Niruriflavone, a New Antioxidant Flavone Sulfonic Acid from *Phyllanthus niruri*

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A new flavone sulfonic acid **1** named niruriflavone was isolated from the 70% ethanolic extract of the whole plant of *Phyllanthus niruri* (Euphorbiaceae), together with 6,10,14-trimethyl-2-pentadecanone, hypophyllanthin, gallic acid, brevifolin carboxylic acid, methyl brevifolin carboxylate, *iso*quercetin, quercetin-3-O-β-D-glucopyranosyl(1→4)-α-rhamnopyranoside, corilagin, and isocorilagin, whose structures were determined by spectroscopic methods and comparison with published data. In an ABTS cation radical reduction assay, niruriflavone (**1**) exhibited potent radical scavenging properties. A biological test system based on bioluminescence of the dinoflagellate *Lingulodinium polyedrum* did not reveal any prooxidant properties of **1** at 50 μM.

Key words: Phyllanthus niruri, Niruriflavone, Antioxidant Activity, Bioluminescent Dinoflagellates, Toxicity

Introduction

The plant *Phyllanthus niruri* (Euphorbiaceae) known as "Taung Zi Phyu" is one of the most important traditional medicines in Myanmar and used as an antiviral and antimalarial remedy and applied for the treatment of jaundice and hepatitis [1]. Phytochemical examinations of this plant have been carried out and several constituents such as lignans [2 – 4], alkaloids [5, 6], flavonoids [7], benzenoids [8, 9], coumarins [10], tannins [8], diterpenes [11], triterpenes [12], sterols [13], phytallates [13] and lipids [14] have been identified and reported. In addition, several pharmacological experiments have also been published [15]. We wish to report here the new flavone sulfonic acid **1** together with 10 known compounds, 6,10,14-trimethyl-2-pentadecanone, hypophyllanthin [2], gallic acid [8], brevifolin carboxylic acid [16], methyl brevifolin carboxylate [17], isoquercetin [18], quercetin-3-O-β-D-glucopyranosyl(1→4)-α-rhamnopyranoside [19], corilagin [20], isocorilagin [20]. The antioxidant activities of **1**, gallic acid, brevifolin carboxylic acid, methyl brevifolin carboxylate, isoquercetin and quercetin-3-O-β-D-glucopyranosyl(1→4)-α-rhamnopyranoside were tested by means of an ABTS cation radical reduction assay. Biological tests for prooxidant effects were performed using the bioluminescent dinoflagellate *Lingulodinium polyedrum* as a test organism.

Results and Discussion

A combination of column chromatography on silica gel and Sephadex LH-20 of the 70% ethanol extract of *P. niruri* gave the new flavone sulfonic acid niruriflavone (**1**) as a yellow powder by repeated column chromatography on Sephadex LH-20 using MeOH as solvent. The molecular formula $C_{16}H_{12}O_8S$ was deduced by (+)-ESI HRMS of the $[M+H]^+$ *pseudo*molecular ion at *m/z* 365.03250 (calcd. 365.03258). After reflux in water, a small additional peak at *m/z* 283 $[M\text{-}SO_3H]^-$ suggested the presence of a sulfonic acid group in the molecule. The UV maxima of band II at 272 nm and band I at 330 nm were in agreement with the general flavone shifts. In addition, the reactions with $AlCl_3$ and $AlCl_3$/HCl confirmed that in the B-ring there were no *ortho*-dihydroxy groups present [21]. The ^{13}C NMR spectrum exhibited 16 signals as demanded by the high resolution, including 6 sp^2 methines, 8 quaternary sig-

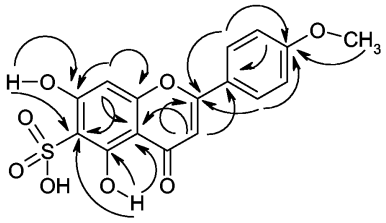
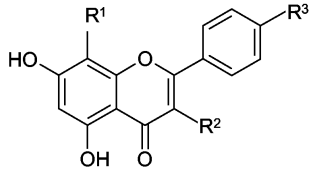


Fig. 1. Important HMBC correlations of niruriflavone (**1**).



2a: $R^1 = H, R^2 = H, R^3 = OCH_3$
2b: $R^1 = SO_3H, R^2 = OH, R^3 = H$
2c: $R^1 = SO_3H, R^2 = OH, R^3 = OH$
2d: $R^1 = SO_3H, R^2 = O\text{-glycosyl}, R^3 = H$

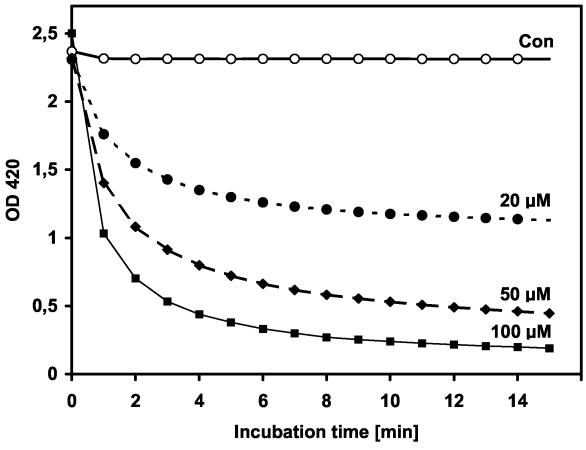


Fig. 2. Radical scavenging by niruriflavone (**1**) in the ABTS cation radical reduction assay.

nals, a methoxy and a carbonyl carbon. The ^{1}H NMR spectrum of **1** showed the presence of an AA'XX' system in the B ring, represented by two pairs of 2H doublets at $\delta = 8.04$ ($J = 8.7$ Hz) and 7.12 ($J = 8.7$ Hz) corresponding to H-2',6',3',5'. Two aromatic singlets at $\delta = 6.87$ and 6.50 (each 1H) were assigned to H-3 and H-8, respectively, on the basis of their long-range correlations with C-2 ($\delta = 163.2$), C-4a ($\delta = 103.1$), and C-1' ($\delta = 122.6$) and with C-7 ($\delta = 160.8$), C-6 ($\delta = 114.1$), C-8a ($\delta = 156.8$), and C-4a ($\delta = 103.1$), respectively. In the aliphatic region, a 3H singlet at $\delta = 3.78$ was assigned to an aromatic methoxy group, which was attached to C-4' ($\delta = 162.3$) due to the HMBC correlation. In the same way, the proton signal at $\delta = 13.78$ (1H, br s, chelated 5-OH) showed a cross peak with the carbon signals of C-5 ($\delta = 159.9$), C-4a ($\delta = 103.1$) and C-6 ($\delta = 114.1$). Furthermore, the 7-OH signal at $\delta = 12.77$ was chelated with a sulfonate oxygen and showed a correlation with the carbon signals at C-7 ($\delta = 160.8$), C-8 ($\delta = 93.9$), C-6 ($\delta = 114.1$) and a ^4J coupling with C-8a ($\delta = 156.8$). The substitution pattern of the B-ring was confirmed by the HMBC spectrum as well. The position of the sulfonate group in compound **1** was confirmed by the HMBC correlation (Fig. 1) as well as the chemical shift of C-6, which appears in acacetin (**2a**) at $\delta = 99.4$ [22], however, by $\delta\ \Delta \sim 15$ downfield shifted in **1** at $\delta = 114.1$ due to the sulfonyl group. Consequently, this compound was identified as **1** and named niruriflavone.

Although there are many reports about flavonoid sulfates from a number of plant families [23, 24], the only reported flavone-sulfonic acids are galangin-8-sulfonic acid (**2b**), kaempferol-8-sulfonic acid (**2c**) and galangin-3-O-β-D-glucoside-8-sulfonic acid (**2d**) isolated from *Phyllanthus virgatus* [25], which are all having the sulfonate group at position 8. This is the first report of a flavone-6-sulfonic acid from nature.

ABTS cation radicals [from 2,2′-azino-bis(3-ethylbenzothiazolone-6-sulfonic acid) and potassium peroxydisulfate] were used for determining the capacity of radical scavenging by **1**, gallic acid, brevifolin carboxylic acid, methyl brevifolin carboxylate, isoquercetin and quercetin-3-O-β-D-glucopyranosyl(1→4)-α-rhamnopyranoside. This assay has the advantage of being independent of several factors interfering in other assays; the reaction mixture is particularly free of iron, so that chelation does not give false-positive results, and the assay can be run at physiological pH. All the substances were capable of efficiently scavenging the ABTS cation radical (for dose- and time-dependent reduction by niruriflavone (**1**) see Fig. 2; other details not shown). Although the final concentration of the ABTS cation radical (105 μM) exceeded all test concentrations of niruriflavone (**1**), this scavenger was capable of reducing approximately half of the cation radical at only 20 μM. This reflects more than molar interactions and therefore multiple scavenging of radicals and remarkable antioxidant potency.

Since a radical scavenger turns into a free radical itself after interaction with a radical, and since a reducing agent may autoxidize, it is important to test for potential prooxidant activity *in vivo*. For this purpose, bioluminescence of the dinoflagellate *Lingulodinium polyedrum* was monitored

as an indicator of oxidative stress. While gallic acid and, to a lesser extent, quercetin-3-O-β-D-glucopyranosyl(1→4)-α-rhamnopyranoside proved to be prooxidant in the assay, niruriflavone (**1**), at 10 μM, did not exhibit any such effect. This demonstrates that niruriflavone (**1**) can efficiently scavenge free radicals without forming prooxidant intermediates.

Experimental Section

Melting points are uncorrected; ^{1}H (300 and 600 MHz) and ^{13}C (75.5 and 125.7 MHz) NMR spectra were measured on a Bruker AMX 300 and on a Varian Inova 600 (599.740 MHz) spectrometer. ESI mass spectra were recorded on a LCQ Finngan Mass Spectrometer, HR-ESI mass spectra were recorded on an APEX IV, 7T FT-ICR mass spectrometer (Bruker Daltonik). GC MS was measured on a TRACE GC-MS ThermoFinnigan mass spectrometer. HPLC MS was run on a LCQ Finnigan, and Flux Instruments Rheos 4000 was used as pump and Linear UVIS-205 was used as detector. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer from KBr pellets. Column chromatography was carried out on silica gel (230 – 400 mesh). Thin layer chromatography (TLC) was performed on Polygram SIL G/UV$_{254}$ (Macherey-Nagel & Co.). R_f values were measured on Polygram SIL G/UV$_{254}$ (Macherey-Nagel & Co.).

Plant material

Phyllanthus niruri (Euphorbiaceae), was collected in Yangon, Myanmar, in March 2002; a voucher specimen, voucher no. Y. H. V. 1003 has been deposited in the Herbarium of the Department of Botany, Yangon University.

Extraction and isolation

The air-dried whole plant material of *P. niruri* (320 g) was extracted with each 96% ethanol and subsequently 70% ethanol (6 × 1 l) at room temperature for 3 days. The extracts were concentrated under reduced pressure at 40 °C. The extract with 70% ethanol (17 g) was dissolved in water and successively partitioned between cyclohexane, ethyl acetate and *n*-butanol. The ethyl acetate fraction was chromatographed on a silica gel column and eluted successively with cyclohexane followed by cyclohexane/EtOAc and EtOAc/MeOH gradients, and finally MeOH. The eluates were monitored by TLC and grouped into 5 fractions. From fraction 1, N-butylbenzenesulfonamide and 6,10,14-trimethyl-2-pentadecanone were identified by GC/MS as impurities. Fraction 2 gave hypophyllanthin (30 mg) by washing with methanol. From fraction 5, by using Sephadex LH-20/MeOH, gallic acid (10 mg) and isoquercetin (5 mg) were obtained. The *n*-butanol extract was dissolved in MeOH and concentrated under reduced pressure. The MeOH soluble fraction was chromatographed repeatedly on Sephadex LH-20 using MeOH to afford niruriflavone (**1**, 50 mg) and known compounds, brevifolin carboxylic acid (10 mg), methyl brevifolin carboxylate (5 mg), quercetin-3-O-β-D-glucopyranosyl(1→4)-α-rhamnopyranoside (22 mg), corilagin (40 mg) and isocorilagin (35 mg), which were readily identified by comparison with the reported literature values.

*5,7-Di hydroxy-2-(4-methoxyphenyl)-4-oxo-4H-chromene-6-sulfonic acid (**1**, niruriflavone)*

Yellow powder, m. p. 297 – 9 °C, $R_{f'} = 0.75$ (CH_2Cl_2/20% MeOH). – UV (DMSO): λ_{max} (lg ε) = 272 (4.11), 294 (3.92), 317 (3.92), 330 (3.93) nm. – UV/vis (DMSO + $AlCl_3$): λ_{max} = 283, 307, 337, 384 nm. – UV (DMSO + $AlCl_3$/HCl): λ_{max} = 281, 307, 339, 389 nm. – IR (KBr): $\tilde{\nu}_{max}$ (cm^{-1})=3414, 3232, 2963, 2928, 2853, 1717, 1649, 1610, 1513, 1465, 1361, 1262, 1229, 1181, 1083. – ^{1}H NMR ([[D$_6$]DMSO, 600 MHz): δ = 13.78 (br s, 1H, 5-OH), 12.77 (br s, 1H, 7-OH), 8.04 (d, ^3J = 8.7 Hz, 2H, 2',6'-H), 7.12 (d, ^3J = 8.7 Hz, 2H, 3',5'-H), 6.87 (s, 1H, 3-H), 6.50 (s, 1H, 8-H), 3.78 (s, 3H, 4'-OCH$_3$). – ^{13}C NMR ([D$_6$]DMSO, 125 MHz): δ = 182.0 (C$_q$-4), 163.2 (C$_q$-2), 162.3 (C$_q$-4'), 160.8 (C$_q$-7), 159.9 (C$_q$-5), 156.8 (C$_q$-8a), 128.3 (CH-2',6'), 122.6 (C$_q$-1'), 114.5 (CH-3',5'), 114.1 (C$_q$-6), 103.3 (CH-3), 103.1 (C$_q$-4a), 93.9 (CH-8), 55.5 (4'-OMe). – (-)-ESI MS: *m/z* (%) = 748.8 ([2M-2H+Na]$^-$, 10), 363.2 ([M-H]$^-$, 100). – (+)-ESI HRMS: 365.03250 (calcd. 365.03258 for [M+H]$^+$, $C_{16}H_{13}O_8S$), 387.01447 (calcd. 387.01452 for [M+Na]$^+$, $C_{16}H_{12}O_8SNa$).

Tests for antioxidant and prooxidant activities

Radical scavenging was tested by means of the ABTS cation radical decoloration assay [26, 27]. Tests for prooxidant effects were carried out using the bioluminescent dinoflagellate *Lingulodinium polyedrum* [28], whose circadian glow maximum is diminished by sublethal oxidative stress, whereas lethal stress causes strong rises of light emission during dying.

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[1] Personal communication with Traditional Medicine Practitioners, Traditional Medicine Hospital, Yangon, Myanmar.
[2] A. Somanabandhu, S. Nitayangkura, C. Mahidol, S. Ruchirawat, K. Likhitwitayawuid, H. L. Shieh, H. P. Chai, M. John, G. A. Cordell, J. Nat. Prod. **56**, 233 (1993).
[3] Y. L. Huang, C. C. Chen, J. C. Ou, Planta Med. **58**, 473 (1992).
[4] M. A. Quader, M. Khatun, M. Mosihuzzaman, J. Bangladesh Acad. Sci. **18**, 229 (1994).
[5] S. A. Hassarajani, N. B. Mulchandani, Indian J. Chem. **29B**, 801 (1990).
[6] N. B. Mulchandani, S. A. Hassarajani, Planta Med. **50**, 104 (1984).
[7] K. Ishimaru, K. Yoshimatsu, T. Yamakawa, H. Kamada, K. Shimomura, Phytochemistry **31**, 2015 (1992).
[8] H. Ueno, S. Horie, Y. Nishi, H. Shogawa, M. Kawasaki, S. Suzuki, T. Hayashi, M. Arisawa, M. Shimizu, M. Yoshizaki, J. Nat. Prod. **51**, 357 (1988).
[9] W. X. Wei, Y. J. Pan, H. Zhang, C. W. Lin, T. Y. Wie, Chem. Nat. Compds. **40**, 460 (2004).
[10] S. Mineo, H. Syunji, T. Satoshi, U. Hajime, H. Toshimitsu, A. Munehisa, S. Shoichi, Y. Masao, M. Naokata, Chem. Pharm. Bull. **37**, 2531 (1989).
[11] B. Singh, P. K. Agrawal, R. S. Thakur, Planta Med. **57**, 98 (1991).
[12] B. Singh, P. K. Agrawal, R. S. Thakur, Phytochemistry **28**, 1980 (1989).
[13] B. Singh, P. Thakur, Indian J. Chem. **25B**, 600 (1986).
[14] M. U. Ahmad, S. K. Husain, S. M. Osman, J. Am. Oil Chem. Soc. **58**, 673 (1981).
[15] J. B. Calixto, A. R. S. Santos, F. V. Cechinel, R. A. Yunes, Med. Res. Rev. **18**, 225 (1998).
[16] T. Yoshida, H. Itoh, S. Matsunaga, R. Tanaka, T. Okuda, Chem. Pharm. Bull. **40**, 53 (1992).
[17] Y.-L. Huang, C.-C. Chen, F.-L. Hsu, C.-F. Chen, Tannins, Flavonol Sulfonates, and a Norlignan from *Phyllanthus virgatus*. J. Nat. Prod. **61**, 1194 (1998).
[18] E. Wong, C. M. Francis, Flavonoids in genotypes of Trifolium subterraneum. I. Normal flavonoid pattern of the Geraldton variety. Phytochemistry **7**, 2123 (1968).
[19] T. Agarwal, J. S. Tiwari, J. Indian Chem. Soc. **68**, 479 (1991).
[20] K. P. Latté, H. Kolodziej, Phytochemistry **54**, 701 (2000).
[21] K. R. Markham, Techniques of Flavonoid Identification, pp. 44 – 45, Academic Press, London (1982).
[22] P. K. Agrawal (ed.): Studies in Organic Chemistry. 39. Carbon-13 NMR of Flavonoids. Elsevier, Amsterdam (1989).
[23] M. A. M. Nawwar, J. Buddrus, Phytochemistry **20**, 2446 (1981).
[24] L. Reinhold, J. B. Harborne, T. Swain (eds.): Progress in Phytochemistry, Vol. 4, Pergamon, Oxford (1977).
[25] L. Huang, C. C. Chen, F. L. C. F. Hsu, Chen, J. Nat. Prod. **61**, 1194 (1998).
[26] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Free Radic. Biol. Med. **26**, 1231 (1999).
[27] B. Poeggeler, S. Thuermann, A. Dose, M. Schoenke, S. Burkhardt, R. Hardeland, J. Pineal Res. **33**, 20 (2002).
[28] A.-R. Ressmeyer, J. C. Mayo, V. Zelosko, R. M. Sáinz, D.-X. Tan, B. Poeggeler, I. Antolín, B. K. Zsizsik, R. J. Reiter, R. Hardeland, Redox Rep. **8**, 205 (2003).