



ORIGINAL ARTICLE

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# Antitumor agent yatein from *Calocedrus formosana* Florin leaf induces apoptosis in non-small-cell lung cancer cells

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## Abstract

*Calocedrus formosana* Florin is a softwood tree species with high economic value in Taiwan. Several bioactivities of the extracts of *C. formosana* have been reported; however, only one study focused on the anti-non-small-cell lung cancer cells' (anti-NSCLC) effect of *C. formosana* extract and its active phytochemical. In the present study, the anti-lung cancer effects of *C. formosana* leaf extract and its active derivative yatein were evaluated. The results revealed that the *n*-hexane fraction of the crude extract exhibited the highest cytotoxicity potential against two non-small-cell lung cancer (NSCLC) cell lines, namely A549 and CL1-5. Yatein, isolated from the *n*-hexane fraction, exhibited the highest cytotoxicity in the A549 and CL1-5 cells. In addition, the CL1-5 cells were more sensitive than the A549 cells after yatein treatment. Flow cytometry results revealed that yatein induced apoptosis in the two cell lines. Furthermore, expression of regulatory proteins related to apoptosis, such as caspase 3, caspase 8, caspase 9, and poly (ADP-ribose) polymerase (PARP), increased in the A549 and CL1-5 cells after yatein treatment. These findings provide insight into the *in vitro* anti-lung tumor efficacy of yatein, thus rendering this phytochemical a potential anticancer lead compound for NSCLC treatment.

**Keywords:** Apoptosis, *Calocedrus formosana*, Extracts, Lung cancer, Yatein

## Introduction

Globally, lung cancer is the second most common cancer among men and women, and causes approximately 1.6 million deaths each year [1]. In 2012, up to 58% of lung cancer cases occurred in less developed [2]. Non-small-cell lung cancer (NSCLC), including lung squamous cell carcinoma, lung adenocarcinoma, and large cell lung cancer, accounts for more than 85% of lung cancer cases [3, 4]. The major cause of lung cancer is tobacco smoking, and more than 80% of lung cancer cases in the United States are related to smoking [1]. Although lung squamous cell carcinoma is strongly associated with smoking, lung adenocarcinoma is the most common form of lung

cancer that is observed in patients who never smoke [5]. In addition to the high incidence rate, the 5-year survival rate of lung cancer is also lower than that of other common cancers [6]. Despite major advances in cancer therapeutics, efficient treatments to improve survival rates among patients with lung cancer are not currently available. Because of the therapeutic limitations of current conventional chemotherapeutic agents, the development of highly effective agents against lung cancer is necessary [7].

Recently, the cytotoxic effects of plant extracts have been widely studied [8, 9]. *Calocedrus formosana* is a popular softwood tree species with a high economic value in craft and construction in Taiwan [10]. In addition to its high industrial value, studies have reported multiple bioactivities of the extract of *C. formosana*, including antimicrobial activity [11], antioxidant activity [12–14], cytotoxicity [15, 16], and immunoregulatory effects [17, 18]. Yuan et al. [16] demonstrated that *C. formosana* leaf

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extracts induce G2/M cell cycle arrest and apoptosis in human bladder cancer cells. In addition, Jayakumar et al. [19] reported that hinokitiol, a monoterpene isolated from *C. formosana*, inhibited the migration of A549 cells by suppressing the expression of matrix metalloproteinases. The finding implied that the *C. formosana* extract and its derived phytochemicals might be potential candidate therapeutic agents against lung cancer.

Previous studies are mainly focused on the anticancer potential of *C. formosana* extract alone or its major compound, hinokitiol [16, 19]. To our knowledge, thus far, no previous study focused on the screening of NSCLC apoptosis inducers from *C. formosana* leaf extract through bioactivity-guided isolation strategy. Therefore, in the present study, the antitumor activities of *C. formosana* leaf extract and its active derivative yatein were assessed in human NSCLC A549 and CL1-5 cells, and the antitumor mechanisms of the active phytochemicals from *C. formosana* were examined.

## Materials and methods

### Plant materials, extraction, and isolation

*C. formosana* leaves were collected from the Hui-Sun experimental forest of National Chung Hsing University. The species was confirmed by Prof. Yen-Hsueh Tseng (Department of forestry, National Chung Hsing University). *C. formosana* leaves were soaked in methanol at room temperature twice for 1 week (each time) to obtain a methanolic extract. The filtered methanolic extract was concentrated and lyophilized using a rotary evaporator and freeze-drying equipment, respectively. The dried samples were divided to various soluble fractions, including *n*-hexane (yield = 14.8%), ethyl acetate (EtOAc) (yield = 9.4%), *n*-butanol (*n*-BuOH) (yield = 20.1%), and water (yield = 47.6%) fractions, using liquid-liquid partition. After a cytotoxicity prescreening, the *n*-hexane fraction of *C. formosana* was further fractionated into 10 subfractions through column chromatography (Geduran Si-60, Merck, Darmstadt, Germany) using a gradient solvent system consisting of EtOAc and *n*-hexane. The cytotoxic phytochemical yatein was isolated and purified from subfraction 4 through semipreparative high-performance liquid chromatography (HPLC) using a PU-2080 pump (Jasco, Tokyo, Japan) equipped with an RI-2031 detector (Jasco) and a 5  $\mu$ m Luna silica column (250  $\times$  10.0 mm internal diameter; Phenomenex, Torrance, CA, USA). The mobile phase consisted of 30% of EtOAc and 70% of *n*-hexane (v/v), and the flow rate was 4 ml/min. The structure of yatein was identified using nuclear magnetic resonance imaging (Bruker Avance 400 MHz FTNMR Spectrometer, Rheinstetten, Germany) and Finnigan MAT-95S ESIMS (San Jose, CA, USA), and all spectral data were consistent with the literature [20].

Additionally, yatein was quantified by HPLC with the same condition as aforementioned. For the calibration curve analysis, the yatein stock solution was dissolved in 30/70 of EtOAc/*n*-hexane (v/v), and then diluted to obtain the desired concentrations (200, 300, 400, 500, and 600  $\mu$ g/ml) for the quantification. The calibration curve was plotted using linear regression method (peak areas versus compound concentrations).

### Cell culture

Human NSCLC A549 and CL1-5 cells were purchased from Bioresource Collection and Research Center (BCRC 60124) and provided by Dr. Jeremy J.-W. Chen (National Chung Hsing University, Taichung, Taiwan), respectively. A549 and CL1-5 cells were cultured in RPMI-1640 and Dulbecco's modified Eagle's medium (DMEM) medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco) and a 1% (v/v) antibiotic-antimycotic agent (Gibco). The cells were incubated in a 37 °C humidified incubator containing 5% CO<sub>2</sub>.

### Cell viability assay

In this study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (Sigma-Aldrich, St. Louis, MO, USA) test was used to evaluate cell viability. To measure the cytotoxicity of the extracts (test samples), the A549 and CL1-5 cells were seeded onto a 24-well plate ( $1 \times 10^4$  cells/well) and incubated overnight. After overnight incubation, 1 ml of fresh medium containing various concentrations of test samples [in 0.1% dimethyl sulfoxide (DMSO)] was added, and the cells were incubated at 37 °C for various durations. The supernatant was removed after incubation and 200  $\mu$ l of MTT reagent (1 mg/ml in serum-free medium) was added. The cells were incubated for 3 h at 37 °C. The MTT reagent was removed and 600  $\mu$ l of DMSO was added to each well. The absorbance at 540 nm of each well was measured using a Tecan Sunrise ELISA reader (Tecan, Chapel Hill, NC).

### Annexin V-FITC binding assay

The apoptotic patterns of lung cancer cells were determined using an Annexin V-FITC Apoptosis Detection Kit (BD Bioscience, San Jose, CA, USA). The treated cells were harvested, washed twice, and resuspended with 2  $\mu$ l of Annexin V-FITC and 2  $\mu$ l of propidium iodide (PI) (BD Bioscience) in 100  $\mu$ l of binding buffer (0.01 M HEPES, pH 7.4; 0.14 M NaCl; 2.5 mM CaCl<sub>2</sub>) (BD Bioscience) for 15 min at room temperature in the dark. The cells were immediately analyzed using flow cytometry (Accuri 5, Accuri Cytometers, Ann Arbor, MI, USA).

**Western blot analysis**

The expression levels of proteins in the cells were determined through Western blot analysis as previously reported [8]. In this study, the primary antibodies were anti-β-actin (Santa Cruz, Dallas, TX, USA), anti-cleaved caspase 3 (Cell Signaling Technology, Danvers, MA, USA), anti-cleaved caspase 8 (Cell Signaling Technology), anti-cleaved caspase 9 (Cell Signaling Technology), and anti-cleaved PARP (Cell Signaling Technology). An enhanced chemiluminescence (ECL) (Sigma-Aldrich) system was used for developing signals of the blots, which were analyzed using an LAS3000 system (Fujifilm, Tokyo, Japan). The experiments were at least duplicate.

**Reactive oxygen species (ROS) assay**

A549 and CL1-5 cells were seeded onto a 6-well plate (1 × 10<sup>5</sup> cells/well). After overnight incubation, cells were treated with 5 μM of yatein for 1, 3, 6, and 12 h. At each time point, the cells were harvested, centrifuged, and stained with 5 μM of 2',7'-dichlorofluorescein diacetate (DCFH-DA) for 10 min at 4 °C. The ROS production levels were measured as FL-1 fluorescence intensity by flow cytometer (BD Biosciences).

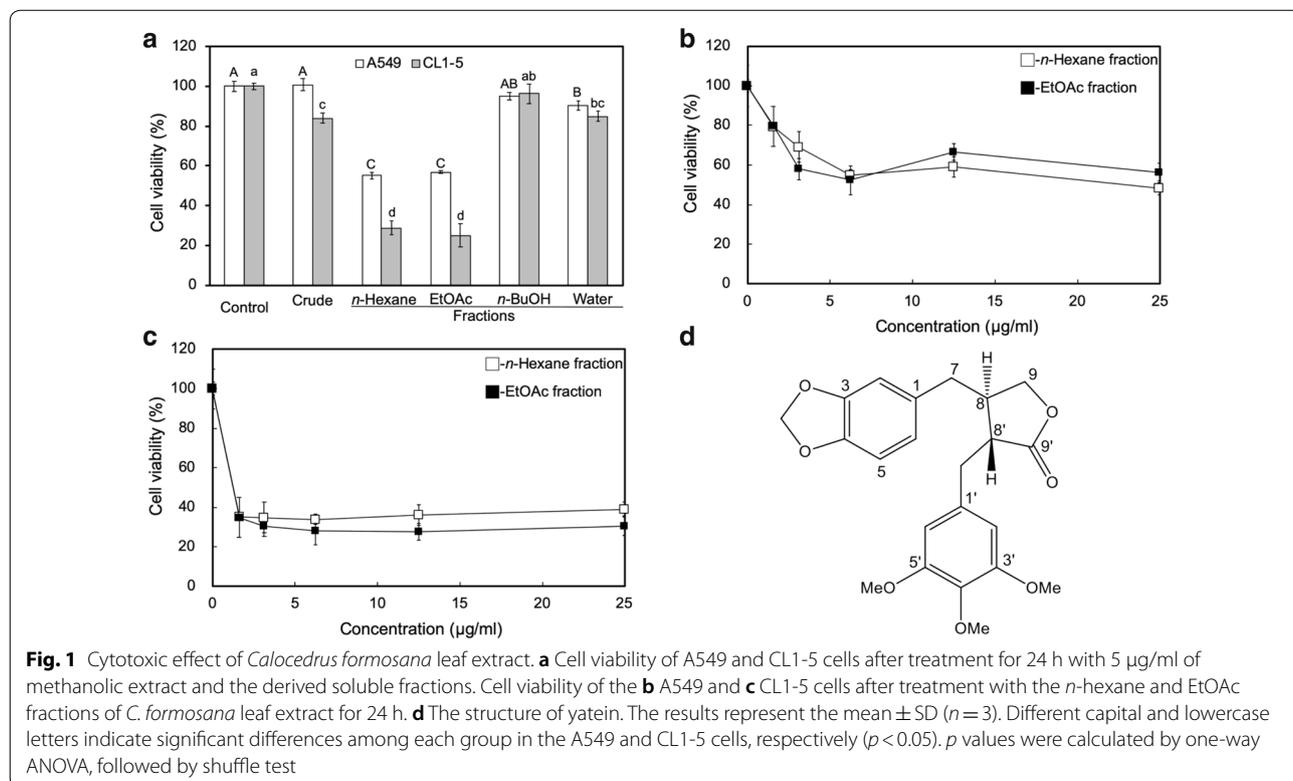
**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the shuffle test or Student's *t* test. A value of *p* < 0.05 was considered statistically significant.

**Results**

**Cytotoxicity of *C. formosana* leaf extract in human NSCLC A549 and CL1-5 cells**

To evaluate the cytotoxic potential of *C. formosana* leaf extract in human NSCLC cells, the cell viability of A549 and CL1-5 cells after treatment with a methanolic extract of *C. formosana* leaf and its derived soluble fractions (*n*-hexane, EtOAc, *n*-BuOH, and water fraction) for 24 h were determined using the MTT assay. The results revealed that at 5 μg/ml, the *n*-hexane and EtOAc fractions exhibited higher cytotoxicity levels in the A549 and CL1-5 cells than the other fractions (Fig. 1a). As shown in Fig. 1b and c, both the *n*-hexane and EtOAc fractions exhibited cytotoxic effects on the human NSCLC cells; the half maximal inhibitory concentration (IC<sub>50</sub>) values of the *n*-hexane fraction in the A549 and CL1-5 cells were 23.1 and 1.2 μg/ml, respectively. The cytotoxic effects of the *n*-hexane and EtOAc fractions were comparable; however, the yield of *n*-hexane fraction (14.8%) was higher than that of the EtOAc fraction (9.4%). Therefore,



**Table 1** The weight fraction and cytotoxicity of *n*-hexane soluble subfractions from the leaf of *C. formosana*

Subfractions	Mobile phase (v/v)	Weight fraction (%)	Cell viability (%) <sup>a</sup>
1	5/95 <sup>b</sup>	50.4	67.4 ± 15.2
2	10/90 <sup>b</sup>	11.1	>80
3	20/80 <sup>b</sup>	3.4	>80
4	20/80 <sup>b</sup>	7.0	43.3 ± 2.6
5	30/70 <sup>b</sup>	2.8	>80
6	50/50 <sup>b</sup>	2.4	>80
7	70/30 <sup>b</sup>	0.6	>80
8	100/0 <sup>b</sup>	1.1	>80
9	10/90 <sup>c</sup>	9.4	>80
10	30/70 <sup>c</sup>	11.8	>80

<sup>a</sup> Cell viability of A549 cells after treatment with 10 µg/ml of each subfraction for 24 h

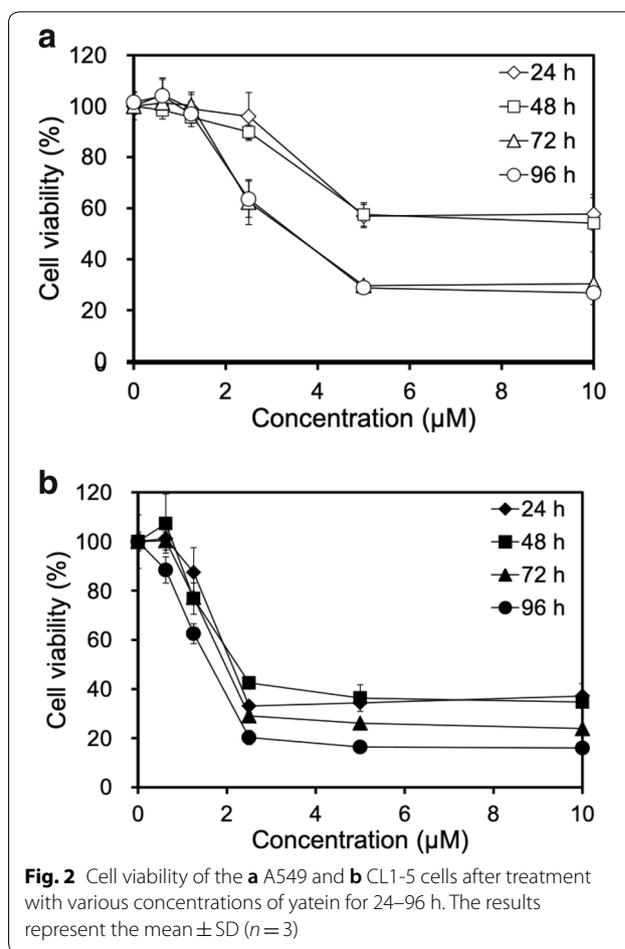
<sup>b</sup> EtOAc/*n*-hexane

<sup>c</sup> MeOH/EtOAc

the active phytochemicals were further isolated, purified, and identified from the *n*-hexane fraction. The *n*-hexane fraction was fractionated into ten subfractions through column chromatography (Si-60 gel). The results of the cytotoxicity screening of the subfractions revealed that subfraction 4 exhibited the highest growth inhibition in the A549 cells (Table 1). Based on the aforementioned results, the bioactivity-guided isolation principle was applied in this study, and the bioactive phytochemical of subfraction 4 is yatein (Fig. 1d) which was isolated and purified through HPLC using a normal phase system (Additional file 1: Fig. S1a). The yield of yatein was 11.1 ± 0.2 µg/g of dry leaf sample based on the calibration curve ( $R^2=0.9963$ , Additional file 1: Fig. S1b). In addition, the purity of yatein was estimated higher than 95% according to the <sup>1</sup>H NMR results (Additional file 1: Fig. S1c).

**Yatein induces cytotoxic effect in human NSCLC A549 and CL1-5 cells**

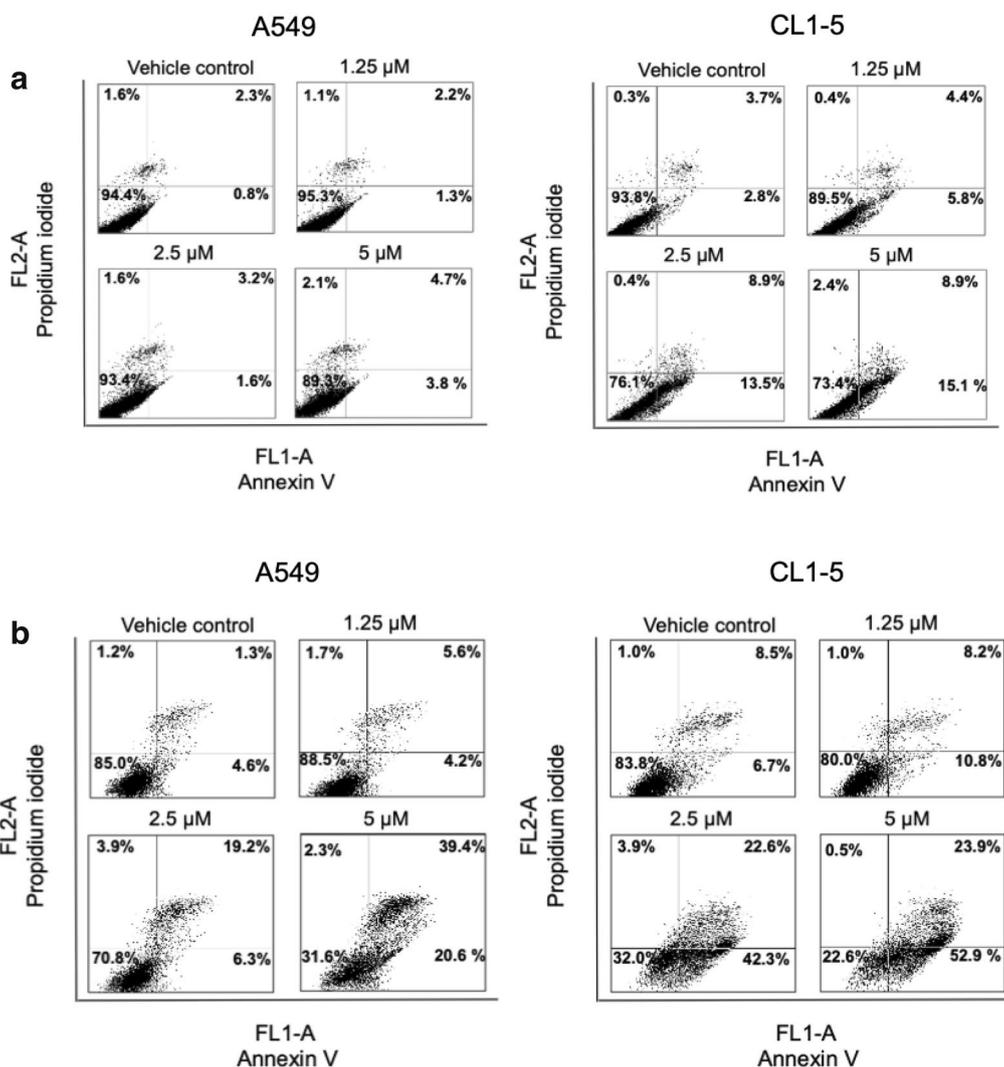
The viabilities of the A549 and CL1-5 cells after treatment with various concentrations of yatein for 24, 48, 72, and 96 h are shown in Fig. 2a and b, respectively. The results revealed that yatein exhibited high cytotoxicity in the A549 and CL1-5 cells, and the IC<sub>50</sub> values of yatein in the A549 and CL1-5 cells were 3.5 and 1.9 µM after treatment for 72 h. The results revealed that yatein was a natural cytotoxic agent that strongly inhibited NSCLC cell growth. Therefore, the mechanism underlying the growth inhibitory effect of yatein in the A549 and CL1-5 cells was further investigated in the present study.



**Fig. 2** Cell viability of the **a** A549 and **b** CL1-5 cells after treatment with various concentrations of yatein for 24–96 h. The results represent the mean ± SD (n = 3)

**Yatein induces apoptosis by enhancing apoptosis-related protein expressions and ROS production in the A549 and CL1-5 cells**

Additionally, we determined whether yatein inhibited the growth of the A549 and CL1-5 cells by inducing apoptosis in these two types of cells. To evaluate the apoptosis features of the A549 and CL1-5 cells, the yatein-treated cells were stained using the Annexin V/PI reagents and then analyzed using a flow cytometer. The results indicated that the population in the early apoptosis stage of CL1-5 cells increased in a dose-dependent manner after yatein treatment for 24 h. As shown in Fig. 3, 15.1% of early apoptotic cells and 8.9% of late apoptotic CL1-5 cells were observed after 5 µM yatein treatment for 24 h. By contrast, the A549 cells exhibited a different trend in the apoptosis stages compared with the CL1-5 cells after yatein treatment for 24 h, both of early and late apoptotic cells were less than 5%. After treatment with yatein for 48 h, the populations of the A549 and CL1-5 cells in the early and late apoptosis stages exhibited a dose-dependent increase (Fig. 3). The aforementioned findings



**Fig. 3** Annexin V/PI staining of the A549 and CL1-5 cells after yatein treatment for **a** 24 and **b** 48 h. The results represent the mean ( $n = 3$ )

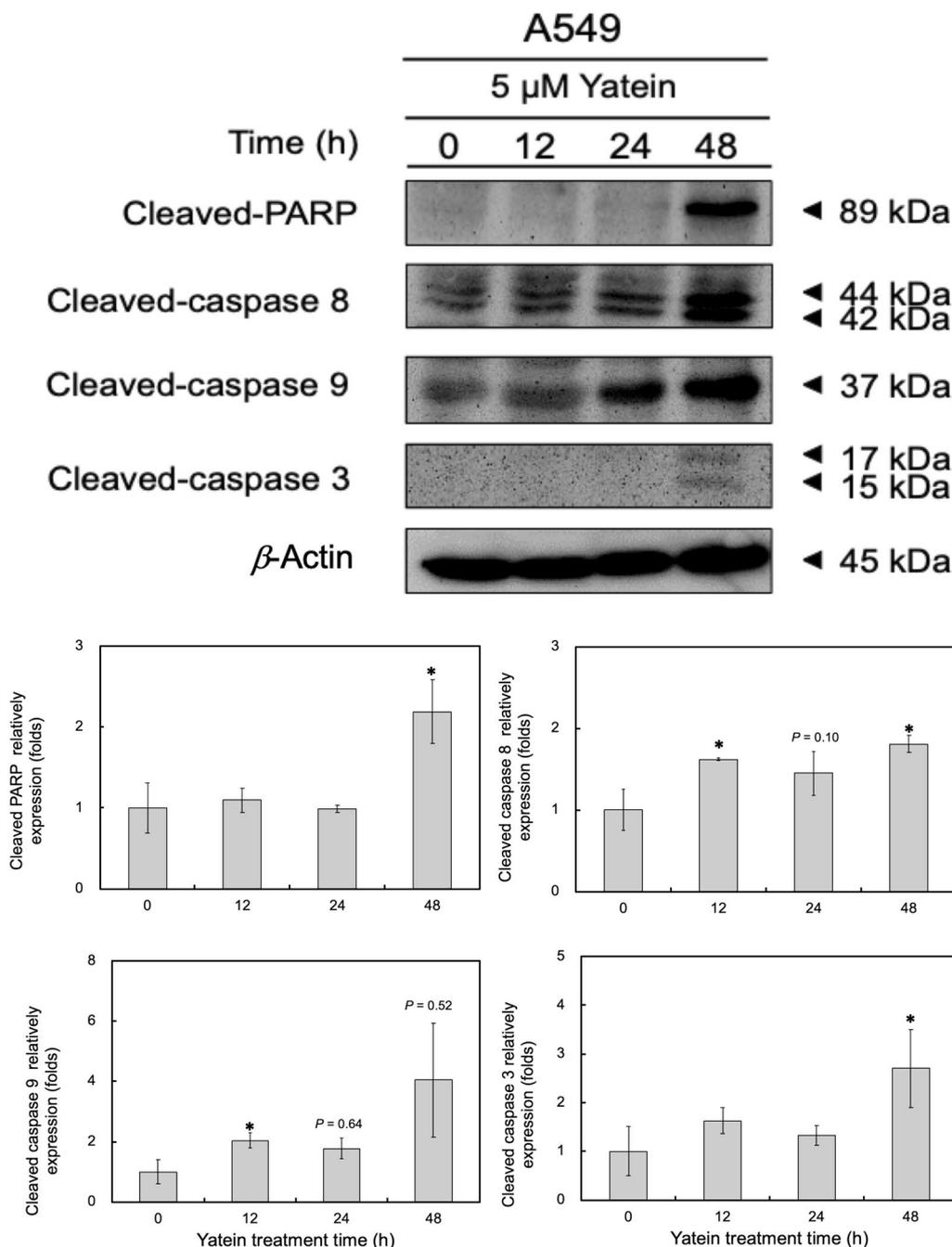
indicate that yatein might have induced apoptosis in these two types of cells for 24 and 48 h treatment.

The expression of apoptosis-related proteins, including cleaved PARP and cleaved caspases 9, 8, and 3, was analyzed after the A549 and CL1-5 cells treated with 5  $\mu$ M yatein for 0, 12, 24, and 48 h. As shown in Figs. 4 and 5, the activation of cleaved PARP and cleaved caspase 3, 8, and 9 increased in these two cell lines after yatein treatment for 48 h. The results implied that yatein induced caspase pathway activation and apoptosis in the A549 and CL1-5 cells. In addition, as shown in Fig. 6, yatein induced ROS formation in the NSCLC

cells at various time points in these two cell lines. Accordingly, we found that the CL1-5 cells were more sensitive for the ROS production than the A549 cells after yatein treatment.

### Discussion

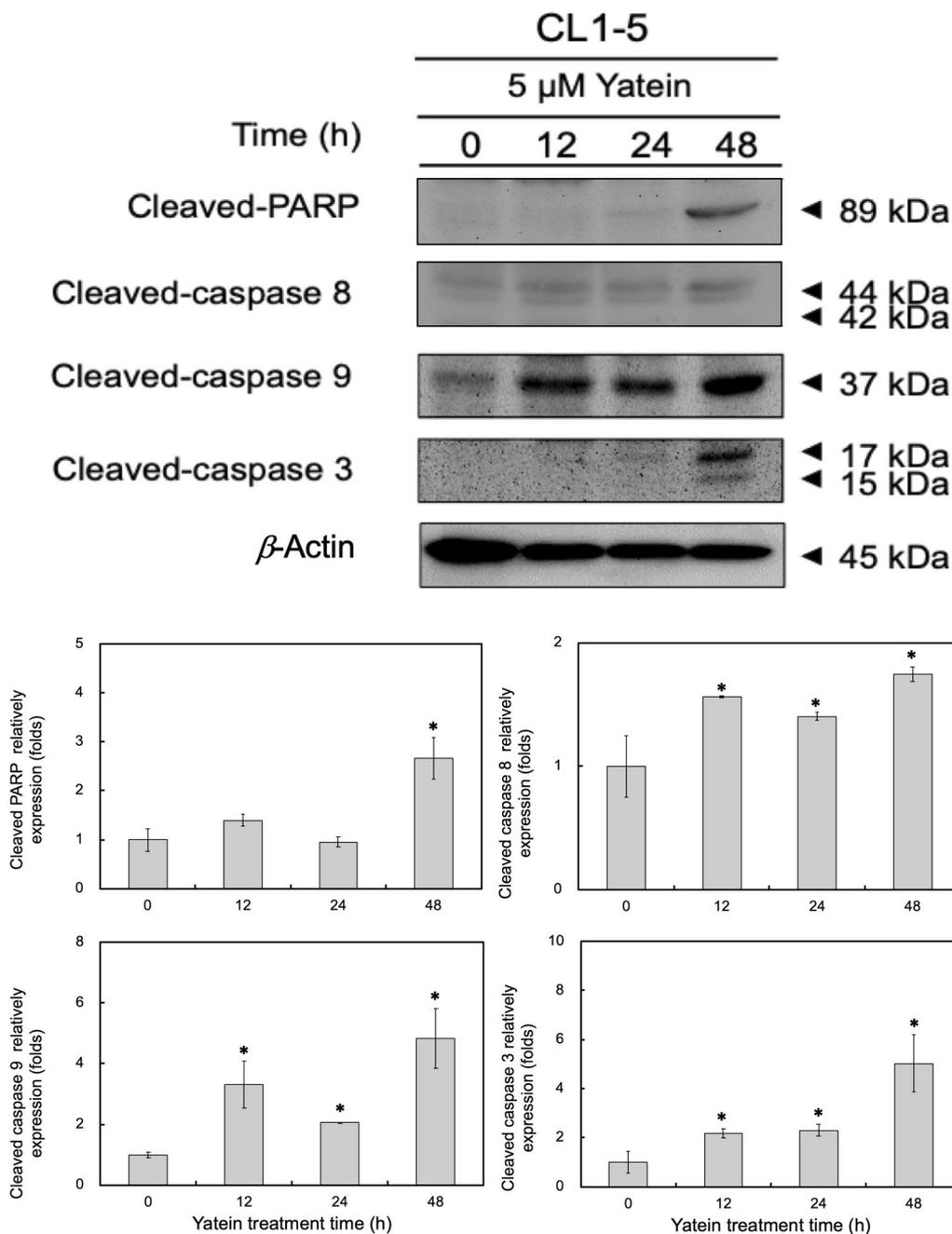
The present study demonstrated for the first time the in vitro anti-NSCLC effects of *C. formosana* leaf extract and its active derivative yatein. Our results indicated that the *C. formosana* leaf extracts exhibited high cytotoxicity ( $IC_{50}$  values of the *n*-hexane fraction in the A549 and CL1-5 cells were 23.1 and 1.2  $\mu$ g/ml,



**Fig. 4** Expression of apoptosis-related proteins in the A549 cells after treatment with yatein (5 μM) for 12, 24, and 48 h. The bands were analyzed using the ImageJ software and normalized to β-actin. All data presented are representatives of three independent experiments with similar results. The results represent the mean ± SD (n = 3). Asterisk indicates a significant difference compared with the control group (p < 0.05). p values were calculated by Student's t test

respectively) in the A549 and CL1-5 cells. We compared the cytotoxic effects of the *C. formosana* leaf extract with those of potential anti-NSCLC plant extracts, such as *Punica granatum* (IC<sub>50</sub> value of A549

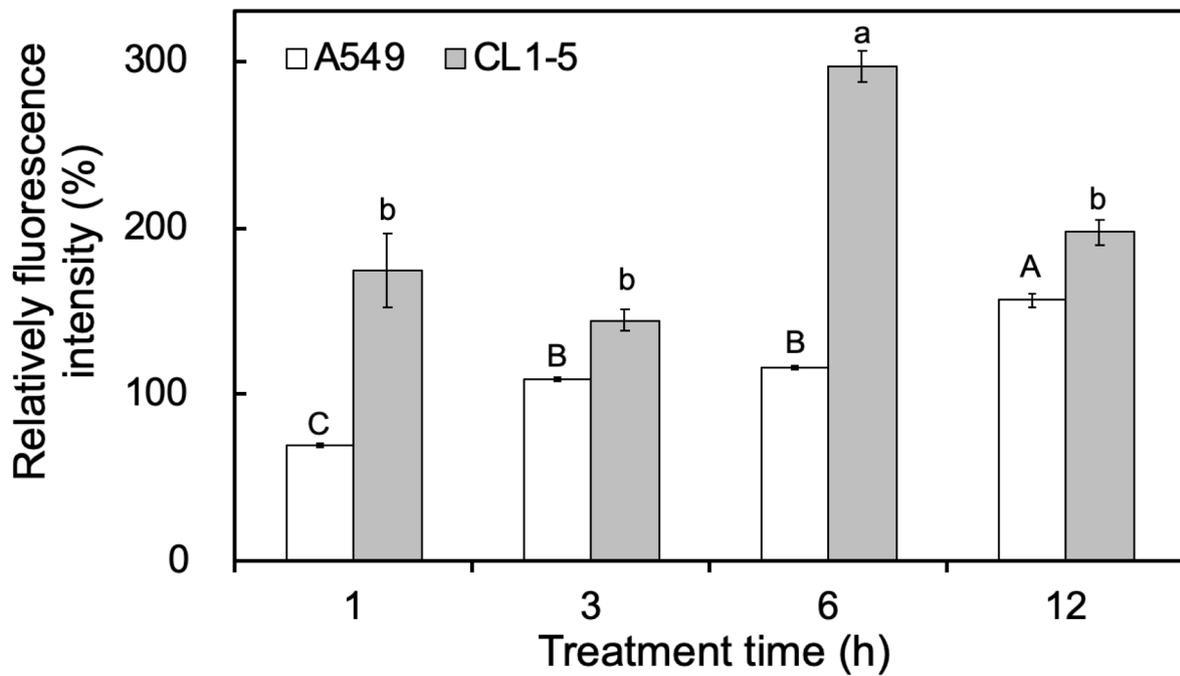
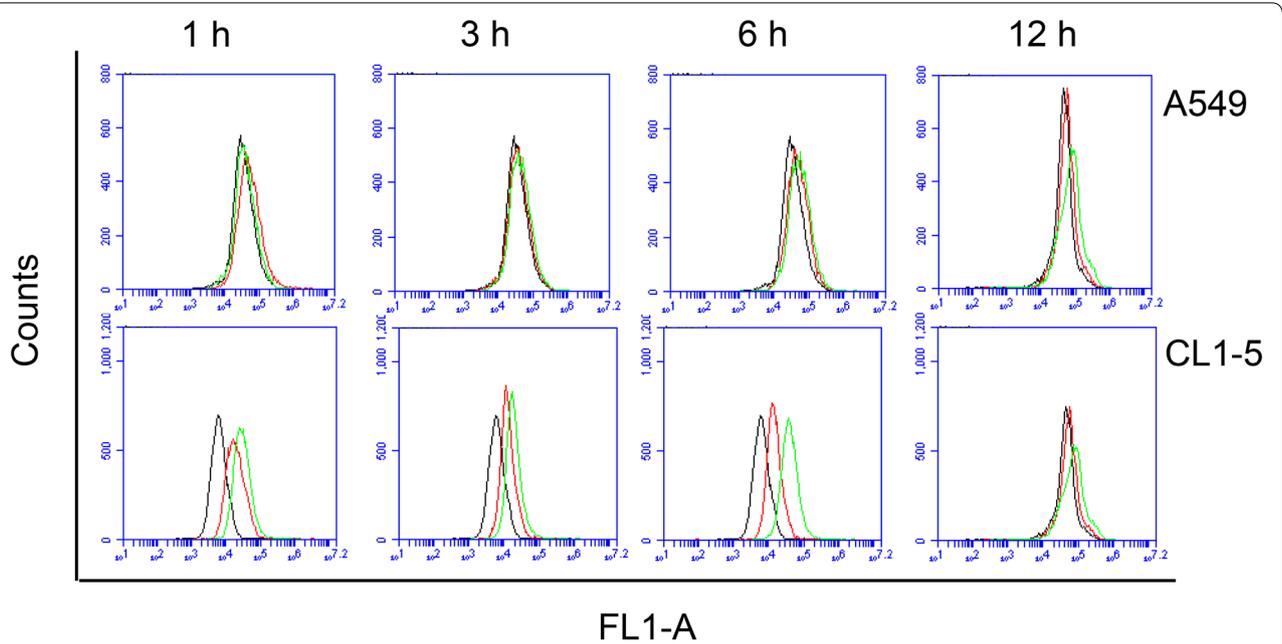
cells > 150 μg/ml), *Brachylaena ramiflora* (IC<sub>50</sub> value of A549 cells = 20 μg/ml), *Vernonia garnieriana* (IC<sub>50</sub> value of A549 cells = 79 μg/ml), *Flacourtia indica* (IC<sub>50</sub> value of A549 cells = 100 μg/ml), and *Rhododendron*



**Fig. 5** Expression of apoptosis-related proteins in the CL1-5 cells after treatment with yatein (5 μM) for 12, 24, and 48 h. The bands were analyzed using the ImageJ software and normalized to β-actin. All data presented are representatives of three independent experiments with similar results. The results represent the mean ± SD (n = 3). Asterisk indicates a significant difference compared with the control group (p < 0.05). p values were calculated by Student's t test

*formosana* (IC<sub>50</sub> value of A549 cells = 80 μg/ml) [21–23]; the cytotoxic potential of the *C. formosana* leaf extract was higher than that of the aforementioned extracts. On the other hand, *C. formosana* leaf extracts also possessed cytotoxicity against bladder cancer cell

growth (IC<sub>50</sub> value ≈ 10 μg/ml for TCCSUP, T24, and TSGH-8301 cells after treatment for 48 h) [16], suggesting that *C. formosana* leaf extracts may exhibit broad-spectrum cytotoxicity against various cancer cells.



**Fig. 6** ROS levels of A549 and CL1-5 cells after treatment with 5  $\mu$ M yatein for 1, 3, 6, and 12 h. Black line: unstained cells, red line: control group, and green line: yatein treatment group. The results represent the mean  $\pm$  SD ( $n = 3$ ); different capital and lowercase letters indicate significant differences among each group in the A549 and CL1-5 cells, respectively ( $p < 0.05$ ).  $p$  values were calculated by one-way ANOVA, followed by shuffle test

As previously reported, some phytochemicals from *C. formosana* leaf extracts were able to inhibit cancer cell growth; for instance, hinokitiol inhibited A549 cell growth at a high dosage (more than 20  $\mu\text{M}$ ) [19]. Additionally, we found that yatein exerted a stronger inhibitory effect on the growth of the A549 cells ( $\text{IC}_{50}$  value = 3.5  $\mu\text{M}$  after treatment for 72 h) than did other lignans that have been reported to have anti-NSCLC growth effects in previous studies. The inhibitory effects of lignan compounds on NSCLC have been reported in previous studies. For example, Min et al. isolated several lignans from *Schisandra chinensis* [24]. Among these phytochemicals, schisantherin C exhibited the highest inhibitory effects on the A549 cell growth with an  $\text{IC}_{50}$  value of 5.6  $\mu\text{M}$ , followed by schizandrin (20.7  $\mu\text{M}$ ) and gomisins N (21.1  $\mu\text{M}$ ). A lignan isolated from *Endiandra anthropophagorum*, (-)-dihydroguaiaretic acid, has been reported to have growth inhibitory effects on A549 cells with an  $\text{IC}_{50}$  value of 7.5  $\mu\text{M}$  [25]. Furthermore, Ou et al. [26] reported that Z-isochailactone (a lignan isolated from *Bupleurum scorzoniferifolium*) displayed cytotoxicity in A549 cells ( $\text{IC}_{50}$  value = 7.5  $\mu\text{M}$ ). Based on the aforementioned reports, lignan compounds have notable potential as new cytotoxic lead compounds for treatment of NSCLC.

To evaluate the anti-NSCLC growth mechanism of yatein, the regulatory effect of yatein on the apoptosis-related pathway was determined using flow cytometry and Western blot analysis. The result of Western blot analysis was consistent with the results of Annexin V/PI staining; we found that prolonged yatein treatment was associated with an increase in apoptosis features in the cells, as observed through flow cytometry and Western blot analysis. Caspase family proteins play a vital role during the apoptotic process; the activated caspase proteins are able to trigger the programmed cell death. Our finding indicated that yatein induced caspase-dependent apoptosis and activated intrinsic and extrinsic apoptotic pathways in the A549 and CL1-5 cells by activating expression of caspase 8 and caspase 9 and then inducing caspase 3 and PARP protein activation. Additionally, yatein induced ROS formation in the NSCLC cells at various time points. ROS has multiple functions in cell biology. At proper doses, ROS are able to regulate cell development functions, including cell cycle regulation, cell death, differentiation, migration, and proliferation. However, excess ROS production would cause severe damage to biomolecules, and then induced apoptosis and/or necrosis [27–29]. Additionally, previous studies also showed that lignan compounds are able to induce apoptosis through producing ROS in various cell types [30–34]. Thus, based

on aforementioned, we speculated that ROS formation was involved in yatein-mediated cell death.

## Conclusions

The present study demonstrated that *C. formosana* Florin leaf extract and its active phytochemical, yatein, suppressed NSCLC growth through the induction of apoptosis. According to the results from flow cytometry and Western blot analyses, we found that yatein initialized the apoptosis cascade in the intrinsic and extrinsic signaling pathways. Additionally, ROS production was also involved in the mechanism underlying yatein-mediated NSCLC growth inhibition. These findings provide insight into the in vitro anti-lung tumor efficacy of yatein, thus rendering this phytochemical a potential anticancer lead compound for NSCLC treatment. However, the in vivo anti-lung tumor effects of yatein are still needed for the future study.

## Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s10086-019-1838-9>.

**Additional file 1: Fig. S1.** HPLC chromatogram of subfraction 4 from *n*-hexane fraction of *C. formosana* leaf extract (a), calibration curve of yatein quantification (b), and  $^1\text{H}$  NMR spectrum ( $\text{CDCl}_3$ , 400 MHz) of yatein (c).

## Abbreviations

BuOH: *n*-butanol; BSA: bovine serum albumin; *C. formosana*: *Calocedrus formosana*; DMEM: Dulbecco's modified Eagle's medium; DMSO: dimethyl sulfoxide; ECL: enhanced chemiluminescence; EtOAc: ethyl acetate; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; NSCLC: non-small-cell lung cancer; PARP: poly (ADP-ribose) polymerase; PBS: phosphate-buffered saline; PI: propidium iodide; ROS: reactive oxygen species; SD: standard deviation.

## Authors' contributions

J-HW and C-CL designed the experiments; S-TH, T-LW, and Y-TT performed the experiments. C-CL, Y-TT, and J-HW supported reagents and kits. S-TH, Y-TT, and J-HW analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

## Funding

This research work was supported in part by the Ministry of Science and Technology of Taiwan and Taipei Medical University under Grant Nos. MOST 107-2628-H-038-001-MY3 and 106-6820-001-112, respectively.

## Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

## Competing interests

The author(s) declared no potential competing interest with respect to the research, authorship, and/or publication of this article.

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Received: 28 May 2019 Accepted: 16 October 2019

Published online: 31 October 2019

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