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Structural Elucidation and Free Radical Scavenging Activity of a new *o*-Orsellinic Acid Derivative Isolated from the Lichen *Cladonia rappii*

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Rappiidic acid, a new *o*-orsellinic acid derivative, was isolated from the lichen *Cladonia rappii*. Its capability to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) was investigated and compared with resveratrol and (+)-usnic acid. Usnic acid at 100 μM was the most efficient ROS scavenger, exhibiting activity 3-fold higher than that of resveratrol. At the same concentration, rappidic acid scavenged 23.1% of ROS formed, demonstrating that this compound is twice as active as resveratrol. Both compounds were shown to be poor RNS scavengers.

Keywords: Rappiidic acid, Orsellinic acid, Natural products, *Cladonia rappii* A. Evans, Free radical scavenging, DPPH, Superoxide anion.

Lichens are mutualistic associations established between fungi and algae/cyanobacteria known to produce specific secondary metabolites [1], such as usnic acid, antranorine, fumarprotocetraric acid and protolichesterinic acid [1,2]. Many of the 17,000 identified lichen species have been used as dyes, pollution monitors, food additives and ingredients for the perfume and pharmaceutical industries [3]. *Cladonia rappii* A. Evans (Cladoniaceae) is widely distributed in Brazil from northeast to south, which includes Pernambuco, Paraíba, Sergipe, Bahia, Espírito Santo, Minas Gerais and Rio Grande do Sul States [4]. Despite its large distribution in Brazil, there are no reports on the constituents of *C. rappii*. Here, we describe the isolation from *C. rappii* and structure elucidation of a novel *o*-orsellinic acid derivative (Figure 1), which was named as rappiidic acid (RAP). The potential of RAP as a free radical scavenger was also addressed.

RAP was isolated as a yellow oil, with a molecular formula of $C_{15}H_{22}O_4$ on the basis of its HR-ESI-TOF-MS (*m/z* 267.1590, for $C_{15}H_{23}O_4$, [M + H^+]). The infrared spectrum showed bands for antisymmetric and symmetric ν(Csp^3-H) stretching, from 2958 to 2856 cm^{-1}. Aromatic ν(Csp^2-H) absorption was observed at 1577 cm^{-1} and ν(C-O) for aryl-alkyl-ethers at 1254 and 1048 cm^{-1}. ^{1}H NMR signals indicated the presence of two methyl triplets at δ_H 0.90 (J = 6.9 Hz, H-5') and δ_H 1.41 (J = 7.2 Hz, H-2''), side chain multiplets (-CH_2-) ranging from δ_H 1.30 to 1.37 (H-3' and H-4'), a triplet at δ_H 2.86 (J = 7.8 Hz, H-1'), a singlet for a methoxy group at δ_H 3.80 and a quartet for a methylene group attached to the oxygen at δ_H 4.40 (J = 7.2 Hz, H-1''). The aromatic hydrogens appear as doublets at δ_H 6.28 (J = 2.7 Hz, H-5) and δ_H 6.33 (J = 2.7 Hz, H-3). A signal for a carboxylic acid was observed as a singlet at δ_H 11.86. Fifteen signals were identified in the ^{13}C NMR spectrum. Particularly the signals at δc 98.8 (C3), 104.8 (C1), 110.6 (C5), 148.0 (C6), 163.8 (C2), 165.6 (C4) and 171.6 (CO_2H) indicated an orsellinic acid moiety [5]. Thus, the alkyl side chain (C5'- δc 14.07, C4'- δc 22.6, C3'- δc 32.1, C2'- δc 31.7 and C1'- δc 37.0), methoxy (δc 55.0) and ethoxy groups (C1''- δc 61.2 and C2''- 14.1) were assigned on the basis of DEPT-135, COSY and HETCOR spectra. The position of the groups attached to the aromatic ring was

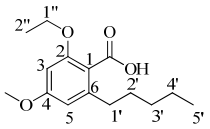

Figure 1: Structure of Rappiidic acid (RAP).

determined by means of NOESY NMR spectroscopy. Specific nOe signals were observed between the alkyl group and CO_2H (δ 11.84). The methoxy position was suggested on the basis of the Overhauser enhancement observed between hydrogen H-5 (δ 6.28) and H-3 (δ 6.33) with a methoxy group (δ 3.80). According to the nOe data, we can assume that the ethoxy and methoxy are linked to C-2 and C-4, respectively (See details in Supplementary Data).

The ability of RAP to scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) was investigated and the results were compared with those for (+)-usnic acid (USN), an important lichen metabolite, and the reference antioxidant resveratrol (Resv). At 100 μM, RAP scavenged 23.1% of the superoxide anion, while USN sequestered 31.4%. Thus, the former was found to be twice as efficient as Resv and the latter around 4-fold more potent than Resv. At 200 μM, compound RAP was less effective (18.1% ROS scavenging) than Resv, while (+)-usnic acid was still around 3-fold more active than the antioxidant reference (Figure 2).

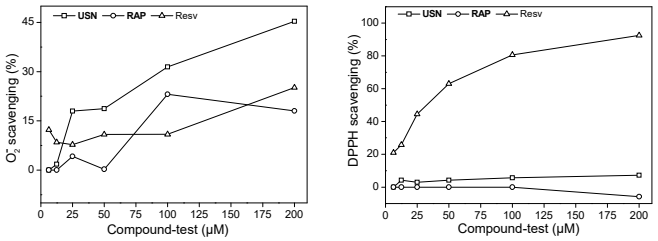

Figure 2: Scavenging effect of rappiidic acid (**RAP**) and (+)-usnic acid (**USN**) on reactive oxygen (ROS) and reactive nitrogen (RNS) species. The ROS and RNS sources were superoxide anion (O_2^-) and 2,2-diphenyl-1-picrylhydrazyl (DPPH), respectively.

Indeed, the *C. rappii* ethanolic extract was effective in reducing the concentration of free radicals [6]. Some lichen substances act as antioxidants, and, for instance, fumarprotocetraric acid was shown to reduce lipid peroxidation in rats [7]. Differently from the observed results for ROS, all the compounds tested failed to scavenge DPPH, an RNS (Figure 2). In fact, Kumar and Müller [8] disclosed that (+)-usnic acid was unable to scavenge DPPH radicals, even when used at concentrations as high as 200 μM.

Experimental

General: The IR, Varian 660-IR gladATR spectrometer; NMR, Varian Mercury 300 MHz spectrometer; HRMS, Shimadzu LCMS-IT-TOF spectrometer; CC, silica gel 60. Fractions were monitored by TLC and compounds were visualized using a vanillin acid solution. Vacuum liquid chromatography [9] was performed using a 2 L sintered funnel and silica gel 60 (53-200 μM).

Lichen material: *Cladonia rappii* was collected in July 2012 at Serra do Brigadeiro State Park located in the city of Araponga, Minas Gerais, Brazil (20º41'26.85''S, 42º28'20.41''W, 1,644 m). The species was identified by Dr Suzana M. A. Martins from the Natural Sciences Museum of Rio Grande do Sul (Pelotas, RS, Brazil) and deposited in the herbarium of the Department of Botany at the Federal University of Viçosa (MG, Brazil) under the voucher specimen number VIC 35,603.

Rappiidic acid extraction and isolation: The powdered and dried lichen (150 g) was extracted with ethanol for 7 days at room temperature to afford a brownish crude extract (18.43 g). This was partitioned using vacuum liquid chromatography [9] with a column (5 x 18.7 cm) packed with silica-gel/60 G. *n*-Hexane, dichloromethane, ethyl acetate and methanol were used as eluents in an increasing order of polarity. The dichloromethane fraction (0.31 g) was loaded onto a silica gel column that was eluted with a *n*-hexane-dichloromethane gradient system (2:1, 1:1, 1:2, 1:3 and 0:1) to obtain 8 fractions (I–VIII). Separation of fraction IV was carried out in a silica gel flash column using *n*-hexane-dichloromethane-acetone (15:1:0.5) to yield fractions IV-A-2, IV-A-2-1 and IV-A-2-2 (0.014 g). Separation of fraction IV-A-2-1 was performed using preparative TLC (toluene/acetic acid – 20:3), which yielded 0.008 g of rappiidic acid. Lichen-derived (+)-usnic acid was acquired from Sigma-Aldrich and used for comparative purposes.

Rappiidic acid

Yellow oil

Rf: 0.6 (Toluene-CH_3CO_2H, 200:30)

IR (neat): 2958, 2932, 2856, 1650, 1577, 1254, 1048 cm^{-1}.

^1H NMR (300 MHz, $CDCl_3$): 0.90 (3H, t, *J* = 6.9 Hz, H-5'), 1.30-1.37 (4H, m, H-3', H-4'), 1.41 (3H, t, *J* = 7.2 Hz, H-2''), 1.57 (2H, pseudo quint., H-2'), 2.86 (2H, t, *J* = 7.8 Hz, H-1'), 3.80 (3H, s, H-8), 4.40 (2H, q, *J* = 7.2 Hz, H-1''), 6.28 (1H, d, *J* = 2.7 Hz, H-5), 6.33 (1H, d, *J* = 2.7 Hz, H-3), 11.84 (1H, s, CO_2H).

^{13}C NMR (75 MHz, $CDCl_3$): 14.07 (C-5'), 14.13 (C-2"), 22.6 (C-4'), 31.7 (C-2'), 32.1(C-3'), 37.0 (C-1'), 55.3 (OCH_3), 61.2 (C-1''), 98.8 (C-3), 104.8 (C-1), 110.6 (C-5), 148.0 (C-6), 163.8 (C-2), 165.6 (C-4), 171.6 (CO_2H).

HR-ESI-TOF-MS: *m/z* 267.1590 [M+H$^+$] (Calcd for $C_{15}H_{23}O_4$, M+H$^+$, *m/z* 267.1591).

Free radicals scavenging activity: The capacity of compounds to scavenge the ROS superoxide anion (O_2^-) was evaluated in reactions containing 60% ethanol, 13.3 mM L-methionine, 75 μM nitroblue tetrazolium, 100 μM EDTA, 2 μM riboflavin and rappiidic acid or (+)-usnic acid or resveratrol in 99.2% ethanol/0.8% dimethyl sulfoxide in the range from 0 to 200 μM. Reaction mixtures were incubated for 10 min at 25ºC in the presence of fluorescent light to induce O_2^- formation. Control reactions consisted of similar systems maintained at 25ºC for 10 min under darkness. The percentage of O_2^- scavenged by each compound was determined by spectrophotometric measurements at 575 nm. The ability of compounds to scavenge the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, a reactive nitrogen species (RNS), was determined according to [10], with modifications. Briefly, each compound was tested at final concentrations that ranged from 0 to 200 μM (in 99.2% ethanol/0.8% dimethyl sulfoxide) in the presence of 100 μM DPPH. Reactions were maintained under darkness for 30 min and the absorbance recorded at 517 nm. Results are from 3 independent experiments, each made in quadruplicate. Resveratrol was used as a reference for a free radical scavenger.

Supplementary Data: All spectral data of RAP are available.

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