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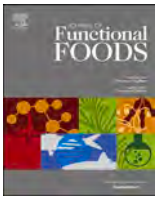

Long-lasting bioactive compounds production from *Malus x domestica* Borkh var. Mela Rosa Marchigiana pulp callus culture with antioxidant activity: *In vitro, ex vivo* and *in vivo* validation

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ABSTRACT

The Mela Rosa Marchigiana (MRM) pulp callus, kept alive since 2017 by monthly sub-cultures, was investigated and morphologically described. In the present paper, the MRM callus ethanolic Extract (MRME) was prepared from one of the 2022 callus sub-cultures and analysed by GC-MS and GC-FID. The investigation was carried out to test whether a long-term culture could affect either the amount or the bioactivity (or both) of the triterpenes produced by the callus. To this aim, the 2022 extract (MRME 2022) was compared to the extract prepared a few years ago (i.e., MRME 2019). Obtained results showed that the composition of both extracts was almost the same, thus revealing that the callus culture efficiency of triterpenes production was nearly unchanged over time. When analysing the biological effect of the extract, all experimental tests, including cell-free, *in vitro*, *ex vivo*, and *in vivo* assays, confirmed that MRME 2022 significantly protected cells, tissues, or whole organisms from oxidative stress-induced damage. Present results could lead to future studies to test MRME as a possible ingredient of a nutraceutical formulation for healthy aging or for the prevention of oxidative stress-associated pathologies.

1. Introduction

Health promotion and chronic diseases prevention are overall goals both at national and European level. Preventive strategies are of paramount importance and one of the potential useful approaches involves medicinal herbs and phytochemicals. Phytochemicals, also known as secondary metabolites (SMs), are chemical non-nutritive compounds which are important for plant metabolism, pest or herbivores defence, growth regulation, communication with neighbouring flora and attracting pollinators (Divekar et al., 2022). In the last decades, phytochemicals have emerged as modulators of critical cellular signalling pathways and health improvement, resulting very attractive for their use in healthcare products as functional (bioactive) compounds. However, to obtain SMs, quite large quantities of fresh plant material or even the use of chemical synthesis is required, which includes particularly expensive systems and some non-negligible environmental negative

Abbreviations: MRM, Mela Rosa Marchigiana; MRME, Mela Rosa Marchigiana ethanolic Extract; MRME 2019, Mela Rosa Marchigiana ethanolic Extract from one of the 2019 MRM pulp callus sub-cultures; MRME 2022, Mela Rosa Marchigiana ethanolic Extract from one of the 2022 MRM pulp callus sub-cultures; OHSC, Organotypic Hippocampal Slice Cultures; SM, Secondary metabolites.

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implications. To address this issue, many efforts (Sohn et al., 2022) have been made in carrying out *in vitro* plant cell or tissue cultures, with particular attention to callus cultures, for producing SMs to apply to pharmaceutical and functional foods fields. Leaves, stems, nodes, internodes and petioles have primarily been used as starting material of callus cultures (Koufan et al., 2020), whereas fruit pulp was only occasionally tested (Verardo, Gorassini, & Fraternale, 2019). This innovative strategy resulted to be an alternative method of "Green Chemistry" for producing large quantities of high quality phytocomplexes. Moreover, one of the advantages of this procedure is that high concentrations of pesticide-free SMs may be continuously obtained, independently of seasonal and environmental conditions. This makes it a promising methodological approach to be exploited in the development of functional products with beneficial health effects.

In 2017, it has been developed in the botanical garden laboratory of the University of Urbino an innovative method of callus culture from the ripe pulp of a local variety of apple, Mela Rosa Marchigiana (MRM). Subsequently, this technique which allows to obtain callus cultures from the ripe pulp of different types of fruit was patented (patent no. 102020000012466).

Malus x domestica Borkh var. Mela Rosa Marchigiana (Rosaceae family) is an ancient apple variety growing at 400 – 900 m above sea level in the Marche region (Italy). Over the last decades its production had almost completely disappeared as it was little known and, therefore, scarcely requested on the market. However, thanks to the preservation of a few remaining trees and the effort of the Marche Region's agri-food services to conserve the ecotype, its production has recently resumed.

Apples are considered an important source of SMs in the human diet and their consumption has been associated to the prevention of degenerative diseases (Vuoso, Porcelli, Cacciapuoti, & D'Angelo, 2020). As in all other apple varieties, in MRM as well, the main SMs found in fresh pulp are phenolic compounds (López et al., 2020), while the amount of triterpenic acids is relatively low (Verardo, Gorassini, Ricci, & Fraternale, 2017). However, triterpenes are noteworthy SMs with multiple biological activities including antioxidant (Fu et al., 2021) and anti-inflammatory (Potenza, Minutelli, Stocchi, & Fraternale, 2020; Schinella et al., 2008; Yousefi-Manesh et al., 2020; Zhao, Zhou, An, Shen, & Yu, 2020) properties. They have also been shown to have a protective effect against cancer and cardiovascular diseases (see for example, Alkholifi,Devi,Yusufoglu,& Alam, 2023; Baldelli et al., 2019; Claro-Cala, Jiménez-Altayó, Zagmutt, & Rodriguez-Rodriguez, 2022; Erdmann, Kujaciński, & Wiciński, 2021; Fang, Deng, Zhou, Hu, & Yang, 2022; Gamede, Mabuza, Ngubane, & Khathi, 2019; López et al., 2019; Olech, Ziemichód, & Nowacka-Jechalke, 2021; Ooi, Poo, Subramaniam, Cordell, & Lim, 2023; Yang et al., 2022; Zhao et al., 2023).

Since 2017 the MRM pulp callus has been kept alive by a series of sub-cultures made every 28 days and the triterpenic acids content of both MRM pulp fruit and callus methanolic extracts have been characterized (Verardo, Gorassini, Ricci, & Fraternale, 2017). As shown in Table 1, the total amount of triterpenes in callus culture products was significantly higher compared to that obtained from fresh fruit pulp. Based on this result, Potenza and co-workers prepared a 70 % ethanol MRM pulp callus extract from one of the 2019 callus sub-cultures (without GC–MS/GC-FID characterization), to test its biological potential. In that study the extract showed antioxidant, geno-protective, anti-inflammatory, stimulating mitochondrial biogenesis, and wound healing activities (Potenza, Minutelli, Stocchi, & Fraternale, 2020).

In the present study, the MRM callus ethanolic extract was prepared from one of the 2022 sub-cultures. The extraction protocol was slightly modified to reduce the debris, and the triterpenic acids composition of the extract was analysed by GC-MS and GC-FID. Considering that the culture was started in 2017, experimental tests were performed to prove if the 2022 extract still had antioxidant capacity and, in that case, to evaluate its effectiveness. After comparing the triterpenic acid content of MRME 2022 and MRME 2019, cell-free, *in vitro, ex vivo* and *in vivo* assays were carried out to assess the potential of the extract against oxidative stress-induced damages. In order to compare present and previous results, we intentionally repeated some of the cell-free (i.e., DPPH, ABTS and ORAC) assays and *in vitro* experiments previously carried out by Potenza et al. (2020).

2. Material and methods

2.1. Chemicals and reagents

Extraction and derivatization solvents were of analytical grade and were obtained from Sigma-Aldrich (Milan, Italy). Cholesterol (GC-FID Internal Standard; IS), β-sitosterol, ursolic and oleanolic acids, used as standards, Sylon BFT [Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1 % of trimethylchlorosilane (TMCS)], employed as a silylating reagent, were also obtained from Sigma-Aldrich. Milli-Q grade water was produced by the Elgastat UHQ-PS system (ELGA, High Wycombe Bucks, UK). Solid-phase extraction (SPE) columns ISOLUTE C18, 1 g, 6 mL were from Biotage (Milan, Italy).

2.2. Callus culture conditions and maintenance

The callus used in this study and in the previous one (Potenza, Minutelli, Stocchi, & Fraternale, 2020) comes from the same culture developed more than six years ago (see Verardo, Gorassini, Ricci, & Fraternale, 2017). In fact, the MRM callus obtained in 2017 was regularly sub-cultured every 28 days and continues to be sub-cultured today. The *in vitro* culture was conducted as described by Verardo et al. (2017). Briefly, we employed the Gamborg B5 medium plus 2.0 mg/L 6-benzylaminopurine (BA, Sigma-Aldrich) and 0.2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid, Sigma-Aldrich), plus 30 g/L sucrose, pH 5.8, as this combination turned out to give the highest biomass production from pulp explants of MRM. Callus culture was conducted in the dark at 25 ± 2 °C and subcultures were made after 28 days in the same medium. Every 28 days, a part of the callus obtained by each sub-culture is sub-

Table 1
Triterpenic acids and β-Sitosterol content (μg/100 mg of dried pulp or callus cultures ± standard deviation, and percentage) of MRM pulp and callus cultures after methanolic and ethanolic extraction, quantified by GC-FID.

Compound	Pulp 2017 (100 % MeOH)[a]		Callus 2017 (100 % MeOH)[a]		Callus 2022 (100 % MeOH)[a]		Callus 2022 (70 % EtOH)[a]	
β-Sitosterol	46.74 ± 1.128	96.39	120.90 ± 2.69	2.24	184.41 ± 2.93	5.59	168.63 ± 2.29	5.16
Oleanolic acid	0.10 ± 0.01	0.21	42.10 ± 1.90	0.78	120.44 ± 2.25	3.65	76.05 ± 1.16	2.33
Ursolic acid	0.38 ± 0.01	0.78	174.43 ± 3.58	3.23	334.36 ± 5.42	10.13	321.69 ± 4.10	9.84
Maslinic acid	0.43 ± 0.01	0.89	963.30 ± 17.62	17.82	361.63 ± 4.97	10.96	378.69 ± 5.19	11.58
Corosolic acid	0.41 ± 0.00	0.84	676.77 ± 3.85	12.52	337.13 ± 6.69	10.21	342.56 ± 3.71	10.48
Pomolic acid	0.16 ± 0.00	0.34	17.10 ± 1.47	0.32	79.60 ± 0.08	2.41	39.76 ± 0.60	1.22
Annurcoic acid	0.07 ± 0.00	0.14	504.57 ± 2.59	9.34	587.51 ± 8.32	17.80	595.61 ± 3.50	18.21
Tormentic acid	0.20 ± 0.01	0.41	2905.63 ± 33.51	53.76	1295.56 ± 9.07	39.25	1347.03 ± 10.04	41.19
Total	48.49 ± 1.12		5404.80 ± 46.69		3300.63 ± 6.04		3270.01 ± 21.96	

[a] Extraction solvent.

cultured again for the maintenance of the material, while the remaining part is harvested and stored at − 20 °C until use. When necessary, part of frozen material is lyophilized and employed for the extraction procedure (for details see paragraph 2.4).

To use a less toxic extraction solvent than methanol, the extraction efficiency of 70 % ethanol following the procedure of Verardo et al. (2017) has been tested. Both 100 % methanol and 70 % ethanol callus extracts were analysed. In addition, a modified protocol of ethanol extraction has been performed, characterized by two extra sequential resuspension (in 70 % ethanol) and centrifugation steps, to minimize the debris as much as possible.

2.3. Morphological and semiquantitative analysis of the callus

The morphological and chemical analysis was performed by a FEI Quanta 200 FEG Environmental Scanning Electron Microscope (FEI, Hillsboro, Oregon, USA) equipped with an Energy Dispersive X-ray Spectrometer (EDAX Inc., Mahwah, NJ, USA) (ESEM–EDS) following standard procedures (Antonini et al., 2018). Briefly, shortly prior to the analysis, the freeze-dried callus, in form of small granules, was deposited onto the aluminium specimen holders of the microscope. The stubs were pre-treated with the application of a double-sided conductive, carbon made tape, specifically realized in clean and pure form for the microscope stubs. (TAAB Ltd., Berks, UK). Samples were analysed using a focalized electron beam in a vacuum electron gun pressure of 5.0^{-6} mbar. The ESEM was settled in low vacuum mode with a specimen chamber pressure ranging from 0.80 to 0.91 mbar, with an accelerating voltage of 15–20 kV, and a magnification ranging between about 50 and 3300x. The images were obtained by means of the secondary and back-scattered electron detectors. The spectrometer unit was equipped with an ECON (Edax carbon oxygen nitrogen) 6 utw (UltraThin Window) X-ray detector and Genesis Analysis software. Each sample was analysed with a time count of 100 sec and an Amptime of 51, while the probe current was 290 μA.

2.4. Mela Rosa Marchigiana callus ethanolic Extract (MRME) preparation

Freeze-dried MRM pulp callus (260 mg) was extracted in a glass potter with 15 mL of 70 % ethanol for 15 min. The homogenate was stirred overnight (ON) at 4 °C and then centrifuged for 45 min at 4 °C x 13,000g. After supernatant recovery, the pellet was extracted a second time with 15 mL of 70 % ethanol and stirred at 4 °C for 30 min. The supernatant was recovered by centrifugation as above, and the two supernatants combined and dried in Savant ON. The day after, 15 mL of 70 % ethanol were added to the residue and, after stirring for 30 min at 4 °C, the tube was centrifuged as above and the recovered supernatant was dried to be subsequently resuspended in 15 mL of 70 % ethanol, re-centrifuged and finally the obtained supernatant aliquoted in Eppendorf (1 mL each) and dried in Savant. After weighting the pellet, each Eppendorf was stored at −20 °C before chemical analysis and biological tests.

2.5. Mela Rosa Marchigiana callus ethanolic extract analyses by GC-MS and GC-FID

The procedure for identification and quantification of triterpenic acids found in MRME used in this study was described in our previous works (De Bellis et al., 2022; Verardo, Gorassini, Ricci, & Fraternale, 2017; Verardo, Gorassini, & Fraternale, 2019). Briefly, each dried extract was spiked with internal standard (cholesterol, IS), suspended in water and extracted twice with EtOAc. The collected organic phases were washed with H_2O, brine, then dried (Na_2SO_4 anhydrous), filtered, and evaporated to dryness in vacuo. The residue was silylated by a mixture (1:1) of pyridine and BSTFA, and the reaction mixture was analysed by GC–MS and GC-FID.

GC–MS analyses were carried out using a Trace GC Ultra gas chromatograph equipped with a split-splitless injector and coupled to an ion-trap mass spectrometer detector Polaris Q (Thermo Scientific). The column was a 30 m × 0.25 mm i.d., 0.1 μm film thickness, fused silica SLB-5 ms (Supelco, Sigma-Aldrich). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 10 min and the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 20 min. Samples were injected in the split (1:10) mode. Injector, transfer line and ion source were set at 280, 280 and 200 °C, respectively. Helium was used as carrier gas at a flow of 1 mL/min. The mass spectra were recorded in electron ionization (EI) mode at 70 eV electron energy with a mass range from m/z 50 to 1000 and a scan rate of 0.8 scan/sec. Identification of metabolites was carried out by comparison of the spectral data and retention times with those of standards or to the spectra from the NIST02 spectral library or with those available in the literature. The data acquisition was under the control of Xcalibur software (Thermo Scientific).

The quantitative analysis was carried out using an Fisons GC 8000 series gas chromatograph, equipped with a split/splitless injector and a flame ionization detector (Fisons Instruments, Milan, Italy). The separation was carried out with a fused silica capillary column DB-5MS UI 30 m × 0.250 mm × 0.25 μm film thickness (Agilent, J&W, Italy). The initial oven temperature was 240 °C programmed to 280 °C at 2 °C/min and kept at 280 °C for 25 min, the temperature was then raised to 310 °C at a rate of 10 °C/min and maintained at this temperature for 13 min. Samples (1 μL) were injected in the split (1:10) mode. Injector and detector were set at 280 °C. Hydrogen was used as carrier gas at a flow of 1.8 mL/min. Peak areas were integrated using a Varian Galaxie Workstation (Agilent Technologies, Italy).

Quantification of the triterpenic acids and β-sitosterol in the samples was performed using the internal standard method based on the relative peak area of analyte to IS (cholesterol) from the average of three replicate measurements.

2.6. DPPH scavenging activity

The antioxidant ability of MRME was determined using the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•) as described by Saltarelli et al. (2019) with minor modifications. In brief, 850 μL of 100 μM DPPH• ethanol solution newly prepared was mixed with 150 μL of MRME (50 mg dw/mL). After half an hour in the dark at room temperature (RT), the absorbance was measured at a wavelength corresponding to 517 nm (UVIKON 930 UV/VIS spectrophotometer); 70 % ethanol was used as blank. Values are expressed as the MRME concentration required to scavenge 50 % of the initial DPPH radicals (IC50 mg dw/mL).

2.7. ABTS scavenging activity

The 2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid (ABTS) assay was carried out according to Loizzo et al. (2019) with some modifications. The ABTS radical is generated after mixing 7 mM ABTS solution with140 mM potassium persulphate ($K_2S_2O_8$) and left to incubate for 12–16 h in the dark at RT. The reaction mixture is then diluted 1:90 v/v with 70 % ethanol and the absorbance is measured and should have a corresponding value of 0.70 ± 0.02 at 734 nm. 10 μL MRME of different solutions ranging from 50 to 500 μg/mL, were added to 1 mL of ABTS ethanolic solution, and incubated 4 min in the dark at RT. The absorbance decrease was measured at 734 nm (UVIKON 930 UV/VIS spectrophotometer); 70 % ethanol was used as blank. Values are expressed as the MRME concentration required to scavenge 50 % of the initial ABTS radicals (IC50 mg dw/mL).

2.8. ORAC assay

The ORAC (oxygen radical absorbance capacity) assay is one of the

most used methods to assess the antioxidant ability of substances for research and industrial purpose. The antioxidant capacity of MRME was tested as described by De Bellis et al. (2019) using a Fluostar Optima Plate reader fluorimeter (BMG Labtech, Offenburg, Germany) equipped with a temperature-controlled incubation chamber (37 °C) and an automatic injection pump. The following compounds were mixed as in the protocol below: 200 μL of 0.096 μM fluorescein sodium salt in 0.075 M Na-phosphate buffer (pH 7.0), 20 μL of sample (MRME) or Trolox or 0.075 M Na-phosphate buffer (pH 7.0) as blank. The reaction was initiated with 40 μL of 0.33 M of 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH). Fluorescence was read at 485 nm excitation and 520 nm emission until complete extinction. Each time, a calibration curve was made using a positive standard, the Trolox, with a concentration ranging from 50 to 500 μM in 0.075 M Na-phosphate buffer (pH 7.0). ORAC values of the tested samples are reported as μmol Trolox Equivalents (TE)/g of callus extract.

2.9. Cell culture, viability, and in vitro antioxidant assays

HaCaT (immortalized human keratinocytes) cells (CLS-Cell Lines Service GmbH, Eppelheim, Germany) were used to investigate the biological properties of MRME. Cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10 % foetal bovine serum, 1 % L-glutamine, 1100 U/mL penicillin/streptomycin, and maintained in a CO_2 incubator at 37 °C and 5 % CO_2. Cell culture reagents were from Sigma-Aldrich (Milan, Italy).

HaCaT cell viability after MRME treatment was analysed by water-soluble tetrazolium (WST)-8 assay to evaluate cell metabolic activity. Briefly, cells ($5x10^3$/well) were seeded in 96-well plates and treated ON with MRME at different concentrations. After incubation the medium was removed, and cell viability analysed. Colour development was monitored at 450 nm in a multiwell plate and data were expressed as cell viability (%) vs untreated control cells.

The antioxidant property of MRME in HaCaT cells was analysed using the cell-permeable fluorogenic probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, Milan, Italy). Cells (1×10^4/well) were seeded in 96-well plates grown at 37 °C for 48 h, washed with PBS and treated ON with different concentrations of MRME (2.24–71.5 μg/mL). A total of 100 μM H_2O_2 was added for 30 min or 1 h at 37 °C. The medium was then removed, and cells incubated with DCFH-DA (5 μM in PBS) for 30 min at 37 °C in the dark. ROS production was determined using the multiwell plate reader FluoStarOptima (BMG Labtech, Ortenberg, Germany) (emission wavelength 520 nm; excitation 485 nm).

2.10. Organotypic hippocampal slice cultures and oxidative treatments

All animal procedures were performed in accordance with the Italian regulation for the care and use of laboratory animals (EU Directive 63/2010; D.L. 26/14; research protocol authorization BEF09.N.D4M). Organotypic hippocampal slice cultures were prepared as previously described (Carloni et al., 2018). Briefly, 7-day-old male and female Sprague-Dawley rat brains (Charles River, Calco, Italy) were removed and gently immersed in an ice-cold dissecting solution. Hippocampi were dissected out on ice and cut into 400-μm thick transverse sections using a McIlwain Tissue Chopper. Slices with even margins and clear, uniform, and well-defined pyramidal cell layers were selected. Slices were plated onto Millicell culture inserts (0.4 μm Millicell-CM, Sigma Aldrich, St. Louis, MO, USA, Z354996-50EA) and pre-incubated with the culture medium (8.4 g/L MEM eagle medium, 20 % horse serum heat inactivated, 30 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mg/L insulin, 25 % ascorbic acid solution, 13 mM D-glucose and 5.2 mM $NaHCO_3$). After preparation, hippocampal cultures were maintained for 2 weeks in a 37 °C humidified incubator gasified with a 5 % CO_2/95 % O_2. Culture medium was changed three times per week.

For the oxidative stress assays, organotypic hippocampal slice cultures were treated with different H_2O_2 concentrations (200 or 400 μM) and incubated at 37 °C for 2 h. To evaluate the MRME antioxidant potential, the extract was added immediately after the end of the 400 μM H_2O_2 treatment and maintained in the medium for 24 h (recovery period). The final concentrations were of 1 μg/mL, 10 μg/mL or 100 μg/mL (H_2O_2 + MRME). Control cultures (CTRL) were also treated with 100 μg/mL MRME (CTRL + MRME) or vehicle (70 % EtOH) (CTRL + vehicle).

Cell damage was assessed by image analysis of propidium iodide (PI) uptake. PI penetrates damaged cells and binds to nuclear DNA to generate a bright red fluorescence. PI (5 μM, Sigma-Aldrich, Milan, Italy, P4170) was added to the cultures 20 min before the end of the incubation period, with MRME or vehicle. PI incorporation into slice cultures was assessed with an Olympus BX-51 microscope using a standard rhodamine filter set (490/590 nm). Camera sensitivity and excitation intensity were standardized the first day of analysis and the same parameters were used for all experiments. Gray-level intensities were measured by using the J-Image Software (https://www.imagej.nih.gov). H_2O_2-induced cell damage was calculated in each slice using the following formula:

$$\text{Cell death (\%)} = \text{Fd / Fo x 100}$$

where Fd is the PI fluorescence detected in the injured areas of the slice, and Fo is the fluorescence detected in the whole slice. Damage observed in hippocampal slice cultures treated with 400 μM H_2O_2 was considered as the maximal cell damage and therefore the PI fluorescence detected in this experimental condition has been assigned as 100 % cell death. Normalized fluorescence intensity values were calculated as follows:

$$\text{Cell death (\%)} = \text{Sample cell death / 400 μM } H_2O_2\text{-induced cell death x 100}$$

2.11. C. elegans Cultivation

We employed standard nematode culture conditions (Maglioni, Schiavi, Runci, Shaik, & Ventura, 2014). Wild-type (N2) nematode strain was maintained at 20 °C on Nematode Growth Media (NGM) agar supplemented with *Escherichia coli* (OP50).

2.12. Preparation of plates containing MRME and pro-oxidants for the stress resistance assays

The MRME was resuspended in EtOH 70 % to a stock solution (2 mg/mL), vortexed and sonicated for 10 min until completely dissolved. A final volume of 150 μL of either apple extract at 400 μg/mL diluted in water or of vehicle (70 % EtOH diluted in water, as control) was then spotted on 6 cm NGM plates spotted with UVB-killed HT115 bacteria.

Juglone containing plates were prepared the day of the experiment by diluting an initial 50 mM stock solution of Juglone in DMSO to reach a final concentration of 200 μM in the pre-cooled NGM (final DMSO concentration must be less than 1 %) before pouring the plates.

DEM containing plates were prepared the day before the experiment by diluting an initial 4 M stock solution of DEM in DMSO to reach a final concentration of 15 mM in the pre-cooled NGM (final DMSO concentration must be less than 1 %) before pouring the plates.

2.13. Stress resistance assays in C. elegans

Juglone sensitivity assay

Fifteen N2 gravid adults were left for 1 h to lay eggs on 6 cm NGM plates (with or without MRME). Plates without the extract and with a small volume (5 μL) of OP50 bacteria were then prepared with a final concentration of 200 μM juglone on the day of the experiment and synchronized 1-day old adult worms (3-day-old animals) were

transferred from MRME-NGM plates on juglone plates and checked for survival every hour for 6 h. Animals not able to move upon pick-prodding and with no pharyngeal pumping were scored as dead.

Diethyl Maleate (DEM) sensitivity assay

Fifteen N2 gravid adults were left for 1 h to lay eggs on 6 cm NGM plates (with or without MRME). Synchronized 1-day old adult worms were then transferred in new 6 cm MRME-NGM plates spotted with UVB-killed HT115 bacteria every day for 4 days. The day before the experiment, 5 mL of HT115 bacteria were incubated ON and the next morning concentrated in a refrigerated centrifuge (10 min; 4000 rpm), spotted on DEM plates and let dry for 1–2 h. The 5-day old adults were then moved on 15 mM DEM plates spotted with concentrated HT115 bacteria (150 μL at OD = 2) and checked after 8, 24, 31 and 48 h, until every worm die. Animals not able to move upon pick-prodding and with no pharyngeal pumping were scored as dead.

2.14. Statistical analyses

Statistical analysis was performed using the computer program Graph Pad Prism (v4.0, GraphPad Software Inc., San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's or Newman-Keuls Multiple Comparison Test was used to determine statistical differences between the experimental groups. The results are expressed as mean ± SEM (Standard Error of the Mean) or SD (Standard Deviation). Differences were considered statistically significant when $p < 0.05$.

Caenorhabditis elegans oxidative stress results were analysed through the online tool OASIS2, an online application for survival analysis (https://sbi.postech.ac.kr/oasis2/) using the statistical tasks Basic survival analysis. Data were introduced following the example given by the program. Survival plots are graphs that show the relationship between the survival and age of the worms where x axis is the time in hours and y axis the % survival at each time point. Survival analysis was performed using the Kaplan Meier estimator and statistical differences were evaluated using the log-rank test, p-values were adjusted for multiple comparisons by Bonferroni method. The mean/median lifespan was instead estimated as the area under the survival curve and extrapolated from the data (i.e., the restricted mean, age in days at % mortality, and the linear interpolation of mortality curve).

3. Results

3.1. Morphological and chemical characterization of MRM callus

In the present study, to test whether a long-term culture could affect the amount of SMs, we characterized the triterpenic acids composition of an extract prepared from a recent (2022) callus sub-culture, following a similar extraction procedure carried out by Verardo et al. (2017). As reported in Table 1, the callus extract composition appeared qualitatively the same, with minor quantitative differences, which was not

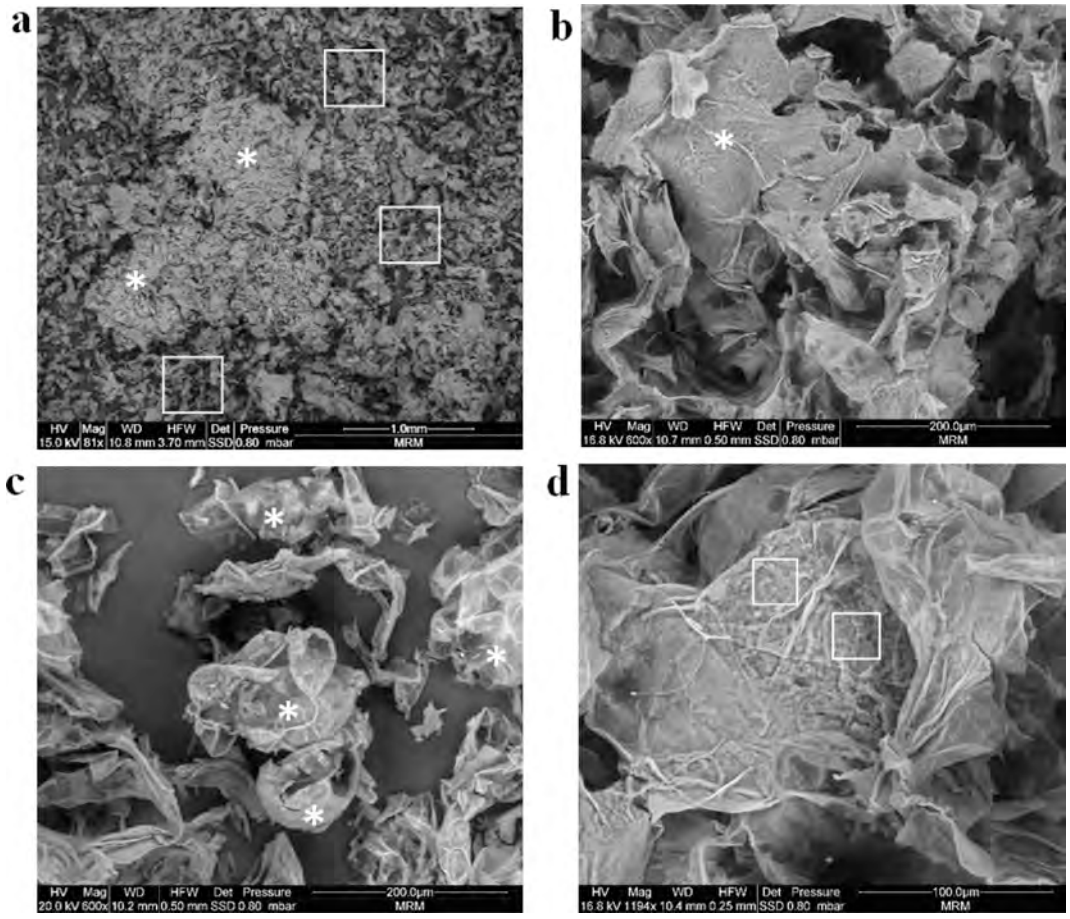

Fig. 1. Environmental scanning electron micrographs (ESEM) showing the morphological analysis of dried Mela Rosa Marchigiana (MRM) callus. a) At low magnification (bar = 1 mm), compact, nodular areas (asterisks) are detectable. This finding is compatible with the remnants after the drying process of meristematic cells. The square areas contain floccular, disaggregated particles, that can be related with the abundant presence of dedifferentiated cells of the callus. b) Fibrotic surface aspect of the dried remnants is easily evident (asterisk) at higher magnification (bar = 200 μm). c) At the same magnification (bar = 200 μm), the dried dedifferentiated cells appear as thin petal shaped particles (asterisks). d) By increasing the magnification (bar = 100 μm), the rough surface of the dehydrated dedifferentiated cells can be detected, and the imprint due to the underlying organelles can be seen as minute surface blebs (square areas).

surprising as the sub-cultures employed to obtain the extracts were not the same. This finding showed that not only the callus can be kept alive for years using a very simple routine of sub-culturing, but also that the production of SMs remains almost constant over time.

Given that ethanol and methanol extraction methods produced equivalent results (Table 1), and that methanol is not recommended in biological tests due to its high toxicity level, all assays were carried out using the 70 % ethanol extract, and the extract antioxidant properties were further investigated.

At ESEM morphological level, the freeze-dried callus samples seem to be constituted by nodular aggregates of particles. These constituents appeared not linked in stable form, showing, around the aggregates, dispersed particles (Fig. 1a, b) with a floccular, fibrotic surface. Dispersed particles (Fig. 1c, d) appeared petal-shaped (100x40 μm long, 50 nm thick). The EDS chemical semi-quantitative analyses revealed the presence of a high amount of K, followed by a significantly small amount of Ca, and, in descending order, Cl, Na, S, P, Mg. The extremely high levels of C and O are most likely from the carbon tape and the low vacuum instrument setting.

3.2. Bioactive compounds content of MRME 2019 and MRME 2022

The bioactive compounds composition of the extracts (MRME 2019 and MRME 2022) was determined by GC–MS and GC-FID analyses. As shown in Table 2, the total amount of triterpenic acids of MRME 2022 was as good as that of MRME 2019.

3.3. Cell free antioxidant activity of the extract is confirmed even after several years

To test the antioxidant ability of MRME 2022, the DPPH and ABTS assays were employed and obtained results were compared to those reported for MRME 2019 (Potenza, Minutelli, Stocchi, & Fraternale, 2020). As shown in Table 3, the antioxidant activity of MRME 2022 was as good as that of MRME 2019.

The Oxygen Radical Absorbance Capacity (ORAC) assay was used to determine the total antioxidant capacity of MRME 2022 which resulted in a mean value of 54.22 ± 1.5 μmol Trolox Equivalent/g of MRM callus extract.

3.4. Overnight MRME incubation led to in vitro antioxidant effects even at low concentrations

HaCaT cells were treated overnight (ON) with different MRME 2022 concentrations (0.36–71 μg/mL) to select non-cytotoxic extract concentrations to be used in the experiments. Cell viability was determined by the WST-8 assay. None of the tested concentrations resulted cytotoxic (Fig. 2a). Previous studies (Potenza,Minutelli, Stocchi, &Fraternale, 2020) carried out in the same cell type (HaCaT) showed that, after a 2 h preincubation with MRME 2019, followed by an oxidative stress (100 μM H_2O_2) for 30 min and 1 h, the extract significantly attenuated the ROS production when supplied at the concentrations of 0.5 and 1 mg/mL. To test whether lower concentrations of the extract could show antioxidant activity, a 2 h-incubation with different concentrations (2.24 to 71.5 μg/mL) of the extract followed by the introduction of H_2O_2 (100 μM for 30 min or 1 h) was tested. No significant results were obtained (data not shown). Then, we extended the pre-treatment time to ON using the same experimental conditions (i.e., same concentrations as above, followed by the same stimulus). The tests showed that when treating cells ON, the extract exerted a significative antioxidant activity, revealed by a reduced ROS amount with respect to the H_2O_2 experimental group, in all the concentration range tested, even after half an hour (Fig. 2b and 2c).

Table 2
Triterpenic acids and β-Sitosterol content (μg/mg of dried extract ± standard deviation, and percentage) found in MRME 2019 and MRME 2022, identified by GC–MS and quantified by GC-FID.

Compound	MRME 2019[a]		MRME 2022[b]	
	μg/mg	%	μg/mg	%
β-Sitosterol	2.29 ± 0.01	6.2	2.24 ± 0.02	5.2
Oleanolic acid	0.90 ± 0.01	2.4	0.96 ± 0.02	2.2
Ursolic acid	4.06 ± 0.08	11.0	4.58 ± 0.04	10.6
Maslinic acid	4.86 ± 0.06	13.2	5.25 ± 0.05	12.2
Corosolic acid	3.56 ± 0.04	9.7	5.10 ± 0.03	11.8
Pomolic acid	0.43 ± 0.02	1.2	0.47 ± 0.01	1.1
Annurcoic acid	8.40 ± 0.10	22.8	7.13 ± 0.04	16.6
Tormentic acid	12.29 ± 0.22	33.4	17.32 ± 0.08	40.2
Total	36.78 ± 0.33		43.05 ± 0.02	

[a] Ethanolic extract obtained as described by Potenza et al. (2020).
[b] Ethanolic extract obtained as described in the present study (see section 2.4).

Table 3
Antioxidant scavenging activity of MRME 2019 (Potenza, Minutelli, Stocchi, & Fraternale, 2020) and MRME 2022 (present study). DPPH and ABTS are reported as mg of dried weight (dw) of callus ethanolic extract/mL with 50% of scavenging ability (IC50).

	MRME 2019 IC50 (mg dw/mL) ± SD	MRME 2022 IC50 (mg dw/mL) ± SD
DPPH	8.43 ± 0.42	5.21 ± 0.04
ABTS	0.90 ± 0.06	0.75 ± 0.02

3.5. MRME reduces H_2O_2-induced cell death in hippocampal slice cultures of neonatal rats

First experiments were aimed at determining the effect of H_2O_2 on cell viability in organotypic hippocampal slice cultures. To this end, hippocampal slice cultures were treated with 200 μM or 400 μM H_2O_2 for 2 h. The PI uptake evaluation showed that H_2O_2 induced cell death in a concentration-dependent manner with a maximal effect after 400 μM H_2O_2 treatment (Fig. 3a). Thus, in the following experiments, PI uptake induced by 400 μM H_2O_2 was considered as 100 % cell death. As shown in Fig. 3, in control hippocampal slices, we found a low, but detectable, PI uptake (8.2 ± 0.7 %; Fig. 3b and 3c), that was not modulated neither by the extract nor by vehicle (70 % EtOH, 11.7 ± 1.8 % and 12.9 ± 0.9 %, respectively; Fig. 3b and 3c). After MRME treatment, no significant difference in PI uptake was observed in slices treated with the extract at the dose of 1 μg/mL compared to H_2O_2-treated slice cultures (91.7 ± 6.9 %; Fig. 3c). Instead, cell death was significantly reduced to 80.0 ± 4.6 % and to 67.2 ± 4.8 % after 10 μg/mL and 100 μg/mL MRME treatment, respectively (Fig. 3c), indicating that the extract reduced H_2O_2-induced PI uptake in a concentration-dependent manner.

3.6. MRME protects against oxidative stress in vivo in C. elegans

To assess the antioxidant property of MRME *in vivo*, a model organism has been considered, the nematode *Caenorhabditis elegans*, widely used to test compounds effects on different health related parameters. Specifically, the ability of the apple extract to protect against lethality induced by oxidative stress has been tested. The animals' survival was first monitored upon treatment with the pro-oxidant juglone (200 μM). The animals fed from embryos to adults (3 days old = 1st day adult) with 400 μg/mL of apple extract supplemented in the food, were slightly but consistently more resistant than animals fed with the vehicle alone (mean survival. CTRL = 5.21 h ± 0.1 h vs MRME = 5.44 h ± 0.11) (Fig. 4a). Longer exposure (>6h treatment) with juglone 200 μM was not further effective since juglone loses its activity when exposed to the air, while higher concentrations were toxic.

To address whether MRME confers selective protection against juglone or a more general protection against oxidative stress, animals' survival upon treatment with the glutathione-conjugating agent

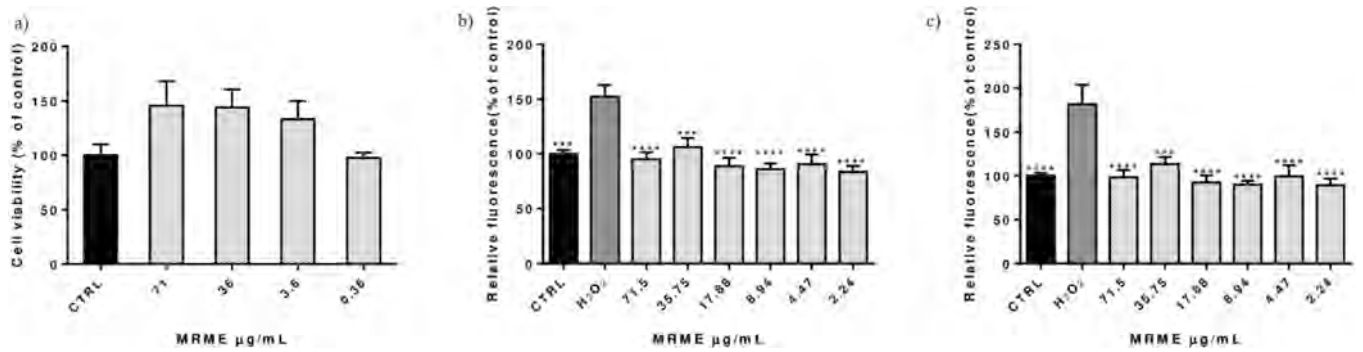

Fig. 2. Cell viability and MRME antioxidant activity. a) Cell viability after MRME 2022 treatment of HaCaT cells for 24 h evaluated by the WST-8 assay. b) and c) MRME 2022 effect on ROS level. Intracellular ROS were evaluated using the DCFH-DA assay in HaCaT cells treated with different concentrations of MRME. ROS production was detected after overnight incubation with 0 (=CTRL), 71.5; 35.75; 17.88; 8.94; 4.47 and 2.24 µg/mL MRME and (b) further introduction of 100 µM H_2O_2 for 30 min or (c) of 100 µM H_2O_2 for 1 h. Results are presented in comparison to H_2O_2. Data represent the mean ± SEM of at least three independent measurements (***p < 0.001; **** p < 0.0001; Dunnett's post hoc test).

dimethyl maleate (DEM) were tested. Consistent with a more general antioxidant effect, feeding with 400 µg/mL of apple extract from embryos for 5 days significantly protected against DEM [15 mM] (mean survival, CTRL = 26.43 h ± 0.96 vs MRME = 29.48 h ± 0.99) (Fig. 4b).

The relatively high concentration of the extract (400 µg/mL, higher than concentrations employed in *in vitro* and *ex vivo* assays) was chosen to overcome uptake problems. *C. elegans* has an impermeable cuticle that forms a strong barrier for the absorption of many drugs.

4. Discussion

The ability of plant cell, tissue, and organ cultures to produce and store many valuable compounds with bioactive effects has been long recognized, but - in the last decades - it has been highly exploited considering undifferentiated callus/cell cultures as potential phytochemical factories (Le et al., 2023). In fact, phytochemicals located in specialized parts of plants (for example, roots, leaves, or fruits) can be obtained in greater amounts in *in vitro* systems. Callus cultures are widely employed (Zaman et al., 2022), but to our knowledge, this is the first study monitoring the same callus culture to test its SMs production ability over time. In addition, for the first time, a morphological and semiquantitative analysis of the callus has been provided.

The study was aimed to (i) test the possibility of a long-lasting bioactive compounds production from *Malus* x *domestica* Borkh var. Mela Rosa Marchigiana (MRM) pulp callus culture; and (ii) investigate the callus ethanolic extract (MRME) antioxidant activity over time. To this end, the MRM fruit pulp callus culture obtained in 2017 and kept alive by monthly sub-cultures was investigated. The results confirmed that the quantity of phytocompounds produced by the callus is much higher than that detected in the fresh fruit, and that the sub-cultures of the same callus continue to produce high amounts of triterpenic acids over time (Table 1).

All experiments were performed using the ethanol callus extract obtained as in (Potenza, Minutelli, Stocchi, & Fraternale, 2020), with slight modifications. To distinguish between the two extracts, we reported them as MRME 2019 (Potenza, Minutelli, Stocchi, & Fraternale, 2020) and MRME 2022 (present study). As in that paper the extract composition was not reported, the MRME 2019 characterization was also performed. As shown in Table 2, the quali-quantitative composition of both extracts was similar. After analysing the composition of MRME 2022 and comparing it with that of MRME 2019, some experiments described in Potenza and colleagues (2020) were intentionally repeated, including cell free and *in vitro* assays, to test the activity of the extract. DPPH and ABTS results confirmed that the antioxidant activity of the extract was maintained (Table 3). When performing the *in vitro* tests, the same experimental conditions as in Potenza et al. (2020) with slight modifications were used. The findings showed that when pre-treating cells with the extract for a longer incubation time (overnight vs 2 h), it was possible to observe a significant antioxidant activity of the extract even at very low concentrations (2.24 µg/mL vs 0.5 mg/mL) after a short time (30 min). Notably, MRME 2022 seemed to be more efficient than MRME 2019. Why so it is hard to say, but this finding could be attributed (at least in part) to a higher amount of tormentic acid (40.2 % vs 33.4 %, Table 2) observed in MRME 2022. We speculate that, considering the broad spectrum of biological activities of this compound (Olech, Ziemichód, & Nowacka-Jechalke, 2021) and its percentage value (>30 %), it is likely to be one of the mayor bioactive ingredients of the extract. We also suspect that the (slightly modified) extraction method employed for MRME 2022 could have played a role in obtaining a more performing product. However, both hypotheses need to be further investigated.

Besides the tormentic acid, the other triterpenic acids of the extract have already been reported as showing a strong anti-inflammatory and anti-tumoral activity and a mild antioxidant ability (Castellano, Ramos-Romero, & Perona, 2022; He et al., 2022; Mlala, Oyedeji, Gondwe, & Oyedeji, 2019; Wei et al., 2022; Zhao, Zhou, An, Shen, & Yu, 2020). Interestingly, when testing the biological effect of the extract, we documented for the first time a good significant antioxidant effect exerted by a mix of triterpenic acids. In our opinion, such a finding is likely attributable to both the synergic action of the ingredients and their high amounts produced by the callus. Please note that, as in previous studies on *P. spinosa* fruit ethanol extract (Coppari et al., 2021), we deliberately did not focus on the single ingredients. All experiments were intentionally carried out aiming at investigating the bioactivity of the whole extract as antioxidant, considering the synergic effect of the compounds, rather than testing the effect (or the contribute) of each component one by one.

The *ex vivo* and *in vivo* models were also employed to investigate the MRME antioxidant effect. Both models, including organotypic hippocampal slice cultures (OHSC) and the nematode *C. elegans*, showed a significative antioxidant effect of the extract at relatively low concentrations (100 µg/mL and 400 µg/mL, respectively), in line with what observed in *in vitro* assays. More specifically, OHSC are a very interesting model suitable for addressing different research questions including studies on neuroprotection (Grabiec, Hohmann, Hammer, & Dehghani, 2017), as in slice cultures the morphological and functional characteristics of both neurons and glial cells are well conserved. Moreover, slices allow the monitoring of time dependent processes, which enabled to investigate the MRME beneficial effect (rescue) after H_2O_2 treatment. *Caenorhabditis elegans* is a powerful model organism largely employed for biological studies to test compounds effects on different health related parameters that may often affect worm longevity. The observed *in vivo* beneficial effects of MRME triterpenic compounds may lead to further studies aiming at addressing the impact on other important stress resistance features and on the molecular mechanisms underlying its

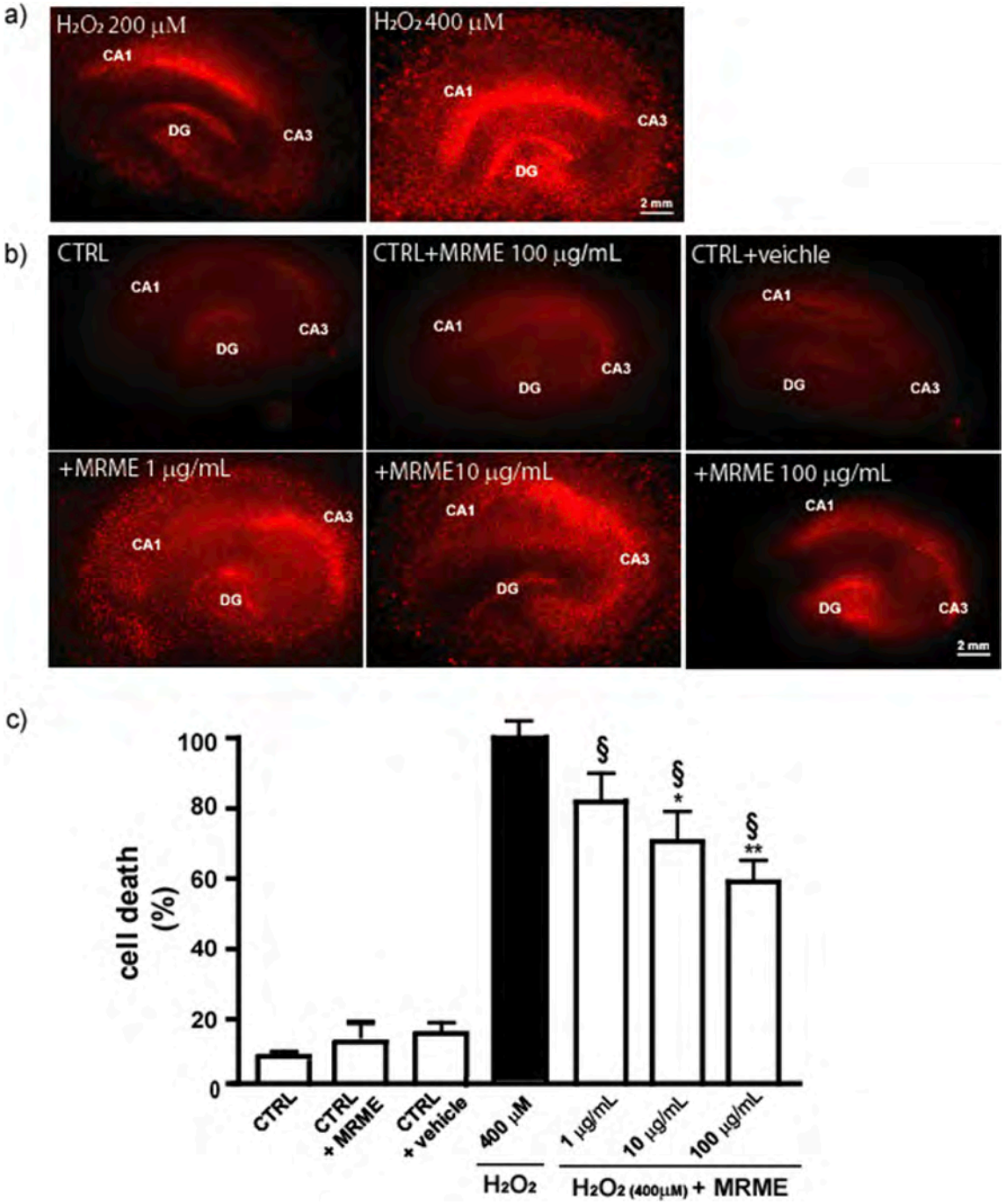

Fig.3. Cell death in hippocampal slice cultures after H_2O_2 and MRME 2022 treatments. a) Representative fluorescence images of cell death using Propidium Iodide (PI) staining in hippocampal cultures treated with H_2O_2 (200 µM or 400 µM) for 2 h to identify the maximal cell death to assign the 100 % value of our experimental condition. b) Representative fluorescence images and quantitative evaluation c) of PI uptake in hippocampal cultures treated with H_2O_2(400 µM) for 2 h and then with 1, 10 or 100 µg/mL MRME for 24 h. Control cultures (CTRL) were treated with 100 µg/mL MRME (CTRL + MRME) or 70 % EtOH (CTRL + vehicle). Normalized fluorescence intensity values are presented as percentage of maximum (i.e., the fluorescence measured in cultures in which the maximal damage was observed, i.e., when using H_2O_2 400 µM). Dentate gyrus (DG) and *Cornu Ammonis* sub-regions CA1 and CA3 represent the three main areas of the hippocampus damaged by H_2O_2 and subjected to quantitative analysis. § $p < 0.001$ vs CTRL; * $p < 0.05$, ** $p < 0.01$ vs H_2O_2 400 µM, One-way ANOVA followed by Dunnett and Newman-Keuls multiple comparison tests (n = 15 slices/experimental condition).

beneficial effects.

5. Conclusions

MRM callus cultures produce triterpenic acids in higher amount than fresh fruit pulp, and callus sub-cultures continue to produce high amounts of triterpenic acids over time. Moreover, phytochemicals bioactivity remains almost unchanged even after years, as demonstrated by *in vitro*, *ex vivo* and *in vivo* experiments. Notably, *in vivo* experiments seem to suggest an interesting antioxidant activity of the extract that confers protection against oxidative stress, regardless of the type of stimulus used. Indeed, in *C. elegans*, stress resistance assays seem to suggest a general protective effect of MRME against different pro-oxidants (H_2O_2, Juglone and DEM), which makes Mela Rosa

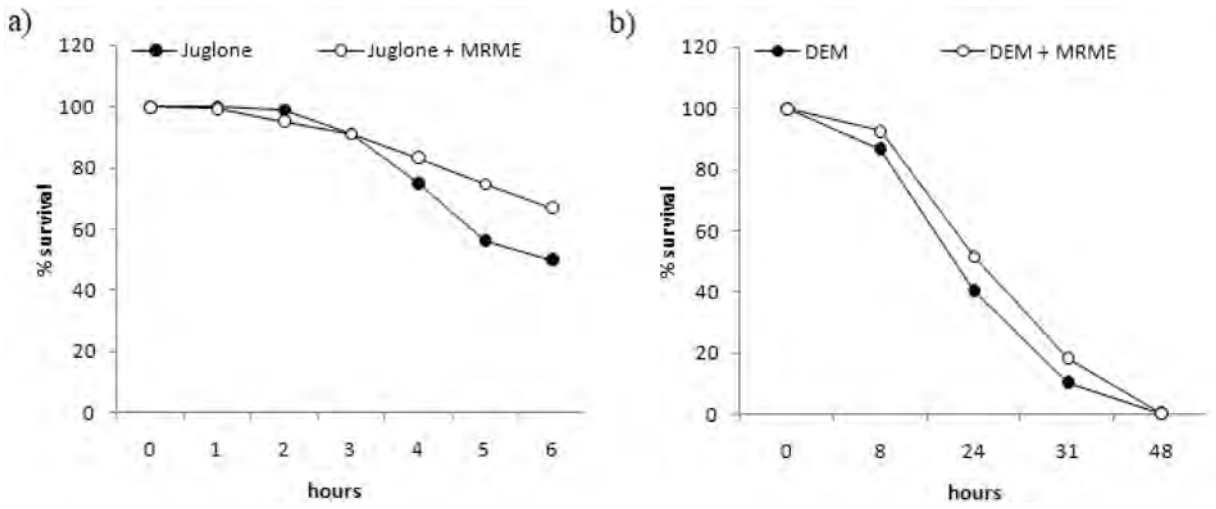

Fig. 4. MRME 2022 significantly increases animalś resistance to oxidative stress. (a) MRME increases resistance to juglone-induced lethality. (b) MRME increases resistance to DEM-induced lethality. Survival curves show combined data from three independent replicas carried out with 50 animals each. Bonferroni *p* value 0.026.

Marchigiana ethanolic extract and pulp callus cultures worthy of attention for further detailed studies. For example, considering also the results obtained in hippocampal slice cultures of neonatal rats showing that the extract could significatively limit H_2O_2-induced cell death, an interesting development of the present research could be to evaluate whether the MRM pulp callus extract and/or the active compounds within it can be proposed as possible ingredients of a nutraceutical formulation for healthy aging or for the prevention of oxidative stress-associated pathologies such as, among others, Alzheimer's, and Parkinson's diseases.

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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. The views and opinions expressedin this manuscript do not represent those of EFSA.

Data availability

No data was used for the research described in the article.

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