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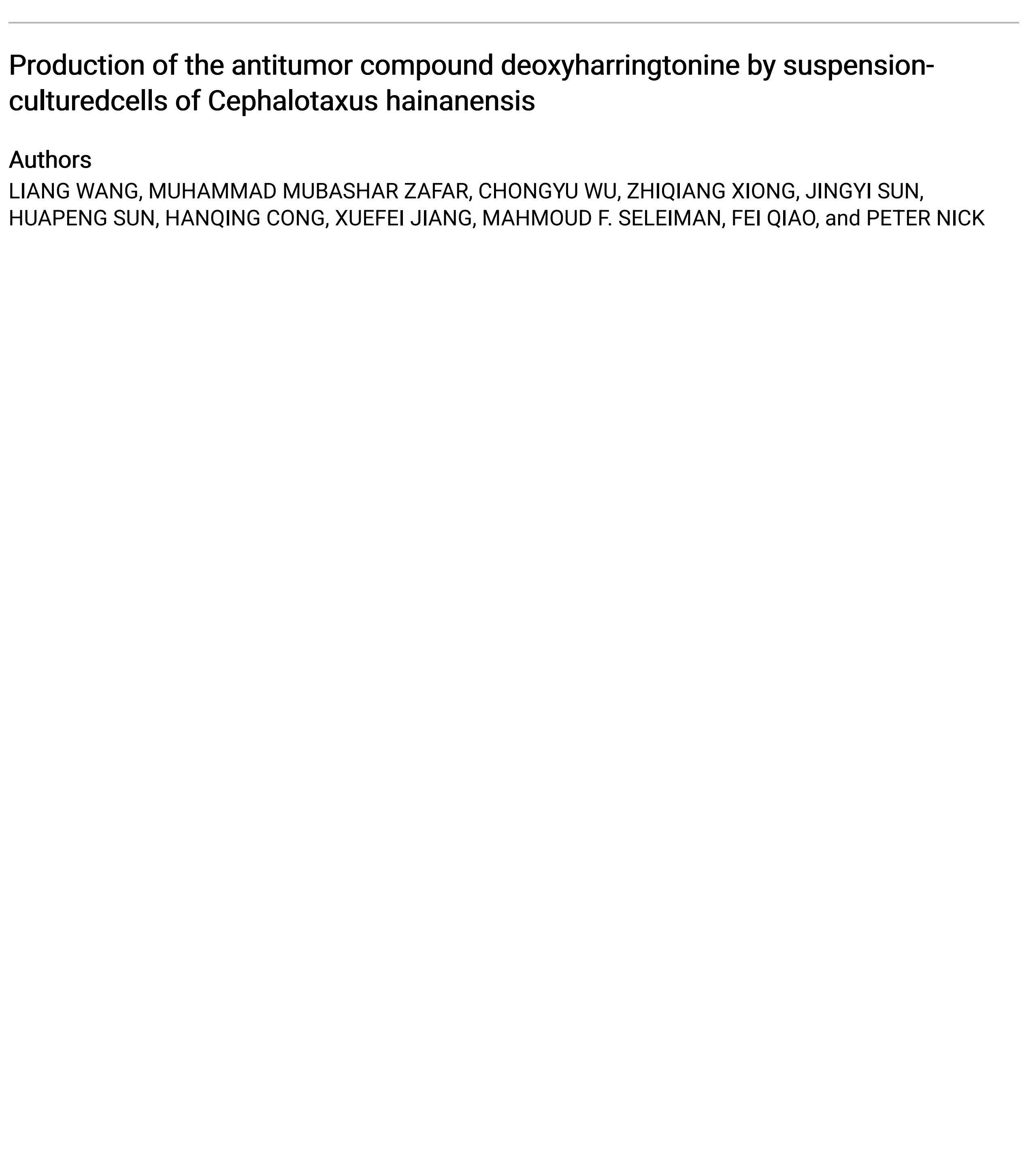

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Production of the antitumor compound deoxyharringtonine by suspension-cultured cells of *Cephalotaxus hainanensis*

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Abstract: Plant cell fermentation can be a sustainable biotechnological alternative to the wild collection of medicinal plants. As a paradigm, we explored the potential of using the highly endangered endemic tree *Cephalotaxus hainanensis*, which accumulates cephalotaxine esters—potent anticancer compounds. We successfully established a suspension cell line able to convert cephalotaxine into its esters, including deoxyharringtonine, harringtonine, isoharringtonine and homoharringtonine at a remarkable maximal conversion rate of up to 86%, when supplemented with L-homoleucine, a precursor for the side chains of these esters. Furthermore, by adding elicitors, we were able to enhance the yield significantly beyond the values expected from the complete conversion of the added cephalotaxine. In particular, the addition of silver nitrate allowed the production of up to 19 mg/L of deoxyharringtonine and 3.3 mg/L of cephalotaxine, more than doubling the expected yield from the complete conversion of the fed precursor (10 mg/L cephalotaxine). Thus, these treatments not only promoted the bioconversion of exogenous cephalotaxine but also stimulated the biosynthesis of endogenous cephalotaxine. This stimulation required more than 5 days to become manifest. This is the first case where deoxyharringtonine could be produced by plant cell culture, paving the way for the commercial production of this valuable pharmaceutical compound while safeguarding the highly endangered and endemic population of the tree itself.

Key words: Antitumor alkaloid, *Cephalotaxus hainanensis*, deoxyharringtonine, silver nitrate, suspension-cultured cells

1. Introduction

Cephalotaxine esters are unique to the family *Cephalotaxaceae*, which is endemic to Southeast Asia (Bagheri et al., 2024; Gul et al., 2024). Cephalotaxine and its esters were first isolated from *Cephalotaxus fortunei* and *Cephalotaxus harringtonii* (Paudler et al., 1963), and subsequently studied extensively with respect to their pharmacology and clinical aspects (Dai, 2006), since the cephalotaxine esters harringtonine, homoharringtonine, deoxyharringtonine and isoharringtonine exhibit significant antitumor activity (Powell et al., 1972; Yang et al., 2009). In contrast, their precursor, cephalotaxine, lacks any biological activity, indicating that the ester side chain is crucial for antitumor activity (Powell et al., 1972).

Among the different species of the genus, *C. hainanensis* accumulates the highest levels of cephalotaxine esters in all parts of the plant (Xu and Zang, 2023). However, even in this species, the levels of these esters remain relatively low. For instance, to obtain 1 g of cephalotaxine esters, between 35 and 60 kg of fresh leaves are required (Table S1). The slow growth rate of this plant, coupled with its endangered status and endemic occurrence on the Chinese island of Hainan, further limits the availability of these valuable compounds, thus prompting exploration into alternative extraction methods.

While a complete total synthesis could be achieved for cephalotaxine by constructing the C-ring using a Friedel–Crafts reaction (Auerbach and Weinreb, 1972) as a prerequisite for the subsequent chemical synthesis of these esters (Mikolajczak et al., 1974), the activities of the products were found to be low due to insufficient reproduction of the numerous chirality

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centers (Mikolajczak et al., 1977). Plant cell fermentation (PCF) offers several advantages over traditional chemical synthesis approaches. Due to the uniformity of cell types, synchronized growth patterns, rapid multiplication rates, and short growth cycles, suspension cultures have the potential for sustainable production of plant-derived pharmaceuticals (Kieran et al., 1997; Mulabagal and Tsay, 2004).

However, so far, neither callus (Delfel and Rothfus, 1977; Wickremesinhe and Arteca, 1993) nor cell suspension cultures (Westgate et al., 1991) from *Cephalotaxus harringtonia* have yielded more than trace amounts of the desired *Cephalotaxus* alkaloids (Rashid et al., 2024; Idohou et al., 2025). Such bottlenecks can be addressed either by promoting the supply with precursors through chemical elicitation, by suppressing competing shunt pathways, or by feeding precursors (Zhao et al., 2005; Namdeo, 2007; Malik et al., 2011).

For instance, elicitation with methyl jasmonate (MeJA) is used to induce the accumulation of the antitumor alkaloid paclitaxel in *Taxus* cells (Phisalaphong and Linden, 1999). Likewise, aluminum ions, through activation of the membrane-located NADPH oxidase respiratory burst oxidase homologue (RboH), can induce the expression of key enzymes of the paclitaxel pathway in *T. chinensis*, stimulating the accumulation of the precursor baccatine VI (Manz et al., 2022). Also, silver ions (Zhang et al., 2000), chitosan (Ketchum et al., 1999), or endophytic fungi (Li et al., 2009) have been used to stimulate paclitaxel accumulation in *Taxus* cells. In the case of *Cephalotaxus mannii*, elicitation with MeJA, and its stable bacterial analogue, coronatine, induced transcripts of the shikimate and phenylpropanoid pathways and also resulted in the accumulation of cephalotaxines (Wang et al., 2021). Elicitation can also be combined with the feeding of limiting precursors (Ishihara et al., 2003). For instance, by feeding the precursor vindoline to a specific strain of *Catharanthus roseus* cells that had been elicited by MeJA, it became possible to obtain the potent antitumor compound vincristine, albeit only in trace amounts (Raorane et al., 2023).

However, any attempt to generate deoxyharringtonine in cell culture has remained futile so far. Therefore, we ventured to establish novel cell suspension cultures from *C. hainanensis* in combination with precursor feeding and elicitation treatments. In this study, we show how this strain of *C. hainanensis* can efficiently convert cephalotaxine to the bioactive esters. This conversion is dependent on the availability of the precursor for the ester side chain. In addition, we provide evidence for positive feedback activating endogenous precursor production, such that the desired product, deoxyharringtonine, accumulates to levels far beyond those expected from the complete conversion of the fed precursors.

2. Materials and methods

2.1. Cell culture

Generative shoots of 10-year-old *C. hainanensis,* which had been cultivated in the Botanic Garden of the Tropical Crops Genetic Resources Institute of the Chinese Academy of Tropical Agricultural Sciences (CATAS), were harvested, and the leaves were removed. The shoots were cut into 2 cm long segments, surface-sterilized, and placed on petri dishes containing 5.32 g/L Driver and Kuniyuki Walnut (DKW) basal medium with vitamins (PhytoTechnology Laboratories, Kansas, USA), 30 g/L sucrose (Sangon Biotech, Shanghai, China), 0.1 g/L casein hydrolysate (Sigma-Aldrich, Shanghai, China), 5 mg/L picloram (Sigma-Aldrich) and 7 g/L agar, pH 5.8–5.9. After 40–45 days of incubation in the dark at 25 °C, callus emerged around the axillary buds. These calluses were excised and subcultured every 2 months. Subsequently, suspension cultures were established by transfer of 2–3 cm^3 callus into 30 mL liquid medium in an Erlenmeyer flask, the liquid medium being the same as the solid medium, just omitting the agar.

The suspension cells were incubated at 25 °C in darkness on an orbital shaker (ISF4-X, Φ = 25 mm; Kuhner Shaker, Basel, Switzerland) at 150 rpm. The cells were subcultured every 10 days by inoculating 5 mL of stationary cells into a 100 mL Erlenmeyer flask containing 30 mL of fresh medium.

2.2. Investigation of cell growth and morphology

To determine the growth of the newly established cell line, 5 mL of fresh cells were inoculated into a 100 mL flask containing 30 mL of medium. Three flasks were harvested at 2-day intervals after inoculation until day 14 of the incubation period to determine the increase in fresh weight after filtration through Whatman no.1 filter paper. The morphology of the suspension-cultured cells was observed by light microscopy (Axio Observer Z1; Zeiss, Oberkochen, Germany) during the stationary phase.

2.3. Precursor feeding

According to the previously deduced biosynthetic pathways for cephalotaxine esters (Parry and Schwab, 1975; Abdelkafi and Nay, 2012), the active compounds derive from a fusion between cephalotaxine and harringtonic acids, a derivative of L-homoleucine. Both cephalotaxine and L-homoleucine are commercially available at low cost, enabling a feasible precursor-feeding strategy. Aliquots of 30 µL of cephalotaxine (10 mg/mL, dissolved in methanol; Yuanye Bio-Technology Co. Ltd., Shanghai, China) and 30 µL of L-homoleucine (10 mg/mL, dissolved in 0.4% hydrochloric acid; Bidepharm Technology Co. Ltd., Shanghai, China) were fed to the cultures on day 10 after subculturing. To generate absorption and conversion curves, samples of both the cells and the medium were harvested daily from day 0 (immediately postfeeding) to day 9.

2.4. Induction by elicitors and other chemicals

To test the effect of elicitation by compounds related to stress signaling on the accumulation of the desired products, either 100 µM abscisic acid (ABA), 100 µM methyl jasmonate (MeJA), 100 µM salicylic acid (SA), or 100 µM ethephon (ETH) was supplemented into the culture directly after the addition of the precursor. To mitigate senescence, 10 µM silver nitrate (SN) was used. In addition, the effects of 20 mg/L chitosan glutamate (CG) as a fungal elicitor activating basal immunity, 200 mg/L casein hydrolysate (CH) as an additional nitrogen source, and 35 µM β-cyclodextrin (CD) as a complexing agent were tested as well. These agents were fed to the cell cultures at the same time as the precursors (10 mg/L cephalotaxine and 10 mg/L L-homoleucine) on day 10 after subculturing, and the cells and medium were harvested 5, 7, and 9 days later.

2.5. Analysis of alkaloid content and profile

Cells and culture filtrate were separated using Whatman no.1 filter paper. Residual alkaloids were removed from the cell surface by two washing steps with water. Aliquots of 0.3 g of cell mass were directly transferred to sterilized 2 mL reaction tubes, while culture filtrate and wash water were collected in aliquots of 0.5 mL, again into sterilized 2 mL reaction tubes.

For both types of samples, each aliquot was complemented with 1.5 mL methanol. The cells were homogenized with two steel balls by a Tissue Lyser at 60 Hz for 60 s. Then, both cells and medium were ultrasonicated at 25 °C with 600 W and 40 kHz for 20 min and spun down for 10 min at 12,000 rpm. The supernatant was filtered through a syringe filter with 0.22 µm pore size and then analyzed by LC-MS.

The *Cephalotaxus* alkaloids were identified and quantified using an Exactive Plus Orbitrap mass spectrometer coupled with a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific, Waltham, MA, USA). The extracts (2 µL) were separated on an Agilent InfinityLab EC-C18 column (2.1 × 100 mm, 2.7 µm particle size) (Agilent Technologies, Santa Clara, CA, USA) and eluted on a gradient of 0.1% v/v aqueous formic acid (HCOOH) (A) and acetonitrile (B) at a flow rate of 0.3 mL/min using a gradient of A:B = 98:2 (v/v) at 0 min, 98:2 at 2 min, 65:35 at 8 min, 15:85 at 10 min, 15:85 at 12 min, 55:45 at 12.8 min, 98:2 at 15 min, and 98:2 at 16 min. For qualitative and quantitative analysis, commercial standards for cephalotaxine, harringtonine, and homoharringtonine (Yuanye Bio-Technology Co. Ltd.) were used, with an injection volume of 2 µL for each (Figures S1 and S2).

An Exactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ionization (HESI) source and supplied with nitrogen as the collision gas was used to identify the *Cephalotaxus* alkaloids. The mass spectrometer was operated in full-scan mass spectrometry/all ion fragmentation (MS/AIF) mode, with a normalized collision energy of 35 eV, a spray voltage set to 3.5 kV, the heated capillary held at 275 °C, and the HESI probe at 350 °C. The sheath gas flow was set to 35 arb, the auxiliary gas flow to 15 arb, and the sweep gas flow to 5 arb. Full-scan MS data were collected with a resolution of 7000 in positive ionization mode, spanning a scan range from 50 to 750 m/z. The all-ion fragmentation routine was conducted at a resolution of 35,000 in the full-scan MS data-dependent mode, and the automatic gain control (AGC) target was set to 3×10^6 for a maximum injection time of 50 ms. The mass accuracy was better than 1 ppm for both full-scan MS/AIF modes, ensuring reliable compound identification.

The accuracy of the MS analysis was ensured by calibrating the detector using commercial calibration solutions provided by the manufacturer, followed by a customized adjustment for small molecular masses. The UPLC-MS analysis platform was controlled by the PC operating software package Thermo Xcalibur version 2.2 SP1.48 (Thermo Fisher Scientific).

2.6. Statistical analysis

Data represent mean values and standard errors from at least three independent experiments. SPSS 25.0.0 software (IBM Corp., Armonk, NY, USA) and Origin 2018 (OriginLab Corporation, Northampton, MA, USA) were used to analyze all experimental data. The significance of differences between control and treated samples was tested by multiple comparison post hoc tests according to Duncan and Dunnett through one-way ANOVA.

3. Results and discussion

3.1. Establishment of a cell-suspension line for *Cephalotaxus hainanensis*

To choose the source material for establishing a cell culture, we monitored the content and composition of cephalotaxine esters in the leaves of young (10-year-old) *C. hainanensis* trees. We found that the precursor cephalotaxine predominated (0.47 mg/g dry weight) over the bioactive ester harringtonine (0.09 mg/g dry weight), homoharringtonine (0.10 mg/g dry weight), isoharringtonine (0.08 mg/g dry weight), and deoxyharringtonine (0.06 mg/g dry weight) (Table S1). This indicates that the condensation of cephalotaxine with side chains represents the limiting step. Using suspension-cultured cells would allow for precursor feeding and thus might remove the bottleneck in the conversion of cephalotaxine to cephalotaxine esters.

Therefore, a callus of *C. hainanensis* was induced from axillary buds, and proliferating calli (Figure 1A) were excised and transferred to liquid medium to establish a suspension cell line. The cells grown in suspension are of elongated shape and form aggregates (Figure 1B). The growth pattern of the suspension culture was monitored

by measuring fresh weight and showed a typical growth curve (Figure 1C) with a lag phase up to day 3, an exponential phase from day 4 to 10, and a stationary phase after day 10. The growth index over the 2-week period was approximately three.

3.2. Toxicity of exogenous cephalotaxine and its esters on cell growth

Plant secondary metabolites are usually produced when a plant is confronted with biotic or abiotic stress. However, these secondary metabolites are often cytotoxic (Heiling et al., 2021). Therefore, plants use several strategies to avoid self-inhibition, such as sequestration in vacuoles or idioblasts (Cai et al., 2011), secretion into the apoplast (Cai et al., 2012), or detoxification by chemical modification, such as glycosylation (Metlen et al., 2009), and deesterification (Hang et al., 2012). From the perspective of plant cell fermentation, accumulation of the product can impair proliferation. We therefore assessed the extent to which culture growth was inhibited by exogenous cephalotaxine and homoharringtonine added to the medium at inoculation, using fresh weight after the subsequent 14-day growth cycle as the readout. We used a concentration of 10 mg/L for both compounds, which is below the maximal accumulation of harringtonine and homoharringtonine found in the leaves. Nevertheless, the growth inhibition was significant. Compared to solvent control and blank control, fresh weight was reduced by 20%–25% (Figure 2). Although the inhibition of cephalotaxine and its ester was not very pronounced, we used a two-step cultivation strategy (Zhong, 2002) for the following experiments. Hereby, the cells were cultured in the absence of any precursors or elicitors until stationary phase (10 days) in order to establish a high biomass, after which they were fed with precursors and elicitors for subsequent conversion.

3.3. *C. hainanensis* cells convert cephalotaxine to deoxyharringtonine

Feeding precursors as a strategy to enhance the yield of value-giving compounds from cell cultures has been a common approach. For example, feeding tyrosol into a cell suspension culture of *Rhodiola imbricata* can significantly improve the yield of salidroside (Rattan et al., 2022). Likewise, the abundance of steroidal alkaloids can increase by 160-fold in response to feeding cholesterol to cell cultures of *Holarrhena antidysenterica*, or the antimalarial compound artemisinin can be produced in suspension cells of *Artemisia annua* by feeding the precursor mevalonic acid lactone (Panda et al., 1992; Baldi and Dixit, 2008).

To verify whether the cells of *Cephalotaxus hainanensis* can convert cephalotaxine into cephalotaxine esters, the accumulation of harringtonine, isoharringtonine, homoharringtonine, and deoxyharringtonine was measured in the cell cultures after feeding cephalotaxine (Figure 3A). Only around 2% of the deoxyharringtonine found in the cells was detected in the medium, indicating that secretion is negligible (Figure 3B). Among these metabolites, deoxyharringtonine was predominant (97.2%), which contrasts with only 17.9% of deoxyharringtonine in the leaves of *Cephalotaxus hainanensis*. Thus, the relative abundance of this ester in cell culture is five times higher than in the leaves (Figure 3C).

L-homoleucine is the common precursor for the ester side chains, i.e. harringtonic acid, homoharringtonic acid, isoharringtonic acid, and deoxyharringtonic acid (Abdelkafi and Nay, 2012). Deoxyharringtonine derives from the condensation of deoxyharringtonic acid with cephalotaxine. This esterification is concurrent with elongation or modification of deoxyharringtonic acid leading to homoharringtonic. In cell culture, deoxyharringtonic acid preferentially fuses with cephalotaxine, while in leaves, this acid preferentially undergoes elongation, such that eventually the fusion with cephalotaxine culminates in the formation of homoharringtonine. Thus, the fusion of the side chain to the cephalotaxine moiety seems to be catalyzed by the rate-limiting enzyme. Why this limitation is removed in cell culture, remains to be elucidated, but cultivation in the absence of light and the lack of chloroplasts might be candidates responsible for the difference.

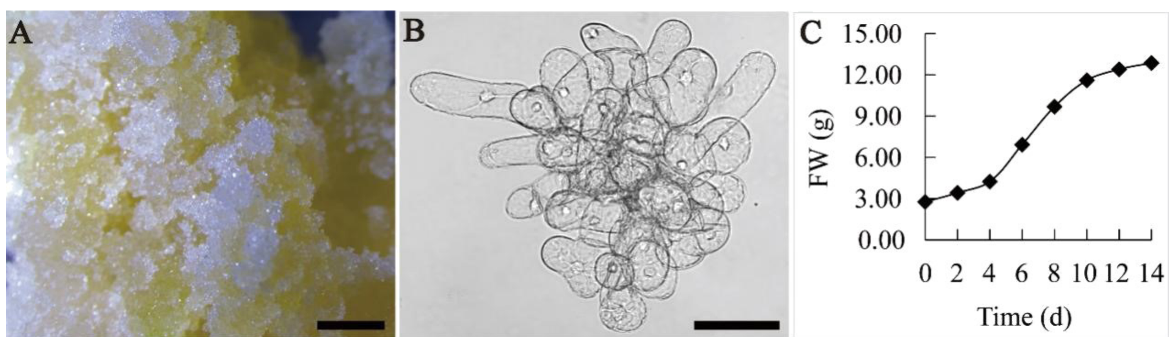

Figure 1. Morphology and growth of cell cultures from Cephalotaxus hainanensis. A. Calli induced from generative shoots. Bar = 2 mm; B. Morphology of suspension-cultured cells. Bar = 20 µm; C. Growth of the cell line monitored by fresh weight (FW). Data represent mean ± SE from three independent experimental series.

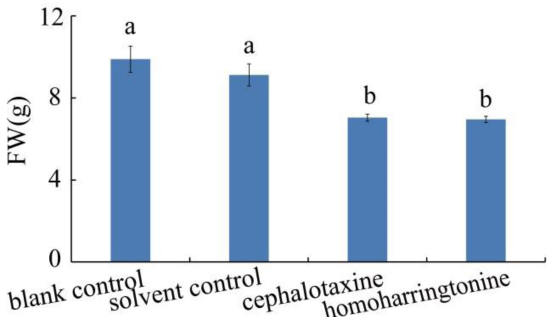

Figure 2. Effects of cephalotaxine and homoharringtonine on cell growth of *C. hainanensis*. Solvent control (0.1% (v/v) methanol); cephalotaxine (10 mg/L); homoharringtonine (10 mg/L). Data represent mean ± SE from three independent experimental series. Different letters indicate significant differences based on Duncan's test at $p < 0.05$.

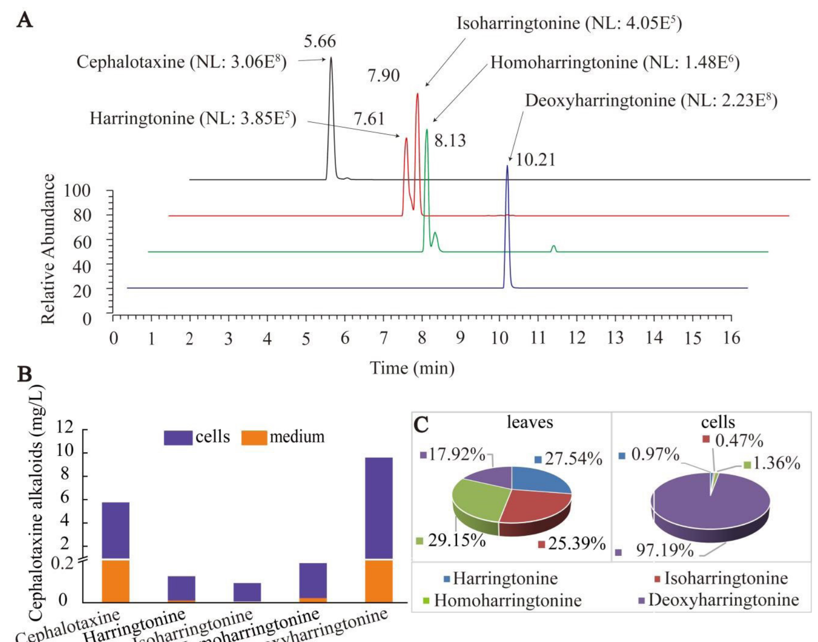

Figure 3. Distribution of *Cephalotaxus* alkaloids in plant leaves and cell cultures. A. Total ion chromatogram (TIC) of cephalotaxine and its esters from *Cephalotaxus hainanensis* cells detected by UPLC-MS (NL: nominal level); B. Distribution of five *Cephalotaxus* alkaloids in cells and culture medium incubated for 5 days after precursor feeding; C. Proportions of harringtonine, isoharringtonine, homoharringtonine, and deoxyharringtonine in leaves and cells. Cell volume is indicated as packed cell volume (PCV).

3.4. L-homoleucine limits the conversion of cephalotaxine to deoxyharringtonine

The side chain of the cephalotaxine esters derives from L-homoleucine, and the availability of this amino acid might represent a bottleneck for the synthesis of bioactive products (Medina-Pérez et al., 2025; Ullah et al., 2025). In paclitaxel biosynthesis, baccatin III, a more abundant precursor accumulating in yew, has to recruit the side chain derived from β-phenylalanine to complete the route towards paclitaxel. This rate-limiting esterification step has been identified and is catalyzed by an acyltransferase named baccatin III:3-amino-3-phenylpropanoyltransferase (BAPT) (Walker et al., 2002).

To address the bottleneck in *C. hainanensis*, we conducted a double feeding experiment, supplementing both cephalotaxine and L-homoleucine. The supplemented cephalotaxine in the medium declined steeply during the first 3 days and subsequently leveled off more slowly

(Figure 4A). At day 9, only 2.4 mg/L of cephalotaxine (i.e. 24% of the added concentration) were left. In contrast, cephalotaxine content in the cells reached 15.23 mg/kg (fresh weight) at day 9. When the accumulated content is related to the added amount of cephalotaxine, the proportion of precursor integrated into the product can be inferred to be around 86%.

Simultaneously, cephalotaxine esters became detectable in both cells and medium. This indicated that the cells not only convert cephalotaxine into its esters but also secrete these esters into the extracellular medium (Figure 4B). This contrasted with the low secretion seen for feeding cephalotaxine without supplemented L-homoleucine (Figure 3B). The ester content in both cells and medium increased rapidly during the first 3 days and stayed at a plateau thereafter. At day 9, esters inside the cells had accumulated to 14.2 mg/kg (fresh weight), whereas 4.8 mg/L was found in the medium (Figure 4B).

To quantify the contribution of L-homoleucine as a precursor of the ester side chain, we plotted the ratio of supplemented cephalotaxine converted to the respective esters over time (Figure 4C). While the final conversion seen at day 9 was comparable (84.7% for cephalotaxine alone and 86.1% for cephalotaxine supplemented with L-homoleucine), the time required to reach half of this final transformation ratio was considerably shortened in response to feeding L-homoleucine (2.6 days instead of 4.0 days). Thus, feeding this side chain precursor removes a bottleneck for the accumulation of the bioactive cephalotaxine esters. It should be mentioned that upon simultaneous feeding of both cephalotaxine and L-homoleucine, other *Cephalotaxus* alkaloids were also formed, albeit in minute amounts only (Figure 5).

3.5. Modulation of ethylene, ABA and jasmonates can elicit deoxyharringtonine

Integration of precursor feeding and elicitation is a frequently used strategy to enhance the yield of secondary metabolites in plant cell cultures (Sivanandhan et al., 2014). For example, the yield of L-DOPA from cell cultures of *Mucuna pruriens* could be significantly induced by a combination of feeding the amino acid precursor and elicitation (Rakesh and Praveen, 2022). Likewise, using a combination of elicitation by MeJA and feeding the precursor vindoline in a particular cell strain of *Catharanthus roseus*, it became possible to detect, for the first time in cell culture, the valuable alkaloid vincristine (Raorane et al., 2023). We, therefore, tested various elicitors in combination with feeding the precursors cephalotaxine and L-homoleucine, treating the cells at day 10 after subculture, when the cells were already in their stationary phase, such that proliferation was not affected. While most elicitors did not impair cell growth, we even observed a significant, but transient (only at day 5) increase in fresh weight (by 50%) for β-cyclodextrin (Figure 6A). Since

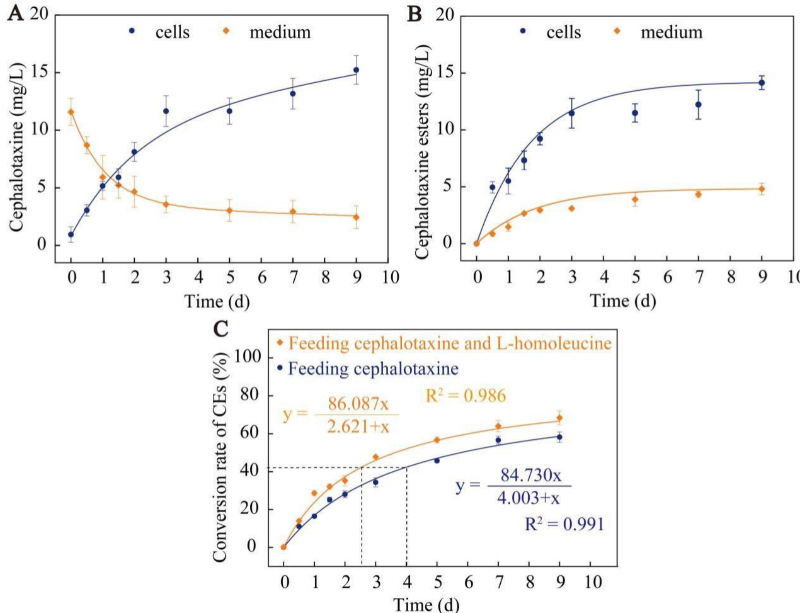

Figure 4. Dynamics of cephalotaxine conversion in the presence of L-homoleucine as determined by UPLC-MS. Both precursors were added at a concentration of 10 mg/L. A. Temporal changes in cephalotaxine levels in the medium and cells. B. Temporal changes in cephalotaxine ester levels in the medium and cells. C. Time course of cephalotaxine conversion into cephalotaxine esters (CEs), either after feeding cephalotaxine alone or in combination with L-homoleucine. Data in A, B, and C represent mean ± SE from three independent experimental series.

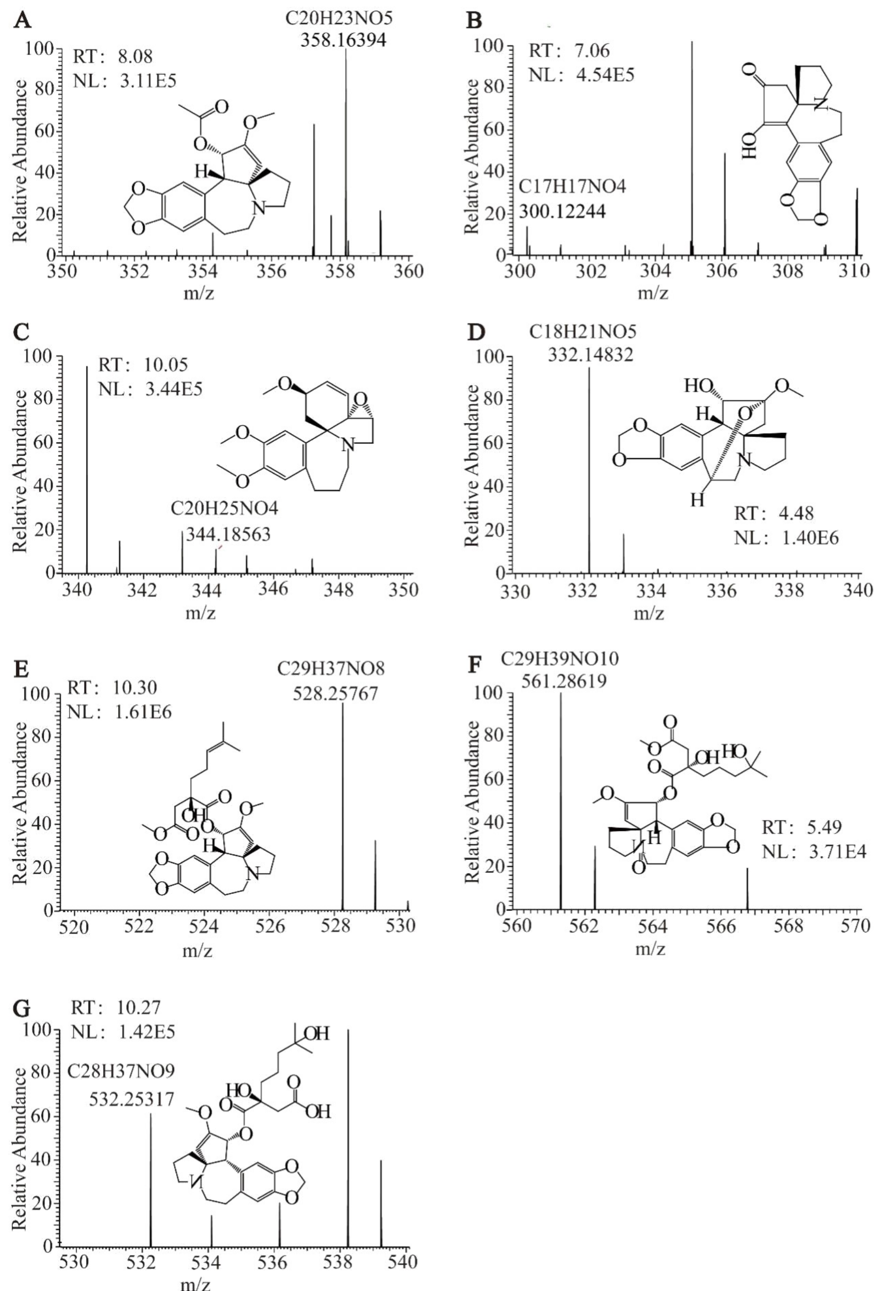

Figure 5. Mass spectra of additional *Cephalotaxus* alkaloids detected by UPLC-MS in cell cultures. A. Extracted ion chromatogram (EIC) from TIC-MS: acetylcephalotaxine, m/z = 358.16394 ([+]-mode, RT = 8.08 min); B. EIC: demethylcephalotaxinone, m/z = 300.12244 ([+]-mode, RT = 7.06 min); C. EIC: 3-epiwilsonine, m/z = 344.18563 ([+]-mode, RT = 10.05 min); D. EIC: drupacine, m/z = 332.14832 ([+]-mode, RT = 4.48 min); E. EIC: dehydrodeoxyhomoharringtonine, m/z = 528.25767 ([+-mode, RT = 10.30 min); F. EIC: homoharringtonine β-N-oxide, m/z = 561.28619 ([+-mode, RT = 5.49 min); G. EIC: 4'-demethyl homoharringtonine, m/z = 532.25317 ([+-mode, RT = 10.27 min).

β-cyclodextrin is known to chelate secondary metabolites (García-Pérez et al., 2019; Su et al., 2020), it might mitigate their negative impact on cell growth.

While at day 5 the total alkaloid content was not stimulated beyond the level of the fed precursor cephalotaxine, contents were induced above the control at day 7, irrespective of the elicitor (Figure 6B). Specifically, for deoxyharringtonine (Figure 6C, Table S2), the highest stimulation compared to the control was observed for ABA and silver nitrate, followed by ethylene. The time-

dependence of elicitation can be easily understood by taking into account that it requires signal transduction, culminating in the induction of biosynthetic genes that need to be translated into proteins, which, in turn, require time to convert significant amounts of the precursor. A similar time-dependence of secondary metabolite production has also been observed in other cases of alkaloid production in cell cultures, such as colchicine and thiocolchicoside accumulation in *Gloriosa superba* (Mahendran et al., 2018). While the elicitors improved the abundance of the cephalotaxine esters, they did not produce any obvious effect on their composition. Specifically, deoxyharringtonine remained the dominant ester (>97%).

The strong accumulation of deoxyharringtonine in response to ABA elicitation (18.6 mg/L) was accompanied by a relatively high level of residual precursor cephalotaxine (4.4 mg/L). Even a complete conversion of the consumed 5.6 mg/L cephalotaxine would not suffice to sustain the observed high level of product. Thus, the elicitation must have mobilized additional cephalotaxine in addition to the exogenous precursor. Elicitation by ABA must, therefore, induce cephalotaxine synthesis de novo (Figure 6B). The fact that both ABA and ethylene induce the accumulation of deoxyharringtonine to similarly high levels (18–19 mg/L), may be related to the integration of stress-related signals in cells. Interestingly, silver nitrate (SN), a well-known inhibitor of ethylene activity classically used in plant tissue culture (Beyer, 1979), has the same effect, which appears paradoxical at first sight. This paradox can be resolved, because ethylene is a negative regulator of ABA, while at low concentrations it stimulates ABA responses (Ghassemian et al., 2000). Silver nitrate removes endogenous ethylene, thereby promoting ABA responses, similar to the effect of elicitation with exogenous ABA. Release of ethylene from ethephone would be expected to impair the ABA response and, thus, impact the accumulation of cephalotaxine esters. However, the opposite is observed. The reason may be that the signal transduction deployed by the two hormones is partially shared, which under long-term treatment can lead to the opposite effect. Treatment with ethephon has

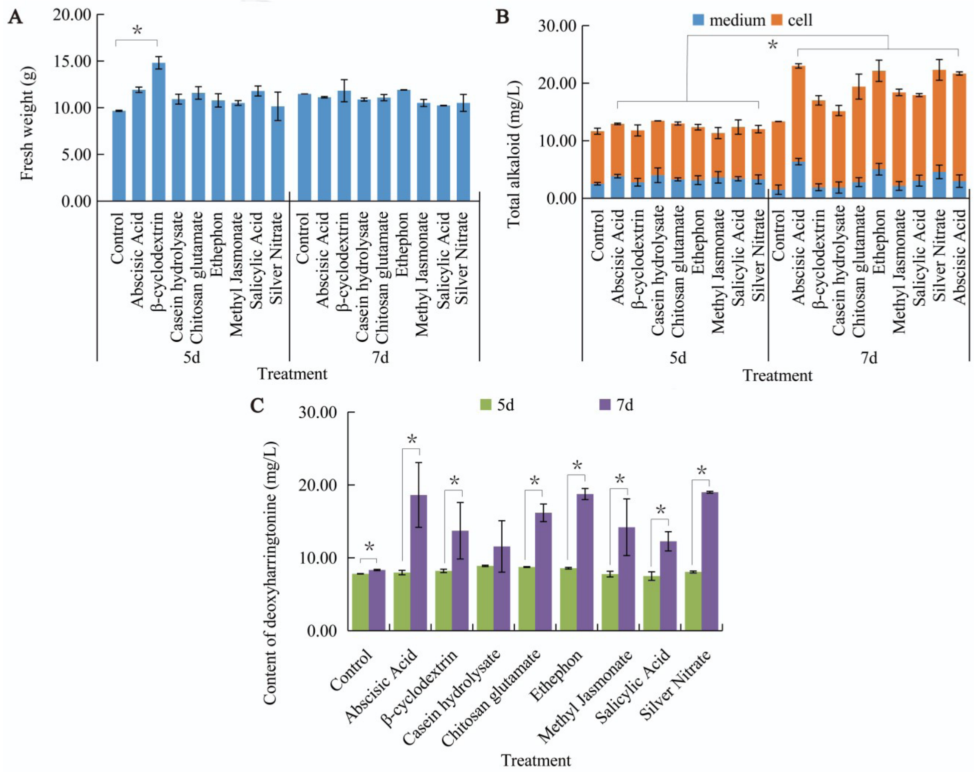

Figure 6. Effect of precursor feeding combined with elicitation on the accumulation of deoxyharringtonine. A. Cell growth in response to different elicitors, indicated by fresh weight. B. Total alkaloid content, including cephalotaxine, harringtonine, homoharingtonine, isoharringtonine, and deoxyharringtonine, measured in both cells and medium using UPLC-MS. Volumes refer to the alkaloids present in either cells or medium from 1 L of culture. C. Deoxyharringtonine levels in cells and medium, measured at day 5 and day 7 after precursor feeding and elicitation. Cephalotaxine and L-homoleucine were added at a concentration of 10 mg/L each, and elicitors were applied at day 10, followed by incubation for 5 or 7 days. "Control" refers to cells treated only with precursors (10 mg/L L-homoleucine and 10 mg/L cephalotaxine) without elicitation. Data points represent mean ± SE from three independent experimental series. * indicates significant difference at $p < 0.05$.

recently been demonstrated to induce the accumulation of ABA (Lee et al., 2021), which also suggests that exogenous ethylene signaling has distinct physiological effects compared to endogenous ethylene signaling.

Suspension-cultured cells of *C. hainanensis* can actively uptake cephalotaxine from the medium into cells (Figure 4A); however, this phenomenon did not occur in the tobacco suspension-cultured cell line bright yellow 2 (BY2) (data not shown). Since cephalotaxine uniquely exists in the plant family Cephalotaxaceae, its uptake may be mediated by an alkaloid-specific transporter. This type of transport could also be located in the tonoplast, where such compartmentalization could minimize autotoxicity. The physiological function of cephalotaxine and its esters on *C. hainanensis* is still unknown. However, the formation of these complex compounds can be elicited. The esters can be detected in both the medium and the cells, but it is unclear whether they are actively secreted into the medium or simply leak from the cells due to cell death after long-term cultivation, or because of changes in cell wall integrity or osmotic conditions following the addition of exogenous elicitors (Iqbal et al., 2024; Matra et al., 2025). The total amount of cephalotaxine in a single Erlenmeyer flask exceeded the amount that had been fed, indicating that cephalotaxine must have been synthesized de novo. This finding will contribute to future work on identifying the enzyme genes and distinctive precursors involved in the biosynthetic pathway, which remains poorly characterized (Qiao et al., 2023).

4. Conclusion

In conclusion, this study demonstrated for the first time that the integration of precursor feeding and different elicitors can promote the biosynthesis of cephalotaxine and cephalotaxine esters in suspension-cultured cells of *Cephalotaxus hainanensis*. It was found that cephalotaxine can be effectively converted into its esters, and the presence of L-homoleucine can promote this conversion. Furthermore, elicitors such as abscisic acid, ethephon, and silver nitrate can even trigger the de novo biosynthesis of cephalotaxine, and their effect strongly depends on the coincubation time.

Availability of data and materials

The data obtained from the present study have been presented in the form of figures and tables, as well as in supplementary files.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

XJ and FQ planned and designed the research. LW, HS, HC, CW, ZX, MMZ, and JS performed the laboratory experiments. LW, MMZ, PN, MFS, and FQ analyzed the data and wrote the paper. All authors read and approved the final manuscript.

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Supplementary file

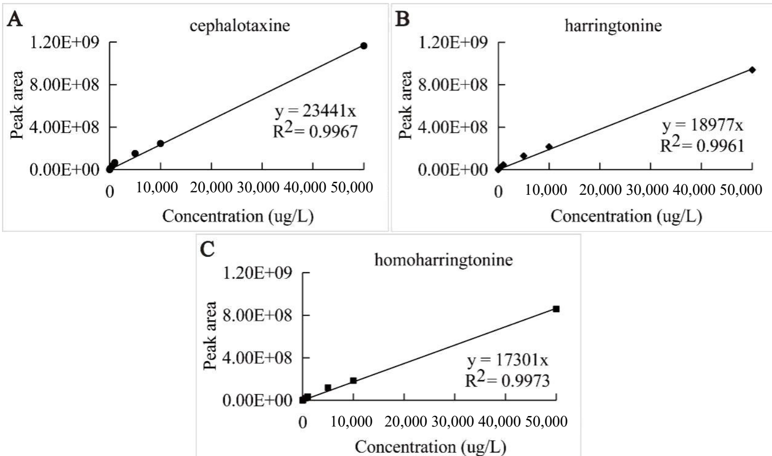

Figure S1. Standard curves of the mixed standards were determined by UPLC-MS. Dissolve 1 mg of cephalotaxine, harringtonine and homoharringtonine standards (Yuanye Bio-Technology Co. Ltd.) in 1 ml of methanol to obtain a mixed standard stock solution with a concentration of 1 mg/ml. Gradient dilution was performed with methanol as the solvent, and the final concentrations of 50,000 μg/L, 10,000 μg/L, 5,000 μg/L, 1,000 μg/L, 500 μg/L, 100 μg/L, 50 μg/L, 10 μg/L, 5 μg/L and 1 μg/L mixed standard solutions were made. Then they were filtered by syringe filter with 0.22 μm pore size before UPLC-MS detection. The qualitative analysis of isocharringtonine and deoxyharringtonine in the samples was estimated by using the harringtonine standard curve in the mixed standards since they are not commercially available.

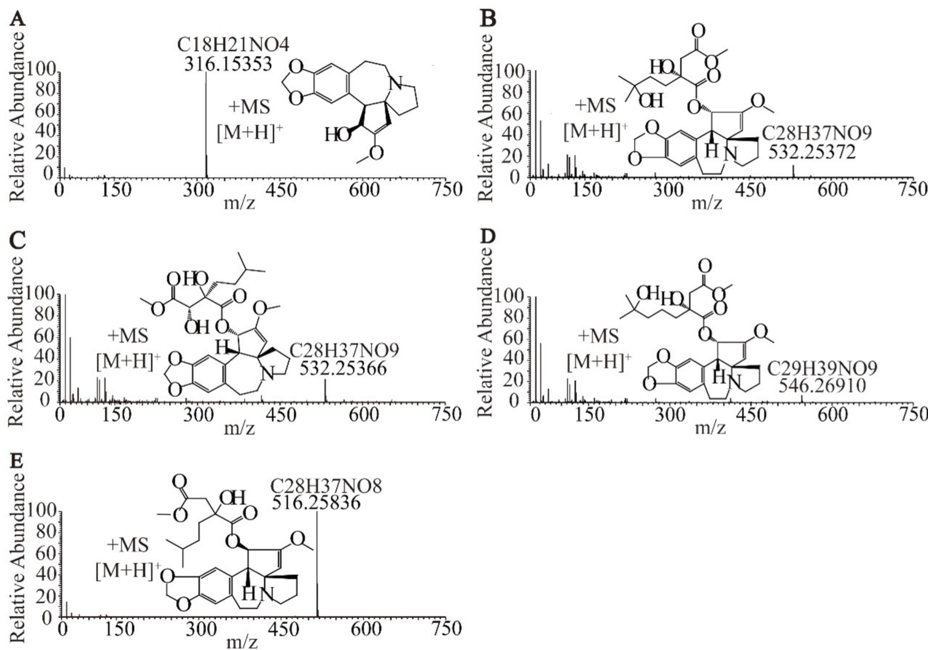

Figure S2. Mass spectra of cephalotaxine, harringtonine, isoharringtonine, homoharringtonine, and deoxyharringtonine detected by UPLC-MS in cell cultures. A. Extracted ion chromatograms (EIC) from TIC-MS: cephalotaxine m/z = 316.15313 ([(+)-mode], RT = 5.66 min); B. EIC from TIC-MS: harringtonine m/z = 530.25372 ([(+)-mode], RT = 7.61 min); C. EIC from TIC-MS: isoharringtonine m/z = 530.25366 ([(+)-mode], RT = 7.90 min); D. EIC from TIC-MS: homoharringtonine m/z = 546.26910 ([(+)-mode], RT = 8.13 min); E. EIC from TIC-MS: deoxyharringtonine m/z = 516.25836 ([(+)-mode], RT = 10.21 min).

Table S1. Contents of cephalotaxine and its esters in leaves of *C. hananensis*.

Tissue	Alkaloids	Content (mg/g dry weight)	Fresh leaves needed for 1 g alkaloid (kg)
Leaves	Cephalotaxine	0.47	7.39
	Harringtonine	0.09	38.19
	Homoharringtonine	0.10	36.07
	Deoxyharringtonine	0.06	58.68
	Isoharringtonine	0.08	41.42

Leaves were harvested from artificially cultivated, 10-year-old trees. The samples were first heated at 105 °C for 30 min and then dried at 60 °C to a constant weight. The dry-to-fresh weight ratio of the leaves was 29.11% (standard error = 0.93%). One gram of leaf powder was ultrasonically extracted with 10 mL of methanol for 45 min (600 W, 40 kHz). After cooling and filtration, an additional 10 mL of methanol was added to the residue for a second ultrasonic extraction. The resulting supernatants were combined, and the solvent was removed using a rotary evaporator at 40 °C. The extracts were dissolved in 10 mL of 0.5% ammonia solution and 20 mL of dichloromethane, and the organic phase was collected using a separatory funnel. This step was repeated 3 times, and the combined organic phases were dried using a rotary evaporator at 40 °C. The final extract was dissolved in 1 mL of methanol and filtered through a 0.22 μm microporous membrane for UPLC-MS analysis.

Table S2. The contents of total alkaloids and deoxyharringtonine in cell cultures incubation for 5 and 7 days.

Treatment	5 days				7 days			
	Total alkaloid		Deoxyharringtonine		Total alkaloid		Deoxyharringtonine	
	mg/L	nmol/flask	mg/L	nmol/flask	mg/L	nmol/flask	mg/L	nmol/flask
Control	11.95	814.41	7.81	454.13	13.34	933.44	8.33	484.41
ABA	11.86	804.15	7.97	463.86	23.00	1448.00	18.62	1083.41
β-CD	12.99	883.50	8.20	477.09	17.01	1061.31	13.71	797.72
CH	12.75	851.63	8.89	517.42	15.13	977.29	11.56	672.51
CG	12.85	861.49	8.74	508.29	19.40	1191.82	16.17	941.03
ETH	12.58	847.74	8.59	498.54	22.15	1353.86	18.74	1090.56
MeJA	11.09	741.45	7.76	451.43	18.39	1177.80	14.19	825.74
SA	12.26	841.93	7.50	436.02	17.92	1211.49	12.26	713.58
SN	11.33	762.53	8.05	468.60	22.31	1357.05	19.00	1105.20

The total alkaloids comprise five *Cephalotaxus* alkaloids: cephalotaxine, harringtonine, isoharringtonine, homoharringtonine, and deoxyharringtonine. The precursor solution included 10 mg/L cephalotaxine and 10 mg/L L-homoleucine.