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Use of yeast extract to elicit a pulp-derived callus cultures from Annurca apple and potentiate its biological activity

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ABSTRACT

Today, plant cell cultures represent a valid alternative method to produce secondary metabolites. Here, we developed a protocol to obtain callus cultures from the pulp of the Italian apple variety, named Annurca. To increase the accumulation of bioactive compounds, yeast extract was used as elicitor. This elicitor induced a remarkable increase in total polyphenol content, with chlorogenic acid, procyanidin B2, and epicatechin being the most abundant. The antioxidant potential of extracts from the callus cultures was investigated and results showed that the use of the elicitor improved the protective antioxidant effect of the extracts on UVA-stressed keratinocytes. Furthermore, the extracts from the elicited callus cultures were able to speed up the process of healing after 24 h. Our results suggest that extracts obtained from elicited apple callus cultures can represent a promising alternative to chemically synthesised bioactive compounds for their use in the pharmaceutical, nutraceutical and cosmetic industries.

1. Introduction

The majority of plant-derived phytochemicals that impart beneficial effects on human health are specialized metabolites including polyphenols, terpenes, and alkaloids. These compounds are physiologically produced by plants as defence agents against pathogen infections (Leicach and Chludil, 2014). The accumulation of these molecules in plant tissues is often insufficient to support their exploitation in industrial formulations (Lange, 2018). In practice, large quantities of raw plant material are frequently required to obtain extracts rich in bioactive molecules, and in most cases, the extraction protocols employed are not appropriately optimized. Thus, the entire extraction process turns out to be economically and environmentally unsustainable (Wu et al., 2021).

The *in vitro* culture of plant tissues is a well-established alternative to the soil-based cultivation. In particular, plant cells can be grown under controlled conditions, starting from different plant's organ explants, that constantly and progressively divide into undifferentiated cells with remarkable metabolic activity (Alvarez, 2014). Plant cell cultures (PCCs) represent an important tool for the sustainable maximization of natural compound production and are a proven system for the production of bioactive molecules to be used in pharmaceutical, nutraceutical, and cosmetic industries (Eibl et al., 2018).

Despite the key role that PCCs play in the production of high-value molecules, the development of a stable cell line and its industrial applicability is not always guaranteed. This depends on the starting material and on the capability of the obtained cell line to maintain the

Abbreviations: YE, Yeast Extract; PCC, Plant Cell Culture; fr. wt, fresh weight; d. wt, dry weight; UVA, ultraviolet A.

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same metabolic profile of the mother plant.

Verardo et al. (2017) analysed the metabolomic profile of apple-pulp-derived cell culture showing that these cultures retained the same metabolites of the apple tissue, such as phloridzin, ursolic acid and oleanolic acid (Verardo et al., 2017). Hence, if the metabolic composition is maintained or further improved, PCCs can serve as a valuable tool for producing specialized metabolites with biological activity (Arya et al., 2020; Davies and Deroles, 2014; Gubser et al., 2021).

A quantitative enrichment of PCC extract in bioactive compounds could be achieved by using elicitors, which are chemical or physical factors capable of triggering signals in plant cells, leading the cells to accumulate a greater amount of phytochemicals. Usually, the most used elicitation methods are abiotic (chemical and physical stressors, salts of heavy metals, gaseous substances, intracellular signalling molecules) or biotic (either crude extracts or partially purified products derived from microorganisms, such as fungi, bacteria, yeast) (Halder et al., 2019).

When considering large-scale metabolite production, the incorporation of elicitors into cell cultures may escalate costs. However, alternatives such as crude extracts or partially purified products, could provide a more economical strategy, especially when these products effectively enhance the yield of valuable compounds. Among the biotic elicitors, yeast extract (YE) is natural and low-priced (Cai et al., 2012). Nevertheless, the successful use of this elicitor can vary significantly and is highly dependent on the plant variety and tissue type used. Indeed, the interaction of elicitors with the type of PCC may trigger the production of different classes of molecules, and their synergistic effects can result in varying bioactivities.

In light of the above considerations, in the present study we developed PCCs from the pulp of the apple Annurca (*Malus pumila* cv Miller), a native variety of Southern Italy, listed as a PGI (Protected Geographical Indication) product from the European Council [Commission Regulation (EC) No.417/2006)]. The interest for this local variety resides in the high levels of polyphenolic compounds produced both in the pulp and in the peel (Fratianni et al., 2007.; Maisto et al., 2023; Tenore et al., 2013). The polyphenolic fraction of the Annurca apple showed significant nutraceutical potential, with demonstrated benefits in managing lipid plasma levels (Tenore et al., 2017), anti-inflammatory activity (Riccio et al., 2017) and antioxidant and antidiabetic properties (Maisto et al., 2022). An Annurca-based formulation (AnnurMetS) was also proved to induce an intense hair-inductive activity, increasing hair tropism and keratin content at once (Piccolo et al., 2019). Furthermore, Annurca extract was demonstrated to drastically reduce the triple-negative breast cancer, killing the MDA-MB-231cells, typically causing this tumour (Vuoso et al., 2020). Once established callus cultures from Annurca pulp, in this study we aimed to assess the value of the obtained materials in terms of secondary metabolites production and bioactivity of their extract. The specific objectives of this interdisciplinary study were: 1) to develop callus cultures starting from the Annurca apple pulp, which is known to contain a higher content of polyphenols than other apple varieties, and at the same time to optimise growth parameters, 2) to increase the total amount of polyphenols accumulated by using YE as elicitor 3) to analyse the antioxidant activity of the PCC-extracts and assess their impact on wound healing using a cell-based model.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, reagents, and standards used were from Sigma-Aldrich, unless differently specified. Water was purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use. Chemicals for Gamborg B5 medium, plant growth regulators and sucrose were purchased from Duchefa Biochemie (RV Haarlem, Netherlands). Yeast extract (YE) was purchased from Thermo Fisher Scientific (Whaltam, Massachusetts, US).

2.2. Plant material and callus cultures

Pieces of pulp from Annurca apple were washed with 20 % Tween in Milli-Q water and then sterilized with 70 % ethanol for 2 min and 1.5 % sodium hypochlorite for 15 min. Thereafter, the pulp was washed three times with sterilized Milli-Q water and cut into tiny pieces (1x1 mm) in sterile conditions. The pieces were placed on Gamborg B5 medium (Dixon, 1985) which was prepared mixing 30 g L^{-1} sucrose, 2.0 mg L^{-1} 2, 4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg L^{-1} 6-benzylaminopurine (BAP) and 0.8 % agar and autoclaved at 121 °C for 20 min after adjusting the pH to 5.8. Cultures were incubated in the dark at 25 ± 2 °C. The complete formation of calli was recorded after three months of culture. Subcultures were performed every 28 days. Calli from exponential developmental stage (15^{th} day) were inoculated either on control medium (Gamborg B5 + 2.0 mg L^{-1} 2,4-D + 0.5 mg L^{-1} BAP) or on medium supplemented with YE 300 or 500 mg L^{-1}. Both the control and the YE-treated set of calli were incubated in the dark at 25 ± 2 °C for three weeks. At the end of the treatment, calli were freeze-dried and the powder was stored at −80 °C before the following analysis.

2.3. Analysis of callus culture biomass after YE treatment

After YE treatment, the increase in callus biomass was measured as fresh weight (fr. wt) and dry weight (dry wt). Calli were carefully taken out from culture Petri dishes, washed with sterile water, and weighed for fr. wt determination. Thereafter, the harvested calli were oven dried at 60 °C for 48 h to determine the dry wt.

2.4. Polyphenolic extraction

For the quantitative polyphenol determination, the extraction from calli was performed according to a method previously described with slight modifications (Maisto et al., 2021). Practically, a volume of 1 mL of 80 % aqueous methanol containing 1 % formic acid solution was added to 40 mg of lyophilized samples; the mixture was mixed on a vortex for 1 min. Then, the samples were placed in an ultrasonic bath (Branson Fisher Scientific 150 E Sonic Dismembrator) for 10 min, shaken on an orbital shaker (Sko-DXL, Argolab, Carpy, Italy) at 51 *g* for 10 min, and centrifuged at 765 *g* for 10 min. The supernatants were collected and stored away from light at 4 °C. The obtained pellets were re-extracted with the same procedure using 0.5 mL of the same extraction solvents. Eventually, the extracts obtained were stored at − 20 °C until further analysis. For the analysis of callus culture extract bioactivity on eukaryotic cells, polyphenolic compounds were extracted in ethanol, a GRAS solvent, as previously reported (Petruk et al., 2016), with some modifications. Briefly, 0.5 g of lyophilized biomass was suspended in 12.5 mL of ethanol, and extractions were carried out in an ultrasonic bath for 1 h on ice. Then, the samples were centrifuged at 5000 *g*, supernatants collected and dried under N_2 flux, and stored at −20 °C.

2.5. HPLC-DAD quantitative polyphenols determination

Analyses were run on a Jasco Extrema LC-4000 system (Jasco Inc., Easton, MD, USA) provided with photo diode array detector (DAD) and a fluorescence detector, coupled with an autosampler and a binary solvent pump. The column selected was a Kinetex®C18 column (250 mm × 4.6 mm, 5 μm; Phenomenex, Torrance, CA, USA). Analyses were performed at a flow rate of 1 mL min^{-1}, using solvent A (2 % formic acid) and solvent B (0.5 % formic acid in acetonitrile and water 50:50, v/v). According to a chromatographic method, previously validated (Maisto et al., 2023), the elution was carried out as follows, 5 min of isocratic elution at 10 % solvent B, from 10 % (B) to 55 % (B) in 50 min and to 95 % (B) in 10 min, followed by 5 min of maintenance. Flavonols, dihydrochalcones, and hydroxycinnamic acids were monitored at 280 nm, while flavan-3-ols at 360 nm. Specifically, the used method has a limit of

detection and limit of quantification (LOQ) for chlorogenic acid determination of 0.0010 and 0.0020, for phlorizin of 0.001 and 0.002, for procyanidin B1 of 0.001 and 0.0025, for procyanidin B2 of 0.0020 and 0.0025, catechin 0.001 and 0.005, epicatechin 0.0020 and 0.0025 and, and for rutin of 0.005 and 0.001 mg mL^{-1}, respectively.

For quantitative analysis, standard curves for each polyphenol standard were prepared over a concentration range of 0.1–1.0 mg mL^{-1} with six different concentration levels and duplicate injections at each level. The identity of polyphenols was confirmed by comparison of the retention time of analytical standard and by internal standard analysis.

2.6. Total phenolic content determination

The total phenol content (TPC) was performed by spectrophotometry, according to Folin–Ciocalteau's assay and using gallic acid as the reference standard (Sigma-Aldrich, St. Louis, MO, USA) ((Iannuzzo et al., 2022). Briefly, 0.125 mL of polyphenolic extract underwent an addition of: 0.5 mL of distilled water, 0.125 mL of Folin–Ciocalteau's (Sigma-Aldrich, St. Louis, MO, USA) reagent and 1.25 mL of an aqueous solution of Na_2CO_3 7.5 % (w/v%), bringing the final volume to 3 mL with water. Then, the samples were shaken and incubated for 90 min in the dark. After the reaction time, the absorbance was measured at 760 nm using a V-730 UV–visible/NIR spectrophotometer operated by Spectra Manager™ Suite (Jasco Inc., Easton, MD, USA). The analysis was performed in triplicate, and the total polyphenols concentration was expressed in gallic acid equivalents (GAEs).

2.7. Antioxidant activity analysis

ABTS analysis was performed to establish the antioxidant potential of extracts. The ABTS radical cation was produced adding 7 mM stock solution of ABTS to 140 mM potassium persulfate and placing the mixture in the dark at 4° C for 12 h before use. The obtained ABTS radical cation solution was diluted with ethanol up to an absorbance of 0.70 ± 0.010 at 734 nm. A mixture containing 1 mL of ABTS radical cation and 100 μL was added to 2 mL centrifuge tube. Ethanol was used as reference antioxidant compound. The absorbance was measured spectrophotometrically at 734 nm after 2.5 min. The ABTS radical cation scavenging capacity of the extract was calculated as described by Ali and collaborators (2018).

2.8. Cell culture and MTT assay

Immortalized human keratinocytes (HaCaT), obtained from Innoprot (Biscay, Spain), were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % foetal bovine serum (HyClone), 2 mM L-glutamine and antibiotics, under a 5 % CO_2 humidified atmosphere at 37 °C. To evaluate the biocompatibility of the Annurca pulp-derived callus extracts, HaCaT cells were seeded in 96-well plates at a cell density of 2 x 10^3 cells $well^{-1}$. After 24 h, cells were incubated with increasing concentration (from 1 to 200 μg mL^{-1}) of either control extract or elicited extract. After 48 h incubation, cell viability was evaluated by the MTT assay as previously described. Cell survival is expressed as the percentage of viable cells in the presence of samples compared with control cells (represented by the average obtained between untreated cells and cells supplemented with the highest concentration of buffer).

2.9. DCFDA assay

The antioxidant activity of the extracts obtained from Annurca pulp-derived callus, was evaluated by measuring the intracellular reactive oxygen species (ROS) in stressed HaCaT cells as previously reported (Petruk et al., 2016). Briefly, HaCaT cells were seeded at a cell density of 2×10^4 cells cm^{-2}. After 24 h, cells were incubated with 100 μg mL^{-1} of both extracts for 2 h. Then, oxidative stress was induced by exposing cells to UVA radiations (100 J cm^{-2}). Immediately after, ROS production was estimated by using 2,7-dichlorodihydrofluorescein diacetate (H_2-DCFDA) as probe.

2.10. Intracellular GSH determination

The 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) assay was carried out using the above mentioned experimental system. Immediately after UVA exposure, intracellular Glutathione (GSH) levels were determined according to the protocol reported by (Petruk et al., 2016).

2.11. Scratch test assay

The effect of the extracts on cell re-epithelization was assessed by a scratch assay as previously reported (Liberti et al., 2023). HaCaT cells were seeded at a cell density of 3 x 10^5 cells cm^{-2} for 24 h, to allow cells to reach confluence. Then, cells were scratched manually with a 200 μL pipet tip and incubated with either 100 μg mL^{-1} of control extract or 100 μg mL^{-1} elicited extract. The scratch area was monitored immediately after the scratch formation and after 24 h by acquiring images using optical microscopy (Zeiss LSM 710, Zeiss, Germany) at 10X magnification. The width of the wound was measured by using Zen Lite 2.3 software (Zeiss, Germany). Results are expressed as a reduction of the area (fold) compared with untreated cells.

2.12. Experimental design and statistical analysis

The experimental results of calli biomass and quantitative polyphenol determination were subjected to the analysis of variance using a one-way ANOVA using SPSS statistics version 28.0. To separate means within each parameter, the Tukey's multiple range test was performed. Biological results are presented as the mean of results obtained after three independent experiments (mean ± SD) and compared by one-way ANOVA according to Bonferroni's method (post hoc) using GraphPad Prism for Windows, version 6.01 (Dotmatics, California, USA).

3. Results

3.1. Development of apple pulp-derived callus cultures and biomass accumulation

The complete formation of callus cultures from *Malus pumila* Miller cv. Annurca pulp occurred in approximatively three months and calli displayed a yellow and friable appearance (Fig. 1). Thereafter, callus cultures were grown in a medium supplemented in the presence or absence of YE, tested at 300 or 500 mg/L and the biomass growth analysis was performed (Fig. 2). The amount of YE added was chosen according to Ali et al. (2018). After three weeks of treatment, the biomass accumulation significantly increased up to 1.3-fold compared to non-elicited samples.

3.2. Secondary metabolites accumulation and antioxidant activity in apple pulp-derived callus culture

To evaluate whether YE treatment was able to increase the accumulation of the most representative apple polyphenols in the pulp-derived callus cultures, a quantitative HPLC-DAD analysis was performed. As shown in Table 1, a significant increase in chlorogenic acid concentration was observed in extracts from the plant callus culture, ranging from 0.95 mg g^{-1} (control) up to 2.06 mg g^{-1} dry wt after elicitation with 500 mg L^{-1} YE. The same trend was observed for catechin, which was detected at a concentration of 0.018 mg g^{-1} dry wt in the control culture, and reached the value of 0.216 mg g^{-1} dry wt after treatment with 300 mg L^{-1} YE. Also, epicatechin was detected after the treatment with 500 mg L^{-1} YE at a concentration of 0.01 mg g^{-1} dry wt. No significant change was observed in dihydrochalcone accumulation,

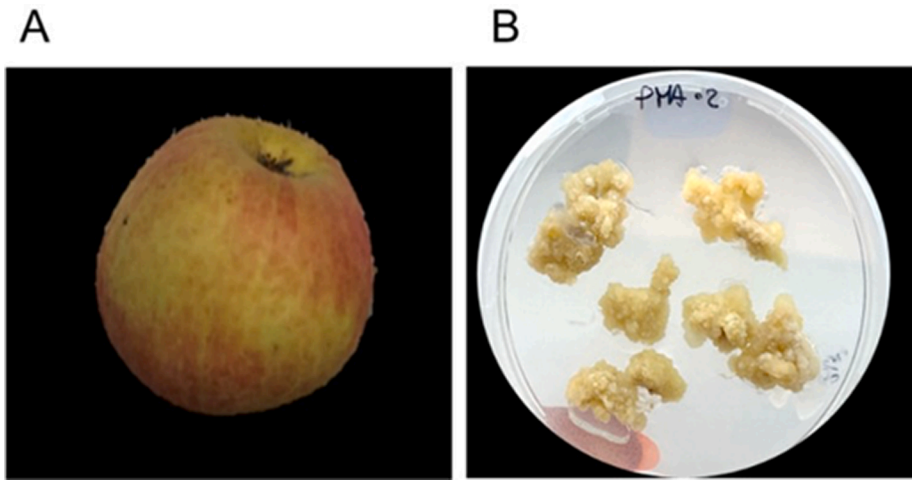

Fig. 1. A, *Malus pumila* cv Annurca; B, Apple pulp-derived calli.

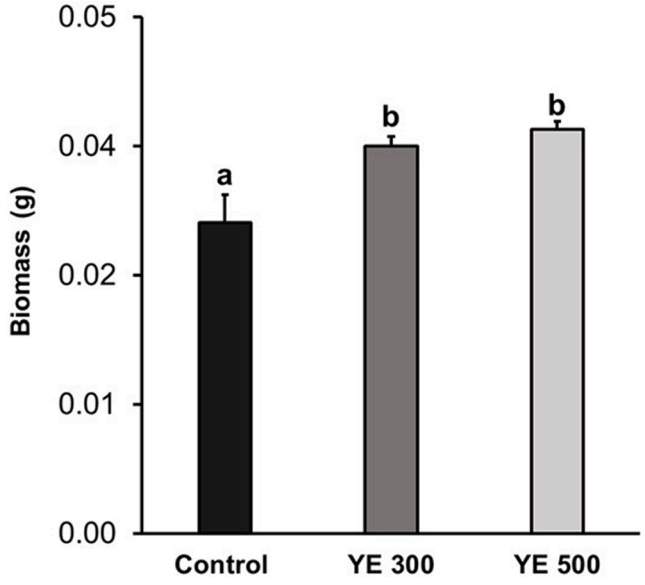

Fig. 2. The effect of yeast extract on the growth of the apple callus cultures. Control, YE300 and YE500 indicate non-elicited and YE-elicited callus cultures, respectively. Data are expressed as mean value ± SE (n ≥ 3). Mean values with different superscript letters are significantly different by Tukey's multiple comparison test ($p < 0.005$).

Table 1
Polyphenol content in the apple pulp-derived cell cultures. Data are expressed as mean value (mg component) g^{-1} dry wt of callus ± SD of three repetitions. Mean values with different superscript letters are significantly different by Tukey's multiple comparison test ($p < 0.005$) calculated along the lines. Control, 300 and 500 mg L^{-1} indicate non-elicited and YE-elicited callus cultures. ND, not detected; LOQ, limit of quantification.

Compounds	Control	300 mg/L	500 mg/L
Chlorogenic acid	0.953 ± 0.077^{a}	1.23 ± 0.006^{a}	2.06 ± 0.046^{b}
Phloridzin	0.523 ± 0.042^{a}	0.321 ± 0.009^{a}	0.520 ± 0.001^{a}
Procyanidin B1	ND	ND	ND
Procyanidin B2	ND	LOQ	0.151 ± 0.000
Catechin	0.018 ± 0.020^{a}	0.216 ± 0.019^{b}	0.203 ± 0.001^{b}
Epicatechin	ND	LOQ	0.013 ± 0.001
Rutin	ND	ND	ND
Total polyphenol content	10.00 ± 0.338^{a}	17.53 ± 1.042^{a}	22.31 ± 1.106^{b}

such as phloridzin. In all the analyzed samples, procyanidin B1 was not detected. Procyanidin B2 was found only in the samples elicited with the highest YE concentration, with a detection below the previously calculated LOQ (0.002 mg g^{-1}) in other samples. Overall, the treatment with YE 500 mg L^{-1} was the most effective, as it induced a significant increase in total polyphenol content (22.31 mg g^{-1} dry wt). Apple pulp-derived callus extracts were investigated to determine their *in vitro* total antioxidant activity using the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay and the inhibition % against free radicals was calculated. Results are showed in Fig. 3 and indicate that the total antioxidant activity was significantly increased after the treatment with YE 500 mg L^{-1} (84.59 %) compared to the control (67.94 %).

Considering the notably favourable results achieved with the 500 mg L^{-1} YE elicitation in terms of secondary metabolite accumulation and *in vitro* antioxidant activity, we proceeded to conduct cell-based experiments. These experiments involved a comparison between extracts obtained from callus cultures elicited with YE 500 mg L^{-1} and non-elicited callus cultures.

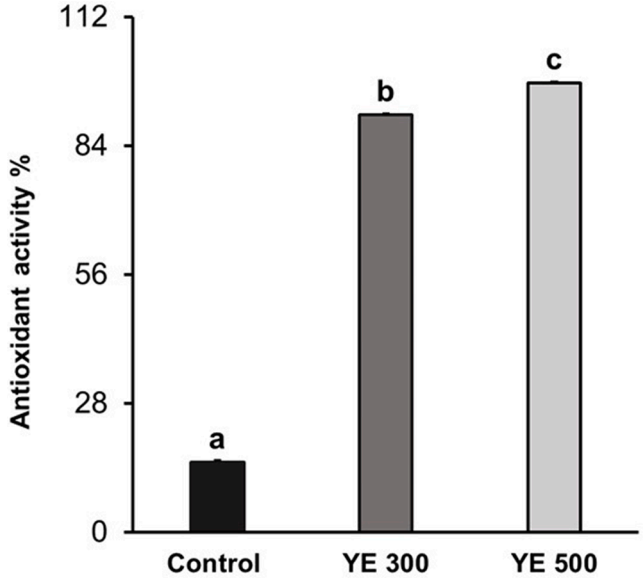

Fig. 3. Total antioxidant activity of extracts from apple pulp-derived calli. Control, YE300 and YE500 indicate non-elicited and YE-elicited callus cultures, respectively. Data are expressed as mean value ± SE (n ≥ 3). Mean values with different superscript letters are significantly different by Tukey's multiple comparison test ($p < 0.005$).

3.3. Effect of the extracts from apple pulp-derived callus culture on cell viability

The biocompatibility of the extracts from the apple pulp-derived callus cultures was evaluated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on immortalized human keratinocytes (HaCaT cells). Cells were plated and after 24 h incubated with increasing concentration of the extracts. Upon 48 h incubation, cell viability was evaluated by the MTT assay and cell survival was expressed as the percentage of viable cells in the presence of the extracts compared to that of control samples. As reported in Fig. 4, both extracts (i.e. from YE-elicited and non-elicited calli) were fully biocompatible as no effect on cell viability was observed at any concentration tested.

3.4. Protective effect of the extracts from apple pulp-derived callus culture against UVA-injury

As the chemical characterization revealed that elicitation induced an increase in polyphenol content, the antioxidant activity of the extracts from the apple callus cultures was investigated on a cell-based system. HaCaT cells and a UVA lamp were chosen as experimental system. Cells were incubated with 100 μg mL^{-1} of each extract for 2 h, then cells were stressed by UVA irradiation (100 J cm^{-2}). Immediately after the irradiation, intracellular ROS levels were measured by the DCFDA (dichlorofluorescin) assay. As shown in Fig. 5A, in the absence of oxidative stress, no alteration in intracellular ROS levels was observed when cells were treated with both extracts. However, upon exposure to UVA irradiation, a significant increase ($p < 0.05$) in ROS levels was observed (2-fold). When cells were pretreated with the control (non-elicited) extract prior the oxidative insult, only a slight inhibition of ROS production was observed (1.5-fold). Interestingly, pretreatment with extract from YE-elicited calli resulted in a stronger protective effect, as the intracellular ROS levels were comparable to the physiological ones (Fig. 5A). Oxidative stress is able to increase intracellular ROS production and to oxidize intracellular GSH, leading to the formation of oxidized glutathione (Daré et al., 2023). Thus, in the presence of oxidative stress, a decrease in intracellular GSH levels is expected. To this purpose, intracellular GSH levels were measured under the same experimental procedure and the results are reported in Fig. 5B. When cells were incubated with control extract, a slight but significant alteration in GSH levels was observed, also in the absence of oxidative stress. When HaCaT cells were irradiated with the UVA lamp, a significant decrease ($p < 0.005$) in GSH levels (40 %) was observed, compared to control cells, indicative of GSH oxidation. Noteworthy, when cells were treated with the elicited extract, and then irradiated by UVA, the intracellular GSH levels were similar to those observed under physiological conditions, as statistical analysis did not evidence any significant difference. It is interesting to notice that the control extract was able to slightly prevent from GSH oxidation with respect to the elicited one.

3.5. Wound healing activity of the extracts from apple pulp-derived cell culture

Antioxidants can play a key role in wound healing processes, thus the effect on wound repair of the Annurca apple PCC extracts was evaluated by carrying out a scratch test. Cells were treated as described in the experimental section and the results are reported in Fig. 6. Under normal physiological conditions (i.e. untreated cells), re-epithelialization spontaneously occurred (Fig. 6B, 2-fold increase, black bar). When cells were treated with control extract, no differences with the untreated cells were observed (Fig. 6B, dark grey bar). Interestingly, the treatment with the extract from the elicited cell culture resulted in a 4-fold reduction of wound area (Fig. 6B, light grey bar), thus suggesting a role in wound healing by the biomolecules present in the elicited extract.

4. Discussion

Plant cell cultures (PCCs) hold much promise as safe and ecological approach to obtain enriched patterns of molecules from different plant species and in particular local varieties. Moreover, there are numerous advantages in using these biotechnological platforms, such as the absence of contaminations, independence from seasonality and water and soil conservation (Georgiev et al., 2018; Krasteva et al., 2021; Tripathi et al., 2019).

In this work, PCCs were produced starting from the pulp of Annurca apple. This Italian local variety is known to accumulate a high content of polyphenolic compounds and exert beneficial effects on human health. A study conducted on individuals with mild hypercholesterolemia who consumed two Annurca apples per day showed a significant reduction in total and low-density lipoprotein cholesterol levels, along with an increase in high-density lipoprotein cholesterol levels when compared to those who consumed Red Delicious, Granny Smith, Fuji, or Golden Delicious apples (Tenore et al., 2017). The Annurca apple was also tested for its properties against microorganisms causing food-borne diseases (Fratianni et al., 2011). The polyphenolic components of this peculiar apple cultivar are classified as hydroxycinnamic acids,

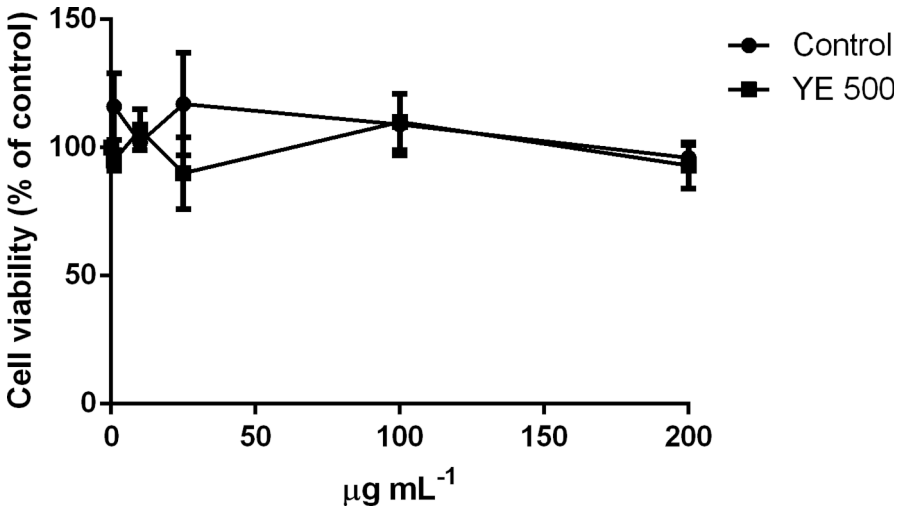

Fig. 4. Effect of apple pulp-derived calli extracts on cell viability. Dose-response curve of HaCaT cells incubated for 48 h with increasing concentration (1–200 μg mL^{-1}) of extract from control (black circles), or extract from YE-elicited (black squares). Cell viability was assessed by the MTT assay and expressed as described in Materials and Methods section. Values are given as means ± SD ($n \geq 3$).

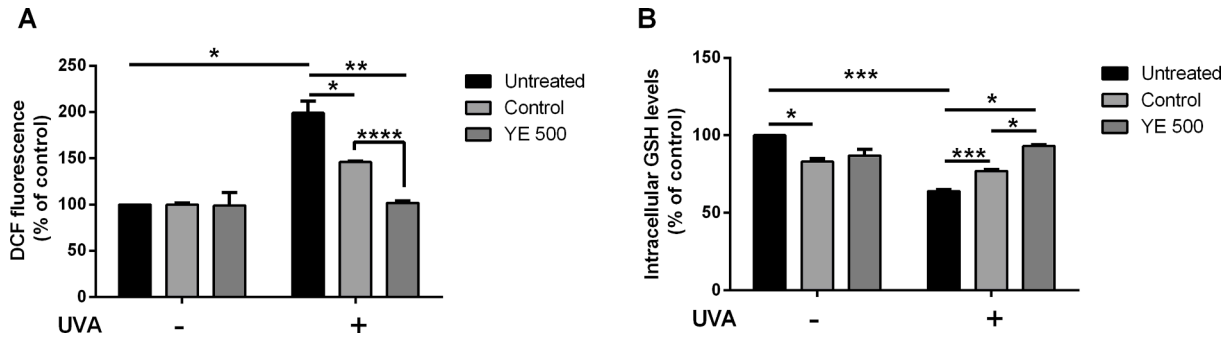

Fig. 5. Antioxidant activity of control and elicited extracts on UVA-stressed cells. A, Intracellular ROS levels measured by DCFDA assay, and B, intracellular GSH levels determined by DTNB assay. Cells were pretreated with 100 μg mL^{-1} of each extract for 2 h prior to UVA irradiation (100 J cm^{-2}). ROS levels and GSH levels were measured immediately after UVA irradiation. Black bars refer to untreated cells, dark grey bars refer to cells incubated with control extract, and light grey bars refer to cells incubated with extract from YE-elicited, in the absence (-) or in the presence (+) of UVA stress. Values are expressed as percentages compared with untreated cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, and **** $p < 0.001$. The lines above the bars indicate the samples compared for statistical analysis.

dihydrochalcones, procyanidins, flavonols and flavan-3-ols, with catechin and epicatechin being the predominant representatives (Fratianni et al., 2007). Additionally, it has been showed that the pulp of Annurca contains a high polyphenolic content namely procyanidins and phloridzin (Tenore et al., 2013; Fratianni et al., 2007). In view of this, a protocol for the production of PCCs from Annurca pulp was developed. The results showed the complete formation of yellow and friable calli after three months. As reported by Mustafa and colleagues, calli from different explants may appear within two months (Mustafa et al., 2011). In this study, the callus formation took longer, and this most probably depend on the starting material (the Apple pulp) and the apple cultivar (the Annurca apple) used. To the best of our knowledge, a cell line from Annurca pulp has never been produced. Furthermore, our protocol led to the production of a cell line that efficiently preserve the polyphenol reservoir typical of Annurca fruit. This success was not a foregone conclusion. Indeed, the metabolomic profile and the quantity of compounds present within PCCs are not always comparable to the starting material (Ali et al., 2018).

Thereafter, callus cultures were elicited with a commercial YE for the elicitation of the bioactive compounds. Yeast cell wall is made up of mannoproteins, β-1,3- and β-1,6-glucans and chitin while its cell membrane encompasses lipids, sterols, and proteins. These compounds can trigger plant defense activating various biosynthetic pathways (Ferrari, 2010; Portu et al., 2016). Two different concentrations were tested, 300 and 500 mg L^{-1}, respectively. These concentrations were chosen according to previous studies that described the use of YE, at concentrations no higher than 500 mg L^{-1}, in PCCs from *Ocimum basilicum* L. var purpurascens, *Oryza sativa* L., *Zingiber officinale* Rosc., *Malus domestica* Borkh and *Glycyrrhiza glabra* (Zaman et al., 2022; El-Beltagi et al. 2022; Ali et al., 2018; Cai et al., 2014; Vijayalakshmi and Abhilasha, 2019).

Our findings show a slight, but significant, increase in biomass weight when calli were elicited with either YE 300 or 500 mg L^{-1}. This could be due to the high content of glucan, chitin, ergosterol, vitamin B complex, and glycopeptides present in the YE that can stimulate cell proliferation and growth (Cai et al., 2012). Results obtained from Cai et al. (2014) demonstrated that the addition of YE to the culture medium did not improve the biomass accumulation of *Malus domestica* Borkh leaf-derived suspension cell culture (Cai et al., 2014). In contrast, treatment of leaf-derived cell suspension cultures obtained from *Glycyrrhiza glabra* (Vijayalakshmi and Abhilasha, 2019) and *Ocimum basilicum* (Zaman et al., 2022) with YE induced a statistically significant increase in biomass weight.

In our experimental system, YE induced the increase of chlorogenic acid, catechin and procyanidin B2, accordingly to a previously published study on cell cultures from *M. domestica* leaves (Sarkate et al., 2017). Particularly, the authors described that chlorogenic acid is one of the most responsive to YE treatment, as its concentration significantly increased in elicited apple cell cultures.

Although Tenore et al. (2013) indicated a high content of procyanidins in Annurca pulp, neither procyanidin B1 nor procyanidin B2 were detected in non-elicited pulp cell cultures. Despite this, a concentration of 0.151 mg g^{-1} dry wt for procyanidin B2 was recorded only after YE 500 mg L^{-1} treatment. This can be explained considering that YE may influence the metabolic biosynthetic pathway leading to the production of procyanidin B2 as defense compound. Indeed, one study showed that the use of 1.69 g L^{-1} of YE sprayed on *Vitis vinifera* cv. Tempranillo stimulated the plant response by enhancing the procyanidin B2 content in the grape berries (Portu et al., 2016).

Despite phloridzin is one of the most representative apple polyphenols, its accumulation was not significantly affected by the different YE concentrations used. Previously, it was reported that the synthesis of phloridzin in the cell culture model was particularly sensitive to the growing conditions or the apple cultivar used. Verardo et al. (2017), who analyzed the polyphenol composition of cell culture deriving from the pulp of two different apple cultivars of *M. domestica*, Golden Delicious (GD) and Mela Rosa Marchigiana (MRM), identified phloridzin only in GD-pulp-derived calli (Verardo et al., 2017). Nevertheless, our data clearly indicated that the cell line derived from Annurca pulp was able to preserve this remarkable molecule. Rutin was not detected in either elicited or non-elicited calli. This could be due to the fact that a radical change in cellular metabolism was initiated during the induction and growth of the calli. It could be assumed that the growth conditions used are able to induce growth of the apple cell culture, but do not promote or activate the biosynthesis of quercetin and quercetin-derivatives (such as rutin). Additionally, although rutin is the main quercetin-derivative contained in Annurca apple (both in the peel and pulp) its relative amount (total quercetin-glycoside estimate of 0.68 ± 0.03 mg/100 FW of Annurca apple) is much lower than other polyphenols detected in this cultivar, such as chlorogenic acid (11.50 mg/100 FW of Annurca apple), procyandin B2 (27.97 ± 1.69 mg/100 FW), and epicatechin (4.56 ± 0.23 mg/100 FW) (Panzella et al., 2013). Reasonably, in the undifferentiated cell mass, mainly the principal metabolic pathways of Annurca apple responsible for the synthesis of the most representative Annurca polyphenols were maintained.

Given the valuable spectrum of specialized compounds found in Annurca cell culture extract, the antioxidant activity was also examined. The activity was significantly enhanced up to 20 % higher with the use of YE 500 mg L^{-1} compared to the non-elicited pulp-derived callus extract. Antioxidant activity of apple is correlated with the amount of secondary metabolites, such as phenolic acid, flavonoid and dihydrochalcone present in the fruit (Giomaro et al., 2014). Specifically, the antioxidant potential of Annurca apple is mainly related to its valuable polyphenolic composition (Tenore et al., 2013). The treatment with YE can induce and stimulate the production of these metabolites and, as a

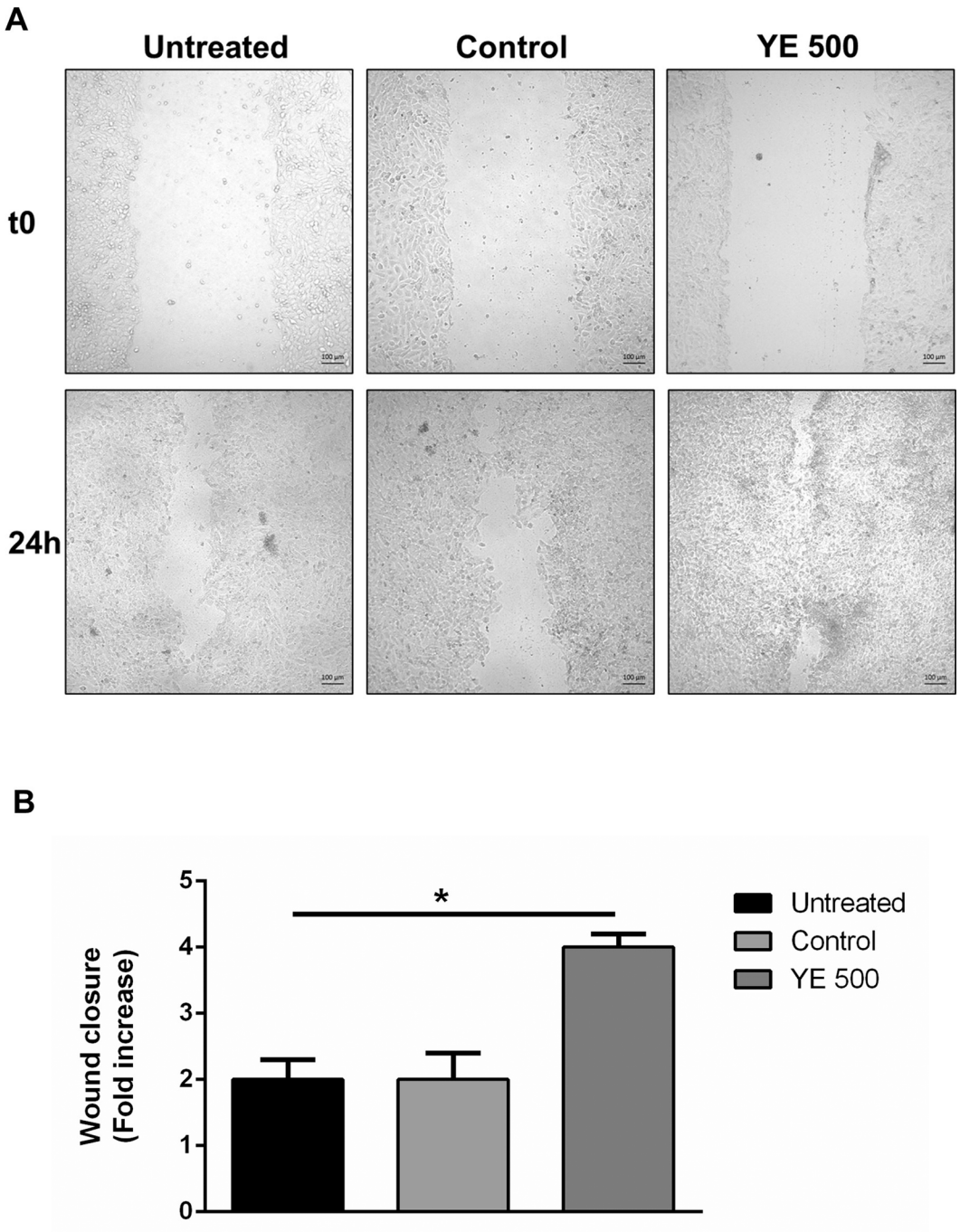

Fig. 6. Effect of control extract and elicited extract on cell re-epithelialization. A, Confluent HaCaT cells were scratched and treated with 100 μg mL^{-1} of either control extract or extract from YE-elicited calli for 24 h. Optical microscopy images were acquired at 10X magnification at the beginning (t0) and end (24 h) of the incubation. B, Reduction of area (fold) of wound closure upon 24 h of incubation. Black bars are referred to untreated cells, dark grey bars are referred to cells treated with 100 μg mL^{-1} of control extract, and light grey bars are referred to cells treated with 100 μg mL^{-1} of extract from YE-elicited calli. Data shown are means ± S.D. of three independent experiments. * indicates $p < 0.05$. The lines above the bars indicate the samples compared for statistical analysis.

consequence, enhance their protective effects. Accordingly, it was showed that *M. domestica* leaf-derived cell cultures elicited with YE exert a higher antioxidant activity than the non-elicited ones (Sarkate et al., 2017). On the contrary, a low antioxidant activity was recently demonstrated in apple pulp-derived calli accumulating a low level of polyphenols (Potenza et al., 2020).

Taking in consideration the metabolomic results, to assess the potential application of the Annurca callus cultures, the extract obtained from the most effective elicitation condition (i.e., YE 500 mg L^{-1}) was tested on a cell-based system in which oxidative stress was induced by UVA lamps. It is known that oxidative stress is able to increase intracellular ROS production and to oxidize intracellular GSH, leading to the formation of oxidized glutathione. YE 500 was able to protect cells from oxidative stress, because, in the presence of this stress, the elicited extract was able to inhibit intracellular ROS production and GSH oxidation. These results are in line with those usually reported for extracts enriched in antioxidants (Halliwell, 2024).

Moreover, the elicited extract was also able to accelerate the re-epithelization processes on scratched HaCaT cells. According to the literature, the plant metabolites most related to the wound healing are

phenolic acids, flavonoids, and terpenes (Álvarez-Santos et al., 2023; Juszczak et al., 2022). Our findings were in line with these studies, since the metabolic analysis indicated the presence of chlorogenic acid, procyanidin B2, catechin and epicatechin within the pulp-derived callus cultures.

Future analyses might help to deeply understand the specific components involved in the process of re-epithelization. Interestingly, no literature is present on the wound healing ability of polyphenolic extract from Annurca apple based-PCC, with the exception of those reported by Potenza and colleagues (Potenza et al., 2020), who investigated the wound healing activity of MRM pulp-derived callus extract, but without the use of elicitors. Other studies conducted on the extracts from cell cultures of *Hibiscus syriacus* (di Martino et al., 2017) and *Fitzroya cupressoides* (Carvajal et al., 2020) leaves showed an improvement in the wound healing activity in terms of epithelium formation, thus extending the use of extracts from PCCs to human health care.

5. Conclusions

Fruits and vegetables contain a broad spectrum of secondary metabolites which can find different applications in cosmeceutical or nutraceutical fields. In this context, the *in vitro* cultures of their tissues have long been adopted. Annurca apple is recognized as a distinctive variety from Southern Italy as its extract is considered to be a versatile agent to be used against various diseases, such as diabetes and cancer. Our study clearly indicates that callus cultures obtained from Annurca pulp contain a valuable set of high-value molecules. Moreover, their content can be increased by the use of natural elicitors, such as YE which has been shown to be safe on human cells. In addition, extract from the callus cultures showed a remarkable protective effect against UVA injury and a positive effect on wound healing. Altogether, these results suggest that Annurca pulp-derived callus extract could be beneficial for human skin and used in cosmetic industry as an alternative source of high-value added molecules.

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Ethics statement

The research was not carried out with animals and humans.

CRediT authorship contribution statement

Carmen Laezza: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Paola Imbimbo:** . **Vincenzo D'Amelia:** . **Adua Marzocchi:** Investigation, Formal analysis. **Daria Maria Monti:** Conceptualization, Resources, Writing – review & editing, Supervision. **Antonio Di Loria:** Writing – review & editing, Writing – original draft. **Simona Maria Monti:** . **Ettore Novellino:** Writing – review & editing, Resources. **Gian Carlo Tenore:** Conceptualization, Resources, Writing – review & editing, Supervision. **Maria Manuela Rigano:** Conceptualization, Resources, Writing – review & editing, Supervision.

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.

Data availability

Data will be made available on request.

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