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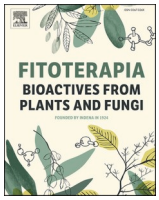

Targeting human skin health: Bioactive evaluation of acorn shell and red grape pomace extracts against pathogens and skin cells

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ABSTRACT

The agricultural and food sectors generate substantial amounts of by-products that are usually discarded as waste. However, agri-food by-products, rich in many bioactive compounds (BCs), can find several applications while promoting sustainability. Acorn shell (AS) and red grape pomace (RGP) stand out as valuable sources of BCs. To be used in human skin, the biological properties of these by-products need to be investigated. Therefore, this study explored the chemical composition of AS and RGP extracts and evaluated their concentration-dependent effects on key pathogenic (*Staphylococcus aureus*) and non-pathogenic (*Staphylococcus epidermidis*) bacteria of the skin microbiota, as well as on resident skin cells (keratinocytes) and skin cancer cells. The AS extract, rich in phenolic acids and gallotannins, and RGP extract, abundant in flavonoids, demonstrated significant antimicrobial activity by inhibiting bacterial growth and biofilm formation ($p < 0.001$). Notably, RGP extract exert species-specific effects on biofilm development. In parallel, both extracts showed concentration-dependent effects on cell viability, cell cycle, and cell death in keratinocytes and skin cancer cells. Despite limited effects on IL-10 secretion, their BCs profiles suggest potential anti-inflammatory benefits. AS and RGP extracts show promise for antimicrobial, antibiofilm, anticancer, and anti-inflammatory skin applications, though further research is needed to clarify their molecular mechanisms.

1. Introduction

The agricultural and food sectors contribute to the generation of large quantities of by-products, which are often discarded as waste, posing a pressing environmental concern. Therefore, significant efforts are of utmost importance for a sustainable agro-waste management. To face such challenge, both research institutions and industries are directing substantial efforts towards recycling and reusing these by-products, thus embracing a circular bioeconomy perspective [52]. In this sense, intensive investigation has explored a diversity of agri-food by-products aiming at a plethora of smart strategies to create sustainable products that offer specific benefits to consumers while mitigating environmental impacts [5,17,59].

Agri-food by-products serve as rich reservoir of bioactive compounds (BCs) with relevant biotechnological, nutritional, and medicinal potential [40,79]. Primarily sourced from agro-industrial transformations, such as grains, legumes, fruits, and vegetables, these by-products are abundant in macronutrients (carbohydrates, protein, and fatty acids) and micronutrients (vitamins, iron, calcium, and potassium) [40]. Moreover, they contain substantial amounts of BCs, including phenolic compounds, carotenoids, and fibers, which exert various biological activities, such as antioxidant, antimicrobial, prebiotic, anti-inflammatory, anticancer, and regenerative effects [40,59,79].

Initially utilized as nutraceuticals or additives to improve food quality and shelf-life, agri-food by- products have attracted interest from the cosmetic, textile, and pharmaceutical industries due to their rich bioactive content and potential for incorporation into innovative formulations and functional products [5,50,63]. Notably, the by-products from animal food and wine industries are receiving special attention due to their significant amount of production within limited timeframes.

For instance, acorn, a fruit from *Quercus* species, is traditionally employed as animal feed and medicinally for the treatment of various conditions, including diabetes, hemorrhoids, and wound healing [13]. Among its benefits, some studies have highlighted its promising antimicrobial properties due to its high polyphenol content [18,24,38,67]. However, while the acorn fruit has been extensively used, its shell is usually discarded as a by-product. Interestingly, it has been demonstrated that the acorn shell (AS) presents still a higher phenolic content

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when compared to the acorn fruit [65]. This highlights the potential of this by-product to be explored as source of specific BCs. Nevertheless, this property of the AS has long been superficial and needs further investigation not only to clarify its antimicrobial and potential, but also other biological properties.

Regarding the wine industry, the large quantity of by-products resulting from the world's red wine production, is of particular interest. In fact, during the winemaking process, after alcoholic fermentation, about 20 % of the processed grapes (by weight) remains as solid organic waste, namely pomace. Grape pomaces (GP) are composed by seeds, stalks, and skins being rich in high-added-value compounds, mainly polyphenols, including phenolic acids, flavonoids, and anthocyanins [1,53]. Similar to grapes, they present numerous health-promoting benefits, including cardiovascular and metabolic health, cancer prevention, skin and gut health, due to their antimicrobial, antioxidant and anti-inflammatory properties [1,4]. In this line, recent studies have demonstrated that specific BCs, particularly phenolic acids and flavonoids, interact with skin cells through well-defined molecular mechanisms. These interactions are often concentration-dependent and support the potential application of such compounds in dermatological and cosmetic formulations [12,39,48].

Phenolic acids mitigate oxidative stress by scavenging reactive oxygen species (ROS) and enhancing endogenous antioxidant enzymes, thereby protecting skin cells and preserving barrier function [32]. They also exhibit anti-inflammatory effects by downregulating pro-inflammatory cytokines via NF-κB pathway inhibition. Flavonoids, further modulate cellular responses by influencing signaling pathways such as MAPK and PI3K/Akt, leading to reduced inflammation, enhanced wound healing in keratinocytes, and induction of apoptosis and cell cycle arrest in skin cancer cells [82].

Together, the rich content in BCs of both AS and red grape pomace (RGP) by-products emphasizes that they are a promising source for the development of high-added-value products, including nutraceuticals, functional foods products, skincare, and several products for biomedical applications [17,53,65]. However, at the skin level, specific biological effects of these agri-food by-product extracts require further investigation to clarify their concentration-dependent activities and guide their optimal application in skin-related formulations. In this sense, this study aimed to evaluate the direct effect of different concentrations of extracts from AS and RGP on key components of the human skin, particularly, on both pathogenic (*Staphylococcus aureus*) and non-pathogenic (*Staphylococcus epidermidis*) bacteria from skin microbiota, as well as on resident skin cells (keratinocytes), and skin cancer cells. To achieve this, antimicrobial and antibiofilm activity, cell viability, cell cycle, and cell death mechanisms were systematically assessed to elucidate their concentration-dependent bioactivities.

2. Materials and methods

2.1. Sample preparation

Acorn shell (AS - *Quercus suber* L.) was kindly provided by Herdade do Freixo do Meio (Foros de Vale de Figueira, Montemor-o-Novo, Portugal). The AS was stored in polyethylene bags and transported to the laboratory and kept under controlled conditions to carry out the analysis. Red grape pomace (RGP - seeds, skins, pulp) from grape cultivar "Vinhão" grape was kindly provided by a farm in the north of Portugal that produces red wine. This by-product was collected after production and transported under refrigeration until they reached the laboratory. After collecting, the samples were homogenized, packed in polyethene flasks, and stored at −80 °C before any manipulation. In the next step, the samples of by-products were dried in an oven at 55 °C until levels of moisture of ca. 5 % were attained, and then milled in a kitchen robot Thermomix TM5, to obtain a flour with a particle size of <1 mm. Lastly, 100 g of each sample was stored in sampling bags in a dark and dry place at room temperature (RT) until further analysis.

2.2. Extraction of AS and RGP

To obtain extracts of AS and RGP, 50 % (*v*/v) absolute ethanol (ET) (CH_3CH_2OH, Honeywell, Portugal) and deionized water (H_2O) were used as solvents following the extraction protocol previously described by Melo et al. [38]. For this, each sample was mixed with 50 % aqueous ethanol at a 1:10 (*w/v*) solid-to-solvent ratio and homogenized with an Ultraturrax T18 (Ika, Germany) at 20,000 rpm for 1 min at 25 °C. Sequentially, each sample was placed in an orbital shaker at 200 rpm for 60 min at 25 °C to promote the extraction (solid–liquid extraction). After the extracts were centrifuged at 10,000 rpm, then kept at 4 °C for 10 min, the supernatants were then filtered through a filter paper (4–7 μm, Prat Dumas, Couze St. Front, France) to eliminate any remaining solid waste. After that, ethanol was evaporated at 37 °C using a Büchi R-210 rotary evaporator (Flawil, Switzerland) and the extracts were lyophilized and used for activity measurements.

2.3. Identification of phenolic compounds by LC-ESI- UHR-QqTOF-MS/MS

The identification of the phenolic compounds present in the extracts was carried out with an LC-ESI-UHR-QqTOF-MS system (Bruker Daltonics, Billerica, MA, USA) following the previous methodology [49] with some modifications. Adjustments were made in the gradient elution program (mobile phase A: 0.1 % aqueous formic acid and mobile phase B: acetonitrile with 0.1 % formic acid) to obtain a good separation of phenolic compounds, namely: 0–5 min (5 % B), 5–25 min (15 % B), 25–35 min (30 % B), 35–40 min (95 % B), 40–41 (5 % B) and 41–42 min (0 % B). Before injection, extracts were solubilized in (a) ultra-pure water and (b) Phosphate-buffered saline (PBS) followed by the addition of Dulbecco's Modified Eagle Medium (DMEM) (1:1), to a final concentration of 20 mg (dry extract)/mL. To remove potential interferents, an equal volume of cold methanol (−80 °C) was added (1:1, *v*/v), and the mixture was centrifuged. The resulting supernatants were then diluted to 400 mg/L and filtered through a 0.22 μm nitrocellulose membrane before LC-MS analysis.

The separation was performed in a Bruker Elute series liquid chromatograph, equipped with an UHR-QqTOF mass spectrometer, with 50,000 full-sensitivity resolution (FSR) (Impact II, Bruker Daltonics, Bremen, Germany) and a BRHSC18022100 intensity Solo 2 C18 column (100 × 2.1 mm, 2.2 μm, Bruker). The injection volume was 5 μL and the parameters for MS analysis were defined using negative ionization mode, with spectra acquired from *m/z* 20 to 1000 in an Auto MS scan mode. Post-acquisition internal mass calibration used sodium formate clusters, with the sodium formate delivered by a syringe pump at the start of each chromatographic analysis.

Data analysis was carried out using Compass DataAnalysis and MetaboScape® software (both from Bruker Daltonics). The elemental composition of the compounds was confirmed using accurate mass measurements and isotope distribution analysis (mSigma, Bruker Daltonics). Mass accuracy was within 5 mDa of the assigned elemental formula, and mSigma values under 20 provided confirmation. Compound identification was achieved by assessing the accurate mass [M − H], and the mass spectral patterns were compared with metabolomic databases such as the Human Metabolome Database (HMDB) and Chemical Entities of Biological Interest (ChEBI). Additionally, in silico fragmentation predictions using MetFrag and information from relevant publications were employed for further verification. Areas of each compound were calculated using extracted ion chromatograms at the corresponding deprotonated molecular ion.

2.4. Microbiology assays

Frozen *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermidis* (DSH 20044) were resuspended and inoculated in sterile tubes containing Mueller-Hinton (MH) broth (Biokar Diagnostics) and left to

grow at 37 °C for 24 h. From the resulting bacterial suspension, a small aliquot was collected with a sterile loop and streaked in MH agar. Bacteria were then left to grow in optimal conditions and once visible colonies were formed, a single isolated colony was collected and inoculated in MH agar. For all the assays, fresh inocula for each bacterial strain were generated from the previous inoculum. The bacteria concentration (CFU/ mL) in the inocula was determined in the mid-log growth phase by optical density (OD 600 mn) and CFU counting.

2.5. Determination of minimum inhibitory concentration

The antimicrobial activity of the by-product extracts was evaluated using a microdilution assay as previously described [33]. Briefly, a series of dilutions of the extracts ranging from 0 to 40 mg/mL were prepared and plated in a 96 well plate with 50 μL of each bacteria (inoculum concentration ~ 8 log CFU/mL). After 24 h of incubation, the minimum inhibitory concentration (MIC) was defined as the lowest concentration at which no microbial growth was observed. The minimum bactericidal concentration (MBC) was determined by sub-culturing the test dilutions on agar plates to evaluate the microbial growth. The MBC was considered the lowest concentrations at which 99.9 % of the microbial population was killed. Three independent assays were performed and results expressed as mg/mL.

2.6. Impact of by-product extracts on bacterial growth kinetics in skin-like conditions

The effects of the extracts on the selected bacteria were evaluated through the determination of growth curves of *S. aureus* and *S. epidermidis* in the absence and presence of by-product extracts. Succinctly, aliquots of the extracts (sub-MIC concentrations) were prepared and 50 μL of each concentration (in distilled water) was added to a (round bottom) 96-well microplate (Sarstedt, Germany). For AS, 1.75, 0.87, 0.43, and 0.21 mg/mL (as final concentration/ well/ condition) were tested; while for the RGP, 10, 5, 2.5, and 1.25 mg/ mL were tested. Afterwards, bacterial inoculum of 10^8 colony-forming units (CFU) mL^{-1} was prepared in three different solutions as follows: MH broth, 0,1 % peptone in sterile water, and minimal medium containing 0,9 % NaCl. Herein, we used: *i*) MH broth as a positive control; *ii*) peptone water (containing some key components found in human skin, such as proteins, amino acids, and water as a strategy to mimic the composition of the human skin); and *iii*) minimal medium (0.9 % NaCl in sterile water +0.003 % of MB broth) to mimic the physiological conditions of the human sweat [31]. For this, 50 μL of each bacteria inoculum was transferred to the respective 96-well plate already prepared with specific concentration of the extracts. The plate was then put in a microplate reader (Multiskan GO, Thermo Scientific, Vantaa, Finland) to read the OD at 600 nm for a 24 h period at 37 °C (1 h intervals). A punctual read at 48 h was also performed to understand the impact of such by-extracts on bacterial growth over prolonged periods. Aliquots of the extract solutions without inoculum were used as negative controls while aliquots of the different bacterial inocula in MB broth were used as positive controls of bacterial growth. In addition, aliquots of the different bacterial inoculum from peptone solution and NaCl were also considered as control (without extract). The increase or decrease in OD was calculated and used to evaluate the bacterial growth in the presence/absence of those by-product extracts. Each condition was tested in triplicate in three independent assays.

2.7. Antibiofilm assay

S. aureus and *S. epidermidis* were grown in MH broth overnight. Afterwards, the bacteria concentration of each inoculum was determined by densitometry and 100 μL of each bacterial inoculum of 10^8 colony-forming unit (CFU) mL^{-1} was added to a 96-well (flat-bottom) microplate together with the different concentrations of AS (0, 0.87, 1.75 and 2.5 mg/mL), and RGP extracts (0, 10, 20, and 40 mg/mL). The plates were incubated for 24 h at 37 °C to allow the biofilm formation. After that time, the content of each well was removed and washed with ringer solution to ensure only the adhered biofilm would be measured. For the measurement of biofilm formation, we followed the methodology of Stepanovic et al. 2020 with some modifications [69]. Briefly, the biofilms were treated with 0.1 % crystal violet for 15 min at RT. The plates were then washed with distilled water 3 times and left to dry at RT for 24 h. The wells were resuspended in glacial acetic acid (30 %), and the OD was measured at $\lambda = 595$ nm using a microplate reader. All experiments were carried out in triplicate. Bacteria cultured in MH broth without the addition of extracts served as the positive control for biofilm formation. The antibiofilm activity of the extracts was evaluated by comparing the OD595 values of treated wells to those of the positive control.

2.8. Cell culture lines and assays

The immortalized human keratinocyte line (HaCaT) obtained from Cell lines Service (Oppenheim, Denmark) was defrosted and maintained in Dulbecco's Modified Eagle Medium (DMEM, high glucose), with 10 % of Fetal Bovine Serum (FBS, BioWest), and 1 % of Penicillin-Streptomycin-Fungizone solution (Pen-strep, Lonza). The skin cancer cells, i.e., malignant melanoma line SK-MEL-3 (ATCC HB 69) were purchased from the American Type Culture Collection, USA. The cells were initially thawed according to recommendations for this cell line. SK-MEL-3 were maintained in McCoy's 5 A (Modified) Medium (Gibco), containing 15 % of FBS and 1 % of Pen-strep solution. Both cell lines were maintained at 37 °C in 5 % CO_2 humidified atmosphere during all the experimental time.

2.9. Cell viability, cell cycle, and cell death assays

To perform the cell viability assay, keratinocytes and skin cancer cells were plated onto a 96-well plate at 1×10^5 cells/mL of density and allowed to attach overnight. Cells were then incubated with various concentrations of the extracts (AS: 0, 0.25, 0.5, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5 and 4 mg/mL) or (RGP: 0, 0.25, 0.5, 1, 1.25, 1.5, 1.75, 2, 2.5, 3, 3.5, 4, 5, 6, 8,10 mg/mL) in 100 μL of complete medium. After 24 h of incubation, the metabolic activity of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. For this purpose, the culture media was removed and replaced with 90 μL of DMEM and 10 μL of MTT solution (5 mg/mL) for 3–4 h at 37 °C in a CO_2 incubator. Following incubation, the culture media was discarded and 100 μL of DMSO per well was added to dissolve the formazan crystals. Subsequently, the plates were kept under agitation for 10 min at RT and the absorbance was measured at 570 nm using a microplate reader (Synergy 4, Biotek). The cell viability was calculated in percentage as follows:

$$\text{Cell viability percentage} = \text{Abs of treated cells} \times 100$$

$$\text{Abs of untread cells (cells cultured without extract)}$$

Each sample tested in triplicate, and each experiment (per cell line) repeated at least three independent times.

For cell cycle assay, both cell lines were plated onto 6 well-plates at 3×10^5 cells/well of density and allowed to attach overnight. The medium was discarded and the cells were incubated with different concentrations of the extracts, as follows: AS (keratinocytes: 0, 0.25, 0.5, 1, 1.5, 2 mg/mL; skin cancer cells: 0, 0.5, 1, 2, 3, 4 mg/mL) and RGP (keratinocytes: 0, 0.5, 1.25, 2.5, 3, 4 mg/mL; skin cancer cells: 0, 0.5, 1.25, 2.5, 5, 10 mg/mL) in 2 mL of complete medium. After 24 h of incubation, the protocol for the cell cycle analysis by quantitation of DNA content was performed as previously described [37]. Briefly, after incubation with the extracts, both floating and attached cells were collected, washed with PBS and incubated with 70 % cold ethanol for 15 min.

Subsequently, cells were washed twice with PBS, incubated with RNase A (200 μg/mL) for 15 min in the dark at 37 °C and with PI (0.5 mg/mL) for 30 min at RT. Samples were immediately analysed using a BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Three independent assays per cell line and by-product extract were performed.

For the cell death assay, we used a FITC Annexin V apoptosis detection kit (BD Pharmingen). For this, both cell lines were plated onto 24 well plates at 1×10^5 cells/well and allowed to attach overnight. The media was the discarded and the cells were incubated with different concentrations of the extracts, as follows: AS (keratinocytes: 0, 0.5, 2, 4 mg/mL; skin cancer cells: 0, 0.5, 2.5, 5 mg/mL) and RGP (keratinocytes: 0, 0.5, 2.5, 4 mg/mL; skin cancer cells: 0, 0.5, 2.5, 5 mg/mL) in 1 mL of complete medium. 10 % DMSO was used as positive control to induce cell death in both cell lines. After 24 h of incubation, both floating and attached cells were collected, washed with PSB, and centrifuged. Afterwards, the supernatant was discarded and resuspended in 100 μL 1 x binding buffer and incubated with 5 μL of FITC Annexin V and 4 μL of PI for 15 min in the dark at RT. Samples were analysed immediately using a BD Accuri™ C6 flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). A dot plot with FL1 channel for detection of FITC Annexin staining and FL4 channel for detection of PI staining enabled the following discrimination: viable cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), late apoptotic cells (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+).

2.10. Quantification of interleukin-10 (IL-10)

To quantify cytokines release, both keratinocytes and skin cancer cell were seeded under conditions similar to those used for the MTT assay. After 24 h of exposure to different concentrations of AS and RGP extracts, 100 μL of the supernatant from each well was collected from three independent experiments and stored at −20 °C until analysis. The levels of IL-10 were quantified using an *enzyme-linked immunosorbent assay* (ELISA) with a commercially available human IL-10 ELISA kit (Invitrogen), following the manufacturer's recommendations. Absorbance was measured at 450 nm using a Synergy 4 microplate reader (Biotek).

2.11. Statistical analysis

Data are presented as means ± standard deviation of the mean and were analysed using the GraphPad Prism software. When appropriate, multiple comparisons were performed using the one-way ANOVA or two-way ANOVA. Statistically significant differences were considered as follows: $^{*,\#} = p < 0.05$, $^{**,\#\#} = p < 0.01$, $^{***,\#\#\#} = \mathrm{p} < 0\ 0.001$, $^{****,\#\#\#\#} = p < 0.0001$.

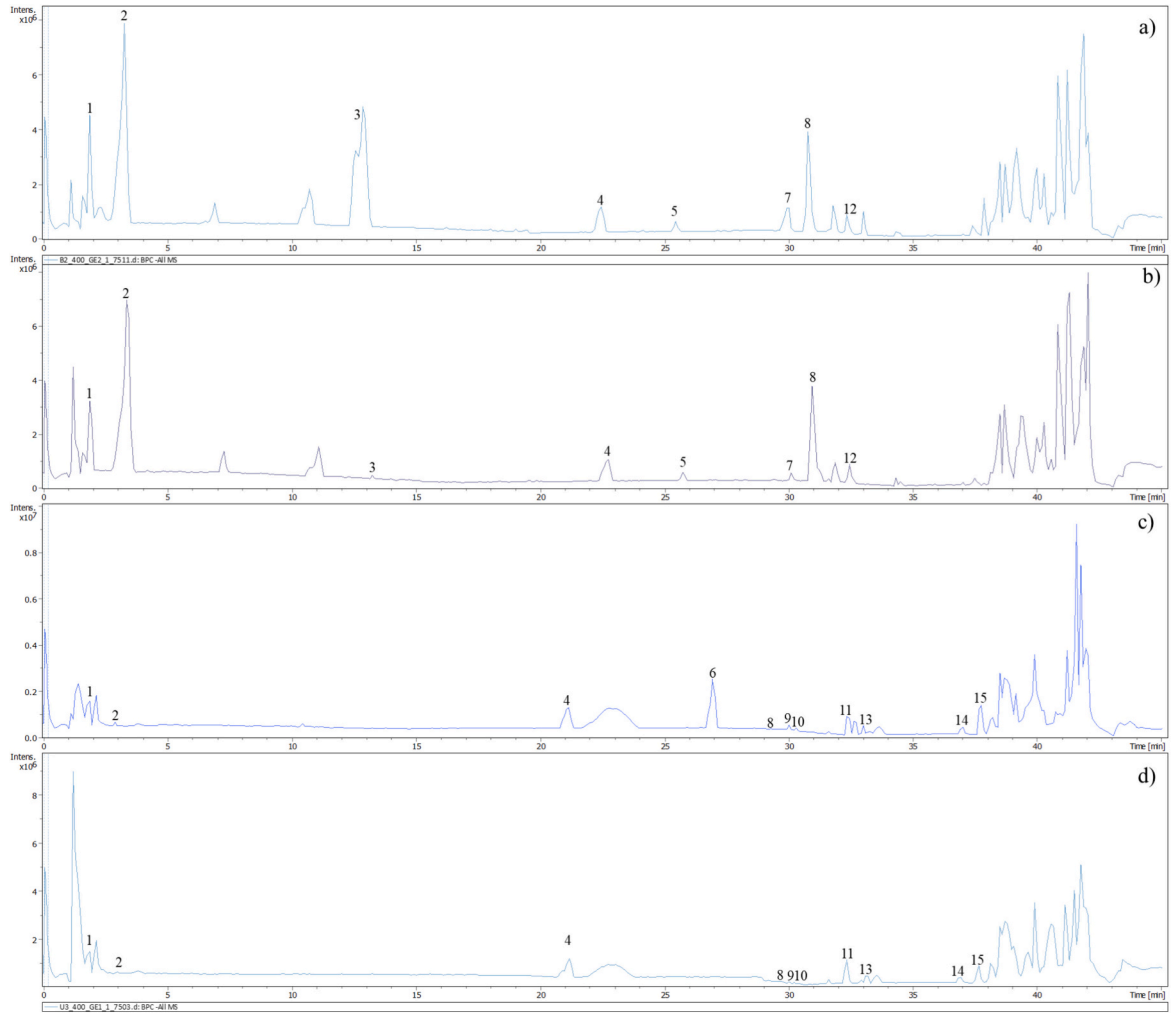

Fig. 1. Chromatogram of aqueous (a) and methanolic (b) acorn shell extract and aqueous (c) and methanolic (d) red grape pomace extract. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results and discussion

3.1. Phenolic profile of the extracts: AS and RGP

The phenolic compounds present in AS and RGP extracts were analysed by LC-ESI- UHR-QqTOF-MS/MS (Fig. 1). Compounds were identified based on their retention time (RT), accurate mass, and MS/MS fragmentation patterns, compared with reference standards and literature data. A complete list of identified compounds is provided in Supplementary Table S1. Table 1 presents a selection of the most representative or differentiating compounds, grouped by class, with emphasis on those known for relevant biological activities.

The phenolic analysis revealed a rich diversity of phenolic acids, flavonoids, and tannins, contributing to the unique bioactive properties of the extracts. In the literature, the predominant phenolic compounds reported in AS include bioactive phenolic acid derivatives, such as gallic and ellagic acid, and their derivatives [66]. In agreement with the literature, the main phenolic compounds of AS were gallic acid and ellagic acid, regardless of the solvent used. These phenolics are known for their potent antioxidant properties [66]. In contrast, the RGP extract contained higher levels of flavonoids, notably catechin and quercetin derivatives, which are associated with anti-inflammatory effects.

Gallic acid (peak 2) was identified as the primary phenolic compound in AS, presenting a deprotonated molecular [M − H] − ion at *m/z* 169.0142, with primary fragmentation characterized by the loss of the CO_2 group (m/z 125.0244). Regarding hydroxybenzoic acid derivatives, *p*-hydroxybenzoic acid was the third most abundant phenolic compound identified in AS extracts, followed by protocatechuic acid (Table S1). Both compounds have previously been identified in *Quercus* acorns. However, *p*-hydroxybenzoic acid has primarily been associated with acorn leaves and wood [55,61]. On the other hand, protocatechuic acid, besides being reported in leaves and wood, has also been detected in beech achene kernels [66] and red oak (*Quercus rubra* L.) seeds [54].

Ellagic acid (peak 8) was the second most abundant phenolic compound identified in AS extracts, presenting a deprotonated molecular [M − H] − ion at *m/z* 300.9990, with a daughter peak at *m/z* 229.0142 [M-H]−, consistent with the loss of CO_2 and CO groups [34]. Ellagic acid has previously been identified in the fruits of several *Quercus* species [54]. The peak with a deprotonated molecular [M − H] − ion at *m/z* 463.0518, with primary fragmentation characterized by the loss of a glucose unit (m/z 300.998) was tentatively identified as an ellagic acid glucoside (peak 5). This ellagic acid derivative was recently found in red oak (*Quercus rubra* L.) seeds [54].

Regarding gallotannins, several compounds were identified in substantial amounts, especially tetragalloyl-hexoside (peak 7), which exhibited a deprotonated molecular [M − H] − ion at *m/z* 787.9999, with MS/MS fragmentation at *m/z* 617.0784, 465.0675, 295.0459, and 169.0142 [54]. Other gallates, such as methyl gallate (peak 3) and ethyl gallate (peak 4), were also identified in significant quantities. The peak with a deprotonated molecular [M − H] − ion at *m/z* 183.0299 and a major fragment ion at m/z 124 due to the loss of - CO_2CH_3 was identified as methyl gallate [15]. The peak identified as ethyl gallate showed a [M − H] − ion at m/z 197.0455 and main fragments at m/z 169 and m/z 124, the typical fragments that determined the presence of gallic acid [15]. Tetragalloyl-hexoside and methyl gallate were both recently reported in the acorn fruits of Spanish *Quercus coccifera* and northern red oak (*Quercus rubra* L.) [43,54]. More recently, ethyl gallate was identified as a prevalent phenolic compound in an ethanolic-water extract of oak bark (*Quercus robur* L.) [64].

Besides phenolic compounds, organic acids such as quinic (*m/z* 191.0561), citric (m/z 191.0197) and azelaic acid (m/z 187.0976) were found in considerable amounts in AS extracts. Quinic and citric acid were found also in significant amounts in acorn fruits of Spanish *Quercus coccifera* [43]. On the other hand, azelaic acid (peak 12) was reported in acorn (*Quercus variabilis* Blume) kernel as one of the most predominant compounds detected [77].

Concerning the RGP extracts, organic acids such as malic acid and isopropylmalic acid were identified in significant amounts (Table S1). These organic acids are commonly present in grapes and their by-products [57]. In terms of phenolic compounds, ethyl gallate (peak 4), gallic acid (peak 2), syringetin 3-O-hexoside (peak 11) and resveratrol dimer (peak 15) were the leading phenolic compounds detected in the

Table 1
Key phenolic and organic compounds identified in acorn shell (AS) and red grape pomace (RGP) extracts by LC-ESI-UHR-QqTOF-MS/MS, listed by retention time (Rt).

Peak	Compound	Rt (min)	Formula	Ms^1 ([M-H]$^-$), *m/z*	Present in	Phenolic Class	Reported Activity	Ref.
1	Citric acid	1.92	$C_6H_8O_7$	191.0197	Both	Organic acid	pH buffering, metabolic effects	Wu et al., [77], Vorobyova et al., [76]
2	Gallic acid	3.35	$C_7H_6O_5$	169.0142	AS > RGP	Phenolic acid	Antioxidant, antimicrobial, anti-inflammatory	Oracz et al., [54]
3	Methyl gallate	12.86	$C_8H_8O_5$	183.0299	AS	Phenolic acid derivative - Gallate ester	Antioxidant, antimicrobial	Dorta et al., [15], Oracz et al., [54]
4	Ethyl gallate	22.63	$C_9H_{10}O_5$	197.0455	Both	Phenolic acid derivative - Gallate ester	Antioxidant, antimicrobial	Dorta et al., [15], Oracz et al., [54]
5	Ellagic acid glucoside	25.5	$C_{20}H_{16}O_{13}$	463.0518	AS	Tannin - Ellagitannin	Antioxidant, anticancer	Oracz et al., [54]
6	Myricetin 3-O-hexoside	26.44	$C_{21}H_{20}O_{13}$	479.0831	RGP	Flavonoid - Flavonol glycoside	Antioxidant, anti-aging	Lantzouraki et al., [30]
7	Tetragalloyl-hexoside	30.1	$C_{34}H_{28}O_{22}$	787.9999	AS	Tannin - Gallotannin	Antibiofilm, antimicrobial	Oracz et al., [54]
8	Ellagic acid	30.87	$C_{14}H_6O_8$	300.9990	Both	Tannin - Ellagitannin	Antioxidant, anticancer, cell cycle arrest	Oracz et al., [54]
9	Quercetin-3-O-hexuronide	31	$C_{21}H_{18}O_{13}$	477.0675	RGP	Flavonoid - Flavonol glycoside	Anti-inflammatory; antiproliferative	Sukovic et al., [70]
10	Malvidin 3-O-glucoside	31.3	$C_{22}H_{22}O_{13}$	493.0988	RGP	Flavonoid - Anthocyanin	Antioxidant, skin protection	Sukovic et al., [70]
11	Syringetin 3-O-hexoside	32.3	$C_{23}H_{24}O_{13}$	507.1144	RGP	Flavonoid - Anthocyanin	Anti-inflammatory, antioxidant	Bouzaida et al., [6]
12	Azelaic acid	32.4	$C_9H_{16}O_4$	187.0976	AS	Organic acid	Skin healing, anti-inflammatory	Wu et al., [77], Vorobyova et al., [76]
13	Myricetin	33.2	$C_{15}H_{10}O_8$	317.0303	RGP	Flavonoid - Flavonol	Antioxidant, antiproliferative	Bouzaida et al., [6]
14	Quercetin	36.9	$C_{15}H_{10}O_7$	301.0354	RGP	Flavonoid - Flavonol	Anticancer, anti-inflammatory	Bouzaida et al., [6]
15	Resveratrol dimer (ε-viniferin)	38.1	$C_{28}H_{22}O_6$	453.1344	RGP	Stilbene - Dimer	Anti-aging, skin barrier repair	Sukovic et al., [70]

The table includes each compound's molecular formula, ion mass ([M − H]$^-$), origin, and reported biological activity relevant to skin applications. References indicate sources for compound identification.

red GP extract. Ethyl gallate (peak 4), which exhibited a deprotonated molecular [M − H] − ion at *m/z* 197.0455 was detected as the second most abundant compound following malic acid (m/z 133.0142). Similar findings were reported for grape pomace extracts from the Prokupac red grape [41] and Sangiovese varieties [28]. According to Milinčić et al. [41], the highest amount of ethyl gallate probably could result from the alcoholic fermentation to which the grape pomace was subjected before analysis [41].

Furthermore, RGP was characterized by a remarkable richness in flavonoids. Syringetin 3-O-hexoside (peak 11) arose as the most predominant compound from this class. This peak exhibited deprotonated molecular [M − H] − ion at *m/z* 507 and a characteristic fragment ion at *m/z* 345 corresponding to syringetin aglycone and the loss of a hexose moiety (*m/z* 162). But, other flavonoids were also identified namely myricetin 3-O-hexoside (peak 6, m/z 479.0831), quercetin-3-O-hexuronide (peak 9, m/z 477.0675), malvidin 3-O-glucoside (peak 10, m/z 493.0988), myricetin 3-O-hexoside (peak 6, m/z 479.0831), myricetin (peak 13, m/z 317.0303) and quercetin (peak 14, *m/z* 301.0354). All these compounds were reported in the literature to be present in grape pomace with similar fragmentation patterns [41,57,70]. At least, regarding stilbenes, a resveratrol dimer (peak 15, m/z 453.1344) was found in RGP samples in analogous amounts to syringetin 3-O-hexoside and gallic acid. This polymeric stilbene derivative was common for the genus *Vitis* and as reported before in samples of grapes and wine [41,70], but also on grape pomace from Granache grapes [6].

While both extracts shared core phenolics such as gallic and ethyl gallate, their overall profiles differed noticeably. The overall phenolic content was considerably higher in the AS extract than in the RGP extract. For instance, ethyl gallate was the most abundant phenolic in RGP, though it appeared in similar quantities in both extracts, ranking fourth in AS. In contrast, gallic acid was markedly more abundant in AS, representing the most dominant phenolic, whereas in RGP it was less concentrated. Moreover, AS was notably richer in phenolic acids and hydrolysable tannins, particularly gallotannins and ellagitannins. RGP, on the other hand, showed a higher abundance of flavonoids and stilbenes, particularly anthocyanins and resveratrol derivatives. The presence of unique phenolic compounds in each extract further highlights their distinct chemical compositions, supporting their potential for different biological activities and applications.

3.2. Differential effects of the by-product extracts on selected skin bacteria

The minimal inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs), and sub-MICs for AS and RGP were determined through microdilution assays and agar plate tests using various concentrations on selected skin bacteria, as detailed in Table 2.

Based on these values, we utilized sub-MIC concentrations of AS and RGP extracts to evaluate bacterial growth kinetics, mimicking crucial physiological conditions found in the human skin microenvironment, using a nutrient medium (as positive control for general bacteria growth), peptone water that contains key components found in human skin, such as proteins, amino acids, and water, and 0,9 % NaCl solution to mimic human sweat.

Table 2
Representation of MIC and MBC values found for AS and RGP extracts in *S. aureus* and *S. epidermidis*.

	Acorn shell (AS) (mg/mL)	Red grape pomace (RGP) (mg/mL)
S. aureus	2.5	40
S. epidermidis	1.75	20
	(MIC/MBC)	(MIC/MBC)
S. aureus	1.75	10
	0.87	5
	0.43	2.5
	0.21	1.25
S. epidermidis	0.87	10
	0.43	5
	0.21	2.5
		1.25

(Sub-MIC concentrations).

Interestingly, exposure to sub-MICs of AS extracts resulted in distinct patterns of growth curve for *S. aureus* and *S. epidermidis* (Fig. 2). While in the absence of AS extract, *S. aureus* displayed a stable growth curve in MH broth, exhibiting an increase after 12 h, in the presence of AS, a noticeable decrease in the growth curve is shown (Fig. 2A, black curves). In comparison to peptone water, *S. aureus* demonstrated a slight increase in growth under all tested conditions, including the bacteria control in peptone (Fig. 2A, orange curves). However, when cultured in the presence of NaCl, *S. aureus* exhibited an increased growth, both in the absence and presence of AS extract (Fig. 2A, green curves). Notably, the growth of *S. aureus* in NaCl was more pronounced compared to MH broth and peptone water. This emphasizes the strongest preference of *S. aureus* to growth under sweat physiological conditions and its high tolerance to high NaCl concentrations, even in the absence of nutrients. This behaviour was confirmed by Swaney et al. [72] which revealed a sweat-concentration-dependent increase in growth for several strains of *S. aureus* which support that these bacteria are highly adapted to the skin environment conditions [72].

In contrast, *S. epidermidis* displayed distinct patterns of growth curves, with decreased growth in the presence of AS extracts across all conditions (MH broth, peptone water, and NaCl) compared to their respective controls (Fig. 2B). Similar to *S. aureus*, *S. epidermidis* demonstrated tolerance and capacity to growth in the presence of NaCl. However, the addition of sub-MICs of AS extracts significantly inhibited its growth, underscoring the antimicrobial potential of this extract even at lower concentrations in *S. epidermidis*.

Although some reports have demonstrated the antibacterial activity of AS extracts in several Gram-positive bacteria, most of them focus on different strains of *S. aureus* usually using rich media in nutrients for general bacterial growth which does not mimic the composition of the human skin microenvironment [65,85]. In general, the MICs and MBCs defined for AS extracts in *S. aureus* after 24 h of incubation range from 0.625 to 10 mg/mL [65,85]. This variation can be correlated with the type of extraction/solvent, the storage conditions after extraction, the bacterial strain, the culture media to perform in vitro evaluation, and the assay used to determine the MICs and MBCs. For instance, [65] demonstrated through time-death curves, that AS extracts exhibited antibacterial activity against both methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *Staphylococcus aureus* MSSA strains of *S. aureus* cultured in MH broth [65]. MICs of 10 and 5 mg/mL were found for fresh and heat-treated AS extracts, respectively, for MRSA and MSSA *S. aureus* cultured in MH broth. However, in a recent study, [38] found MIC values of 50 mg/mL for two *Staphylococcus* strains (MRSA and ATCC 6538) also cultured in MH broth. Regarding the antimicrobial effects of AS extracts on *S. epidermidis,* there is still a dearth in literature.

In the case of RGP extract, both *S. aureus* and *S. epidermidis* displayed growth inhibition curves when exposed to the extract, showing lower growth rates compared to their respective controls (Fig. 2C and D). These findings underscore the antibacterial effect of RGP, even under sub-MIC concentrations, against both Gram-positive bacteria, independently of the culture media. According to the literature, most studies have evaluated the antibacterial effect of RGP against foodborne and clinical pathogens, including Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella* spp), and Gram-positive bacteria (*S. aureus*, *Enterococcus faecalis*, *Listeria monocytogenes*, and *Bacillus cereus*) [21,63,78]. In this sense, MIC and MBC values ranging from 40 to 250 mg/mL have been reported for *S. aureus* cultured in nutritive media [21,29,78]. However, similarly to AS, there is no report about the effect of RGP extract in *S. epidermidis*, with focus on skin applications. Besides, to the best of our knowledge, no reports have evaluated the effect of the AS and RGP extracts using such different conditions to mimic the skin

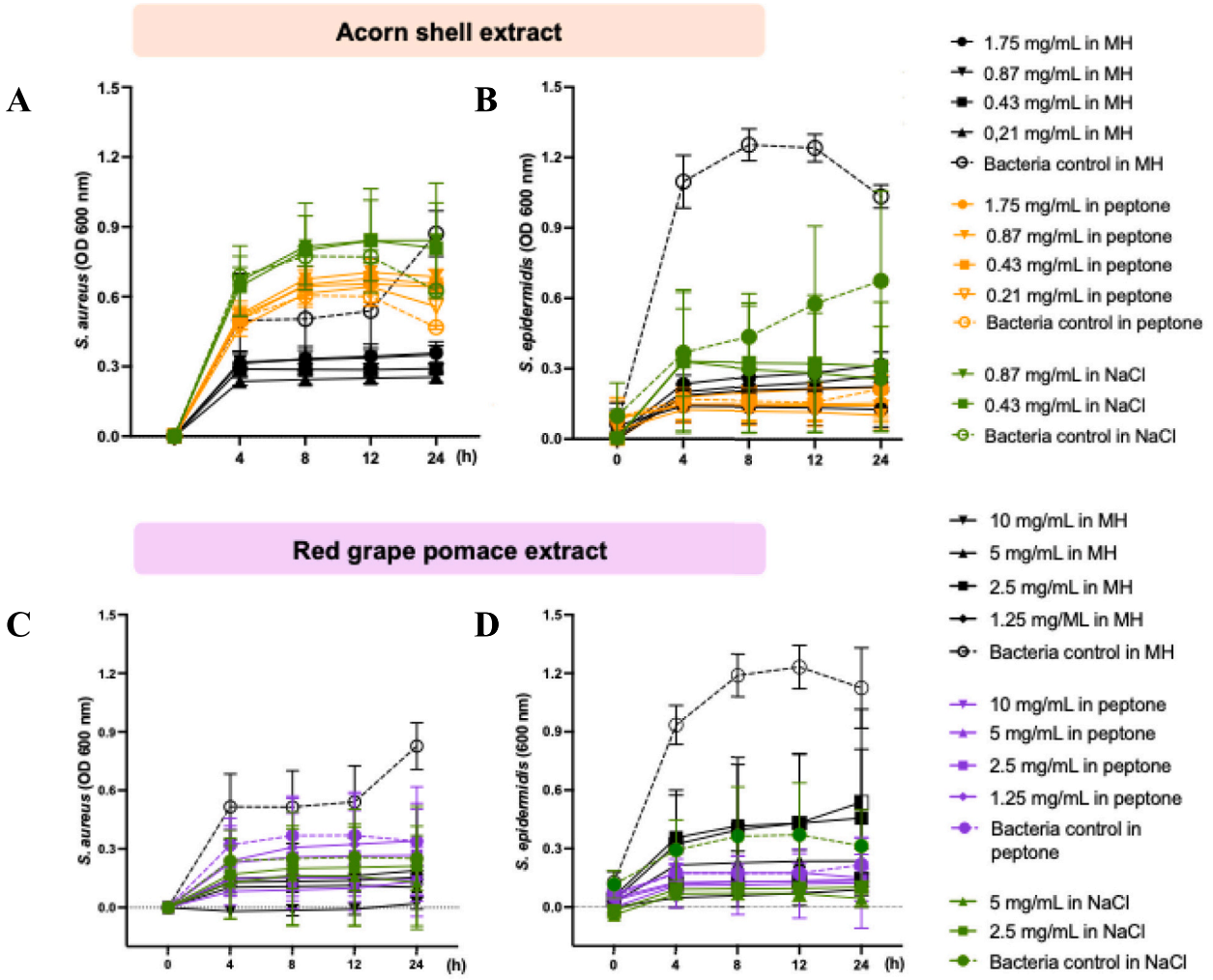

Fig. 2. Growth curves of selected bacteria (*S. aureus* and *S. epidermidis*) in the absence and presence of sub-MICs concentrations of AS (A, B) and RGP extracts (C, D) cultured in MH broth, 0,1 % peptone water, and 0,9 % NaCl in 0.003 % MH broth. Optical density (OD) values detected by absorbance reading at a wavelength of 600 nm are shown and data are reported as the mean ± S.E.M.

physiological conditions. Moreover, the results highlight that the lower MIC/MBC values found for the AS extract may be attributed to its higher content of phenolic compounds compared to the RGP extract.

3.3. Antibiofilm properties

Among the members of the skin microbiota, *S. epidermidis* serves as a natural barrier against the colonization of pathogenic *S. aureus*, engaging in a competitive relationship for the same ecological niche

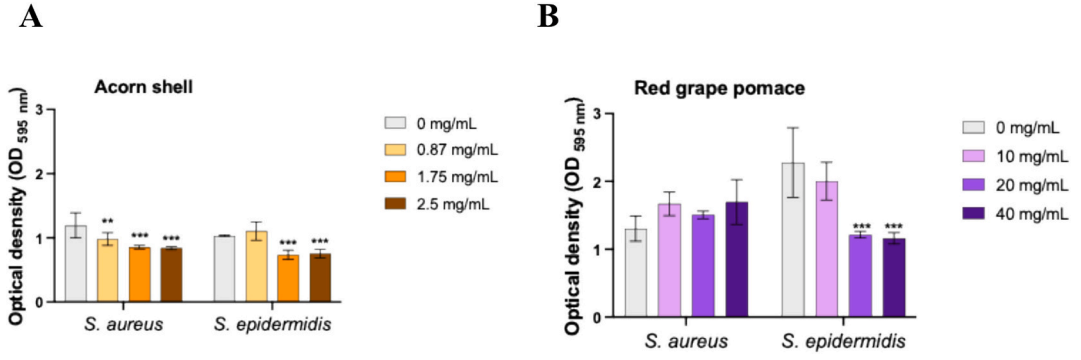

Fig. 3. Adherence levels of *S. aureus* and *S. epidermidis* measured in the absence and presence of sub-MICs and MICs of AS (A) and RGP (B) extracts in mg/mL. Optical density (OD) values, determined by absorbance readings at a wavelength of 595 nm, are presented. Comparison with the untreated bacteria (0 mg/mL) was used to assess the level of adherence, which is directly correlated with biofilm formation or inhibition under each experimental condition. Data are reported as the mean ± S.E.M. Asterisks indicate statistical significance with respect to the uncontrol (0 mg/mL): ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

[20]. However, disruptions in the balance of the skin microbiota, characterized by a decrease in beneficial commensal microorganisms, particularly *S. epidermidis*, significantly increase the risk of *S. aureus* colonization and the formation of biofilms [46]. Consequently, the presence of biofilm-forming *S. aureus* has been extensively documented in various skin conditions, since it is closely associated with disease severity [14,83]. This directly contributes to the occlusion of sweat ducts, skin inflammation, and itching [83].

Given these considerations, we aimed to assess the impact of AS and RGP extracts on biofilm formation or inhibition by *S. aureus* and *S. epidermidis*, using previously established sub-MICs and MICs values. Fig. 3A shows that AS extracts significantly inhibited the biofilm development of both *S. aureus* and *S. epidermidis* ($p < 0.01$, and $p < 0$ 0.001). Specifically, a gradual inhibition in adhesion of *S. aureus* was observed with increasing concentrations of AS extracts. In contrast, for *S. epidermidis*, the lowest concentration of AS extract (0.87 mg/mL) stimulated adhesion and biofilm formation, whereas the two higher concentrations significantly reduced bacterial adhesion and inhibited biofilm formation ($p < 0$ 0.001).

To the best of our knowledge, few studies have explored the anti-biofilm capacity of *Quercus* species against *Staphylococcus* spp. [25,65]. More specifically, it was demonstrated that both ½ and ¼ MBCs (ranging from 1.25 to 5 mg/mL of AS extract from *Q. ilex* and *Q. suber*) inhibited biofilm formation in both MSSA and MRSA [65]. However, there are no reports on the effect of such by-product extracts on the biofilm formation capacity of *S. epidermidis*.

Interestingly, while AS extract induced a similar inhibitory effect on biofilm formation in both gram-positive bacteria, RGP extract exhibited markedly different behaviors between *S. aureus* and *S. epidermidis* (Fig. 3B). Specifically, compared to the control (0 mg/mL of RGP extract), *S. aureus* showed increased adhesion at all tested concentrations of RGP extract. In contrast, *S. epidermidis* displayed a notable and significant inhibition ($p < 0.001$) in adhesion, which was dependent on the concentration of RGP. These findings demonstrate that RGP at concentrations of 10 to 40 mg/mL stimulates biofilm formation in *S. aureus* but inhibits biofilm formation in *S. epidermidis*. Hereupon, the opposing effects suggest that the mechanisms underlying biofilm formation in *S. aureus* and *S. epidermidis* are distinct and respond differentially to the bioactive compounds present in RGP extract.

The contrasting behaviors observed with AS and RGP extracts highlight the complexity of biofilm regulation in both gram-positive bacteria species. Similarly to the antibacterial activity previously reported, the antibiofilm capacity of such extracts, namely AS, can be explained by the presence and elevated concentration of phenolic compounds, namely gallic and ellagic acid. These compounds have the capability of reducing biofilm synthesis by suppressing the activity of the quorum sensing system functional for cell-to-cell communication [63,73]. However, together, the results underscore the need for further investigation into the molecular pathways involved in the biofilm inhibition/formation process and how such extracts (AS and RGP), interact with these mechanisms in different bacterial species. These insights could be valuable for developing target strategies to prevent biofilm-associated infections.

3.4. The impact of by-product extracts on skin resident cells and skin cancer cells

While we have gained insight into various biological properties of by-product extracts, there is a notable lack of data regarding their effects on human skin resident cells and skin cancer cells across different concentrations. To address this knowledge gap, we conducted a thorough assessment of cell viability following exposure to varying concentrations of AS and RGP extracts after 24 h incubation. This evaluation included MTT assay, cell cycle analysis, and cell death characterization to provide a detailed understanding of their cellular impact.

In general, keratinocytes exhibited a higher sensitivity to both by-products extracts when compared to skin cancer cells, as illustrated in Fig. 4. Notably, AS extracts reduced keratinocytes viability by 50 % even at low concentrations ($IC_{50} = 0.24$ mg/mL) demonstrating a significant effect at all tested concentrations ($p < 0.0001$) (Fig. 4A). However, achieving the same effect in skin cancer cells, required an increased concentration of AS extract up to 4 mg/mL to define the IC_{50} (2.12 mg/mL) (Fig. 4B). It is important to note that, to observe a more prominent anti-proliferative effect of the AS extract in skin cancer cells, an increase of the AS concentration to 4 mg/mL, was needed.

In comparison to the AS extract, RGP extract exhibited superior biocompatibility in both cell lines (Fig. 4C, D). The IC_{50} for keratinocytes was 3.08 mg/mL, while skin cancer cells displayed the same reduction at 7.79 mg/mL. Similar to AS extract, a more pronounced anti-proliferative effect of RGP extract in skin cancer cells required an increase in concentration up to 10 mg/mL to define the IC_{50} for this cell line (Fig. 4D).

Presently, the assessment of cytotoxicity is crucial, but equally significant is comprehending the influence of by-product extracts and their components, on the cell cycle of normal and cancer cells - a paramount consideration for potential skin applications. In this context, we investigated the impact of various concentrations, ranging from low to high, of the by-product extracts in study on the cell cycle of keratinocytes and skin cancer cells.

To assess the influence of these by-product extracts on the cell cycle, our attention was directed towards analysing the proportion of cells in the sub-G1 phase (indicative of cell death with cell membrane and DNA damage) and the S phase (representing the proliferative phase of cells) (Fig. 5).

In response to AS extract, keratinocytes displayed a dose-dependent increase in the sub-G1 phase and a corresponding decrease in the S phase, particularly evident up to 1 mg/mL, compared to untreated cells. Statistically significant differences were observed from 0.5 to 1.5 mg/mL ($* = p < 0.05$, $^{\#\#} = p < 0$ 0.01, $^{\#\#\#} = p < 0$ 0.001) (Fig. 5A). Conversely, this effect was not observed in skin cancer cells. However, concentrations of 0.5, 1, and 4 mg/mL of AS extract induced a significant increase in the sub-G1 phase compared to untreated cells ($^{\#} = p < 0.05$) (Fig. 5B). Interestingly, only the highest concentration of AS extract demonstrated a significant reduction in the S phase in skin cancer cells compared to untreated cells ($** = p < 0$ 0.01).

In contrast, the proportion of cells in sub-G1 phase for both keratinocytes and skin cancer cells treated with RGP extract is significantly concentration-dependent, exhibiting a statistical variance compared to their respective untreated cells (0 mg/mL of extract) ($^{\#} = p < 0$ 0.05, $^{\#\#} = p < 0$ 0.01, $^{\#\#\#\#} = p < 0$ 0.0001) (Fig. 5C, D). Remarkably, a statistical disparity in the heightened proportion of cells in the sub-G1 phase is noticeable from concentrations of 2.5 mg/mL and 1.25 mg/mL, respectively, for keratinocytes and skin cancer cells. Simultaneously, this phenomenon is accompanied by a reduction in the S phase and a significant arrest in the G0/G1 phase for both cell lines, in a concentration-dependent fashion.

To complement these findings, we evaluated whether the observed increase in the sub-G1 cell population following treatment with both by-product extracts is associated with apoptotic cell death. Indeed, exposure to AS and RGP extracts induced a concentration-dependent increase in apoptotic and necrotic markers, particularly in keratinocytes (Fig. 6).

At the highest concentration tested (4 mg/mL for both AS and RGP), a significant decrease in the percentage of viable keratinocytes (Annexin V^-/PI^-) was observed, along with a corresponding increase in early apoptosis (Annexin V^+/PI^-), late apoptosis (Annexin V^+/PI^+), and necrosis (Annexin V^-/PI^+). These effects were statistically significant across all parameters, reinforcing the cytotoxic potential of both extracts at elevated concentrations for the keratinocytes.

In contrast, a less pronounced pro-apoptotic effect was observed in skin cancer cells treated with equivalent concentrations. The viability of cancer cells significantly decreased with increasing extract concentrations, with a strong induction of early and late apoptosis, particularly at 5 mg/mL (Fig. 6). Notably, RGP exhibited slightly greater cytotoxicity

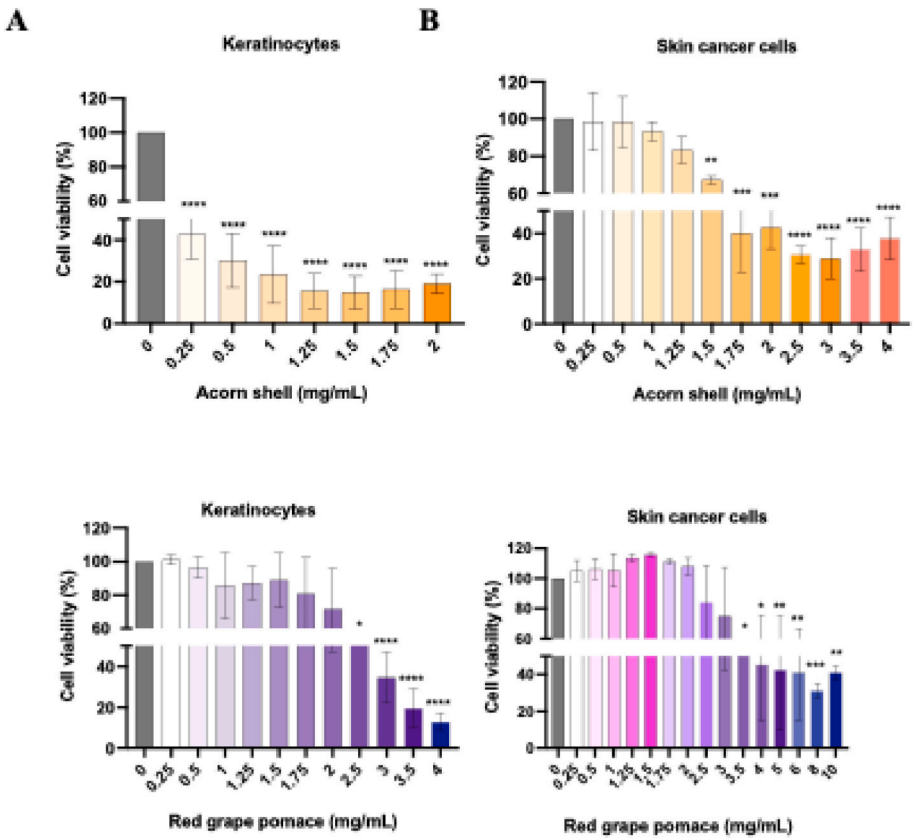

Fig. 4. Cell viability of keratinocytes (HaCat cell line) and skin cancer cells (SK-MEL3 cell line) after 24 h of incubation with varied concentrations of AS (A, B) and RGP (C, D) extracts. Data are reported as the mean ± S.E.M. Asterisks indicate statistical significance with respect to untreated cells (0 mg/mL): * = $p < 0.05$, ** = $p < 0.01$, *** = p < 0 0.001, **** = $p < 0.0001$.

compared to AS at equivalent concentrations. The statistical markers (*, #, +) emphasize significant differences among conditions, indicating potential toxicity of the extracts towards skin cancer cells.

Although acorns (*Quercus* spp.) are commonly used in animal and human diets and have been investigated for their potential in treating gastrointestinal disorders [10,51], there are no studies exploring the impact of acorn extracts, particularly from its shell, on components of the skin microenvironment, including normal skin resident cells and skin cancer cells.

Nevertheless some studies have indicated a high rate of cell viability in keratinocytes without cytotoxic effects, these findings are associated with relatively low concentrations of acorn fruit extracts, ranging from 5 to 100 μg/mL [26]. Regarding the effect of such extracts on cancer cells, only a handful of in vitro studies have previously explored the anti-cancer properties of *Quercus* spp. extracts [16,47,51]. Generally, these investigations suggest that acorn extracts exhibit promising effects on breast, lung, cervical, and colon cancer cell lines, leading to reduced cell viability, dependent on concentration and time.

The anti-proliferative effect in such cancer cells has been demonstrated for different parts of acorn, namely, fruit, cup, leaves, galls, and shell [45,81]. For instance, an intriguing study delved into the comparison of extracts sourced from various components of acorns, evaluating their impacts on different cancer cell lines [22]. Herein, ethanolic extracts derived from the cup parts of acorns exhibited heightened anticancer potential, demonstrating IC_{50} values ranging from 5.04 ± 0.02 to 18.04 ± 0.16 μg/mL across breast, lung, and cervical cancer cell lines. Remarkably, while breast cancer cells (MCF-7) showcased heightened sensitivity, lung cancer cells (A549) displayed greater resilience, underscoring the varied cytotoxic responses observed across different cancer types.

Despite some studies indicating stronger anti-proliferative effects with low concentrations of acorn extracts in different cancer cells, to date, only two studies explored the anticancer potential of acorn extracts in skin cancer cells [47,81]. The first one demonstrated that both ethanol and water extracts from acorns exhibit potent anti-proliferative activity against the human skin epidermoid cancer cell line (A431). In this case, the aqueous extract demonstrated superior efficacy when compared to the ethanolic extract. According to the authors, this variation in the effects of the tested extracts may stem from differences in the phenolic compounds (such as phenolic acids, flavonoids, ellagitannins, and ellagic acid derivatives) and the extraction conditions (including extraction temperature, solvent type, and concentration). Like our study, the second study evaluated the cytotoxic effects of galls *of Q. brantii* in both skin cancer cells (A375 and SK-MEL3) and normal human fibroblasts (AGO-122). At 0.05 mg/mL, after 24 h of incubation, increased cytotoxicity, reactive oxygen species (ROS) formation and cytochrome *c* release in both skin cancer cells compared to fibroblasts, was observed.

In addition, the anti-proliferative activity observed in cervical cancer and adenocarcinoma cell lines associated with the *Quercus* genus is frequently attributed to the presence of ellagic acid, kaempferol and its glycosides, quercetin, and myricetin [7,44]. Studies have shown that these compounds inhibit cell proliferation by inducing early apoptotic cell death, a process associated with the generation of species ROS and release of cytochrome *c* [44,81]. These cellular mechanisms are likely correlated with the effects of AS on cell cycle progression and cell death markers observed in both keratinocytes and skin cancer cells.

Apart from its traditional uses in composting and animal feed, numerous studies have investigated the bioactive potential of RGP extract for applications in the food and pharmaceutical industries, particularly in cosmeceuticals ([36], F. [35,58]). RGP extract contains BCs such as anthocyanins, flavonoids, flavan-3-ols, and stilbenes, which

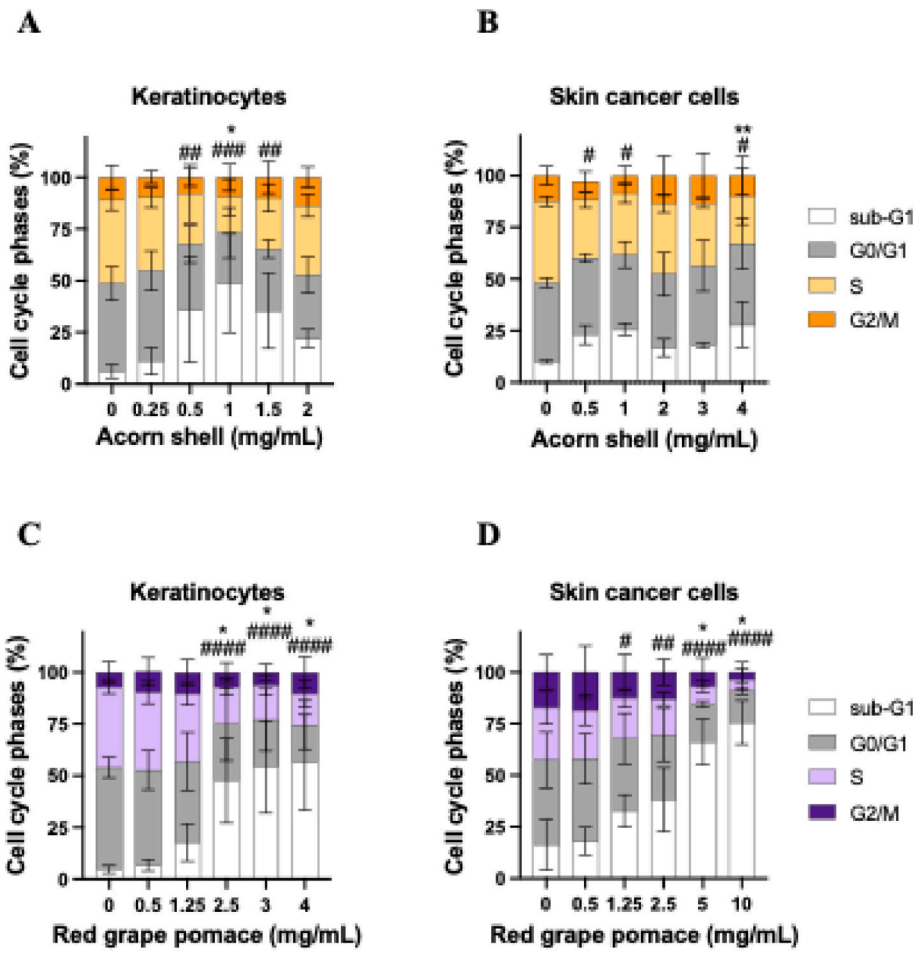

Fig. 5. Quantification of the cell cycle phases of keratinocytes and skin cancer cells after 24 h of incubation with varied concentrations of acorn shell (AS) (A, B) and red grape pomace (RGP) (C, D) extracts. Data are reported as the mean ± S.E.M. The significance between the sub-G1 and S phase in comparison to untreated cells (0 mg/mL of extract) are represented as # and *, respectively for sub-G1 and S phase. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exhibit various biological activities including antioxidant, anti-inflammatory, and anticancer properties (Perez-[2,56]). For topical applications, the evaluation of different dilutions of RGP extract using keratinocytes demonstrated no cytotoxicity, indicating its safety for skin usage [58]. Similarly, cytotoxicity results demonstrated the safety of grape pomace extracts for skin applications, even at the highest concentration tested (200 mg·mL^{-1}) in fibroblasts (3 T3 cell line) (F. [35]).

Regarding its anticancer potential, RGP extracts have received more attention for their impact on colon cancer compared to skin cancer. Purified extracts of RGP showed reduced cell viability and proliferation in colon cancer cells (Caco-2 and HT-19) in a dose-dependent manner, tested between 25 and 225 μg/mL [56]. Similarly, the anticancer activity of red grape seed extract (GSE) on a skin cancer cell line (A431) was explored using concentrations ranging from 10 to 500 μg/mL with an IC_{50} value of 480 μg/mL. However, this study did not investigate the effects of GSE on human skin normal cells, such as keratinocytes or fibroblasts. Consequently, there is no insight into the potential impact of GSE on these essential components of the skin microenvironment.

The anti-proliferative effects of RGP extracts have been extensively explored in human colon cancer, lung cancer, breast cancer, and murine melanoma using both in vitro and in vivo models [2,9,42]. Consistent with our findings, when treated with RGP extracts, normal cells, particularly keratinocytes, have shown greater sensitivity to fresh and fermented GP extracts (abundant in polyphenol content) compared to cancer cell lines such as A549, MDA-MB-231, and B164A4 [2]. In fact, RGP extracts contain significant quantities of phytochemicals, including anthocyanins, catechins, resveratrol, phenolic acids, and procyanidins, among others, which have the potential to influence various cellular mechanisms such as proliferation, cell cycle arrest, and cell death [9,19]. However, their effects are contingent upon specific factors, namely presence and concentration of BCs, cell lines, and environmental conditions [2].

While both extracts showcased in this study exhibited a dose-dependent reduction in skin cancer cell viability, it is essential to highlight the divergent sensitivity of keratinocytes compared to cancer cells, as evidenced by the distinct IC_{50} values for each cell line. This difference needs special consideration for the development of novel topical application strategies, emphasizing the potential of these by-product extracts as a bioactive source for incorporation in several products for direct skin applications.

3.5. Anti-inflammatory potential

There is a growing interest in natural approaches to alleviate inflammation in various skin pathological conditions. Particularly, the use of extracts from plants and their by-products has shown promising anti-inflammatory properties [23,74]. However, the anti-inflammatory effects of AS and RGP extracts remain largely unexplored. While many studies focus on assessing the antimicrobial, cytotoxic, and anti-cancer properties of these by-product extracts, it's crucial to explore their biological potential, specifically their capacity to alleviate skin inflammation by promoting the secretion of essential anti-inflammatory

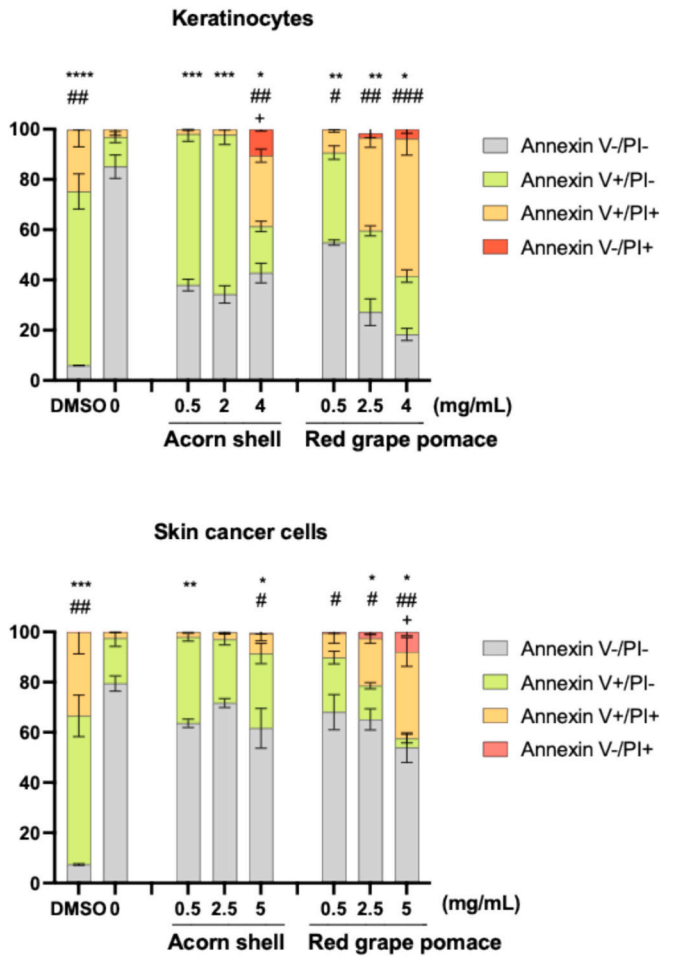

Fig. 6. Effect of acorn shell (AS) and red grape pomace (RGP) extracts on cell viability and cell death in keratinocytes and skin cancer cells after 24 h of incubation.
Cells were treated with increasing concentrations of AS and RGP extracts (0.5, 2/2.5, and 4/5 mg/mL, respectively). 10 % DMSO was used as a positive control for cell death. Following treatment, cell viability and death were evaluated using FITC Annexin V and propidium iodide (PI) staining by flow cytometry. Bars represent the percentage distribution of viable cells (Annexin V^-/PI^-), early apoptotic cells (Annexin V^+/PI^-), late apoptotic cells (Annexin V^+/PI^+), and necrotic cells (Annexin V^-/PI^+). Statistical significance relative to untreated cells (0 mg/mL) is indicated as follows: * for Annexin V^+/PI^-, # for Annexin V^+/PI^+, and $^+$ for Annexin V^-/PI^+. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

factors.

In this regard, interleukin-10 (IL-10) emerges as a significant immunomodulator with multifaceted functions. Produced by various cell types, including keratinocytes, immune cells, and cancer cells, IL-10 plays a pivotal role in regulating immune responses and reducing inflammation [68]. In the skin microenvironment, IL-10 may act as a well-known anti-inflammatory mediator, with ability to modulate the inflammation and contribute to tumor regression and the suppression of metastasis [62,84].

Considering this, we evaluated the release of IL-10, employing non-toxic concentrations of AS extracts (0, 0.5 and 2 mg/mL) and RGP extracts (0, 0.5 and 2.5 mg/mL) in both keratinocytes and skin cancer cells following a 24 h of exposure. Our findings reveal no statistically significant differences in IL-10 release between treated and untreated cells (0 mg/mL of extract) for either AS or RGP extracts in both keratinocytes and skin cancer cells (Fig. 7). However, it is of notice that skin cancer cells exhibited increased secretion of IL-10 compared to both untreated cells and keratinocytes. This effect was observed for both AS and RGP extracts. Together, these results are just a preliminary screening of the anti-inflammatory potential of such by-products extracts.

Regarding the anti-inflammatory potential of acorn extracts, Yin et al. (2029) demonstrated that phenolic compounds derived from *Q. mongolica* bark extracts exert anti-inflammatory effects by promoting IL-10 secretion in ultraviolet B (UVB)-irradiated keratinocytes [80]. These findings highlight the potential of acorn-derived phenolics to modulate inflammatory responses in skin cells. Although there is no information about the anti-inflammatory potential of acorn extracts in skin cancer cells, there are some studies that have explored the anti-inflammatory properties of isolated BCs from plant extracts, namely gallic acid [27,71]. Gallic acid, one of the most relevant compounds found in both extracts, is well-known for its anti-inflammatory activity [27]. In this regard, it was demonstrated that gallic acid suppresses the release of pro-inflammatory cytokine IL-6 in IL-31-treated eosinophils-dermal fibroblasts co-culture [75]. This finding suggests that gallic acid can reduce inflammation through the inhibition of eosinophils, activated in skin inflammatory conditions, such as atopic dermatitis and skin cancer.

In contrast, both in vitro and in vivo studies have demonstrated the anti-inflammatory properties of RGP extracts in both normal and in cancer cells [2,4,58]. Although significant differences in the secretion of IL-10 in comparison to untreated cells (0 mg/mL) were not observed, RGP extracts have been closely associated with their ability to modulate inflammatory responses. For instance, it has been shown that RGP extracts reduce the levels of IL-1β, IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 in intestinal and endothelial cells [8], alveolar epithelial cells [3], colon cancer cells [60] and human keratinocytes [58].

Between the RGP extract BCs, resveratrol has attracted attention for its anti-inflammatory, anticancer, and anti-aging properties [4]. Although resveratrol itself was not detected in the RGP extract, derivatives of resveratrol, such as the resveratrol dimer (ε-viniferin), can exert similar biological activities [11]. ε-Viniferin, identified in substantial amounts within RGP, is a stilbene polyphenol formed by two resveratrol subunits and is noticed to have greater anti-inflammatory potential than resveratrol [11]. According to Cheng et al. [11], topically applied ε-viniferin accumulated at higher levels than resveratrol (0.067 versus 0.029 nmol/mg) in psoriasis-like mouse skin with impaired barrier capacity. Additionally, ε-viniferin was more effective than resveratrol in alleviating psoriasiform symptoms and reducing IL-23 secretion (58 % reduction compared to 37 %). These findings suggest that ε-viniferin holds promise as an anti-inflammatory agent for the prevention or treatment of psoriasis [11].

4. Conclusion

This study highlights the distinct chemical compositions and biological properties of AS and RGP extracts, namely their impacts in key components of the human skin microenvironment. While AS extract is rich in phenolic acids, such as gallic and ellagic acid, as well as gallotannins, RGP extract is abundant in flavonoids, including catechin and quercetin derivatives. Both by-product extracts showed inhibitory effects on bacterial growth and biofilm formation against gram-positive bacteria under conditions mimicking the human skin microenvironment. Although RGP extract also inhibited the growth of both bacteria, its effects on biofilm formation were species-dependent, stimulating biofilm formation in *S. aureus* but inhibiting it in *S. epidermidis*. These findings indicate potential applications for target modulation of skin microbiota. However, further studies are necessary to clarify the underlying mechanisms of action.

In addition, both by-product extracts displayed concentration-dependent effects on cell viability and cell cycle, with AS extracts leading to increased cell death in keratinocytes and skin cancer cells, while RGP extracts induced cell cycle arrest in both cell types at higher

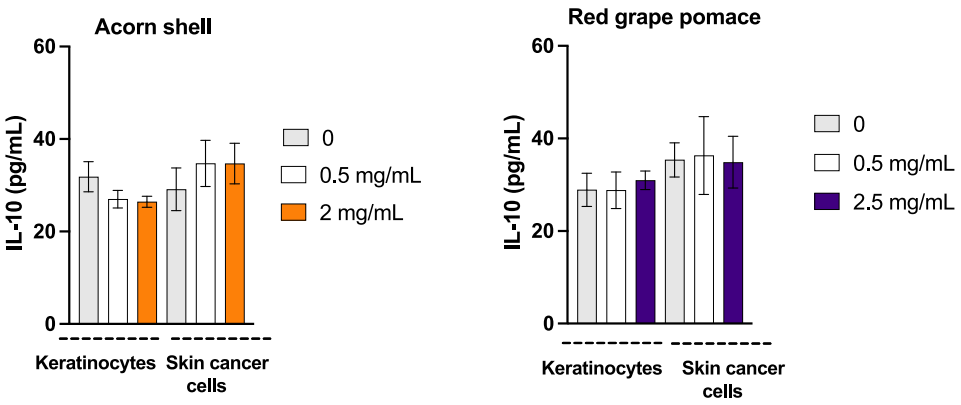

Fig. 7. IL-10 secretion by keratinocytes and skin cancer cells after 24 h of exposure to acorn shell (AS) and red grape pomace (RGP) extracts. Cells were treated with 0 (untreated control), 0.5, and 2 mg/mL of AS extracts, and 0 (untreated control), 0.5, and 2.5 mg/mL of RGP extracts. Data are presented as mean ± S.E.M. from three independent experiments. Statistical comparisons were made relative to the untreated control group (0 mg/mL). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrations. Although both by-product extracts demonstrated limited impact on IL-10 secretion, their potential anti-inflammatory effects require intensive investigation due to the presence of key bioactive compounds with recognized anti-inflammatory properties in both extracts.

Overall, our findings suggest that AS and RGP extracts present promising potential as bioactive compounds source for diverse skin applications. Dependent on the concentration, these by-product extracts can be used as antimicrobial, antibiofilm, anticancer and anti-inflammatory. However, further studies are needed to explore their specific molecular mechanisms involved with these biological properties.

In vitro evaluation revealed concentration-dependent effects of both AS and RGP extracts on cell viability, cell cycle progression, and cell death in keratinocytes and skin cancer cells. Keratonocytes were more sensitive to the extracts compared to skin cancer cells, indicating cell type-specific responses. Although the extracts exhibited limited impact on IL-10 secretion, the presence of bioactive compounds with known anti-inflammatory potential supports the need for further investigation into their immunomodulatory effects.

In summary, AS and RGP extracts represent promising candidates for the development of skin-targeted formulations with antimicrobial, antibiofilm, and antiproliferative properties. Nevertheless, additional studies—particularly those elucidating molecular mechanisms and evaluating safety and efficacy in complex biological models, e.g., advanced three dimensional (3D) skin models—are required to clarify and support their potential therapeutic applications.

CRediT authorship contribution statement

Cláudia S. Oliveira: Writing – original draft, Methodology, Investigation, Conceptualization. **Adma Melo:** Writing – original draft, Methodology, Investigation. **Tania Ribeiro:** Writing – original draft, Methodology, Investigation. **Manuela Pintado:** Writing – review & editing, Supervision, Funding acquisition. **Freni K. Tavaria:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The present work did not involve human participants, human data or human tissue.

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Declaration of competing interest

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

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The graphical abstract was created in BioRender. Oliveira et al. [50] https://BioRender.com/undefined.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2025.106798.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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