Rubiothiazepine a Novel Unusual Cytotoxic Alkaloid from *Ixora undulata* Roxb. Leaves

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**Abstract**

Leaves of *Ixora undulata* Roxb. were investigated phytochemically for the first time for its alkaloidal content, which resulted in the isolation of a novel unusual thiazepine alkaloid glycoside identified as 7-[(β-D-glucopyranosyloxy)-6-hydroxy-2-methoxy-4,5-dihydro-1,3-thiazepine and trivially named as Rubiothiazepine. Its structure was determined on the basis of 1D and 2D NMR (1H-, 13C-NMR, 1H−1H COSY, 1H−13C HETCOR, 1H−13C HMBC, 1H−15N HMBC and DIFNOE), high resolution ESI-CID-MS/MS, UV and IR spectroscopy. The isolated compound showed cytotoxic activity against EL4 (Murine Leukemia) with IC_{50} >100 µg/mL, and also showed cytotoxic and HIV-1 activity against MT-4 and HIV-1IB with CC_{50} >100 µg/mL and EC_{50} >100 µg/mL, respectively.

**Keywords:** *Ixora undulata* Roxb.; Rubiaceae; Cytotoxic and HIV-1 activity; Thiazepine Alkaloids.

**Introduction**

*Ixora undulata* Roxb. is native to North Africa, Southern Europe and Asia, and it can be cultivated in moderate climates. *I. undulata* is an evergreen shrub 6 to 8 ft., it belongs to family Rubiaceae which comprises of 400 genera, from which the genus *Ixora* consists of 300 species; three of which were collected in Egypt: *Ixora coccinea, Ixora finlaysoniana* and *Ixora undulata* [1]. The genus *Ixora* has been used in the Ayurvedic system of medicine for a variety of ailments e.g., leaves in diarrhea, antimalarial and anti-inflammatory; roots in hiccough, fever, scores, chonic ulcers and skin diseases; flowers in case of bronchitis, dysentery, cytotoxic and antitumor principles. The aerial parts were used as central nerves system (CNS) depressant, hypothermic and semen coagulant activity [2]. Literature survey revealed little information concerning the chemical constituents, Saleh et al. [3] reported the possible presence of tannins in the plant leaves. Mohammed [4] reported the isolation of D-mannitol from *I. undulata* leaves, which was reported to ameliorate some metabolic disorders in infected mice [5]. Thus, our group started the phytochemical investigation searching for the alkaloidal content of *I. undulata* leaves, and then evaluating the cytotoxicity of the isolates against EL4 (Murine Leukemia), and its anti-HIV-1 activity against MT-4 and HIV-1

**Experimental**

**Plants materials**

The leaves of *Ixora undulata* were collected in May 2006, from El-Orman Botanical Garden - Giza Governorate - Egypt. The plant samples were kindly identified by Miss. Tressa Labib-Head of Taxonomist at the garden, a voucher specimen (No.23) of the plant was kept at the Herbarium of El-Orman Botanical Garden.

**Apparatus**

**Melting point:** (uncorrected) was determined on a BUCHI melting point apparatus. 1H-, 13C-NMR, 1H−13C COSY, 1H−13C HETCOR, DEPT and NOE spectra were obtained using a pulse sequence supplied from Varian VXR-Unite-300 MHz spectrometer (in DMSO-d$_6$). Chemical shifts were given in values (ppm) relative to trimethylsiline (TMS) as an internal reference. Nitrogen (15N) chemical shifts are reported against EL4 (Murine Leukemia), and its anti-HIV-1 activity against MT-4 and HIV-1

δ 380.2. Gradient 1H−13C HMBC (J, 10 Hz), and gradient 1H−15N HMBC experiments (J$_{1H,15N}$ 5 Hz) were performed with standard pulse programs on a Bruker Advance DPX 500, ESI/MS: was obtained using Nano-electrospray tandem (MS/MS) mass spectrometry on a hybrid quadrupole time-of-flight (Q-TOF) MS instrument equipped with Protanis Nano-ESI source for HRESI/MS and Nano-spray needles from Proxeon (Applied Biosystems/MDS Sciex) (QSTAR, prototype, PE-Sciex, Canada). Tandem (MS/MS) spectra were interpreted using the programs BioMultiView (PE Sciex, Canada) and GPMAW (Lighthouse Data, Denmark).

For accurate mass measurements the instrument was calibrated using a 10 mM solution of NaI in isopropanol/water. The instrument’s mass scale was calibrated for each determined ion mass using the cluster ions Na$_{n+1}$+ closest to the sought mass. Collision induced dissociation (CID) spectra were obtained using N$_2$ in the collision cell and collision energies between 30 – 40 eV (E$_{col}$). Analyses were first conducted using ESI/MS in positive mode to obtain ionized molecular species. Then tandem MS/MS spectra were obtained by Collision Induced Dissociation (CID) of the [M+Na]$^+$ ion. The product ion spectra were obtained in the continuous mode of acquisition of the quadruple analyzer. Reversed Phase-High Performance Liquid Chromatography (RP-HPLC): Consists of L-6200 Intelligent Pump (Merck-HITACHI) equipped with UV-VIS Detector SPD-10A (SHIMADZU), the column used in HPLC separation is (20 µm × 250 mm, Develosil ODS-HG-5, Nomura Chemicals). Samples of 8 mL volume were injected into 10 mL loop (after prefiltration with Nylon Filter 0.45 µm.) HPLC solvents used for all analyses were of grade M (Sigma-Aldrich chemie, UK) with ultra-pure water. All solvents used were of AR grade. Kiesel

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gel 60 F254 (Merck) was used for analytical TLC.

**Extraction and isolation**

The air-dried leaves (1.3 Kg) of *Ixora undulata* Roxb. leaves were defatted with pet. ether (60-80°C), then the defatted residue was extracted with hot MeOH, which was defatted once again with pet. ether (60-80°C) to completely remove all the fatty components and sterols. The residue was dissolved in distilled H2O and extracted with CHCl3 (discharged) and finally with MeOH to afford 46.66 g of the MeOH extract. 35.2 g of the MeOH extract were dissolved in 120 mL of (1:1) of MeOH – 1% HCOOH, then subjected to fractionation (8 mL injected into 10 mL loop, after prefiltration with Nylon Filter 0.45 μm) using preparative RP-HPLC (20 μm x 250 mm, Develosil ODS-HG-5, Nomura Chemicals) at r.t. Solvent A = 1% HCOOH and Solvent B = 100% MeOH were used in the elution profile 90% A, 50% A and finally washed with 100% B, monitoring at 340 nm, with flow rate 4 mL/min. Fractions eluted with 50% A were mainly phenolics (kept for further investigation). Elution with 90% A resulted with 4 fractions (1 – 4); fraction 1 was precipitated giving D-mannitol, fractions (2 – 4) gave the characteristic orange colour with Dragendorff’s reagent. Rechromatography of these fractions (2 – 4) using the elution profile of 95% A linearly, with Rf = 0.8ml/min and u.v. 340 nm, revealed the isolation of compound 1, which was examined for its purity using TLC with EtOAc-HCOOH-AcOH-H2O (100:11:11:26) and EtOAc-MEK-HCOOH-AcOH-H2O (50:30:7:3:10).

**Assays for cytotoxicity**

The cell line EL (Murine Lymphoma) was purchased from National Cancer Institute (NCI). A standard high-flux anticancer-drug screening method was employed in this study [6]. Briefly, cancer cells were plated in 96-multitwell plate (104 cells/well) for 24 h before treatment with the samples to allow attachment of the cell to the wall of the plate. Different concentrations of the samples under test (0, 1, 5, 10, 25, 50, 100, 250, 500, and 1000 μg/mL) were added to the cell monolayer, triplicate wells were prepared for each individual dose. The monolayer cells were incubated with the test samples at 37 °C for 48h in atmosphere of 5% CO2, 1% O2, 94% N2. The cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [7]. The cytotoxicity of test compounds was evaluated in parallel with the standard limiting dilution method (dilution 1:2, four replica wells per dilution) in 96-well plates. The infectious virus titer was determined by light microscope scoring of syncytia after 4 days of incubation. Virus titers were expressed as CCID50/mL.

**Anti-HIV assays**

The activity of tested compound against multiplication of HIV-1 wild type IIIB, N119, A17, and EFV6 in acutely infected cells was based on inhibition of virus-induced cytopathicity in MT-4 cells. Briefly, an amount of 50 μL of culture medium containing 1.0 x 104 cells was added to each well of flat-bottom microtiter trays containing 50 μL of culture medium with or without various concentrations of test compounds. Then an amount of 20 μL of HIV-1 suspensions (containing the appropriate amount of CCID50 to cause complete cytopathicity at day 4) was added. After incubation at 37°C, cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [7]. The cytotoxicity of test compounds was evaluated in parallel with their antiviral activity and was based on the viability of mock-infected cells, as monitored by the MTT method.

**Results and Discussion**

Compound (1) was isolated as white crystalline powder (MeOH), with m.p. 176–178°C and [α]D25 = +38.5° (c 0.02, EtOH). It gave with Dragendorff’s reagent the characteristic orange colour of alkaloid [7,8]. Micro-analysis of compound (1) revealed the presence of C, H, N, and S. The IR (KBr Disk) revealed the presence of 3368 cm-1 (B−H stretching), 1631 cm−1 (C=N), 1032 − 1073 cm−1 (C–H stretching). Its molecular formula was determined to be C18H14NO5S by (Positive mode) HRESIMS (Figure 1), a quasimolecular ion peak at m/z 360.0727 [M + Na]+ (calcd 360.0729 for C18H14NO5SNa).

**Figure 1: H−H COSY correlations (−−) and selected EIMS (−→) and ESICID-MS/MS ( ) fragmentations of Rubiothiazepine.
The EIMS of compound (1) (Figure 1) showed a molecular ion peak at m/z 337 [M]⁺ corresponding to the molecular formula C₁₈H₁₇NO₅S. The collision induced dissociation tandem MS/MS (CID-MS/MS) of the sodium adduct [M + Na]⁺ of the compound showed a molecular ion peak at m/z 315 (ESIMS) corresponding to [M – (162u + Na)], this was identical with the molecular ion peak at m/z 175 (EIMS) corresponding to [M – 162u], suggested the presence of a hexose moiety, which was confirmed to be β-hexoside moiety from the molecular ion peak at m/z 201. The ¹H-NMR spectrum (Table 1) showed a doublet signal at δ₁H = 4.44 (1H, d, J = 7.8 Hz, H-1') corresponding to the anomic proton of glucose in the β-configuration, which was confirmed from the ¹H–¹H coupling constant [7], a sharp singlet signal at δ₁H = 3.86 (3H, s, OMe) corresponding to the methoxyl group, a broad doublet at δ₁H = 4.39 (1H, brd, J = 9.3 Hz, H-6), a doublet at δ₁H = 5.04 (1H, d, J = 6.6 Hz, H-7), two sets of doublet of doublet signals at δ₁H = 6.12 (1H, dd, J = 3.3, 10.9 Hz, H-5) and δ₁H = 6.21 (1H, dd, J = 1.1, 10.9 Hz, H-4). In accord with the molecular formula C₁₈H₁₇NO₅S, 12 carbon signals were resolved in the ¹³C-NMR spectrum (Table 1), and categorized by DEPT 135° and HETCOR experiments as: one methoxyl, one methylene, nine methines and one quaternary carbon, which can be divided into one methylene and five methines corresponding to glucosyl moiety, and suggested that the other signals (four methines, one methoxyl and one quaternary carbons) belongs to the aglycone part, this suggestion was confirmed from ¹H–¹H COSY (Figure 1), which showed three cross-peaks represented the correlation of the broad doublet signal at δ₁H = 4.39 with the signals at δ₁H = 5.04, 6.12 and 6.21, another cross-peak corresponding to the correlation of the signal at δ₁H = 6.21 with that at δ₁H = 6.12 with J value (10.9 Hz) corresponding to the molecular ion peak at m/z 201. Thus, compound (1) was suggested to be 1,3-thiazepine glucoside with two possible structures I and II (Figure 3). One nitrogen signal at δ₁N = 326.0 correlated with the proton signals at δ₁H = 6.21 (H-4), at δ₁H = 6.12 (H-5) and at δ₁N = 3.86 (3H, s, -OCH₃), as observed in the ¹H–¹⁵N HMBC (Figure 2). The absolute configuration of compound (1) was confirmed via the DIFNOE experiment (Figure 4) [9,10]. The above mentioned data proved that compound (1) is 7-[(β-D-glucopyranosyloxy)-6-hydroxy-2-methoxy-4,5-dihydro-1,3-thiazepine and trivially named as ‘Rubiothiazepine’ (Structure I).

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<th>Position</th>
<th>Compound (1)</th>
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<th>δ C</th>
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Table 1: The ¹H and ¹³C NMR assignments of Rubiothiazepine.
The cytotoxicity of the isolated compound was evaluated against EL4 (Murine Leukemia) and resulted with IC\textsubscript{50} >100 µg/mL, and it showed cytotoxicity and HIV-1 activity against MT-4 and HIV-1\textsubscript{IIIB} with CC\textsubscript{50} >100 µg/mL and EC\textsubscript{50} >100 µg/mL, respectively.

Although the Family Rubiaceae are rich in alkaloids, approximately 677 alkaloids of different structural types e.g., simple amines, piperidine/pyrrolidine, pyridine, quinoline, isoquinoline, indole, monoterpen, triterpene, peptide, and steroidal alkaloids, were isolated and identified from 57 genera/181 species this is the first study reporting the isolation of the thiazepine type alkaloids from \textit{I. undulata} leaves. Moreover, thiazepine type alkaloids are rare in nature and most of the synthesized thiazepine rings were of 1,4-type and fused with benzene, phenyl…etc. and were free of carbohydrate [11-13].

References
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