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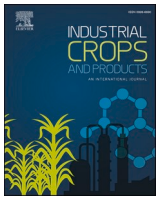

Isolation and identification of components of mother liquor sugar from *Stevia rebaudiana* Bertoni with nematicidal activity

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ABSTRACT

Mother liquor sugar (MLS) is a highly prevalent by-product of processing *Stevia rebaudiana* Bertoni, which is an important cash crop used to prepare a natural sweetener. With an increase in demand for stevia, MLS production has also increased. However, MLS has limited applications owing to its complex sweetness profile and pronounced bitter aftertaste. To explore the potential reuse of MLS, two diterpenoid compounds were gradually separated and identified using polarity-based separation, normal-phase chromatography, reversed-phase chromatography and LH-20 separation followed by an acute toxicity tracking test in *Caenorhabditis elegans*. These compounds were identified as sterebins E and F using high-performance liquid chromatography, mass spectrometry and nuclear magnetic resonance. Sterebins E and F were found to be isomers, and sterebin F exhibited stronger nematicidal activity than sterebin E and fosthiazate at 24 h. The preliminary isolation of sterebin F was achieved via gradient elution using macroporous HPD-100 resin with methanol concentrations of 70 %, 90 % and 100 %. The adsorption process lasted approximately 10 h, and the desorption process was completed in 2 h. To the best of our knowledge, this is the first study to identify specific individual compounds from stevia by tracking their nematicidal activity. The insights gained into the nematicidal properties and isolation process of sterebin F from MLS provide a crucial theoretical and practical foundation for developing eco-friendly pest control solutions and natural alternatives to chemical pesticides in sustainable agriculture.

1. Introduction

Stevia rebaudiana Bertoni is a perennial herbaceous plant of the Asteraceae family. It is widely regarded as one of the world's most important cash crops and is used for the production of natural sweeteners (De Andrade et al., 2024; Saravi et al., 2022). The chemical composition of stevia is complex, primarily comprising steviol glycosides (SGs), flavonoids, diterpenoids and their derivatives, phenols and their derivatives, polysaccharides and volatile oils (Libik-Konieczny et al., 2020; Michael et al., 2016; Miladinova-Georgieva et al., 2023; Molina-Calle et al., 2017; Myint et al., 2020; Oshima et al., 1986, 1988; Schiatti-Sisó et al., 2023; Sharma et al., 2023). These compounds exhibit various biological activities, including anti-diabetic, anti-oxidant, anti-hypertensive, anti-caries, anti-obesity, anti-viral, anti-microbial and anti-tumour properties (Adhikari et al., 2018; Borgo et al., 2021; Chakma et al., 2023; Dias et al., 2021; Fatima, et al., 2023; Hanson and De Oliveira, 1993; Momtazi-Borojeni et al., 2017; Shukla et al., 2013). SGs, extracted from *S. rebaudiana* leaves via water extraction, are the most widely used zero-calorie natural high-intensity sweeteners in the world (Huang et al., 2024; Németh and Jánosi, 2019; Sandra et al., 2020). The global consumption of SG powder is projected to reach 10, 254.93 tonnes by 2027, with an annual growth rate of 7 %–8 % (Ahmad et al., 2020).

Mother liquor sugar (MLS), a by-product of SG extraction, contains approximately 60 % SGs by dry weight, along with other bioactive compounds, including flavonoids and polyphenolics (Xu et al., 2021b). However, MLS does not meet the commercial standards for SG production (Liu et al., 2019). Its complex sweetness profile and bitter aftertaste further hinder its commercial viability and potential applications (Liu et al., 2019; Xu et al., 2021b). With the increase in SG production,

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widespread cultivation of *S. rebaudiana* and accumulation of MLS, the stevia industry faces significant environmental and sustainability challenges.

Currently, overstocking of MLS has become a critical issue in the stevia industry (Liu et al., 2021). Although research has mainly focused on SG recovery, studies focusing on the separation and utilisation of bioactive components of MLS, particularly those with potential nematicidal activity, remain limited (Liu et al., 2019; Liu et al., 2021; Luo et al., 2021; Sun et al., 2024). The extensive utilisation of MLS is further hindered by its complex sweetness profile, the lack of efficient processing technologies and insufficient research on the bioactivity of other compounds. Therefore, developing applications for the bioactive substances in MLS to enhance its commercial value presents a significant challenge.

Plant-parasitic nematodes (PPNs) are major crop pathogens that cause annual global economic losses of approximately USD 358 billion (Oldani et al., 2023; Khanal and Land, 2023). Chemical nematicides, which are known for their rapid effect, high efficacy and well-defined modes of action, remain the primary method for controlling PPNs (Swale, 2019). However, their prolonged use has led to environmental pollution, toxicity to non-target organisms and the emergence of nematode resistance (Desaeger et al., 2017; Huang et al., 2019; Huang et al., 2025; Li et al., 2017; Vanegas et al., 2022; Wang et al., 2024a; Wang et al., 2025), limiting their long-term utility. Therefore, research on natural nematicidal compounds from plants, microorganisms and other natural sources has gained significant momentum. This shift reflects a broader trend towards more sustainable and eco-friendly strategies for controlling PPNs. These natural compounds often offer effective pest management with a minimal risk to non-target organisms and are rapidly biodegraded. Further, PPNs have a lower likelihood of developing resistance to natural nematicidal compounds given their diverse active ingredients and modes of action (Ibrahim et al., 2025). Plant-derived pesticides have gained considerable attention owing to their insecticidal and bactericidal properties, as well as their benefits in regulating plant growth, promoting environmental protection and preventing resistance (Angaye et al., 2024; Saroj et al., 2020; Souto et al., 2021). Thus, there is an urgent need for safer, more sustainable alternatives to chemical nematicides. Natural nematicidal compounds, particularly those with potential for industrial-scale cultivation, may provide promising solutions.

To date, more than 348 plant species from over 81 families have been identified as sources of nematicidal compounds, with the Asteraceae family being a particularly significant source (Aharoni et al., 2005; Mwamula et al., 2022). Key bioactive classes, including alkaloids, terpenes, phenolics, flavonoids and sulfur-containing compounds, are among the secondary metabolites screened for nematicidal activity. Terpenoids, the most structurally diverse class of secondary metabolites, are characterised by multiple isoprene units, which are five-carbon building blocks (Ahmed et al., 2017). Terpenoids can be further subdivided into hemiterpenoids, monoterpenoids, iridoids, sesquiterpenoids, diterpenoids, sesterterpenoids, triterpenoids, tetraterpenoids, polyterpenoids and irregular terpenoids (Ludwiczuk et al., 2017). These terpenoids are also major components of essential oils (EOs) (D'Addabbo and Avato, 2021). Natural products and their residues are rich sources of compounds that contribute not only to agricultural productivity but also to control phytoparasites (Rocha et al., 2022).

Previous studies have identified nematicidal and insecticidal compounds in the leaves and stems of *S. rebaudiana* (Benelli et al., 2020; Ntalli et al., 2020), suggesting that further exploration of MLS derived from *Stevia* leaves could reveal bioactive compounds with targeted efficacy. Notably, the extraction of nematicidal compounds from MLS remains an understudied area. Although terpenoids have been extensively studied for their bioactivity, the specific nematicidal properties of terpenoids from MLS remain unexplored. This makes our investigation into the nematicidal potential of MLS both practical and promising, offering opportunities to discover novel insecticidal compounds. This approach not only promotes the recycling of by-products but also provides a potential solution to the shortage of raw materials.

Caenorhabditis elegans is a widely used model organism for screening nematicides as it shares toxicological mechanisms with PPNs, such as interference with the nervous system, disruption of cell membrane integrity, inhibition of energy metabolism and induction of oxidative stress (Sarri et al., 2024). However, the key difference between *C. elegans* and PPNs is the unique anatomical structures and effector repertoires that plant nematodes utilise for parasitism, which *C. elegans* lacks. This limits the utility of *C. elegans* as a model for studying parasitic processes (Coke et al., 2024). Despite this limitation, *C. elegans* remains valuable for drug development and toxin screening and is ideal for high-throughput screening because of its ease of cultivation and genetic manipulation (Burns et al., 2023; Chen et al., 2020; Giunti et al., 2021; Moya et al., 2022).

This study aimed to isolate and identify nematicidal compounds from MLS, assess their toxicity and establish a scalable purification process. To achieve this, an acute toxicity tracking test was conducted using *C. elegans* as a model organism, and components with nematicidal activity were isolated and identified for further screening. To the best of our knowledge, this is the first study to identify specific individual compounds from stevia. By identifying bioactive compounds with nematicidal properties, this research offers a sustainable alternative to synthetic pesticides, potentially reducing environmental and health risks associated with chemical-based pest control (Han et al., 2024; Xu et al., 2021a). The utilisation of by-products, such as MLS from *S. rebaudiana*, also promotes resource efficiency and waste valorisation, aligning with circular economy principles. In agriculture, these bio-based solutions can enhance soil health and biodiversity, minimising long-term ecological impact (Capanoglu et al., 2022; Dey et al., 2021). Industrially, the development of nematicidal formulations derived from natural sources can drive innovation in green technologies and can support the growing demand for eco-friendly pest management practices (Deguine, et al., 2021; Jiang and Wang, 2025; Xiong et al., 2022; Zhang et al., 2024). This approach could significantly reduce dependency on harmful chemicals, fostering more sustainable agricultural systems globally. This research highlights the potential of exploring the nematicidal properties of MLS, which could offer promising opportunities for discovering novel insecticidal compounds.

2. Materials and methods

2.1. Materials and chemicals

MLS was provided by Shandong Haigen Biotechnology Co., Ltd. (Shandong, China). MLS was obtained as a by-product after the recovery of rebaudioside A (RA) and stevioside (STV) from *Puxing 6* via a process involving water extraction, filtration, macroporous resin extraction and drying. *C. elegans* and *Escherichia coli* OP_{50} were obtained from the Lab of Microbial Secondary Metabolism (College of Life Science, Yangtze University, Jingzhou, China). Analytical-grade chemicals and solvents, including ethyl acetate (batch number: 20221205), n-butanol (batch number: 20230810), methanol (batch number: 20221011), chloroform (batch number: 20230505), dichloromethane (batch number: 20240902) and petroleum ether (batch number: 20220118), were purchased from Shanghai Chemical Reagents Co., Ltd. (Shanghai, China). Silica gel (normal-phase packing, 200–300 mesh, Qingdao Haiyang Chemical Co., batch number: 20230508), C18 silica gel (reversed-phase packing, 40–60 μm, 100 Å, Qingdao Bangkai High-tech Material Co., batch number: PCH2024060432) and Sephadex LH-20 gel (Amersham Biosciences, Little Chalfont, UK, batch number: JS250176) were used for column chromatography (CC).

2.2. C. elegans cultivation and synchronisation

C. elegans was cultivated at 20°C on nematode growth medium

(NGM) plates containing *E. coli* OP_{50} under standard conditions. The nematode eggs were synchronised to obtain L1 larvae and L4-stage nematodes, as described by Calahorro et al. (2021).

2.3. C. elegans mortality assay (24-h exposure)

Synchronised L4-stage nematodes were selected for the experiment. Then, 200 μL of each test substance solution and approximately 30 nematodes were added to a 96-well plate. Wells containing the same volume of solvent-only solution were used as negative controls. The plates were incubated at 20°C in the dark. After 24 h of exposure, the status of the nematodes in each well was determined, and the number of dead nematodes was recorded. Nematode death was determined based on the cessation of pharyngeal pumping, with the nematodes remaining stiff and motionless.

Mortality (%) = (number of dead nematodes/number of tested nematodes) × 100.

2.4. Assessment of body length and head thrash frequency

Body length and head thrash frequency, which are key indicators of nematode growth and development, were recorded using stereoscopic microscopy (Olympus CX31, Olympus Corporation, Japan). Approximately 30 L1 nematodes were transferred into each well, treated with 200 μL of 0, 0.1, 0.3 and 1 mg/mL sterebins E and F and then incubated at 20°C. The nematodes were collected from the 96-well plates on days 1, 2, 3, 5 and 7 post-treatment, washed 2–3 times and subsequently placed on empty NGM plates. Their body length and head thrash frequency (in 30 s) were recorded (Liu et al., 2023). A head thrash was defined as a change in the direction of bending at the midbody.

2.5. Isolation of bioactive compounds from MLS

A total of 500 g of MLS powder was accurately weighed and then added to 2500 mL of water at 25°C. A separation process of the bioactive compounds in the MLS solution was performed (Fig. 1), targeting the fraction that exhibited the highest activity against *C. elegans*.

2.6. Polar solvent extraction of bioactive compounds from MLS

The MLS solution was extracted using solvents of varying polarities, namely, water, ethyl acetate and n-butanol, ranked in descending order of polarity. The MLS solution was first extracted using 30 mL of water-saturated ethyl acetate (ethyl acetate: water = 5:1). The ethyl acetate layer was separated, and the aqueous phase was re-extracted thrice. The water-saturated ethyl acetate layers were then combined and collected. Next, the aqueous phase was extracted with 30 mL of water-saturated n-butanol (n-butanol: water = 5:1). The n-butanol layer was separated, and the aqueous phase was re-extracted thrice. The water-saturated n-butanol layers were then combined and collected, and the aqueous phase was collected. Finally, the three separated layers were individually dried in a rotary evaporator. The resulting solids were individually dissolved in 3 mL of a solvent mixture (methylene chloride: methanol = 1:1) and extracted thrice; the solutions of the three separated layers were combined separately. These solutions were placed in a fume hood for evaporation and drying. Subsequently, the components of the ethyl acetate, n-butanol and water fractions were obtained, dissolved in sterile water and prepared into 0, 10, 30 and 50 mg/mL solutions for the evaluation of acute toxicity.

2.7. Normal-phase chromatography separation

Methanol and ethanol are the most commonly used polar solvents for obtaining plant extracts (Grauso et al., 2020). Herein, methanol was employed as the solvent for chromatographic separation. Silica gel (5 μm, 30 g) was packed into a column (300 mm × 4.6 mm) using the wet packing method and thoroughly rinsed. Samples (10 g) from the ethyl acetate and n-butanol fractions were individually dissolved in methanol, loaded onto the column and eluted using a gradient of solvents (4–5 column volumes) at a flow rate of 3 BV/h. The first gradient elution was performed using chloroform: methanol = 95:5. The second gradient elution was performed using five column volumes of chloroform: methanol: water = 80:20:1.5. The third gradient elution was performed using chloroform: methanol: water = 50:50:5. The fractions collected during gradient elution were separated, evaporated via rotary evaporation and weighed. Subsequently, the obtained solids were dissolved in 5 mL of a solvent mixture (methylene chloride: methanol = 1:1), extracted thrice, combined and dried at 40°C to obtain the final extract.

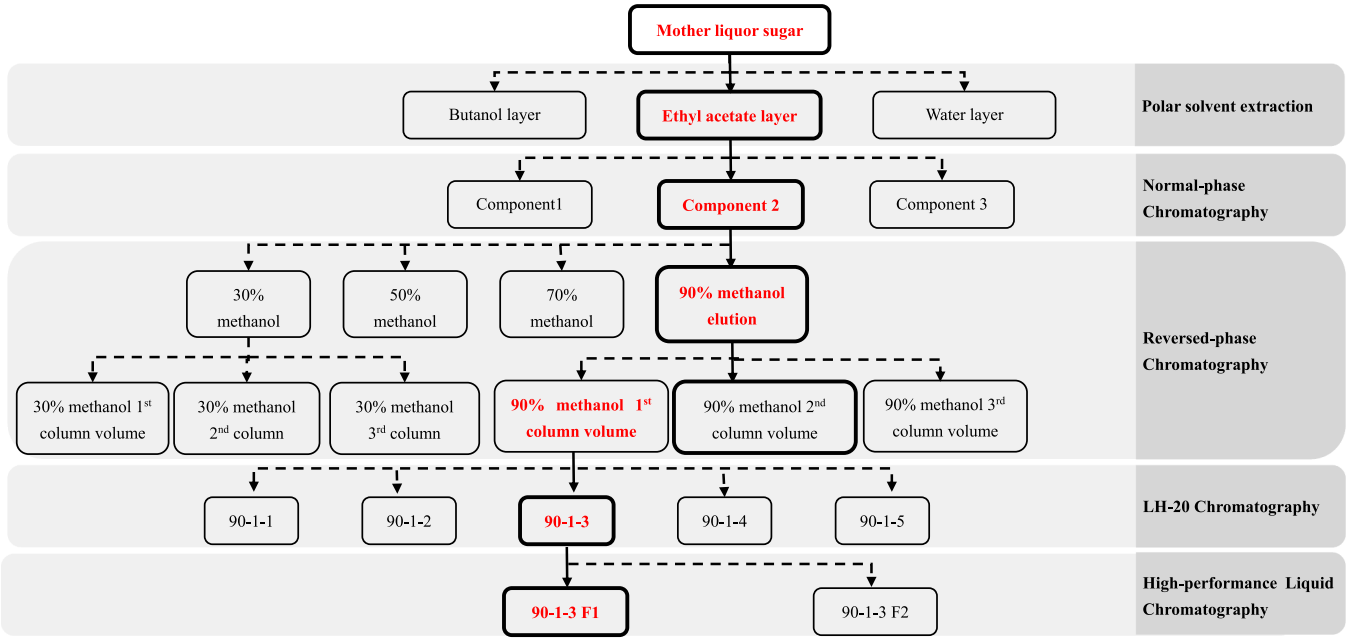

Fig. 1. Isolation of bioactive compounds from MLS.

The components in the n-butanol layer were prepared into 0, 1, 2.14, 4.68 and 10 mg/mL solutions for the evaluation of acute toxicity. The components in the ethyl acetate layer were prepared into 0, 1, 1.26, 1.58 and 2.0 mg/mL solutions for the evaluation of acute toxicity.

2.8. Reversed-phase chromatography separation

The second gradient fraction from the ethyl acetate fraction was dissolved in methanol (20 mg/mL, 250 mL), and reversed-phase liquid chromatography was performed using a C_{18} silica gel (5 μm) column (250 mm × 4.5 mm). Elution was performed using 30 %, 50 %, 70 % and 90 % methanol at a flow rate of 3 BV/h. Each elution was performed for three column volumes, and the fractions were collected. The separated fractions were then evaporated and dried. The collected fractions were assessed for nematicidal activity at concentrations of 0, 1, 3 and 5 mg/mL.

2.9. Sephadex LH-20 CC separation

The sample solutions from the first column volume eluted with 90 % methanol (designated 90–1) were re-dissolved in methanol (20 mg/mL, 25 mL), loaded onto a Sephadex LH-20 column (400 mm × 20 mm, 5 μm) and eluted with anhydrous methanol at a flow rate of 1 mL/min. The total elution volume was approximately 80 mL. The fractions were collected separately, with each fraction collected every 3–4 mL. The fractions were pooled based on similarity and evaporated under reduced pressure at 40°C in a rotary evaporator.

2.10. High-performance liquid chromatography separation

High-performance liquid chromatography (HPLC) analysis was performed using an Agilent 1260 Infinity II system (Agilent Technologies Inc., USA). Chromatographic separation was performed at room temperature on an Eclipse Plus C_{18} column (250 mm × 4.6 mm, 5 μm) following the method described by Cevasco-Contreras et al. (2024). Fraction 90–1–3 (1 mg/mL, 60 mL) was eluted using a gradient of 50 %, 70 %, 90 % and 100 % methanol (*v/v*) at a flow rate of 1.0 mL/min. Two distinct peaks were observed in the HPLC results.

2.11. UHPLC-MS and nuclear magnetic resonance analyses

UHPLC-MS was performed using a Thermo Scientific Dionex Ultimate 3000 system equipped with a Thermo Scientific Acclaim C_{18} column (2.1 × 100 mm, 2.2 μm) (Thermo Fisher Scientific, USA) at Qingdao Stande Testing Co., Ltd. (Qingdao, China). Nuclear magnetic resonance (NMR) data were acquired using a Bruker 600 MHz AVANCE NEO spectrometer (Bruker Physik AG, Germany) at 25 °C in a CD_3OD solvent at the State Key Laboratory of Microbial Technology, Shandong University (Qingdao, China). The samples were prepared by dissolving the dried samples (50 mg) in 100 μL methanol, followed by injection (10 μL) into the instrument. Peaks were identified based on retention time, mass-to-charge ratio (*m/z*) and fragmentation patterns.

2.12. Preparative HPLC separation of sterebin F

Sterebin F was isolated using an UltiMate 3000 HPLC system (Thermo Fisher Scientific Co., Ltd., USA) with a SinoChrom ODS-BP column (250 mm × 10 mm, 5 μm). Separation was performed using a 75 % methanol solution as the mobile phase at a flow rate of 4.0 mL/min. The fractions were collected, evaporated and dried at 40°C to obtain the final extract. These purified fractions were then subjected to further analysis and biological activity testing.

2.13. Macroporous resin pre-treatment

Before use, 20 g of macroporous resin (MRs) were pre-treated following the method described by Liang et al. (2019), with slight modifications. The resins were soaked in anhydrous ethanol for 12 h, followed by washing with anhydrous ethanol and rinsing with distilled water to remove any residual ethanol.

2.14. Standard curve for sterebin F

A standard stock solution of sterebin F with a concentration of 10 μg/mL (purity ≈ 95 %) as obtained and identified in this study was prepared using 100 % methanol. Eight standard solutions (2.5, 5, 10, 20, 60, 130, 250 and 500 μL) were injected into the HPLC system to generate a standard curve according to the corresponding peak areas. The linear regression equation $y = 1173.3x + 17.586$ ($R^2 = 0.9997$, $p < 0.01$; Fig. S1) was derived to determine the concentration of sterebin F.

2.15. Optimisation of MRs for sterebin F enrichment in MLS

The selection of the optimum MR was based on the adsorption and desorption capacities for sterebin F from MLS. To evaluate these capacities, 2 g of pre-treated MRs (listed in Table S1) was placed in a flask and 100 mL of a 200 mg/mL MLS solution was added. The mixture was shaken at 27°C and 120 rpm for 24 h. After shaking, the supernatant was centrifuged and filtered through a 0.45 μm membrane for HPLC analysis. The resins were washed twice with distilled water, followed by desorption in 50 mL of 100 % (*v/v*) methanol at 27°C and 120 rpm for 12 h. The supernatant was then centrifuged, and the adsorption and desorption capacities were calculated according to the method described by Wang et al. (2021).

2.16. Adsorption and desorption kinetics of MLS on HPD-100 resin

To evaluate the adsorption kinetics of MLS on HPD-100 resin, 100 mL of a 200 mg/mL MLS solution was mixed with pre-activated HPD-100 resin in test tubes. The mixture was shaken at 27°C and 120 rpm on a shaker and then transferred into a Buchner funnel and subjected to vacuum filtration (20 μm, 0.07–0.1 MPa). The filtrate was collected at 2, 4, 6, 8, 10, 12 and 24 h. For desorption, 10 mL of anhydrous ethanol solution was added to the adsorbed resin, and the mixture was shaken at 27°C and 120 rpm. The mixture was transferred into a Buchner funnel and subjected to vacuum filtration (20 μm, 0.07–0.1 MPa). The filtrate was collected at 2, 4, 6, 8, 10 and 12 h until a desorption equilibrium was reached. The adsorbed and desorbed quantities of sterebin F per gram of resin were calculated based on HPLC analysis.

2.17. Optimisation of the desorption solvent

Different solvents for desorption were tested, including petroleum ether, ethyl acetate, methylene chloride and methanol. The desorption capacity was evaluated based on the retention times and peak areas of the desorbed samples by HPLC. Methanol was selected as the optimal elution solvent, and desorption efficiency was further optimised by testing methanol concentrations ranging from 50 % to 100 %.

2.18. Statistical analysis

Microsoft Excel 2019 was used to record the experimental data and generate the figures in this study. All experiments were independently repeated thrice, and the results are presented as mean ± standard deviation. DPS 19.05 was used for statistical analysis. If the data met the assumptions of ANOVA, one-way ANOVA was applied, followed by the Student–Newman–Keuls test for multiple comparisons. Welch's test for unequal variances was used when the data did not meet homogeneity of variance assumptions. The data that did not meet the normality assumption were analysed using an independent samples *t*-test with one-sided. A significance level of 0.05 was considered for all statistical

analyses, and results from multiple comparisons are presented using letter markers.

3. Results and discussion

3.1. Isolation and evaluation of the nematicidal activity of bioactive compounds from MLS

The extraction of bioactive compounds is influenced by the polarity of the solvents, which plays a crucial role in the recovery of chemical components (Herrera-Pool et al., 2021; Khan et al., 2023; Kumari et al., 2024; Laquale et al., 2020). Herein, MLS solution was extracted using solvents of varying polarities, namely, water, ethyl acetate and n-butanol, ranked in descending order of polarity. After evaporation, three distinct fractions were obtained: ethyl acetate (3 %), n-butanol (72 %) and water (25 %) fractions (Fig. 2A). In the ethyl acetate fraction, the active components were sequentially eluted in the following proportions: 16 %, 43 % and 41 %. By contrast, the n-butanol fraction showed a distribution of 4 %, 87 % and 9 %, respectively (Fig. 2B).

To evaluate the nematicidal activity of MLS, a 24-h acute toxicity assay was conducted using *C. elegans*. Results indicated that MLS was moderately toxic (Fig. 3A), which is likely attributable to compounds derived from *S. rebaudiana* leaves (Ntalli et al., 2020). The subsequent evaluation of fractions with varying polarities revealed notable differences in their acute toxicity. In particular, treatment with the ethyl acetate fraction resulted in 89 % mortality and that with the n-butanol fraction caused 33 % mortality, both at a concentration of 30 mg/mL (Fig. 3B). These mortality rates were notably higher than the 18 % mortality observed with the original MLS solution at the same concentration (Fig. 3A and 3B), indicating that the ethyl acetate and n-butanol fractions contain more potent toxic components.

Based on these findings, the gradient elution products from the ethyl acetate and n-butanol fractions were further separated using normal-phase chromatography, followed by an assessment of their nematicidal activity. Each of the isolated components exhibited different nematicidal activity (Fig. 3 C and 3D). Notably, the second component derived from the ethyl acetate fraction demonstrated the highest toxicity, with a mortality of approximately 70 % (Fig. 3D).

To further isolate the toxic compounds, the second gradient fraction from the ethyl acetate fraction was subjected to reversed-phase chromatography using 30 %, 50 %, 70 % and 90 % methanol as eluents for three column volumes. The collected fractions were assessed for nematicidal activity at concentrations of 0, 1, 3 and 5 mg/mL. The volume of the fractions eluted with 30 % (the second and third column volumes), 50 % and 70 % methanol were low and they exhibited reduced toxicity, whereas the other fractions displayed stronger toxicity (Fig. S2). The fractions eluted with 90 % methanol (the first and second column volumes) exhibited the highest toxicity, whereas the fractions from the first column volume of 30 % methanol and the third column volume of 90 % methanol showed low toxicity (Fig. 4A). These results suggest that the most toxic substances were concentrated in the first and second column volumes of the 90 % methanol elution. Based on these findings, the fraction from the first column volume eluted with 90 % methanol (designated 90–1) was selected for further purification.

Five distinct fractions (designated 90–1–1, 90–1–2, 90–1–3, 90–1–4 and 90–1–5) were obtained using Sephadex LH-20 chromatography. Among them, the fraction 90–1–3 exhibited significantly higher toxicity compared with the other fractions (Fig. 4B). Notably, the toxicity of the fraction 90–1–3 was similar to that of the parent fraction 90–1, indicating that the toxic components were effectively concentrated in the fraction 90–1–3 during Sephadex LH-20 fractionation (Fig. 4B). This finding suggests that further purification of fraction 90–1–3 could facilitate the isolation of individual toxic compounds with potent nematicidal activity. Consequently, the fraction 90–1–3 was subjected to HPLC for further separation.

Two individual compounds, provisionally designated 90–1–3F1 and 90–1–3F2, were acquired via HPLC isolation. These compounds were eluted at retention times of 37–38 min (Fig. 5A) and 35–36 min (Fig. 5B), respectively, using gradient elution with 40 %–100 % methanol at a detection wavelength of 230 nm. The purity levels of 90–1–3F1 and 90–1–3F2 were approximately 95 % and 91 %, respectively, confirming successful isolation. Considering the well-documented nematicidal activities of fosthiazate and avermectin (Li et al., 2020; Liu et al., 2023; Liu et al., 2024; Zhang et al., 2022a), these two compounds were used as positive controls for assessing the toxicity of the isolated substances. Toxicity assays revealed that both 90–1–3F1 and 90–1–3F2 exhibited nematicidal activity, with 90–1–3F1 demonstrating higher toxicity (Fig. 5C). In particular, 90–1–3F1 had an LC_{50} value of 1.57 at 24 h, which was significantly lower than that of fosthiazate but higher than that of avermectin (Fig. 5C). These results highlight the potential of 90–1–3F1 as a promising candidate for nematicidal applications.

3.2. Structural identification of sterebins E and F

The structures of 90–1–3F1 and 90–1–3F2 were determined using MS and NMR analyses. 90–1–3F1 was isolated as a white powder, which turned brownish-yellow upon treatment with 10 % H_2SO_4–EtOH. Based on the MS and $^{1\ 3}C$ NMR spectra (Fig. 6A and S3–S8 and Table 1), the molecular formula of 90–1–3F1 was determined to be $C_{20}H_{34}O_4$ (*m/z* 303.21 [M-2H_2O+1] +), with a degree of unsaturation of 4. The $^{1\ 3}C$ NMR data (150 MHz, CD_3OD) were as follows: δ 42.3 (t, C-1), 19.3 (t, C-2), 45.1 (t, C-3), 34.9 (s, C-4), 58.8 (d, C-5), 73.0 (d, C-6), 85.8 (d, C-7), 76.5 (s, C-8), 65.4 (t, C-9), 39.0 (t, C-10), 128.2 (t, C-11), 132.9 (t, C-12), 135.7 (s, C-13), 128.6 (t, C-14), 58.5 (d, C-15), 20.9 (q, C-16), 19.3 (q, C-17), 37.0 (q, C-18), 22.5 (q, C-19) and 17.8 (q, C-20). The obtained spectral data were consistent with previously reported values (Oshima

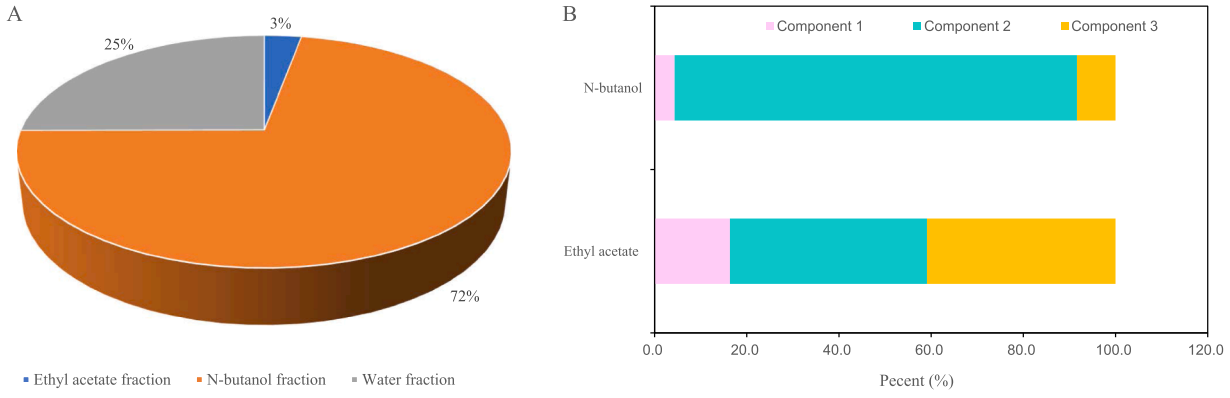

Fig. 2. The content of three distinct fractions (the ethyl acetate, n-butanol and water fractions) from MLS (A), Contents of different active components in the ethyl acetate and n-butanol fractions (B).

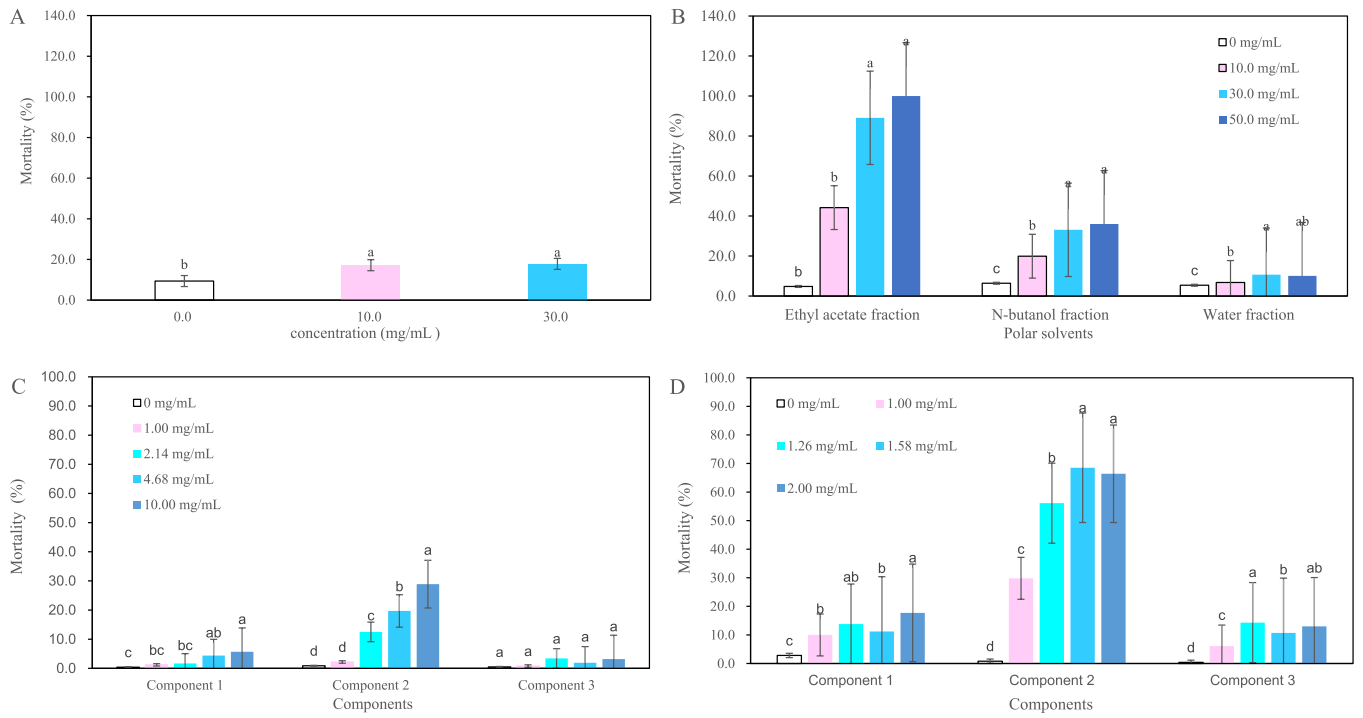

Fig. 3. 24 h acute toxicity assay for different concentration of MLS on *C. elegans* (A), 24 h acute toxicity assay for three polar fractions (the ethyl acetate, n-butanol and water fractions) from MLS (B), 24 h acute toxicity assay for components in n-butanol layer (C), 24 h acute toxicity assay for components in ethyl acetate layer (D). Note: In Fig. 3B, the statistical analysis of water fraction between different concentration was performed using an independent samples *t*-test with one-sided. In Fig. 3 C, the statistical analysis of component 3 between different concentration in n-butanol layer was performed using Welch's *t*-test. The statistical analysis of others was performed using ANOVA with SNK for multiple comparisons.

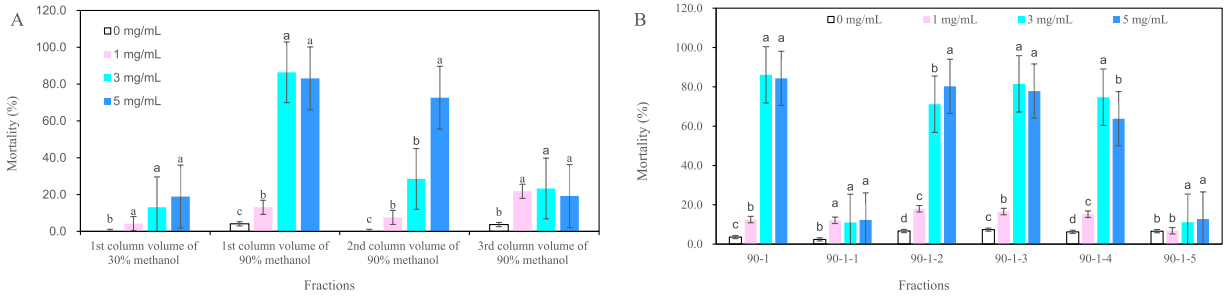

Fig. 4. 24 h acute toxicity assay for different column volumes and methanol concentration from the second gradient eluent of ethyl acetate by reversed-phase chromatography (A), 24 h acute toxicity assay for 90–1 separated tubes by Sephadex LH-20 (B). Note: In Fig. 4 A, the statistical analysis of different column volumes and methanol concentration was performed using an independent samples *t*-test with one-sided. In Fig. 4B, the statistical analysis of separated tubes by Sephadex LH-20 between different concentration was performed using an independent samples *t*-test with one-sided.

et al., 1988), confirming the identity of 90–1–3F1 as sterebin F.

In addition, 90–1–3F2 was isolated as a white powder, which turned brownish-yellow upon treatment with 10 % H_2SO_4–EtOH. Its MS and ^{1} ^{3}C NMR spectra (Fig. 6B and S9–S14 and Table 1) revealed the same molecular formula, $C_{20}H_{34}O_4$ (*m/z* 303.21 [M-2H$_2$O+1] +), as that of 90–1–3F1. Similar to 90–1–3F1, 90–1–3F2 contained a conjugated diene system. A comparison of the NMR spectra indicated that the two compounds were isomers with the same parent structure, differing primarily in the configuration of their double bonds. Notable differences were observed in the chemical shifts of H-11, H-12, H-14 and H-15 in the C-9 side chain: δH 6.13 (1 H, d, J = 15.3 Hz), 5.70 (1 H, dd, J = 15.3, 9.9 Hz), 5.56 (1 H, dd, J = 6.8 Hz), 4.20 (1 H, dd, J = 6.8, 2.5 Hz) and 1.81 (3 H, s). In addition, shifts in the signals for the C-16 methyl group and C-12 olefinic carbon (ΔΔδ, −8.1 and +7.8 ppm, respectively) further confirmed the changes in the double-bond configurations. In particular, the two double bonds in 90–1–3F2 were identified as 11E and 13E. This evidence confirmed that 90–1–3F2 is an isomer of 90–1–3F1 at C-13. Based on these findings and in accordance with the physicochemical properties and NMR data reported in the literature (Oshima et al., 1988), 90–1–3F2 was identified as sterebin E.

Sterebins E and F are isomers, both containing a conjugated diene system and classified as diterpenoids. Labdane diterpenoids are well known for their diverse biological activities, including anti-inflammatory, anti-cancer, cytotoxic, anti-lipoxygenase and herbicidal properties (Marcos et al., 2012). However, the bioactivity of sterebins has not yet been extensively explored.

3.3. *Nematicidal activity of sterebins E and F*

Sterebins E and F, two diterpenoids isolated from MLS, exhibited nematicidal activity (Fig. 5C). To evaluate their nematicidal effects, nematode body length and head thrash frequency were measured at 1, 2, 3, 5 and 7 days post-treatment with sterebins E and F. The nematode body length increased significantly over time, but was notably reduced

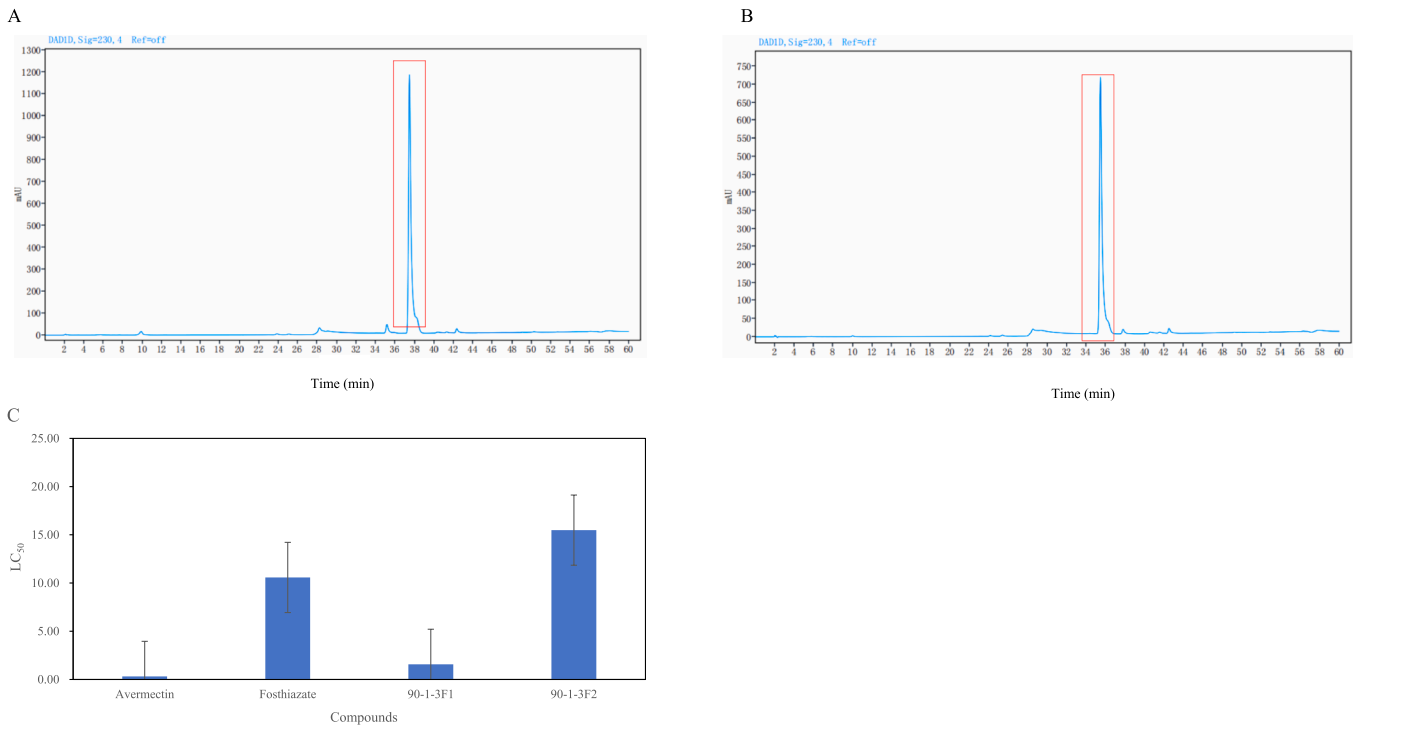

Fig. 5. Separation and toxicity assay for 90–1–3 F1 and 90–1–3 F2. HLPC analysis of 90–1–3 F1 under 230 nm(A), HLPC analysis of 90–1–3 F2 under 230 nm(B), LC_{50} value of 90–1–3F1 and 90–1–3F2 at 24 h (C).

as the treatment concentration increased (Fig. 6C and 6D). The body lengths of the nematodes treated with sterebin F were shorter than those of the nematodes treated with sterebin E. As the exposure period increased, head thrash frequency decreased (Fig. 6E and 6F). Compared with the control group (CK), both sterebins E and F treatments resulted in a notable reduction in head thrash frequency, with this effect being more pronounced at higher concentrations. This indicates a marked inhibition of nematode head thrash. Notably, the inhibition of head thrash by sterebin F was more significant than that by sterebin E, suggesting that sterebin F exhibited stronger toxicity towards the nematodes. In summary, these results highlight the higher nematicidal activity of sterebin F.

The differences in nematicidal activity between sterebins E and F are likely due to variations in the configuration of their double bonds. Nematicidal activity in certain alkyl alcohols and enols has been shown to correlate with factors such as the length of the carbon chain or the position of the ethylenic bond (Chen and Song, 2021). The mortality of pinewood nematodes (PWNs) caused by 25 linear and cyclic pure compounds in EOs and extracts has been strongly linked to specific functional groups, isomerism or the length of the carbon chain (Faria et al., 2021). Isomers such as carvacrol and thymol exhibit similarly high nematicidal toxicity (D'Addabbo et al., 2021). Significant variations in bioactivity, ranging from 3.37 to 1578 times, have been observed among the four stereoisomers of isopyrazam (Wang et al., 2024b). The geometric isomers of citral, neral and geranial show different potencies against *Trichophyton rubrum*, with geranial (trans-citral) demonstrating considerably stronger activity than neral (cis-citral) (Zheng et al., 2021). Menaquinone-7 (MK-7) exists in cis, trans and cis/trans isomeric forms, but only the all-trans form has biological significance (Lal and Berenjian, 2020). The unnatural stereoisomer (+)-trans-cannabidiol (CBD) and its derivatives show considerable therapeutic potential for treating epileptic seizure disorders compared with the corresponding (−)-trans isomers (Rao et al., 2024). Trans-2-hexenal has been shown to influence nutrient metabolism and digestive enzyme activity in PWNs, significantly enhancing glutathione S-transferase activity and disrupting normal metabolic processes (Miao et al., 2012; Zhao et al., 2017). Moreover, trans-2-hexenal exhibits inhibitory effects against *Heterodera avenae* and *Meloidogyne incognita* (Saroj et al., 2020). The position and length of the double bond in aldehydes have been shown to directly affect their nematicidal activity (Seo et al., 2010). In addition, alterations in the positions of the alkenyl, epoxy and ketone groups have been reported to influence nematicidal activity (Kimbaris et al., 2017). The stereochemical configuration, presence of unsaturated double bonds and position of the epoxy groups are critical factors that affect the nematicidal properties of these compounds (Ntalli et al., 2010; Caboni et al., 2013).

Herein, the cis-olefin structure of sterebin F demonstrated superior nematicidal activity, with an LC_{50} value of 1.57, which is approximately one-eighth of that of sterebin E (LC_{50} = 15.5) at 24 h. These results suggest that chirality may play a role in modulating nematicidal activity. However, the underlying mechanism requires further investigation.

Fosthiazate, a typical commercial organophosphorus nematicide, primarily acts as an acetylcholinesterase inhibitor (Li et al., 2020). Herein, it was used as a reference to assess the potential application of sterebins E and F. Research indicates that the toxicity of the natural terpenoid compounds to nematodes, bacteria and microbial systems is influenced by the type and position of the functional groups in their molecular structure. The nematicidal activity of the terpenoid compounds is closely associated with the presence of oxygen-containing functional groups (such as aldehyde, ketone or alcohol) and the existence of double-bond systems promotes biological processes involving electron transfer, thereby enhancing the reactivity of terpenoids towards nematodes (D'Addabbo and Avato, 2021). Both sterebins E and F contain aromatic rings, hydroxyl groups and olefinic side chains that may disrupt cell membrane integrity. By binding to proteins and cell membranes, these structures interfere with metabolic enzymes in nematode cells, affecting energy production or disrupting the anti-oxidant system, leading to oxidative stress and subsequent cell damage (Álvarez-Martínez et al., 2021). The olefinic double-bond chain may interact with neurotransmitters or receptors, disrupting nerve signal transmission (Tamfu et al., 2021). The difference between the bioactivities of sterebins E and F is primarily attributed to stereochemical

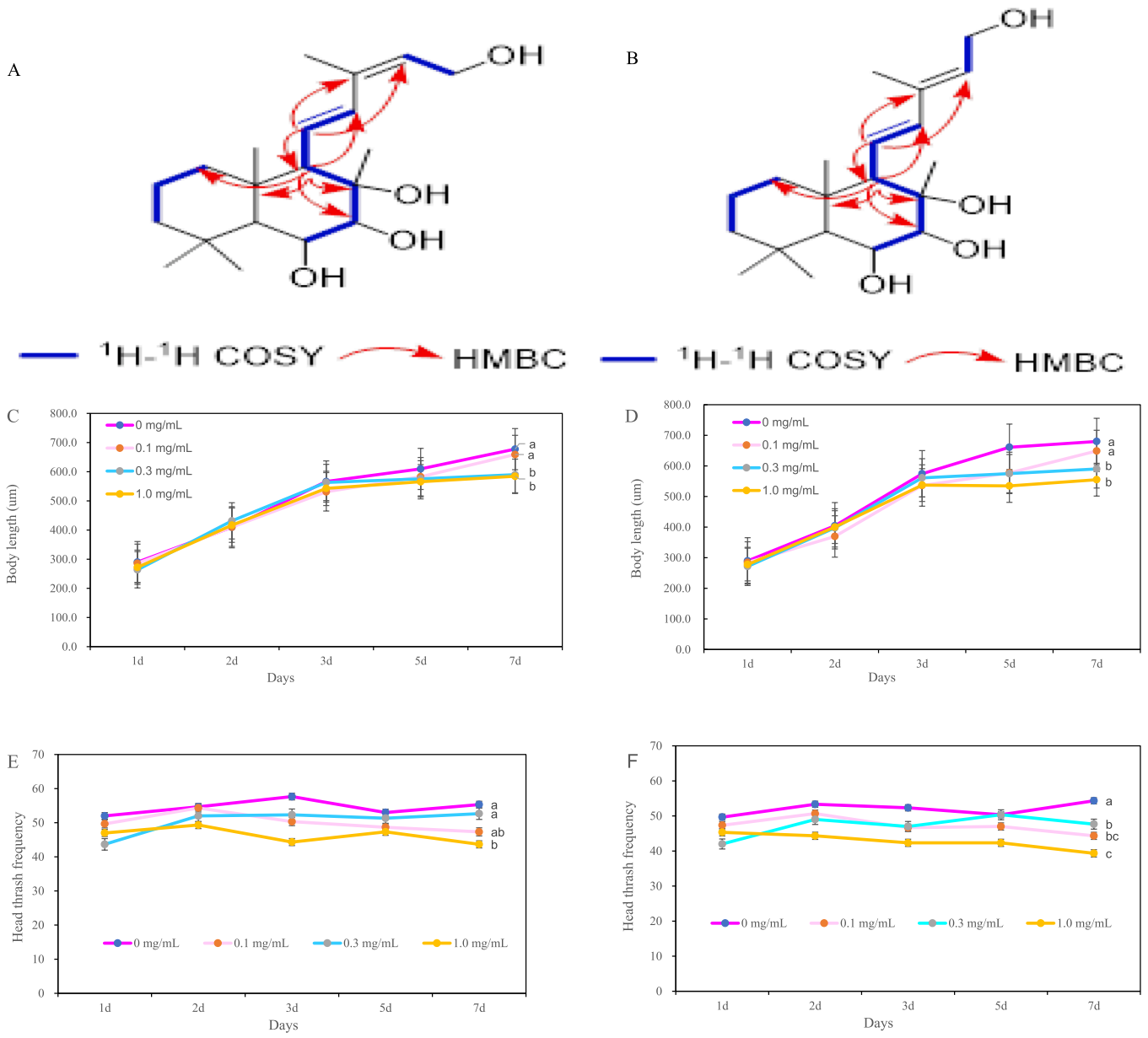

Fig. 6. Structures and toxin assay of sterebins E and F. Structures of 1H-1H COSY and the key HMBC correlations of sterebins F (A) and E (B), Body length at 1, 2, 3, 5, and 7 days post-treatment with sterebin E (C) and sterebin F (D), Head thrashes frequency at 1, 2, 3, 5, and 7 days post-treatment with sterebin E (E) and sterebin F (F).

differences, with the cis-olefin structure of F possibly being biologically more active. The specific mechanisms of the biological activity require further investigation.

The nematicidal activity of sterebin F against *C. elegans* has been identified, and variability among nematode species limits the generalisability of the findings. Therefore, future studies should focus on specific parasitic nematodes. However, toxicity assays against root-knot nematodes are currently being conducted by our group. However, its potential activities, such as anti-diabetic, anti-oxidant, anti-hypertensive, anti-caries, anti-obesity, anti-viral, anti-microbial and anti-tumour properties, remain to be further investigated.

3.4. MR optimisation for sterebin F purification

Sterebin F is a potential natural alternative to chemical pesticides used in sustainable agriculture. In this context, we first explored its extraction process. Extraction methods play a critical role because the bioactivity of the resulting products can vary depending on the technique employed (Kovačević et al., 2018; Latridis et al., 2022; Rafi et al., 2020). MR chromatography is particularly advantageous for industrial applications because of its low cost, high stability, scalability, ease of regeneration and absence of secondary pollution (Shen et al., 2022; Yang et al., 2022; Zhang et al., 2022b). This method has been widely used for separating and enriching bioactive natural products (Chen et al., 2019; Jiang et al., 2020; Liu et al., 2016; Song et al., 2022; Wu et al., 2016; Zhu et al., 2018). Herein, MR chromatography was employed to isolate and concentrate sterebin F for industrial use.

The optimal MR was selected based on an evaluation of the adsorption and desorption capacities of 20 resins with varying polarities and pore sizes (Table S1). Results indicated that the adsorption capacities of sterebin F were not significantly different among most of the resins tested. However, the non-polar resins, namely, HPD-100, HZ-816, X5 and DM1180S, demonstrated superior enrichment effects on sterebin F compared with the other resins (Fig. 7A). This could be attributed to

Table 1
1H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectroscopic data for 90–1-3 F1 and 90–1-3 F2 in CD_3OD.

90–1-3 F1			90–1-3 F2		
No.	**δ_H (*J* in Hz)**	**δ_C**	**No.**	**δ_H (*J* in Hz)**	**δ_C**
1	1.36, m; 0.84, td (13.0,12.6,3.5)	42.3	1	1.36, m; 0.84, td (13.0,12.6,3.5)	42.3
2	1.59, qt (14.8,3.6); 1.36, m	19.3	2	1.59, qt (14.8,3.6); 1.36, m	19.3
3	1.36, m; 1.23, m	45.1	3	1.36, m; 1.23, m	45.1
4		34.9	4		34.9
5	1.11, d (11.0 z)	58.8	5	1.11, d (11.0 z)	58.8
6	3.63, dd (11.0, 9.4)	73.0	6	3.63, dd (11.0, 9.4)	73.0
7	3.31, s	85.8	7	3.31, s	85.8
8		76.5	8		76.5
9	1.85, d (11.0)	65.4	9	1.85, d (11.0)	65.4
10		39.0	10		39.1
11	5.77, dd (7.3, 6.3, 1.2)	128.2	11	5.70, dd (15.3, 9.9)	125.5
12	6.48, d (15.3)	132.9	12	6.13, d (15.3)	140.7
13		135.7	13		136.7
14	5.47, dd (15.3, 10.0)	128.6	14	5.56, dd (6.8)	130.5
15	4.23, d (6.5)	58.5	15	4.20, dd (6.8, 2.5)	59.4
16	1.87, s	20.9	16	1.81, s	12.8
17	1.19, s	19.3	17	1.19, s	19.3
18	1.17, s	37.0	18	1.17, s	37.0
19	1.03, s	22.5	19	1.03, s	22.5
20	1.01, s	17.8	20	1.01, s	17.8

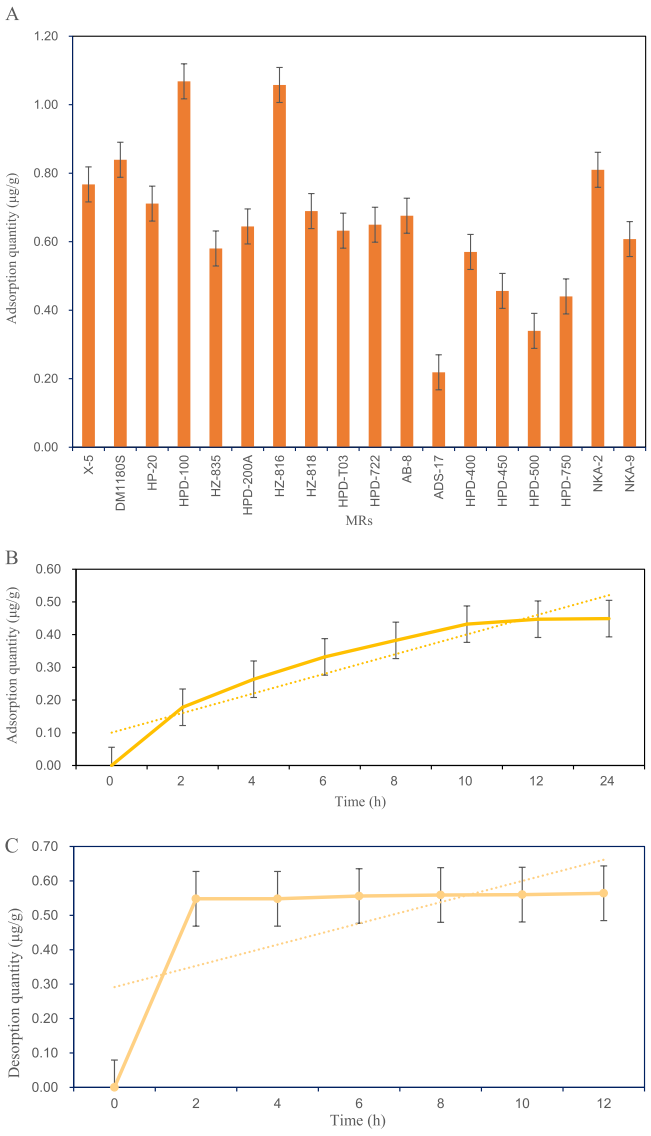

Fig. 7. Adsorption and desorption of macroporous resin for sterebin F. Adsorption quantity (μg per gram MR) of the 20 types of macroporous resins assessed (A), Adsorption curve of HPD-100 over time (B), Desorption curve of HPD-100 over time (C).

the strong binding of sterebin F via hydrophobic interactions, van der Waals forces and potential π–π stacking interactions between the naphthalene rings of sterebin F and the styrene-based aromatic groups of the resin (Liu et al., 2019; Pan et al., 2003; Suresh et al., 2012). Furthermore, it is possible that other resins may exhibit excellent adsorption capacities under different experimental conditions. Desorption capacity should also be considered. By comparing the adsorption and desorption ratios of sterebin F on the various resins, HPD-100 was identified as the most suitable resin for the purification of sterebin F among the tested options (Table S2). From an economic perspective and scalability, HPD-100 was selected as the preliminary enrichment resin, consistent with its use in the adsorption of notoginsenoside Fc from *Panax notoginseng* leaves (Guo et al., 2023).

Studying adsorption and desorption kinetics can provides insights such as rate changes, equilibrium time and overall process dynamics over time. Adsorption and desorption curves were obtained based on experimental data. As shown in Fig. 7B, the adsorption quantity increased over time, with a rapid increase observed during the initial phase. The adsorption curve plateaued after 10 h, indicating that saturation had nearly been achieved, after which the adsorption rate slowed significantly. Beyond this point, even with prolonged exposure, the adsorption of sterebin F on HPD-100 resin approached equilibrium because most of the resin's binding sites had been occupied. Therefore, the equilibrium time for the adsorption of sterebin F on HPD-100 resin in aqueous solution was determined to be 10 h. As shown in Fig. 7C, the maximum desorption quantity of sterebin F was observed at 2 h, after which the desorption rates stabilised. Thus, the equilibrium time for the desorption of sterebin F on HPD-100 resin in aqueous solution was determined to be 2 h.

The polarity of the elution solvent plays a crucial role in both enrichment and purification processes (Guo et al., 2023). Commonly used solvents with varying polarities, including petroleum ether (non-polar), ethyl acetate (moderately polar), dichloromethane (moderately polar) and methanol (polar), were tested in static desorption assays. The HPLC results showed that petroleum ether could not elute sterebin F and methanol and that dichloromethane and ethyl acetate could elute sterebin F from the MR (Fig. S15). Among these solvents, methanol yielded fewer solvent peaks compared with the other two, leading to a higher content of sterebin F with minimal impurities (Fig. S15 and Fig. 8). In our experiments, methanol was found to be the most effective solvent for the purification and desorption of sterebin F from MLS. This is likely because sterebin F, a Labdane-type diterpenoid (Jyoti et al., 2018), contains multiple hydroxyl groups and isopentenyl alcohol units (Fig. 6A), which weaken the intermolecular forces between the resin and sterebin F, thereby facilitating its entry into the methanol

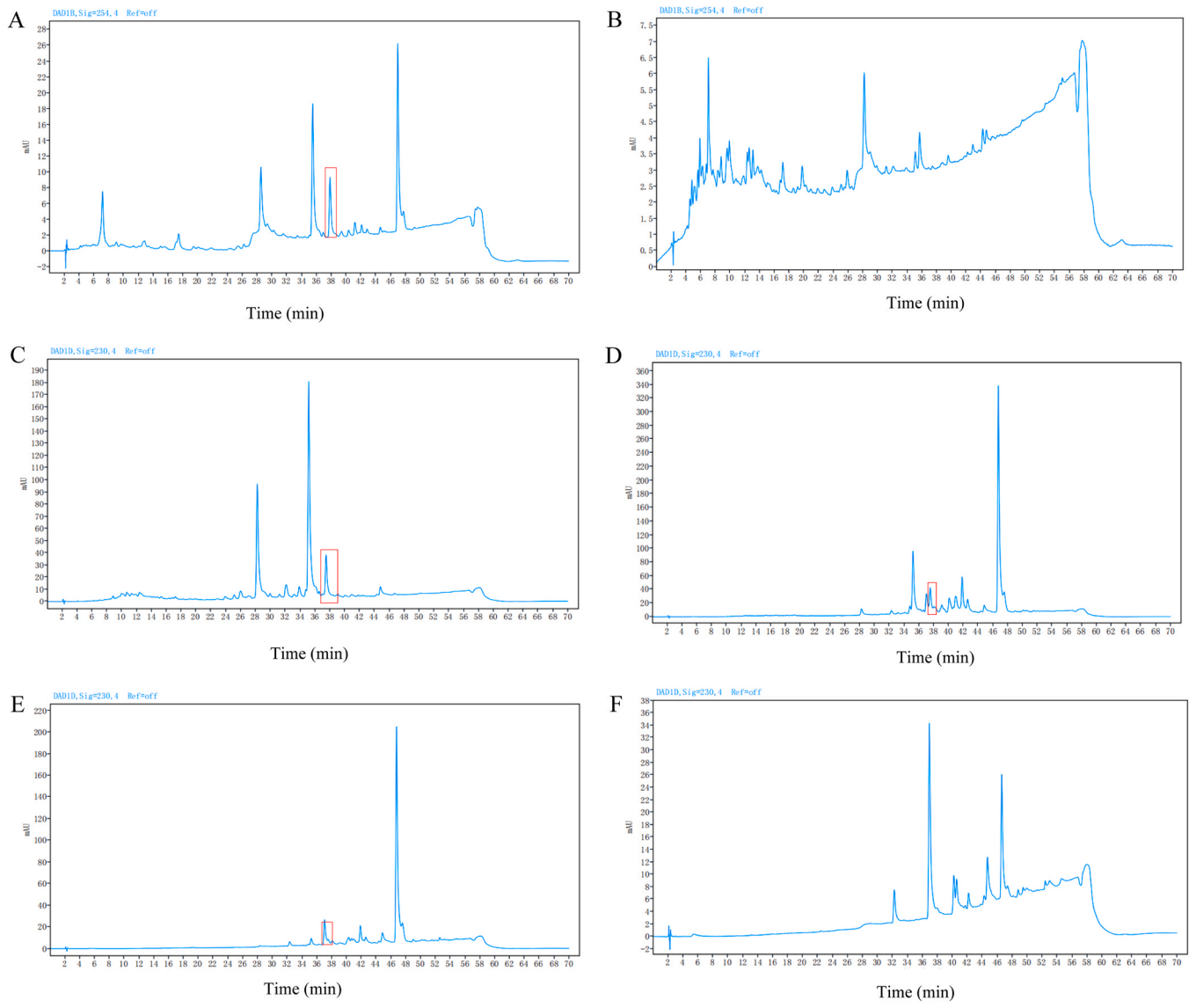

Fig. 8. Methanol concentration of desorption elution for sterebin F by HPLC at 230 nm. HPLC analysis with direct 100 % methanol desorption (A), HPLC analysis of 1st column volume with 50 % methanol desorption (B), HPLC analysis of 1st column volume with 70 % methanol desorption (C), HPLC analysis of 1st column volume with 90 % methanol desorption (D), HPLC analysis of 2nd column volume with 90 % methanol desorption (E), HPLC analysis of 1st column volume with 100 % methanol desorption (F).

solution. Therefore, methanol was selected as the optimal eluent based on its superior elution efficiency and purity profile. The concentration of the solvent is a critical factor in impurity removal (Yang et al., 2018). As shown in Fig. 8 and Table S3, the dynamic desorption ratio increased with gradient elution using 50 %, 70 %, 90 % and 100 % methanol, resulting in an increase in sterebin F purity from 3 % to 33 %.

Adsorptive MRs are employed to separate and enrich bioactive compounds using mechanisms such as electrostatic interactions, hydrogen bonding, complex formation and size-exclusion effects between resins and natural products in solution (Zhang et al., 2018; Zhou et al., 2023). As a result, MRs are widely used to isolate and concentrate bioactive compounds with similar polarities and structures, such as vitexin and isovitexin (Fu et al., 2007), flavonoids (Wang et al., 2019) and baicalin and wogonoside (Du et al., 2012). Although sterebins E and F are isomers, their retention times differ, and sterebin F can be effectively isolated using MR. Liu et al. (2019) removed high-polarity impurities using 25 % ethanol, followed by 100 % ethanol to recover SG with high purity from non-polar MR from MLS. Based on this approach, we propose a scalable separation strategy that is driven by polarity principles. By utilising methanol solutions of varying concentrations and optimising the process design, high-polarity impurities can be effectively removed, SG can be recovered as sweeteners and natural insecticidal components, such as sterebin F, can be separated and collected. Further detailed experimental design and process optimisation will be undertaken in subsequent studies. This strategy not only enhances the economic value of MLS but also aligns with sustainable and green production principles.

The overstocking of MLS would provide an adequate supply of raw materials for the production of sterebin F. In our experiment, sterebin F appeared stable in a dry and sealed environment. However, additional studies on its compatibility and storage, especially as a biological insecticide, are needed. Currently, the purity of sterebin F obtained via MR separation is low, and preparative liquid chromatography typically achieves purities exceeding 90 %. Owing to the inherent complexity of the MR purification process, further refinement is required for large-scale production. The preliminary development of an MR separation and enrichment process for sterebin F from MLS can significantly enhance production efficiency and cost-effectiveness, offering a

promising strategy for industrial-scale production.

3.5. Efficacy and challenges of sterebin F as a natural nematicide

Extracts from neem, *Chenopodium* species and orange oil have demonstrated the feasibility of using natural compounds as terpene-based biopesticides in agricultural practices (Ninkuu et al., 2021). Biopesticides act via mechanisms such as multi-site inhibition, neuromuscular disruption, metabolic interference, growth regulation and intestinal disruption, effectively reducing pest populations (Khursheed et al., 2022). Sterebin F shows superior 24-h nematicidal activity compared to fosthiazate, highlighting its potential as a safer and more efficient alternative to traditional chemical nematicides. With it being derived from stevia by-products, it aligns with circular economy principles, reducing production costs and supporting sustainable agriculture.

However, natural active compounds face challenges such as rapid degradation, requiring frequent applications and stable formulations, which increase costs and lead to inconsistent field performance (Srivastava et al., 2020). Despite these limitations, the eco-friendly nature, unique composition and modes of action of biopesticides hold significant promise. Future efforts should focus on multi-target testing, field validation, formulation optimisation and industrial-scale development of these active compounds to enhance their stability, efficacy and practical applicability, paving the way for broader adoption in sustainable pest management and agricultural innovation (Khursheed et al., 2022).

Despite the promising results obtained in this study, several limitations need to be acknowledged. Although the sample size used for bioassays was sufficient to demonstrate significant activity, it may limit the generalisability of the findings to various nematode species. In addition, potential biases during the compound isolation and purification processes may have influenced the relative activity levels of sterebins E and F. Furthermore, this study did not comprehensively evaluate the stability of compounds under various environmental conditions (e.g. light, temperature and pH), which could affect their feasibility and efficacy in field applications. Future studies should focus on increasing sample sizes, optimising isolation methods, thoroughly assessing compound stability and incorporating additional control experiments to validate and expand upon these findings.

4. Conclusion

We successfully identified and characterised highly active diterpenoids, namely, sterebins E and F, from stevia MLS using an activity-guided screening method targeting *C. elegans*. This is the first report of a specific compound with significant nematicidal activity isolated from stevia. Further, the cis-olefin structure at the C-13 position of sterebin F exhibited greater nematicidal activity than that of the trans structure of sterebin E. HPD-100 resin, coupled with gradient elution using 70 %, 90 % and 100 % methanol, proved effective for separating sterebin F isomers from MLS. Saturated adsorption was achieved in 10 h, and the desorption process reached equilibrium after 2 h. We have laid the foundation for providing a more economical, effective and environment-friendly separation process. However, challenges in scalable production, including sustainable sourcing and minimising the environmental impact of extraction and purification, must be addressed to ensure its feasibility for sustainable agricultural applications.

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CRediT authorship contribution statement

liu anbang: Writing – original draft, Methodology, Data curation. **wang jianghao:** Methodology, Data curation. **Cannon Nicola:** Writing – review & editing. **chang xianmin:** Writing – review & editing. **guo xinmei:** Writing – review & editing, Supervision, Conceptualization. **gao mengxiang:** Writing – review & editing, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.120848.

Data availability

Data will be made available on request.

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