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RESEARCH ARTICLE

Urease inhibitors from *Hypericum oblongifolium* WALL.

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Abstract

The bioassay-guided fractionation of *H. oblongifolium* has led to the isolation of potent urease inhibitors **1–3**. The structures were elucidated by NMR and mass spectroscopic techniques. Compound **2** showed a potent enzyme inhibition activity (IC_{50} 20.96 ± 0.93), which is comparatively higher than that for the standard thiourea (IC_{50} 21.01 ± 0.51 μM). Compounds **1** and **3** also showed a significant activity, with IC_{50} 37.95 ± 1.93 and 138.43 ± 1.23 μM, respectively. The sub crude fractions (**F1**, **F2**, **F3**, and **F4**) were tested *in vitro* for their urease inhibition activity. Fractions **F2** and **F4** showed significant activity with IC_{50} 140.37 ± 1.93 and 167.43 ± 3.03 μM, respectively.

Keywords: Hypericum oblongifolium*; guttiferae; urease inhibitors*

Introduction

Hypericum oblongifolium WALL. is an erect evergreen shrub, 6–12 m high, that occurs on Khasia Hill at an altitude of 5000–6000 m, in China, and in the Himalayan hills[1]. It has been used in traditional Chinese medicine for the treatment of hepatitis, bacterial infections, and nasal hemorrhage, and as a remedy for dog-bites and bee-stings. The plants of the *Hypericum* genus have shown antidepressant, anxiolytic, antiviral, wound healing, and antimicrobial activities[2]. Due to their widespread use in folk medicine, the chemical constituents and sub crude fractions of *Hypericum oblongifoliun* were evaluated for their urease inhibitory activities. Urease (urea amidohydrolase, EC 3.5.15) catalyzes the hydrolysis of urea to ammonia and carbon dioxide[3] and has been shown to be an important factor in the pathogenesis of many clinical conditions[4]. Urease inhibitors have attracted major attention as potent anti-ulcer drugs[5]. Due to the diverse functions of this enzyme, its inhibition by potent and specific compounds could lead to the treatment of infections caused by urease-producing bacteria[6]. A number of synthetic compounds including imidazoles, hydroxamic acids, and phosphazenes are effective urease inhibitors, but limited studies have been conducted on natural products[5,7]. Herein we report the urease inhibitory activity of various fractions and compounds isolated for the first time from *H. oblongifolium*.

Experimental

General

Ultraviolet (UV) spectra were obtained on a Hitachi-U-3200 UV-visible spectrometer with $CHCl_3$ as solvent. Infrared (IR) spectra were recorded on a Vector 22 (Bruker) Fourier-transform infrared (FT-IR) spectrometer using CH_2Cl_2 as solvent. 1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance AV-500 spectrometer. Chemical shifts (δ) are expressed in ppm relative to tetramethylsilane (TMS) as internal standard and coupling constants are given in Hz. ^1H NMR spectra were referenced against the $CHCl_3$ + CH_3OH signal at δ_H 7.27, 4.9 and ^{13}C NMR spectra against the corresponding signal at δ_C 77.04. Mass spectra (electron ionization (EI-) and high resolution (HR)-EI-MS) were measured in electron impact mode on Finnigan MAT-312 and MAT-95 XP spectrometers, and ions are denoted as m/z (%). Thin layer chromatography (TLC) was performed on pre-coated silica gel F-254 plates (E. Merck); the detection was done at 254 nm, by spraying with ceric sulfate reagent. Column silica gel (E. Merck, 70–230 mesh) and flash silica gel (E. Merck, 230–400 mesh) were used for column chroma-

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tography. Melting points were determined on a Buchi-535 apparatus and are uncorrected.

Plant material

Hypericum oblongifolium was authenticated by Professor Dr Habib Ahmad, Dean of the Faculty of Science, Hazara University. The plant material was collected during the flowering period in June 2007 from Buner District, Northwest Frontier Province (NWFP). A voucher specimen (HUH-002) was retained for verification purposes in the Department of Botany, Hazara University, NWFP, Pakistan.

Extraction and isolation

The air-dried, powdered twig material (12 kg) was exhaustively extracted with hexane, ethyl acetate, and methanol (3 × 25 L, each for 3 days) at room temperature. Extracts were concentrated under vacuum to obtain a residue, as fractions **F1** (hexane) and **F2** (ethyl acetate). The concentrated methanolic fraction was suspended in water and extracted with *n*-butanol to afford fractions **F3** (butanol) and **F4** (water). The ethyl acetate fraction (**F2**, 260 g) was subjected to column chromatography over silica gel and eluted with *n*-hexane–ethyl acetate and ethyl acetate–MeOH, in increasing order of polarity, to afford 200 fractions that were combined according to similarity on TLC profiles, and 30 major fractions were obtained. Fraction 14 was further subjected to column chromatography over flash silica gel (chloroform–hexane, 30:70) and led to the isolation of compound **2** (22 mg). Fractions 15 and 16 were combined and loaded over a flash silica gel chromatography column (chloroform–hexane, 40:60) to afford **1** (6 mg). Similarly, fractions 18 and 19 were combined and subjected to flash silica gel column chromatography (methanol–chloroform, 1:99) to afford **3** (11 mg).

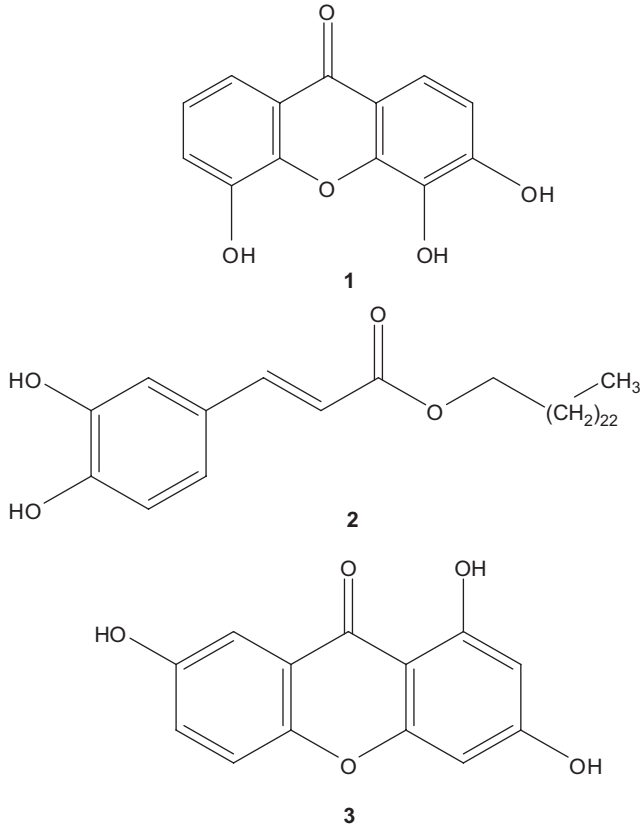

Figure 1. Structure of compounds **1–3**.

*3,4,5-Trihydroxy xanthone (**1**)*

Yellow amorphous powder solid; m.p. 280–283°C; IR, ν_{max} (chloroform) cm^{-1} 3599, 3512, 3462 (O-H), 2928, 2843, 1644 (C=O), 1580, 1443, 1328, 1257, 1137, 1047; λ_{max} ($CHCl_3$) nm (log ε): 240 (4.38), 308 (3.81), 346 (3.71); EI-MS (70 eV) *m/z* 244.0 (calc. for $[C_{13}H_8O_5]^+$); ^{1}H NMR (400 MHz, CD_3OD + $CDCl_3$): δ_H 7.7 (1H, *dd*, *J* = 7.9 Hz, *J* = 1.4 Hz, H-8), 7.39 (1H, *dd*, *J* = 7.9 Hz and 1.45 Hz, H-6), 7.35 (1H, *d*, *J* = 8.9 Hz, H-2), 7.28 (1H, *t*, *J* = 7.9 Hz, H-7), 6.96 (1H, *d*, *J* = 8.9, H-1); ^{13}C NMR (100 MHz, CD_3OD + $CDCl_3$): δ_C 183.0 (C-9), 161.0 (C-4), 149.0 (C-3), 148.0 (C-4), 147.0 (C-5a), 141.0 (C-4a), 124.0 (C-2, 7), 122.0 (C-8a), 121.0 (C-6), 116.0 (C-8), 110.0 (C-1a), and 107.0 (C-1). The physical and spectral data showed complete agreement with those reported in the literature[8].

*Tetracosyl 3-(3,4-dihydroxyphenyl) acrylate (**2**)*

White solid; m.p. 202–205°C; UV, λ_{max} (MeOH): 235 (log ε 4.1), 325 nm (4.0); IR bands (KBr): 3500, 1700, 1670, 1600, 1510, 1460, 1280, 1160, 715 cm^{-1}; EI-MS (70 eV) *m/z* 516.0 (calc. for $[C_{33}H_{56}O_4]^+$); ^{1}H NMR (400 MHz, $CDCl_3$): δ_H 7.6 (1H, *d*, *J* = 15.0 Hz, H-7), 7.2 (1H, *s*, H-6), 7.1 (1H, *d*, *J* = 10.0 Hz, H-3), 6.98 (1H, *d*, *J* = 10.0 Hz, H-2), 6.26 (1H, *d*, *J* = 15.0 Hz, H-8), 4.1 (2H, *m*, H-10), 1.0–1.3 (44H, *m*, H-11-24), 0.97 (3H, brs, Me-25); ^{13}C NMR (100 MHz, $CDCl_3$): δ_C 168.5 (C-9), 148.5 (C-5), 147.4 (C-7), 146.27 (C-4), 123.49 (C-1), 122.61 (C-6), 120.41 (C-2), 119.43 (C-8), 118.54 (C-3), 63.4 (C-10), 24.44–30.4 (C-11-32), 23.92 (C-25). The physical and spectral data showed complete agreement with those reported in the literature[9].

*1,3,7-Trihydroxy xanthone (**3**)*

Yellow solid; m.p. 318–320°C; IR, ν_{max} (chloroform) cm^{-1} 3519, 3502, 3442 (O-H), 2928, 2843, 1654 (C=O), 1580, 1443; λ_{max} ($CHCl_3$) nm (log ε): 244 (4.38), 318 (3.81), 356 (3.71); EI-MS (70 eV) *m/z* 244.0 (calc. for $[C_{13}H_8O_5]^+$; ^{1}H NMR (400 MHz, CD_3OD + $CDCl_3$): δ_H 7.45 (1H, *d*, *J* = 8.9, H-8), 7.35 (1H, *d*, *J* = 8.9 Hz, H-5), 7.25 (1H, *dd*, *J* = 8.9 Hz and 2.8 Hz, H-6), 6.39 (1H, *d*, *J* = 1.9 Hz, H-4), 6.19 (1H, *d*, *J* = 8.9 Hz, H-2); ^{13}C NMR (100 MHz, CD_3OD + $CDCl_3$): δ_C 176.7 (C-9), 167.3 (C-3), 164.6 (C-1), 159.3 (C-4a), 155.8 (C-7), 151.0 (C-5a), 125.0 (C-6), 122.0 (C-8a), 119.8 (C-5), 109.0 (C-8), 98.8 (C-2), and 96.9 (C-4) The physical and spectral data showed complete agreement with those reported in the literature[10].

Urease inhibition assay

Reaction mixtures comprising 25 μL of enzyme (jack bean urease) solution and 55 μL of buffer containing 100 mM urea were incubated with 5 μL of test compound (0.5 mM) at 30°C for 15 min in 96-well plates. Urease activity was determined by measuring the ammonia production using the indophenol method, as described by Weatherburn[11]. Briefly, 45 μL of each phenol reagent (1% (w/v) phenol

and 0.005% (w/v) sodium nitroprussside) and 70 μL of alkali reagent (0.5% (w/v) NaOH and 0.1% active chloride, NaOCl) were added to each well. The increasing absorbance at 630 nm was measured after 50 min, using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). All reactions were performed in triplicate in a final volume of 200 μL. The results (change in absorbance per min) were processed using SoftMax Pro software (Molecular Devices). The entire assay was performed at pH 6.8. Percentage inhibitions were measured from the formula 100 – ($OD_{testwell}$/$OD_{control}$) × 100. Thiourea was used as the standard inhibitor of urease[12]. For kinetic studies, the concentration of compounds **1** and **2** that inhibited the hydrolysis of substrates (jack bean urease) by 50% (IC_{50}) was determined by monitoring the inhibition effect of various concentrations of both compounds in the assay. The IC_{50} (inhibitor concentration that inhibits 50% activity of enzyme) values were then calculated using the EZ-Fit Enzyme Kinetics program (Perrella Scientific Inc., Amherst, MA, USA). Graphs were plotted using the GraFit program[13]. Values of correlation coefficients, slopes, intercepts, and their standard errors were obtained by linear regression analysis using the same program. Each point in the constructed graphs represents the mean of three experiments. The K_i values were calculated from the slopes of each line in the Lineweaver–Burk plot versus different concentrations of both compounds.

Results and discussion

The bioassay-guided fractionation of *H. oblongifolium* led to the isolation of potent urease inhibitors. Various fractions (**F1**, **F2**, **F3**, and **F4)** were obtained from the air-dried, powdered twigs of *H. oblongifolium* (see "Experimental" section). These fractions (**F1**, **F2**, **F3**, and **F4)** were tested *in vitro* for their urease inhibition activity. Among the fractions, **F2** and **F4** showed significant activity with IC_{50} 140.37 ± 1.93 and 167.43 ± 3.03 μM, respectively. Therefore, **F2** was subjected to column chromatography over silica gel, eluting with *n*-hexane–ethyl acetate and ethyl acetate–MeOH in increasing order of polarity, to afford compounds **1–3**. All these compounds were evaluated for urease inhibitory activity. The IC_{50} values with percent inhibition of urease by various fractions and compounds are summarized in Table 1. Compound **2** showed potent activity (IC_{50} 20.96 ± 0.93), which is comparatively higher than that for standard thiourea (IC_{50} 21.01 ± 0.51 μM). Compounds **1** and **3** also showed significant activity, with IC_{50} 37.95 ± 1.93 and 138.43 ± 1.23 μM, respectively. The activities of **1**, **2**, and **3** can be attributed to their co-ordinating capabilities with the metallocenter (i.e. nickel) of the enzyme[14]. The greater activity of compound **2** can be conceived to be due to the presence of two aromatic hydroxyl groups and α,β unsaturated carboxylic in the backbone of the molecule, which can strongly bind to the active sites of the enzyme[15]. Compounds **1** and **2** inhibited the urease enzymes (Figure 2) in a concentration-dependent manner, with K_i value of 31 ± 0.010 and 18 ± 0.014 mM against the jack bean ureases, respectively. Lineweaver–Burk plots and their replots indicated that **2** is a mixed type of inhibitor of jack bean urease, as a change in both V_{max} and affinity (K_m value) of urease toward the substrate (urea) was observed. On the other hand, compound **1** showed a competitive type of inhibition (Figure 2), causing an increase in K_m without affecting the V_{max} value. Mechanistic studies of both compounds are expected to provide useful information about the design of new inhibitors of jack bean urease.

Table 1. The IC_{50} values by percent inhibition of urease to the fractions and compounds.

Compound/extract	% Inhibition at 1000 μg/ml	IC_{50} (μM) ± SEM
F1	26.9	—
F2	68.3	140.37 ± 1.93
F3	23.7	—
F4	67.5	167.43 ± 3.03
1	99.3	37.95 ± 1.93
2	96.96	20.96 ± 0.93
3	71.4	138.43 ± 1.23
Thiourea	98.82	21.01 ± 0.51

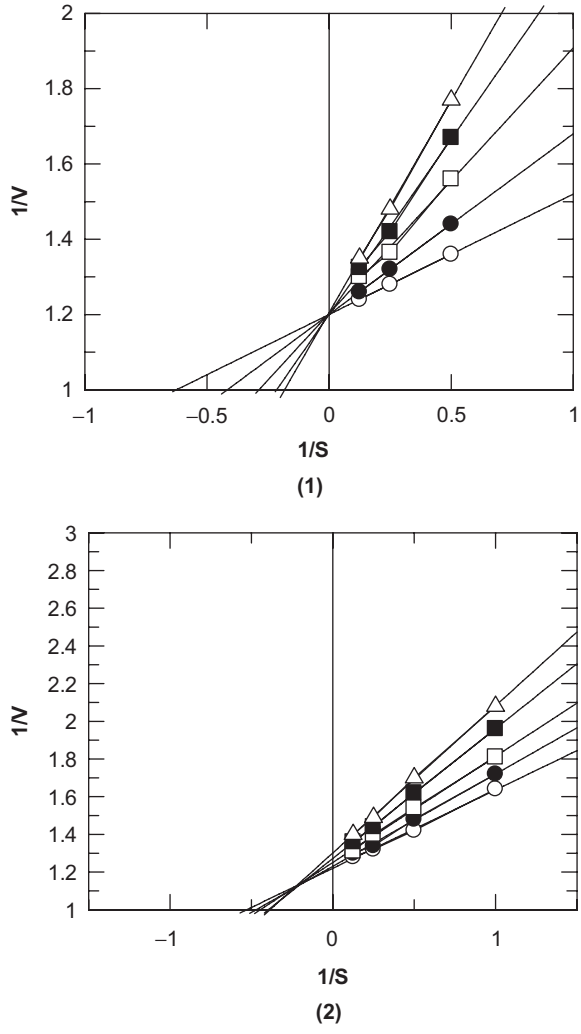

Figure 2. Inhibition of jack bean urease by compounds **1** and **2**. Lineweaver–Burk plots of the reciprocal of initial velocities vs. reciprocal of four fixed substrate concentrations in absence (○) and presence of 100 mM (▲), 80 mM (△), 60 mM (■), 40 mM (□), 20 mM (●).

Table 2. *In vitro* inhibition of urease by compounds **1** and **2**.

Compound	Enzyme	Type of inhibition	IC_{50} (μM) ± SEM	K_i[a] ± SEM[b] (mM)
1	Jack bean urease	Mixed type	37.50 ± 0.94	31 ± 0.010
2	Jack bean urease	Competitive	20.96 ± 0.47	18 ± 0.014

[a]K_i is the mean of three values calculated using Lineweaver–Burk secondary plots.
[b]Standard mean error of three assays.

Acknowledgements

Declaration of interest

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