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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-glucopyranosyl)oxy]-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 (^{1}H-, ^{13}C-NMR, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HETCOR, ^{1}H-^{13}C HMBC, ^{1}H-^{15}N HMBC and DIFNOE), high resolution ESI-CID-MS/MS, UV and IR spectroscopy. The isolated compound showed cytotoxic activity against EL_4 (Murine Leukemia) with IC_{50} >100 µg/mL, and also showed cytotoxic and HIV-1 activity against MT-4 and HIV-1IIIB 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 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 hiccough, fever, scores, chonic ulcers and skin diseases; flowers in catarrhal 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 *schistosomal mansoni* 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_{IIIB}.

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

Melting point: (uncorrected) was determined on a BÜCHI melting point apparatus. ^{1}H-, ^{13}C-NMR, ^{1}H–^{1}H COSY, ^{1}H–^{13}C 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 trimethylsilane (TMS) as an internal reference. Nitrogen (^{15}N) chemical shifts are reported relative to liquid ammonia using a nitromethane chemical shift of δ 380.2. Gradient ^{1}H-^{13}C HMBC (J, 10 Hz), and gradient ^{1}H-^{15}N HMBC experiments (J_{NH}, 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 Protana'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 $Na_{n+1}I_n$+ 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_{lab}). 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-10AV (SHIMADZU), the column used in HPLC separation is (20ϕ 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 chemie, UK) with ultra-pure water. All solvents used were of AR grade. Kiesel

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gel 60 F_{254} (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 H_2O and extracted with $CHCl_3$ (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 (20ϕ 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. Re-chromatography of these fractions (2 – 4) using the elution profile of 95% A linearly, with R_f = 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-H_2O (100:11:11:26) and EtOAc-MEK-HCOOH-AcOH-H_2O (50:30:7:3:10).

Assays for cytotoxicity

The cell line EL_4 (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 (10^4 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% CO_2. Cultures were fixed with trichloroacetic acid, then stained with sulforhodamine B and the colour intensity was measured at 490 nm by ELISA reader. All experiments were performed at least two times in triplicates. Thapsigargin (T-9033, >96%, Sigma) was used as positive potent cytotoxin with IC_{50} 1.9 ± 0.5 µM/mL. Data were given as IC_{50} (µM/mL) mean ± SEM from 4 different experiments.

Assay for antiviral

It was performed at department of science and biomedical technology. Cittadella University, Monserrato, Italy Samples were solubilized in DMSO at 100.000 γ and then diluted in culture medium.

Virus and cells: MT-4, C8166, and H9/IIIB cells were grown at 37°C in a 5% CO_2 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 4.5×10^6 50% cell culture infectious dose ($CCID_{50}$)/mL. The Y181C mutant (NIH N119) was derived from an AZT-sensitive clinical isolate passaged initially in CEM and then in MT-4 cells in the presence of nevirapine (10 µM). The double mutant K103N+Y181C (NIH A17) was derived from the IIIB strain passaged in H9 cells in the presence of BI-RG 587 (1 µM). The triple mutant K103R+V179D+P225H (EFV^R) was derived from an IIIB strain passaged in MT-4 cells in the presence of Efavirenz (up to 2 µM). N119, A17 and EFV^R, stock solutions had titers of 1.2×10^8, 2.1×10^7 and 4.0×10^7 $CCID_{50}$/mL, respectively.

HIV titration: Titration of HIV was performed in C8166 cells by 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 EFV^R 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 10^4 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 $CCID_{50}$ 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 $[\alpha]^{20}_D$ = +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 $C_{12}H_{19}NO_8S$ by (Positive mode) HRESIMS (Figure 1), a quasimolecular ion peak at *m/z* 360.0727 $[M + Na]^+$ (calcd 360.0729 for $C_{12}H_{19}NO_8SNa$).

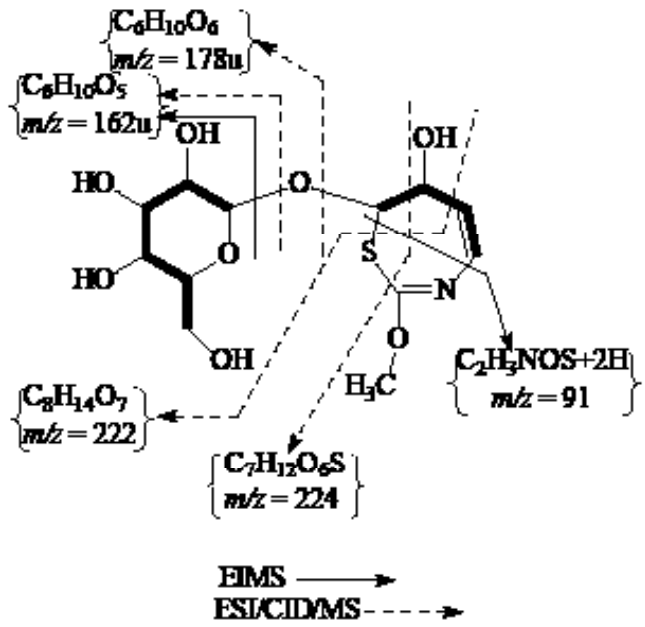

Figure 1: $^1H-^1H$ COSY correlations (—) and selected EIMS (→) and ESI/CID-MS/MS ( ) fragmentations of Rubiothiazepine.

The EIMS of compound (1) (Figure 1) showed a molecular ion peak at *m/z* 337 $[M]^+$ corresponding to the molecular formula $C_{12}H_{19}NO_8S$. 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 ^{1}H-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 $^3J_{H1,H2}$ coupling constant [7], a sharp singlet signal at δ_H 3.86 (3H, *s*, OMe) corresponding to the methoxyl group, a broad signal at δ_H 4.39 (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 $C_{12}H_{19}NO_8S$, 12 carbon signals were resolved in the ^{13}C-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 ^{1}H–^{1}H 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 $C_7H_{12}O_6$ SNa 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-thiaza group (HS–C(OCH_3)=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.

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) 3.47 (1H, *dd*, 4.4, 4.8)	60.8	CH_2
OCH_3	3.86 (3H, *s*)	62.1	CH_3

Table 1: The ^{1}H and ^{13}C NMR assignments of Rubiothiazepine.

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 ^{1}H–^{15}N 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).

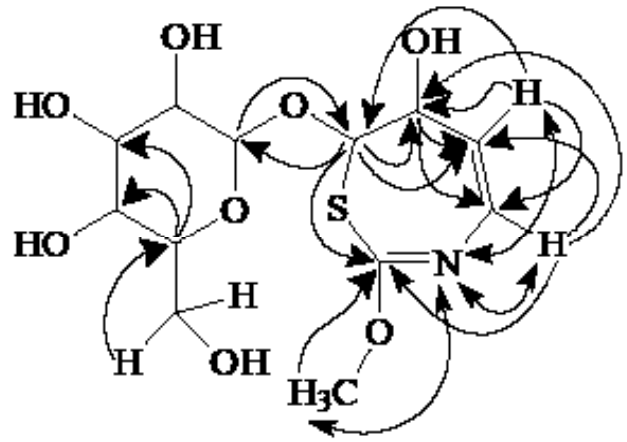

Figure 2: Full ^{1}H–^{13}C HMBC (→) and ^{1}H–^{15}N HMBC (↔) correlations of Rubiothiazepine.

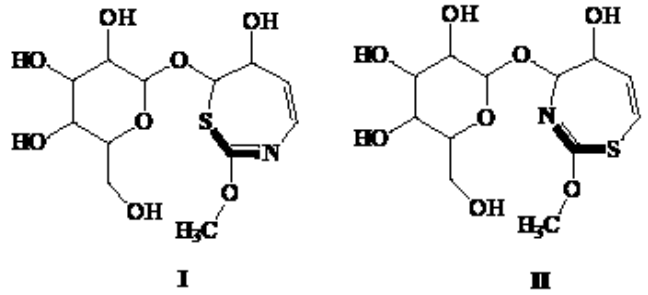

Figure 3: The postulated structures of compound (2).

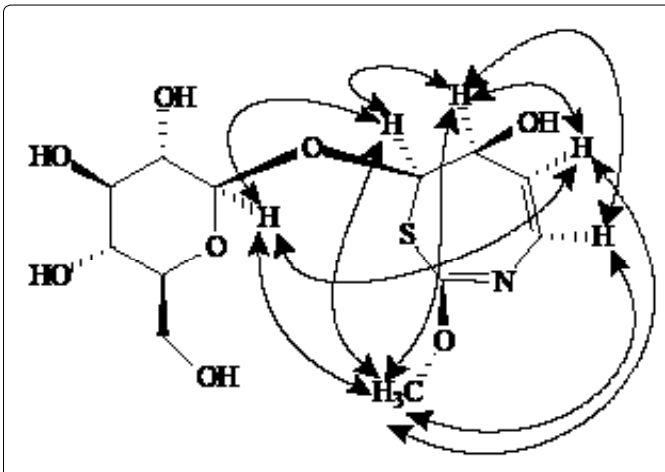

Figure 4: Key NOE (↔) enhancements of Rubiothiazepine.

The cytotoxicity of the isolated compound was evaluated against EL_4 (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, monoterpene, 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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