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Research Article

ISOLATION AND IDENTIFICATION OF α-GLUCOSIDASE, α-AMYLASE AND LIPASE INHIBITORS FROM HORTIA LONGIFOLIA

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ABSTRACT

Phytochemical investigation from branches of *Hortia longifolia* (Rutaceae) yielded the amide N-[2-(4-prenyloxyphenyl)ethyl]tigliamide (**1**), (*E*)-methyl-*O*-prenyl ferulate (**2**), limonin (**3**), cinnamic acid derivatives [(*E*)-methyl-5′-hydroxy-*O*-prenyl cinnamate **4** and integrifoliodiol **7**], scopoletin (**5**) and skimmianine (**6**). The compound **4** showed moderate α-amylase inhibitory activity, the coumarin **5** presented strong activity on α-glucosidase (IC_{50} of 0.89 µg/mL). The compounds **1**, **2**, **4** and **5** exhibited significant inhibitory activity on lipase with IC_{50} at 6.91, 8.14, 3.86 and 5.07 µg/mL, respectively. The results demonstrate potential use for these compounds in development of drugs of natural origin in the prevention or treatment of metabolic syndrome.

Keywords: *Hortia longifolia*, Rutaceae, Phenylpropanes, α-glucosidase, Lipase, α-amylase

INTRODUCTION

Hortia longifolia Benth. Ex Engl (Rutaceae) occurs in Brazil [1], and previous study reported the isolation of alkaloids, coumarins and flavonoid from bark [2]. In this paper we describe the isolation and structural identification of seven compounds from branches and evaluate their α-glucosidase, α-amylase and lipase inhibition properties. The importance of the inhibitors of these enzymes have attracted interest because the inhibition of the catalytic activity of α-glucosidase leads to retardation of the absorption of glucose and the reduction of postprandial blood glucose level [3,4], therefore they play a significant role in the therapy of diabetes and obesity. Similarly, inhibitors of α-amylases may be used in treating obesity, reducing postprandial levels of insulin and increasing the sensation of satiety due to the retardation of gastric emptying [5,6]. Lipases are fat-digesting enzymes, the lipid metabolism is balanced to maintain homeostasis, and when this balance is lost, obesity or hyperlipidemia develops, leading to a variety of serious diseases, including atherosclerosis and diabetes [7,8]. Considering the low availability of inhibitors of enzymes in the pharmaceutical industry, the search for models of secondary metabolites from natural sources is promising.

MATERIAL AND METHODS

General experimental procedures

Column chromatography (CC) was performed with silica gel 60 (70-230 and 230-400 mesh, Merck). Analytical thin layer chromatography (TLC) was carried out on plates precoated with silica gel 60 F_{254} (0.20 mm; Merck). NMR spectra were measured in a Bruker DRX 400 apparatus; chemical shifts (d) were expressed in ppm, and coupling constants (*J*) in Hertz; TMS was used as internal standard.

Plant material

Hortia longifolia was collected in the Forest Reserve Adolfo Ducke, Amazonas, Brazil, and identified by J.R. Pirani (Universidade de São Paulo). A voucher no. 209963 is deposited in the Herbarium of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, AM.

Extraction, fractionation and isolation

Powdered air-dried branches (735 g) were macerated at room temperature with *n*-hexane and then MeOH. The *n*-hexane extract (2.1 g) was fractionated over silica gel column (3.5 x 18.5 cm; 70-230 mesh), eluted with hexane, hexane:EtOAc and acetone, to yield twelve fractions. The fractions 8-9 (559.5 mg) after column chromatography over silica gel (2 x 22.5 cm; 230-400 mesh), eluted with hexane:EtOAc (2-100%), yielded compound **1** (91.6 mg) [R_f 0.52 (hexane:acetone, 7:3)]. The MeOH extract (12.2 g) was suspended in H_2O and partitioned successively with hexane, CH_2Cl_2 and EtOAc. The hexane phase yielded a mixture of β-sitosterol and stigamasterol and the CH_2Cl_2 phase (F-2; 4.1 g) was fractionated over silica gel (2.8 x 60 cm; 70-230 mesh), eluted with CH_2Cl_2, CH_2Cl_2:EtOAc (10-100%), EtOAc:MeOH (10-50%) and MeOH, yielding twenty nine fractions. The combined fractions 7-9 (F-2.7), 12-13 (F-2.12) and 14-18 (F-2.14) were submitted to further chromatographic fractionations. Frs. F-2.7 (72 mg) was subjected to column chromatography over silica gel (1.7 x 50 cm; 230-400 mesh), eluted with hexane:EtOAc (5-100%), to give **2** (4 mg) [R_f 0.55 (hexane:EtOAc, 7:3)]. F-2.12 (348 mg) was purified with a acetone to give **3** (13 mg) [R_f 0.50 (CH_2Cl_2:EtOAc, 85:15)]and the mother liquor was fractionated over silica gel column (1.7 x 50 cm; 230-400 Mesh), eluted with CH_2Cl_2, CH_2Cl_2:EtOAc (10-100%), EtOAc:MeOH (10-50%), to give sixty seven fractions; and the frs. 39-43 (F-2.39), 44-48 (F-2.44) and 49-52 (F-2.49) were submitted to further chromatographic fractionations. F-2.39 (69 mg) was chromatographed on silica gel (1 x 31 cm; 230-400 mesh), eluted with hexane:EtOAc (5-50%), to give **4** (27 mg) [R_f 0.59 (CH_2Cl_2:EtOAc, 85:15)] and **5** (6 mg) [R_f 0.67 (CH_2Cl_2:EtOAc, 9:1)]. The clearing of F-2.44 (25 mg) with MeOH and F-2.49 (29 mg) with EtOAc yielded compounds **6** (3 mg) [R_f 0.43 (CH_2Cl_2:EtOAc, 93:7)] and **3** (13 mg), respectively. F-2.14 (933 mg) was fractionated over silica gel column (1.5 x 26 cm; 230-400 mesh), eluted with CH_2Cl_2, CH_2Cl_2: EtOAc (2-100%) to give **7** (4 mg) [R_f 0.50 (CH_2Cl_2:EtOAc, 7:3)].

Compound 1

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.11 (2H, dd, J = 6.5, 2.1 Hz, H-2, H-6), 6.87 (2H, dd, J = 6.5, 2.1 Hz, H-3, H-5), 6.37 (1H, dd, J = 13.8, 6.9 Hz, H-5''), 5.49 (1H, m, H-2'), 5.40 (s, N-H), 4.50 (2H, d, J = 6.8, H-1'), 3.53 (2H, dd, J = 12.8, 6.9 Hz, H-2''), 2.78 (2H, t, J = 6.9 Hz, H-1''), 1.79 (3H, m, H-5'), 1.78 (3H, m, H-6''), 1.73 (3H, m, H-4'), 1.73 (3H, m, H-4', H-7''). ^{13}C NMR ($CDCl_3$,100 MHz): literature [9]. HSQC and HMBC ($CDCl_3$, 400/100 MHz,): Text.

Compound 2

^{1}H NMR ($CDCl_3$, 400 MHz): 7.62 (1H d, J = 16.0, H-1''), 7.07 (1H, ddd, J= 8.4, 2.4, 0.4 Hz, H-6), 7.04 (1H, d, J =2.8 Hz, H-2), 6.87 (1H, d, J = 8 Hz, H-5), 6.32 (1H, d, J = 16.0, H-2''), 5.68 (sl, N-H), 5.51 (1H, m, H-2'), 4.63 (1H, d, J = 8 Hz, H-1'), 3.89 (3H, s, OMe), 3.79 (3H, s, H-4''), 1.78 (3H, dl, H-5'), 1.77 (3H, sl, H-4').^{13}C NMR ($CDCl_3$, 100 MHz): 167.7 (C-3''), 150.7 (C-4), 149.8(C-3), 144.9 (C-1''), 138.1 (C-3'), 127.5 (C-1), 122.5 (C-6), 119.6 (C-2'), 115.5 (C-2''), 112.9 (C-5), 110.3 (C-2), 65.9 (C-1'), 56.0 (OMe), 51.5 (C-4''), 25.8 (C-5'), 18.3 (C-4'), HSQC and HMBC ($CDCl_3$, 400 MHz): Text.

Compound 3

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.41 (1H, m, H-21), 7.40 (1H, m, H-23), 6.34 (1H, m, H-22), 5.47 (1H, s, H-17), 4.78 (1H, d, J = 13.0 Hz, H-19a), 4.48 (1H, d, J = 13.0 Hz, H-19b), 4.04 (1H, s, H-3), 4.04 (1H, s, H-15), 3.00 (1H, dd, J = 16.8, 4.0 Hz, H-2a), 2.86 (1H, dd, J = 15.6, 0.8 Hz, H-6a), 2.70 (1H, dd, J = 16.8, 2.0 Hz, H-2b), 2.49 (1H, dd, J = 14.4, 3.2 Hz, H-6b), 2.55 (1H, dd, H-9), 2.23 (1H, dd, H-5), 1.80 (1H, m, H-12a), 1.91 (1H, m, H-11a), 1.77 (1H, m, H-11b), 1.52 (1H, m, H-12b), 1.29 (3H, s, H-28), 1.18 (3H, s, H-29), 1.17 (3H, s, H-18), 1.07 (3H, s, H-30). ^{13}C NMR ($CDCl_3$,100 MHz,): literature [10].

Compound 4

^{1}H NMR ($CDCl_3$, 400 MHz): 7.66 (1H, d, J = 16.0 Hz, C-1''), 7.48 (2H, d, J = 8.8 Hz, C-2, C-6), 6.92 (2H, d, J = 8.8 Hz, C-3, C-5), 6.30 (1H, d, J = 16.0 Hz, C-2''), 5.77 (1H, m, C-2'), 4.63 (1H, dd, J = 6.8, 0.8 Hz, C-1'), 4.09 (2H , d, J = 4.8 Hz, C-5'), 3.79 (3H, s, H-4''), 1.77 (3H, s, C-4'). NMR ^{13}C (100 MHz, $CDCl_3$): literature [12].

Compound 5

^{1}H NMR (CD_3OD, 400 MHz): δ 7.84 (1H, d, J = 9.6 Hz, H-4), 7.04 (1H, s, H-5), 6.70 (1H, s, H-8), 6.13 (1H, d, J = 9.2 Hz, H-3), 3.88 (OMe). ^{13}C NMR (CD_3OD, 100 MHz): 165.0 (C-2), 157.0 (C-4a), 148.3 (C-7), 146.4 (C-4), 131.0 (C-6), 111.0 (C-8b), 110.8 (C-3), 109.4 (C-5), 104.3 (C-7), 56.7 (OMe).

Compound 6

^{1}H NMR (CD_3OD, 400 MHz): 8.03 (1H, d, J = 9.2 Hz, H-5), 7.59 (1H, d, J = 2.8 Hz, H-2'), 7.25 (1H, d, J = 9.2 Hz, H-6), 7.05 (1H, d, J = 2.8 Hz, H-1'), 4.43 (s, OMe-4), 4.11 (s, OMe-8), 4.03 (s, OMe-7). ^{13}C NMR (CD_3OD, 100 MHz): literature [13].

Compound 7

NMR ^{1}H (400 MHz, MeOD): 7.31 (d, J = 8.4 Hz, C-2 and C-6), 6.87 (d, J = 8.4 Hz, C-3 and C-5), 6.57 (d, J = 16.0 Hz, C-1''), 6.24 (dt, J = 15.6 and 6.0 Hz, C-2''), 5.77 (m, C-2'), 4.60 (dd, J = 6.4 and 0.8 Hz, C-1'), 4.29 (dd, J = 6.0 and 1.6 Hz, C-3''), 4.09 (t, J = 0.4 Hz, C-5'), 1.78 (sl, C-4'). NMR ^{13}C (100 MHz, MeOD): literature [14].

Enzymatic assays *in vitro*

The α-amylase and lipase inhibitory assays were performed as previously described, with some modifications [15,16]. The α-glucosidase inhibitory activity was determined according to Andrade-Cetto et al (2008) [17].

α-Amylase inhibitory activity

In this colorimetric test, 20 µL of α-amylase enzyme from human saliva (A1031, Sigma) at 0.5 mg/mL was incubated with 20 µL of the compounds **1-3** and **5** (500 µg/mL), compound **4** (7.8-500 µg/mL), or with control drug Acarbose (A8980, Sigma), for 5 minutes at 37 °C. After adding 50 µL of the Amylase Substrate (starch at 0.5 mg/mL, Labtest), the plate was incubated for approximately 8 minutes. Then 100 µL of the reactive α-amylase (iodide potassium at 0.5 mg/mL, Labtest), and 150 µL of distilled water were added. The microplate was incubated for another 5 minutes at 37 °C, and the absorbances were measured at 630 nm.

α-Glucosidase inhibitory activity

This was determined by incubation of 20 µL of the compounds (6.25-100 µg/mL), solvent or Acarbose (A8980, Sigma) with 180 µL of the α-glucosidase enzyme from *Saccharomyces cerevisiae* (G0660, Sigma) for 2 minutes, at 37 °C. Then, after the addition of 150 µL of the color reagent PNPG (*p*-nitrophenyl-α-D-glucopyranoside, Sigma), it was incubated for 15 minutes, at 37 °C. The assay media contained 10 mM potassium phosphate buffer (pH 6.9), 5 mM PNPG and alpha-glucosidase (0.5 mg/mL). The reading of the microplate was performed at 405 nm.

Pancreatic lipase inhibitory activity

The activity was determined by incubation of 20 µL of the compounds (1.56-50 µg/mL), diluent or control drug Orlistat® (O4139, Sigma) with 180 µL of the enzyme. The porcine pancreas lipase, type II (L3126, Sigma) was incubated for 2 minutes at 37 °C. After the addition of 200 µL of Tris, the absorbances values were measured at 405 nm and the second reading were made 15 minutes after addition of 20 µL of the PNP (Sigma). The assay media contained 75 mM Tris buffer (pH 8.5), 2.5 mM PNP and 250 mU of pancreatic lipase.

Each test was performed three times and the IC_{50} values were determined by nonlinear regression using the program Microcal™ Origin ® version 6.0 (Microcal Software Inc).

RESULTS AND DISCUSSION

Isolated compounds

The compounds 1-5 and 7 are reported for the first time from this species. The ^{1}H and ^{13}C NMR spectra of compound **1** are consistent with N-[2-(4-prenyloxyphenyl) ethyl]tigliamide [9]. The HSQC experiment showed the correlations of hydrogen at δ 7.11 and 6.87 with carbon at δ 129.7 (C-2/6) and 114.8 (C-3/5), respectively. The HMBC experiment showed the correlations between the methylene hydrogens at δ 4.50 and the ^{13}C signals at δ 157.5 (C-4), 119.7 (C-2') and 138.1 (C-3'), the olefinic hydrogen at δ 6.37 with carbonyl at δ 169.3, methyl carbon at δ 12.3 and 13.9 of the tigliamide group.

The ^{1}H and ^{13}C NMR spectra of **2**, **4** and **7** showed typical signals of prenylated ferulate or cinnamate. The ^{1}H NMR spectrum of **2** exhibited signals typical of trisubstituted aromatic ring, prenyl group and α,β-unsaturated ester. In the HSQC, the methylene signal at δ 4.63 (H-1'), correlates with the ^{13}C signals at δ 65.9. The olefinic signal at δ 7.62 (H-1"), showed a long-range correlations with the ^{13}C signals at δ 167.8 (C-3"), 110.3 (C-2), 122.5 (C-6) and 115.5 (C-2') further the correlations between methoxyl hydrogens at δ 3.89 with ^{13}C signal at δ 167.8 (C-3"). Compound **2** is thus (*E*)-methyl-*O*-prenyl ferulate which differs from boropinic acid [11] by the presence of methoxyl group at C-3" instead of the carboxyl. The ^{1}H and ^{13}C NMR data of **4** and **7** were similar to compound identified as know *(E)*-methyl-5'-hydroxy-*O*-prenyl cinnamate [12] and integrifoliodiol [13], respectively. The ^{1}H and ^{13}C NMR data of **7**, were identical to those reported to the cinnamic acid derivative known as integrifoliodiol [14].

The other compounds were identified as limonin (**3)** [10], scopoletin (**5**) [18] and skimmianine (6) [13] by comparing their ^{1}H and ^{13}C NMR data with those reported previously.

Effects of the compounds on α-amylase, α-glucosidase and lipase *in vitro*

The α-amylase activity of compounds **1-3** and **5** evaluated at 500 µg/mL, presented low percentage inhibition (21.74-31.57%). The cinnamic acid derivative (**4**) tested for concentrations from 7.8-500 µg/mL showed IC_{50} of 156.77±7.57 µg/mL (Table 1). This may be considered a moderate effect on digestive enzyme α-amylase which is desirable since many side effects of α-glucosidase inhibitors as acarbose, for example, are due to the effect on α-amylase that generates incomplete digestion of carbohydrates [19]. Scopoletin (5) showed strong α-glucosidase inhibition activity (IC_{50} 0.89±0.02 µg/mL), this was about 50 times greater than the acarbose used in our test as control and reference drug in the treatment of type 2 diabetic patients. The amide (**1**), ferulic acid (**2**), cinnamic acid derivatives (**4**), and the coumarin (**5**) exhibited significant inhibitory activity on lipase when compared to the control drug (Orlistat).

The literature shows a variety of plant species used as antidiabetics in various traditional systems of medicines including species in the family Rutaceae [20], but few examples of assays with substances inhibiting enzymes that can promote the therapy of diabetes and obsesity. The observed results for compounds of *H. longifolia* demonstrate their potential for developing drugs of natural origin for the prevention or treatment of metabolic syndrome in which the inhibition of digestive enzymes is desirable. These compounds differentiate from those currently used in therapy due to their ability to inhibit more than one enzyme complex.

Table 1: Inhibitory activity of compounds 1-5 on α-amylase, α-glucosidase and lipase

Compounds	IC_{50} (µg/mL)		
	α-Amylase	α-Glucosidase	Lipase
1		55.77±4.52	6.91±0.11
2		26.82±1.78	8.14±1.37
3		47.57±3.97	26.51±0.13
4	156.77±7.57		3.86±0.66
5		0.89±0.02	5.07±0.03
Acarbose	33.12±4.09	51.5±5.6	
Orlistat			0.84±0.12

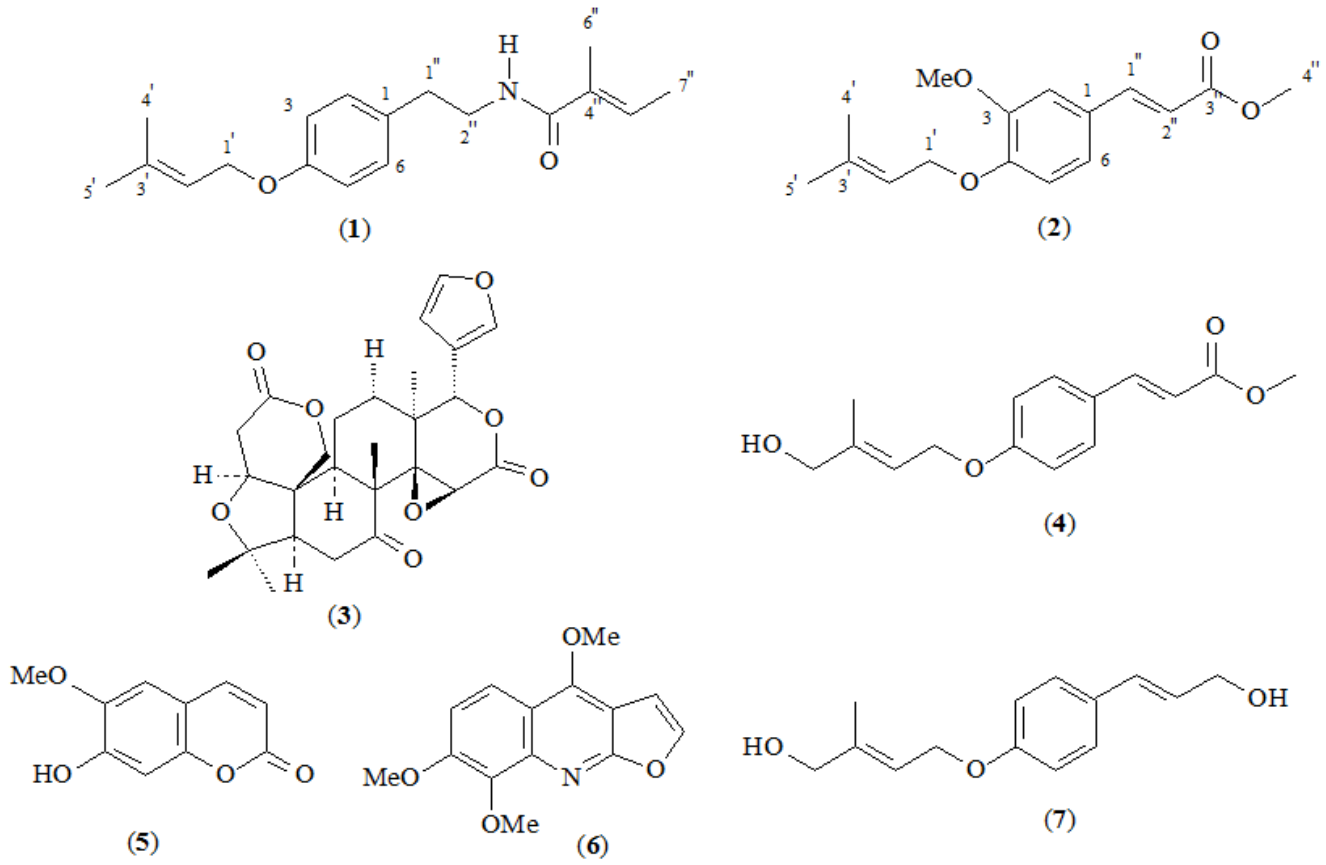

Fig. 1: Structures of compounds isolated from *Hortia longifolia*

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