

Antioxidant and Antimicrobial capacities of complex organic extracts containing stilbenes derivatives obtained from *Vitis vinifera* Pinot Noir Hairy Roots cultures for potential application in cosmetics

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Abstract

Stilbenes are bioactive molecules widely studied for their health benefits. A previously established *Vitis vinifera* hairy root line was used for its ability to produce several stilbenes, including *trans*-resveratrol, *trans*-piceatannol, *cis*-vitisin B, *trans*-piceid, pallidol, and *trans*- ϵ -viniferin. The influence of methyl- β -cyclodextrin (β CD) and Linecaps® maltodextrin was assessed during the growth phase, prior to elicitation (induced after a complete medium renewal). Stilbenes were extracted 7 days after elicitation from both the culture medium and the roots as crude extracts. Their antioxidant activities were evaluated using four *in vitro* assays and their antimicrobial activities were tested on three skin-associated bacteria. Cytotoxicity was assessed using an immortalized human keratinocyte line. Correlations between stilbenes content and bioactivity were examined. Roots pre-treated with β CD produced significantly more stilbenes and increased dimer formation, suggesting a potentializing effect. In contrast, Linecaps-treated roots produced similar stilbenes levels than control one but showed a significant increase in roots biomass. Crude extracts from the culture medium, rich in secreted stilbenes, exhibited the strongest antioxidant and antimicrobial effects, with similar cytotoxicity to less enriched extracts. This hairy root system proves to be a promising platform for producing bioactive plant compounds with potential cosmetic applications.

1- Introduction

The grapevine (*Vitis vinifera*) is a plant widely studied for its economic and cultural importance (Grassi and De Lorenzis, 2021), particularly through winemaking, which plays an important role in the cultural heritage of many countries. It has also been associated with potential health benefits, especially in relation to the “French Paradox,” which links moderate wine consumption to reduced cardiovascular risk (Catalgol et al., 2012).

These observations have led to numerous studies aimed at identifying these compounds in plants (Goufo et al., 2020),(Chaher et al., 2014), as well as the biological mechanisms that might explain their effects (Jeandet et al., 2010),(Akinwumi et al., 2018). Among these compounds, stilbenes such as resveratrol have attracted particular interest for their biological properties (Chaher et al., 2014). Grapevine is recognized worldwide for its richness in different specialized metabolites specially phenolic compounds such as flavonoids (Braidot et al., 2008) or stilbenes (Goufo et al., 2020), which include well-known molecules as resveratrol (Kiselev, 2011) viniferin (Šikuten et al., 2020; Tříška et al., 2017) pallidol (Gutiérrez-Escobar et al., 2021) or pterostilbene (Duke, 2022).

It was already shown that the antioxidant properties of stilbenes allow neutralizing free radicals and thus protect plant cells against oxidative stress caused by abiotic factors (Tabeshpour et al., 2018). They also help fight against biotic stress (Langcake and Pryce, n.d.; Tabeshpour et al., 2018), especially against pathogenic bacteria or fungi such as *Botrytis cinerea* (Elmer and Reglinski, 2006). For several decades, these compounds have attracted growing interest for their antioxidant (Ndiaye et al., 2011)(Biais et al., 2017)(Sáez et al., 2018), antimicrobial (Gabaston et al., 2017) and antitumoral (Balasubramani et al.,

2019),(Giovannelli et al., 2014) properties. These activities are under consideration for their use in cosmetic applications in order to prevent cellular photoaging (Petruk et al., 2018) or in the protection against certain bacteria involved in skin pathologies, such as *Cutibacterium acnes* (Aneklaphakij et al., 2022). These biological properties have also interest in pharmacological or nutraceutical fields (Duke, 2022),(Navarro et al., 2018), to prevent the development of cancers (De Filippis et al., 2017) or various chronic pathologies such as cardiovascular disease (Kershaw and Kim, 2017).

However, stilbenes present in grapevines offer potential benefits for human health, their traditional extraction from the plant poses challenges in terms of yield due to a generally low concentration of these compounds in grapevine tissues depending on factors like the variety (Aliaño-González et al., 2020), the growing conditions, and the post-harvest handling (Houillé et al., 2015). Stilbenes from grapevines also face limitations related to their extraction efficiency when using conventional methods (Gabaston et al., 2018). These techniques, which often rely on solvent-based extraction from grape skins, seeds, or canes, may be time-consuming and require large volumes of solvent (Choi et al., 2003). In addition, obtaining sufficient quantities often requires significant biomass amount, raising concerns regarding the sustainability of this approach. These limitations remains relevant in the context of constrained access to natural resources and environmental changes associated with climate change, which may affect grapevine metabolism and stilbene content (Singh et al., 2023).

In recent years, plant biotechnologies have been seeking to offer innovative solutions to overcome some of these limitations. Thanks to advances in grapevine cell cultures and *in vitro* cultivation techniques (Donnez et al., 2011, 2009) it is now possible to produce these natural molecules in a controlled and repeatable way. Culture systems such as cell suspensions (Jeandet et al., 2016, 2014) or hairy roots (Tisserant et al., 2016a),(Hoseinpanahi et al., 2020),(Hosseini et al., 2017) allow optimal growing conditions and enable the application of chemical elicitors that activate plant defense responses and plays a key role in enhancing the biosynthesis of specialized metabolites like stilbenes (Doran, 1997; Goel et al., 2011). These processes are facilitating their extraction on both small-scale and large-scale without the constraints associated with vine harvesting. These biotechnologies advances pave the way for a sustainable and standardized production of these valuable molecules thanks to a year-round continuous production while compacting the process, reducing wastes and the global impact of extraction (Gutierrez-Valdes et al., 2020).

In the case of *Vitis vinifera*, the production of complex extracts rich in stilbenes and other bioactive compounds through biotechnological approaches offers opportunities for less energy-intensive valorization of co-products from the wine industry. In this way, the integration of biotechnologies in the production of compounds from grapevines could meet growing market needs while helping to conserve natural resources for other circular economic purposes and reduce production costs (Martin et al., 2021). The raw extracts obtained by these means are reproducible (Gutierrez-Valdes et al., 2020), can be perfectly characterized, and their cytotoxicity can be assessed for cosmetic applications. This makes possible the use of these complex extracts without purification if their biological properties are

equivalent or superior to those of the pure molecule thus offering innovative and sustainable solutions to public health and environmental challenges.

In this study we used a *Vitis vinifera* hairy root line (Tisserant et al., 2016a, 2016b) as a source for stilbene production regarding methyl jasmonate and β -cyclodextrin elicitation conditions. The aim was both to produce crude extracts, one from medium (MCE) and the other from roots (RCE), that could be used in the cosmetic field. We then studied their biological activities without purification steps. To achieve this, we first tried to optimize the biomass production of the *Vitis vinifera* hairy root clone and to determine the best production conditions for stilbenes, in particular, *trans*-Resveratrol, ϵ -Viniferin, *cis*-Vitisin B, Pallidol, Piceid and Piceatannol. Secondly, various *in vitro* cell-free antioxidant and antimicrobial activity tests on three microorganisms related to the skin flora (*Acinetobacter calcoaceticus*, *Corynebacterium xerosis* and *Staphylococcus epidermidis*) were performed. Ultimately, we evaluated the cytotoxicity of the different extracts on an immortalized keratinocyte cell line (HaCaT), to determine their putative impact on human skin cells. These elements suggest that plant biotechnologies may represent a promising and sustainable approach for the controlled production of bioactive natural extracts, while reducing reliance on conventional plant harvesting methods.

2- Material and Methods

2.1 Materials

2.1.1 Chemicals

MURASHIGE & SKOOG medium including modified vitamins (MS), Yeast extract, Tryptone, Sodium chloride and Sucrose were purchased from Duchefa Biochemie. Dimethyl sulfoxide (DMSO), Methyl- β -cyclodextrins (β CD's) and Methyl jasmonate (MeJA), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), TPTZ (2,4,6-tris-2,4,6-tripyridyl-2-triazine), ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt, DPPH (2,2-diphenyl-1-picrylhydrazyl), potassium persulfate, glacial acetic acid, sodium acetate, copper chloride, neocuproine, ferric chloride, 37% hydrochloric acid and resveratrol, were purchased from Sigma-Aldrich®. Maltodextrine Kleptose Linecaps was purchased from Roquette®. 100% Ethanol was purchased from Carlo Erba reagents. Ethyl acetate, Acetonitrile, Formic acid were purchased from Biosolve.

HPLC standards were purchased from TargetMol (*trans*-Resveratrol 99.9%, *trans*-Piceatannol 99.57% and *cis*-Vitisin B), Cayman Chemical Company (*trans*-Piceid *trans*-Polydatin 98%) and Merck (Pallidol 90% and *trans*- ϵ -Viniferin 95%). Chelex® 100 Resin was purchased from BIO-RAD. Gibco™ DMEM was purchased from Fischer Scientific.

2.1.2 - Plant material

The hairy root line was generated in 2016 from *in vitro* plantlets of *Vitis vinifera* cv. Pinot Noir PN 40027 cultivar using *Rhizobacterium rhizogenes* ATCC 15834 (Tisserant et al., 2016), and was cultured on ½ MS

medium supplemented with 3% sucrose on a shaker under dark conditions

2.1.3 Keratinocyte cell line

Assays to determine the cytotoxicity of the extracts were performed on the HaCaT immortalized keratinocyte line n°300493, (Cell Lines Service GmbH, Eppelheim, Germany).

2.1.4 - Bacterial strains

Antimicrobial tests were carried out on three skin related strains bacteria: *Acinetobacter calcoaceticus* CIP 103625 (Gram-negative aerobic), *Corynebacterium xerosis* CIP 100653 T (Gram-positive aerobic), *Staphylococcus epidermidis* CIP 81.55 T (Gram-positive facultative anaerobic).

2.2 – Obtention of crude extracts: Stilbene production & extraction

2.2.1 Culture condition

The *Vitis vinifera* hairy root line was first cultured for 21 days for root development in 500 mL Erlenmeyer flasks containing 100 mL ½ MS medium including modified vitamins and 3% sucrose at pH 5.8, in darkness at 24°C, and under shaking (90 rpm)

Four different culture conditions were tested for root growth. A positive control which will be elicited after 21 days and a negative control that won't be. They both contain ½ MS medium including modified vitamins. The other two conditions used the same medium each supplemented with a different type of dextrin.

The first dextrin tested is the methyl-β-cyclodextrins - Sigma-Aldrich® (βCD), and the second one is the Kleptose Linecaps maltodextrin supplied by Roquette®.

A 7-day elicitation phase is then induced for stilbene production in which we change the root culture medium to ½ MS medium with modified vitamins, 3% sucrose, containing 50 mM methyl-β-cyclodextrins and 100μM methyl jasmonate (MeJA) at pH 5.8. The negative control condition medium is not supplemented with βCD's and MeJA to avoid triggering stilbene production.

2.2.2 Medium crude extract

Stilbenes were extracted from the culture medium with ethyl acetate (v/v).

The organic phase was then firstly evaporated at 40°C using a rotavapor, followed by nitrogen evaporation on a dry bath at 40°C to obtain the medium crude extract (MCE).

2.2.3 Root crude extract

Stilbene extractions from the roots were carried out by grinding them with liquid nitrogen using a RetschMM200 ball mills at 30 frequencies per second for 3 minutes.

The obtained root powder was then mixed with 100% ethanol at a ratio of 10 mL for 2.5g of powder. The suspension is placed in darkness and stirred for 1 hour at 40 rpm. After centrifugation for 5 min at 8,000 rpm, the supernatant was collected. This extraction step is carried out a second time to extract as much stilbene as possible, at a ratio of 10 mL for 5g of powder. The supernatants from both extractions are pooled, and the ethanolic phase is evaporated on a dry bath at 40°C using a nitrogen evaporator to obtain the root crude extract (RCE).

Each of the conditions applied during the growth phase: negative control, positive control, Linecaps, and β -cyclodextrin, have 6 biological replicates of *in vitro* cultures labelled from A to F. A medium crude extract (MCE) and a root crude extract (RCE) has been obtained from each biological replicate.

The MCE and RCE, were kept in a dry at 15°C. They were then solubilized in 100% ethanol, LB-DMSO10% or DMEM-DMSO2%, depending on their use in the various experiments.

2.3 – LC-UV Analysis

The LC-UV analysis were adapted from Tisserant et al. (2016) (Tisserant et al., 2016).

Samples were analyzed using a HPLC system PROMINENCE UFLC UV (SHIMADZU CORPORATION, Kyoto, Japan). Separation was carried out from a 10 μ L injection volume at 1mL/min flow rate and 30°C on an ALLTECH APOLLO C18 reversed-phase column (5 μ m, 250 $\times$ 4.6 mm i.d.) (GRACE DAVISON DISCOVERY) using an elution gradient composed of ultrapure water (mobile phase A) and acetonitrile HPLC grade (mobile phase B).

The 60 min gradient started at 10% (B) and increased up to 60% in 40 min, then reached 100% in 3 min and remained 5 min followed with a 1 min return to 10% kept 14 min for equilibration.

Quantification was performed using calibration curves realized from standards *trans*-Resveratrol *trans*-Piceatannol, *cis*-Vitisin B, *trans*-Piceid, Pallidol, *trans*- ϵ -Viniferin at 320nm except for Pallidol and *cis*-Vitisin B which were quantified at 284nm. As samples, all standards were prepared at 1 mg.mL⁻¹ in Ethanol absolute HiPerSolv CHROMANORM HPLC 97% (VWR Chemicals), diluted at 0.5, 0.2, 0.1, 0.075, 0.05, 0.025, 0.01 mg.mL⁻¹.

2.4 Determination of antioxidant capacity

Antioxidant assays were carried out in 96-well microplates. Each extract condition is tested in biological triplicates and technical triplicates. Biological triplicates are randomly selected from six available biological replicates for each condition. All the antioxidant capacities are compared to the activity of Trolox which is a vitamin E derivative.

2.4.1 ABTS.

This antioxidant assay method was adapted in microplate from Re et al. (1999) (Re et al., 1999).

The ABTS^{•+} radical stock solution was generated by reacting 10 mM ABTS with 10 mM potassium persulfate and allowed to stand for 12 to 16 hours at room temperature in the dark.

The fresh reactive solution for the analysis is diluted from the stock solution with ethanol to obtain a 0,700 $\pm$ 0,02 absorbance solution at 734 nm. The reaction was carried out by mixing the ABTS^{•+} working solution with the sample in a microplate and measuring the absorbance at 734 nm.

2.4.2 DPPH.

This antioxidant assay method was adapted in microplate from J. Xie and K. M. Schaich (2014) (Xie and Schaich, 2014). The DPPH stock solution was prepared at a final concentration of 10 mM in ethanol and stored at 4°C. The fresh reactive solution for the analysis is diluted from the stock solution with ethanol to obtain a 0,800 $\pm$ 0,05 absorbance solution at 517 nm. The test was performed by mixing the working solution with the sample in a microplate and measuring the absorbance at 517 nm.

2.4.3 FRAP.

This antioxidant assay method was adapted in microplate from Benzie and Strain (1996) (Benzie and Strain, 1996).

The FRAP fresh reactive solution is composed of a solution of 300 mM sodium acetate buffer (pH 3.6) with 280 mM glacial acetic acid and adjusted with distilled water. A 10 mM TPTZ solution (2,4,6-Tri(2-pyridyl)-s-triazine) with 0,4% of 37% HCl and adjusted with distilled water. A 20 mM ferric chloride solution adjusted with distilled water.

The reactive solution is made with a ratio of 10:1:1 of each solution (Sodium acetate buffer: TPTZ: FeCl₃). The reaction mixture was combined with the sample in a microplate to run the test at 593 nm.

2.4.4 CUPRAC.

This antioxidant assay method was adapted in microplate from Özyürek et al. (2011) (Özyürek et al., 2011).

The CUPRAC fresh reactive solution is composed of a solution of 1 M ammonium acetate buffer (pH 7) adjusted with distilled water. A copper 10 mM chloride solution was made and adjusted with distilled water. A 7.5 mM neocuproine solution was made in ethanol. The reactive solution is made with a ratio of 1:1:1. (Ammonium acetate buffer: CuCl₂: Neocuproine). The test was carried out by combining the working solution with the sample in a microplate, followed by measurement at 450 nm.

2.4.5 TEAC calculation

The antioxidant activity of the extracts was determined using the following standardized method. A calibration curve was made using Trolox solutions, and absorbance was measured at the test

wavelength. The directing coefficient (a) and y-intercept (b) of the regression line were determined from the absorbance and trolox concentration values in the well. The TEAC of the samples was calculated according to the following formula:

$$\text{TEAC} = \frac{\frac{\text{Abs}-b}{a}}{S.C.}$$

Abs is the mean absorbance of the sample, a and b are the parameters of the Trolox calibration curve and S.C. is the sample concentration in mg.ml^{-1} . Results are expressed as Trolox equivalents in grams of Trolox per gram of extract.

2.5 Determination of antimicrobial activity

Antimicrobial assays were performed in 96-well Greiner BIO-ONE microplates. Each extract condition is tested in biological triplicates and technical triplicates. Biological triplicates extracts were randomly selected from six available biological replicates for each condition. Prior to analyzing, concentration/absorbance correlations were performed on each strain from liquid cultures incubated for 24 hours at 37°C in LB medium containing 10g.L^{-1} NaCl.

For antimicrobial activity assays, bacteria were first cultured for 24h at 37°C in liquid LB medium with 10g.L^{-1} NaCl, then each was adjusted to a concentration of 10^7 bacteria. mL^{-1} . Successively, the adjusted culture was added to the well, followed by LB medium, then tested extract solubilized in 10%DMSO LB medium to obtain a final concentration of 10^6 bacteria. mL^{-1} and 1% DMSO in the well. The concentration range tested for all extracts is 0.5, 1 and 2 mg.mL^{-1} in different wells.

After 24 h incubation at 37°C , the absorbance was read at 600 nm on a Id3 Spectramax.

2.6 – Maintenance and IC50 determination of HaCaT - keratinocyte cell line.

Prior to culture, the calcium present in FBS is chelated under agitation for 1h at 4°C using a ratio of 3.8g of Chelex® resin to 500mL of serum. The serum is then filtered through $0.22\ \mu\text{m}$ filters.

HaCat cells are cultured in low-calcium DMEM (LC DMEM) medium supplemented with 10% chelated FBS, 1% antibiotic streptomycin and penicillin, 1% L-glutamine and 1 mL mycozap. Cells were cultured in T75 flasks adapted to the growth of adherent cells, at 37°C and 5% CO_2 .

Two passages per week were performed according to the following protocol: the LC DMEM was removed from the flasks, the cells were washed with PBS and incubated for 5–10 min with trypsin to detach the cells from the support. The trypsin action is then inhibited by adding LC DMEM. The cells are centrifuged at 1500 rpm for 5 min, and the pellet is resuspended in DMEM LC. Cell viability is then determined by Trypan blue staining and counting with the LUNA II automatic cell counter. Cells are seeded at a density of 300,000 cells per flask.

The determination of extracts cytotoxicity on HaCaT cells was carried out in 96-well microplates. Plates were seeded with 3500 cells per well in 200 µl of LC-DMEM medium (0.03 M CaCl₂, 1% calcium-free FBS, 1% glutamine, 1% penicillin/streptomycin).

Extracts are solubilized in the same DMEM supplemented with 2% DMSO. The range of final concentrations of the extracts tested in the well was 0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1 and 2 mg.mL⁻¹. HaCaT Cell viability is determined after 24 h of contact with the extracts using CCK8 (added 1h before the end of contact). Absorbance is measured at 450nm using a spectrophotometer (Spectramax Id3).

2.7 - Statistical analysis

Statistical analyses were carried out using JAMOV software for Fisher's, Welch's or Kruskal - Wallis ANOVA tests. Differences in the data are considered significant for p-value < 0.05 in each statistical test. Correlation tests were carried out between the antioxidant capacity of extracts (measured by ABTS, CUPRAC, DPPH and FRAP tests) and their stilbene concentration. Analyses were performed using R software. Coefficients of determination (R²) were calculated from linear regressions using the `stat_poly_eq` function in the `ggpmisc` package, integrated with graph visualizations produced with `ggplot2`.

3- Results

3.1 Optimization of the root biomass increase during the preculture phase.

The biomass production of this *Vitis vinifera* hairy root line was studied prior to elicitation phase particularly through the addition of potential carbon substrate (methyl-β-cyclodextrin and maltodextrin).

Results are expressed in grams of fresh mass gained by the hairy roots in 21 days. In the preculture phase, CTL- and CTL + culture conditions are identical.

The results indicate that medium supplemented with methylated β-cyclodextrins have no effect on the hairy root growth (Fig. 1). Medium supplementation of Linecaps maltodextrin at a concentration of 50 mM significantly increased biomass production after 21 days of culture compared to control condition (Fig. 1).

Application of a two-fold lower concentration of Linecaps maltodextrin (25 mM) does not appear to be sufficient to induce a statistically significant increase in biomass (data not shown).

3.2 Stilbenes production

To characterize the stilbenes production after elicitation on hairy roots, six of the stilbenes predominantly produced by this clone (*trans*-Resveratrol, ε-Viniferin, *cis*-Vitisin B, Pallidol, Piceid and Piceatannol) were evaluated by HPLC analysis of the crude extracts based on a previous LC-MS and

NMR study (Tisserant et al., 2016b). The study of MCE (Fig. 2) shows that *trans*-Resveratrol, ϵ -Viniferin and *cis*-Vitisin B are absent in the negative control condition (no elicitation). Only a small amount of pallidol was detected in the absence of elicitation (less than 0.5 mg per 100 mg extract). Following culture elicitation with a combination of MeJa (100 μ M) and β CD (50 mM), a significant increase in the production of *trans*-Resveratrol, ϵ -Viniferin, *cis*-Vitisin B and Pallidol is observed in the MCE (Fig. 2). This production also increased significantly for all the stilbenes measured in the MCE produced from the culture medium of roots pre-treated with β CD during their growth phase.

Regarding RCE, greater diversity can be observed in the concentrations of stilbenes found in hairy roots. The stilbenes present in the RCE of the Linecaps and positive control conditions are all significantly identical.

The β CD condition has significantly more ϵ -Viniferin and Pallidol than the other extracts but shows a similar *cis*-Vitisin B concentration than the Linecaps and positive control conditions (Fig. 3).

In contrast to MCE, resveratrol levels in RCE are significantly lower in the β CD condition than in the CTL + and Linecaps conditions (Fig. 3). CTL- RCE remains the extract condition with the lowest total stilbene concentration. The stilbene concentrations found in the RCEs are all lower than those found in the same conditions for the MCEs. (Fig. 2, 3)

The study of the six predominant stilbenes production, expressed as the percentage extracted from the culture medium and roots after elicitation, provides a clearer picture of resveratrol oligomerization mechanisms in this study. In the medium crude extracts (MCE), a high proportion of Pallidol in the non-elicited condition is recovered (Fig. 4, A). It is the only stilbene to have been quantified in significant quantities in the medium (Fig. 2, D) (quantity below 0.5 mg/100mg dry extract). Resveratrol represents around 80% of total stilbenes in the positive control and Linecaps MCE. ϵ -Viniferin and Pallidol, the two resveratrol dimers studied here, represent 15% of the stilbenes quantified in these extracts. In the MCE pre-treated during their growth phase with β CD, these proportions differ. The proportion of resveratrol is reduced to 57%, while the proportion of the two dimers measured (Pallidol and ϵ -Viniferin) is increased to 36%. (Fig. 4, A)

Likewise, for the stilbenes recovered in the RCE, a similar proportion profile between the CTL + and Linecaps conditions (Fig. 4, B) is observed. The β CD RCE has a much higher proportion of ϵ -Viniferin, around 60% compared to around 26% in the CTL + and Linecaps extracts (Fig. 4, B). However, this quantity is in similar proportions to the one found in CTL- RCE of around 44%. In contrast, resveratrol is more than twice as present in CTL + RCE and Linecaps extracts as in RCE β CD (Fig. 4, B). *cis*-Vitisin B is around 5 to 10% more present in the total stilbenes of RCE CTL + and Linecaps than in CTL- and β CD RCE (Fig. 4, B).

3.3 Antioxidant response

Four *in vitro* antioxidant activity assays were performed: CUPRAC, FRAP, ABTS and DPPH. Results are expressed as the equivalent (in g) of standard trolox antioxidant activity per gram of dry extract tested

(TEAC g/ extract g). A high TEAC value within a test indicate a good sample response to the test mechanisms and a potential antioxidant capacity.

Each antioxidant test is based on a different mechanism of action - trapping or reduction of complexes or ions (Benzie and Strain, 1996; Özyürek et al., 2011; Re et al., 1999; Xie and Schaich, 2014). The MCE results indicate that no antioxidant activity is found in the negative control culture medium condition (Fig. 5). Antioxidant activity measured in the CTL + and Linecaps MCE is significantly similar. For these extracts, antioxidant activity is higher for the ABTS assay, with values close to 0.2 TEAC g/extract g. This corresponds to an antioxidant activity equivalent to 20% of the antioxidant activity of trolox.

These same extracts are measured at 0.1, 0.04 and 0.02 TEAC g/extract g respectively for the CUPRAC, FRAP and DPPH assays. This represents respectively 10%, 4% and 2% of the activity measured for trolox in these tests. (Fig. 5)

The highest MCE antioxidant activity is achieved for each test by the β CD extract. MCE activity measured in DPPH is equivalent to 5% of trolox activity (approx. 0.05 TEAC g/extract g), 10% for the FRAP assay, and 30% for the CUPRAC assay.

The antioxidant activity of the same MCE measured in ABTS tests reaches around 40% of trolox activity. This is the highest value found for culture media extracts (Fig. 5). In comparison with pure resveratrol, the antioxidant activity of β CD MCE has been determined to be similar for the ABTS assay and 3 to 6 times lower for CUPRAC, FRAP and DPPH assays (result not shown).

The antioxidant activities measured for the RCE do not follow the same trend as those for MCE. For these extracts, each one has a biological activity, even if it is often low. The lowest activity found in the RCE for each test belongs to the β CD condition. The antioxidant activities measured in DPPH and FRAP are around 0.01 and 0.015 TEAC g/extract g respectively, i.e. 1% and 1.5% of trolox activity. The CUPRAC and ABTS tests respectively show assay values of around 0.05 and 0.1 TEAC g/extract g, representing somewhat higher dosages, with activities equivalent to 5% and 10% of trolox activity. (Fig. 5)

The antioxidant activities of RCE measured by CUPRAC, FRAP and ABTS tend to be higher for the CTL + and Linecaps conditions than for the β CD condition, but no significant difference is found. Only CTL + and Linecaps RCE recovered with DPPH are significantly more antioxidant than β CD RCE, with a double value approximately.

The highest values of antioxidant activity in the RCE for each test were found in the CTL- condition. This represents 3% of trolox activity in DPPH, 5% in FRAP, between 10 and 15% in CUPRAC and 40% in ABTS.

Globally, the antioxidant assay results show similar activity trends within each test, for each type of extract tested (Fig. 5).

3.4 Antimicrobial activity

The results of the antimicrobial tests show the effect of a 24-hour contact between the extracts and a bacterial culture of microorganisms inhibiting or potentially infecting the skin flora. *Acinetobacter calcoaceticus*, *Corynebacterium xerosis* and *Staphylococcus epidermidis* were chosen for this study.

Results are expressed as a percentage reduction in viability compared to a control condition with no extract. The control condition is subjected to a final DMSO concentration corresponding to the tests with extracts. (Fig. 6)

The MCE conditions tested include the positive control, Linecaps and β CD conditions.

The MCE negative control condition could not be tested due to a too low quantity of extract obtained after extraction for this condition specifically.

The effect of CTL + and Linecaps MCE is similar on each flora. The effect of these two extracts on *Acinetobacter calcoaceticus* begins to be significant at the highest concentration, 2 mg.mL^{-1} . On *Staphylococcus epidermidis*, a significant effect of around 20% reduction in viability was observed at 1 and 2 mg.mL^{-1} for CTL + MCE and at 2 mg.mL^{-1} for Linecaps MCE. (Fig. 6)

For the viability of *Corynebacterium xerosis*, strong standard deviations were found, even for the control. Both CTL + and Linecaps MCE had no significant effect on this species at any of the concentrations tested. (Fig. 6)

The study of the effect of MCE β CD seems to indicate a stronger effect starting from 0.5 mg.mL^{-1} on *Staphylococcus epidermidis*, and from 1 mg.mL^{-1} on *Acinetobacter calcoaceticus* and *Corynebacterium xerosis*. This effect reached a 80% drop in the viability of all flora tested at the 2 mg.mL^{-1} concentration. This inhibition was already significantly achieved at 1 mg.mL^{-1} for *Corynebacterium xerosis* and *Staphylococcus epidermidis*. (Fig. 6)

The RCE conditions tested included all four conditions, negative control, positive control, Linecaps and β CD. The effect of RCE had no significant impact on the viability of the three tested microorganisms (Fig. 6). A decreasing trend seems to be observed for *Staphylococcus epidermidis* when the extract concentration is increased, but as results do not follow a normal distribution, only non-parametric statistical tests could be performed, requiring more differences in the results to observe a significance.

3.5 Keratinocytes cellular response

IC50 results on HaCaT cells are expressed in mg.mL^{-1} . Resveratrol was used as a control in order to observe the potential synergistic effect of stilbene mixtures present or absent in MCE and RCE. Analysis of the IC50 values for the MCE extracts shows that no significant difference was found between the different conditions, indicating that they all have the same overall effect on the cells (Table 2). IC50 values for these extracts range from 0.6 to 0.9 mg.mL^{-1} . The IC50 value for resveratrol is around 0.1 mg.mL^{-1} . This value is significantly different from the results obtained for Linecaps and β CD MCE,

indicating that pure resveratrol is cytotoxic at a lower concentration than these extracts. This does not apply to the IC50 value calculated for CTL + MCE, which is statistically comparable to resveratrol.

Table 2

50% inhibitory concentration (IC50) of MCE and RCE on HaCaT cells (immortalized keratinocytes). Significant differences were demonstrated with Fischer's ANOVA tests ($p > 0.05$) (MCE and RCE). Nd (not defined) indicates that the IC50 could not be reached at the maximum values tested in the study. "X" indicates insufficient extract to perform this test. Data are expressed as the means $\pm$ SD of 3 independent experiments

Resveratrol ^a	CTL-MCE	CTL + MCE ^{ab}	Linecaps MCE ^b	β CD MCE ^b
0.099 $\pm$ 0.065 mg.mL ⁻¹	X	0.597 $\pm$ 0,335 mg.mL ⁻¹	0.9 $\pm$ 0.188 mg.mL ⁻¹	0.864 $\pm$ 0.105 mg.mL ⁻¹
	CTL-RCE	CTL + RCE	Linecaps RCE	β CD RCE
	Nd	Nd	Nd	Nd

3.6 Stilbene and biological activities correlations

The study of biological activities from natural compounds is generally carried out on purified compounds. This study aims to investigate the antioxidant and antimicrobial biological activities of crude extracts. MCE and RCE are composed of the measured stilbenes, as well as other compounds extracted from the organic phase during extraction with ethyl acetate for MCE, or ethanol for RCE which haven't been characterized. A correlation study between TEAC results and stilbene concentrations in the extracts was carried out to observe whether the observed activity is due to the presence of stilbenes or not. Medium to strong correlations between antioxidant capacities measured in CUPRAC (Fig. 7) of MCEs for each of the stilbenes measured were observed. This is also true for the other tests (Fig. 1 supp data). The correlation results for RCE (Fig. 1 supp data) indicate more variable and overall weaker correlations. The highest correlations found in the RCEs are for the antioxidant activities of CTL- RCEs in the FRAP, DPPH and CUPRAC tests, where low activities are correlated with low stilbene concentrations.

4- Discussion

4.1 Optimizing Hairy Root Growth Conditions

The optimization of culture parameters represents a crucial step in the development of plant biotechnologies. Various physical processes have been described in the literature to increase both the root biomass of hairy root cultures and the amount of produced specialized metabolites. This includes the use of processes such as ultrasound (Liu et al., 2012), light exposure (Abbasi et al., 2007), or changes in pH, temperature and / or oxygenation (Zhu et al., 2024). Although the use of physical treatments may turn out to be more sustainable, the difficulty of scaling up this process for industrial bioreactor applications can sometimes be too challenging. Exposure to light, which is the simplest method to implement, even on a laboratory scale, cannot be studied here due to the light and UV

sensitivity of stilbenoids (Neyra Recky et al., 2024). In order to maintain a degree of repeatability with previous work carried out on this hairy root clone (Tisserant et al., 2016a, 2016b), changes in pH, temperature, and agitation were minimized during incubation. Few studies have focused on the use of non-eliciting molecules as growth agents to be added to the hairy root culture medium. Overall, the focus is on selecting the most suitable culture medium, without necessarily studying further optimization (Gai et al., 2015) (Pakdin Parizi et al., 2015). The previous study on this *Vitis vinifera* hairy root line indicates that it has a doubling time (DT) of 14.9 days (Tisserant et al., 2016a). This growth rate does not represent a biotechnological advantage, when other hairy root lines such as *Withania somnifera*, *Gmelina arborea* or *Vitis rotundifolia* have DTs under 10 days (Dhakulkar et al., 2005; Murthy et al., 2008; Nopo-Olazabal et al., 2013). This previous result justified a growth optimization step for this *Vitis vinifera* hairy root. Beyond the classic study of culture media, there is no mention in the bibliography of the specific use of maltodextrins as growth agents. Maltodextrins are polysaccharides derived from the hydrolysis of starch. They are generally found in the food industry. However, the literature mentions a study on the use of these molecules to encapsulate phenolic compounds, notably resveratrol, present in plant extracts produced by *Vitis vinifera* (Boonchu and Utama-ang, 2015).

The increase in root biomass (Fig. 1.) induced by 50 mM Linecaps maltodextrin suggests a putative use of these sugars by the root for its primary metabolism. However, the hairy root clone showed in a previous study little tolerance to sucrose concentrations in excess of 3%. (Tisserant et al., 2016a). The use of 50 mM Linecaps maltodextrins, in addition to a sucrose concentration of no more than 3%, would therefore enable it to increase its growth without generating osmotic stress and thus premature browning of the roots. A lower concentration of Linecaps (25mM) seems to be insufficient for the hairy root clone to significantly increase the root growth. This may indicate that a threshold concentration is required to access or metabolize this compound in the culture media. Once potentially hydrolyzed by the roots, the glucose units derived from maltodextrin could be metabolized by the plant for its growth. Interestingly, this result could indicate that this hairy root clone of *Vitis vinifera* could metabolize glucose better than sucrose for its growth. However, all dextrans do not show a root biomass increase. A 50 mM β CDs could also represent a potential carbon source for the growth of the roots but show no improvement in the growth increase. This could be explained by the very stable cyclic structure of β CDs and the presence of methyl groups, which make enzyme-substrate binding more difficult (Loftsson and Brewster, 2010), making it impossible to hydrolyze β CDs and consume their glucose units.

4.2 Optimizing Stilbene production and oligomerization.

Resveratrol and its oligomeric derivatives are widely studied for their biological activities. The production of these molecules in an axenic *in vitro* system represents a challenge in terms of optimization and method validation. The literature includes studies on the coupled use of MeJA and β CD to increase stilbene excretion from biotechnological cultures like cell suspension or hairy roots (Jeandet et al., 2016; Tisserant et al., 2016b). β CDs are cyclic molecules able to improve resveratrol solubilization (Duarte et al., 2015) by transiently capturing it within their non-polar cavities. The results of HPLC analyses indicate that an overall increase in stilbene concentration is observed in the MCE of roots pre-treated during their

growth phase with β CD (Fig. 2). The culture media is completely changed between growth phase and elicitation phase suggesting that a potentializing phenomenon occurred during growth phase by the application of β CD. Interestingly, a study by Nopo-Olazabal et al. (2013) on stilbene production by an hairy root of *Vitis rotundifolia* Michx showed a very low or even absent secretion of stilbenes (trans-Resveratrol, ϵ -Viniferin, Piceid, Piceatannol) when elicited only with 100 μ M MeJa after 21 days of culture (Nopo-Olazabal et al., 2013). This may seem to indicate that β CDs coupled with MeJa significantly increase the secretion of stilbenes in the culture medium of *Vitis* hairy roots during the elicitation phase.

Multiple occurrences of the use of β CD as an elicitor for *Vitis vinifera* cell cultures have been reported (Almagro et al., 2015),(Almagro et al., 2020),(Belchí-Navarro et al., 2012). However, no study on this cell culture or on the hairy root model suggests that pre-treatment with β CD prior to elicitation with MeJA or the MeJA β CD pairing could significantly increase the total amount of stilbenes produced. A main problem with β CD remains its very high price and the ecological impact of its production. This does not make it the best candidate for potential biotechnological applications. Nevertheless, other dextrins could be investigated as alternative pretreatments for root or cell with the aim of enhancing the stilbene production ratio in plants.

This potentialization of the roots by β CD seems to increase both the total concentration of stilbenes and the proportion of resveratrol dimers in the extracts (Fig. 4). The dimerization of resveratrol into ϵ -viniferin and / or pallidol has been studied in *Vitis vinifera* cell cultures as being mainly the result of an oxidative reaction by an intracellular peroxidase (Marant et al., 2024). However, these plant peroxidases are not specific. A dozen have already been identified, while new ones are currently being studied (Marant et al., 2025, 2024). The results of this study seem to provide a first clue regarding where ϵ -viniferin is produced in *in vitro* root systems. The presence of ϵ -viniferin is significantly higher in β CD RCE indicating that this increase in dimerization following potentialization does not only take place with the excretion of resveratrol in the culture medium, but also occurs directly inside the roots with the presence of resveratrol. This also justifies the lower concentration of resveratrol in β CD RCE as it was potentially either more secreted (Fig. 2A) or more dimerized (Fig. 3A, B). The β CD condition results in an overall increase in resveratrol monomer units of around 40% in both the roots and the culture medium (calculated between the CTL + and β CD conditions from the results in Figs. 2 and 3).

Furthermore, the low concentration of resveratrol in CTL- RCE and MCE (Fig. 2A, 3A) and the high proportion of dimer in these extracts (Fig. 4, A and B) would indicate the presence of a constitutive conversion rate in the roots due to the potential presence of both extracellular and intracellular peroxidases or laccases (Marant et al., 2024) in these *Vitis vinifera* hairy root cultures. These enzymes are both likely to be extracellular (Marant et al., 2024) but further studies needs to be performed to understand the multifactorial oligomerization of resveratrol. The putative potentialization of hairy roots by β CD and the significant increase in resveratrol oligomerization secreted into the culture medium could make this hairy root a good object of transcriptomic and proteomic study for understanding the resveratrol dimerization mechanism under biotechnological conditions *in vitro*.

ϵ -Viniferin is a stilbene that has been much studied for its biological properties, particularly in the field of pharmacology, where it has shown anti-tumor (Giovannelli et al., 2014), obesity preventive (Gómez-Zorita et al., 2023) neuroprotective (Zhang et al., 2020) and cardioprotective activities (Wu et al., 2020). In the field of cosmetics, this molecule has been extensively studied by the French group CAUDALIE ©, from its extraction from vine shoots to its use in the Vinoperfect product line. These products are designed to reduce skin spots thanks to its inhibitory effect on tyrosinase, an enzyme involved in skin melanization (Ismail et al., 2016).

The significant enrichment of ϵ -Viniferin in the β CD MCE opens the way to various applications for these complex extracts, which could benefit from potential applications in particular in cosmetics. Performing tyrosinase inhibition tests on these MCE's would further characterize these extracts while enhancing their potential as cosmetic extracts.

Resveratrol tetramer, measured here as cis-Vitisin B, was also produced in the extract in quantities of 1.5%. This compound has recently been studied for its wide range of biological activities that can be exploited in the pharmaceutical field (anti-cancer, anti-inflammatory, cardio and neuroprotective) (Bakrim et al., 2025). The study of the production of this compound by biotechnological means in hairy root via different elicitation way could also enable extracts to be used for pharmaceutical applications.

The review published by Jeandet *et al.*, (2014) (Jeandet et al., 2014) brings together information from dozens of studies on the production of resveratrol or stilbene derivatives using *Vitis vinifera in vitro* cell suspensions. The majority of the resveratrol production presented in this cited review are expressed as resveratrol produced per liter of culture media, in flask or bioreactor. The production range was found to be between 150 and 5000 mg of resveratrol per liter of culture medium, for all grape varieties and production methods. When compared to the results of the present study, a concentration of approximately 445 mg of total stilbenes per liter of culture medium was reached under the most productive condition (β CD MCE results not shown). Although this value does not place the system among the highest *in vitro* producers, it can nonetheless be related to the observed proportions (Fig. 4).

A complex extract composed for one third of resveratrol dimers (ϵ -viniferin and pallidol) (Fig. 4) with potentially known biological activities (Biais et al., 2017; Gómez-Zorita et al., 2023; Zhang et al., 2020) was produced. The added value of this study therefore concerns the ability of the hairy root to produce these dimers. When compared to a study that aimed to enrich cell suspension extracts with ϵ -viniferin (Giovannelli et al., 2014), a maximum concentration of 7.28 mg·g⁻¹ in the dry extract was reported, whereas a concentration of 77.3 mg·g⁻¹ in the extract was achieved in this presented work (converted from Supplementary Table 1). These results suggest that this production method is a suitable approach for obtaining resveratrol oligomers of interest.

4.3 Determination of biological activities

4.3.1 Antioxidant capacity

The analysis of the antioxidant activity of the extracts was carried out using four *in vitro* assays. Antioxidant activity tests are based on different mechanisms of action. The ABTS and DPPH tests makes possible to observe the ability of a compound or extract to scavenge a radical. The CUPRAC and FRAP tests assess the ability of a compound or extract to reduce an ion or a complex. As a result, the analysis carried out by these four tests enables us to visualize the overall antioxidant activity of our extracts, and to identify the mechanisms of action involved.

The results presented in Fig. 5 tend to show that the extracts do not have a specific anti-oxidation mode of action. Indeed, the strongest activity of the extracts is found for the β CD MCE extract with the ABTS assay. However, the activity of the DPPH assay is around 10 times lower. This indicates that the compounds present in MCE β CD are able to scavenge the ABTS radical, but little or none of the DPPH radical. This is most likely reported as a usual result for the study of phenolic compounds (Tan and Lim, 2015). Furthermore, the steric accessibility of the radical usually impact the result of the test since the DPPH radical is bigger than the ABTS radical. Finally, some stilbenes have already shown undetectability from the DPPH radical like piceid and pterostilbene (Tan and Lim, 2015).

Similarly, the highest antioxidant activity measured with the CUPRAC test is found for MCE β CD (Fig. 5). This FRAP-assayed activity is also the highest for this assay, but is again 10-fold lower than the CUPRAC-assayed activity. These compounds therefore have the ability to trap compounds or reduce complexes or ions, but in a context-specific manner.

The CUPRAC test developed by Apak et al., (2004) (Apak et al., 2004) is used in particular for the analysis of molecules of interest, as the test's reagent solution approaches physiological pH (pH7), in contrast to FRAP, which is performed at acidic pH (3.6). CUPRAC is described as reflecting the total antioxidant power of a physiological sample, particularly in a mixture containing polyphenols which can be in some case less active in acidic conditions (Apak et al., 2008; Bibi Sadeer et al., 2020).

This hypothesis is supported by the correlation results indicating that the activity is due to the presence of stilbenes in the extracts (Fig. 7). A subtlety of the CUPRAC test was taken into account, which is the reactivity time of complex extracts with the CUPRAC solution, which can vary up to 60 minutes for complex mixtures (Karadag et al., 2009). The results shown in Fig. 5 are derived from 90 min assay kinetics in each test. The results shown are those after 30 min of reaction between the fresh CUPRAC solution and the sample, to allow time for the compounds in the mixture to react with the (Cu(II)-Nc) complex. In this way, maximum consideration was given to the reaction time of the mixture as a key factor influencing the test's reactivity.

The low antioxidant activity observed in the FRAP assay (Fig. 5) may be attributed to the specific nature of the test, which does not effectively detect all types of antioxidant molecules particularly those that act through radical-trapping mechanisms (Karadag et al., 2009). This limitation could explain the difference with the ABTS results, especially for the β CD MCE condition, where higher activity was observed (Fig. 5).

The ABTS test is the one for which we obtained the highest antioxidant activity values. However, it should be remembered that this test is based on the trapping of a synthetic radical, absent from biological systems, which limits the direct extrapolation of these results to physiological conditions. These high values are nevertheless obtained with one of the most commonly used tests for assessing antioxidant activity *in vitro*, giving these data comparative relevance regarding literature (Karadag et al., 2009).

The results of the antioxidant activity evaluation in ABTS indicate that CTL- RCE has an antioxidant activity equivalent to that of β CD MCE, i.e. around 40% of the activity of trolox. This result is not correlated with the stilbene concentration in these extracts (Fig. 1 Supp data), compared with the results for MCE, which have activities correlated with stilbene concentrations (Fig. 1 Supp data). These correlations are verified for the 4 antioxidant assays: FRAP, ABTS, DPPH and CUPRAC.

The high antioxidant activity observed in CTL- RCE extracts can be explained by the complexity of these extracts, derived from ethanol-extracted root crushed material, rich in diversified metabolites. The only difference with the other conditions is the absence of treatment with the MeJa- β CD couple. These results suggest that, in the absence of elicitation, other, non-stilbene compounds could contribute significantly to this antioxidant activity (Fig. 1 Supp data). Upon elicitation, this activity seems to decrease, in favor of an antioxidant activity more specifically associated with the production and accumulation of stilbenes in MCE (Fig. 1 Supp data).

Hypotheses as to the presence of antioxidant activity in CTL- RCEs focus on the potential basal presence of other antioxidant compounds in the roots such as flavonoids, tannins and phenolic acids (Goufo et al., 2020). Non-stilbene molecules in the extract may have a synergistic effect on each other, enhancing activity without necessarily being present in large quantities. In addition, MeJa elicitation may also repress other metabolic pathways through competition or reallocation of metabolic resources, leading to a decrease in certain initial antioxidant compounds. It cannot be ruled out that the extractability of these potential antioxidant molecules was altered by MeJa treatment, impacting their production by the hairy root or their availability during ethanol extraction.

Finally, reaction time was also taken into account when interpreting ABTS results. The values presented are derived from a kinetic analysis, and the point chosen at 10 minutes enables antioxidant capacity to be assessed over a short reaction time. In the context of a complex mixture, it also enables the speed of reactivity to be observed. The activities found here suggest a high reactivity of the extracts and their ability to rapidly trap the ABTS $\cdot^+$ radical.

The DPPH test, although widely used, has certain limitations that make its results sometimes difficult to interpret. It is sensitive to light, oxygen and the nature of the sample solvent. Its limited solubility in aqueous media restricts the evaluation of hydrophilic antioxidants (Karadag et al., 2009). Furthermore, reaction kinetics are not always representative of physiological mechanisms, and some compounds may interfere with the measurement or react reversibly, leading to an underestimation of actual antioxidant activity. Nevertheless, as with the other tests, the activities obtained for all MCE are strongly correlated by the presence or absence of stilbenes (Fig. 1 Supp data).

The antioxidant test results show the activity of non-purified extracts. In comparison with the results for pure molecules (result not shown) on resveratrol, it can be seen that the overall activity of β CD MCE is equivalent to that of pure resveratrol, despite the fact that it's composed only around 25% of stilbenes (Fig. 2A, B, C, D). However, this equivalence was not found in the activity measurements of the other tests (FRAP, CUPRAC and DPPH), indicating that a potential synergistic effect between the stilbenes in the mixture or between all the other potential antioxidant molecules in this complex extract may have neutralized the $ABTS^{\bullet+}$ radical. The aim of this work is to observe the antioxidant activity of complex extracts for potential cosmetic applications. With this in mind, although observing satisfactory antioxidant activity is a positive point, the cosmetic field does not necessarily require the application of active molecules with high activity. A moderate level of activity may also be valid in this field, and this antioxidant activity should be considered in relation to other biological activities and impacts, such as antimicrobial capacity or cellular cytotoxicity. The results show that the extracts tested have interesting antioxidant activities, with potential applications in cosmetics. However, these activities, which are sometimes correlated with stilbene concentration and sometimes not, require further investigation to be fully characterized.

4.3.2 Impact of stilbene on the skin flora

Natural products are a valuable source of inspiration for drug development. Resveratrol is one of the molecules studied in the fight against antibiotic resistance, notably for its antimicrobial activities (Vestergaard and Ingmer, 2019). However, the aim of this study is to understand the link between the presence of stilbenes in extracts and biological activities. Moreover, in the context of cosmetic applications, a high level of antimicrobial activity is not necessarily required. The key consideration is the potential effect of the extract when used as an active ingredient in a formulation.

The bacteria chosen for this study are part of the resident commensal flora of the skin (*Staphylococcus epidermidis* and *Corynebacterium xerosis*) or may be potential pathogenic contaminants of the skin (*Acinetobacter calcoaceticus*) (Aucken et al., 1999).

The bacterium most affected by one of the extracts was *Staphylococcus epidermidis*, with an IC_{50} of $0.310 \pm 0.0269 \text{ mg.mL}^{-1}$. This microorganism is still being studied for its beneficial and deleterious role in skin health (Brown and Horswill, 2020). In fact, although *Staphylococcus epidermidis* is well-known to colonize the skin and contributing to its proper functioning (Severn and Horswill, 2023), certain strains of this opportunistic pathogen can have a negative impact on skin health (Brown and Horswill, 2020; Claudel et al., 2019; Severn and Horswill, 2023). The antimicrobial activity of these extract should therefore be studied for its use as a cosmetic active ingredient, and its impact should be considered in relation to the different flora constituting the skin barrier.

Results for the second skin microorganism, *Corynebacterium xerosis*, were highly variable (Table 1). This may be explained by the metabolism of this species and the nature of the microplate test. This bacteria is able of producing biosurfactants on the surface of liquid cultures (Bernard and Funke, 2015). This modifies the optical density reading by the microplate reader. The results presented for this bacterium

should therefore be interpreted with caution and require further validation. However, the tendency of β CD MCE to inhibit its growth at concentrations from 1 to 2 mg.mL⁻¹, indicating an effect of stilbenes on this skin flora can be discussed.

Table 1

50% inhibitory concentration (IC50) of β -CD MCE on 3 bacterial cultures belonging to the cutaneous commensal flora. (Significant differences were demonstrated with Fischer's ANOVA tests ($p > 0.05$) (MCE and RCE). Data are expressed as the means $\pm$ SD of 3 independent experiments

	<i>Acinetobacter calcoaceticus</i>	<i>Corynebacterium xerosis</i>	<i>Staphylococcus epidermidis</i>
β CD MCE	1,04 $\pm$ 0,257 mg.mL ⁻¹	0,500 $\pm$ 0,728 mg.mL ⁻¹	0,310 $\pm$ 0,0269 mg.mL ⁻¹

Acinetobacter calcoaceticus can be found in soil, water and hospital environments. It can be implicated in opportunistic infections (pneumonia, septicemia, urinary tract infections, wound infections) and possesses a certain resistance to antibiotics (Glover et al., 2022). Due to the context of rising antibiotic resistance, it is interesting to study the ability of natural extracts such as stilbene to inhibit this flora for cosmetic or pharmacological applications.

The antimicrobial effect on various flora of the extract with the highest stilbenes concentration (β CD MCE) opens the way to the potential use of this active ingredient in the care of oily skin with acneic tendencies or for specific skin disorders (Claudel et al., 2019). However, the inhibition of commensal flora needs to be assessed over the long term, to avoid a phenomenon of dysbiosis, notably between the cutaneous bacteria *Staphyloccocus epidermidis* and *Cutibacterium acnes*. A change in the colonization of these different bacteria can have a deleterious impact on skin health, with the appearance of overproduction of sebum or acne, for example (Fournière et al., 2020).

It is therefore necessary to understand the impact of these extracts on other species of skin flora. However, the final concentration of these extracts as active ingredients in potential cosmetic formulations needs to be considered. They will not be used at concentrations close to the IC50 of human keratinocyte cells (HaCaT) (between 0.6 and 0.9 mg.mL⁻¹ Table 2). In view of the results on these cells, the IC50s in Table 2 and the bacterial viabilities in Fig. 6 only *Staphyloccocus epidermidis* appears to be affected at concentrations lower than the IC50 value for human keratinocytes.

4.3.3 Impact of the extracts on skin cell viability

Cellular response after 24 hours exposure to the extracts was assessed on an immortalized human keratinocyte line (HaCaT) and on three bacterial strains associated with skin flora. The results obtained (Tables 1 and 2) show measurable cytotoxicity, but this remains comparable between bacterial and cellular models, with no cell type appearing particularly more sensitive to the concentrations tested.

One specificity found for viability results on HaCaT cells is the wave effect of cytotoxicity. A concentration-dependent decrease in viability is observed, followed by an increase and a further decrease (results not shown). This phenomenon is known as the “U” shaped dose response or hormetic effect (Calabrese et al., 2022). It can be found in the literature for complex mixtures of molecules (Barreto-Diaz et al., 2023) and is an indicator of the complexity of the reaction between extracts and cells. This shows that the extract can sometimes be more or less beneficial depending on concentrations (Barreto-Diaz et al., 2023; Calabrese et al., 2022). Once this phenomenon is known, it will be important to always acknowledge the impact of the extract on these skin cells at the concentrations envisaged for use as a cosmetic active ingredient, in order to define the risks and benefits for human health.

This study focused on the biotechnological production of stilbenes from *Vitis vinifera* hairy root in the form of complex extracts. The application of different culture conditions during the root growth phase enabled us to produce extracts with different concentrations of stilbenes, and in particular to significantly increase both the stilbenes concentration and the oligomerization of resveratrol into ϵ -viniferin, pallidol and *cis*-vitisin B. This method enables a reliable and repeatable production of these extracts.

Several *in vitro* biological activities of these extracts were tested for potential cosmetic applications. The extracts with the highest stilbenes concentration appears to have the overall strongest antioxidant and antimicrobial biological activities. A high proportion of stilbenes in extracts derived from the culture medium offers good antioxidant activity and has a negative impact on opportunistic pathogenic bacterial flora of the skin which is a promising result for cosmetic applications. These results are validated by correlations that confirm the role of stilbenes in these activities, even in unpurified complex extracts.

The *in vitro* cytotoxicity of the extracts was tested on a human keratinocyte cell line (HaCaT line). The results show that the cytotoxicity of MCE is similar whatever the stilbenes concentration, indicating that an increase in bioactive compound does not necessarily increase the cytotoxicity of the crude extracts. This result is also promising for potential cosmetic uses. The biological activities of the RCEs were generally low, with the exception of the CTL- condition, which seemed to offer a decent antioxidant activity despite a very low stilbenes concentration.

These extracts have the particularity of being obtained using solvents that are more environmentally friendly than those usually used for stilbene extractions (Soural et al., 2015). Obtaining and studying these extracts demonstrates the interest of plant biotechnologies for a consistent confined controlled and environmentally-friendly production of molecules of interest in various fields of application.

Abbreviations

MeJA : Methyl Jasmonate

MCE : Medium Crude Extract

RCE : Root crude extract

βCD : Methyl-β-cyclodextrin

Linecaps : Kleptose Linecaps maltodextrin

DMEM LC : Dulbecco's Modified Eagle medium Low Calcium

DT : Doubling time.

Declarations

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All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Alban COQUEL, Romain ROULARD and Sandy THEYSGEUR. The supervision of this study and project were set up by Elodie CHOQUE, Rozenn RAVALLEC Isabelle GAILLARD and Nathalie JULLIAN. The first draft of the manuscript was written by Alban COQUEL and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability statement

The datasets generated and/or analyzed during the current study are not publicly available because they contain information considered partially confidential and are related to an ongoing research project that has not yet been disclosed. However, data may be made available from the corresponding author upon reasonable request.

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Figures

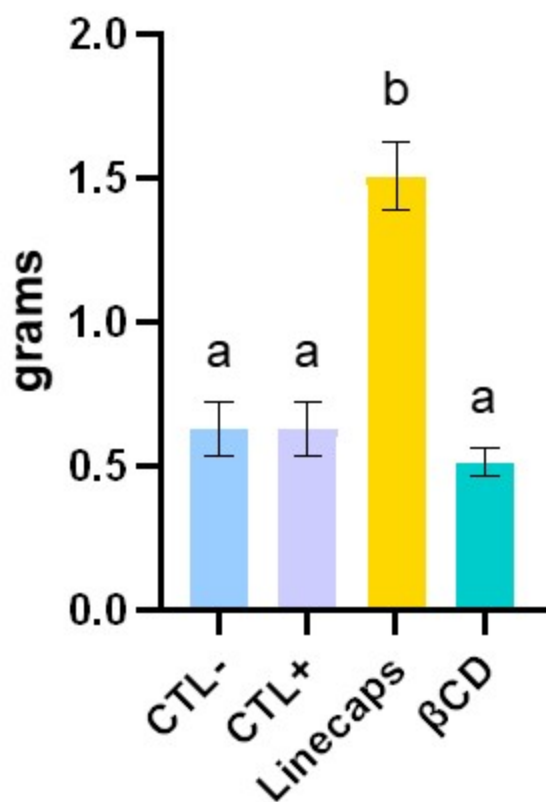


Figure 1

Effect of dextrins on the increase of fresh biomass of a hairy root line of *Vitis vinifera* cv Pinot Noir after 21 days of culture in ½ MS medium, 3% sucrose, pH5.8. Conditions were compared to control using Welch's ANOVA test ($p < 0.05$).

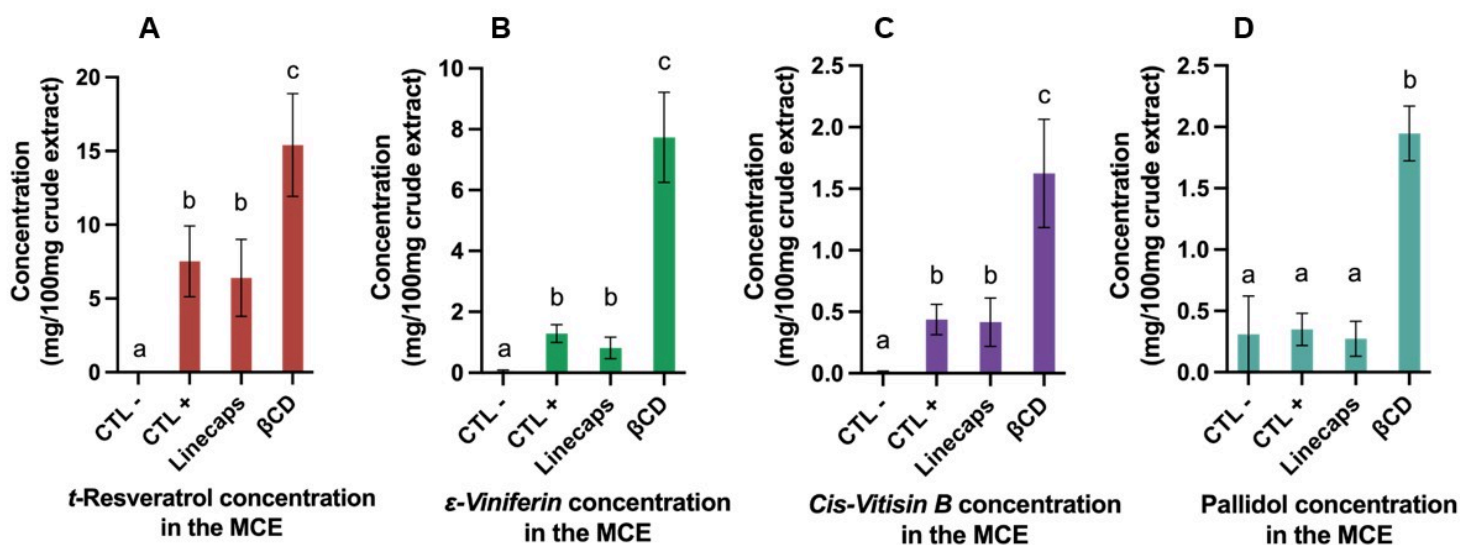


Figure 2

Stilbene concentration in the Medium Crude Extract (MCE) (A) *trans*-Resveratrol, (B) ϵ -Viniferin, (C) *cis*-Vitisin B, (D) Pallidol. Means are calculated from 6 biological replicates, standard deviations shown demonstrate deviation from the mean. Significant differences were demonstrated with Welch's ANOVA tests ($p>0.05$)

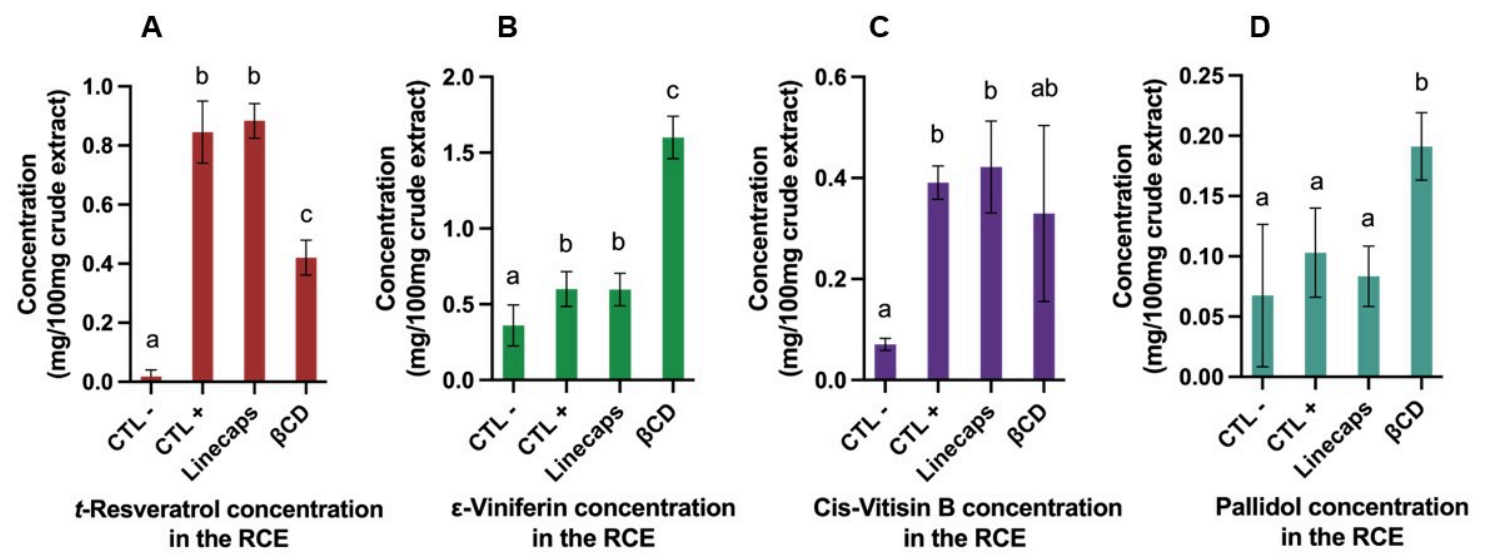


Figure 3

Stilbene concentration in the Roots Crude Extract (RCE) (A) *trans*-Resveratrol, (B) ϵ -Viniferin, (C)*cis*-Vitisin B, (D)Pallidol. Means are calculated from 6 biological replicates, standard deviations shown demonstrate deviation from the mean. Significant differences were demonstrated with Fisher's or Welch's ANOVA tests ($p>0.05$).

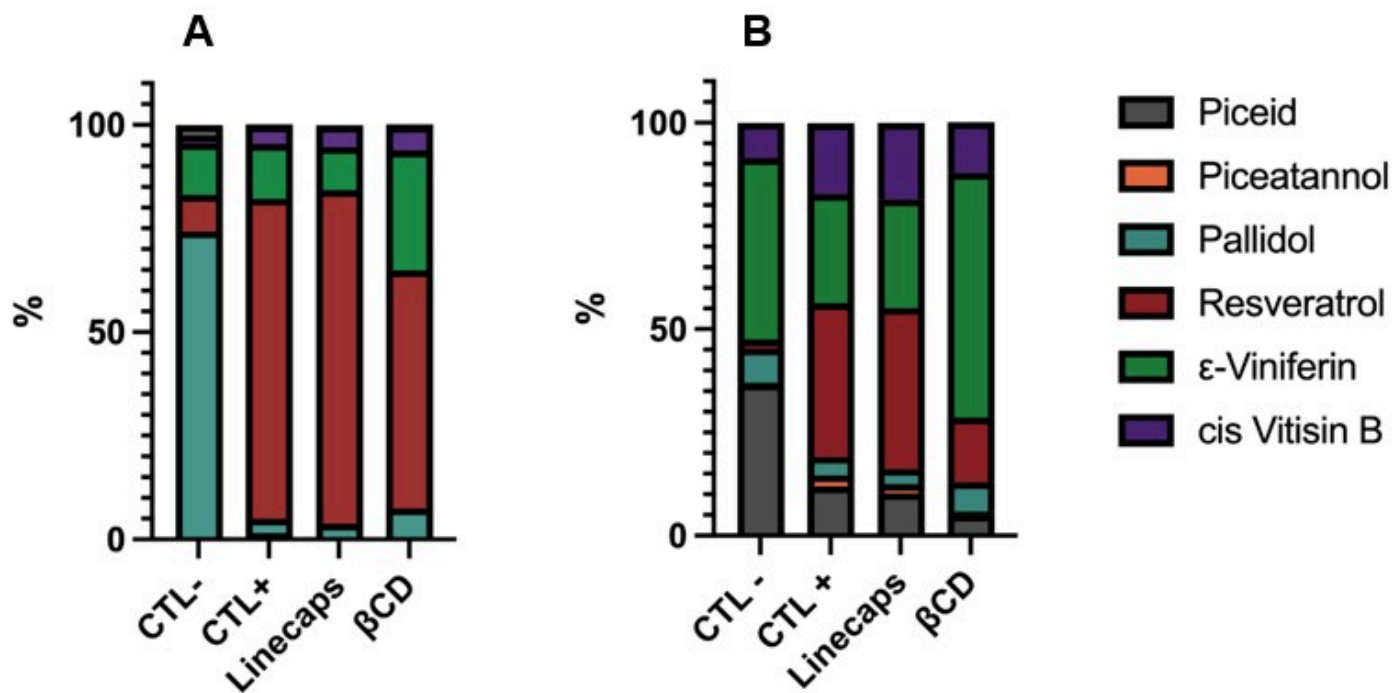


Figure 4

Proportionality in percentage of the 6 major stilbenes of the medium (A) and root (B) crude extracts analyzed by HPLC after 21 days of cultivation and 7 days of elicitation (or 21 + 7 days of conventional cultivation for the negative control condition without elicitation).

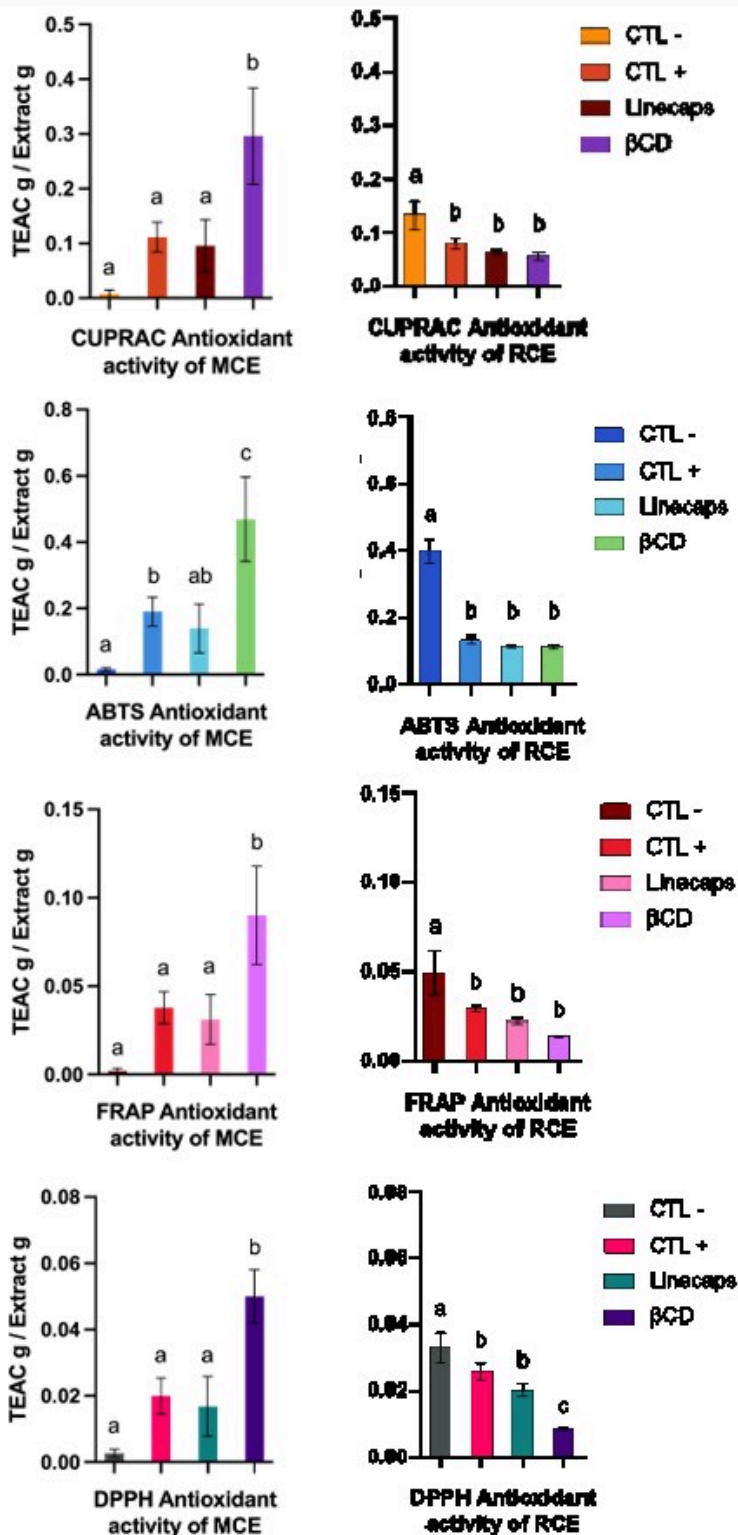


Figure 5

Trolox equivalent capacity (TEAC) expressed as gram of trolox per gram of extract obtained from four antioxidant assay methods (CUPRAC, FRAP, ABTS, DPPH). Significant differences were demonstrated with ANOVA tests ($p > 0.05$).

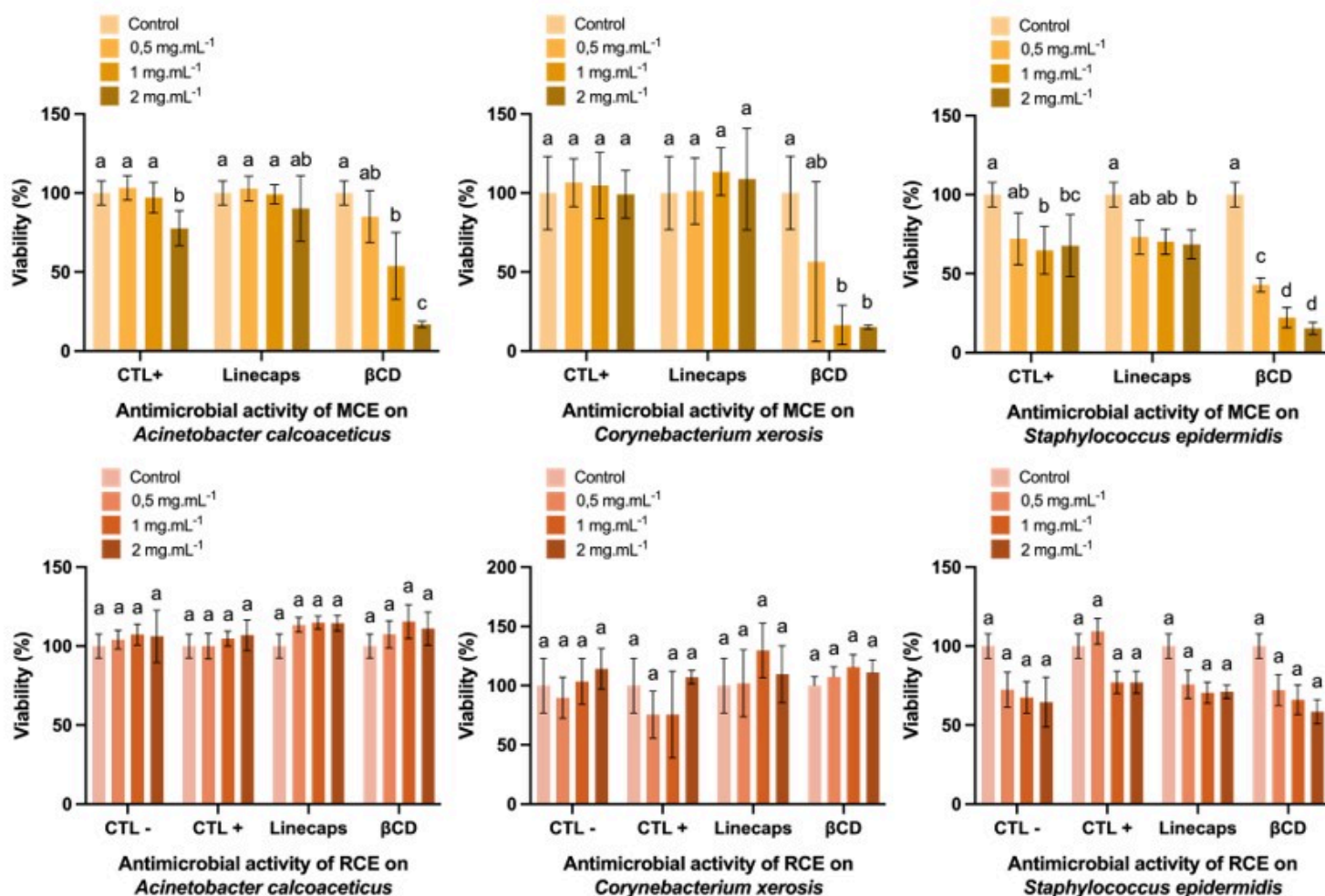


Figure 6

Effect of crude extracts containing different stilbenes content tested at different concentrations (0.5, 1 and 2 mg.mL⁻¹) on the viability of bacterial cultures related to the cutaneous commensal or pathogenic flora. Significant differences were demonstrated with Welch's ANOVA ($p > 0.05$) (MCE) and Kruskal and Wallis ANOVA ($p > 0.05$) (RCE) tests.

MCE Stilbene concentration and CUPRAC antioxidant capacity correlation

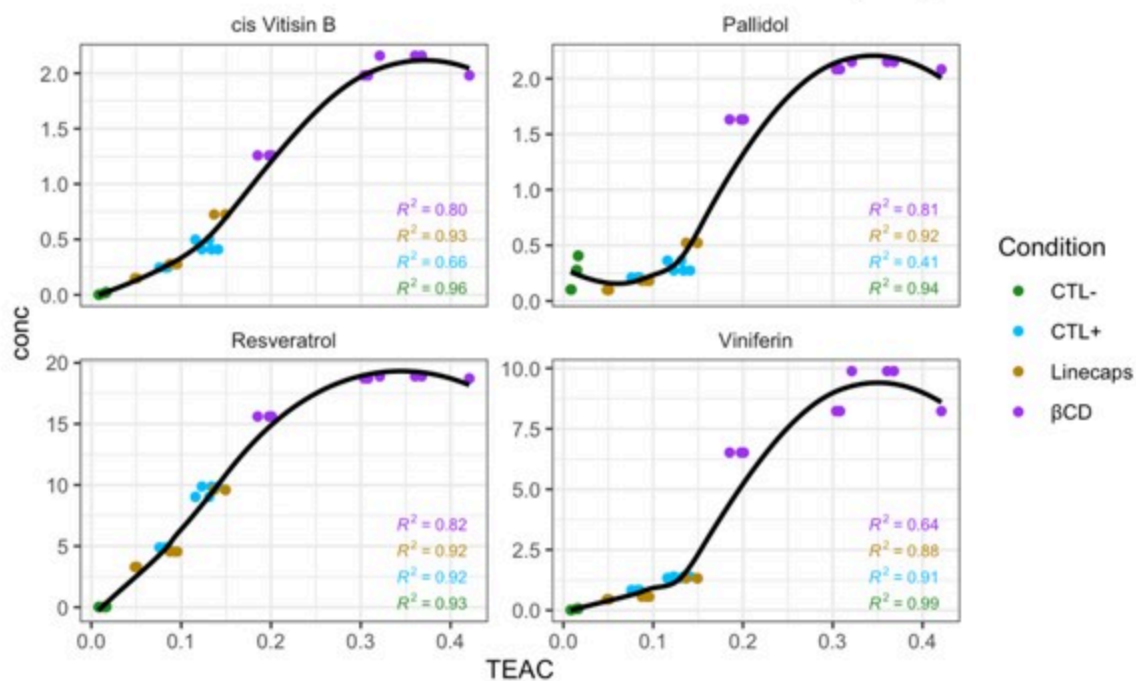


Figure 7

MCE Stilbene concentration and CUPRAC antioxidant capacity correlation. R^2 were calculated using the linear regression from the `stat_poly_eq` formula in the `ggmisc` package on R.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Supplementarydataarticlestilbene.docx](#)