ANTITUMOR ACTIVITIES OF THE FOUR SESQUITERPENE LACTONES FROM ELEPHANTOPUS SCABER L.

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Aim: To evaluate antitumor activity of sesquiterpene lactones (scabertopin (ES-2), isoscabertopin (ES-3), deoxyelephantopin (ES-4), isodeoxyelephantopin (ES-5)) isolated from Elephantopus scaber L. in vitro and in vivo. Methods: SMMC-7721, Caco-2 and HeLa cell lines were treated with ES-2,3,4,5. Cell viability was determined by MTT assay. Agarose gel electrophoresis was used to detect DNA fragmentation. To evaluate in vivo antitumor activity of ES-4, experimental murine tumor model was used. Results: It was shown that ES-2, ES-4, ES-5 exhibited significant antitumor effect in vitro in a concentration-dependent manner. However, the effect of ES-3 on the growth of tested cell lines was relatively weak. In HeLa cells exposed to ES-4 for 48 h, morphological changes and DNA ladder pattern evidencing on apoptosis were detected. ES-4 revealed in vivo antitumor activity. Conclusion: Antitumor activity of studied sesquiterpene lactones may be due, at least in part, to induction of apoptosis in vitro. ES-4 possesses also antitumor activity in vivo. Key Words: Elephantopus scaber L., sesquiterpene lactones, antitumor activity.

Presently it is generally realized that plants are a powerful source of clinically relevant compounds for the treatment of a number of diseases. Elephantopus scaber L. grows mainly in South China. It has been used for stomach ailment, hepatitis, nephritis, and bronchitis in folk medicine. Some minority ethnic groups in the southwest district of China also use this medicinal herb [1].

Chemical study started from 1960’s and showed that constituents of Elephantopus scaber L. include flavonoids, triterpenoids, flavonoid esters and sesquiterpene lactones. Sesquiterpene lactones are most important due to their antitumor activity [2–3]. In spite of this, several tumor cell lines have been investigated, but the mechanism of antitumor activity remains unclear. For that reason, our study focused on investigating whether sesquiterpene lactones — scabertopin (ES-2), isoscabertopin (ES-3), deoxyelephantopin (ES-4), isodeoxyelephantopin (ES-5) — might act as anticancer agents in vitro and in vivo.

Recently, it was shown that the mechanism of action of many antineoplastic drugs is based on apoptosis induction [4–5]. Apoptosis is a regulated cell death used by multicellular organisms to dispose redundant cells. It is morphologically and biochemically characterized by cell shrinkage, cell membrane blebbing, nuclear chromatin condensation, and nonrandom DNA fragmentation. This type of death is altered in many pathological states and is indispensable for elimination of “sick” or not normal cells in organisms [6]. In view of the unclear mechanism of sesquiterpene lactones action, this study also attempts to reveal whether they might function as antitumor agent via induction of apoptosis.

MATERIALS AND METHODS

Preparation of extracts. All plant samples were collected from South China. The material was identified by professor Z.N. Gong, and a voucher specimen was deposited at the Department of Pharmacology, China Pharmaceutical University, Nanjing, P. R. C. for reference. ES-2, ES-3, ES-4, ES-5 were obtained according to [7, 8]. The chemical structures of the four compounds are shown in Fig. 1.

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**Cell lines and conditions of cultivation.** Human hepatocarcinoma SMMC7721 cells were inoculated at a density of $1 \times 10^5$ cells in 96-well plate and cultured in DMEM (GIBCO BRL, USA), supplemented with 10% heat-inactivated fetal bovine serum (Hangzhou Si Ji Qing Co. Ltd, China), 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C and 5% CO₂.

Human cervical carcinoma HeLa cells were cultured at same conditions as SMMC7721 except that the culture medium was replaced by RPMI-1640 (GIBCO BRL, USA).

Human colon carcinoma Caco-2 cells were maintained in DMEM, containing 10% heat-inactivated fetal bovine serum (Hangzhou Si Ji Qing Co. Ltd, China), 1% L-glutamine, 1% nonessential amino acids and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37 °C and 5% CO₂.

**MTT assay.** The cell viability was measured by MTT assay [9]. 20 μl of the stock 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide solution (MTT, 5 mg/ml) (Fluka, USA) was added to each well and cells were incubated for 4 h. 150 μl of the solution was added to each well. The optical density was determined by measuring light absorbance at 570 nm with an ELISA plate reader (Hua Dong Electronic Co., China). All MTT assays were performed in six parallels.

100 μl of cell suspension was added into 96-well culture plates at $5 \times 10^4$ cells per well, and incubated for 24 h at 37 °C. The medium was replaced with fresh medium containing different concentrations of extracts (ES-2/ES-3/ES-4/ES-5, 100, 50, 10, 5, 1μM/L) or the vehicle. Then the extract, containing medium, was removed and cell proliferation was determined by MTT assay. Experiments were performed in triplicate and the results were expressed as inhibitory rate (IR):

$$IR = \frac{1 - \frac{A_{570(chemical)}}{A_{570(control)}}}{100}.$$  

Morphological features of the cells were visualized under a phase-contrast inverted microscope and photographed with a Nikon camera.

**DNA fragmentation.** Experiments were performed as described previously [10]. Cells ($10^5$) were incubated in a digestion buffer containing 0.2 mg/ml proteinase K at 50 °C for 5 h. The cellular DNA was extracted and digested with RNase (final concentration 0.6 mg/ml) at 37 °C for 30 min, the sample was then subjected to electrophoresis on a 1.8% agarose gel in Tris-acetate buffer (40 mM/L Tris-acetate, 2 mM/L EDTA, pH 8.0). The gel was then stained with ethidium bromide and photographed on UV transilluminator.

**In vivo antitumor effect of ES-4 on stably transfected HeLa cells.** A total of 10 athymic 6-week-old nude mice (BALB/c nu/nu, Animal Laboratories, Shanghai, China) underwent subcutaneous implantation of HeLa cells in the mammary line near the axilla. For tumor implantation, $1.0 \times 10^5$ HeLa cells were injected in 0.1 ml of PBS buffered solution. These mice were divided into two groups: group 1 — experimental (ES-4 treatment) ($n = 5$); and group 2 — control (received phosphate-buffered saline solution) ($n = 5$). Treatment began at 8th day after tumor transplantation. The mice underwent intraperitoneal injection of ES-4 (10 mg/kg body weight) once a day for 21 days. Tumor size and body weight were measured every 7 days. Tumor weight measurement was performed routinely [11]. The mice were killed after 3 weeks since initiation of treatment, and the tumors were extracted and weighed. All animal experiments were performed using approved protocols in accordance with the recommendation for proper care.

**Statistical analysis.** Statistical analysis was performed, using Student’s criterium. A value $P < 0.05$ was considered significant.

**RESULTS**

**Effect of ES-2, ES-3, ES-4, ES-5 on proliferation of SMMC7721, HeLa, Caco-2 cell lines.** In order to evaluate the antitumor effect of four isolated compounds from *Elephantopus scaber* L., antiproliferative assay with cell lines SMMC7721, HeLa, Caco-2 was performed.

One from four compounds, ES-4 revealed significant antiproliferative effect (inhibitory rate > 75% at > 50 μM/L) in three cell lines (Table 1), whilst ES-3 has the lower antiproliferative effect (inhibitory rate 41.94% < 50%) at all studied concentrations. For SMMC7721 cell line antiproliferative activity of four compounds in incremental manner looks as: ES-4 > ES-2 > ES-5 > ES-3. For HeLa: ES-2 > ES-4 > ES-5 > ES-3. For Caco-2: ES-4 > ES-2 > ES-5 > ES-3. Generally, the Caco-2 cells were much more sensitive to the antiproliferative effects of the test compounds than other cell lines.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Concentration (M/L)</th>
<th>SMMC7721</th>
<th>HeLa</th>
<th>Caco-2</th>
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<tbody>
<tr>
<td>ES-2</td>
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Notes: inhibitory rate vs. control “−” — indicates 0–25%; “+” — 25–50%; “++” — 50–75%; “+++” — 75–100%. The results are calculated as average mean of three independent experiments with less than 10% standard deviations.

**ES-4 effect on morphology of HeLa cells in vitro.** To show whether these compounds might function as antitumor agents via induction of apoptosis in tumor cells, we selected ES-4, the most active agent,
to observe its effect on morphology and DNA fragmentation in HeLa cells.

In morphological experiment, HeLa cells were inoculated at the density of 1 × 10^5 cells in 96-well plates. After cells reached subconfluence, medium was changed to fresh one, contained appropriate concentration of ES-4, and incubated for 0.5 h, 2 h, 12 h, 24 h, 48 h respectively. As shown on Fig. 2, when cells were incubated with 1 × 10^4 M/L ES-4 for 0.5 h, cells remained unaltered and looked like control cells in RPMI-1640 medium. The exposure of HeLa cells to 1 × 10^4 M/L ES-4 for 2 h caused marked decrease in cell viability, assayed by MTT. Some cells became round and floated in the culture supernatant. As incubation period increased from 12 h to 48 h, more and more cells shrank and detached from the dish. The condensation of nuclei, membrane blebbing and formation of apoptotic bodies were observed by microscopy examination. All these changes are typical indications of apoptosis. At 48 h of incubation, almost all cells had undergone above-mentioned morphological changes. Generally, viability of cells incubated with ES-4 decreased as incubation time increased (Fig. 2), contrary to control samples (with vehicle).

**DNA fragmentation.** In order to detect DNA fragmentation, DNA samples were isolated from HeLa cells after 12, 24, 48 h of incubation with the agent, separated by electrophoresis in 1.8% agarose gel, containing ethidium bromide, and visualized under UV transillumination.

As shown in Fig. 3, after incubation of cells with ES-4 for 12–48 h, oligonucleosome ladder pattern was displayed and became gradually more pronounced as incubation time extended. The data indicated that cellular DNA was cleaved into multiple fragments of 180–200 bp upon apoptosis induced by ES-4.

**Treatment with ES-4 in vivo.** Administration of ES-4 (see Materials and Methods) significantly reduced the growth rate of primary tumors in vivo (Fig. 4).

**DISCUSSION**

A variety of plant extracts have been investigated for their ability to influence programmed cell death or arrest proliferation of tumor cells [12]. The whole plant of *Elephantopus scaber L.*, which belongs to the *Elephantopus* genus of the Compositae family, is used in Chinese medicine as a diuretic, antifebrile, antiviral and antibacterial agent. The plant is rich in novel antitumor substances—sesquiterpene lactones. It was reported that elephantopin from *Elephantopus scaber L.* has inhibitory effect on human nasopharyngeal carcinoma KB cells and murine leukaemia P388 cells with IC50 of 0.28–20 µg/ml. In addition, deoxyelephantopin can significantly arrest the growth of human sarcoma W256 cells. Other sesquiterpene lactones, found in this plant, also have potent antitumor and cytotoxic activity [13–15]. For example, now Argrabin (sesquiterpene lactone), established in Kazakhstan, is successfully used as antitumor drug [16].

Based on our previous work, this paper further investigates antiproliferative effect of four sesquiterpene lactones, isolated from *Elephantopus scaber L.*, scabertopin (ES-2), isoscabertopin (ES-3), deoxyelephantopin (ES-4), isodeoxyelephantopin (ES-5) in vitro. The results showed that compounds ES-2, ES-4, ES-5 have antiproliferative effects and inhibit the growth of SMMC-7721, HeLa and Caco-2 cells in time and concentration-dependent manner. After 48 h treatment the IC50 values for ES-2, ES-4, ES-5 on SMMC-7721, HeLa and Caco-2 cells were 18.20, 14.08, 9.53 µM/L; 12.85, 17.40, 25.85 µM/L and 18.28, 14.59, 18.28 µM/L respectively. All these values are comparable to those reported elsewhere [13–15]. Moreover, we also showed significant inhibition of tumor growth with ES-4 in a human cervical cancer xenograft model, which is in accordance with in vitro data. Our results demonstrated that antiproliferative activity of ES-4 was manifested via apoptosis, as it is shown by morphologic analysis and DNA fragmentation assays.

Medicinal natural resources can contribute to pharmaceutical and health services [17]. The results in this study point to the possibility of development of ES (2–5) as potential agents for cancer chemo-

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**Fig. 3.** Gel electrophoresis analysis of DNA from HeLa cells treated with ES-4. Lane M — DNA DL 2000 marker; lane 1 — DNA from control cells; lane 2–4: DNA from cells exposed to ES-4 for 12 h, 24 h, and 48 h respectively

**Fig. 4.** Dynamics of growth of primary tumors. Data represent means ± SD (n = 5). *p < 0.05 compared with control group on day 7; **p < 0.01 compared with control group on days 14 and 21
therapy. More research concerning the possible utility of *Elephantopus scaber* L. in cancer treatment is warranted.

**ACKNOWLEDGEMENT**

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**REFERENCES**


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**ПРОТИВООПУХОЛЕВЫЕ СВОЙСТВА ЧЕТЫРЕХ ЛАКТОНОВ СЕСКВИТЕРПЕНА ИЗ *ELEPHANTOPUS SCABER* L.**


**Методы:** клеточные линии SMMC-7721, Caco-2 и HeLa обрабатывали ES-2,3,4,5. Инспецифичность клеток определяли при помощи MTT-метода. Для детекции фрагментации ДНК использовали метод электрофореза в агарозном геле. Противоопухолевое действие ES-4 было исследовано в экспериментальной модели in vivo на мышах. Результаты: установлено, что in vitro ES-2, ES-4, ES-5 проявляют противоопухолевое действие в концентрационно-зависимом режиме, причем ES-3 имеет наиболее слабый эффект. При 48 ч инкубации клеток линии HeLa с ES-4 отмечены морфологические изменения клеток и фрагментация ДНК, характерные для апоптоза. В экспериментах in vivo препарат ES-4 проявил противоопухолевую активность. Выводы: индукция апоптоза при действии лактонов сесквитерпена in vitro определяет наличие противоопухолевой активности у этих соединений. Препарат ES-4 также обладает противоопухолевой активностью in vivo.

**Ключевые слова:** *Elephantopus scaber* L., лактоны сесквитерпена, противоопухолевое действие.