# Cytotoxicity of 3-*O*-(*β*-D-Glucopyranosyl) Etioline, a Steroidal Alkaloid from *Solanum diphyllum* L.

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In continuation of our interest in phytochemical screening of the Egyptian flora for potential drugs, the reinvestigation of the methanolic extract of the roots of *Solanum diphyllum*, which grows naturally in the south of Egypt and is recorded as new to the Egyptian flora, afforded an interesting, highly cytotoxic compound, named 3-*O*-(*β*-D-glucopyranosyl) etioline [(25*S*)-22,26-epimino-3*β*-(*β*-D-glucopyranosyloxy) cholesta-5,22(*N*)-dien-16*α*-ol]. The chemical structure of this compound was determined by comprehensive NMR studies, including DEPT, COSY, HMQC, and MS. The compound exhibited high cytotoxic effects against the cervical cancer cell line, Hela cells, with an $IC_{50}$ value of 150 *μ*g/mL.

*Key words: Solanum diphyllum*, Steroidal Alkaloid, Cytotoxicity

## Introduction

*Solanum* L. (Solanaceae) is distributed mainly throughout the tropical and subtropical regions of the world and is the largest and most complex genus of the family Solanaceae. The Solanaceae plant family contains members that are relevant to human nutrition and health. These include capsicum (peppers), eggplant, tomato, and potato as well as black nightshade and jimson weed seeds and tobacco. These plants produce beneficial as well as potentially toxic compounds, both during growth and during post-harvest marketing. These compounds include alkaloids and glycoalkaloids (Friedman, 2006). Solanaceous plants are important sources used as food and in folk medicine. *Solanum lyratum* and *S. nigrum* are used as anticancer and antiherpes agents. Extensive investigations of 45 *Solanum* plant species revealed that a considerable amount of glycosides, such as spirosolane, solanidane, spirostane and furostane, are found in these plants, and some of the isolated glycosides showed strong antiproliferative activity against various cancer cell lines and antiherpes activity (Nohara *et al*., 2007). Steroidal alkaloids with an unaltered cholestane carbon skeleton, which generally occur as glycosides, have been isolated from numerous species of the Solanaceae and Liliaceae (Ripperger and Schreiber, 1981; Hegnauer, 1973, 1990). Among these alkaloids spirosolane-type structures prevail but compounds with other heterocyclic structures have also been found (Ripperger and Schreiber 1981; Hill *et al*., 1991). Previous studies centered their attention on the isolation of steroidal alkaloids of the spirosolane type used as starting materials in the industrial production of hormonal steroids, on the search for steroidal alkaloids of novel structure, as well as on the biological activity of these natural products (Ripperger and Schreiber, 1981; Mann, 1979; Wink, 1993). More recently, the inactivation of *Herpes simplex* virus and the inhibition of fungal growth by *Solanum* glycoalkaloids has been demonstrated (Thorne *et al*., 1985; Fewell *et al*., 1994). On the other hand, some studies reported that solasodine glycosides are clinically and his-

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tologically effective in the treatment of skin cancers (Cham *et al*., 1987, 1991). Although a huge number of phytochemicals has been isolated and identified from solanaceous plants, very few compounds have been screened for their biological activities so far. Herein we report on the isolation and identification of an interesting steroidal alkaloid, recently recorded *Solanum diphyllum* from the Egyptian flora and its high, cytotoxic activity from.

## Material and Methods

### *General*

In the $^{1}$H NMR (400 MHz, $CDCl_3$) and $^{13}$C NMR (100 MHz, $CDCl_3$) experiments TMS was used as an internal standard. EIMS was performed on a JEOL SX102A mass spectrometer.

### *Plant material*

The roots of *S. diphyllum* were collected in 2005, from plants naturally grown on a fruit farm in the Nile Island (Elephentene), Aswan area, Egypt. A voucher specimen has been deposited at the Herbarium of the Faculty of Science, Aswan, Egypt.

### *Extraction and isolation*

The air-dried roots (100 g) of *S. diphyllum* were powdered and extracted with MeOH (100%) at room temperature. The extract was concentrated *in vacuo* to give a residue of 12 g. The residue was fractionated on a silica gel column (6 × 120 cm) eluted with $CH_2Cl_2$ (2 L) followed by a gradient of MeOH up to 15% MeOH (2 L of each solvent mixture). The $CH_2Cl_2$/MeOH (9:1) fraction was chromatographed on a Sephadex LH-20 column eluted with *n*-hexane/$CH_2Cl_2$/MeOH (7:4:2) to afford 3-*O*-(*β*-D-glucopyranosyl) etioline (12 mg).

*3-O-(β-D-Glucopyranosyl) etioline* [(25*S*)-22,26-epimino-3*β*-(*β*-D-glucopyranosyloxy) cholesta-5,22(*N*)-dien-16*α*-ol]: Yellowish powder; $[\alpha]_D^{25}$ = –47.78° (*c* 1.35, MeOH). – $^{1}$H NMR (400 MHz, $CDCl_3$, TMS): $\delta_H$ = 4.42 (d, *J* = 8.0 Hz, H-1'), 3.23 (t, *J* = 8.0 Hz, H-2'), 3.41 (overlapped signals, H-3', H-4'), 3.29 (m, H-5'), 3.75 (dd, *J* = 12, 4.8 Hz, H-6'a), 3.84 (dd, *J* = 12.4, 2.8 Hz, H-6'b). – $^{13}$C NMR (100 MHz, $CDCl_3$, TMS): $\delta_C$ = 102.48 (C-1'), 73.70 (C-2'), 76.45 (C-3'), 70.25 (C-4'), 76.66 (C-5'), 61.36 (C-6'). – EIMS (70 eV): *m/z* (rel. int.) = 575 $[M]^+$, 557 $[M–H_2O]^+$, 542 $[M–H_2O–CH_3]^+$, 396 $[M–C_6H_{11}O_6]^+$.

### *Cytotoxicity assay*

#### Cell lines

Hela cells were maintained in DMEM supplemented with 2 mM L-glutamine and 10% FCS (Sigma, USA).

#### Viability assay

Cell viability was detected using a cell counting kit (CCK-8) (Dojindo, Japan). Briefly, cells were precultured in a 96-well plate (3,000 cells/well) for 24 h. 2, 3 and 4 d after 3-*O*-(*β*-D-glucopyranosyl) etioline treatment at the indicated doses, culture media were replaced by the WST-8 reagent. WST-8 reduced by cellular dehydrogenases turns into orange formazan. The produced formazan is directly proportional to the number of living cells. Absorbance was measured at 450 nm by a microplate reader equipped with a computer (NEC, Tokyo, Japan).

#### Flow cytometry analysis

Hela cells were cultured in 3-cm$^2$ dishes for 24 h. Following treatment, cells were trypsinized, washed twice in phosphate-buffered saline (PBS) and the cell cycle phases were analyzed as described by Nicoletti *et al*. (1991) with a minor modification. Briefly, cells were fixed at 4 °C overnight in 70% ethanol. After washing with $Ca^{2+}$-$Mg^{2+}$-free Dulbecco's PBS, cells were treated with 0.1 *μ*g/mL RNase (Type I-A, Sigma, St. Louis, MO, USA), stained with 100 *μ*g/mL propidium iodide (PI; Sigma) for 20 min, filtered and kept on ice until measured. Cells were acquired by a fluorescence activated cell sorter (FACS; BD Biosciences) and then analyzed using the CellQuest software. Cell fractions with a DNA content lower than G0/G1, the sub-G0/G1 peak, were quantified and considered as marker of the number of apoptotic cells.

#### Annexin V staining

After harvesting and washing as described above, the cells were stained directly with PI at a final concentration of 10 *μ*g/mL and 2% annexine-V flous (Roche) in incubation buffer [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$] for 10 min. Cells were acquired with the FACS af-

ter calibrating the controls to non-treated, stained cells and after two washes in PBS. Apoptotic cells were analyzed using the CellQuest software.

## Results and Discussion

The methanolic extract of the roots of *S. diphyllum* was chromatographed on silica gel and Sephadex LH-20 columns to give 3-*O*-(*β*-D-glucopyranosyl) etioline (Fig. 1). 3-*O*-(*β*-D-Glucopyranosyl) etioline was isolated as a yellowish powder. It was positive to Dragendorff's reagent, revealing to be an alkaloid compound. The low-resolution EI mass spectrum showed the molecular ion peak $[M]^+$ at *m/z* 575, in accordance with the molecular formula $C_{33}H_{53}NO_7$. Its characteristic fragment at *m/z* 125 was in accordance with a 22(*N*)-unsaturated 22,26-epiminocholestane structure (Ripperger and Schreiber, 1981). The $[M–C_6H_{11}O_6]^+$ ion indicated the presence of a hexose. The anomeric proton which appeared at $\delta_H$ 4.42 ($J$ = 8.0 Hz) is certainly due to a *β*-D-hexose. The $^1H$ and $^{13}C$ NMR chemical shifts are fully compatible with a *β*-D-glucose structure for this hexose unit. The structure of the compound was determined from careful investigation of the 1D and 2D NMR data. The $^1H$ NMR spectrum revealed the presence of the olefinic proton as a doublet signal at $\delta_H$ 5.37 ($J$ = 4.9 Hz, H-6), which showed clear correlation in the $^1H$-$^1H$ COSY spectrum with a multiplet signal at $\delta_H$ 1.95 (H-7). Moreover, the examination of the connectivity in the $^1H$-$^1H$ COSY spectrum of 3-*O*-(*β*-D-glucopyranosyl) etioline indicated strong correlations were observed between the multiplet signal at $\delta_H$ 1.44 (H-8) with the multiplets at $\delta_H$ 1.95 (H-7) and 1.00 (H-9), suggesting the presence of a $=C_6(H)$-$C_7H(H)$-$C_8(H)$-$C_9(H)$ moiety. Additionally, the $^1H$ NMR spectrum showed the oxygenated proton of the aglycone part located at C-3 as a multiplet signal at $\delta_H$ 3.60 (m, 1H, H-3), correlated in the $^1H$-$^1H$ COSY spectrum with multiplet signals at $\delta_H$ 2.41 (H-4a), 2.30 (H-4b), 1.90 (H-2a), and 1.61 (H-2b). The latter two protons showed clear correlations with the multiplets at $\delta_H$ 1.08 (H-1a) and 1.86 (H-1b), indicating the presence of a $C_1H(H)$–$C_2H(H)$–$C_3H(O)$–$C_4H(H)$ moiety. Moreover, it indicated the other oxygenated proton of the aglycone part located at C-16 as a multiplet signal at $\delta_H$ 3.88 (m, 1H, H-16), correlated in the $^1H$-$^1H$ COSY spectrum with multiplet signals at $\delta_H$ 1.52 (H-15a), 1.58 (H-15b), and 1.51 (H-17), suggesting the presence of a $C_{15}H(H)$-$C_{16}H(O)$-$C_{17}(H)$ moiety. Furthermore, the $^1H$ NMR spectrum revealed the presence of the methyl groups as a singlet signal at $\delta_H$ 0.76 (s, 3H, H-18), a doublet at $\delta_H$ 0.94 (d, 3H, $J$ = 6.8 Hz, H-27), a singlet signal at $\delta_H$ 1.02 (s, 3H, H-19), and a doublet at $\delta_H$ 1.14 (d, 3H, $J$ = 6.8 Hz, H-21). The methylene protons ($CH_2$-N, H-26) appeared as a doublet of doublets at $\delta_H$ 2.92 (dd, 1H, $J$ = 10.0, 16.5 Hz, H-26a) and a multiplet signal at $\delta_H$ 3.60 (m, 1H, H-26b), correlated in the $^1H$-$^1H$ COSY

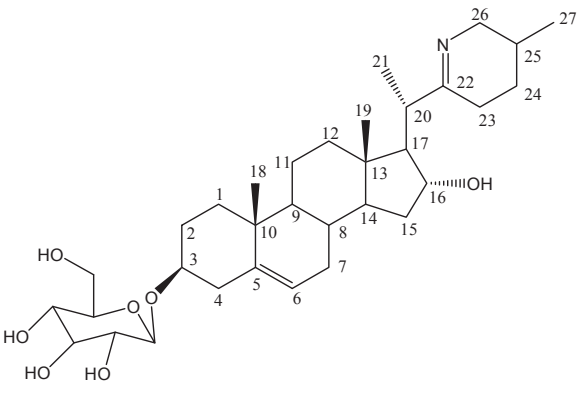


Fig. 1. Chemical structure of 3-*O*-(*β*-D-glucopyranosyl) etioline.

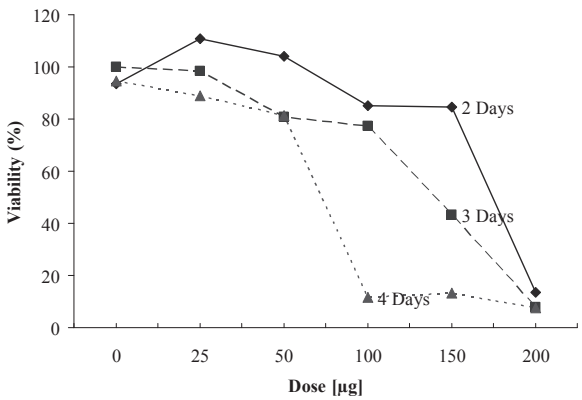


Fig. 2. Comparison of relative viability of Hela cells treated with different doses of 3-*O*-(*β*-D-glucopyranosyl) etioline for different periods of time. Hela cells were precultured in a 96-well plate (3,000 cells/well) for 24 h. Two, three and four days after addition of the compound at the indicated dose, culture media were replaced by the WST-8 reagent (see Materials and Methods). 100 *μ*g of the compound showed significant reduction of cellular viability especially after three days. Each data point represents the mean of three measurements.

spectrum with a multiplet signal at $\delta_H$ 1.61 (H-25), which showed strong correlation with a doublet signal at $\delta_H$ 0.94 (d, 3H, $J$ = 6.8 Hz, H-27) and with the multiplet signals at $\delta_H$ 1.62 (H-24a) and 1.90 (H-24b), indicating the presence of a $C_{24}H(H)$–$C_{25}H(CH_3)$-$C_{26}N(CH_2)$ moiety. The $^{13}C$ NMR data revealed the presence of 33 carbon atoms and their multiplicities (by DEPT analysis) confirmed the number of hydrogen atoms of the formula given above. The carbon atoms were assigned as

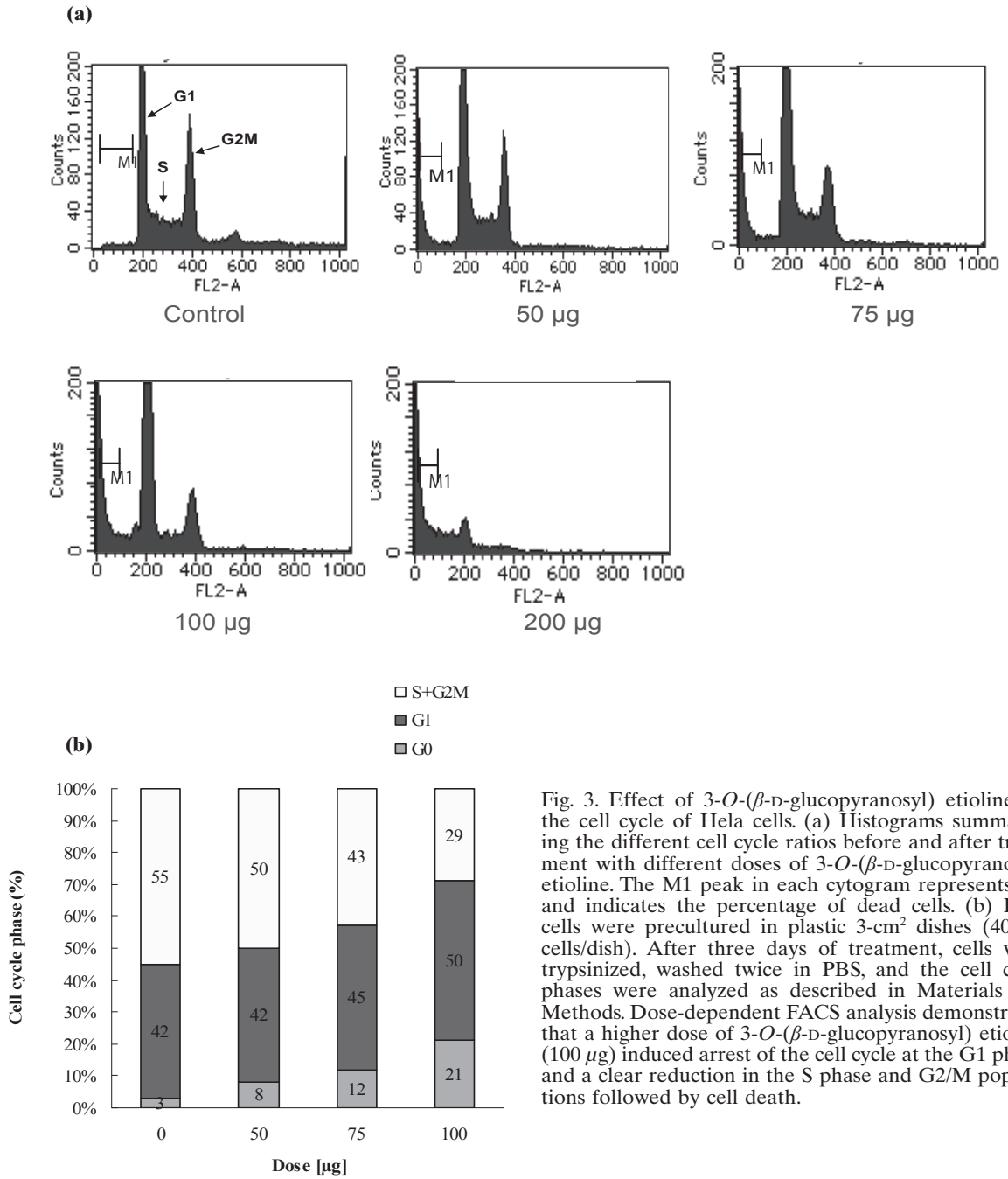


Fig. 3. Effect of 3-*O*-(*β*-D-glucopyranosyl) etioline on the cell cycle of Hela cells. (a) Histograms summarizing the different cell cycle ratios before and after treatment with different doses of 3-*O*-(*β*-D-glucopyranosyl) etioline. The M1 peak in each cytogram represents G0 and indicates the percentage of dead cells. (b) Hela cells were precultured in plastic 3-cm$^2$ dishes (40,000 cells/dish). After three days of treatment, cells were trypsinized, washed twice in PBS, and the cell cycle phases were analyzed as described in Materials and Methods. Dose-dependent FACS analysis demonstrated that a higher dose of 3-*O*-(*β*-D-glucopyranosyl) etioline (100 *µ*g) induced arrest of the cell cycle at the G1 phase, and a clear reduction in the S phase and G2/M populations followed by cell death.

four methyl carbon atoms, eleven methylene carbon atoms, fourteen methine carbon atoms and four quaternary carbon atoms. Moreover, all proton and carbon signals were determined by $^{1}H$-$^{1}H$ COSY, HMQC and comparison with the literature. The spectra indicated 3$\beta$- and 16$\alpha$-hydroxy groups as well as a $\Delta^5$-double bond. On the basis of these results, the structure of the compound was assigned as 3-*O*-($\beta$-D-glucopyranosyl) etioline [(25*S*)-22,26-epimino-3$\beta$-($\beta$-D-glucopyranosyloxy) cholesta-5,22(*N*)-dien-16$\alpha$-ol] (Fig. 1). It was for the first time isolated from *Solanum diphyllum* and previously isolated from *S. spirale* (Ripperger, 1996).

The cytotoxic effect of 3-*O*-($\beta$-D-glucopyranosyl) etioline on the viability of the cervical cancer cell line, Hela cells, has been studied. The viability of Hela cells significantly reduced when treated with 100 $\mu$g of the compound or higher doses for three days or longer. Importantly, doses lower than 100 $\mu$g did not show a significant effect on the cellular viability during shorter time, two days, treatment (Fig. 2). To show how 3-*O*-($\beta$-D-glucopyranosyl) etioline does affect the different cell cycle populations, we treated Hela cells with different doses of the compound (50 $\mu$g, 75 $\mu$g and 100 $\mu$g) for three days, and then cells were acquired by a FACS. Analysis of both control and treated cells by CellQuest software revealed that 3-*O*-($\beta$-D-glucopyranosyl) etioline caused cellular arrest in the G1 phase. Also, cells were released from the S and G2/M phase gradually without accumulation in the G1 phase but they appeared finally in the subG0/G1 phase. Besides showing

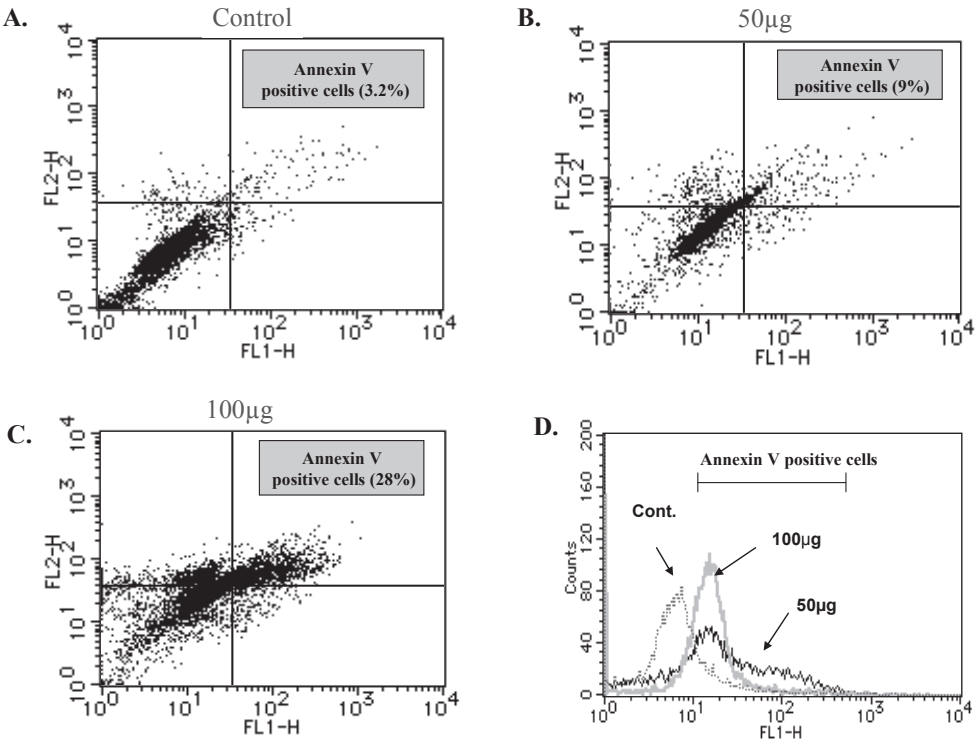


Fig. 4. 3-*O*-($\beta$-D-Glucopyranosyl) etioline-induced cell death by apoptosis. Hela cells were precultured in plastic 3-cm$^2$ dishes (40,000 cells/dish). After three days of treatment with (A) 0 $\mu$g, (B) 50 $\mu$g and (C) 100 $\mu$g of 3-*O*-($\beta$-D-glucopyranosyl) etioline, annexin V staining of cells was performed. The percentage of annexin V-stained cells increased gradually with increasing the compound dose indicating that the cell death induced by this compound is apoptosis. The clouds indicate the distribution pattern of the cells. Annexin V negative cells are distributed to the left while positive cells are shifted to the right side. The cells shifted down are propidium idodid (PI) negative cells and those shifted up are PI positive cells. Shifting the cells to the up-right means death of the cells by apoptosis. (D) Summary of the effect of different doses of the compound showing an increased number of stained cells upon increasing the compound's dose.

how 3-*O*-(*β*-D-glucopyranosyl) etioline affects the human cell cycle, this result confirmed the toxic effect of 3-*O*-(*β*-D-glucopyranosyl) etioline to human cervical cancer cells (Fig. 3). To confirm the toxic effect of 3-*O*-(*β*-D-glucopyranosyl) etioline on Hela cells and also determine if cell death by the compound is apoptosis or necrosis, we estimated the cellular death by annexin V staining. As shown in Fig. 4, the number of annexin V-stained cells increased with increasing the dose of the compound verifying the apoptotic effect of it to human cells. Importantly, 3-*O*-(*β*-D-glucopyranosyl) etioline-induced apoptosis was mitochondria-independent (not intrinsic mechanism of apoptosis) as indicated by Western study of the proapoptotic Bax, the major proapoptotic protein. Hela cells showed no change in the Bax amount before and after 3-*O*-(*β*-D-glucopyranosyl) etioline treatment (data not shown). Further experiments are still needed to prove the precise apoptotic mechanisms and cell cycle regulators involved in the 3-*O*-(*β*-D-glucopyranosyl) etioline-induced cell death.

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