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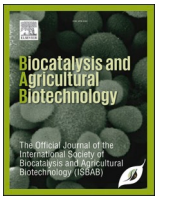

Capsicum chinense cell cultures: A biotechnological platform for the sustainable production of bioactive metabolites for the cosmetics market

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1. Introduction

The genus *Capsicum* (family Solanaceae) comprises more than 40 species of peppers, five of which are cultivated for their fruits (Carrizo García et al., 2022). *C. chinense* is widely grown due to the popularity of several highly pungent cultivars, such as Habanero and Scotch Bonnet (Elibox et al., 2017). More than 10 pungent and highly aromatic *C. chinense* cultivars have been identified on the Caribbean island of Trinidad, along with a single highly aromatic but non-pungent cultivar known locally as Pimento, the metabolic and bioactive properties of which have not been investigated in detail (Koeda et al., 2014).

The pungency and nutritional quality of peppers is determined by the metabolic profile, and these metabolites are also useful as cosmetic ingredients. Chili peppers contain characteristic alkaloids known as capsaicinoids (such as capsaicin), capsinoids (such as capsiate), health-promoting fatty acids (e.g., oleic and linoleic acids), carotenoids (e.g., capsanthin, capsorubin and lutein), ascorbic acid, tocopherols, phenolic acids and polyphenols (e.g., quercetin and luteolin), and also volatiles such as terpenes (Alonso-Villegas et al., 2023). The metabolomic profiles and biological activities of *Capsicum* fruits have been widely investigated, and the same techniques applied to *C. chinense* leaf extracts have revealed that these tissues also contain abundant phenolic amides, with caffeoyl putrescine identified as one of the principal compounds responsible for antioxidant activity (Assefa et al., 2021; Herrera-Pool et al., 2021).

Capsinoids are fatty acid esters of vanillyl alcohol, contrasting with capsaicinoids where a fatty acid amide is linked to vanillylamine (Vázquez-Espinosa et al., 2020). These compounds and their analogs possess antioxidant, anti-inflammatory, antitumor and anti-obesity properties. Capsinoids are ~1000-fold less pungent than capsaicinoids, allowing their use in diverse applications, including foods, dietary supplements, and medicinal products (Gupta et al., 2021). In the cosmetic industry, several antioxidant and anti-inflammatory *Capsicum*-based cosmeceuticals are available as dermatological creams (AG Cosmética natural™), soaps (S-SKIN Naturals™), facemasks (Gipsy vibes™) and other beauty products (Alonso-Villegas et al., 2023; Baenas et al., 2019). The demand for

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natural antioxidants is driven by the potential toxicity of synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene and *tert*-butylhydroquinone (Khezerlou et al., 2022).

Although *Capsicum* species are excellent candidates for the production of capsinoids as natural antioxidants, they are more labor-intensive to cultivate than other solanaceous crops such as tomato, potato and tobacco, and most domesticated chili cultivars are poorly adapted to stress (Cervantes-Hernández et al., 2022). Furthermore, valuable compounds such as capsaicin and capsiate make up only 0.1–1% of the fresh weight (FW) of pepper plants, an average of ~300 μg/g (Antonio et al., 2018). They accumulate in particular organs at certain developmental stages and the yield is influenced by the environment (Bae et al., 2014). This makes it difficult to produce consistent levels of these compounds for pharmaceutical, cosmetic and food applications. Cell cultures, although more expensive than traditional cultivation, offer a more consistent and reliable supply of capsinoids and other bioactive metabolites, and can be tuned to produce combinations of specific metabolites that are not found in large quantities by the corresponding plants. We therefore set out to establish *C. chinense* cv. Trinidad Pimento suspension cell cultures from callus tissue and to analyze the phytochemical profile of extracts, focusing on those showing antioxidant and antimicrobial activity that may be suitable as cosmetic ingredients. We carried out comprehensive metabolomic profiling to identify the primary and secondary metabolites present in different biomass extracts followed by antimicrobial, antioxidant and cytotoxicity assays to demonstrate the potential of these cells as a sustainable source of bioactive cosmetic ingredients. Our approach emphasizes the potential of crude extracts, which may show synergistic biological activity and align with sustainable practices by minimizing the waste associated with the isolation of pure bioactive compounds.

2. Materials and methods

2.1. Overall experimental workflow

The *C. chinense* cell suspension cultures were established from leaf-derived callus tissue that was grown in darkness or under photoperiodic conditions. Cell suspension cultures were established and progressively scaled up to 2-L flasks (500 mL working volume) for biomass generation, and extracts were prepared using ethyl acetate or a mixture of water and ethanol for metabolic profiling (targeted and untargeted metabolomics) and bioactivity assays. The workflow is summarized in Fig. 1.

2.2. Plant material and establishment of leaf-derived callus

We obtained *C. chinense* cv. Trinidad Pimento seeds from Refining Fire Chiles, USA (Koeda et al., 2014). The seeds were surface sterilized with 70% (v/v) ethanol for 1 min, soaked in 10% sodium hypochlorite in sterile water containing 1–1.5% active chlorine and a drop of Tween-20 (Sigma-Aldrich, USA) for 20 min, and finally rinsed with sterile water three times. Seeds were allowed to germinate for 3 days at 24 ± 1 °C in darkness on sterile moist filter paper. Seedlings were then placed on MS basal medium M0222 (Duchefa Biochemie, Netherlands) with 3% sucrose solidified with 0.3% Gelrite (Carl Roth, Germany) in 9-cm Petri dishes. They were incubated at 24 ± 1 °C under a standard cool white fluorescent light with a flux rate of 30–40 μmol mm^{-2} s^{-1} and a 16-h photoperiod for 1 week. Before adding Gelrite, the medium was adjusted to pH 5.8 and sterilized by autoclaving at 121 °C for 20 min. The seedlings were transferred aseptically to sterile plastic boxes containing the same solid MS medium. After 4 weeks, leaf explants (1 cm in length) were excised and transferred to solid MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid and 0.5 mg/L kinetin, and were then incubated with a 16-h photoperiod (P) or in darkness (D). Callus material was subcultured at intervals of 3–4 weeks.

2.3. Initiation of cell suspension cultures

After several passages on solid medium, cell suspensions were established from 2 g fresh weight (FW) of callus material inoculated into 50 mL MS liquid medium supplemented with phytohormones as above in 125-mL Erlenmeyer flasks. The cultures were incubated on a rotary shaker (130 rpm) at 24 ± 1 °C under either P or D conditions. Cell suspensions were subcultured every 2 weeks by filtering through autoclaved Miracloth and inoculating fresh medium at a concentration of 40 g/L. We also transferred 100-μL aliquots of the culture at the beginning and end of cultivation to plates containing plate count agar (PCA, Difco), potato dextrose agar (PDA, Difco),

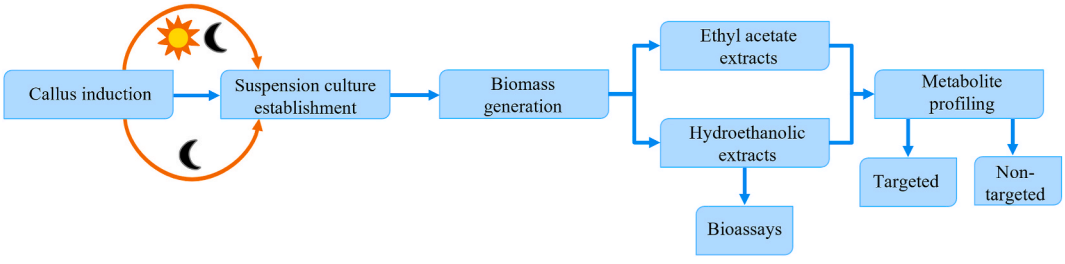

Fig. 1. Schematic workflow for the establishment, scale-up and extraction of *Capsicum chinense* cell suspension cultures and the subsequent metabolic analysis and bioactivity assays.

half-strength tryptic soy agar (½TSA) or half-strength tryptic soy broth (½TSB) prepared in house, and incubated them for 2 days for microbiological evaluation to exclude contamination.

2.4. Growth profile investigation

The growth characteristics of cells cultivated under P conditions were determined by measuring the FW and dry weight (DW) following 10 days of cultivation in 125-mL Erlenmeyer flasks as described above. The cells were then combined and filtered, and 1 g of biomass was transferred to a fresh 125-mL Erlenmeyer flask containing 25 mL MS medium supplemented with phytohormones as above (40 g/L cell inoculum). Cells were harvested at 4-day intervals over a period of 32 days followed by Büchner vacuum filtration using Miracloth (22–25 μm pore size) and two washes with Milli-Q water before measuring the FW. The cells were then lyophilized using an Alpha 1–4 LD plus device (Martin Christ Gefriertrocknungsanlagen, Germany) at −40 °C and 0.2 mbar for 7 days before measuring the DW. All experiments were performed in triplicate.

2.5. Biomass generation

Cell pools were prepared from suspension cultures grown under D and P conditions for 10 days in 250-mL flasks (70 mL working volume). The suspensions were passed through a 500-μm sieve to remove excess medium and 20 g of wet biomass was transferred to two parallel 2-L Erlenmeyer flasks containing 500 mL fresh culture medium. The suspension cultures were incubated as described above for 9–11 days and the FW of the harvested biomass was determined as described above. New suspension cultures were initiated by transferring cells from the first flask to two new 2-L flasks at an inoculum density of 40 g/L. The cells harvested from the second flask were lyophilized to determine the DW as described above. Cultivations under D and P conditions were carried out for eight consecutive growth cycles.

2.6. Chemical analysis

2.6.1. Preparation of extracts

Lyophilized biomass was powdered in an MM301 ball mill (Retsch, Germany) for 2 × 1 min at 29 Hz. Semi-polar compounds were extracted twice with 70% (v/v) ethanol (w/v 1:20) for 48 h at room temperature with constant stirring in darkness. These hydroethanolic extracts were then passed through a Büchner funnel lined with Grade 1 Whatman filter paper and centrifuged at 3220 g for 30 min. The ethanol was removed using a rotary evaporator (Heidolph, Germany) at 40 °C and the water fractions were combined and freeze-dried. Non-polar compounds were extracted from dried and powdered cells with 1:10 (w/v) ACS-grade ethyl acetate (VWR International, France) followed by ultrasonication at 50–60 Hz using a Laborette 17 device (Fritsch, Germany) for 30 min. The mixtures were filtered and centrifuged as described above and the clear extracts were evaporated to dryness at 40 °C using the rotary evaporator. All extracts were stored at −20 °C.

2.6.2. LC-HRMS analysis

The hydroethanolic and ethyl acetate extracts were fractionated by liquid chromatography high-resolution mass spectrometry with photo diode array detection (LC-PDA-HRMS) using an Ultimate HPLC (Dionex) and a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA). All solvents were LC-MS grade (Merck, Germany). For semi-polar metabolites, 10 mg of dried extract was resuspended in 1 mL 0.1% formic acid in 75% methanol spiked with 0.5 μg/mL formononetin (Sigma-Aldrich, USA) as an internal standard. The samples were then analyzed as previously described (Cuciniello et al., 2023). For non-polar metabolites, 10 mg dried ethyl acetate extract was resuspended in 1 mL ethyl acetate spiked with 25 μg/mL of the internal standard DL-α-tocopherol acetate (Sigma-Aldrich) then vortexed, shaken, centrifuged and filtered as above. We injected 5-μL samples into a Carotenoid C30 column (100 × 3.0 mm, 3 μm) (YMC, Germany) set at 25 °C followed by LC-MS as previously described (Sulli et al., 2023).

2.6.3. Untargeted metabolomics analysis

Raw LC-HRMS files were processed using Compound Discoverer 3.3 (Thermo Fisher Scientific) to screen mass spectral libraries in the ChemSpider, KEGG, PubChem and mzCloud databases. The following parameters were set: retention time (RT) alignment using the ChromAlign algorithm (Sadygov et al., 2006), original peak rating cut-off ≥6 in at least two samples, features (peak/signal) mass tolerance of 5 ppm, and centroid filtering with S/N threshold 1.5. Compounds were identified using ChemSpider (formula or exact mass), and mzCloud (ddMS2). Peak areas were calculated for preferred ions. LC-HRMS features were filtered by peak quality (peak rating) ≥ 7 in at least three samples, reference ion charge ±1, percentage of coefficient variation (group CV [%]) ≤ 25 in at least one sample group, using three replicates each group, annotation source at least full match in source predicted composition (chemical formula), mass error (Δppm) ≤ 4, RT between 0.8 and 25 or 15 min for LC-HESI-HRMS and LC-APCI-HRMS, respectively, and group area ≥4×10^5. Only peaks detected in all three replicates were selected. MS^2 annotated compounds were assigned using mzCloud (match value > 60/100 between the experimental and reference spectra, see mzCloud match column in Tables S1 and S2). The most abundant compounds were assigned in terms of calculated area, ordering features by maximum area values (≥6×10^7) for both semi-polar and non-polar compounds. Manual curation was applied to assign the preferred name or identify additional compounds not detected by *in-batch* analysis, searched by full MS and MS^2 spectra using Xcalibur Qual v4.4 or MetFrag for *in silico* fragmentation (Ruttkies et al., 2016), and Massbank peak list search (https://massbank.eu/MassBank). The list of tentatively identified metabolites with detailed information, including the accumulation levels (group area and D/P ratio, $\log_2$ fold change and *p* value) is reported in

Tables S1 and S2, for semi-polar and non-polar metabolites, respectively.

2.6.4. Targeted analysis of secondary metabolites

Targeted metabolomic analysis was based on literature and *in-house* data. Metabolites were identified based on accurate masses (PubChem and KEGG), MS^2 fragmentation (MetFrag and MassBank), and comparison with authentic reference substances, when available. For capsaicin, a calibration curve was prepared for absolute quantification ($R^2 = 0.991$) using the analytical standard (Merck), and data were interpolated with capsaicin signal intensities in hydroethanolic extracts analyzed by LC-HESI-HRMS. Other compounds were quantified relative to the internal standard (Fold-IS) by calculating the ion peak areas using Xcalibur Quan, normalized to the ion peak area of the internal standard (formononetin and α-tocopherol acetate for hydroethanolic and ethyl acetate extracts, respectively).

2.7. Antioxidant activity assay

The antioxidant activity of hydroethanolic extracts was evaluated using a diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay as previously described (Brand-Williams et al., 1995). DPPH scavenging activity was calculated as shown in Eq. (1).

$$\text{DPPH scavenging activity, FRS } (\%) = \frac{\text{Abs}^{\text{BL}}_{\text{corr}-517\ \text{nm}} - \text{Abs}^{\text{sample}}_{\text{corr}-517\ \text{nm}}}{\text{Abs}^{\text{BL}}_{\text{corr}-517\ \text{nm}}} x100\% \quad \text{(Eq. 1)}$$

The linear part of the free radical scavenging curve, defined as FRS (%), was plotted against the concentration of ascorbic acid in the range 0.52–2.1 mg/L to calculate ascorbic acid equivalents (AAE) producing the same free radical scattering effect as 100 g of *C. chinense* cell culture extracts, expressed as mg AAE/100 g extract.

2.8. Assessment of antimicrobial activity

Antimicrobial activity in microbial cultures spiked with hydroethanolic extracts was tested as previously described (Häkkinen et al., 2021) with two extract concentrations (1 and 5 mg/mL). Antimicrobial activity was primarily tested against *Staphylococcus aureus* VTT E−70045 (ATCC 6538). Selected extracts were tested also against *Pseudomonas aeruginosa* VTT E−84219 (ATCC 15692), *Escherichia coli* VTT E−94564T (ATCC 11775), *Candida albicans* VTT C-85161 (ATCC 10231) and methicillin-resistant *S. aureus* (MRSA) VTT E−183582 (DSM 11822). The half-maximal effective dose (ED_{50}) was tested using a broader range of extract concentrations. Chloramphenicol and hygromycin were used as positive controls for bacterial and *C. albicans* cultures, respectively.

2.9. Toxicity assays

BALB/c-3T3 clone A31 cells (ATCC, USA) were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 100 μg/mL streptomycin, 100 μg/mL penicillin and 10% (v/v) calf serum (Sigma-Aldrich). HaCaT human keratinocytes (Cell Lines Services, Germany) were grown in the same medium, with 10% (v/v) fetal bovine serum (Sigma-Aldrich) instead of calf serum. The cells were cultivated at 37 °C in a humidified 5% CO_2 atmosphere. Cells were detached and passaged by incubating with 0.25% trypsin/EDTA (Sigma-Aldrich). We determined *in vitro* phototoxicity according to OECD Test Guideline 432 (OECD, 2019). Cytotoxicity was tested essentially according to OECD Test Guideline 129 (OECD, 2010). Cell viability was calculated as a percentage relative to the medium control using Eq. (2) and, where applicable, IC_{50} and IC_{20} values were also determined.

$$\textit{Viability } (\%) = \frac{Abs_{540}(treatment) - Abs_{540}(background)}{Abs_{540}(untreated\ control) - Abs_{540}\ (background)} x100\% \quad \text{(Eq. 2)}$$

2.10. Statistical analysis

For comparative untargeted metabolomics analysis, p values were calculated using a two-tailed Student's t-test in Compound Discoverer, and significant differences in abundance were defined at $p < 0.05$ with a $\log_2$ fold change (FC) ≥ 1. The enrichment of major classes of metabolites was analyzed using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca/MetaboAnalyst/home.xhtml) with thresholds of $\log_2$ FC > 1 and $p < 0.05$. The top 25 enriched metabolites were represented as a dot plot. For targeted analysis, Student's t-test was used to evaluate significant differences in metabolite abundance between cultures grown under P and D conditions. Cytotoxicity and phototoxicity data were assessed by two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test in GraphPad Prism v9 (GraphPad Software, USA) to determine statistically significant differences between treatments.

3. Results and discussion

3.1. Callus induction and establishment of cell suspension cultures

Callus induction from *C. chinense* cv. Trinidad Pimento leaf explants was observed after 5–7 days in culture. Four weeks of incubation under P conditions yielded fully developed, soft and friable light-yellow callus, whereas D conditions required 6–8 weeks for callus formation, and the color was brighter (Fig. 2). Friable callus was cultured on medium supplemented with phytohormones as

above to induce rapid proliferation. The cultures were maintained on the same medium for >3 years, during which there were no discernible visual changes.

The growth characteristics of *C. chinense* cell suspension cultures were investigated in 125-mL Erlenmeyer flasks under P conditions. Slow growth was observed until day 8, followed by a rapid increase in FW and DW with a doubling period of 4 days between days 6 and 20. The maximum DW (11.7 ± 0.6 g/L, n = 3) was attained on day 28, as previously shown for *C. chinense* Jacq. cv. Umorok and cv. Naga King Chili with the same medium and phytohormones (Kabita et al., 2019; Kehie et al., 2014). However, our cultured cells achieved a higher FW compared to cv. Umorok.

To generate adequate biomass for chemical analysis, we cultivated the cells in 2-L flasks under P and D conditions. The biomass accumulation under P conditions (n = 8) was 160.4 ± 49.0 g/L FW and 7.1 ± 2.0 g/L DW, whereas the equivalent results under D conditions (n = 8) were 168.9 ± 27.6 g/L FW and 8.2 ± 2.7 g/L DW. The difference between the P and D conditions was not statistically significant. Notably, the P cultures required time to adjust to the higher cultivation volume. The first four growth cycles resulted in an average biomass yield of 116.2 ± 3.9 g/L FW, whereas the seventh cycle resulted in a biomass accumulation of 217.1 g/L FW. No adjustment period was necessary under D conditions.

3.2. Untargeted metabolic analysis

Extracts of the *C. chinense* cell cultures were prepared using 70% (v/v) ethanol (hydroethanolic extract) or 100% ethyl acetate. The hydroethanolic extract represented 30% of the cell DW under P conditions and 15.6% of the cell DW under D conditions, whereas the ethyl acetate extract represented 1.14% of the DW for both culture conditions.

The untargeted metabolomic analysis of semi-polar compounds in the hydroethanolic extracts revealed 186 highly abundant compounds (area max. $\geq 6 \times 10^7$) from a total of 567 detected. About 200 compounds were annotated by exact mass, whereas 45 were annotated by MS^2 fragmentation pattern matching in mzCloud. We identified 477 compounds with differential accumulation based on the criteria $\log_2$ FC ≥ 1 and $p < 0.05$, 216 more abundant under P conditions and 261 more abundant under D conditions (Fig. 3A).

Principal component analysis (PCA) clearly separated the cell cultures grown under the two conditions, with ~98% of total variance explained by the first two principal components (Fig. 3B). We observed two peaks indicating the most abundant metabolites. The first, *N*-feruloyl-putrescine, migrated at 3.9 RT and was more abundant under P conditions (D/P = 0.20), whereas the second peak at 5.35 RT showed higher levels under D conditions (D/P = 2.06). Both peaks yielded hydroxycinnamic mono-substituted amine MS^2 fragments with the characteristic feruloyl (*m/z* 177.05) and amine (*m/z* 89.11) ions (Li et al., 2018). Only *N*-feruloyl-putrescine at RT 5.35 and *N*-feruloyl-octopamine at RT 10.00 were more abundant under D conditions (D/P = 1.69). All other hydroxycinnamic amides, such as *N*-caffeoyl-putrescine, *N*-coumaroyl-putrescine, *p*-coumaroyl-agmatine and another isomer of *N*-feruloyl-octopamine, were more abundant under P conditions (D/P = 0.001 and 0.24 for *N*-caffeoyl-putrescine and *N*-feruloyl-octopamine, respectively). Because *cis*-isomers are eluted by RPLC earlier than *trans*-isomers, the more abundant peaks under D conditions were annotated as *trans*-isomers, suggesting that only *trans*-feruloyl-octopamine (#296, Table S1) and *trans*-feruloyl-putrescine (#318) were more abundant under D conditions.

We also identified a set of manually annotated hydroxycinnamic acid (HCA) conjugates of ferulic acid showing MS^2 fragmentation with the predominant ion at *m/z* 177 [ferulic acid + H−H_2O]$^+$ and a smaller ion at *m/z* 145 [ferulic acid−H_2O−OCH_3]$^+$ (#77, #134, #286 and #388 in Table S1). The MS^2 fragments in Fig. S1 showed two peaks at *m/z* 373.175 indicating the presence of a putative *cis*-isomer at 11.40 min, which was more abundant under P conditions. Additionally, the *trans*-ferulic conjugate at 12.1 min was 3.55-fold

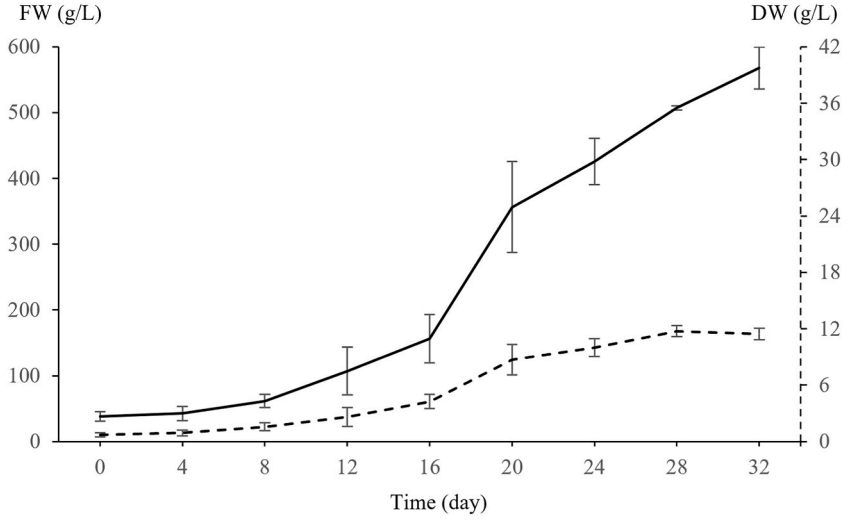

Fig. 2. The growth profile of *C. chinense* cell suspension cultures cultivated in MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid and 0.5 mg/L kinetin for 32 days under P conditions. Data are means ± SD (n = 3). FW = fresh weight (−) and DW = dry weight (– -).

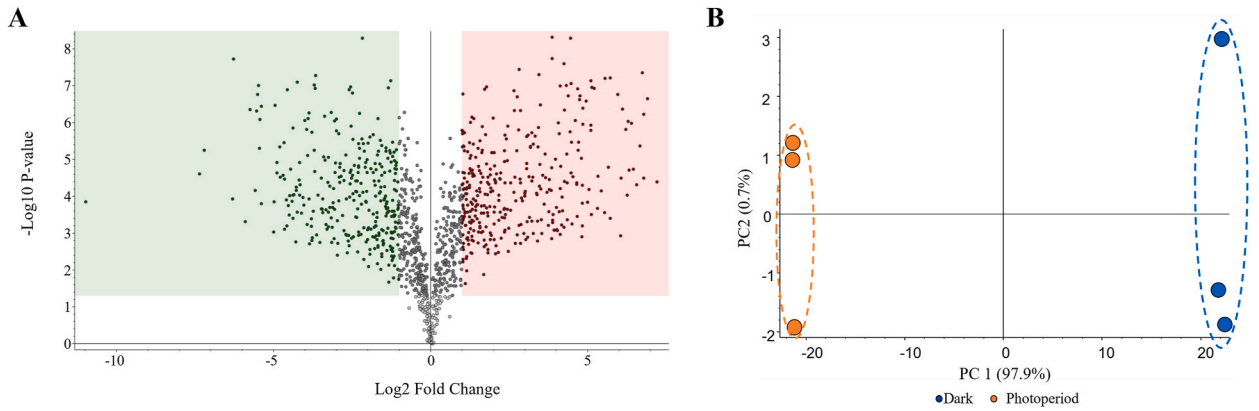

Fig. 3. Comparative untargeted metabolomic analysis of semi-polar compounds in hydroethanolic extracts of *C. chinense* cell cultures. (**A**) Volcano plot showing semi-polar compounds found in the cell cultures grown under D vs P conditions, indicating significant overabundance (green dots) or depletion (red dots) under P conditions ($p < 0.05$, $\log_2$ FC D/P > 1). (**B**) PCA plot (PC1 vs PC2) of cell culture extracts grown under D (blue) and P (orange) conditions.

more abundant under D conditions. The peak at *m/z* 351.1909 (MS^2 fragments, Fig. S2) featured two isomers at RT 6.40 and 7.04 min, with only the first (*cis*-isomer) ~4-fold more abundant under P conditions. Finally, two isomers of feruloyl-β-D-glucose were 3–7-fold more abundant under D conditions.

HCA-substituted polyamines (PAs), such as putrescine, octopamine and agmatine, are associated with the presence of photoreceptor systems (Pál et al., 2021) and PA levels are elevated when photosynthesizing organisms are illuminated (Ioannidis and Kotzabasis, 2007). Furthermore, P and D conditions influence the accumulation of PAs (Gondor et al., 2021; Takács et al., 2016). PA levels (putrescine, spermidine and spermine) initially increase when dark-induced senescence occurs in detached barley leaves, and diaminopropane accumulates during senescence along with putrescine conjugates (Sobieszczuk-Nowicka et al., 2016). This suggests that genes involved in the synthesis of PAs and their condensation with HCAs to generate phenolamides (Pál et al., 2021), as well as the corresponding transcription factors (Onkokesung et al., 2012), are regulated by different light conditions in our *C. chinense* cultures. Polyamine oxidases (PAOs) generate putrescine from spermidine (Pál et al., 2021), potentially explaining the dominance of putrescine conjugates in our cell cultures. Furthermore, the expression or activity of PA transporters could be modulated by light regimes to control the distribution of PAs (Gondor et al., 2021).

Although traces of *p*-coumaric acid were detected in both hydroethanolic extracts, its derivative 7-hydroxycoumarin (umbelliferone) was 25-fold more abundant under P conditions. Umbelliferone is a common ingredient in sunscreens because it absorbs UV radiation at several wavelengths and is an antioxidant with minimal toxicity, enhancing its suitability for cosmetic applications (Molnar et al., 2017; Sarkar et al., 2013). Recently, umbelliferone scaffold-based glycoconjugates were shown to inhibit the growth of Gram-negative and Gram-positive bacteria (Jatav et al., 2023).

Untargeted analysis revealed the accumulation of sinapoyl-glucosides in the dark (#484, #527 and #532 in Table S1), as well as

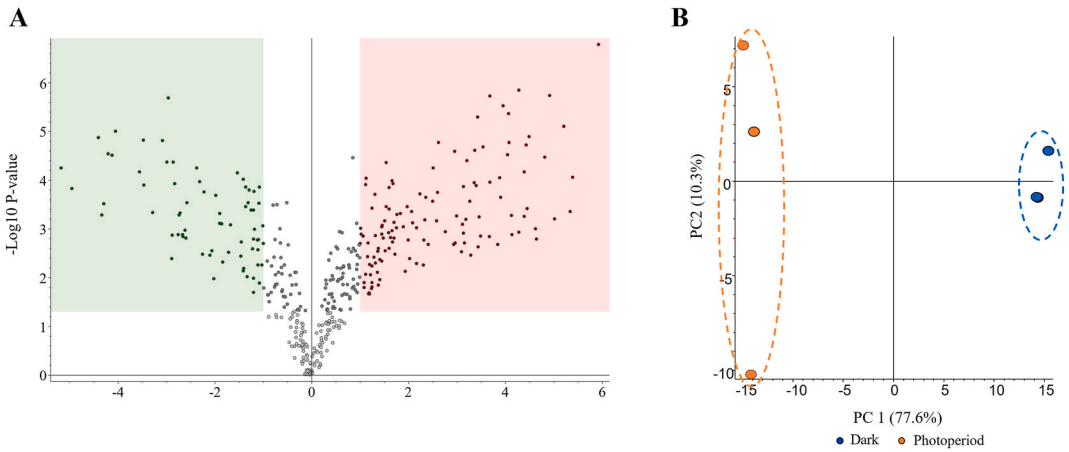

Fig. 4. Comparative untargeted metabolomic analysis of non-polar compounds in ethyl acetate extracts of *C. chinense* cell cultures. (**A**) Volcano plot showing non-polar compounds found in the cell cultures grown under D vs P conditions, indicating significant overabundance (green dots) or depletion (red dots) under P conditions ($p < 0.05$, $\log_2$ FC D/P > 1). (**B**) PCA plot (PC1 vs PC2) of cell culture extracts grown under D (blue) and P (orange) conditions.

sinapine (*O*-sinapoyl-choline, #524), identified as an MS^2 fragment ion at *m/z* 310.1645, which showed a D/P ratio of 22.2. Sinapic acid and its glucose esters have been detected in chili (de Aguiar et al., 2019) as well as *C. chinense* cv. Carolina Reaper callus and *in vitro* plants (Sherova et al., 2019). HCA derivatives such as caffeic acid and sinapic acid are known to mitigate UV-induced skin photo-aging effects (Jeon et al., 2019; Nićiforović and Abramovič, 2014; Thiyam et al., 2006). We also identified procyanidin B2 (#211), with a D/P ratio of 0.5. This biflavonoid and B-type proanthocyanidin is known for its anti-inflammatory and antioxidant effects in cosmeceutical products (Ferreira et al., 2021).

The analysis of non-polar compounds as described above for the semi-polar compounds revealed 325 features, 172 of which were identified as highly abundant (area max. $\geq 6 \times 10^7$). These included 91 identified by exact mass and 54 annotated by MS^2 fragmentation pattern, with a majority showing a MS^2 match score >60/100 (Table S2). We found that 43 were more abundant under P conditions and 93 were more abundant under D conditions ($p < 0.05$, $\log_2$ FC ≥ 1) (Fig. 4A). PCA clearly separated the distribution of non-polar compounds based on growth conditions (Fig. 4B), with the first two principal components explaining 88% of the total variance. We identified many fatty acids, their conjugated forms and derivatives, quinones, and mono-, di- or tri-acylglycerols. A group of compounds was also manually annotated using our *in-house* library based on authentic standards (labeled ** in Table S2) whereas other isomers were annotated as fragments.

The most abundant non-polar compounds in the ethyl acetate extracts were linoleic acid (#264) and palmitic acid (#249), with group areas of up to 6.6×10^{10} under D conditions, followed by derivatives of linoleic acid such as 9(*Z*),11(*E*)-conjugates (#102, #180, #189, #205), α-eleostearic acid (#187), decanoic acid (#199), stearic acid (#212) and oleic acid (#266). Linoleic and oleic acid were about three times more abundant under D conditions, and margaric acid was twice as abundant, indicating that cultivation in darkness encourages the accumulation of fatty acids. Similar observations have been reported for soybean cell suspension cultures, although the levels of linoleic acid and total trienoic fatty acid levels declined in darkness (Collados et al., 2006).

The analysis of 75 pepper fruits revealed that seven fatty acids were present in all samples, namely palmitic, stearic, oleic, linoleic, arachidic, margaric and caproic acid, with linoleic, oleic and palmitic acid present at the highest levels (Li et al., 2022). We observed the same trend, although we did not detect caproic acid. Myristic acid is also regarded as one of the major fatty acids in *Capsicum* spp. (Ananthan et al., 2018; Kim et al., 2019) and this compound was abundant in our cell cultures, along with its derivative isopropyl myristate. A compound tentatively identified as eugenol (#315) was 22-fold more abundant under D conditions. This allylbenzene compound is known for its radical-scavenging and anti-inflammatory activities, with potential applications in cosmetics (Ulanowska and Olas, 2021). Furthermore, cells grown in the dark accumulated 24-fold more α-tocopherol (#317), 10-fold more vanillyl ethyl ether (#301), 4-fold more 11(*Z*),14(*Z*)-eicosadienoic acid (#277) and twice as much erucic acid (#250) as cells under P conditions.

We detected two abundant ceramide compounds, namely glucosylceramide (d18:1/16:0) (#253) and its precursor C16-

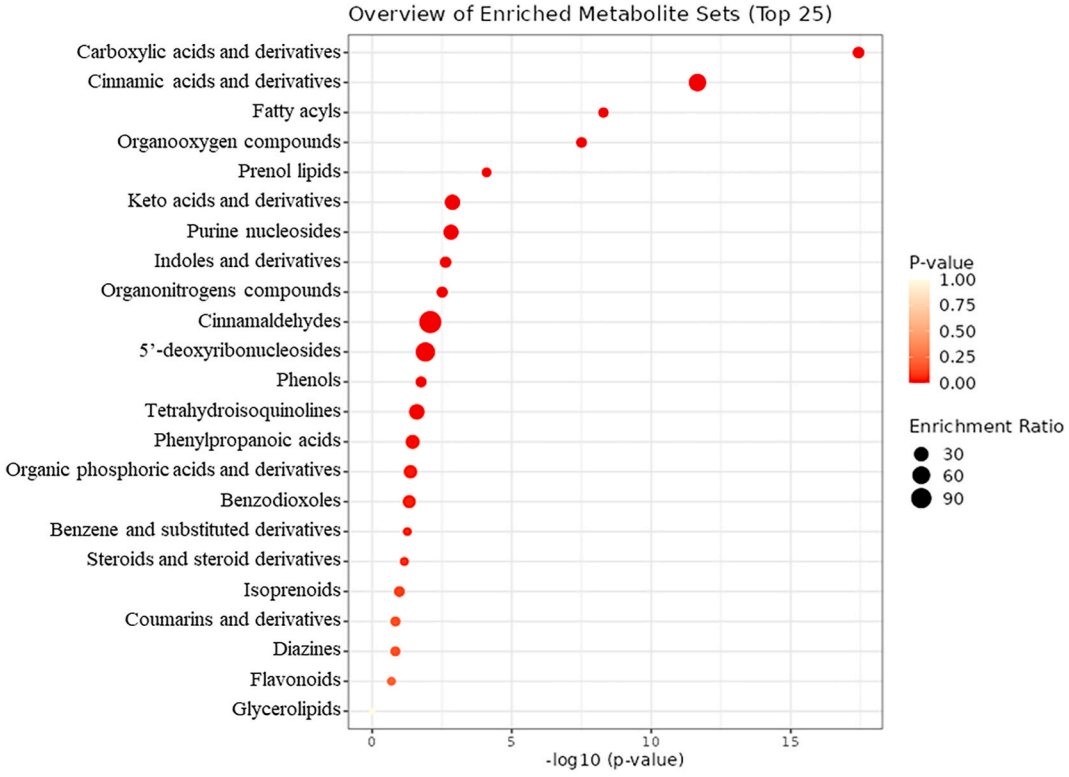

Fig. 5. Enrichment analysis of metabolites that accumulate to different levels in *C chinense* cell cultures under different conditions. The dot plot (top 25) was generated using MetaboAnalyst 6.0 and shows metabolites with significant differences in abundance ($\log_2$FC > 1, $p < 0.05$).

dihydroceramide (#67), with the glycosylated form being 2.6-fold more abundant under D conditions. Ceramides are sphingolipids that enhance skin and hair hydration at low doses, preventing dryness and protecting against irritation by synthetic surfactants. Interestingly, ceramides from plants are regarded as safer than and therefore preferable to animal ceramides in pharmaceutical and cosmetic applications (Tessema et al., 2017).

Finally, we used MetaboAnalyst to find enriched metabolic classes differing in abundance between growth conditions in both extracts ($\log_2 FC > 1, p < 0.05$). We found that carboxylic acids and cinnamic acids in the semi-polar extracts, as well as fatty acids and organic oxygen compounds in the non-polar extracts, were the most enriched metabolic classes (Fig. 5).

3.3. Targeted metabolomic analysis

3.3.1. Targeted analysis of the hydroethanolic extracts

Hydroxycinnamic acids (HCAs) are known for their antioxidant, antibacterial and anti-inflammatory properties, helping to filter UV light and reduce the risk of cancer, neurodegenerative diseases and diabetes (Neelam et al., 2020). Caffeic and ferulic acid, which are widely used in cosmetics due to their anti-aging and antimicrobial activity and ability to stimulate collagen production (Magnani et al., 2014), were 1.8-fold and 2.8-fold more abundant under D conditions, respectively ($p < 0.05$) whereas cinnamic acid levels remained constant (Table S3). Traces of *p*-coumaric acid were detected under both conditions, whereas 4-hydroxybenzoic acid was only found under P conditions and the concentration was negligible. Glucosides of sinapate were ~20-fold more abundant under D conditions but other glucosides were not detected. Sinapoyl glucosides have also previously been detected in chili (de Aguiar et al., 2019), and their antioxidant activities were similar to those of synthetic antioxidants (Nguyen et al., 2021). Although cinnamic acid, *p*-coumaric acid, vanillic acid, and gallic acid were present in our *C. chinense* cv. Trindidad Pimento cell cultures, they were not detected in cultures of *C. chinense* cv. Carolina Reaper (Sherova et al., 2019). Conversely, chlorogenic acid and protocatechuic acid were present in Carolina Reaper but not detected in our cultures.

Phenolamides or hydroxycinnamic amides (HCAAs) are HCA derivatives that help plants respond to stress. They are widely used in food, pharmaceuticals and cosmetics (Roumani et al., 2020), and have been detected before in pepper leaves (Assefa et al., 2021) and fruits (Domokos-Szabolcsy et al., 2023). The primary HCAAs in pepper plants were coumaroyl, feruloyl and caffeoyl substituted amines. In addition, putrescine conjugates are potent α-glucosidase enzyme inhibitors, particularly caffeoyl-putrescine from chili, suggesting their potential for the management of type 2 diabetes (Assefa et al., 2021). Five phenolamides (the two feruloyl-putrescine isomers, *N-cis*-caffeoyl-putrescine, *N-trans*-caffeoyl-putrescine and coumaroyl-putrescine) were among the most abundant compounds detected by untargeted analysis, with feruloyl-putrescine isomers accumulating to the highest levels. Individual phenolamide accumulation generally favored P conditions, except the *trans*-isomers of feruloyl-octopamine and feruloyl-putrescine. The latter contributed to an overall increase in total phenolamides under D conditions, despite other compounds being more abundant when the cells were illuminated, including the *cis*-isomer of caffeoyl-putrescine. In addition to the phenolamides already identified by untargeted analysis, we found another *cis*-isomer of *N*-caffeoyl-putrescine at higher levels under P conditions (Table S3). Phenylpropanoid pathway intermediates, specifically *p*-coumaroyl-CoA, caffeoyl-CoA and feruloyl-CoA, are presumably competitive precursors for the synthesis of capsaicinoids, flavonoids, lignins, coumarins and phenolamides in *Capsicum* spp. The presence of phenolamides as major compounds in our cell cultures implies that these intermediates are actively synthesized and channeled towards the pathway. A rational metabolic-flow switching approach could enhance the capacity of plant cell cultures to produce these valuable compounds. For example, feruloyl-putrescine accumulates to high levels when bamboo (*Phyllostachys nigra*) is grown in media inducing lignification, along with a smaller amount of *p*-coumaroyl-putrescine. The transformation of bamboo cells with barley agmatine coumaroyltransferase (ACT) resulted in the primary production of *p*-coumaroyl-agmatine. The biosynthesis of cinnamoyl-putrescines has been reported in tobacco (*Nicotiana tabacum*) cell cultures, including caffeoyl and feruloyl putrescine (Negrel, 1989). Furthermore, the depletion of phosphate levels enhanced the synthesis of caffeoyl and feruloyl putrescine in these cultures, correlating with higher phenylalanine ammonia lyase activity (Knobloch and Berlin, 1981).

The mean capsaicin content was 6.01 ± 1.49 and 8.50 ± 1.01 ng/g DW under D and P conditions, respectively, which was not a statistically significant difference. Its water-soluble glycosylated derivative, capsaicin-*β*-D-glucopyranoside, was present at the same concentration as capsaicin, but exclusively under P conditions. Interestingly, capsinoids such as capsiate and its two derivatives, dihydrocapsiate and nordihydrocapsiate, were detected in chili cell cultures for the first time (Table S3) but levels did not differ significantly between the two growth conditions ($p < 0.05$). However, dihydrocapsiate and nordihydrocapsiate were more abundant than capsiate under both conditions, and other derivatives, such as dihydrocapsaicin, were absent. The capsiate/dihydrocapsiate ratio varies between 2 and 6 in different *Capsicum* accessions, also depending on the growth stage of the fruit (Fayos et al., 2019), contrasting with the higher relative abundance of dihydrocapsiate in our cultures. Vanillylamine was only detected under P conditions, whereas vanillic acid was 2.7-fold more abundant under D conditions, and neither vanillin nor vanillyl alcohol were detected under either condition. The impact of light on capsaicinoid accumulation in natural *Capsicum* habitats varies according to the availability of precursors and other environmental factors (Jiménez-Viveros et al., 2023). Nevertheless, in contrast with our findings, light inhibits capsaicin production in callus cultures of *C. annuum* var. Jalapeno (Kwon et al., 2001; Weathers et al., 1992).

Numerous studies have reported higher levels of flavonoids, phenolic acids, and total phenolic compounds in pepper leaves and stems compared to other vegetative components, including fruits (Jiménez-Ortega et al., 2023). The predominant flavonoids in pepper leaves are myricetin, quercetin, luteolin, apigenin and kaempferol, principally as glycosides and aglycones. Total flavonoid and polyphenol levels have also been determined in *Capsicum* cell cultures (Ferri et al., 2018). Even so, only a single report describes the identification of five specific flavonoids in in *C. chinense* cv. Carolina Reaper callus cultures (Sherova et al., 2019), and our cell cultures contained only low levels of catechin and kaempferol-3-*O*-robinobioside as well as traces of quercetin 3-*O*-rhamnoside, exclusively

under P conditions (Table S3). The inverse trend between phenolamides and flavonoids in our *C. chinense* cell cultures may reflect the highly branched phenylpropanoid pathway, leading to competitive interactions favoring phenolamide production.

Finally looking at sesquiterpenes, we detected capsidiol, aromadendrene and solavetivone in our cultures under both conditions. There was no significant differential abundance in aromadendrene levels, but capsidiol was significantly ($p < 0.05$) more abundant under P conditions and solavetivone was significantly ($p < 0.05$) more abundant under D conditions (Table S3). Aromadendrene and solavetivone were previously detected only in the culture medium of elicited *C. annuum* cell cultures (Sabater-Jara et al., 2010), whereas capsidiol was reported in elicited cell cultures (Ma, 2008) as well as pepper fruits (De Marino et al., 2006; Yang and Song, 2021). Recently, the orchestrated regulation of solavetivone metabolism was shown to confer resistance to abiotic stress, such as waterlogging, in pepper plants (Gong et al., 2022). The anti-neuroinflammatory, antifungal and bacteriostatic activities of capsidiol, solavetivone and aromadendrene have also been documented (Carev et al., 2023; Chen et al., 2013; De Marino et al., 2006; Yang and Song, 2021). Interestingly, aromadendrene can inhibit the growth of MRSA (Mulyaningsih et al., 2011).

3.3.2. *Targeted analysis of the ethyl acetate extracts*

Carotenoids have proven to be effective against premature skin aging caused by oxidative damage (Biskanaki et al., 2023) and can penetrate into the upper epidermis following topical application to strengthen antioxidant defenses (Lademann et al., 2009). To our best knowledge, we are the first to report the carotenoid composition of *Capsicum* cell cultures. We identified 18 carotenoids in the ethyl acetate extracts, including colorless precursors, provitamin-A carotenes and xanthophylls. Overall, higher titers of carotenoids were found under P conditions (Fig. 6), and hydroxyphytoene, ζ-carotene, α-carotene, β-cryptoxanthin, antheraxanthin and luteoxanthin were exclusive to the P cultures (Table S3). Lutein was 5.35-fold more abundant under P conditions, β-carotene 2.29-fold, and zeaxanthin 2.17-fold, and all have been shown to boost the antioxidant capacity of the skin following topical or oral application, reducing UV-induced skin damage caused by free radicals (Palombo et al., 2007). Accordingly, β-carotene is widely used in skincare products due to its effective anti-wrinkle and anti-aging activities (Cho et al., 2010). The levels of neoxanthin and violaxanthin did not significantly vary between conditions. Notably, phytoene and phytofluene were 23-fold and 3-fold more abundant under P conditions, with phytoene emerging as the predominant carotenoid. High concentrations of colorless carotenoids, particularly phytoene, in the P

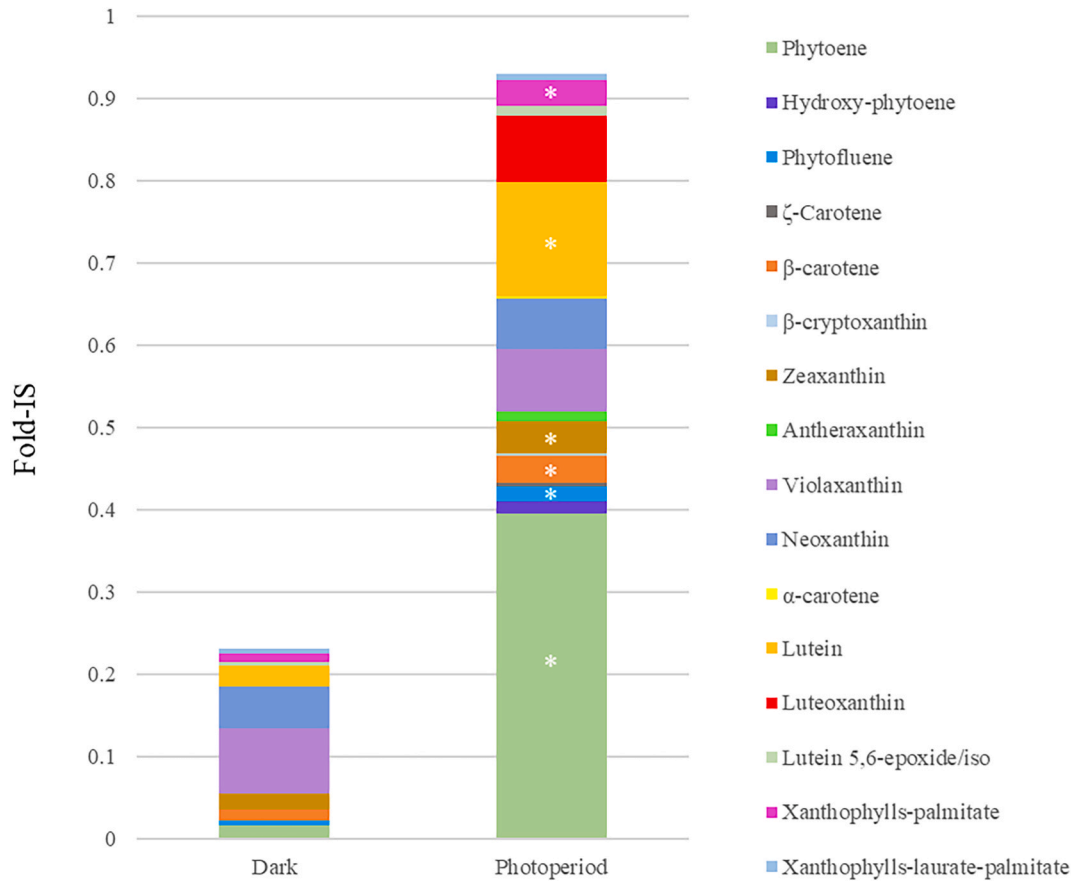

Fig. 6. Carotenoids and carotenoid esters in ethyl acetate extracts of *C. chinense* cell cultures grown under dark and photoperiodic conditions. The carotenoids were detected by LC-PDA-APCI-HRMS and their abundance is expressed relative to the internal standard (fold-IS, α-tocopherol acetate). Asterisks indicate significant differences between growth conditions ($p < 0.05$) according to Student's *t*-test.

cultures is promising, given their documented safety and benefits such as protection against UVB-generated lipoprotein oxidation products following topical application (Miras-Moreno et al., 2019). Finally, we did not detect capsorubin, capsanthin or their laurate, palmitate and myristate esters regardless of the culture conditions. Similarly, capsorubin and capsanthin have not been detected in *C. chinense* plants (Giuffrida et al., 2013). In contrast, we detected low levels of esterified xanthophylls, probably luteoxanthin and lutein with palmitate and laurate groups, identified by accurate mass and detected as $[M+H]^+$ ions at 839.6541 and 1021.8233 *m/z*, respectively, with major peaks at 420–440 nm (Sulli et al., 2017).

Light plays a pivotal role in the regulation of carotenoid biosynthesis and accumulation (Gómez-García and Ochoa-Alejo, 2013). Red pepper fruits accumulate six primary pigments: β-carotene, β-cryptoxanthin, zeaxanthin, antheraxanthin, capsorubin, and capsanthin, with lutein present in smaller quantities. Notably, β-carotene, β-cryptoxanthin and zeaxanthin accumulate at significantly lower levels than capsanthin in red pepper fruits (Venkatesh et al., 2023). Typically, carotenoid accumulation depends on genotype, tissue and developmental stage, as well as environmental conditions. Capsanthin and capsorubin are exclusive to red-fruited pepper genotypes. Capsanthin and violaxanthin arise from antheraxanthin and capsorubin, respectively (Venkatesh et al., 2023). In contrast, the most abundant carotenoids in our cell cultures were phytoene, lutein, luteoxanthin, violaxanthin, and neoxanthin. The absence of capsanthin and capsorubin, coupled with the presence of pathway intermediates, suggests the enzymes (and corresponding genes) responsible for their synthesis are inactive. It is notable that 8-methyl-6-nonenoic acid, a fatty acid exclusively synthesized in *Capsicum* spp., was also found in our cell cultures. Conversely, its analog nonanoic acid was only detected under P conditions.

3.4. Assessment of bioactivity

3.4.1. Antioxidant activity

The antioxidant activity of hydroethanolic extracts prepared from cell cultures under P conditions (38.1%) was slightly higher than that of their counterparts prepared under D conditions (25.9%) when each was tested at a concentration of 1.67 mg/mL. In terms of AAE, 100 g of P culture extract was equivalent to 84.6 mg AA, whereas 100 g of D culture extract was equivalent to 57.7 mg AA (Table 1). Antioxidant activity was therefore significantly higher when cells were cultured under P rather than D conditions ($p = 0.001$, Student's equal variance *t*-test with a two-tailed distribution).

The primary source of antioxidant activity in *Capsicum* fruits and leaves is the total phenolic content, with additional antioxidant effects associated with capsaicinoids and capsinoids (Rosa et al., 2002). The antioxidant properties of *C. chinense* plant extracts have been attributed to the presence of phenolic compounds such as ferulic, caffeic and sinapic acid derivatives, particularly putrescine conjugates (Sang et al., 2007). These were the most abundant compounds in our cell culture extracts, which nevertheless exhibited lower antioxidant activity than extracts of *Capsicum* fruits and leaves (Cho et al., 2020). This may reflect the synergistic effects of metabolites in the various differentiated cells of plant organs, in contrast to our homogenous undifferentiated cells. Interestingly, our P culture extracts conferred greater antioxidant activity than cloudberry, stone berry, arctic bramble, and strawberry cell culture extracts (Rischer et al., 2022). However, lingonberry cultures were superior to all the cell cultures discussed above, including our *C. chinense* cell cultures (Rischer et al., 2022).

3.4.2. Antimicrobial activity

The hydroethanolic extracts inhibited the growth of Gram-positive *Staphylococcus aureus*, showing a bacteriostatic effect at 3 mg/mL and a bactericidal effect at 4–5 mg/mL (Fig. 7A and B). There was no significant difference between the extracts prepared from cells under P conditions (Fig. 7A) and D conditions (Fig. 7B), although the P culture extracts were somewhat more active at concentrations of 2–3 mg/mL perhaps due to the higher content of caffeoyl and coumaroyl putrescine (Table S1). However, several phenolic derivatives were more abundant in the D culture extracts, suggesting the antimicrobial activity was probably based on the effects of multiple compounds. The synergistic action of plant secondary metabolites in extracts is a common phenomenon (Abreu et al., 2012; Stermitz et al., 2000; Vaou et al., 2022). The same extracts were also assessed against MRSA, and were bacteriostatic at 2 mg/mL and bactericidal at $\geq$4 mg/mL, similar to the results with the standard *S. aureus* strain (Fig. 7C and D). The extracts showed no activity against Gram-negative *Escherichia coli* and *Pseudomonas aeruginosa*, or against the yeast *Candida albicans*. Ethyl acetate extracts of D cultures were also assessed against *S. aureus*, *E. coli* and *P. aeruginosa* but showed no activity, and the experiments with these extracts were therefore discontinued.

Capsicum bioactive compounds such as phenolics and capsinoids provide health-promoting effects, including antimicrobial

Table 1
Antioxidant activity of *C. chinense* cell culture hydroethanolic extracts following cell cultivation under photoperiodic (P) conditions or in the dark (D conditions). Data are means ± SD (n = 4). Ascorbic acid was used as the positive control (EC_{50} = 2.01 mg/L). The difference in DPPH scavenging activity between P-grown and D-gown cells, expressed in mg AAE/100 g of extract and in FRS%, is statistically significant (p = 0.001, Student's equal variance *t*-test, with a two-tailed distribution).

	DPPH (mg AAE/100 g of extract)[a]	DPPH scavenging activity (FRS%) at 1.67 mg/mL
Photoperiod	84.6 ± 8.5	38.1 ± 3.8%
Dark	57.7 ± 3.7	25.9 ± 1.7%

Abbreviations: AAE, ascorbic acid equivalent; DPPH, diphenyl-1-picrylhydrazyl; FRS, free-radical scavenging activity.
[a] Antioxidant activity of 100 g of extract in mg AAE per 100 g of dry extract.

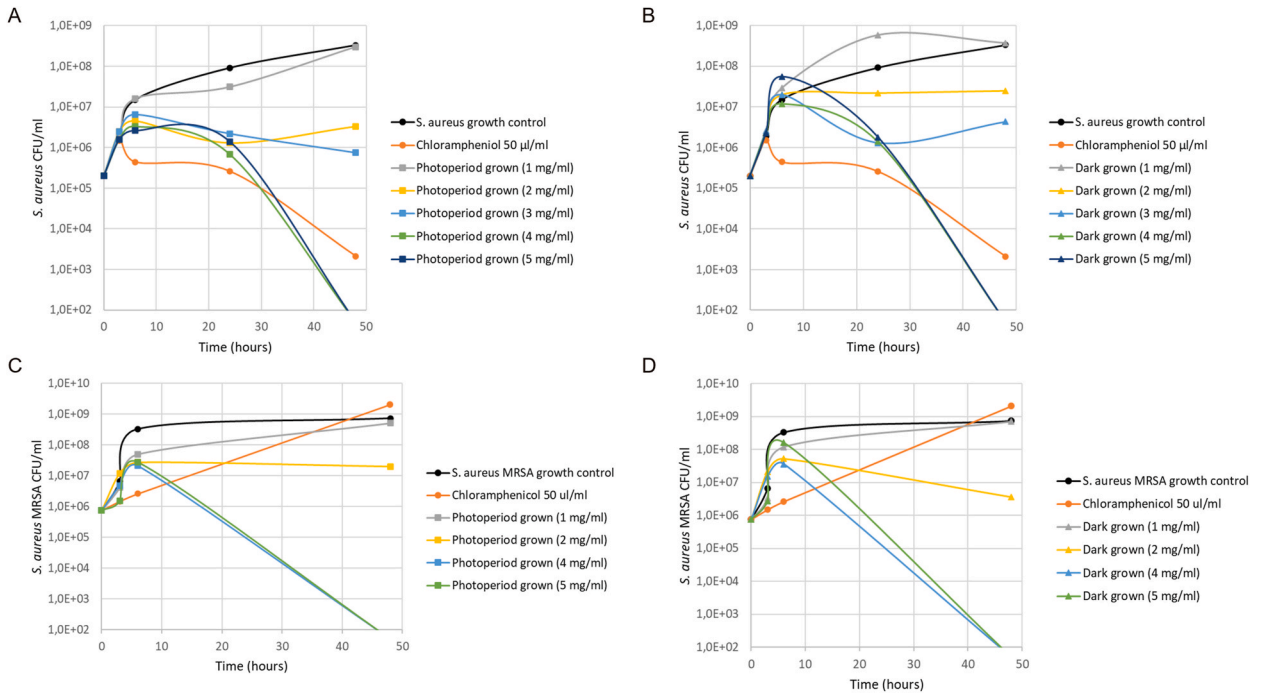

Fig. 7. Antimicrobial activity of hydroethanolic extracts (1–5 mg/mL) of *C. chinense* cell cultures against (**A**,**B**) *S. aureus* VTT E−70045; (**C**,**D**) *S. aureus* MRSA VTT E−183582. **A** and **C** present the extracts from P cultures whereas **B** and **D** present those derived from D cultures.

activity, making them promising candidates as pharmaceutical and cosmetic ingredients (Romero-Luna et al., 2023). These include HCA and its derivatives, such as ferulic, caffeic, *p*-coumaric and sinapic acids and their esters, as well as phenolamides: for example, feruloyl-putrescine is known for its antimicrobial and antioxidant activity (Neelam et al., 2020) particularly in combination with capsaicinoids (Mokhtar et al., 2017). The evaluation of polar extracts from wounded potato tubers revealed antibacterial activity against *Erwinia carotovora* and a non-pathogenic strain of *E. coli*, attributed to the presence of ferulic acid and feruloyl-putrescine, as well as derivatives such as dihydroferuloyl-putrescine (Dastmalchi et al., 2019).

3.4.3. Cytotoxicity

The cytotoxicity of the extracts was evaluated against mouse fibroblasts (BALB/c 3T3) and human keratinocytes (HaCaT) with loss of ≥20% viability considered cytotoxic (Fig. 8). Hydroethanolic extracts of cell cultures grown under P conditions did not affect the viability of BALB/c 3T3 cells in the concentration range 0.156–2.5 mg/mL, whereas the corresponding extracts of cells grown under D conditions were cytotoxic at concentrations above 1.25 mg/mL. The opposite profile was observed for HaCaT cells, where no toxicity was observed for the D extract in the concentration range 0.156–2.5 mg/mL but the P extracts were cytotoxic at concentrations above 1.25 mg/mL. At extract concentrations above 2.5 mg/mL, the P extracts were significantly more cytotoxic than the D extracts against BALB/c 3T3 cells and the opposite was true for the HaCaT cells. Furthermore, the viability of the HaCaT cells declined steadily in the presence of the P extract (>2.5 mg/mL) but remained unaffected at higher concentrations of the D extract until a sudden and

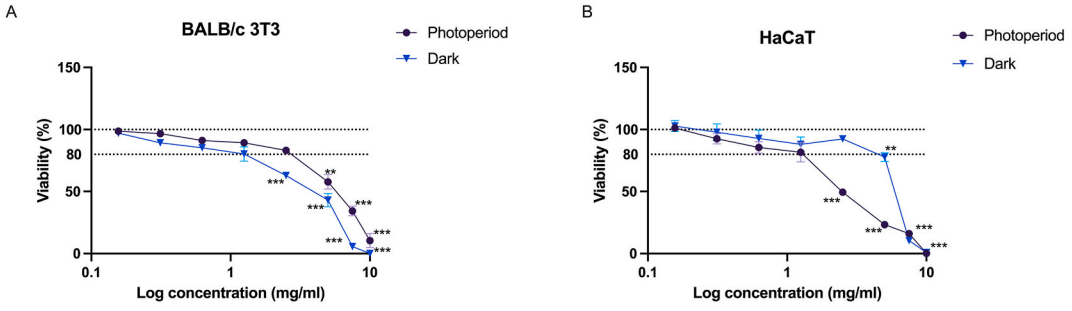

Fig. 8. Viability of (A) BALB/c 3T3 and (B) HaCaT cells after 48 h in the presence of hydroethanolic extracts of *C. chinense* cell cultures grown under photoperiodic (P) or dark (D) conditions. Data are mean viabilities determined in neutral red uptake assays ± SD (n = 3). Statistical significance compared to the solvent control was determined by two-way ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

precipitous loss of viability occurred at the highest three concentrations.

IC_{20} and IC_{50} values were determined for both extracts and used to calculate therapeutic indices (Table 2). IC_{20} values were necessary because lower cell viability was observed at the highest solvent concentrations. This reduction corresponds to the amount of solvent present in the extract solutions that reduce cell viability by 50%. However, the viability at high extract concentrations differed significantly from the solvent control, indicating the extract-specific cytotoxic effects of these elevated concentrations.

Although the concentration-dependent effects were similar for both extracts, differences in cytotoxicity were observed between the cell lines, indicating differential sensitivity. Capsaicin and its derivatives have selective cytotoxicity against different cell lines (Luján-Méndez et al., 2023). *Capsicum* is generally considered safe, but some adverse effects have been reported, especially in cases of prolonged use or high capsaicin levels (Batiha et al., 2020; Bode and Dong, 2011). *Capsicum* compounds such as capsaicin also have a long half-life in skin tissue compared to other tissues, implying topical applications would result in stronger effects compared to less effective systemic administration due to metabolic instability and short half-life in circulation (Zhang et al., 2024). Data concerning the toxicity of capsaicin and its derivatives in different cell lines and rodent models are contradictory. Indeed, concentration-dependent cytotoxic effects have been observed in several cancer cell lines (Chel-Guerrero et al., 2022) but proliferation has been even slightly stimulated in other cell lines. The effects of different capsaicin derivatives on cell viability may therefore vary widely (Santos et al., 2023). *Capsicum* extracts containing diverse plant metabolites are more cytotoxic than capsaicin alone (Chilczuk et al., 2021; Hernández-Pérez et al., 2020). *Capsicum* extracts are already used as topical applications and novel topical products are under development (Truong et al., 2022).

To the best of our knowledge, this is the first time that the toxicity of *C. chinense* extracts has been tested against BALB/c 3T3 and HaCaT cells. The BALB/c 3T3 cell line was chosen as the standard test system recommended by *in vitro* safety testing protocols, including OECD guidelines. In a study with the mouse embryonic fibroblast cell line NIH 3T3, *C. annuum* methanolic extracts showed no evidence of cytotoxicity in the concentration range 0.125–1.0 mg/mL whereas concentration-dependent cytotoxicity was observed in lung adenocarcinoma cell line A549 (Hu et al., 2021). When IC_{50} values are used for calculations, a therapeutic index >1.0 indicates low cytotoxicity and the higher the therapeutic index the safer the ingredient (Muller and Milton, 2012). Although the therapeutic indices in this study were relatively low, our *C. chinense* cell culture extracts appear to confer antimicrobial activity at doses that do not affect the viability of mammalian cells.

3.4.4. Phototoxicity

Extracts of the cells grown under P conditions showed no phototoxic effect against BALB/c 3T3 cells within the concentration range 0.156–5 mg/mL, with cell viabilities remaining between 81.01% and 106.08% relative to irradiated controls (Fig. 9). However, cell viability in the presence of the extract was slightly higher than controls, especially at low concentrations, perhaps reflecting the antioxidant activity of the extract being more pronounced under UV induced stress. In contrast, the extracts prepared from cells grown under D conditions were phototoxic at concentrations above 2.5 mg/mL (Fig. 9). At lower extract concentrations (0.078–1.25 mg/mL), cell viability varied between 80.64% and 100.65% relative to irradiated controls. Reduced cell viability was also observed in non-irradiated cells pre-incubated with the highest concentration of the extract. Cell viability was slightly higher in the presence of low concentrations of the D culture extract, again probably due to its antioxidant activity.

Both extracts had similar effects when incubated with HaCaT cells. The D culture extract was phototoxic at concentrations above 2.5 mg/mL, but cell viability was higher in irradiated cells that had been pre-incubated with the P culture extract compared to irradiated controls. Interestingly, phenolic glycosides (e.g., sinapoyl-glucosides) extracted from peppers protect lymphocytes from damage caused by X-rays due to their antioxidant activity (Materska et al., 2015) and these compounds may also contribute to the UV-protective effect of our extracts. Capsiate, which has similar biological effects to capsaicin but is non-pungent and does not induce irritation, has been shown to inhibit UV-induced inflammatory responses in HaCaT cells as well as angiogenesis *in vitro* and *in vivo* (Lee et al., 2010). The skin-related positive effects of capsiate have also been shown in studies with immune cells and atopic dermatitis models (Lee et al., 2015). Phenolamides, including *N*-caffeoyl-putrescine, are present in our cell culture extracts and might be associated with the low phototoxicity toward BALB/c 3T3 cells and even the increased viability of HaCaT cells after UV treatment given previous studies reporting the protective effects of putrescine derivatives in HaCaT cells (Kim et al., 2023; Lee et al., 2010).

4. Conclusions

The cosmetic industry is responding to consumer demands for safe and sustainably produced ingredients with proven efficacy by focusing on bioactive compounds produced using plant cell suspension cultures. *Capsicum* species produce a broad spectrum of

Table 2
IC_{20} and IC_{50} values, and therapeutic indices (TI), of *C. chinense* cell culture extracts following growth under photoperiodic (P) or dark (D) conditions. Therapeutic indices were calculated from $IC_{20,}$ IC_{50} and MIC (minimal inhibitory concentration) values against *Staphylococcus aureus.* Data are means ± SD (n = 3).

	BALB/c 3T3				HaCaT			
	IC_{50} mg/mL	IC_{20} mg/mL	TI IC_{50}/MIC	TI IC_{20}/MIC	IC_{50} mg/mL	IC_{20} mg/mL	TI IC_{50}/MIC	TI IC_{20}/MIC
Photoperiod	5.71 ± 0.16	2.31 ± 0.03	1.43 ± 0.04	0.58 ± 0.01	4.03 ± 0.17	1.07 ± 0.29	1.01 ± 0.04	0.27 ± 0.07
Dark	4.20 ± 0.05	1.24 ± 0.06	1.05 ± 0.01	0.31 ± 0.01	5.37 ± 0.11	2.49 ± 0.21	1.34 ± 0.03	0.62 ± 0.05

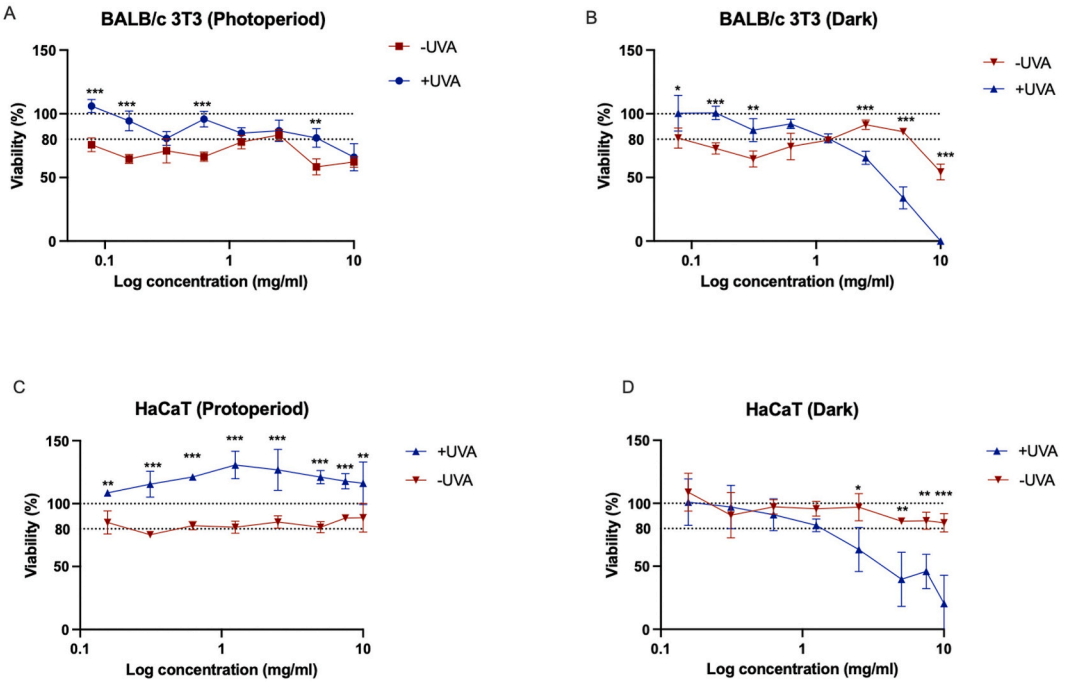

Fig. 9. Phototoxicity of *C. chinense* cell culture-derived extracts in (**A, B**) BALB/c 3T3 and (**C, D**) HaCaT cell cultures 24 h after UVA irradiation. Cell viability data are means ± SD based on neutral red uptake assays (n = 3, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-way ANOVA).

bioactive compounds with properties relevant for cosmetic products, including antioxidant and antimicrobial activities. We induced callus from *C. chinense* cv. Trinidad Pimento leaf tissue and established cell suspension cultures that were scaled up for biomass production. The cells were cultivated in darkness (D) or with a photoperiodic light source (P) and we prepared hydroethanolic and ethyl acetate extracts to allow the analysis of a broad range of diverse metabolites. Comprehensive untargeted metabolomic profiling followed by PCA clearly separated the extracts according to the cell growth conditions and revealed phenolamides and fatty acids were the most abundant compounds in our extracts. Targeted analysis, focusing on metabolic classes widely reported in *Capsicum* extracts, identified capsiate, dihydrocapsiate and nordihydrocapsiate as key bioactive products, along with 18 different carotenoids, dominated by the colorless compound phytoene. The hydroethanolic extracts were subject to a range of bioactivity assays, revealing moderate antioxidant activity and the ability to inhibit the growth of *S. aureus*, including MRSA. There was no evidence of cytotoxicity toward BALB/c 3T3 and HaCaT cell lines at concentrations below 1.25 mg/mL, and no evidence of phototoxicity in the same cells at concentrations below 1.25 mg/mL and 2.5 mg/mL, respectively.

Our findings provide valuable insights into the dynamics of biomass accumulation and the synthesis of bioactive metabolites as adaptive responses of *C. chinense* cv. Trinidad Pimento cell cultures to different cultivation conditions. This will facilitate the development of *Capsicum* cell suspension cultures as an inexpensive and sustainable source of safe and efficacious ingredients for the cosmetics industry. However, our study addressed only a limited range of cultivation conditions and it is likely that the testing of a broader range of parameters would yield extracts with unique metabolic profiles and bioactive properties. In terms of industrial translation, it is important to note that the ability of cell cultures to produce valuable ingredients in larger quantities and with greater consistency than cultivated plants must be traded off against the higher overall production costs, meaning that detailed cost-benefit analysis is necessary. On the same topic, it may also be challenging to scale up our cell suspension cultures while maintaining the same metabolic profiles and bioactive properties, and this work is currently underway. Several companies, including Vytrus Biotech, Innova BM, Arterra Bioscience, and Lumene Oy, have developed natural active ingredients derived from plant stem cells, and in some cases directly incorporate plant cell cultures into their products, offering innovative solutions for skin and hair care based on large-scale plant cell cultivation. The incorporation of plant cells or their extracted compounds into cosmetic products also requires standardized downstream processes that ensure the stability and activity of plant-derived ingredients over the anticipated shelf-life of cosmetic products.

CRediT authorship contribution statement

Amir Akhgari: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Maria Sulli:** Writing – review & editing, Methodology, Investigation. **Anna Ramata-Stunda:** Methodology, Investigation. **Suvi T. Häkkinen:** Methodology, Investigation. **Liisa Nohynek:** Methodology, Investigation. **Aleksander Salwinski:** Methodology, Investigation. **Olivia Costantina Demurtas:** Methodology, Investigation. **Mārtiņš Borodušķis:** Methodology, Investigation. **Maria Pajumo:** Methodology,

Investigation. **Richard M. Twyman:** Writing – review & editing. **Kirsi-Marja Oksman-Caldentey:** Writing – review & editing, Supervision, Funding acquisition. **Gianfranco Diretto:** Writing – review & editing, Supervision. **Heiko Rischer:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

None of the authors has any conflict of interests regarding this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2024.103478.

Data availability

Data will be made available on request.

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