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Antimetastatic activities of *Selaginella tamariscina* (Beauv.) on lung cancer cells *in vitro* and *in vivo*

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Abstract

Selaginella tamariscina is a traditional Chinese herb for the therapy of chronic trachitis and has been approved some anti-tumor activity. However, the anti-metastasis effects of *Selaginella tamariscina* in the lung cancer have not been understood clearly. The objectives of study were to investigate the effects of the *Selaginella tamariscina* extracts (STE) on the invasion and motility of highly metastatic A549 and Lewis lung carcinoma (LLC) cells. To further investigate the precise involvement of STE in tumor metastasis, A549 and LLC cells were treated with STE at various concentrations (0–100 μg/mL) for a specified period. The results from zymography showed that a STE treatment decreased ($p < 0.05$) the expressions of matrix metalloproteinase (MMP)-2, -9 and urokinase plasminogen activator (u-PA) in a dose-dependent manner in the A549 and LLC cell. Meanwhile, their endogenous inhibitors, which are tissue inhibitor of metalloproteinase-2 (TIMP-2) and plasminogen activator inhibitor-1 (PAI-1), were increased in the A549 cell. Furthermore, the inhibitory effect of STE on the growth and metastasis of LLC cells *in vivo* was also proven. These results demonstrated that STE could be a candidate antimetastatic agent against lung cancer.
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Keywords: *Selaginella tamariscina*; Invasion; Motility; MMP-2; u-PA

1. Introduction

Selaginella tamariscina (Beauv.) is a traditional Chinese herb for the therapy of chronic trachitis, thrombocytopenic purpura and several forms of cancers in the orient (Zheng et al., 1998; Dai et al., 2005). The major constituents in *Selaginella tamariscina* are flavonoids (e.g. amentoflavone, hinokiflavone, sotetsuflavone and apogenin) (Cheong et al., 1998; Zheng et al., 1998) and saccharides (e.g. trehalose, D-glucose, D-fructose and D-rhamnose) (Shimada et al., 1984). The crude extracts of *Selaginella tamariscina* were evidenced to reduce the gene expression and proinflammatory cytokines, including interleukin-1β and TNF-α production in human mesangial cells (Kuo et al., 1998). In addition, the tumoricidal effects of *Selaginella tamariscina* were demonstrated via an expression of p53 tumor suppressor gene and an induction of G1 arrest in the cell cycle to against human tumor cell lines (Lee et al., 1999).

Cancer metastasis, the spread of cancer cells from the primary neoplasm to distant sites and their growth there, is the major cause of death in various cancer patients (Weiss, 1990). Metastasis of cancer cells involves multiple processes and various cytophysiological changes, including changing adhesion capability between cells and extracellular matrix (ECM) and damaging intercellular interaction. Thus, a degradation of the ECM and components of the basement membrane caused by a concerted action of proteinases, such as matrix metalloproteinases (MMPs), cathepsins, and plasminogen activator (PA), play a critical role in tumor invasion and metastasis (Westermarck and Kahari, 1999; Yoon et al., 2003). Among these enzymes, MMP-2, MMP-9 and urokinase-PA (u-PA) could degrade most components of the ECM directly and deeply involved in cancer invasion and metastasis (Bjorklund and Koivunen, 2005; Yang

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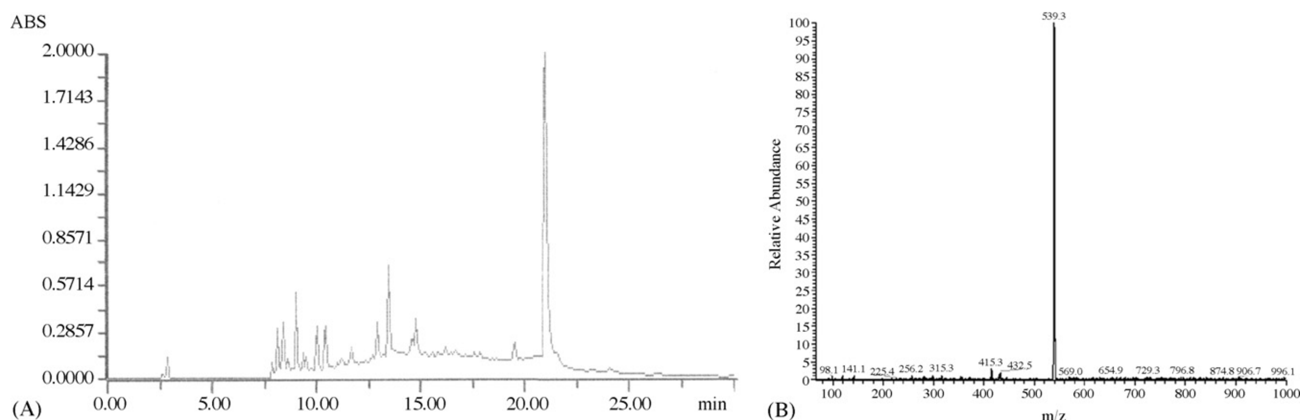

Fig. 1. The chemical profile of STE was analyzed by HPLC-mass spectrometer. (A) Chromatographic patterns from HPLC analysis (254 nm) of STE extracts showed peaks corresponding to the retention times (min). (B) The main product peak (with a retention time of 21.3 min as shown in (A)) was then subjected to mass spectrometer.

et al., 2005a). Therefore, the inhibition of migration or invasion mediated by MMP-2, -9 or u-PA could be a preventive way of cancer metastasis (Bjorklund and Koivunen, 2005).

Some pharmacological studies have demonstrated that *Selaginella tamariscina* possesses anti-bacterial, anti-hypertensive, anti-hyperglycemic (Miao et al., 1966; Zheng et al., 1998) and anti-tumoral activities (Lee et al., 1999). Based on our knowledge, the antimetastatic activities of *Selaginella tamariscina* were not well studied previously. Therefore, in this study, the antimetastatic activities of STE on the cell invasion were examined *in vitro* on A549, a highly metastatic human lung cancer cells. An *in vivo* study of LLC (Lewis lung carcinoma)-bearing mice, was also conducted to understand the antimetastatic activities of STE.

2. Materials and methods

2.1. Preparation of Selaginella tamariscina extracts (STE)

Selaginella tamariscina (Beauv.) leaves were purchased from local herb stores in Taichung, Taiwan and the *Selaginella tamariscina* extracts (STE) were prepared as described previously (Kuo et al., 1998). Briefly, 100 g of air-dried leaves were boiled at 70 °C for 24 h with 500 mL of 50% ethanol. The extraction procedure was repeated twice. Then, solvent was removed from the combined extract with a vacuum rotary evaporator. The filtrate was then lyophilized and stored at −20 °C until further studies. A voucher specimen is deposited in The National Research Institute of Chinese Medicine, Taiwan (Kuo et al., 1998). Furthermore, the extraction yield was 2.8% (w/w) and the chemical profile of *Selaginella tamariscina* extracts was analyzed by using high-pressure liquid chromatograms (HPLC)-mass spectrometer and shown in Fig. 1. Briefly, STE were analyzed by HPLC-mass spectrometer using a HPLC (Hitachi L-6200 with an L-4500 Diode Array detector) with a PE Sciex Qstar Pulsar ESI-TOF mass spectrometer. Samples (10 μl) were injected onto a Merck LiChrospher 100 RP-18 column (4 mm × 250 mm). The column was equilibrated in 0.05% acetic acid/water (solution A) and elution of the components was achieved by increasing the concentration of solution B (100% acetonitrile) from 0 to 100% in 30 min at a flow rate of 1 ml/min. Absorbance was monitored at 254 nm. The molecular masses of the peaks were determined from electrospray ionisation mass spectra using multiply-charged ion profile based on the modified method of Chang et al. (2005). For subsequent experiments, the STE powder was dissolved in distilled water to achieve designed concentrations (0, 10, 25, 50, 75, and 100 μg/mL).

2.2. Cell and cell culture

A549 and LLC cell lines obtained from ATCC (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM non-essential amino acid and 1mM sodium pyruvate (Sigma chemical Co., St. Louis, MO, USA). All cell cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.3. Determination of cell viability (MTT Assay)

To evaluate the cytotoxicity of STE, a MTT colorimetric assay was performed to determine the cell viability (Mosmann, 1983). Cells were seeded in 24-well plates at a density of 3.5×10^4 cells per well and treated with 0, 10, 25, 50, 75, and 100 μg/mL of STE at 37 °C for 24 h. At the end of the exposure period, cells were washed with PBS and then incubated with 20 μL MTT (5 mg/mL) (Sigma chemical Co., St. Louis, MO, USA) for 4 h. The viable cell number is directly proportional to the production of formazan following solublization with isopropanol, which can be measured spectrophotometrically at 563 nm (Beckman Spectrophotometer DU 640, Beckman Instruments, Fullerton, CA, USA).

2.4. Cell invasion and motility assays

The cell invasion and motility were assayed according to the methods described by Chu et al. (2004). After being treated

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