Total Alkaloids from *Solanum lyratum* Thunb. Inhibited HeLa Cells Proliferation Through Induction of Apoptosis and Cell Cycle Arrest

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**SUMMARY.** The object of the present study was to investigate the anticancer properties of total alkaloids from *Solanum lyratum* Thunb (SLT-A), including the inhibitory effect of SLT-A on HeLa cells and the apoptosis-inducing capacity in vitro. In our study, cytotoxicity was measured by the growth inhibition assay and detection of apoptosis was performed by Hoechst33342 and Tdt-mediated dUTP nick end labeling (TUNEL) staining assays. The in vitro cytotoxic studies were complemented by the cell cycle analysis and determination caspase-3 activity. Reverse transcription-polymerase chain reaction (RT-PCR) assay was applied on the expression of apoptosis-associated genes. The result showed that treatment of HeLa cells with SLT-A resulted in the growth inhibition effect, and the IC₅₀ value was approximately 82 µg/mL. SLT-A (80 µg/ml) induced more cell apoptosis of HeLa cells and accumulated the cells in the G2/M phase compared with the control cells. On the other hand, the expression of p53 and Bax gene was increased in the cells treated with SLT-A (80 µg/ml), with an increase in the activity of caspase-3, while Bel-2 expression was not changed compared to the control cells. Our results demonstrated that SLT-A presented antiproliferative activity in HeLa cells and might be a potential anticancer drug.

**INTRODUCTION**

China was a rich source of medicinal plants and many plant extracts were used against diseases in folk medicine, but only a few of these have been scientifically investigated. Plant-derived natural products such as alkaloids, flavonoids, polysaccharides, and so on have received considerable attention in recent years because of their various pharmacological properties, including cytotoxic and cancer chemopreventive effects.

For better or for worse, hundreds of thousands if not millions of patients around the world were experimenting with natural compounds in their efforts to heal themselves of cancer. According to report of Newman and Cragg 1, over 50% of the drugs in clinical trials for anti-tumor activity were isolated from natural sources or are related to them. Several plant products have been tested for antitumor activity and some of these, such as vincristine and taxol are now available as drugs of choice. Moreover, although the future would look bright for eventual success in the fight against cancer, we were not there yet. Much more work remained to be done. As a science, the field of natural compound research could contribute to a greater understanding of cancer and a faster development of successful therapies. One of the best approaches in the search for anti-tumor agents from plant resources was the selection of plant based on ethnomedical leads, and testing the selected plants’ efficacy and safety through modern scientific methods 2.

*Solanum lyratum* Thunb. was a widely occurring plant species in some regions and it had several popular names according to the place where it was found. In China it is widely used as folk medicine by indigenous people to treat a variety of illnesses including digestive tract cancers, malaria, edema, icterus, gonorrhea, rheumatism and ursipelas 3. Some reports have

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shown that aqueous extract of *S. lyra*um can inhibit HeLa cell and SPC-A-1 cell line. In addition, alcoholic extract of it induced MCF-7 cell and BEL-7404 cell apoptosis. In *vivo*, anti-oxidant effect of water extract of *S. lyra*um was found, and the production of nitric oxide was increased in mice by treatment of it. Furthermore, Hepatoprotective activity of it was also found. Phytochemical analyses of *S. lyra*um revealed a broad chemical constitution, and it was previously believed that this fact could explain its wide use. The main compounds already isolated from the plant were steroidal alkaloids and polysaccharides. The alkaloids present in *S. lyra*um have not been fully elucidated, although tomatidenol, olasodine, soladulcidine and some others have been isolated so far. Interestingly, the same genus plant *Solanum dulcamara* L. has been shown to suppress tumors in mice.

Taking these findings into account, the present work was carried out to unravel the anti-cancer activity of alkaloids of *S. lyra*um against the HeLa cells.

**MATERIALS AND METHODS**

**Chemicals**

Fetal bovine serum (FBS), penicillin G, and streptomycin were from Gibco BRL. Dimethylsulfoxide (DMSO), RNase A, DNase, diethyl pyrocarbonate (DEPC), 1,3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and Hoechst33342 were purchased from Sigma Chemical Co. (St Louis, MO, USA). In Situ Cell Death Detection Kit and Caspase-3 Fluorometric Immunosorbent Enzyme Assay Kit were obtained from Roche Diagnostics (Mannheim, Germany). Dubecco's Modified Eagle's Medium (DMEM) was obtained from Thermo-Fisher Biochemical Products (Beijing) Co., Ltd., (Thermo Fisher, USA). TRIzol and SuperScript® III Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) were purchased from De Yi Yi Bei Jing Science and Technology Development Co., Ltd., (Beijing, China). Other chemicals used were of analytical grade.

**Sample preparation and extraction**

According to the extraction method of alkaloids by Li et al., 300 g air-dried *Solanum lyra*um Thunb. was cut in small pieces, further percolated with 4000 ml 65 °C distilled water under ultrasonic for 90 min, then concentrated under reduced pressure. Adjusted this aqueous extract with sulfuric acid to pH 2-3, then centrifuged the resultant under 3000 rpm for 10 min, collected the supernatant, and concentrated it under reduced pressure at 65 °C. The resulting extract was further extracted with n-butanol under ultrasonic for bedding, collected the water layer, and repeated this step for 20 times. Adjusted it to pH 9-10 with sodium hydrate, and centrifuged under 3000 rpm for 10 min, collected supernatant, and concentrated it under reduced pressure at 65 °C. Again, extracted it with n-butanol under ultrasonic for bedding, collected the n-butanol layer, and extracted the alkaloid portion from n-butanol with sulfuric acid solution of pH 2-3, repeated this step for 25 times. After that, adjusted the acid solution to pH 7.2-7.5, and concentrated it under reduced pressure at 65 °C, then got the crude alkaloids from *Solanum lyra*um Thunb. Added methanol to dissolve crude alkaloid, subsequently filtered it through paper (Whatman No.3) to get rid of the sulfate. Finally, the collected filtered part was concentrated with a rotary evaporator and further frozen and dried to give 1.53 g (0.51% of initial amount), named by alkaloids of *S. lyra*um (SLT-A). SLT-A was prepared as a stock of 1 mg/ml in basal medium DMEM and kept at 4 °C.

The qualitative analysis of SLT-A was carried out with Mayer, Draggendorff and Sonnenschein precipitation reagent (these three reagents were giving positive tests for detecting the presence of alkaloids) and the color of precipitation showed light yellow, tangerine and brown yellow, which revealed that SLT-A was mainly consisted of alkaloids.

**Cell cultures**

HeLa cell line, the human cervical cancer cells, was obtained from the Cell Center of Chinese Academy of Medical Sciences. Normal human lymphocytes were separated by the Ficol Hypaque method. Both HeLa cells and normal cells lymphocytes were incubated in DMEM medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and 5% CO₂ atmosphere.

**MTT assay**

The cytotoxicity assay was performed according to the MTT colorimetric assay. The cells were seeded within a 96-well culture plate (104 cells/well). After 24 h incubation, the cells were treated with different concentrations of SLT-A (20, 40, 60, 80, 100, 120 μg/ml) for 48 h. Cell viability was measured using the MTT assay. Ab-
sorbance in control and drug-treated wells was measured by an Automated Microplate Reader (Multiskan MK3, Finland) at 570 nm. The cytotoxicity of SLT-A and normal human lymphocytes was expressed as IC₅₀ (concentration of 50% cytotoxicity, which was extrapolated from linear regression analysis of experimental data).

**Hoechst33342 staining assay**

HeLa cells were treated with SLT-A (80 µg/ml) for 48 h, then washed, fixed with 4% paraformaldehyde, and subsequently stained with Hoechst33342 (0.5 µg/ml). The cells were observed under a fluorescence microscope (Nikon, Japan), and blue cells with apoptotic nuclear characteristics, such as nuclear condensation and fragmentation, were scored as apoptotic. Results are expressed as the percentage of the number of apoptotic cells compared with the total number of cells in 10 random equal-sized fields.

**TUNEL staining assay**

Apoptosis was also determined by assessment of TUNEL staining assay in parallel to Hoechst33342 staining using the In situ Cell Death Detection kit, according to the manufacturer's instructions. HeLa cells were treated with or without SLT-A (80 µg/ml) for 48 h, and then fixed in 10% neutral buffered formaldehyde for 10 min. After rinsing slides with phosphate buffered saline (PBS), the slides were incubated with blocking solution (3% H₂O₂ in methanol) for 10 min at room temperature. The slides were rinsed with PBS, and then incubated slides in permeabilisation solution (0.1% TritonX-100 in 0.1% sodium citrate) for 2 min on ice. TUNEL reaction mixture was added on the samples after rinsing the slides with PBS, subsequently incubated for 60 min at 37 °C in a humidified atmosphere in the dark. Finally, a fluorescence microscopy (Nikon, Japan) was used to identify the apoptotic cells nuclei, as indicated by their distinct green color change. The number of cells undergoing apoptosis was counted in ten equal-sized fields and expressed as a percentage of the total number of HeLa cells in the same field.

**Caspase-3 activity assay**

Caspase-3 activity was analyzed using a fluorometric immunosorbent enzyme assay kit according to the manufacturer's instructions. Briefly, caspase-3 from cellular lysates (2×10⁶ cells) was captured by a monoclonal anti-caspase-3 antibody precoated to the microtiter plate. Following the washing step, added the fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate (AC-DEVD-AFC) was cleaved proportionally to the amount of activated caspase-3. Generated free fluorescent AFC was determined fluorometrically at 505 nm. Results are reported as formed AFC (µM/L) as determined against a standard curve.

**Cell cycle analysis**

HeLa cells (2×10⁶) were seeded in 6-well culture plate and treated with SLT-A (80 µg/ml) for 48 h. Both floating and attached cells were collected, and poured together in the centrifuge tubes. Cells were washed with PBS, re-suspended and fixed in 70% ice-cold ethanol for 1 h at 4 °C. Subsequently they were treated with RNase A for 30 min. Finally, cells were analyzed in a FACScan flow cytometer (Becton Dickinson, USA). The distribution of cells in the different phases of the cell cycle was analyzed from the DNA-histograms using CELLQuest software.

**RT-PCR assay**

Total RNA was isolated from cells treated with SLT-A (80 µg/ml) by Trizol according to the manufacturer's instructions. After treating the samples with DNase, 1 µg of total RNA was used for cDNA synthesis. In brief, the reverse transcription reaction was carried out in 20-µl volumes containing RNA, 5×First-Strand buffer, 0.1 mM DTT, Oligo(dT)₁₂₋₁₈, 10 mM dNTPs, RNase out, DEPC-H₂O, and Reverse transcriptase at 50 °C. PCR was performed in 50-µl volumes containing Reaction buffer, Taq, 25 mM MgCl₂, 2 mM dNTP, DEPC-H₂O, cDNA and 100 pmol specific primers. The primer pairs (Table 1) were used for PCR. The PCR conditions were as follows: 5 min at 95 °C, followed by cycles of 45 s at 94 °C for denaturation, 45 s at 56 °C for annealing, and 1 min at 72 °C for extension. PCR products were analyzed with ethidium bromide-stained 1% agarose gels electrophoresis, viewed under ultraviolet illumination. Total gray were estimated by Glyko BandScan 4.5 for RT-PCR products and the data were presented as the ratio of samples densitometric units to the β-actin units for each condition.

**Statistical analysis**

The data were expressed as mean ± standard deviation (SD). A one-way analysis of variance (ANOVA) using Origin7.0 software and p < 0.05 was considered statistically significant.
RESULTS

Cytoxicity of SLT-A to HeLa cells

For most anticancer agents, cytotoxicity is measured by a standard MTT assay following a brief drug exposure. HeLa cells and normal human lymphocytes were treated with various concentration of SLT-A for 48 h. The results showed that the IC₅₀ of SLT-A towards HeLa cells was approximately 82 μg/ml. On the other hand, the IC₅₀ of SLT-A towards normal human lymphocytes exceeded 300 μg/ml. The cytotoxic activities of SLT-A were substantially lower towards normal human lymphocytes. On the basis of the results, SLT-A (80 μg/ml) was used in the next experiments with 48 h incubation.

Analysis of apoptosis induced by SLT-A

To determine the contribution of cell death to SLT-A-induced reductions in cell growth, we employed two different methods to detect apoptotic cells: Hoechst33342 staining and TUNEL labeling. Hoechst33342 staining, by fluorescence detection, enables more direct visualization of apoptotic cells, which undergo compaction and fragmentation of the nuclear chromatin, shrinkage of the cytoplasm, and appearance of apoptotic body. As shown in Figure 1, condensed nuclei and apoptotic body were more frequently observed in the cells by treated with SLT-A and were only rarely seen in the control cells. These findings were confirmed by TUNEL assay, in which labeled uridine bases are attached to the DNA nicks characteristic of apoptotic cells showing green fluorescence. Many cells were apoptotic compared with the control group. Moreover, most of the cells counted apoptotic showed stronger intensity of green in the HeLa cells by treatment of SLT-A (Fig. 2).

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward primer (5'-3')</th>
<th>Reverse primer (5'-3')</th>
</tr>
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<tbody>
<tr>
<td>β-actin</td>
<td>TCATGAAGTGTGGACCTGTCACTCGT</td>
<td>CTCAGAAGCATTGGCCTGACGATG</td>
</tr>
<tr>
<td>p53</td>
<td>TGAAGCCCTCAGGTGTC</td>
<td>AAGGCTTGG AAGGCTCTA</td>
</tr>
<tr>
<td>Bax</td>
<td>GCTCGAACAGACATGAAAGACAG</td>
<td>CAATCCAAAGTGACACTTAGG</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>CCTGGCACCTGCGGATGCG</td>
<td>CGACTGAAAGTGGACCCAGCAGAAC</td>
</tr>
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Table 1. Primer pairs used in RT-PCR.

Figure 1. Hoechst33342 staining assay. A: the control group, B: treatment of SLT-A (80 μg/ml) group. Arrows showed fluorescence of condensed chromatin or apoptotic body; C: percentage of apoptotic cells in experimental group.

Figure 2. TUNEL staining assay. A: the control group; B: treatment of SLT-A (80 μg/ml) group; C: percentage of apoptotic cells in experimental group.
### Table 2. Effect of SLT-A on distribution of cell cycle. Results are expressed as mean ± SD. Significant differences compared with the control, **p < 0.01.

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1</th>
<th>S</th>
<th>G2/M</th>
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<tbody>
<tr>
<td>Control</td>
<td>46.9±1.31</td>
<td>27.8±1.12</td>
<td>25.3±1.57</td>
</tr>
<tr>
<td>SLT-A (80 µg/ml)</td>
<td>27.1±0.87</td>
<td>25.6±1.63</td>
<td>47.3±1.59**</td>
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**Figure 3.** Caspase-3 activity assay. Caspase-3 activity was compared in HeLa cells treated with SLT-A (80 µg/ml) for 48 h vs. the control group using an immunosorbent assay. Compared with the control group, statistical significance was **p < 0.01.

#### Analysis of caspase-3 activity

To investigate potential molecular mechanisms for SLT-A-induced HeLa cells apoptosis, we examined the effect of SLT-A on cell caspase-3 activity, which is the one of apoptosis regulatory proteins. As shown in Figure 3, caspase-3 activity of cells by treatment of SLT-A was markedly elevated compared with the control group.

#### Expression analysis of apoptosis-regulated genes

We further revealed the mechanisms of SLT-A-induced HeLa cells apoptosis by analysis of expression level in the genes (p53, Bcl-2 and Bax). Comparing with the control group, the expression of p53 and Bax gene was increased by treatment of SLT-A. However, the expression of Bcl-2 was not nearly changed (Fig. 4).

### DISCUSSION

Alkaloids were an important group of diversely distributed, chemically, biologically and commercially significant natural products. Among the chemical classes presented in medicinal plant species, alkaloids stood as a class of major importance in the development of new drugs because alkaloids possessed a great variety of chemical structures and have been identified as responsible for pharmacological properties of medicinal plants. Some plant families had the genetic capability of producing more than one alkaloid, reflected in the structural diversity of these compounds. Many reported that plant-derived medicinal alkaloids processed anti-tumor activity, including the well known vinblastine, vincristine, vincamin and camptothecin isolated from Catharanthus roseus L. Don. Recently, some alkaloids have been showed that antiproliferative activity and induction of apoptosis of cancer cells in vitro and received more attention. In our results, the IC₅₀ values showed that the HeLa cell line was much more sensitive to SLT-A than normal human lympho-
cytes, so a relative low toxicity might be expected for SLT-A in further research.

We have indicated that the total alkaloids of *Solanum lyratum* Thunb. could inhibit the growth of HeLa cells and induce HeLa cells to undergo apoptosis. Apoptosis was programmed into the cell at birth and was triggered at old age or under other conditions where cell death benefited the organism as a whole. At any given moment for any given cell, apoptosis was either induced or not induced depending on the relative balance of "do die" and "do not die" signals, involving many genes \(^24\). Among the RT-PCR assay of apoptosis regulatory genes tested in our studies, the proapoptotic gene Bax was found to be up-regulated in HeLa cells treated with SLT-A, with concomitant triggering of apoptotic pathways, including caspase-3 activations. A major regulation of the apoptotic death signal resided with the Bcl-2 and Bax genes, the former an apoptosis-suppressing and the latter an apoptosis-promoting protein \(^25\). In our studies, Bcl-2 expression was not changed in the HeLa cells, while p53 expression was increased. Thus, SLT-A treatment may result in increased Bax protein expression, leading to an increased apoptotic rate. p53 mediated at least two important cellular events: it could induce cell cycle arrest in G1 or it could promote apoptosis, and p53 probably functioned by regulating the ratio of Bax/Bcl-2 \(^26\). SLT-A belonged to the class of steroidal alkaloids and the steroid molecules could bind to intracellular receptor proteins by direct diffusion across the plasma membrane of target cells \(^3,17\). This binding might further activate the receptors, which then regulate the transcription of apoptosis-relative genes. Then, finally, changes in the products of apoptosis-associated genes resulted in the apoptosis of HeLa cells.

Cancer cells were no different from normal cells in their need to be stimulated before entering the cell cycle, except that stimulation was excessive in cancer cells. This excessive stimulation provided a number of targets for inhibiting cancer cell proliferation. In addition to preventing proliferation, keeping cells from entering the cell cycle might have the long-term effect of inducing apoptosis. When cells were not able to divide, they eventually died of apoptosis in most cases. Thus, control of cell cycle progression in cancer cells was considered to be a potentially effective strategy for the control of tumor growth as the molecular analyses of human cancers \(^27\). In the study, treatment of HeLa cells with SLT-A could affect processes in the cell cycle proper, arresting in the G2/M phase. Hence, one mechanism of anticancer effect of SLT-A could be an inhibition of the malignant proliferation of cancer cells.

Alkaloids presented in a variety of plant species used in traditional medical systems. Biomedical examination revealed that alkaloids were bioactive, distinct in its anticancer properties. In many aspects the anticancer effect of alkaloids was relevant to the development of new anticancer medications. The total Alkaloids of *S. lyratum* needed to be further isolated and characterized the structure of an active compound. Meanwhile, the active compound would be examined \textit{in vivo}. Consequently, we expected that anticancer mechanisms of alkaloids of *S. lyratum* would be completely revealed in the future.

**CONCLUSION**

Our data suggested that SLT-A might act as an inhibitor of Hela cells proliferatin \textit{in vitro} and be a potential, natural apoptosis-inducing anticancer agent.

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**REFERENCES**