

Article

Salt Elicitation of *Centella asiatica* Enhances Antimicrobial and Keratinocyte Wound-Closure Activity: From Germplasm Screening to Nicotinamide Accumulation

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Abstract

Centella asiatica (L.) Urb. is valued for skin repair. Salt (NaCl) treatment is an inexpensive way to alter its metabolome, but its effect on bioactivity across germplasm is unknown. Thirty-nine Thai accessions were grown with or without 100 mM NaCl for 10 days, and leaf extracts were screened against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans* using a growth ratio (GR), growth with the NaCl extract relative to the untreated one. The most responsive accession, PHE, was profiled by untargeted LC-HRMS and tested in kinetic growth, HaCaT viability, and scratch-wound closure assays. NaCl extracts reduced bacterial growth to 0.88 (*E. coli*) and 0.90 (*S. aureus*) of the untreated level, but responses were genotype-dependent. In PHE, nicotinamide was the most strongly induced metabolite (9.3-fold by LC-HRMS, 9.6-fold by HPLC). The NaCl extract inhibited *E. coli* by 87% (untreated extract 33%), was non-cytotoxic, increased keratinocyte metabolic activity, and raised scratch-wound closure to 84% of the positive control. Pure nicotinamide reproduced this closure (83%), but only at a concentration far above its content in the extract. Salt elicitation therefore enhances antimicrobial and wound-closure activity in responsive genotypes, with nicotinamide as a marker of the response and a plausible but not sole contributor.

Keywords: *Centella asiatica*; NaCl elicitation; untargeted metabolomics; nicotinamide; antimicrobial activity; wound closure



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1. Introduction

Centella asiatica (L.) Urb. (Apiaceae), known in Thailand as Bua Bok, is one of the most widely used medicinal herbs of tropical Asia and a common ingredient of skin-care products. Its reputation rests largely on the pentacyclic triterpenoids asiaticoside, madecassoside, asiatic acid and madecassic acid, which stimulate collagen synthesis, keratinocyte migration and wound closure in cellular and animal models [1,2]. These properties underlie the wide use of the plant in dermatology and cosmetology [3]. Extracts of the plant also inhibit

a range of bacteria and fungi, an activity attributed to triterpenoid acids and phenolic constituents [4,5]. Because wound infection and delayed re-epithelialisation are linked problems, an extract that combines antimicrobial and keratinocyte-stimulating properties would be particularly useful for topical applications.

The chemical composition of a medicinal plant is dynamic and can be substantially influenced by environmental conditions. Abiotic stress redirects carbon and nitrogen into defensive and protective metabolites, and mild salinity is one of the cheapest stresses that can be applied under cultivation [6–8]. In *C. asiatica*, NaCl increases centelloside and phenolic contents and antioxidant capacity while the plant tolerates concentrations up to 100 mM [9,10]. We recently showed, in the same accession panel used here, that a short 100 mM NaCl treatment for 6 days enhances phytochemical accumulation and antioxidant activity in a genotype-dependent manner [11]. Salt stress also triggers the accumulation of compatible solutes such as glycine betaine and proline and reorganises NAD and amino acid metabolism, changes that are usually studied as markers of tolerance rather than as sources of bioactivity [12,13].

Thai *C. asiatica* germplasm is chemically and morphologically diverse, and accessions differ several-fold in centelloside content and biomass [14,15]. This diversity implies that the response to an elicitor will also vary, so that a single accession cannot represent the species. A screen across many accessions is needed to identify genotypes in which salt elicitation actually improves the desired bioactivity, before investing in metabolomic and functional characterisation of a selected line.

Three research gaps still remained. The effect of salt elicitation on the antimicrobial activity of *C. asiatica* extracts, and the consistency of that effect across germplasm, had not been examined. The metabolites induced in a responsive accession, and their contribution to any gain in activity, were unknown. It was also unclear whether an elicited extract retains the keratinocyte-stimulating properties expected of *C. asiatica* or whether the stress-altered chemistry compromises them. Previous studies of salt-treated *C. asiatica* measured bulk phytochemical indices rather than bioactivity, and none linked a germplasm screen to metabolomics and functional assays.

Here we addressed these questions in four linked steps. We screened extracts of 39 Thai accessions grown with or without NaCl against *E. coli*, *S. aureus*, and *C. albicans*, using a growth ratio that expresses the effect of elicitation within each accession. We then profiled the metabolome of the most responsive accession, PHE, by untargeted LC-HRMS, examined the changed metabolites both as fold changes and as shares of the total signal, and quantified nicotinamide in the extracts by HPLC. We tested the hypothesis emerging from the metabolomics, namely that nicotinamide contributes to the enhanced activity, by comparing the NaCl extract, the untreated extract, and pure nicotinamide in 24 h growth kinetics. Finally, we evaluated cytotoxicity, metabolic activity, and scratch-wound closure in HaCaT keratinocytes. The results show that salt elicitation enhances two distinct bioactivities in a responsive accession of *C. asiatica*, and that nicotinamide, although strongly induced, is a marker and plausible contributor rather than the sole cause of either.

2. Results

2.1. NaCl Elicitation Shifts Extracts from Growth Promotion Toward Inhibition

In the 4 h antimicrobial screening, extracts of untreated plants tended to promote rather than inhibit microbial growth, and elicitation removed this effect (Figure 1). The pattern was clearest for the two bacteria. Untreated extracts stimulated *E. coli* (Figure 1a) and *S. aureus* (Figure 1b) by a mean of 14 to 15%, whereas NaCl extracts of the same accessions were neutral, and the number of accessions with net inhibition roughly tripled for *E. coli* (7 to 22) and nearly doubled for *S. aureus* (11 to 19). For *C. albicans* (Figure 1c) the

responses of individual accessions were much more scattered in both directions, and the condition means did not differ.

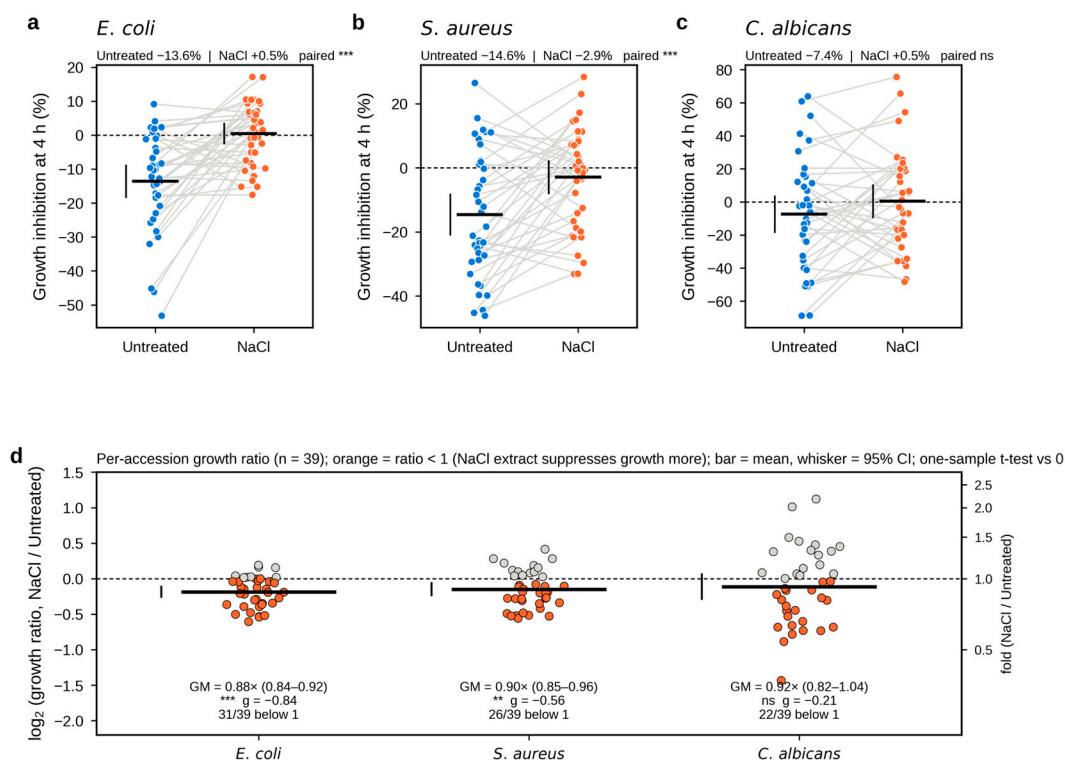


Figure 1. Effect of NaCl elicitation on the antimicrobial activity of extracts from 39 *C. asiatica* accessions. (a–c) Growth inhibition (%) at 4 h by extracts of untreated (blue) and NaCl-treated (orange) plants for *E. coli*, *S. aureus*, and *C. albicans*. Points are accession means (Grubbs-filtered, $n = 4$ to 5 replicates), grey lines connect the two conditions of the same accession, the black bar and whisker show the mean and 95% CI, and the dashed line marks 0% (no effect). Headings give the condition means, and the paired test is on accession means. (d) Growth ratio (NaCl extract/untreated extract) per accession on a log₂ scale with the fold scale on the right. Orange points indicate GR < 1 and grey points GR ≥ 1; the black bar and whisker show the geometric mean (GM) with 95% CI, and the dashed line marks GR = 1. g, Hedges' effect size, from a one-sample test against zero. *** $p < 0.001$, ** $p < 0.01$, ns not significant.

To express the effect of elicitation within each accession, we used the growth ratio (GR), the growth of the microorganism with the NaCl extract relative to that with the untreated extract of the same plant, so that GR < 1 indicates stronger suppression after elicitation (Figure 1d). Confidence intervals (CI) and false discovery rates (FDR) were used throughout to summarise uncertainty. NaCl extracts reduced bacterial growth to about nine tenths of the untreated level, with a large effect size for *E. coli* (geometric mean GR 0.88, Hedges' $g = -0.84$) and a moderate one for *S. aureus* (0.90, $g = -0.56$), and most accessions fell below unity (31 and 26 of 39). For *C. albicans* the mean shift was small and not significant (0.92, 22 of 39 below unity) because accessions responded in opposite directions. These conclusions held for every statistical approach applied (Table S2) and were unaffected by whether outliers were removed (Figure S2, Table S6).

2.2. The Response to Elicitation Is Accession Dependent and Identifies PHE as the Most Responsive Line

Behind the overall shift lay a wide spread of individual responses (Figure 2). For *E. coli* (Figure 2a) more than one third of the accessions responded significantly, all in the direction of stronger suppression (GR 0.66 to 0.92), and a single accession (TW) responded

in the opposite direction. For *S. aureus* (Figure 2b), only PLK and NBP responded significantly, and TW again reversed, which marks TW as a genotype in which elicitation weakens antibacterial activity. For *C. albicans* (Figure 2c), no accession reached significance after correction, yet the growth ratios spanned a six-fold range (0.37 in PHE to 2.18 in LPG), which shows that the absence of a mean effect reflects opposing responses rather than insensitivity.

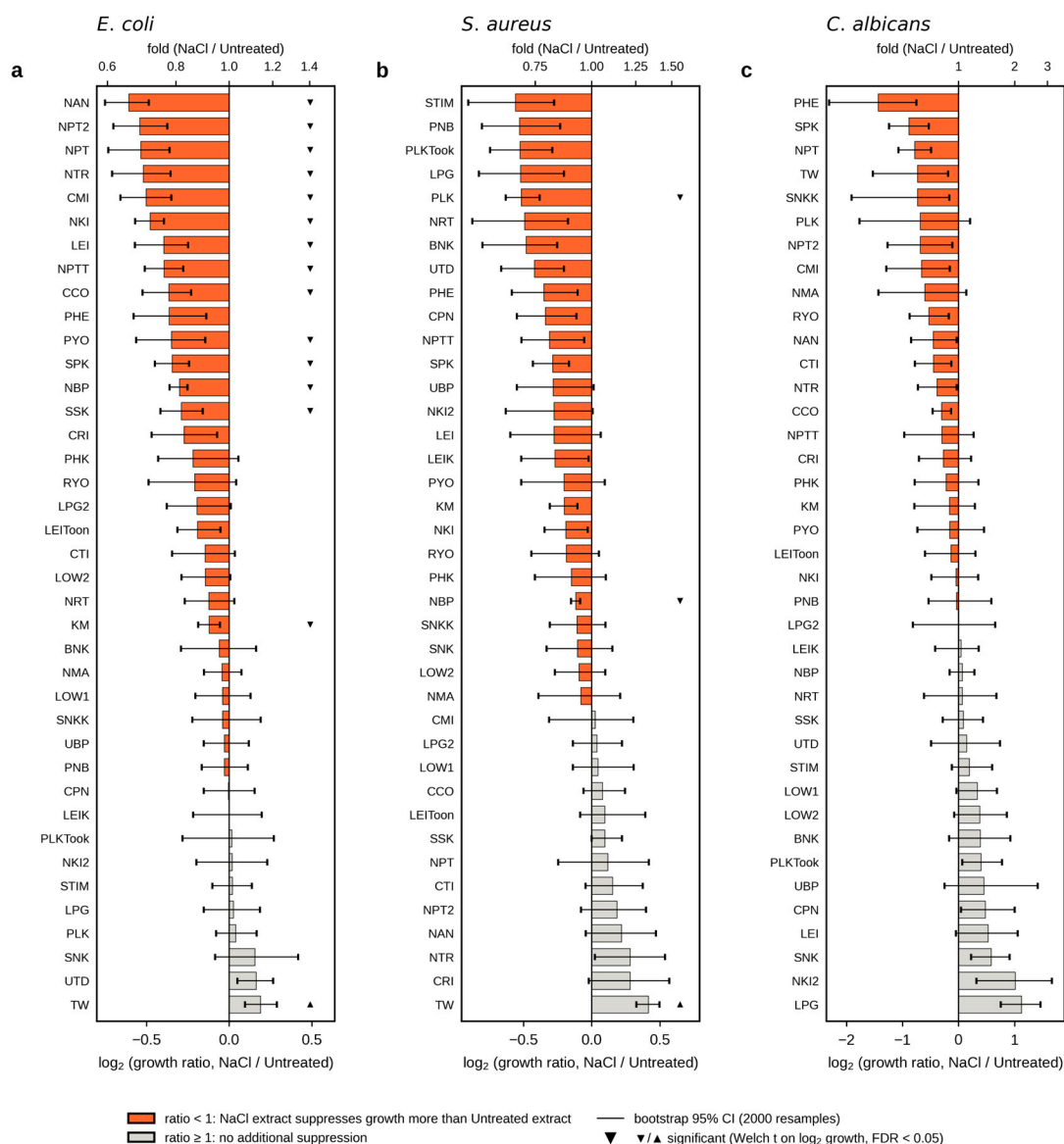


Figure 2. Growth ratio (NaCl extract/untreated extract) of each of the 39 accessions for (a) *E. coli*, (b) *S. aureus* and (c) *C. albicans*, ordered from the strongest to the weakest suppression by the NaCl extract. Bars show log₂GR with the fold scale above and bootstrap 95% CI (2000 resamples). Orange, GR < 1 (stronger suppression by the NaCl extract), and grey, GR ≥ 1. Triangles mark accessions with FDR < 0.05 (Welch *t*-test on log₂ growth with Benjamini and Hochberg correction within organism).

Clustering of the three-organism profiles (Figure 3a) separated accessions that responded mainly for *E. coli* and *C. albicans* from those that responded for *S. aureus*, in which the yeast response was often reversed. Averaging the responses across organisms (Figure 3b) placed PHE clearly first (mean log₂GR −0.72, equivalent to 0.61-fold growth), followed by SPK, NPT, PLK, CMI and NPT2. Ranking by activity under NaCl alone (Figure 3c) also placed PHE first, followed by PLK, NPTT and SPK. The two rankings

differed for accessions such as NPT and CMI, whose high ratio-based scores arose because their untreated extracts were strongly growth-promoting while their NaCl extracts were merely neutral. PHE was the only accession ranked first by both criteria, and its extract inhibited all three organisms after elicitation (66% inhibition of *C. albicans* and about 20% of each bacterium), so it was selected for the subsequent work.

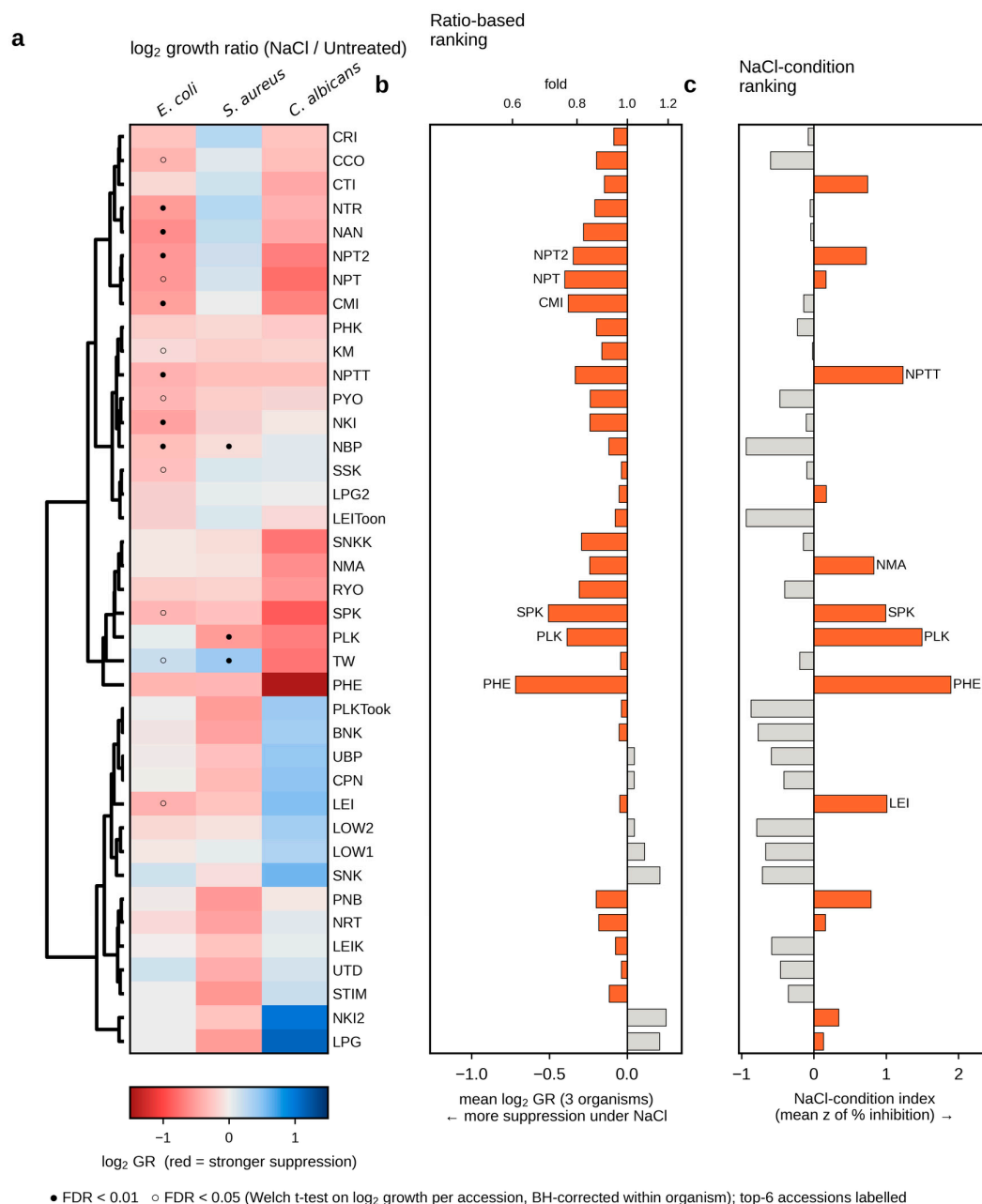


Figure 3. Ranking of accessions by the response to elicitation. (a) Heat map of $\log_2$ GR for the 39 accessions and three microorganisms with hierarchical clustering. Red indicates stronger suppression by the NaCl extract. Filled and open circles mark FDR < 0.01 and < 0.05. (b) Ratio-based ranking, the mean $\log_2$ GR over the three organisms, and (c) NaCl-condition ranking, the mean z-score of % inhibition under NaCl. The six leading accessions are labelled in each ranking.

2.3. Gram-Negative and Gram-Positive Responses Are Inversely Related

The three organisms did not respond to elicitation in parallel (Figure 4). Under untreated conditions (Figure 4a), accessions active against *E. coli* were also active against *C. albicans* ($r = 0.55$), whereas under NaCl (Figure 4b), the strongest association was between

S. aureus and *C. albicans* ($r = 0.62$). The growth ratios (Figure 4c) showed the underlying structure. The *E. coli* and *S. aureus* responses were negatively correlated (Figure 4d, $r = -0.42$), the *E. coli* and *C. albicans* responses were positively correlated (Figure 4e, $r = 0.44$), and the *S. aureus* and *C. albicans* responses were again negatively correlated (Figure 4f, $r = -0.38$). Accessions whose elicited extracts gained activity against the Gram-negative bacterium therefore tended to lose activity against the Gram-positive one, which points to different induced constituents acting on the two bacteria.

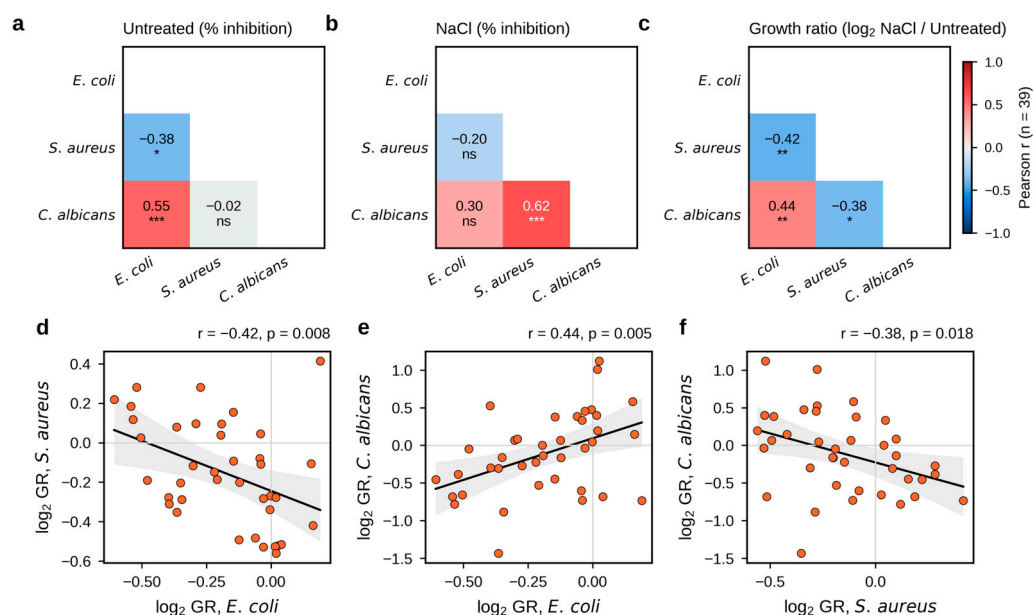


Figure 4. Correlations between organisms. (a–c) Pearson correlation matrices for % inhibition under untreated and NaCl conditions and for log₂GR ($n = 39$ accessions). Cell colour indicates the correlation coefficient (r) according to the colour bar, and the symbol below each coefficient gives the significance of the correlation: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns not significant. (d–f) Scatter plots of log₂GR between organism pairs with the regression line and 95% confidence band.

2.4. Untargeted Metabolomics of Accession PHE: Nicotinamide Is the Most Strongly Induced Metabolite

Leaf extracts of untreated and NaCl-treated PHE plants ($n = 3$) were profiled by LC-HRMS in positive ionisation mode. A total of 783 features were annotated. A plausibility filter first removed annotations attributable to microbial, synthetic or animal sources (Table S13), and a stricter filter then retained features annotated as known plant or primary metabolites or matching a plant flavonoid database, leaving 126 plant-derived metabolites after removal of duplicate annotations. All results below refer to this set after probabilistic quotient normalisation (PQN).

Elicitation reorganised the metabolome extensively. The two conditions separated completely along the first principal component, which captured three-quarters of the variance (Figure 5a), and about 30% of the metabolites changed by at least two-fold (37 of 126, 14 increased and 23 decreased, Figure 5b and Table S7). Nicotinamide, whose identity was confirmed with an authentic standard, showed by far the largest increase (9.3-fold, confirmed by targeted HPLC quantification, Section 2.5), followed by the osmoprotectant glycine betaine (5.1-fold). Smaller increases occurred among phenolics, including the flavonol glucuronide miquelianin, a dicaffeoylquinic acid isomer and a pentahydroxyflavone isomer (2- to 3-fold each). The decreased group was dominated by nitrogen-containing primary metabolites and their derivatives, including pipercolic acid, N-acetylornithine, the dipeptide alanyl-proline and, with lower annotation confidence, aspartate and adenosine (0.16- to 0.47-fold), together with abscisic acid and raffinose. The

heat map (Figure 5c) shows that these changes were consistent across replicates, and two-thirds of them were also significant on raw, un-normalised peak areas. Several further features annotated as alkaloids of other plant families also changed, but because their spectral matches were weak or the compounds are not known from *C. asiatica*, they are listed in Table S7 without interpretation.

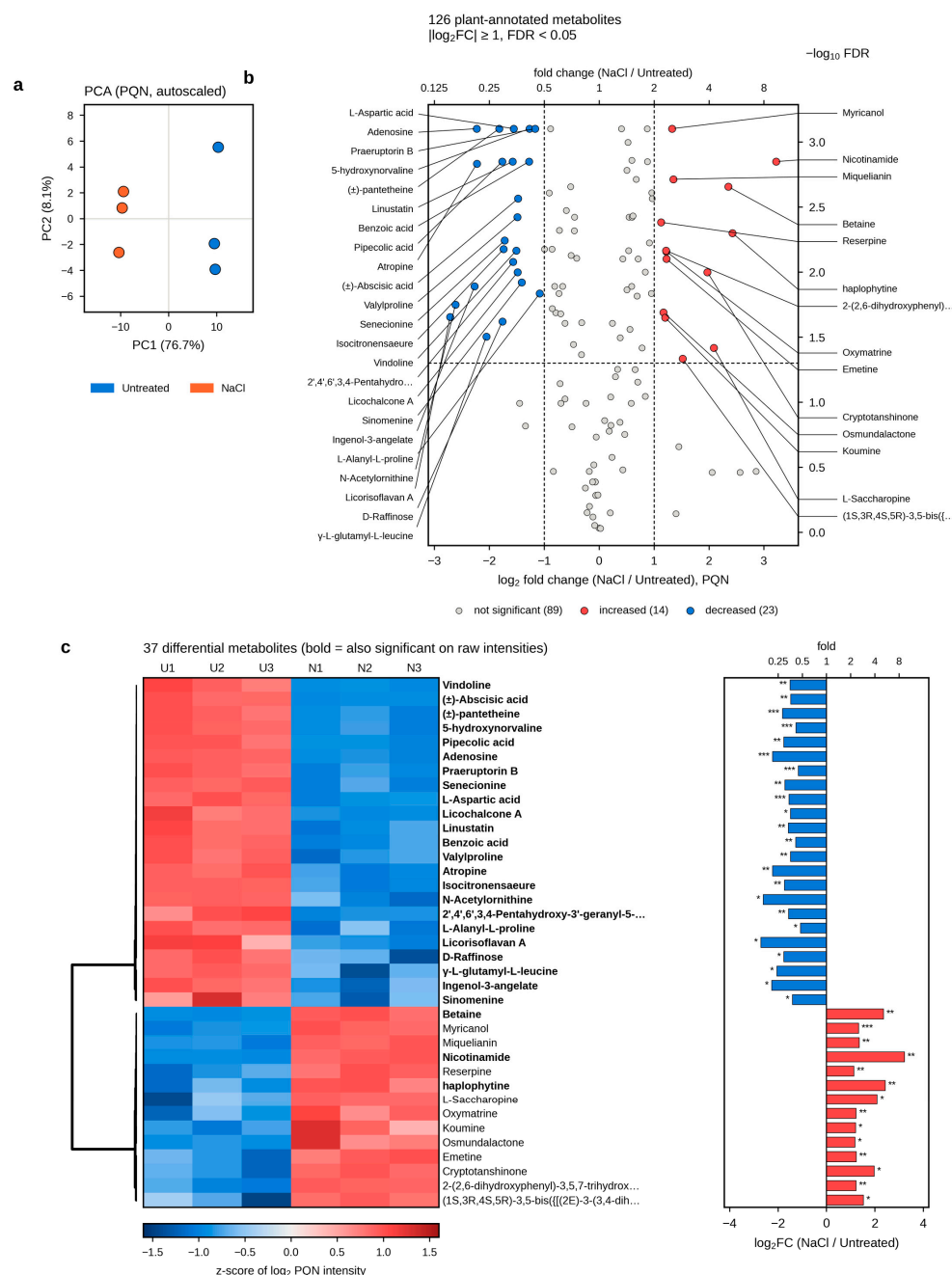


Figure 5. Untargeted metabolomics of accession PHE. (a) PCA of autoscaled log₂ PQN intensities of the 126 plant-derived metabolites. (b) Volcano plot of log₂ fold change (NaCl/untreated) against $-\log_{10}$ FDR. Red, increased, and blue, decreased, at $|\log_2\text{FC}| \geq 1$ and $\text{FDR} < 0.05$, with the fold scale above. Significant metabolites are labelled outside the plot. (c) Heat map of z-scored log₂ intensities of the 37 differential metabolites (U1 to U3 untreated, N1 to N3 NaCl) with hierarchical clustering, and log₂ fold change with FDR asterisks on the right. Bold names were also significant on raw intensities. Asterisks indicate the significance of the difference between NaCl and untreated extracts (Welch's *t*-test with FDR correction): *** $\text{FDR} < 0.001$, ** $\text{FDR} < 0.01$, * $\text{FDR} < 0.05$. Long systematic names are truncated (...); full names are given in Table S7.

2.5. Nicotinamide Is Strongly Induced but Remains a Minor Constituent of the Extract by Mass

Because a large fold change in a trace compound has little practical meaning, each metabolite was also expressed as its share of the total plant-derived LC-HRMS signal (Figure 6). Most differential metabolites were minor contributors to the signal, but nicotinamide, glycine betaine, pipecolic acid and adenosine were exceptions, being both prominent in signal and strongly changed (Figure 6a). At the class level (Figure 6b), elicitation increased the share of alkaloids and other nitrogen-containing metabolites (46% to 54% of the signal) at the expense of sugars, flavonoids and nucleoside-like compounds, and this class was also over-represented among the differential metabolites (odds ratio 3.4, Figure S4 and Table S8). Among the ten metabolites with the largest signal (Figure 6c), nicotinamide rose from 1.4% to 10.3% of the signal and glycine betaine from 3.8% to 14.9%, whereas pipecolic acid, adenosine and γ -aminobutyric acid, which had dominated the untreated extract, fell to a fraction of their former share. Signal shares depend on ionisation efficiency and do not translate into mass. To place the nicotinamide response on an absolute scale, the compound was quantified in the crude extracts by HPLC against an authentic standard (Table S14). Nicotinamide increased from 0.25 ± 0.03 mg/g in the untreated extract to 2.43 ± 0.12 mg/g in the NaCl extract (9.6-fold, Welch's *t*-test $p < 0.001$, $n = 3$), in close agreement with the LC-HRMS fold change. In absolute terms, however, nicotinamide made up only 0.24% (*w/w*) of the elicited extract, so its LC-HRMS signal share overstated its mass abundance by roughly 40-fold. Elicitation thus strongly and reproducibly induced nicotinamide, but the compound remained a minor constituent of the extract by mass.

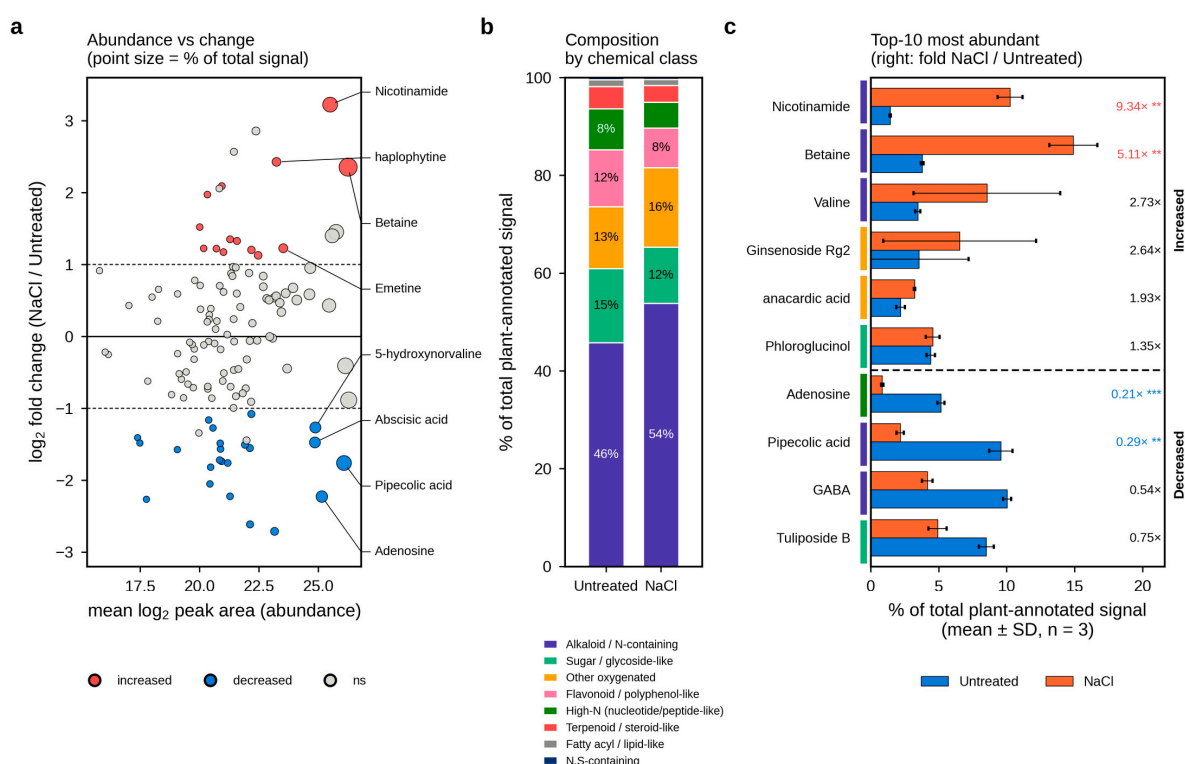


Figure 6. Abundance of plant-derived metabolites in PHE extracts. (a) Mean log₂ peak area against log₂ fold change, with point size proportional to the share of the total signal. (b) Composition of the plant-derived signal by chemical class in untreated and NaCl extracts. (c) The ten metabolites with the largest share of the signal, split into increased (top) and decreased (bottom) groups, with the fold change on the right. Coloured ticks give the chemical class as in (b). Mean $\pm$ SD, $n = 3$. Asterisks indicate the significance of the difference between NaCl and untreated extracts (Welch's *t*-test with FDR correction): *** FDR < 0.001, ** FDR < 0.01.

2.6. Pathway View: NAD Salvage and Glycine Betaine Synthesis

Placing the changed metabolites on their pathways gave a coherent picture (Figure 7). In the NAD pathway (Figure 7a), the de novo precursor aspartate declined while the salvage-cycle intermediates nicotinamide and, to a lesser and non-significant extent, nicotinic acid rose, a pattern consistent with, although not proof of, accelerated NAD⁺ turnover in which nicotinamide is released faster than it is recycled. In the glycine betaine pathway (Figure 7b), choline was unchanged while glycine betaine accumulated, a pattern compatible with increased conversion of choline to glycine betaine by choline monooxygenase and betaine aldehyde dehydrogenase. Phosphorylated intermediates and NAD⁺ itself are poorly retained under the reversed-phase conditions used and were not detected.

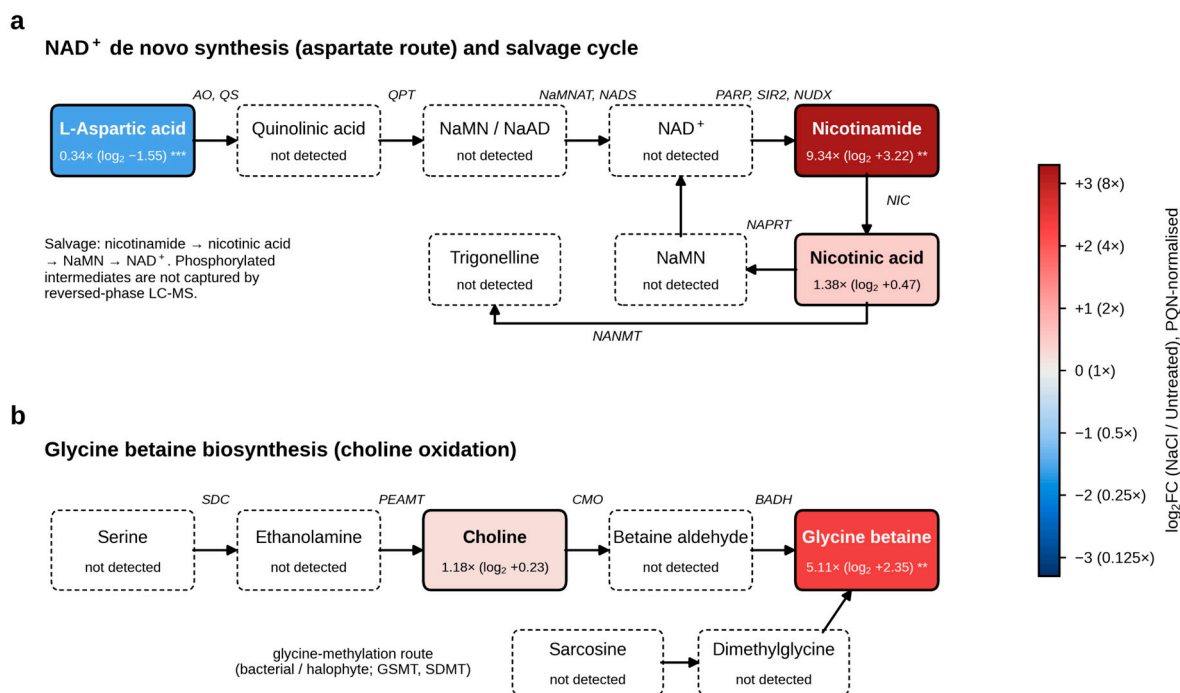


Figure 7. Pathway context of the two most strongly induced metabolites. (a) NAD⁺ de novo synthesis from aspartate and the nicotinamide salvage cycle. (b) Glycine betaine synthesis by choline oxidation. Arrows indicate the direction of enzymatic conversion between metabolites, with the enzymes shown in italics beside each arrow. Solid boxes, detected metabolites coloured by log₂ fold change (NaCl/untreated) with the fold and FDR shown; *** FDR < 0.001, ** FDR < 0.01. Dashed boxes, not detected.

2.7. Kinetic Validation: The NaCl Extract Is a Stronger Bacterial Growth Inhibitor than the Untreated Extract or Nicotinamide Alone

Because the 4 h screen captures only an early effect on a dense inoculum, the PHE extracts and pure nicotinamide were compared over a full 24 h growth cycle at 10 mg/mL by optical density at 600 nm (OD₆₀₀), with growth summarised as the area under the curve (AUC) (Figure 8). All treatments and the negative control contained the same final DMSO concentration (1%, v/v), so the negative control served as the vehicle control.

Against *E. coli* (Figure 8a,d) the three treatments produced markedly different growth profiles. The NaCl extract maintained *E. coli* growth close to baseline throughout the 24 h period, whereas the untreated extract permitted approximately half of the growth observed in the negative control. In contrast, nicotinamide exhibited a growth profile closely comparable to that of the negative control. Based on AUC analysis, the NaCl extract produced 87% growth inhibition, compared with 33% inhibition for the untreated extract, while nicotinamide showed no inhibitory activity. Kanamycin, used as the positive control

for this ampicillin-resistant strain, suppressed growth by 94%, and the inhibition produced by the NaCl extract did not differ significantly from that of the antibiotic (Tukey HSD).

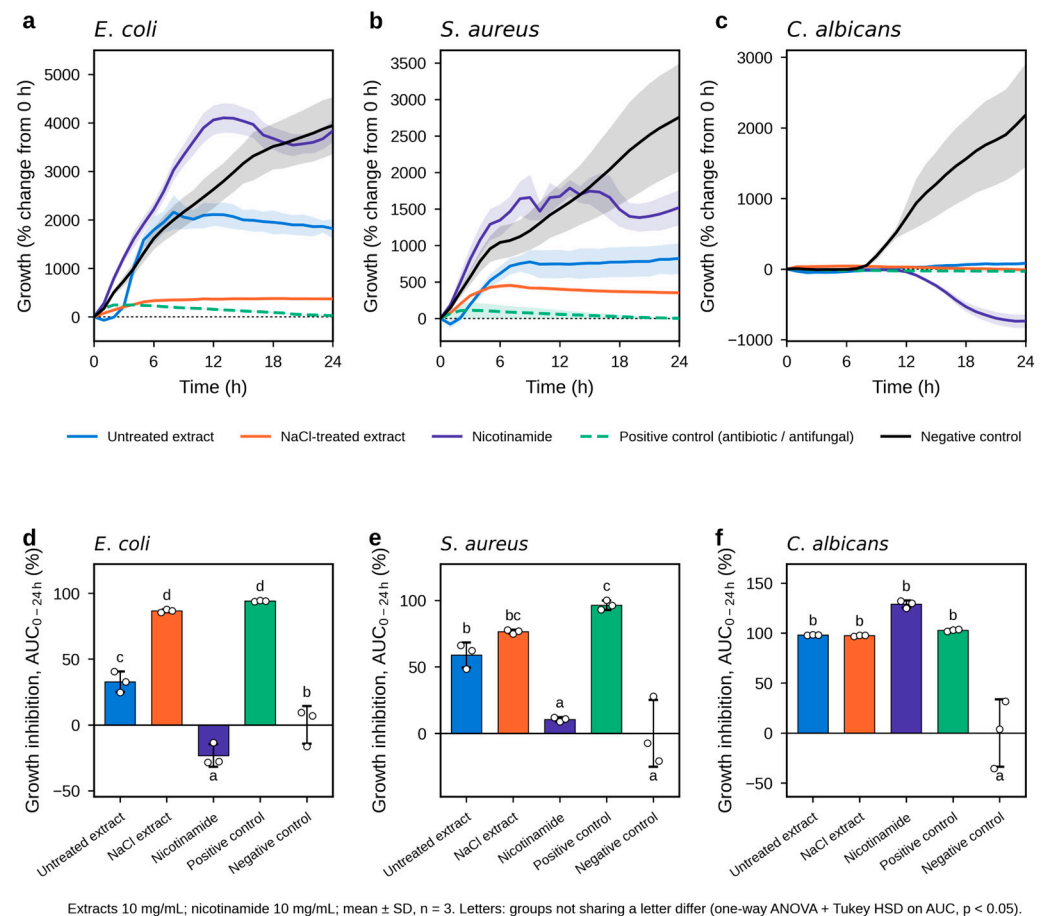


Figure 8. Growth kinetics of *E. coli*, *S. aureus* and *C. albicans* with PHE extracts and nicotinamide. (a–c) OD₆₀₀ expressed as % change from 0 h over 24 h, mean with SD band, $n = 3$. (d–f) Growth inhibition calculated from the AUC (0 to 24 h) relative to the negative control. Extracts and nicotinamide at 10 mg/mL. Positive controls: kanamycin 20 μ g/mL for *E. coli*, amoxicillin 20 μ g/mL for *S. aureus* and ketoconazole 50 μ g/mL for *C. albicans*. DMSO 1% (v/v) in all treatments and in the negative (vehicle) control. Letters indicate groups that differ (one-way ANOVA with Tukey HSD, $p < 0.05$).

Against *S. aureus* (Figure 8b,e), the NaCl extract again showed stronger antibacterial activity than the untreated extract, with 76% versus 59% inhibition based on AUC. Nicotinamide showed a distinct time-dependent effect. Growth initially tracked the negative control but plateaued after approximately 12 h, whereas the control continued to increase. Thus, nicotinamide produced only 11% inhibition based on AUC but reached 45% inhibition at 24 h. Amoxicillin completely suppressed *S. aureus* growth, confirming its effectiveness as a positive control. Against *C. albicans* (Figure 8c,f), both extracts almost completely suppressed growth, with activity comparable to ketoconazole. In contrast, nicotinamide caused OD₆₀₀ to fall below the initial baseline from approximately 12 h onward, suggesting loss of viable yeast biomass through cell lysis rather than simple growth inhibition.

2.8. The NaCl Extract Is Non-Cytotoxic, Increases HaCaT Metabolic Activity and Accelerates Scratch-Wound Closure, an Effect Reproduced by Nicotinamide

None of the treatments was cytotoxic to HaCaT keratinocytes at any concentration up to 500 μ g/mL in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 9a). Instead, the NaCl extract increased MTT reduction in a bell-shaped manner, with the signal rising to a plateau of about 180% of untreated cells at 100 to

250 µg/mL before declining toward baseline at the highest dose. Because MTT reduction reflects reductase activity as well as cell number, this increase is reported as metabolic activity rather than proliferation. The untreated extract showed the same shape at a lower amplitude (about 140%) that did not remain significant after correction, and nicotinamide had no effect at any concentration. At the peak concentrations, the NaCl extract exceeded both nicotinamide and the untreated extract (Table S11).

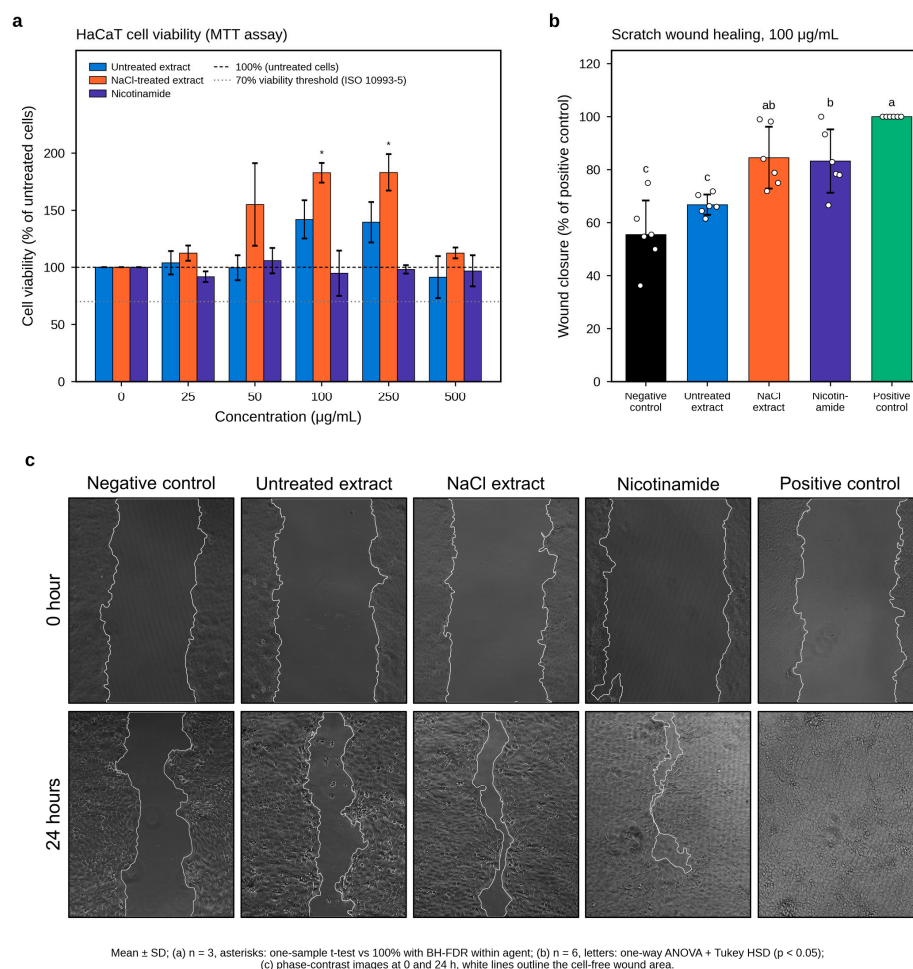


Figure 9. Effects of PHE extracts and nicotinamide on HaCaT keratinocytes. **(a)** Cell viability (MTT, % of untreated cells) after 24 h exposure to 0 to 500 µg/mL. The dashed line marks 100%, and the dotted line marks the 70% cytotoxicity threshold. Asterisks: one-sample t -test against 100% with FDR correction, $n = 3$. **(b)** Scratch-wound closure at 24 h as % of the positive control at 100 µg/mL, $n = 6$. DMSO 0.5% (v/v) in all wells, including the controls. Letters indicate groups that differ (ANOVA with Tukey HSD). **(c)** Representative phase-contrast images at 0 and 24 h. White lines outline the cell-free area.

In the scratch-wound closure assay, in which closure integrates cell migration and proliferation, treatment with the NaCl extract at 100 µg/mL, the concentration showing the strongest effect, significantly improved wound closure. Wound closure reached 84% of the positive control, compared with 55% in the negative control, and was not significantly different from the positive control (Figure 9b). The untreated extract showed only a small and non-significant improvement, reaching 67%. Nicotinamide at 100 µg/mL, a concentration about 400-fold higher than its content in 100 µg/mL of the NaCl extract (Section 2.5), produced a similar effect (83% closure), and both treatments were more effective than the untreated extract. The representative images (Figure 9c) show a smaller

remaining gap after 24 h in the NaCl extract and nicotinamide groups, compared with the wider gaps observed in the negative control and untreated extract groups.

3. Discussion

3.1. Salt Elicitation Converts Growth-Promoting Extracts into Inhibitory Ones in a Genotype-Dependent Way

The clearest effect of elicitation across the germplasm was not the appearance of a strong new antimicrobial activity but the disappearance of an old one: the tendency of untreated extracts to feed microbial growth. Methanol extracts of unstressed leaves carry a large load of sugars, free amino acids and nucleosides. When such an extract is added to a rich medium, it acts as a supplement rather than an inhibitor, and a dense inoculum grows faster than the vehicle control. Salt stress reversed this balance. Nutrient-like metabolites were drawn down, nitrogen-containing secondary metabolites accumulated, and the extract shifted from promoting to restraining bacterial growth. The average shift was modest, about one tenth of the untreated growth level, but in the most responsive accessions it reached one third, and the effect for *E. coli* was large by conventional standards and independent of how the data were treated.

The screening also makes clear why one accession cannot stand for the species. Responses ranged from strong suppression to outright reversal, and in one accession elicitation weakened activity against both bacteria. For *C. albicans*, the responsive and non-responsive genotypes cancelled each other, so that no overall effect was visible even though a strong anti-Candida response emerged in the most responsive line. This picture matches the genotype-dependent phytochemical response of the same panel to short-term salinity [11] and the chemotype diversity of Thai *C. asiatica* [15]. Genotype- and provenance-dependent adjustment of antioxidant enzymes and metabolites under salinity and drought has likewise been documented in woody and medicinal species such as argan (*Argania spinosa*), where seed origin and propagation method shape the stress response [16,17]. Germplasm-level variation in abiotic-stress adaptation is therefore a general feature, and elicitation protocols for medicinal plants should be developed on selected genotypes rather than on the species as a whole.

An unexpected feature of the screening was that the *E. coli* and *S. aureus* responses moved in opposite directions. Gram-negative and Gram-positive bacteria differ in their outer permeability barrier and in the classes of plant compounds that reach their targets [18]. A shift in the balance of induced metabolites within an accession could therefore favour activity against one group at the expense of the other. This pattern argues against a single broad-spectrum active principle and points instead to a mixture of constituents whose proportions vary among genotypes. The kinetic experiments with PHE support this interpretation.

3.2. Nicotinamide Marks a Nitrogen-Centred Metabolic Response

The metabolome of the PHE accession responded to NaCl in the way expected of a salt-stressed glycophyte. Glycine betaine accumulated while its precursor choline did not, a pattern consistent with increased conversion of choline through choline monooxygenase and betaine aldehyde dehydrogenase [12,19]. Glycine betaine stabilises proteins and membranes and supports osmotic adjustment [20], and its accumulation is associated with salinity resistance in other species [21]. Its role is therefore protective for the plant rather than antimicrobial. Bacteria themselves take up glycine betaine as an osmoprotectant [22], so it was not pursued as a candidate for the bioactivities described here.

Nicotinamide, whose identity was confirmed against an authentic standard, was the most strongly induced metabolite, and its rise was mirrored by a fall in aspartate,

the starting substrate of de novo NAD synthesis in plants. Together, these changes are consistent with accelerated NAD⁺ turnover, although steady-state metabolite levels cannot demonstrate flux, and NAD⁺ quantification, enzyme or transcript measurements or isotope tracing would be needed to establish it. Stress-activated NAD⁺-consuming enzymes such as poly(ADP-ribose) polymerases and sirtuins release nicotinamide, which is recycled through the salvage pathway or methylated to trigonelline [23,24]. Nicotinamide has long been regarded as an early signal of oxidative stress in plant cells and accumulates under UV-B and oxidative stress [25,26], and exogenous nicotinamide exerts antioxidative and DNA-hypomethylating effects in plant cells [27]. The parallel loss of adenosine, pipecolic acid, N-acetylornithine and dipeptides is compatible with the remobilisation of nitrogen from amino acid and nucleoside pools that accompanies salt acclimatisation [13]. The elicited metabolome is thus one in which nitrogen appears to have been redirected from storage and transport forms toward a small number of protective compounds.

Viewing the same changes as shares of the LC-HRMS signal, rather than as fold changes, suggested that the elicited extract had become dominated by a few nitrogen-containing compounds, but the targeted HPLC measurement put this picture into proportion. Nicotinamide accounted for about one tenth of the plant-derived signal yet for only 0.24% of the extract by mass, a 40-fold discrepancy that reflects the high ionisation efficiency of this small, basic molecule in positive-mode electrospray. Signal shares therefore describe relative composition and must not be read as mass abundance, and the same caution applies to glycine betaine and to the other compounds discussed here. The value of the HPLC data is twofold. It confirms the roughly ten-fold induction of nicotinamide by an independent method with an authentic standard, so that nicotinamide can serve as a robust chemical marker of the salt response in this accession, and it provides an absolute content (2.4 mg/g extract, about 0.2 mg/g dry leaf) against which elicited raw material can be standardised. Whether the induced nicotinamide is also large enough to affect bioactivity is a separate question, which the functional assays address below.

3.3. The Antibacterial Gain of the Elicited Extract Is Not Explained by Its Nicotinamide Content

The kinetic experiments answered the question raised by the metabolomics, namely how much of the enhanced activity nicotinamide alone can explain. Nicotinamide is an antimicrobial agent in its own right. It arrests the growth of *E. coli*, *S. aureus*, *Pseudomonas aeruginosa* and *C. albicans* by binding DNA and blocking cell division [28]. The concentration needed for complete inhibition is high, however, and it differs between species, being 30 mg/mL for *E. coli* and 25 mg/mL for *S. aureus*. Against *C. albicans*, the compound is far more potent in other test systems, with a half-maximal inhibitory concentration of about 2.4 mg/mL. Its antifungal action involves cell wall remodelling and inhibition of the NAD⁺-dependent histone deacetylase Hst3, and it is active against fluconazole-resistant isolates and in a mouse model of candidiasis [29,30].

The concentration used here, 10 mg/mL, therefore lay below the threshold for complete bacterial inhibition but several-fold above the anti-*Candida* threshold, and the results followed these thresholds closely. Nicotinamide drove the yeast OD below its starting value, which indicates lysis rather than growth arrest. Against the bacteria, its effect was partial and delayed. *S. aureus* grew normally at first and then levelled off in the second half of the growth cycle, whereas *E. coli* was not inhibited at all. Nicotinamide therefore does restrain the bacteria, but only late and only partly, exactly as expected of a sub-inhibitory dose.

The NaCl extract behaved very differently. At 10 mg/mL it contains only about 24 µg/mL nicotinamide, 400-fold less than the pure compound tested, yet it suppressed the growth of both bacteria throughout the growth cycle, and it did so far more strongly

than the untreated extract. The antibacterial gain must therefore come from other induced constituents, possibly acting together with nicotinamide at its low content, rather than from nicotinamide itself. Such combined action is common in plant extracts, in which different constituents act on different targets, improve one another's uptake, or block efflux and resistance mechanisms [31]. Alkaloid-type compounds were enriched among the induced metabolites, and this class is a well-documented source of antibacterial, antibiotic-enhancing and antivirulence activity [32]. The flavonol glucuronide and the dicaffeoylquinic acid that increased are also antibacterial in other systems [33]. *C. asiatica* extracts already inhibit Gram-negative and Gram-positive bacteria through triterpenoid acids and phenolics [5,34], and elicitation appears to add a set of nitrogen-containing metabolites to this existing repertoire. We refrain from naming specific alkaloids as active principles, because their annotations rest on spectral similarity to compounds not known from *C. asiatica*. Bioassay-guided fractionation of the elicited extract, and tests of defined mixtures of nicotinamide with the induced phenolics, are the logical next steps.

The two antimicrobial assays also illuminate each other. In the 24 h assay, the untreated PHE extract was clearly active, whereas in the 4 h screening the same extract appeared neutral. The screening measures an early effect on a dense inoculum and is designed to rank many genotypes quickly, while the kinetic assay uses a lower inoculum and follows a complete growth cycle, so it captures delayed and cumulative effects. The two are therefore complementary rather than contradictory. For *E. coli* ATCC 25922, which carries an ampicillin-resistance gene, kanamycin was used as the positive control, and the elicited extract matched the antibiotic in AUC-based inhibition.

3.4. The Elicited Extract Stimulates Keratinocytes and Accelerates Scratch-Wound Closure, an Effect That Nicotinamide Phenocopies at High Concentration

Neither extract nor nicotinamide was cytotoxic to HaCaT cells at any concentration tested, which places the elicited extract comfortably within the safety range required for topical use. Instead of reducing viability, the NaCl extract raised MTT reduction well above the untreated level in a bell-shaped manner, with a peak at intermediate concentrations. MTT reduction reflects mitochondrial and cytosolic reductase activity as well as cell number [35], so the increase is reported here as metabolic activity and not as proliferation, and a direct cell count or DNA-based assay would be needed to separate the two. Even so, a response of this size in serum-free medium indicates a strong trophic effect. Nicotinamide alone did not increase MTT reduction, so this trophic effect comes from other constituents of the elicited extract. Asiaticoside and madecassoside, the classical wound-healing triterpenoids of *C. asiatica*, stimulate keratinocyte and fibroblast growth and migration [36,37] and are plausible contributors, although they were not among the plant-derived features that changed here and their levels in this accession remain to be quantified.

In the scratch-wound assay, the elicited extract accelerated closure to a level that no longer differed from the positive control, whereas the untreated extract produced only a small improvement. Nicotinamide alone matched the elicited extract almost exactly (83% versus 84% of the positive control). This correspondence shows that nicotinamide can reproduce, or phenocopy, the wound-closure effect. It does not show that the nicotinamide present in the extract causes it, because the pure compound was tested at 100 µg/mL whereas 100 µg/mL of the elicited extract contains only about 0.24 µg/mL (2 µM) nicotinamide. Nicotinamide is an established dermatological active. It improves barrier function by increasing ceramide and other stratum corneum lipid synthesis in keratinocytes [38], improves the appearance of ageing skin in clinical use [39], promotes fibroblast migration, proliferation and collagen synthesis in vitro [40], and accelerates epithelialisation and tissue regeneration in full-thickness wounds [41]. It also has a favourable safety profile [42]. At its

actual content in the extract, nicotinamide is therefore a plausible contributor rather than the established cause of the enhanced closure, and other induced constituents, or nicotinamide acting together with them, must account for most of the effect. *C. asiatica* already contains well-studied wound-healing triterpenoids, and the present data add nicotinamide as a further, salt-inducible compound with the same activity rather than replacing them. A concentration-matched test of nicotinamide, and depletion or add-back experiments, would settle its contribution. Glycine betaine is a humectant used in skin care and may add to the cosmetic value of the extract, but it was not tested separately here.

Taken together, the two functional assays show that the elicited extract gains both antibacterial and keratinocyte-stimulating activity, and that nicotinamide reproduces the second of these when supplied at high concentration but is present in the extract at a level far below that. The extract therefore cannot be replaced by nicotinamide alone, and the two activities may depend on different sets of induced constituents. Because closure in a scratch assay integrates migration and proliferation, and no antiproliferative pre-treatment such as mitomycin C was applied, the assay is reported here as scratch-wound closure rather than wound healing. Proliferation is unlikely to dominate the readout, because the cells were kept in serum-free medium and the 24 h observation period is shorter than the doubling time of HaCaT cells under these conditions, but a migration-specific assay would confirm this. For product development, the practical implication is that elicitation should be evaluated on the extract as a whole, with nicotinamide content as a convenient marker of a successful treatment.

3.5. Practical Implications

A 10-day treatment with 100 mM NaCl in a sub-irrigation system is simple and inexpensive, and it is compatible with the hydroponic and soil systems already used for *C. asiatica* [14]. The present data suggest that such a treatment can be used deliberately to raise the nicotinamide content and the antibacterial and keratinocyte-stimulating activity of the harvested leaves. Extraction yield was similar for untreated and NaCl-treated PHE leaves (9.8% and 9.4% *w/w* of dry leaf), so the higher activity per gram of extract translates almost directly into higher activity per gram of dry leaf. Two cautions apply. Salt reduces biomass in this species even at tolerable concentrations [9], so the gain in activity per gram must be weighed against the loss of yield, and residual sodium in the raw material may need to be controlled for some applications. Genotype selection is the second lever. Because responses ranged from strong suppression to reversal, an elicitation programme should begin with a screening of the available germplasm, as done here. PHE is the primary candidate from this panel, and SPK and PLK, which ranked next by both criteria, are the natural accessions to examine next.

3.6. Limitations and Outlook

Some aspects of this work define the scope of the conclusions and point to useful next steps. The identity of nicotinamide was confirmed with an authentic standard, whereas the other metabolites discussed are putatively annotated by spectral and formula matching, which is the usual level of confidence in untargeted profiling [43,44]. Targeted HPLC quantification of nicotinamide confirmed its induction and provides a basis for standardising the elicited raw material, but the other induced metabolites remain unquantified, and LC-HRMS signal shares should be read as relative composition only. The metabolomic comparison was made on one accession selected for its strong response, with three biological replicates per condition from a planting made for this purpose, so the metabolic signature of less responsive accessions remains to be described, and the small sample size limits the precision of variance estimates and the power to detect smaller changes among

the 126 metabolites. The fold-change threshold, the FDR correction, the confirmation of key results on raw intensities, and the independent HPLC measurement mitigate but do not remove this limitation. No pooled quality-control sample was included in the LC-HRMS sequence, so analytical reproducibility was judged from the biological replicates rather than from repeated QC injections. The 4 h screening was designed for throughput across 39 accessions and was complemented by the 24 h kinetic assay for the selected accession, and minimum inhibitory concentrations of the elicited extract are a natural next measurement. The functional assays used a single keratinocyte line and one concentration for the scratch assay, and confirmation in primary keratinocytes and in vivo wound models would strengthen the translational case. Finally, the contribution of individual induced metabolites was tested with one pure compound at a single concentration far above its content in the extract, so the present data establish that nicotinamide can reproduce the wound-closure effect but not that it is responsible for it. Concentration-matched assays, depletion or add-back experiments and bioassay-guided fractionation are the next steps to identify the constituents that carry the two bioactivities.

4. Materials and Methods

4.1. Plant Material and Cultivation

Thirty-nine accessions of *C. asiatica* (L.) Urb. (Table S1) collected from different regions of Thailand and maintained as living germplasm at Phichit Agricultural Research and Development Center, Phichit, Thailand [11] were propagated from stolon explants in pots of homogeneously mixed commercial potting soil, with the stolon placed at three-quarters of the pot height. Pots were grown in a modified sub-irrigation system in which each pot stood in a container of half-strength Hoagland solution kept at about one quarter of the pot height. The solution was topped up to this level daily and replaced completely every three days, and plants were raised for 40 days under greenhouse conditions before treatment. Plants of uniform size and developmental stage were then selected to reduce physiological variation among replicates.

4.2. NaCl Treatment

The selected plants were transferred to a controlled-environment growth chamber (30 °C, 50 to 70% relative humidity, 12 h light and 12 h dark, 300 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density from plant-growing LED lamps). NaCl-treated plants received nutrient solution containing 100 mM NaCl for 10 days, and untreated plants received nutrient solution without added salt. Throughout the treatment, the solution in each container was topped up to the set level daily and replaced completely every three days, and its pH was adjusted to 5.8 daily. The experiment followed a completely randomised design with five biological replicates per accession and treatment, and treated and untreated plants were harvested at the same time. Because the leaf mass required for LC-HRMS exceeded that available from the five screening replicates, a separate set of PHE plants propagated from the same stock was grown and treated at the same time under identical conditions for metabolomics, and three plants per treatment were analysed, the number being set by the cost of the analysis.

4.3. Extraction

The youngest fully expanded leaves were dried in a hot-air oven at 40 °C for 24 h and ground to a fine powder. Powder (50 mg) was extracted with 1 mL methanol for 48 h, and the extract was used for the antimicrobial screen and for metabolomics. For the kinetic, cell viability, scratch-wound and HPLC assays, larger extracts of accession PHE were prepared in three biological replicates per condition, each replicate consisting of

the pooled leaf powder of 30 plants. Leaf powder (25 g per replicate) was extracted with 250 mL methanol for 48 h, the extract was dried using a rotary evaporator and the crude extract was weighed (2.458 g from untreated and 2.345 g from NaCl-treated leaves per 25 g of powder, i.e., extraction yields of 9.8% and 9.4% *w/w* of dry leaf) and redissolved in DMSO. All extract concentrations given for these assays refer to the mass of dried crude extract per volume.

4.4. Antimicrobial Screen at 4 h

The strains *Escherichia coli* ATCC 25922 carrying an ampicillin-resistance plasmid, *Staphylococcus aureus* ATCC 25923 and *Candida albicans* ATCC 10231 were obtained from the Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand. A 100 μ L portion of each methanol extract (equivalent to 5 mg leaf powder) was transferred to a well of a 96-well plate, and the solvent was evaporated under a stream of nitrogen. Overnight cultures in nutrient broth were adjusted to $OD_{600} = 0.1$, and 200 μ L of the suspension was added to each well and mixed with the extract residue. Plates were incubated at 37 °C and OD_{600} was read at 0 and 4 h using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA). Growth inhibition (%) was calculated as $100 \times (1 - \Delta OD_{\text{extract}} / \Delta OD_{\text{control}})$, where ΔOD is the change in OD_{600} between 0 and 4 h and the control contained microorganisms without extract, so that negative values indicate growth stimulation. Each accession and treatment was tested with five biological replicates per organism.

4.5. Growth Ratio and Statistics for the Screen

Within each accession, treatment and organism, replicate values were screened for single outliers with Grubbs' test ($\alpha = 0.05$, at most two iterations) [45], which removed one value in 19 of 234 groups. Relative growth was defined as $G = 1 - \text{inhibition}/100$, with a floor of 0.02 to avoid zero or negative values, and the growth ratio as $GR = \text{mean } G(\text{NaCl}) / \text{mean } G(\text{untreated})$ for each accession and organism. Effects across accessions were tested on $\log_2 GR$ ($n = 39$) with a one-sample *t*-test and a Wilcoxon signed-rank test, summarised as the geometric mean GR with 95% CI and Hedges' *g* [46], and confirmed with a linear mixed model of replicate-level $\log_2 G$ with treatment as fixed effect and accession as random effect. For each accession, a 95% CI of GR was obtained by bootstrap (2000 resamples) [47], and the treatment effect was tested with Welch's *t*-test on $\log_2 G$ [48] with Benjamini and Hochberg correction within organism [49]. Condition means were compared with zero by one-sample *t*-tests and between conditions by paired *t*-tests on accession means. The ratio-based index was the mean $\log_2 GR$ over the three organisms and the NaCl-condition index the mean *z*-score of % inhibition under NaCl. Correlations were Pearson's *r*. The influence of replicate handling was assessed by repeating the analysis on all five replicates (Table S6).

4.6. Untargeted LC-HRMS Metabolomics

Methanol extracts of untreated and NaCl-treated PHE leaves (three biological replicates per condition from the separate planting described in Section 4.2, prepared as in Section 4.3) were analysed on an UltiMate 3000 UHPLC system coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Separation used a Hypersil GOLD aQ C18 column (2.1 $\times$ 100 mm, 1.9 μ m; Thermo Fisher Scientific, Waltham, MA, USA) held at 60 °C with 0.1% formic acid in water (A) and in acetonitrile (B) at 0.325 mL/min. The gradient held 5% B for 2 min, rose linearly to 95% B over 25 min, held for 2 min and re-equilibrated, for a total run of 30 min. The injection volume was 2 μ L and the autosampler was kept at 8 °C. Blank injections of 0.1% formic acid in water followed each sample to limit carry-over. Electrospray ionisation was operated in positive

mode at 3.5 kV with the capillary at 325 °C. Acquisition alternated between full-scan MS (m/z 75 to 800, resolution 180,000) and data-dependent MS/MS (resolution 22,500) using dynamic exclusion, under Xcalibur version 3.1 (Thermo Fisher Scientific, Waltham, MA, USA).

Raw files were processed in Compound Discoverer version 3.3 (Thermo Fisher Scientific, Waltham, MA, USA). Retention times were aligned with the ChromAlign algorithm [50] with a tolerance of 0.5 min and a mass tolerance of 2 ppm. Features were extracted with predefined adduct rules, a mass deviation of 2 ppm, 30% intensity tolerance and a signal-to-noise threshold of 2, and quantified by peak area. Molecular formulas were predicted from accurate mass, and compounds were annotated by MS/MS spectral matching against mzCloud (HighChem LLC, Predmerská, Slovakia) and by mass and formula matching against ChemSpider (Royal Society of Chemistry, London, UK), KEGG (Kanehisa Laboratories, Kyoto, Japan) and in-house lists. Compounds were retained if their original peak rating was at least 2 in at least three samples. Gap filling searched for missing features within 5 ppm and kept centroid peaks with signal-to-noise ≥ 2 . Each biological replicate was injected in duplicate (one untreated replicate once), and technical injections were combined as the median area per biological replicate. No pooled quality-control sample was included in the sequence, so no QC-based drift correction was applied and the SERRF node of the workflow [51] was inactive. Analytical reproducibility was instead assessed from the biological replicates, which gave a median within-group coefficient of variation of 8.6% (untreated) and 11.2% (NaCl) for the 126 metabolites analysed, with 90% and 83% of metabolites below 30%. The export used for statistics contained 783 annotated features with name, formula, calculated mass, m/z , retention time, mzCloud score and peak areas for the six samples.

4.7. Metabolomics Data Processing and Statistics

Annotations were screened for biological plausibility before analysis. Features annotated as microbial natural products, synthetic drugs, industrial chemicals, animal-specific metabolites or catalogue identifiers without a plant identity were removed, with the reason recorded for each feature (Table S13), leaving 421 plant-plausible features. A stricter set of 129 features annotated as known plant or primary metabolites or matching the Arita laboratory flavonoid database [52] was then selected, and three pairs of features with identical peak areas across all samples (isobaric duplicate annotations) were merged, keeping the annotation with the higher mzCloud score, to give 126 metabolites. Peak areas were normalised by probabilistic quotient normalisation [53] using the median sample as reference and $\log_2$ -transformed. Differences between conditions were tested with Welch's t -test and corrected by the Benjamini and Hochberg procedure, and metabolites with $|\log_2\text{FC}| \geq 1$ and $\text{FDR} < 0.05$ were considered differential. The same tests were repeated on raw peak areas, and metabolites significant in both analyses were flagged as robust. PCA was performed on autoscaled $\log_2$ intensities. Metabolites were assigned to chemical classes from their formulas and names, and class enrichment among differential metabolites was tested with one-sided Fisher's exact tests with FDR correction. The share of each metabolite was calculated from raw peak areas as a percentage of the summed area of the 126 metabolites in each sample. The identity of nicotinamide was confirmed by comparison of retention time, accurate mass and MS/MS spectrum with an authentic standard (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan). Other annotations correspond to confidence levels 2 and 3 [43,44].

4.8. HPLC Quantification of Nicotinamide

Nicotinamide was quantified in the dried crude extracts of untreated and NaCl-treated PHE leaves (three biological replicates per condition, prepared as in Section 4.3) by reversed-phase HPLC following the method of Ozaki et al. [54] on a Shimadzu system fitted with an LC-30AD pump and an SPD-20AD UV detector (Shimadzu Corporation, Kyoto, Japan). Separation used a COSMOSIL 3PBr column (3.0 mm i.d. $\times$ 150 mm, 3 μ m; Nacalai Tesque, Kyoto, Japan) at 40 °C and 0.4 mL/min with UV detection at 260 nm. The mobile phase was prepared from 20 mM ammonium formate (pH 6.4, filtered through a 0.45 μ m membrane) mixed with methanol at 95:5 (*v/v*, solvent A) and 80:20 (*v/v*, solvent B). After equilibration in solvent A, samples (1 μ L) were eluted with 0% B for 5 min, a linear gradient to 100% B at 20 min and re-equilibration at 0% B from 20.1 to 30 min. Extracts were dissolved in ultrapure water and filtered (0.45 μ m) before injection. Nicotinamide (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) dissolved in ultrapure water (5 mM stock) was used to confirm the peak shape and retention time and to prepare the external calibration curve (0 to 500 μ M), and a blank was injected before each series. Contents were expressed as mg per g of dried extract, and the two conditions were compared with Welch's *t*-test.

4.9. Kinetic Antimicrobial Assay

Each of the three microorganisms was pre-cultured for 24 h under its respective growth conditions. Subsequently, 1% (*v/v*) of each stock culture was inoculated into freshly prepared nutrient broth. For the assay, 990 μ L of the inoculated medium was dispensed into sterile microtubes and supplemented with 10 μ L of the extract or nicotinamide stock solution in DMSO, resulting in a final concentration of 10 mg/mL and 1% (*v/v*) DMSO. The negative control consisted of inoculated nutrient broth containing the same volume of DMSO (1%, *v/v*), so that all treatments and controls were vehicle-matched, while the blank contained the corresponding test substance in the absence of microorganisms. Kanamycin (20 μ g/mL) for *E. coli* ATCC 25922, which carries an ampicillin-resistance gene, amoxicillin (20 μ g/mL) for *S. aureus* and ketoconazole (50 μ g/mL) for *C. albicans* were included as positive controls. Mixtures (200 μ L per well) were transferred to 96-well plates, and OD₆₀₀ was recorded hourly from 0 to 24 h in a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C. Three replicates were run per treatment. Growth was expressed as % change in blank-corrected OD₆₀₀ from 0 h, the AUC from 0 to 24 h was calculated by the trapezoidal rule [55], and inhibition was calculated as $100 \times (1 - \text{AUC}_{\text{treatment}} / \text{AUC}_{\text{negative control}})$. Treatments were compared by one-way ANOVA with Tukey's HSD test [56] and pairwise with Welch's *t*-test.

4.10. HaCaT Culture and MTT Assay

Human HaCaT keratinocytes (Cellosaurus accession CVCL_0038) were kindly provided by Asst. Prof. Dr. Jukkarn Srivilai, School of Pharmaceutical Sciences, University of Phayao, Phayao, Thailand. HaCaT cells were cultured in DMEM/F-12 medium supplemented with 10% FBS, penicillin 100 U/mL and streptomycin 0.1 mg/mL at 37 °C in a 5% CO₂ incubator. Cells were seeded at 10,000 per well in 96-well plates and incubated for 24 h. The medium was then replaced with serum-free DMEM/F-12 containing the extracts or nicotinamide at final concentrations of 0, 10, 25, 50, 100, 250 and 500 μ g/mL with the DMSO concentration fixed at 0.5% in all wells, including the untreated control. After 24 h, 10 μ L of 5 mg/mL MTT was added for the final 2 h of incubation; the formazan was dissolved in DMSO (200 μ L), and absorbance was read at 540 nm using a Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Waltham, MA, USA) [35]. MTT reduction (referred to as viability or metabolic activity) was expressed as % of the untreated control, and a value below 70% was taken as cytotoxic [57]. Three independent

experiments were performed. Viability at each concentration was compared with 100% by one-sample *t*-tests with FDR correction within each agent, and agents were compared at each concentration by ANOVA with Tukey's HSD.

4.11. Scratch-Wound Closure Assay

HaCaT cells were seeded at a density of 150,000 cells per well in 24-well plates and cultured for 24 h until a confluent monolayer. A linear scratch was then generated across the cell monolayer using a sterile P200 pipette tip. Detached cells and debris were removed by washing the wells twice with 1 mL PBS. Cells were subsequently cultured in serum-free medium supplemented with the respective extracts or nicotinamide at 100 µg/mL with DMSO fixed at 0.5% (*v/v*). Medium containing 2% fetal bovine serum (FBS) and 0.5% DMSO was used as the positive control, whereas serum-free medium containing 0.5% DMSO served as the vehicle (negative) control. No antiproliferative pre-treatment was applied, so the readout integrates migration and proliferation and is reported as scratch-wound closure. Images of the scratched areas were captured immediately after scratching (0 h) and following 24 h of treatment using an inverted phase-contrast microscope. The cell-free area was quantified using ImageJ version 1.54m (National Institutes of Health, Bethesda, MD, USA) [58]. Wound closure was calculated as the percentage reduction of the cell-free area from 0 to 24 h and expressed relative to the positive control (set to 100%). Six replicates were analysed per group. Groups were compared by one-way ANOVA with Tukey's HSD and by Welch's *t*-test against the negative control.

4.12. Software, Generative AI Use and Data Availability

Analyses were performed in Python version 3.11 (Python Software Foundation, Wilmington, DE, USA) with the open-source packages pandas 2.2, NumPy 1.26, SciPy 1.13, statsmodels 0.14, scikit-learn 1.4 and Matplotlib 3.9. Compound Discoverer 3.3 (Thermo Fisher Scientific, Waltham, MA, USA) and ImageJ version 1.54m (National Institutes of Health, Bethesda, MD, USA) were used as described above. During the preparation of this study, the authors used Claude model Fable 5.1 (Anthropic, San Francisco, CA, USA) to generate code for data visualisations and to improve the clarity of the writing. The authors have reviewed and edited all output and take full responsibility for the content of this publication.

5. Conclusions

A 10-day treatment with 100 mM NaCl shifted the antimicrobial activity of *C. asiatica* leaf extracts toward stronger bacterial suppression on average, but the response was strongly genotype-dependent, was reversed in some accessions and was not significant for *C. albicans* across the panel. The most responsive accession, PHE, gained activity against all three test organisms. In this accession, elicitation induced nicotinamide about ten-fold, as confirmed by LC-HRMS and by HPLC, together with the osmoprotectant glycine betaine and a broader reorganisation of nitrogen and NAD metabolism, although nicotinamide remained a minor constituent of the extract by mass. The elicited PHE extract was non-cytotoxic, increased keratinocyte metabolic activity, accelerated scratch-wound closure and inhibited bacterial growth more strongly than the untreated extract. Pure nicotinamide reproduced the wound-closure effect at a concentration far above its content in the extract, so nicotinamide is a marker of the salt response and a plausible contributor, alongside the known triterpenoids and other induced constituents, rather than the demonstrated cause of either bioactivity. Salt elicitation combined with accession selection is thus a practical route to *C. asiatica* raw material with enhanced antimicrobial and wound-closure properties in

responsive genotypes, and concentration-matched tests and bioassay-guided fractionation are the next steps to identify the active constituents.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/stresses6040071/s1>, Figure S1: Ranking of the 39 accessions by % growth inhibition within the untreated and NaCl conditions for each organism (Grubbs-filtered means with 95% CI); Figure S2: Sensitivity of the growth-ratio statistics to replicate handling. Mean $\log_2$ GR with 95% CI for each organism using all five replicates (A) or Grubbs-filtered replicates (B); Figure S3: Summary of Grubbs outlier filtering ($\alpha = 0.05$, at most two iterations) across the 234 accession by treatment by organism groups; Figure S4: Chemical-class enrichment among the 37 differential metabolites (one-sided Fisher exact test with FDR correction). Numbers give significant/total metabolites per class; Table S1: Accession codes used in this study. Table S2: Growth-ratio statistics across the 39 accessions for each organism: geometric mean GR with 95% CI, mean $\log_2$ GR, one-sample *t* and Wilcoxon *p*, Hedges *g*, mixed-model effect and *p*, number of accessions with GR < 1 and with FDR < 0.05, condition means of % inhibition with tests against zero, and paired *t*-test on accession means. Grubbs-filtered replicates. Table S3: Growth ratio per accession and organism with bootstrap 95% CI (2000 resamples), Welch *t*-test on $\log_2$ growth and Benjamini-Hochberg FDR within organism. Table S4: Ratio-based index (Ratio_index = $-\text{mean } \log_2\text{GR over three organisms}$), NaCl-condition index and untreated-condition index (mean *z*-score of % inhibition). Table S5: Pearson correlations between organisms for % inhibition (untreated, NaCl) and $\log_2$ GR ($n = 39$). Table S6: Sensitivity of the overall growth-ratio statistics to replicate handling (A, all five replicates; B, Grubbs-filtered). Table S7: Statistics for the 126 plant-derived metabolites: PQN-normalised $\log_2$ FC (NaCl/untreated), Welch *p*, FDR, significance, raw-intensity results, robust flag, mzCloud best match, class and $\log_2$ PQN intensities per sample. Table S8. (a) Chemical-class enrichment among differential metabolites (one-sided Fisher exact test, FDR). (b) Share of the total plant-derived signal by chemical class in untreated and NaCl extracts (%). Table S9: Share of the total plant-derived signal (% of summed raw peak area of the 126 metabolites) per metabolite and condition, with ranks and fold change. Table S10. (a) Kinetic antimicrobial assay: AUC (0–24 h) and 24 h growth, inhibition relative to the vehicle (negative) control, Welch *p* and Tukey letters ($n = 3$). *E. coli* data from the repeated experiment with kanamycin 20 $\mu\text{g/mL}$ as positive control. (b) Kinetic antimicrobial assay: pairwise Tukey HSD comparisons on AUC and 24 h growth. Table S11. (a) HaCaT viability (MTT, % of untreated cells) per agent and concentration with one-sample *t*-test vs. 100% and FDR within agent ($n = 3$). (b) HaCaT viability: Tukey HSD comparisons between agents at each concentration. Table S12. (a) Scratch wound healing at 100 $\mu\text{g/mL}$ (% of positive control, $n = 6$): mean, SD, Tukey letter, Welch *p* vs. negative control. (b) Scratch wound healing: pairwise Tukey HSD comparisons. Table S13: Features removed by the plant-origin plausibility filter (783 to 421) with the reason for removal. Table S14: HPLC quantification of nicotinamide in dried crude extracts of untreated and NaCl-treated PHE leaves (mg/g extract, $n = 3$ biological replicates, each from the pooled leaf powder of 30 plants; 25 g powder extracted in 250 mL methanol for 48 h), with derived values.

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Abbreviations

The following abbreviations are used in this manuscript:

AUC	Area under the growth curve
CI	Confidence interval
FDR	False discovery rate
GR	Growth ratio (growth with NaCl extract/growth with untreated extract)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAD ⁺	Nicotinamide adenine dinucleotide
OD ₆₀₀	Optical density at 600 nm
PCA	Principal component analysis
PQN	Probabilistic quotient normalisation
HPLC	High-performance liquid chromatography

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