S)	0.0 ± 0.0	0.25
4 + INH	1/4 +1/4 (S)	10.0 ± 11.3	0.50

[a] MIC. [b] ΔGI value (mean ± SD) of the control vial (10^{-2}) was 32.0 ± 2.6 for the sensitive strain. [c] FIC. [d] Susceptible. [e] Not determined.

Table 3. ^{1}H and ^{13}C NMR Data of (*E*)-3,2′,4′-Trihydroxy-3′-methoxychalcone (**4**)[a]

position	^{13}C	^{1}H
1	137.2	
2	116.1	7.26−7.30 (1H, m)[b]
3	158.8	
4	118.7	6.94 (1H, ddd, J = 8.0, 2.5, 1.0 Hz)
5	130.8	7.26−7.30 (1H, m)[b]
6	121.2	7.33 (1H, dt, J = 7.7, 1.0 Hz)
1′	115.2	
2′	159.8	
3′	135.8	
4′	157.9	
5′	108.4	6.52 (1H, d, J = 9.1 Hz)
6′	127.7	7.94 (1H, d, J = 9.1 Hz)
CO	193.5	
α	121.6	7.87 (1H, d, J = 15.4 Hz)
β	145.1	7.80 (1H, d, J = 15.4 Hz)
3′-OCH_3	60.5	3.85 (3H, s)
3-OH		8.60−9.10 (1H, brs)[c]
2′-OH		13.67 (1H, s)
4′-OH		8.60−9.10 (1H, brs)[c]

[a] 600 MHz for ^{1}H and 150 MHz for ^{13}C in acetone-d_6. [b] These signals are overlapped. [c] These signals are overlapped.

(108.0 mg) and (*E*)-3,2′,4′-trihydroxychalcone (**2**) (53.3 mg), respectively. Fractions 4 (201.0 mg) and 5 (28.3 mg) were subjected to preparative HPLC using $CHCl_3$−MeOH (4:1) to give (*E*)-2′,4′-dihydroxychalcone (**3**) (24.0 mg) and (*E*)-3,2′,4′-trihydroxy-3′-methoxychalcone (**4**) (28.0 mg), respectively.

Determination of Antimycobacterial Activity. The EtOH extract of *G. africana* and the isolated compounds (**1**−**4**) were tested against a nonpathogenic strain of Mycobacteria (*M. smegmatis* MC^2 155) using the microplate dilution method as described previously.[14] These samples were also evaluated using the drug-susceptible strain of Mycobacteria (*M. tuberculosis* H37RV, ATCC 27264) obtained from American Type Culture Collection using the radiometric method as reported previously.[13] The compounds were further tested for synergistic, intracellular activity, cytotoxicity, and subversive properties. Synergistic activity of compounds **1**, **2**, **3**, and **4** (compound in combination with INH) was evaluated at their MIC and sub-MIC levels. Analysis of the drug combination data was achieved by calculating the fractional inhibitory concentration (FIC) index as described.[15] Cytotoxicity of the EtOH extract of *G. africana* and compounds was investigated in the U937 human macrophage cell line in order to evaluate their antimycobacterial activity as described previously.[16]

Mechanistic Studies. *M. tuberculosis* Mtr and the mycothiol substrate analogue benzyl-2(*N*-acetyl-L-cysteinyl) amino-2-deoxy-α-D-glucopyranoside (BnOMSH) were prepared as previously described.[12,17] Enzyme activity was determined by the increase in absorbance at 470 nm, due to reduction of Ellman's reagent by BnOMSH. Control assays were carried out in the absence of inhibitor.

Acknowledgment. This work was supported by a grant from the National Research Foundation, South Africa. The authors wish to thank the technical assistance of the South African Medical Research Council (TB Epidemiology and Intervention Research Unit, Pretoria).

References and Notes

(1) Snider, S. H.; Libonati, J. P.; Middlebrook, G. *J. Clin. Microbiol.* **1981**, *18*, 689–696.
(2) Ibrahim, M.; Andries, K.; Lounis, N.; Chauffour, A.; Truffot-Pernot, C.; Jarlier, V.; Veziris, N. *Antimicrob. Agents Chemother.* **2007**, *51*, 1011–1015.
(3) Vries, F. A.; Bitar, H. E. L.; Green, I. R.; Klassen, J. A.; Mabusela, W. T.; Bodo, B.; Johnson, Q. *11th Napreca Symposium Book of Proceedings* **2005**, 123–131.
(4) Watt, C.; Breyer-Brandwijk M. G. *The Medicinal and Poisonous Plants of Southern and Eastern Africa*; E & S Livingstone Ltd.: Edinburg, 1962; p 449.
(5) Miyaichi, Y.; Imoto, Y.; Tomimori, T.; Lin, C. C. *Chem. Pharm. Bull.* **1987**, *35*, 3720–3725.
(6) Bohlmann, F.; Jakupovic, J. *Phytochemistry* **1979**, *18*, 1189–1194.
(7) Severi, F.; Benvenuti, S.; Costantino, L.; Vampa, G.; Melegari, M.; Antolini, L. *Eur. J. Med. Chem.* **1998**, *33*, 859–866.
(8) Miyaichi, Y.; Morimoto, T.; Yaguchi, K.; Kizu, H. *J. Nat. Med.* **2006**, *60*, 157–158.
(9) Su, B. N.; Park, E. J.; Vigo, J. S.; Graham, J. G.; Cabieses, F.; Fong, H. S. F.; Pezzuto, J. M.; Kinghorn, A. D. *Phytochemistry* **2003**, *63*, 335–341.
(10) Svetaz, L.; Aguero, M. B.; Alvarez, S.; Luna, L.; Feresin, G.; Derita, M.; Tapia, A.; Zacchino, S. *Planta Med.* **2007**, *73*, 1074–1080.
(11) Patel, M. P.; Blanchard, J. S. *Biochemistry* **2001**, *40*, 5119–5126.
(12) Hamilton, C. J.; Saravanamuthu, A. H.; Fairlamb, A. H.; Eggleston, I. M. *Bioorg. Med. Chem.* **2003**, *11*, 3683–3693.
(13) Mativandlela, S. P. N.; Meyer, J. J. M.; Hussein, A. A.; Houghton, P. J.; Hamilton, C. J.; Lall, N. *Phytother. Res.* **2008**, *22*, 841–845.
(14) Mativandlela, S. P. N.; Meyer, J. J. M.; Hussein, A. A.; Lall, N. *Pharm. Biol.* **2007**, *45*, 645–650.
(15) Bapela, N. B.; Lall, N.; Fourie, P. B.; Franzblau, S. G.; Van Rensburg, C. E. J. *Phytomedicine* **2006**, *3*, 630–635.
(16) Passmore, J. S.; Lukey, P. T.; Ress, S. R. *Immunology* **2001**, *102*, 146–156.
(17) Stewart, M. J. G.; Jothivasan, V. K.; Rowan, A. S.; Wagg, J.; Hamilton, C. J. *Org. Biomol. Chem.* **2008**, *6*, 385–390.

NP800778B