

Extracts from the Roots of *Lindera strychnifolia* Induces Apoptosis in Lung Cancer Cells and Prolongs Survival of Tumor-bearing Mice

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Abstract: *Lindera strychnifolia*, a scandent shrub Lauraceous medicinal plant, has been used in Chinese traditional medicine as a palliative and an anti-spasmodic. It also shows cytotoxic effects against several tumor cell lines and inhibits macromolecule biosynthesis. This study investigated the anti-tumor effects of *L. strychnifolia* extract against lung cancer cells using *in vitro* and *in vivo* models. Two human lung cancer cell lines A549 (adenocarcinoma) and SBC-3 (small cell carcinoma), and a non-tumor cell line 3T3-L1 (mice fibroblasts) were subjected to *L. strychnifolia* extract treatment. On lung cancer cells, *L. strychnifolia* induced cell growth inhibition in a dose-dependent manner. Conversely, the extract did not show any significant cytotoxic effect on 3T3-L1 cells. Therefore, the extract is specific for tumor cells. Tumor cells treated with *L. strychnifolia* extract showed typical morphological appearance of apoptosis including nuclei fragmentation and cell condensation. The *in vivo* effects of *L. strychnifolia* extract were investigated in C57BL/6 mice transplanted with Lewis lung cancer (LL-2) cells, and in BALB/c nude mice transplanted with A549 or SBC-3 human lung cancer cells. Oral administration of *L. strychnifolia* extract prolonged survival time and inhibited tumor growth in a dose-dependent manner by inducing apoptosis in the LL-2 cell mice model. Furthermore, in A549 or SBC-3 cell nude mice models, oral administration of *L. strychnifolia* extract also significantly inhibited tumor growth at the 5.0 mg/ml concentration. These findings suggested that the components of *L. strychnifolia* have anticancer activity and may contribute to clinical applications in the prevention and treatment of lung cancer.

Keywords: *Lindera strychnifolia*; Lung Cancer; Apoptosis; Anticancer Activity.

Introduction

Lung cancer is still one of the leading causes of neoplasia-related fatalities worldwide. Despite improvement in the detection and treatment of lung cancer in the past decades, the prognosis for lung cancer remains very poor and only less than 15% of patients have long-term survival for greater than five years after initial diagnosis (Greenlee *et al.*, 2000). In addition, the response rates to chemotherapy have not changed substantially and many lung cancer cells have showed significant resistance to chemotherapeutic drugs. Therefore, development of new therapeutic anti-lung cancer drugs is clinically important.

Apoptosis is a form of cell death, and plays an essential role in maintaining tissue homeostasis (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Since the balance between mitosis and cell death determines the growth of cancer tissue, the induction of apoptosis in cancer cells is one of the major concerns in cancer therapy. A number of studies have explored the attractive idea that tumor cells could be eliminated by artificially triggering death through apoptosis (Thompson, 1995). The cell death that is induced by irradiation, hyperthermia and a range of chemotherapy drugs, has been found to be apoptosis (Sellins and Cohen, 1987; Kaufmann, 1989; Yanagisawa-Shiota *et al.*, 1995).

The pharmacology and clinical application of medicinal plants has been well documented. Recently, a bioassay-guided investigation of the active compounds from medicinal plants has led to the discovery of many new anticancer agents. *Lindera strychnifolia* (Sieb. & Zucc.) f. *Vills* (Lauraceae) (Tomita *et al.*, 1969), a scandent shrub Lauraceous medicinal plant, has a strong fragrance and is used in Chinese traditional medicine as a palliative and an anti-spasmodic. Beverages made from *L. strychnifolia* extract have also been consumed in some areas of Japan for promoting good health and vitality. *L. strychnifolia* was found to contain aporphine-type alkaloids, which accounted for the numerous biological activities in its extract (Kondo *et al.*, 1990; Chen *et al.*, 1991; Teng *et al.*, 1991). It was reported that extracts from the roots of *L. strychnifolia* show cytotoxic effects against several tumor cell lines and inhibited macromolecule biosynthesis (Wu *et al.*, 1989; Kondo *et al.*, 1990). It was also reported that extracts from the roots of *L. strychnifolia* exert anti-platelet aggregation (Chen *et al.*, 1991; Yu *et al.*, 1992a) and α_1 -adrenoceptor antagonist (Teng *et al.*, 1991; Yu *et al.*, 1992b; Yu *et al.*, 1993). The wide acceptability and potential medicinal value of *L. strychnifolia* have attracted intense interest in the search for pharmacological compounds from *L. strychnifolia* (Tomita *et al.*, 1969; Kouno *et al.*, 2001).

In this study, we investigated the effects of *L. strychnifolia* extract against lung cancer. *In vitro* study showed that the extract could inhibit cancer cell growth and induce apoptosis. Oral administration of *L. strychnifolia* extract prolonged survival time of tumor-bearing mice by inhibiting cancer cell proliferation and/or inducing apoptosis. Our data indicated that *L. strychnifolia* has anticancer activity and may contribute to the clinical application in lung cancer prevention and treatment.

Materials and Methods

Preparation and Analysis of Aqueous Extracts of L. strychnifolia Roots

L. strychnifolia was harvested in Shingu City, Wakayama Prefecture, Japan in July 1999. The roots of *L. strychnifolia* were cut to small pieces and extracted by boiling water (100 mg/ml for 60 minutes at 100°C). The extract powder, obtained from spray-drying the extracts was weighed, and a stock solution of the aqueous extract was used for subsequent experiments by adding to the culture medium for *in vitro* studies or drinking water for *in vivo* animal experiments. Spray-dried extract powder was prepared by Tsumura & Co. (Tokyo, Japan).

Tumor Cell Lines and Culture

Two human lung cancer cell lines (SBC-3: small cell carcinoma, A549: adenocarcinoma), a mouse lung cancer cell line (Lewis lung cancer cell line, LL-2) and a non-tumor cell line 3T3-L1 (mice fibroblasts) were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). These cell lines were grown in DMEM medium, with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂/95% air at densities that promoted exponential proliferation.

Cell Viability Assay

In vitro cell viability was measured using the tetrazolium dye (MTT) assay, as originally described (Mosmann, 1983), with minor modifications. In brief, 100 µl of 2.0×10^5 cells/ml A549 and SBC-3 cells or 5.0×10^4 cells/ml 3T3-L1 cells per well were seeded in 96-multiwell plates and pre-cultured for 24 hours before drug treatment. The medium was changed to fresh medium with or without (control) various concentrations of *L. strychnifolia* extract. After 48 hours of incubation, 10 µl of MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium] solution (5 mg/ml) was added to each well and plates were incubated at 37°C for 4 hours. The MTT product in viable cells was solubilized in 100 µl dimethylsulfoxide (DMSO) and absorbance was measured using an automated microplate reader at a wavelength of 560 nm, each representing the average of six wells. The cell survival was expressed as a percentage of the control. The 50% inhibitory concentration (IC₅₀) value was determined graphically from the dose-response curve at ten drug concentration points. The experiments were repeated three to five times for each cell line.

Cell Morphological Analysis

For cell morphological examination, cells were harvested after a 48-hour treatment with 250 µg/ml of *L. strychnifolia* extract and fixed with methanol, then stained with Hoechst 33258 (final concentration 10 µM) for 10 minutes. Stained cells were observed with an

Olympus IX 70 microscope equipped with a filter set. Cells with condensed and/or fragmented nuclei were defined as apoptotic cells.

In Vivo Mouse Experiments

Five-week-old female C57BL/6 mice and BALB/c nu/nu nude mice (CLEA Japan, Tokyo, Japan) used in murine and human tumor-bearing mice models, respectively. Animals were housed under specific pathogen-free conditions and maintained in our animal facility for at least 1 week before use. Mice were kept on a 12-hour light/12-hour dark cycle and provided standard mouse chow.

To establish the murine tumor-bearing mice model, LL-2 cancer cells were grown in culture, harvested and injected as a suspension (1.0×10^5 cells in 0.1 ml of serum-free medium) subcutaneously into the seven-week-old female C57BL/6 mice. Drinking water was replaced every 3 days. In these experiments, the mice were divided into four groups with 11 mice per group. Mice of the control group were administered tap water. Mice of other groups were orally administered *L. strychnifolia* extracted solution from drinking water. Tap water containing *L. strychnifolia* extract, at concentrations of 0.5 mg/ml, 2.5 mg/ml or 5.0 mg/ml, was provided *ad libitum*. Drug administration was started from 1 week before transplantation of tumor cells and continued until the end of the experiment. Body weights were recorded once per week. Mice were sacrificed 4 weeks after transplantation. Their tumors were removed, weighed and the short and long diameters were measured. Tissue that would be used for molecular biological analysis were preserved in neutral-buffered formalin at 4°C before embedding in paraffin.

For human tumor-bearing mice models, either 1.0×10^7 of A549 or SBC-3 cells were subcutaneously injected into the BALB/c nude mice in the proximal midline of the dorsa. Mice of the treatment groups were orally administered *L. strychnifolia* extracted solution (5.0 mg/ml) from drinking water. Mice of the control group were administered autoclaved distill water (six mice in each group). Treatment point was continued for 2 months, at which all mice were sacrificed and the tumors were removed and weighed.

The survival time of tumor-bearing mice was also monitored up to 60 days after transplantation in both C57BL/6 and BALB/c nude mice. All animals were treated in accordance with the institution's guidelines for the care and use of laboratory animals.

Determination of Cancer Cell Proliferation

Immunohistochemical stains for proliferating cell nuclear antigen (PCNA), which is known to play an important role in DNA replication and to be necessary for cellular replication, were performed with anti-PCNA monoclonal antibodies PC10 (1:100 Dakopatts) using the avidin-biotin peroxidase complex method (Hsu *et al.*, 1981). Diaminobenzidine (Dakopatts) was used for coloring. For the quantification of PCNA expression, positive cells were counted in 20 random 0.159 mm² fields at $\times 200$ magnification. Greater than 1000 tumor cells were counted, and the fraction of positive cells was determined.

DNA Nick End-labeling of Tissue Sections

The biochemical and morphological method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin (biotin-16-deoxyuridine triphosphate) nick end-labeling (TUNEL) was performed using Apop Tag Plus *In Situ* Apoptosis Detection Kit (Oncor, Inc. Ref 22) with the following modifications (Gavrieli *et al.*, 1992). The nuclei of tissue sections were stripped of protein by incubation with 20 mg/ml of proteinase K for 10 minutes and immediately fixed in 40% paraformaldehyde at room temperature for 5 minutes. The slides were then washed with 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO) in PBS for 5 minutes at room temperature and incubated in a 1% glycine-PBS solution. After being rinsed with distilled water, the slides were immersed in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate, 1 mM cobalt chloride). TdT (Gibco BRL, Gaithersburg, MD) (0.3 U/ml) and biotin-16-dUTP (Boehringer Mannheim) in TdT buffer were then added to cover the sections and incubated in a humid atmosphere at 37°C for 60 minutes. The slides were washed with PBS and incubated with streptavidin-alkaline phosphatase conjugate (Gibco BRL) at a concentration of 1 U/ml for 30 minutes at 37°C. After rinsing with PBS, the slides were washed three times for 5 minutes each in 0.5% Triton in PBS, followed by three 5-minute washes in PBS, and three 5-minute washes in Ap 9.6 (0.1M Tris HCL < pH 9.6 >, 0.1M NaCl, 0.1M MgCl₂). They were then immersed in Ap 9.6 containing 5-bromo-4-chloro-3-indole phosphate (Sigma), and nitro blue tetrazolium (Sigma), and left in the dark for 30 minutes. The color reaction was stopped by washing with Na₂EDTA, and the slides were examined under a light microscope. For the quantification of TUNEL expression, positive cells were counted in 20 random 0.159 mm² fields at × 200.

Statistical Analysis

All values were expressed as means ± SD. All data were analyzed by analysis of variance (ANOVA). Statistical analyses were performed with a one-tailed Student's t-test. Survival rates were calculated by the Kaplan-Meier method, and differences in the survival rates were evaluated using the log-rank test. Differences were considered to be significant at $p < 0.05$.

Results

In Vitro Cell Survival Assay

Figure 1 shows the effect of the *L. strychnifolia* extract on cell viability of A549 and SBC-3 measured by the MTT assay, which revealed a dose-dependent decrease in cell viability after a 48-hour treatment with *L. strychnifolia* extract. Proliferation of A549 and SBC-3 was inhibited by the treatment with the *L. strychnifolia* extract, with IC₅₀ value of approximately 250 µg/ml and 100 µg/ml, respectively. At a concentration of 1000 µg/ml of *L. strychnifolia* extract, cell viability of A549 and SBC-3 cell lines was suppressed to less than 15% of the

control after the 48-hour treatment. To clarify whether the *L. strychnifolia* extract anti-proliferative action is specific to tumor cells, the effect of this extract on one kind of non-tumor cells, 3T3-L1 mice fibroblasts, was tested. The results demonstrated that *L. strychnifolia* extract did not show any significant cytotoxic effect on the non-tumor cells (Fig. 1).

Assessment of Apoptosis in Culture Cells

Morphological changes of lung cancer cells after treatment with *L. strychnifolia* were assessed using staining dye, Hoechst 33258, under a fluorescent microscope. Treatment of cells with *L. strychnifolia* (250 $\mu\text{g/ml}$) for 24 hours resulted in nuclear fragmentation and condensation, which are characteristic of apoptosis (Fig. 2). Further evidence to suggest apoptotic change induced by *L. strychnifolia* was provided by the characteristic of DNA fragmentation ladder pattern (data not shown). Thus, loss of cell viability was suggested to be mainly due to the induction of apoptosis.

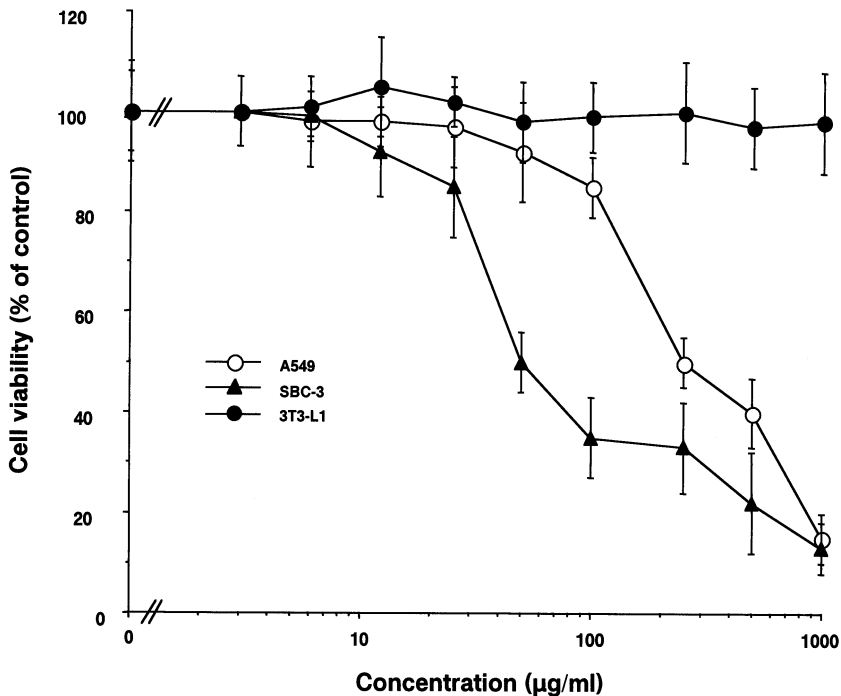


Figure 1. Dose-response effects of *L. strychnifolia* on the cell viability of lung cancer cells and non-tumor cells. Lung cancer cells, adenocarcinoma (A549) and squamous cell carcinoma (SBC-3), non-tumor cells 3T3-L1 were cultured for 48 hours in medium containing various concentrations of *L. strychnifolia* extract. Cell viability was assessed using MTT assay and the values were plotted as a percentage of non-treated control culture. Values are presented as the means \pm SD from six wells.

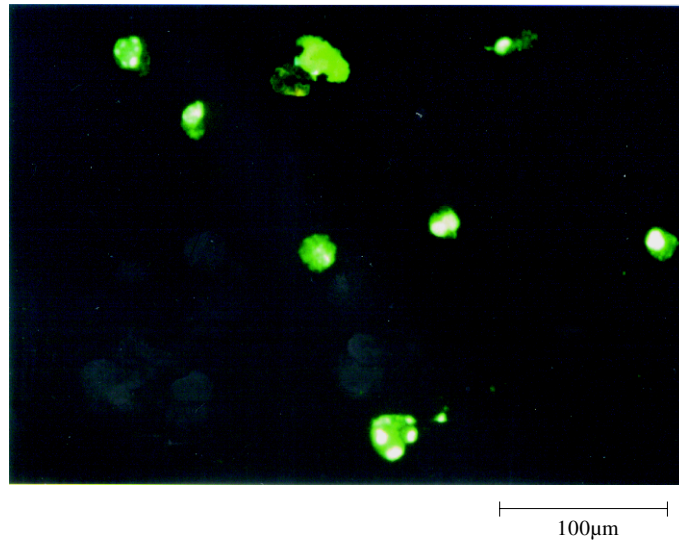


Figure 2. Apoptotic features of cancer cells induced by *L. strychnifolia* extract. SBC-3 cells were treated with 250 $\mu\text{g/ml}$ of *L. strychnifolia* extract. Cells were harvested after a 48-hour treatment, stained with Hoechst 33258 (10 μM) for 10 minutes and photographed under a fluorescence microscope.

Effects of Oral Administration of L. strychnifolia Extract for Transplanted Lung Cancer Cells in C57BL/6 Mice and BALB/c Nude Mice

The anti-tumor activity of *L. strychnifolia* was first evaluated against the subcutaneously implanted murine lung cancer LL-2 cells. *L. strychnifolia* extract was administered *ad libitum* in drinking water from 1 week before implantation of LL-2 cancer cells and continued until the end of the experiments. At 4 weeks after LL-2 cell transplanted, tumor-bearing mice were provided with drinking water containing *L. strychnifolia* extract at 5.0 mg/ml, 2.5 mg/ml or 0.5 mg/ml, tumor weight was inhibited by about 75%, 58% and 33% in the different concentration treatment groups compared with the control group. Significant suppression of tumor growth compared with the control group was observed in mice provided with drinking water containing 5.0 mg/ml or 2.5 mg/ml *L. strychnifolia* extract (Fig. 3a).

The anti-tumor activity of *L. strychnifolia* was then investigated in human lung cancer transplanted nude mice models. Since in murine tumor model the optimal anti-tumor effect dose of *L. strychnifolia* in this experiment was found to be at the concentration of 5.0 mg/ml, this concentration of *L. strychnifolia* was used in nude mice. We found that transplanted tumors were significantly smaller in mice treated with *L. strychnifolia* than those of the control mice. The average weight of the tumor at 60 days after transplantation was 401.6 ± 210.5 mg in the *L. strychnifolia* treatment group versus 819.8 ± 306.9 mg in the control group of A549 cell transplanted nude mice ($p = 0.036$), and 1138.8 ± 414.4 mg in the *L. strychnifolia* treatment group versus 2073.2 ± 754.0 mg in the control group of SBC-3 cell nude mice model ($p = 0.041$) (Fig. 3b).

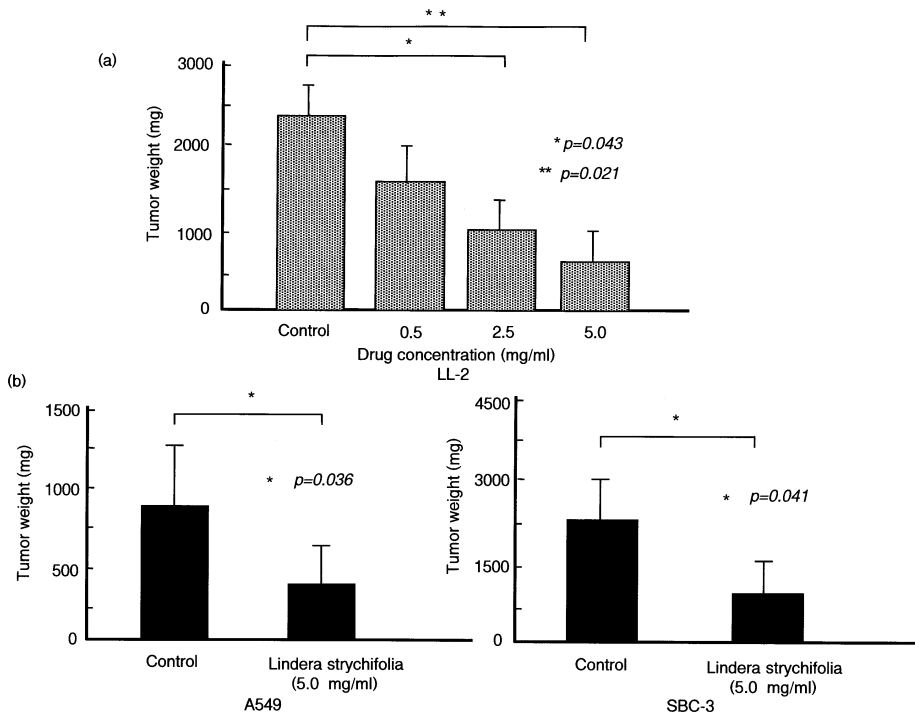


Figure 3. (a) After 29 days LL-2 cell transplantation, tumors were weighed for the control group, 0.5 mg/ml *L. strychnifolia* extract, 2.5 mg/ml *L. strychnifolia* extract and 5.0 mg/ml *L. strychnifolia* extract treatment groups, respectively. Tumor weights were significantly higher in the control group than in the treatment of 2.5 mg/ml *L. strychnifolia* extract ($p < 0.05$), and 5.0 mg/ml *L. strychnifolia* extract group ($p < 0.05$). Tumor weights were higher in the control group than in the 0.5 mg/ml *L. strychnifolia* extract group, but not significantly ($p > 0.05$). (b) Anti-tumor effects of *L. strychnifolia* extract in A549 or SBC-3 human lung cancer cell transplanted BALB/c nude mice. The mice received 5.0 mg/ml concentration of *L. strychnifolia* extract starting 1 week before tumor cell transplantation and continued for 2 months, at which all mice were sacrificed and the tumors were removed and weighed. Each value is the mean from five to six mice (bars SD).

To investigate the effect of *L. strychnifolia* versus the mouse subsist effect, we also used the LL-2 cell transplanted tumor-bearing mice model. The survival rates were calculated using the Kaplan-Meier method, and differences in the survival rates calculated using the log-rank test. High survival rates were observed in the groups that received a concentration of 5.0 mg/ml ($p < 0.01$) or 2.5 mg/ml ($p < 0.05$) *L. strychnifolia* compared with the control group. Survival rate was higher in the group treated with a concentration of 0.5 mg/ml *L. strychnifolia* than the control group but it was statistically insignificant ($p > 0.05$) (Fig. 4).

In the case of human tumor-bearing, A549 cell transplanted nude mice, no mice died both in the *L. strychnifolia*-treated and control groups. In SBC-3 cell transplanted nude mice, one of six mice died on day 56 after tumor cell transplantation in the control group, and no mice died in the *L. strychnifolia* treatment group during the 60-day experiment period. So the difference in survival rates in nude mice was not calculated.

The toxicity of the drugs was investigated at the maximum dose of 5.0 mg/ml in drinking water in tumor-free C57BL/6 mice. No gross or histological abnormalities were found 2 months after administration.

Oral administration of 0.5 mg/ml, 2.5 mg/ml and 5.0 mg/ml of *L. strychnifolia* extract did not significantly influence body weight, but slight increases in food and fluid consumption were observed during the 4-week observation period (data not shown).

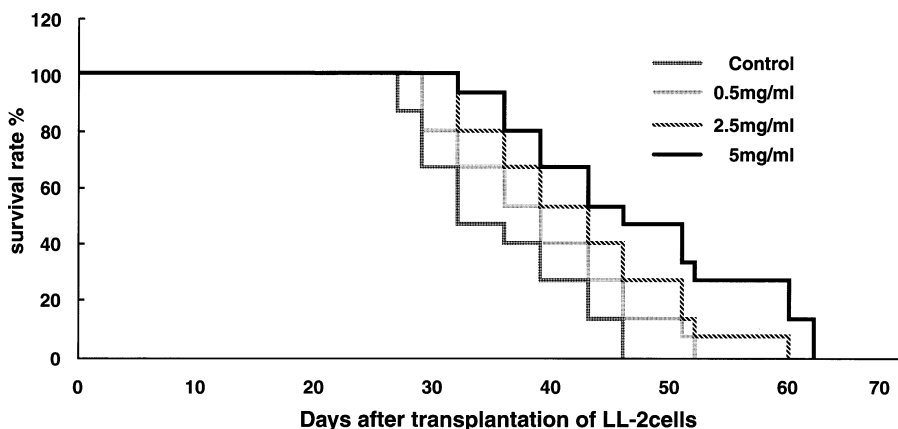


Figure 4. Survival curves of mice following s.c transplantation of LL-2 lung cancer cells. Various concentrations of *L. strychnifolia* extract were orally administered 1 week before and after the transplantation. Survival time was monitored every day (n = 11 per group).

Expression of PCNA in Tumor Tissue

Immunohistochemical staining of PCNA showed a selective nuclear pattern. Tumor tissues of the control mice showed strong staining of PCNA, indicating high cell proliferation activity. Staining intensity was significantly weaker in the tumors of mice treated with *L. strychnifolia* extract (5.0 mg/ml in drinking water) compared with the control ($p < 0.01$) (Figs. 5a and b).

Apoptosis in Lung Tissues Detected by TUNEL

Figures 5c and d show immunohistochemical findings of tumor specimens stained with TUNEL, which detects DNA strand breaks in apoptotic cells. The average TUNEL positive signals were significantly higher in tumors treated with *L. strychnifolia* ($p < 0.01$) than in the control tumor.

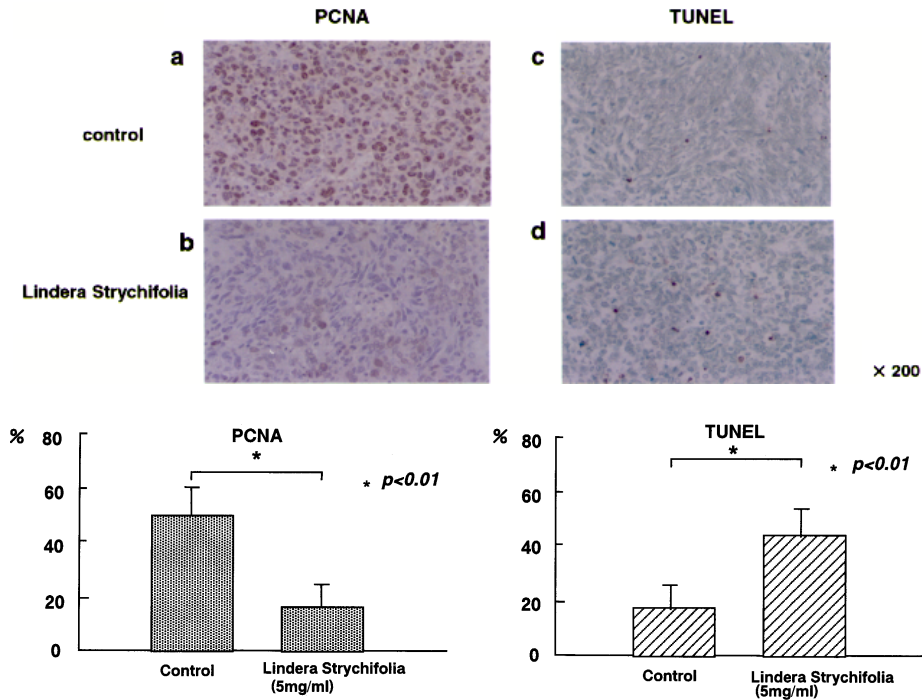


Figure 5. Immunohistochemical staining of PCNA and TUNEL in LL-2 cell s.c. transplanted in mice. At 1 week before transplantation of LL-2 cancer cells, mice received *L. strychnifolia* extracted solution daily until the end of the experiment. At 35 days after *L. strychnifolia* treatment, tumors were resected and examined histologically ($n = 11$ /group). Photographs show the typical immunohistochemical appearance in tumor tissues from mice of control (a and c) and *L. strychnifolia* extract (5.0 mg/ml) treated mice (b and d). Cell proliferation and apoptosis were detected using anti-PCNA antibody (a and b) and a modified TUNEL method (c and d), respectively. Graphs show change in PCNA-positive and TUNEL-positive cell number in the tumor tissue. Values represent the means \pm SD.

Discussion

Apoptosis is a genetically encoded programmed cell death defined by characteristic morphological and biochemical changes (Sarih *et al.*, 1993; Smeyne *et al.*, 1993; Corcoran *et al.*, 1994). Apoptosis is different from necrosis both morphologically and biochemically (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Searle *et al.*, 1982). Apoptosis plays a role opposite to that of mitosis in cell population kinetics in almost all tissues, and apoptosis abnormalities are closely associated with multistep carcinogenesis (Wyllie, 1997). Growth of tumor tissue is determined by the balance between cell proliferation and apoptosis. The inability of cancer cells to undergo apoptosis may provide a growth advantage, and cells with defective apoptosis may therefore have a better chance of surviving. Thus, induction of apoptosis was recognized as an ideal strategy for cancer chemotherapy.

In the present study, we showed that aqueous extract of *L. strychnifolia* has potent growth-inhibitory effects on lung cancer cell lines. Various anticancer drugs such as cisplatin, camptothecin, VP16 and all-trans retinoic acid were reported to induce apoptosis as well as differentiation in many tumor cell types (Kaufmann, 1989; Barry *et al.*, 1990; Bertrand *et al.*, 1993; Evans and Dive, 1993; Onishi *et al.*, 1993; Yoshida *et al.*, 1993; Wyllie, 1997). We examined, in the present study, whether *L. strychnifolia* can also induce apoptosis in lung cancer cells. We demonstrated that *L. strychnifolia*-induced typical apoptosis in the lung cancer cell lines in a dose-dependent manner. Lung cancer cells treated with *L. strychnifolia* acquired apoptotic morphological features, extracted DNA showed a ladder pattern when analyzed by gel electrophoresis (data not shown), suggesting an association of the anticancer effect of *L. strychnifolia* on lung cancer cells with the induction of apoptosis. The morphological changes in *L. strychnifolia*-treated lung cancer cells were similar to those found in the aforementioned anticancer drugs, and which were shown to be a dose-dependent process. Although the mechanisms by which *L. strychnifolia* extract induce apoptosis in lung cancer cells require further clarification, it was suggested that components of *L. strychnifolia* might be a candidate of apoptosis-inducing anticancer drug.

We showed that administration of *L. strychnifolia* inhibited tumor growth in murine tumor-bearing C57BL/6 mice. The rate of tumor growth is dependent on the balance between the proliferative activity and death rate of the tumor cells. It was suggested that *L. strychnifolia* induced a high level of apoptotic activity, which may be linked to slower tumor growth and better survival rate in lung cancer mice model. As shown in the *in vivo* experiments, oral administration of an aqueous *L. strychnifolia* extract as the sole source of drinking water to LL-2 cells transplanted subcutaneously to C57BL/6 mice markedly inhibited tumor growth in a dose-dependent manner. Oral administration of an aqueous *L. strychnifolia* extract of 5.0 mg/ml as the sole source of drinking water to A549 or SBC-3 cell transplanted BALB/c nude mice significantly inhibited tumor growth. In addition to inhibiting the tumor growth, oral administration of *L. strychnifolia* extract also significantly prolonged the survival rate in a dose-dependent manner in C57BL/6 mice. The proliferation rate was determined immunohistochemically by using an antibody to PCNA, a key protein in DNA synthesis. The extent of apoptosis was determined by detecting and counting apoptotic cells by TUNEL. The average PCNA positive signals were significantly higher in controls than in those treated with *L. strychnifolia* extract (5.0 mg/ml in drinking water). However, the average TUNEL positive signals were significantly higher in those treated with *L. strychnifolia* extract (5.0 mg/ml in drinking water) than in controls. Thus, our observations of tumor cell apoptosis suggested that *L. strychnifolia* has an anti-cancer activity by inducing apoptosis.

Previous pharmacological studies isolated many bioactive agents from the roots of *L. strychnifolia* (Chen *et al.*, 1991; Teng *et al.*, 1991), and some of them possess anticancer activity (Kondo *et al.*, 1990). In this study, we could not identify the original standards. Despite the anti-lung cancer properties of *L. strychnifolia*, as described above, it remains unclear which agents in *L. strychnifolia* are predominantly responsible for the effects seen in this study. Further studies will be needed to clarify these details.

Toxicity studies have shown that the maximum concentration of *L. strychnifolia* 5.0 mg/ml is achievable with little toxicity, suggesting that higher concentrations may be pharmacologically achievable. The IC₅₀ of *L. strychnifolia* in each human lung cancer cell line fell within or below the range of that administered in mice. At a concentration of 1000 µg/ml, A549 and SBC-3 cell lines showed greater than 85% growth inhibition. Since the maximum tolerated dose of *L. strychnifolia* has not been firmly established in this study, it is feasible that concentrations above 5.0 mg/ml may be achieved with tolerable toxicity. *L. strychnifolia* possesses a secreting effect of gastric liquid, and is used as a gastrointestinal drug. This effect of *L. strychnifolia* may partly explain the increase in consumption of food and fluid in mice during the experimental period (Tomita *et al.*, 1969).

It is noteworthy that a common and interesting feature of medicinal plants is the presence of multiple compounds, which could act either independently or synergistically to elicit their pharmacological effects. We have shown that *L. strychnifolia* inhibits lung cancer growth by inducing apoptosis and prolonging the survival of tumor-bearing mice; further studies to evaluate the application of *L. strychnifolia* for lung cancer treatment are warranted.

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