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 IC50 >100 µg/mL and also showed cytotoxic and HIV-1 activity against MT-4 and HIV-1IIIB with CC50 >100 µg/mL and EC50 >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 cultivated 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, antimicrobial and anti-inflammatory; roots in hiccup, fever, scores, chonic ulcers and skin diseases; flowers in catarhall 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 MT-4 and HIV-1 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-1IIIB.

**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 Specialists at the garden, a voucher specimen (No.23) of the plant was kept at the Herbarium of El-Orman Botanical Garden.

**Apparatus**

Meltling point: (uncorrected) was determined on a BÜCHI melting point apparatus. 1H-, 13C-NMR, 1H-1H COSY, 1H-13C HETCOR, DEPT and NOE spectra were obtained using a pulse sequence supplied from Varian VXR-Unite-300 MHz spectrometer (in DMSO-d6). Chemical shifts were given in values (ppm) relative to trimethylsilyl (TMS) as an internal reference. Nitrogen (15N) chemical shifts are reported relative to liquid ammonia using a nitromethane chemical shift of δ 380.2. Gradient 1H-13C HMBC (J, 10 Hz), and gradient 1H-13N HMBC experiments (J, 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 Protona's 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+ and closest to the sought mass. Collision induced dissociation (CID) spectra were obtained using N2 in the collision cell and collision energies between 30 – 40 eV (Ecol). 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 quadrupole analyzer. Reversed Phase-High Performance Liquid Chromatography (RP-HPLC); Consists of L-6200 Intelligent Pump (Merck-HITACHI) equipped with UV-VIS Detector SPD-10A/V (SHIMADZU), the column used in HPLC separation is (20 ϕ x 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 chemical, UK) with ultra-pure water. All solvents used were of AR grade. Kiesel

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**Received** November 26, 2013; **Accepted** February 05, 2014; **Published** February 07, 2014

**Citation:** Mohammed MMD, Ibrahim NA, Chen M, Zhai L (2014) Rubiothiazepine a Novel Unusual Cytotoxic Alkaloid from *Ixora undulata* Roxb. Leaves. Nat Prod Chem Res 2: 128 doi:10.4172/2329-6836.1000128

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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 (discarded) 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 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 EL4 (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-multiwell plate (104 cells/well) for 24 h before treatment with the samples to allow attachment of the cells to the wall of the plate. Different concentrations of the samples under test (0, 1, 5, 10, 25, 50, 100, 250, 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 48 h in atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 IU/mL penicillin G, and 100 µg/mL streptomycin. Cell cultures were checked periodically for the absence of mycoplasma contamination with a MycoTect Kit (Gibco). Human immunodeficiency viruses type 1 (HIV-1, IIIb strain) was obtained from supernatants of persistently infected H9/IIIb cells. The HIV-1 stock solutions had titers of 4.5 x 105 to 50% cell culture infectious dose (CCID50)/mL. The Y181C mutant (NIH N119) was derived from the IIIB strain passaged in MT-4 cells in the presence of Efavirenz (up to 2 μM). N119, A17 and EFV, stock solutions had titers of 1.2 x 106, 2.1 x 105 and 4.0 x 104 CCID50/mL, respectively.

**HIV titration**

Titration of HIV was performed in C8166 cells by the standard limiting dilution method (dilution 1:2, four replicate 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 EFV 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 105 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-diphenyldtetrazolium 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]D 25 = +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 (Broad OH), 2933 cm−1 (C–H stretching), 1631 cm−1 (C=N), 1032 – 1073 cm−1 (C–O ether stretching). Its molecular formula was determined to be C19H14NO5S by (Positive mode) HRESIMS (Figure 1), a quasimolecular ion peak at m/z 360.0727 [M + Na]+ (calcd 360.0729 for C19H14NO5SNa).

![Figure 1: H+−H COSY correlations (▬) and selected EIMS (→) and ESI/CID-MS/MS ( ) fragmentations of Rubiothiazepine.](image)
The EIMS of compound (1) (Figure 1) showed a molecular ion peak at m/z 337 [M]+ corresponding to the molecular formula C_{19}H_{20}NO_5S. 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 185 (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 O-hexoside moiety from the molecular ion peak at m/z 201. The 1H-NMR spectrum (Table 1) showed a doublet signal at δ_H = 4.44 (1H, d, J = 7.8 Hz, H-1`) corresponding to the anomeric proton of glucose in the β-configuration, which was confirmed from the 1H-{1H,1H} coupling constant [7], a sharp singlet signal at δ_H = 3.86 (3H, s, OMe) corresponding to the methoxyl group, a broad doublet signal at δ_H = 3.39 (1H, brd, J = 9.3 Hz, H-6), a doublet signal at δ_H = 5.04 (1H, d, J = 6.6 Hz, H-7), two sets of doublet of doublet signals at δ_H = 6.12 (1H, dd, J = 3.3, 10.9 Hz, H-5) and δ_H = 6.21 (1H, dd, J = 1.1, 10.9 Hz, H-4). In accord with the molecular formula C_{19}H_{20}NO_5S, 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 methine 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 δ_H = 4.39 with the signals at δ_H = 5.04, 6.12 and 6.21, another cross-peak corresponding to the correlation of the signal at δ_H = 6.12 with that at δ_H = 6.21 with J value (10.9 Hz) corresponding to AM system in the cis-configuration. The CID-MS/MS spectra of the sodium adduct [M + Na]+ of compound (1) showed a molecular ion peak at m/z 247 corresponding to molecular formula C_{19}H_{19}O_5SNa of m/z (224 + 23) confirmed that, the attachment of the glucosyl moiety to be at C-7 adjacent to the sulphur atom. The EIMS of compound (1) showed a fragment ion peak at m/z 91 corresponding to 1,3-thiazepine skeleton (HS−COCH=NH). The above data suggested that compound (1) has 1,3-thiazepine glucoside skeleton, and its backbone was established as shown in the HMBC (Figure 2), which showed the correlations of the methoxyl and four methene protons with each other and their correlations toward the quaternary carbon of the thiazepine ring. The glucosyl moiety was proved to be attached to C-7 of the thiazepine ring as indicated from the cross-peak between H-1` at δ_H = 4.44 and C-7 at δ_C = 82.8, another two cross-peaks revealed the correlations of the quaternary carbon (C-2) at δ_C = 147.9 with the methoxyl signal at δ_H = 3.86 and H-4 at δ_H = 6.21.

Thus, compound (1) was suggested to be 1,3-thiazepine glucoside with two possible structures I and II (Figure 3). One nitrogen signal at δ_N = 326.0 correlated with the proton signals at δ_H = 6.21 (H-4), at δ_H = 6.12 (H-5) and at δ_H = 3.86 (3H, s, -OCH_3), 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-glucopyranosyl)oxy]-6-hydroxy-2-methoxy-4,5-dihydro-1,3-thiazepine and trivially named as ‘Rubiothiazepine’ (Structure I).

### Table 1: The 1H and 13C NMR assignments of Rubiothiazepine.

| Position | Compound (1) | Δ H(mult, J in Hz) | Δ C | DEPT |
|----------|--------------|--------------------|-----|------|
| 2        |              | 147.6              | qC  |      |
| 4        |              | 6.21 (1H, dd, 1.1, 10.9) | 121.9 | CH   |
| 5        |              | 6.12 (1H, dd, 3.3, 10.9) | 135.0 | CH   |
| 6        |              | 4.39 (1H, brd, 9.3)   | 66.4 | CH   |
| 7        |              | 5.04 (1H, d, 6.6)     | 82.5 | CH   |
| Glu      | 1            | 4.44 (1H, d, 7.8)     | 103.7 | CH   |
| 2        |              | 2.99 (1H, brt, 8.0)   | 73.6 | CH   |
| 3        |              | 3.16 (1H, brd, 8.8)   | 76.2 | CH   |
| 4        |              | 3.06 (1H, brd, 9.1)   | 69.6 | CH   |
| 5        |              | 3.11 (1H, brd, 4.8)   | 77.0 | CH   |
| 6        |              | 3.65 (1H, brd, 11.4)  | 60.8 | CH_3 |
|          |              | 3.47 (1H, d, 4.4, 4.8) |      |      |
| OCH_3    |              | 3.86 (3H, s)         | 62.1 | CH_j |

Figure 2: Full 1H−13C HMBC (→) and 1H−15N HMBC (↔) correlations of Rubiothiazepine.

Figure 3: The postulated structures of compound (2).

Figure 4: Key NOE (↔) enhancements of Rubiothiazepine.
The cytotoxicity of the isolated compound was evaluated against EL4 (Murine Leukemia) and resulted with IC$_{50}$ >100 µg/mL, and it showed cytotoxicity and HIV-1 activity against MT-4 and HIV-1 IIIB with CC$_{50}$ >100 µg/mL and EC$_{50}$ >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, monoterpenes, 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 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].

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