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The abietane diterpene taxodione contributes to the antioxidant activity of rosemary by-product in muscle tissue

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ABSTRACT

Research on rosemary antioxidant activity and its potential use in human health and food applications is focused on rosemary leaves and two main bioactive compounds carnosic acid and carnosol. However, many other, not-yet identified molecules could be present, especially in rosemary by-products. In this study, we first showed that rosemary stem extract was the most efficient in protecting human skeletal muscle cells against oxidation. Then, using bioassay-guided fractionation, we identified taxodione, an abietane diterpene, as the main bioactive molecule in the rosemary stem extract. We demonstrated that taxodione protects skeletal muscle cells from hydrogen peroxide-induced cytotoxic damage (by monitoring ROS production, H2AX phosphorylation and *CHOP* gene expression). Moreover, we showed that taxodione reduces lipid and protein oxidation in post-mortem mice and beef muscles during refrigerated storage. In conclusion, our results indicate that taxodione extracted from rosemary stems, a cheap and unused resource of natural antioxidants, limits oxidation in muscle tissue.

1. Introduction

Oxidative processes cause damage to biomolecules and are associated with muscle wasting diseases in humans, and undesirable changes in food systems (Canton, Menazza, & Di Lisa, 2014; Choi, Ow, Yang, & Taneja, 2016; Papuc, Goran, Predescu, & Nicorescu, 2017). In human and animal diseases, accumulation of pro-oxidant molecules derived from reactive oxygen species (ROS) can affect the balance between protein synthesis and degradation, induces muscle fatigue, cell death and skeletal muscle repair dysfunction, resulting in extensive muscle loss over time (Canton et al., 2014; Choi et al., 2016).

Antioxidant compounds can be used to prevent or delay these oxidative processes. However, evidences on their efficacy are very limited (Passerieux et al., 2015), and the antioxidant capacity to delay, prevent, or reverse loss of muscle mass is unclear (Steinhubl, 2008). During animal meat oxidation, changes in a large number of compounds, such as lipid peroxidation and discoloration (myoglobin oxidation), adversely affect meat products and limit their shelf life (Papuc et al., 2017). Synthetic antioxidants, e.g., butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been added to meat and meat products with success, but their use has been discouraged because of their toxic effects and recent consumer interest in natural products.

Abbreviations: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CA, carnosic acid; CO, carnosol; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); H₂O₂, hydrogen peroxide; LDH, lactate dehydrogenase; LOD, limit of detection; LOQ, limit of quantification; qPCR, quantitative polymerase chain reaction; ROS, Reactive oxygen species; RS, rosemary stems extract; RL, rosemary leaves extract; RW, rosemary whole extract; SD, standard deviation; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TNB, thionitrobenzoic acid; TPE, 1,1,3,3-tetraethoxypropane; TX, taxodione

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Therefore, the meat industry is promoting research to identify new inexpensive and effective natural antioxidants (Shah, Bosco, & Mir, 2014). In conclusion, it is of great interest to identify effective and safe antioxidant molecules for human health and food application.

Plants are an important source of bioactive molecules (Newman & Cragg, 2012). Rosemary (*Rosmarinus officinalis* L., *Lamiaceae*) leaf extracts contain many different phenolic compounds, including flavonoids and phenolic diterpenes and triterpenes (Borras-Linares et al., 2014), with many major biological properties (antidiabetic, anti-inflammatory, antioxidant and anticancer) (Altinier et al., 2007; Bakirel, Bakirel, Keles, Ulgen, & Yardibi, 2008; Lo, Liang, Lin-Shiau, Ho, & Lin, 2002; Perez-Fons, Garzon, & Micol, 2010). The antioxidant activities of rosemary leaf extracts can mainly be attributed to phenolic diterpenes carnosic acid (CA) and carnosol (CO), and to a lesser extent to other phenolic compounds, such as rosmarinic acid (Birtic, Dussort, Pierre, Bily, & Roller, 2015; Srancikova, Horvathova, & Kozics, 2013). *Rosmarinus officinalis* extracts have been added as preservatives in processed meat, in fish oil enriched milk, to replace chemical antioxidants and protect from oxidation (Qiu, Jacobsen, & Sorensen, 2018; Shah et al., 2014; Xiong, 2017). Moreover, rosemary-based diets and its active molecules, essentially carnosic acid, can enhance the antioxidant status of animal skeletal muscle (Ortuno, Serrano, Jordan, & Banon, 2016). In 2008, Rosemary leaf extracts have been approved for use in the European Union as food additive E932 under the Regulation 1333/2008 of the European Parliament and Council.

The aim of this study was to identify bioactive molecules from rosemary by-products, by bioassay-guided fractionation approach, to prevent the deleterious effects of oxidation in muscle cells. Firstly, to screen antioxidant properties of putative bioactive molecules, we used a cellular model of H₂O₂-treated human skeletal muscle cells. In a second step, the identified biomolecule was tested on post-mortem muscles to point out its potential application in protecting meat products against oxidation.

2. Materials and methods

2.1. General experimental procedure

Flash column chromatography was performed using a Spot Liquid Chromatography Flash instrument (Armen Instrument, Saint-Avé, France) equipped with an UV/visible spectrophotometer, a quaternary pump and a fraction collector. ¹H NMR, ¹³C NMR and 2D NMR spectra were recorded in the appropriate deuterated solvent on a BRUKER Avance III – 600 MHz NMR spectrometer. HR-ESI-MS was recorded on a Synapt G2-S (Waters) HDMS-Q-TOF mass spectrometer.

2.2. Reagent and standards

DPPH radical (97%), cyclohexane (99.8%), chloroform (99%), dichloromethane (99.9%), deuterated chloroform (99.8%), DMSO (99.9%) and Tempol were purchased from Sigma-Aldrich (Steinheim, Germany). Acetonitrile (99.9%) was purchased from Chromasolv (Seelze, Germany). Formic acid (98%), ethyl acetate (99%) and acetone (99.5%) were from Panreac (Barcelona, Spain). Trolox (98%) was purchased from Fluka Chemicals (Steinheim, Switzerland), and ethanol (99.9%) from VWR BDH Prolabo (Pennsylvania, USA). Carnosic acid (99%) and carnosol (99%) were purchased from Phytolab (Germany).

2.3. Plant material

Rosmarinus officinalis was collected in the North of Montpellier (France) in February 2015. Dry stems and leaves were ground and directly extracted. Voucher specimens were deposited as n° 055150 RL and RS.

2.4. Extraction

150 g of ground rosemary stems were macerated in the dark at room temperature with 900 g of absolute ethanol and 450 g of distilled water, with agitation every 24 h. After 7 days, the stem extract was filtered. Evaporation under reduced pressure to dryness yielded 12.2 g of hydro-ethanolic extract, named RS (Rosemary Stems). The same procedure was used for 150 g of ground leaves and allowed obtaining 69 g of hydro-ethanolic extract, named RL (Rosemary Leaves). The same procedure was used for 150 g of ground leaves and stems, named RW (Rosemary Whole). The dry extracts were kept at –20 °C until analysis and purification.

2.5. Bioassay-guided isolation of taxodione from the rosemary stem extract

At each purification step, fractions were tested using the assay described below (part 2.9). The RS extract (12.2 g) was partitioned in CH₂Cl₂ soluble fraction and aqueous fraction. After evaporation under reduced pressure to dryness, these two fractions yielded 4.41 g of CH₂Cl₂ soluble extract and 7.79 g of aqueous soluble extract. The CH₂Cl₂ soluble extract was separated on normal-phase flash column chromatography (Merck Chimie SVF D26-SI60, 15–40 µm-30 g, flow rate 6.5 mL/min, 25 mL/fraction). Elution was completed with mixtures of cyclohexane:ethyl acetate (100:0 to 0:100), and then chloroform:methanol (100:0 to 80:20 in 1% then 5% stepwise). After thin-layer chromatography (TLC) analysis, the first fractions eluted with 100% cyclohexane (fractions 1–69) were combined and concentrated under reduced pressure, yielding fraction F1 (370 mg). F1 was purified on LH-20 Sephadex gel (2.4 × 38 cm, 40 g LH-20, elution: 100% CH₂Cl₂ to 100% methanol in 50% stepwise, then 100% acetone, 3 mL/fraction). Fractions 17 to 33 eluted with 100% CH₂Cl₂ were combined and concentrated under reduced pressure, yielding fraction F1-2 (160 mg). F1-2 was finally purified on reverse-phase flash column chromatography (Chromabond® Flash, RS4 C₁₈, 4.3 g, flow rate: 5 mL/min, 25 mL/fraction). Elution was completed with a mixture of acetonitrile/water (50:50 to 100:0) and gave 111 fractions. Fractions 17 to 29 eluted with acetonitrile/water (60:40) were combined (F1-2–3) to give 50 mg of pure compound 1 determined by NMR as taxodione. ¹H NMR (CDCl₃, 600 MHz), ¹³C NMR (CDCl₃, 150 MHz) and HMBC data were shown in Table 1. ¹H and ¹³C spectra were presented in supplementary data (Supplementary Fig. s1 A and B).

2.6. High-performance liquid chromatography (HPLC) analysis

Chromatographic separation and detection for quantitative analysis were performed on a SpectroSYSTEM® instrument that included a P4000 pump, a SCM1000 degasser, an AS3000 automatic sampler and an UV6000LP DAD detector (Thermo Fisher Scientific Inc., San José, USA). The system was operated using the ChromQuest software, version 5.0. Chromatographic separation was achieved on an ODS Hypersyl C₁₈ column (250 mm × 4.6 mm, 5 µm, Thermo Fisher Scientific Inc., San José, USA), with a column temperature maintained at 30 °C. Fractions were eluted at a flow rate of 1 mL/min (initial back pressure of approximately 105 bar), using solvent A (water/formic acid 99.9:0.1 v/v) and solvent B (acetonitrile). The gradient used for the analysis of standards and rosemary extracts was: 0–10 min, 85% A; 10–20 min, 85–65% A; 20–25 min, 65–30% A; 25–30 min, 30% A; 30–50 min, 30–20% A; 50–60 min, 20–10% A; 60–70 min, 10–85%; 70–80 min 85% A. The UV/vis spectra were recorded in the 200–400 nm range and chromatograms were acquired at 230, 280 and 330 nm. RS extract was analysed at 5 mg/mL (acetonitrile/water 60:40), RW and RL extracts were analysed at 20 mg/mL for quantification of TX.

2.7. Quantification of taxodione (TX) by HPLC

Linearity/work range: Standard curves were generated with increasing amounts of TX corresponding to a concentration range of

Table 1

NMR data of Taxodione in CDCl₃ (at 600 MHz for ¹H NMR and 150 MHz for ¹³C NMR).

Position	δH (J in Hz)	δC	HMBC
1α	1.73 dd (2.4, 10.6) ^a	37.1	C-2, C-9
1β	2.92 br d (10.6)		C-2, C-5, C-6 (low), C-10, C-20
2α	1.58 m	18.7	C-3, C-4
2β	1.71 q5t (13.6, 2.4) ^a		C-3
3α	1.20 dd (3.5, 13.0)	42.7	C-4
3β	1.40 q (13.2)		–
4		33.0	
5α	2.58 s	63.1	C-4, C-6, C-9, C-10,
6	–	201.1	
7	6.19 s	134.01	C-5, C-9, C-14
8		140.1	
9		125.8	
10	–	43.0	
11		145.1	
12		181.8	
13		145.45	
14	6.87 s	136.3	C-7, C-8, C-9, C-12, C-15
15	3.06 sept (6.8)	27.3	C-12, C-13, C-14, C-16 ^b and/or C-17 ^b
16	1.17 d (7.0)	21.3 ^b	C-13, C-15, C-16 ^b and/or C-17 ^b
17	1.15 d (7.0)	21.8 ^b	C-13, C-15C-16 ^b and/or C-17 ^b
18	1.10 s	33.4	C-3, C-4, C-5, C-19,
19	1.26 s	22.2	C-1, C-4, C-5, C-9, C-10
20	1.26 s	22.0	
11-OH	7.57 s		

^a Partially overlapped signal.

^b These assignments are interchangeable.

0.029 to 1 mg/mL (n = 3). Peak areas of taxodione were integrated and a calibration curve constructed. In regression analysis, curve fitting was deemed acceptable if the regression coefficient *r* was greater than 0.99.

Limit of detection/Limit of quantification (LOD/LOQ): The LOD was defined as the sample concentration resulting in a response three times higher than the noise level. The LOQ was defined as the sample concentration resulting in a response ten times higher than the noise level.

Taxodione recovery was assessed by sample analysis at three different concentrations (0.05, 0.4 and 0.8 mg/mL). Accuracy was expressed as percent error [(mean of measured)/mean of expected] × 100, while precision was the determined coefficient of variation (CV, in %).

Recovery in extract samples after addition of standard known amounts of taxodione: the RS extract was analysed by HPLC to quantify TX concentration and compared with the same extract spiked with known concentrations of pure TX. Recoveries were determined as [(mean value in the spiked extract – mean value in the blank extract)/(expected concentration) × 100].

2.8. Primary cultures of human myoblasts

The quadriceps muscle biopsy was from one healthy adult (AFM-BTR “Banque de tissus pour la recherche”). Myoblasts were purified from the muscle biopsy and were cultured on collagen-coated dishes in DMEM/F12 medium with 10% foetal bovine serum (FBS), 0.1% Ultraser G and 1 ng/ml of human basic fibroblast growth factor (proliferation medium), as previously described (Kitzmann et al., 2006). For cell differentiation, confluent cells were cultured in DMEM with 4% FBS for 3–5 days (differentiation medium).

2.9. Cell death and ROS quantification

Myoblasts: Myoblasts were seeded in 35 mm collagen-coated dishes, cultured in proliferation medium, pre-incubated or not with the tested compounds for 24 h and then incubated or not with a lethal concentration of hydrogen peroxide (H₂O₂), a strong pro-oxidant/pro-

apoptotic compound, for 24 h. The optimal H₂O₂ concentration was the concentration required to kill between 30% and 50% of total cells and was established before each experiment. In general, myoblasts were incubated with 120 μM H₂O₂. Dead myoblasts were identified by staining with the Muse® Count and Viability Kit, and ROS was quantified with the Muse® Oxidative Stress Kit, followed by analysis with a Fluorescence Activated Cell Sorting (FACS) Muse apparatus (Millipore, France).

Myotubes: Myoblasts were seeded in 35 mm collagen-coated dishes, cultured in proliferation medium until confluence, and then switched to differentiation medium for 4 days. At day 2, cells were incubated with TX for 24 h prior to incubation with H₂O₂ for 24 h. The H₂O₂ concentration used in myotube cultures (550 μM) was higher than that used for myoblasts, suggesting that myotubes are resistant to apoptosis inducers (Salucci et al., 2013). As myotubes were too big for FACS analysis, H₂O₂ effect was determined by quantifying lactate dehydrogenase (LDH) activity, which is increased in the culture medium during tissue damage, using the LDH Cytotoxic Kit (ThermoFisher, France). In parallel, myotube cultures were loaded with a ROS-fluorescent probe (CellRox) followed by fluorescence quantification using a TECAN spectrophotometer.

2.10. RT-qPCR assays

Myoblasts were seeded in 35 mm collagen-coated dishes, cultured in proliferation medium, pre-incubated or not with TX for 24 h, and then incubated or not with a sub-lethal concentration of H₂O₂ (80 μM; to avoid interference with dead cells) for 24 h. Then, total RNA was isolated from muscle cells using the NucleoSpin RNA II Kit (Macherey-Nagel, Hoerd, France). The RNA concentration of each sample was measured with an Eppendorf BioPhotometer. cDNA was prepared using the Verso cDNA Synthesis Kit (Thermo Scientific, Ilkirch, France). The expression of the *CHOP* (target) and *RPLPO* (control) genes was analysed by quantitative polymerase chain reaction (qPCR) on a LightCycler apparatus (Roche Diagnostics, Meylan, France), as previously described (El Haddad et al., 2017), using the following primers:

RPLPO (F-TCATCCAGCAGGTGTTCG; R-AGCAAGTGGGAAGGTG TAA)

CHOP (F-AAGGAAAGTGGCACAGC; R-ATTCACCATTGCGTCAATC AGA).

2.11. Western blotting

Myoblasts were seeded in 35 mm collagen-coated dishes, cultured in proliferation medium, pre-incubated or not with TX for 24 h and then incubated or not with 80 μM H₂O₂ for 24 h. Protein extracts were separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes, blocked at room temperature with Odyssey blocking buffer (Eurobio, France) and probed with the rabbit polyclonal anti-Histone H1.4 (Sigma-Aldrich; 1/5000) and rabbit polyclonal anti-gamma H2AX (Cell signalling; 1/3000) antibodies followed by IRDye® 680RD and IRDye® 800RD secondary antibodies (Eurobio, France). Fluorescence was quantified with the Odyssey software. Data were normalized to Histone H1.4 expression.

2.12. Muscle sampling and preparation

The experimental protocol of the mice study was in strict accordance with the European directives (86/609/CEE) and was approved by the Ethical Committee of the Occitanie Region. Gastrocnemius muscles from six-month-old C57BL/6 male mice were removed and immediately placed on ice. Muscles were then minced with sterile scissors for 5 min and divided in 600 mg batches. Each batch of minced muscle was mixed with different amounts of butylated hydroxytoluene (BHT) (0.010%, 0.005%, 0.0025% w/w minced muscle), carnosic acid (CA) (0.015%, 0.0075%, 0.00375% w/w minced

muscle) or taxodione (TX) (0.015%, 0.0075%, 0.00375% w/w minced muscle) dissolved in ethanol (50 μ L/600 mg). A control batch was mixed only with ethanol (50 μ L/600 mg). Different percentages of the three antioxidants were used to correct for the molecular weight differences. Each batch of minced muscle was divided in four portions (150 mg) using a weighing cup, and individually packaged in polypropylene film bags. Three portions were stored at 4 ± 1 °C in the dark for 7 days. The fourth (0 day) was immediately homogenized in 50 mM phosphate buffer (pH 7.0) (1:9) with an Ultra-Turrax homogenizer. The fraction of homogenate needed for thiobarbituric acid reactive substances (TBARS) measurement was quickly frozen, and the rest of homogenate was centrifuged at 1000 g at 4 °C for 15 min before storage at -20 °C for total thiols measurements. The same procedure was adopted for beef meat (“entrecote”). The pieces of meat came from animals slaughtered 1 week before.

2.13. TBARS measurement

The lipid peroxidation index was determined in muscle homogenates by measuring TBARS (Sunderman, Marzouk, Hopfer, Zaharia, & Reid, 1985). Briefly, muscle homogenates were mixed with 154 mM KCl, phosphoric acid (1% v/v) and 30 mM thiobarbituric acid (TBA). The mixture was boiled at 100 °C for 1 h. After cooling, it was extracted with n-butanol and centrifuged at 1000g at room temperature for 15 min. The fluorescence intensity of the organic phase was measured with a spectrofluorometer (Ex: 515 nm; Em: 553 nm). A standard was prepared from 1,1,3,3-tetraethoxypropane (TEP), and results were expressed as nanomoles of TBARS per gram of tissue and were the mean \pm SD of three experiments.

2.14. Protein oxidation assay or sulfhydryl group measurement

Total thiol quantification (Faure & Lafond, 1995) was based on the reaction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) with the samples that produces thionitrobenzoic acid (TNB), a yellow product that can be quantified spectrophotometrically at 412 nm. Results were expressed as nanomoles of total thiols per milligram of protein and were the mean \pm SD of three experiments. Protein concentrations were determined using the BioRad Protein Assay (BioRad, Hercules, CA, USA) and bovine serum albumin as standard.

2.15. Statistical analysis

Statistical analysis was done with the GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA). All experiments were performed in triplicate. Error bars represent the SD of the mean. Statistical significance was determined using one-way ANOVA; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****) were considered significant.

3. Results and discussion

3.1. Rosemary stem extract has a strong antioxidant activity

To screen the antioxidant activity of rosemary extract, we used an *ex vivo* cell model based on primary human myoblasts (skeletal muscle precursor cells). We previously showed that H_2O_2 , a strong pro-oxidant molecule, increases the percentage of apoptotic cells in adherent cultures of human myoblasts (skeletal muscle precursors) (Jean et al., 2011). We tested the effect of pre-incubating human myoblasts with increasing concentrations of *Rosmarinus officinalis* extract from a mixture of leaves and stems (whole rosemary extract, RW) or Tempol, a powerful synthetic antioxidant, for 24 h prior to incubation with a lethal concentration of H_2O_2 . As expected, Tempol protected human myoblasts efficiently against H_2O_2 -induced cell death. RW also efficiently reduced cell death at all tested concentrations (Fig. 1A). Then,

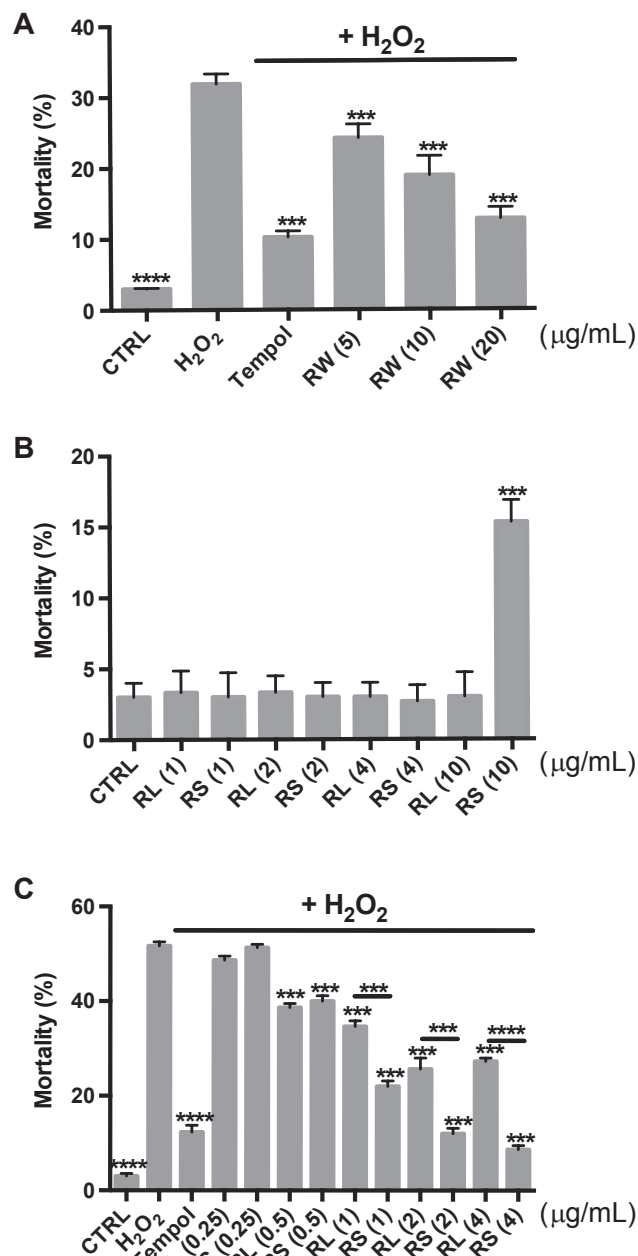


Fig. 1. Rosemary stem extract protects human myoblasts from induced oxidative stress. Cell death quantification (percentage of all cells) in human myoblasts that were incubated with (A) *Rosmarinus officinalis* whole extracts (RW) at indicated concentrations or tempol (50 μ M as control) or with (B, C) different concentrations of *Rosmarinus officinalis* leaf (RL) or stem (RS) extracts prior to incubation with (A, C) 120 μ M H_2O_2 (lethal concentration). CTRL: cells not incubated with H_2O_2 . Cell death was quantified using the Cell Count and Viability Kit and the Muse Cell Analyzer; $p < 0.001$ (***) and $p < 0.0001$ (****) compared with H_2O_2 (A, C) and CTRL (B) (one-way ANOVA).

we prepared *Rosmarinus officinalis* leaf (RL) or stem (RS) extracts and determined their toxicity by incubating myoblasts with increasing concentrations of these extracts for 24 h. Only the concentration of 10 μ g/mL RS extract was significantly cytotoxic (Fig. 1B). Therefore, we chose to incubate myoblasts with increasing concentrations of RL or RS extracts below 10 μ g/mL for 24 h before addition of H_2O_2 and cell death quantification. RS was the most efficient in protecting myoblasts against H_2O_2 -induced cell death at 1, 2 and 4 μ g/mL (Fig. 1C). This result was quite surprising because the two main rosemary antioxidants

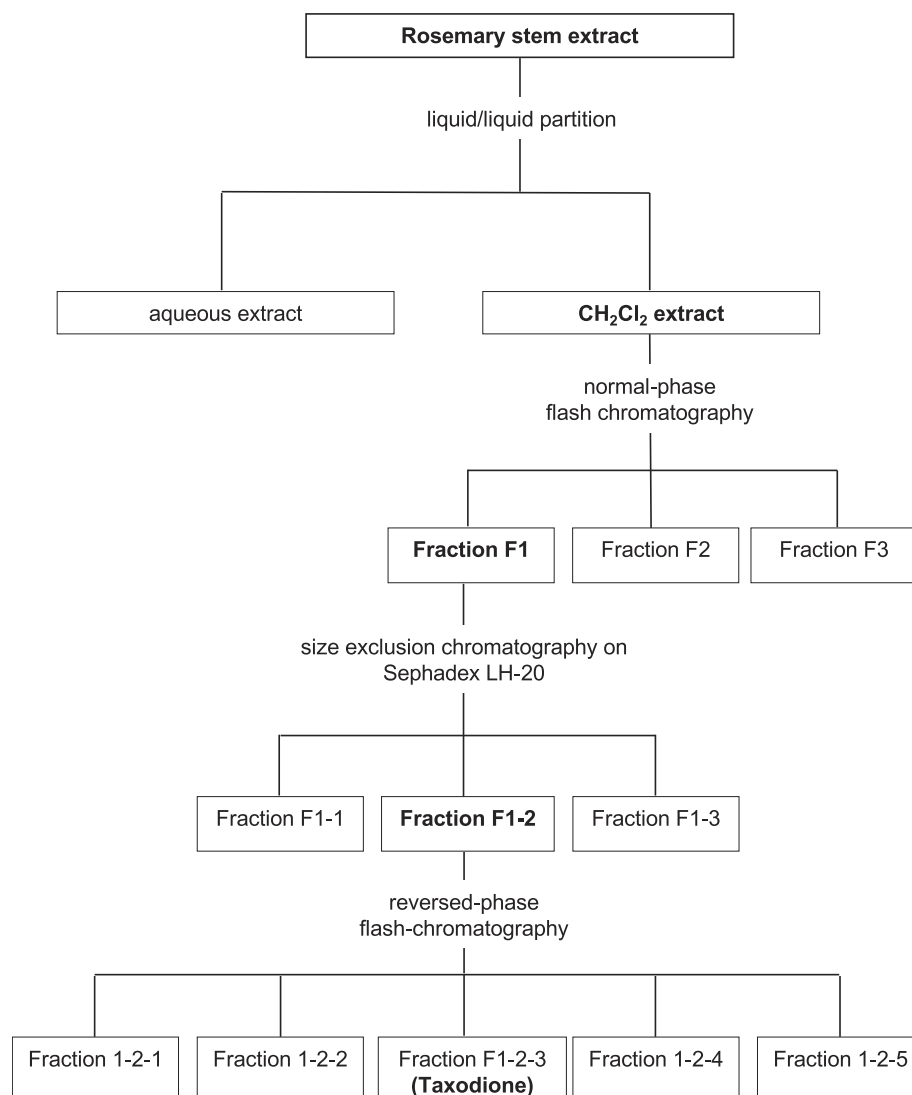


Fig. 2. Different steps of taxodione purification from rosemary stem extract.

carosic acid (CA) and carnosol (CO) are mainly extracted from leaves and are present at very low levels in the woody parts of the plant, such as stems (del Bano et al., 2003). This suggested that other molecule(s) might contribute to RS antioxidant activity.

3.2. Bioassay-guided isolation of the antioxidant compound from the RS extract

To isolate the compound(s) responsible for the antioxidant activity of the RS extract, we used a bioassay-guided fractionation approach. At each purification step, we evaluated the ability of the obtained fractions to protect myoblasts against H₂O₂-induced cell death. The RS extract (12.2 g) was partitioned between water and CH₂Cl₂ (Fig. 2). The CH₂Cl₂ soluble fraction (4.41 g) was responsible for RS antioxidant activity (data not shown). Therefore, we further purified this CH₂Cl₂ fraction on normal-phase flash chromatography (elution with cyclohexane/ethyl acetate then chloroform/methanol) to obtain three fractions. Only the first fraction (370 mg) was active and was chromatographed on Sephadex LH-20 gel eluted with CH₂Cl₂ then methanol yielding three fractions. The second fraction (160 mg) was finally purified on C18 flash-chromatography eluted with acetonitrile/water (see Fig. 2) to obtain 50 mg of pure compound. The compound was obtained as an orange powder, and its molecular formula was deduced as C₂₀H₂₆O₃ from the analysis of its NMR and HRMS data (*m/z* 315.1959 [M+H]⁺,

calculated for C₂₀H₂₇O₃ 315.1960). Analyses of ¹H-, ¹³C- and 2D-NMR experiments (Table 1) identified this compound as taxodione (TX) (Rodríguez, 2003; Tezuka et al., 1998), first isolated from the leaves of *Taxodium distichum* (Kupchan, Karim, & Marcks, 1968). We obtained a purification yield of 0.33 mg of taxodione (TX)/g of dry stems or 4.1 mg/g dry extract with purity of 94.8% determined in ¹H NMR spectrum.

3.3. Quantification of taxodione in rosemary extracts

To quantify taxodione (TX) in rosemary stem (RS), rosemary leaf (RL) and rosemary leaves and stems extracts (RW), an HPLC-based method was developed and validated. All analyses were performed at 330 nm. The method selectivity was assessed by analysis of the RS, RL and RW chromatograms. No peak of interfering compounds was observed within the intervals of TX retention time (*t_R* = 43.1 min). The specificity of the method has been attested by the use of DAD detector and confirmation of UV spectra of all detected peaks (UV spectrum of TX was characteristic with λ_{max} = 322 and 333 nm). Furthermore, no degradation interfering compound was observed after 24 h in solution. The samples were analysed in the day following their preparation to avoid degradation. Linearity was evaluated from the calibration curves by triplicate analysis of TX at five concentrations (0.029, 0.125, 0.250, 0.5 and 1 mg/mL). Linearity was expressed as the coefficient of linear

correlation (r) from the slope of the calibration curve. The linearity of the analytical response across the studied range (0.029–1 mg/mL) was excellent, with a correlation coefficient of 0.9985. The signal to noise (S/N) ratio was evaluated for the time window in which taxodione was expected, by injecting ten blank samples. The LOD was evaluated to be three times higher than the S/N ratio, corresponding to 8 µg/mL. The standard deviation (SD) of 10 known low concentrations of taxodione ($6 \times$ estimated LOD, 50 µg/mL) was calculated. Then, the limit of detection (LOD) value was calculated as $3 \times$ SD, and the limit of quantification (LOQ) as $10 \times$ SD. At 330 nm, the LOD for taxodione was 8.73 µg/mL and the LOQ was 29.1 µg/mL. Accuracy and precision were assessed by sample analysis of taxodione at three different concentrations (0.05, 0.4 and 0.8 mg/mL, $n = 5$). The TX recovery was in the acceptable range: 85.6–101.6% (Table 2). Rosemary extract samples (5 mg/mL) were spiked with known amount of taxodione (0.4 mg/mL) to evaluate the matrix effect. Recovery, calculated in 15 extract samples, was in the accepted range: 90.4–99.9% (mean: 96.4%).

This method indicated that in the RS extract, TX concentration was 11.7 mg/g dry extract. Quantification by HPLC suggested that TX concentration in the RS extract was higher than what suggested by the purification yield (4.1 mg/g dry extract), implying that the conditions of extraction and purification could be improved. The analysis of RL and RW extracts shows the absence of TX in these extracts ($<$ LOD).

The abietane terpene taxodione (TX) was previously isolated from *Rosmarinus officinalis* roots with a purification yield of 0.14 mg/g of dry roots (Abou-Donia, Assaad, Ghazy, Tempesta, & Sanson, 1989) and isolated in stems (purification unspecified) (El-Lakany, 2004). Taxodione was also detected in mixture with [9]-shogaol in leaves (Borras-Linares et al., 2015). However, no precise information is giving regarding to content of TX in the crude extract obtained. Indeed, TX was only detected in an enriched fraction. In addition, TX was undetectable in our RL extracts ($<$ LOD).

TX was also described in different plants: *Taxodium distichum*, *Taxodium ascendens*, *Cupressus sempervirens*, *Volkameria eriophylla* (synonym: *Clerodendrum eriophyllum*), *Plectranthus barbatus*, *Premna obtusifolia*, and several *Salvia* sp. Most of these studies reported TX enrichments lower than the 0.33 mg/g dry stem isolated in the RS extract. Few studies have focused on obtaining large TX quantities: from *Taxodium distichum* seeds and cones (3–3.4 mg/g of dry matter) (Hirasawa et al., 2007; Kupchan et al., 1968), from *Salvia phlomoides* roots (3.72 mg/g of dry roots) (Hueso-Rodríguez, Jimeno, Rodríguez, Savona, & Bruno, 1983) and from transformed *Salvia austriaca* hairy roots (0.43 mg/g of dry roots and 1.15 mg/g) (Kuzma, Kaiser, & Wysokinska, 2017; Kuzma et al., 2016). However, the seeds of *Taxodium distichum* or the roots of *Salvia* sp. are not easily exploitable plant by-products on an industrial scale.

3.4. Taxodione protects human myoblasts and myotubes against H₂O₂ induced stress

Before assessing TX antioxidant activity, we tested its toxicity by incubating myoblasts with increasing concentrations of TX for 24 h. We quantified dead cells (Fig. 3A) and accumulation of ROS (Fig. 3B), highly reactive molecules associated with apoptotic cells. Based on both parameters, TX was significantly toxic starting from 1 µg/mL (Fig. 3A and B). Therefore, to avoid cytotoxicity, we chose to incubate myoblasts with 0.125 µg/mL, 0.250 µg/mL and 0.5 µg/mL of TX for 24 h before H₂O₂ addition. All three concentrations had similar and strong

Table 2
Taxodione recovery measurements at 330 nm.

Level	Observed concentration (CV in %)	Range of recovery (%)
0.8 mg/mL	0.72 (7.3)	85.6–101.6
0.4 mg/mL	0.39 (1.4)	94.6–98.2
0.05 mg/mL	0.049 (3.1)	89.8–93.0

protective effect against H₂O₂-induced cell death (Fig. 3C). We then compared TX antioxidant activity with that of the main bioactive compounds of rosemary: carnosic acid (CA) andarnosol (CO) (Fig. 3D). TX was significantly more efficient at all tested concentrations. The antioxidant activity of a molecule could be the result of free radical scavenging. Indeed, Kolak et al. showed an important antioxidant capacity of TX derived from *Salvia barrelieri* using different assays (β -carotene bleaching, DPPH and ABTS free radical scavenging activity, and cupric reducing antioxidant capacity) (Kolak et al., 2009).

In many cases, transient exposure to H₂O₂ triggers apoptosis through the mitochondrial pathway involving, sequential loss of mitochondrial membrane potential, ROS accumulation, endoplasmic reticulum (ER) stress, cytochrome C release, effector caspase-3 activation, and DNA damages (Redza-Dutordoir & Averill-Bates, 2016)). Therefore, we assessed TX capacity to efficiently protect myoblasts against H₂O₂-induced damages. To measure H₂O₂-induced cellular damages, we quantified the level of ROS (Fig. 4A), of γ H2AX, a protein phosphorylated upon DNA double-strand break formation (Fig. 4B), and of the *CHOP* gene, a marker of endoplasmic reticulum stress (Fig. 4C). After pre-incubation with TX for 24 h and exposure to H₂O₂ for 24 h, as expected, H₂O₂ treatment increased the levels of ROS, γ H2AX proteins and *CHOP* mRNA. Pre-treatment with TX reduced H₂O₂ effects, whereas TX alone did not have any effect. During muscle cell differentiation, myoblasts, the progeny of satellite stem cells, exit the cell cycle and spontaneously differentiate into myotubes that are quiescent multinucleated cells expressing muscle-specific structural proteins. To determine whether TX displayed antioxidant activity also in more mature skeletal muscle cells, we switched confluent human primary myoblasts to differentiation medium for 4 days. At day 2, we incubated cells with TX for 24 h, followed by H₂O₂ for another 24 h. LDH activity and ROS level were increased in myotubes incubated only with H₂O₂ (Fig. 4D, E). Conversely, pre-incubation with TX significantly reduced H₂O₂ effects. In the literature, only one manuscript described TX antioxidant activity *ex vivo*. Specifically, TX presented antioxidant and protective effects against serum/glucose deprivation-induced ischemic injury in a dose dependent manner in PC12 cells (Shafaei-Bajestani, Emami, Asili, & Tayarani-Najaran, 2014).

We reported that high concentrations of rosemary stem extract (Fig. 1B) or taxodione (Fig. 3A, B) induce cell cytotoxicity. It is now well accepted that, although the antioxidant activity of phytochemicals is proven, they can also display pro-oxidant activities under certain conditions. In most cases, the pro-oxidant or antioxidant activity intimately depends on their concentrations (Bouayed & Bohn, 2010). Uchihara and collaborators (2018) have shown that taxodione induces cell toxicity at concentrations above 5 µM (Uchihara et al., 2018). This cytotoxicity has been already reported by Tayarani and collaborators in 2013. Their results demonstrated that taxodione exhibited high cytotoxic activity against apoptosis-proficient HL-60 and apoptosis-resistant K562 cells, at concentrations above 3 µM (Tayarani-Najaran et al., 2013). We have performed experiments showing that high doses of rosemary stem extract (data not shown) or taxodione at concentrations greater than 3 µM (Fig. 3B) induce ROS accumulation in muscle cells. Therefore, high concentrations of taxodione induced apoptosis through ROS accumulation in skeletal muscle cells as previously demonstrated in cancer cells (Uchihara et al., 2018). In the RS extract, the taxodione concentration is below the cytotoxic threshold. This suggests that the toxicity of the rosemary stem extract is also due to other compounds present in the extract.

Here, we demonstrated that at low concentrations (from 0.4 to 1.6 µM), taxodione exhibits antioxidant activities in human skeletal muscle cells. Taxodione has an antioxidant activity at similar concentrations (0.2–1.5 µM) in PC12 cells (Shafaei-Bajestani et al., 2014). We postulate that such low concentrations of taxodione could be considered as “physiological” with beneficial effects as opposed to deleterious effects at high doses.

Besides its antioxidant property, TX was reported to have various

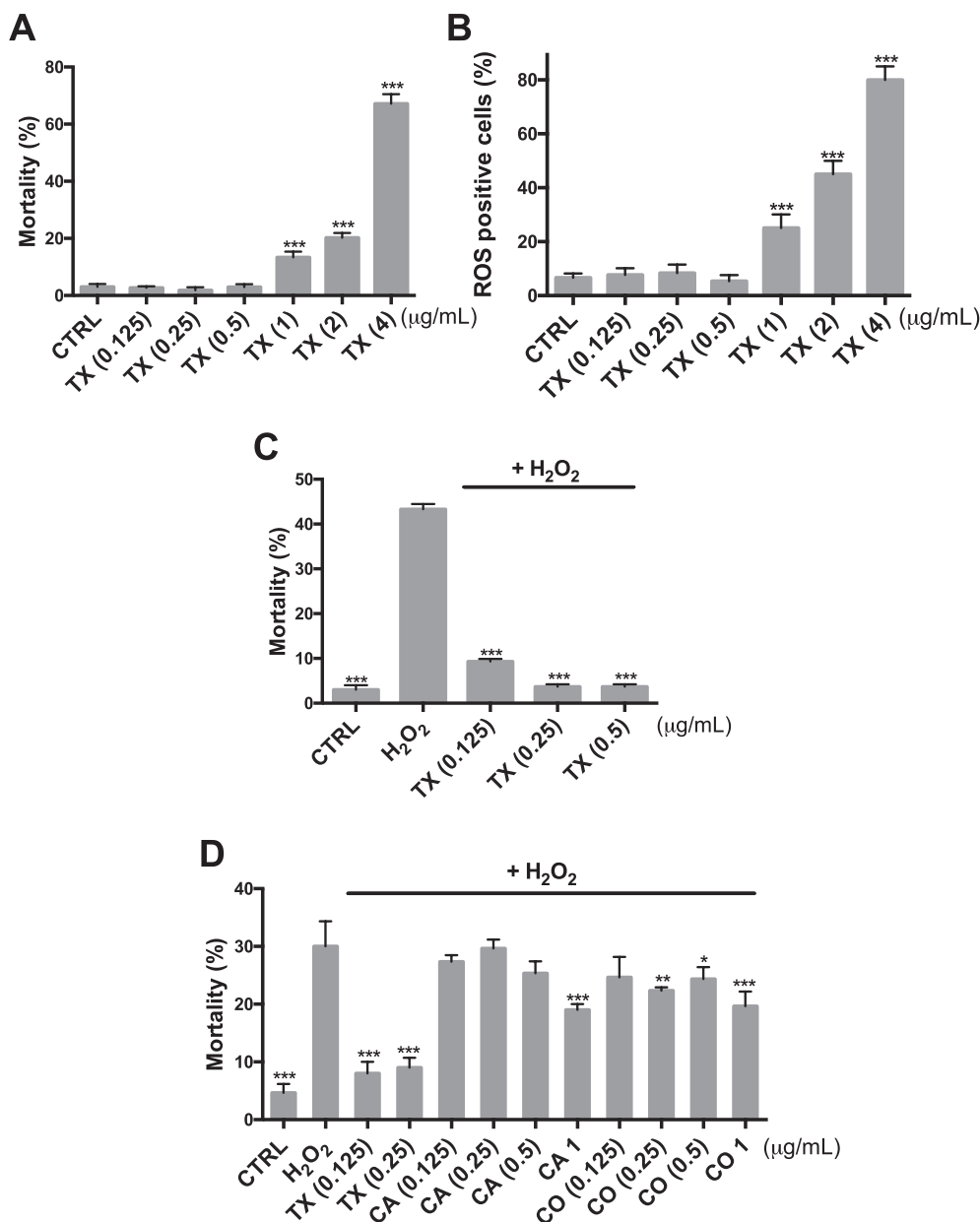


Fig. 3. Taxodione has a strong anti-oxidant activity on human muscle cells. Cell death (A, C, D) and ROS (B) quantifications (percentage of all cells) in human myoblasts upon incubation with (A, B, C) the indicated concentrations of taxodione (TX) or (D) of the main bioactive compounds of rosemary, carnosic acid (CA) and carnosol (CO), prior to exposure to (C, D) 120 μM H₂O₂ (lethal concentration). CTRL: cells not incubated with H₂O₂. Cell death was quantified using the “Cell Count and Viability Kit” and the Muse Cell Analyzer; ROS was quantified with the “oxidative stress kit” and the Muse Cell Analyzer. $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) compared with H₂O₂ (B, C) and CTRL (A) (one-way ANOVA).

bioactivities: Tumor inhibitor, human cholinesterase inhibitor, anti-bacterial, DNA binding, anti-termite, anti-fungal, anti-protozoal, and anti-leishmanial (Bufalo et al., 2016; Hanson, Lardy, & Kupchan, 1970; Kupchan et al., 1968; Kusumoto et al., 2009; Kusumoto, Ashitani, Murayama, Ogiyama, & Takahashi, 2010; Mothana et al., 2014; Yang et al., 2001; Zaghloul, Gohar, Naiem, & Abdel Bar, 2008). Recently, using *in silico* and *in vitro* screening methods, TX was identified as a candidate farnesyl synthase inhibitor, potentially useful in patients with Hutchinson-Gilford progeria syndrome (Liu et al., 2014). However, using a more complex biological system, we found that TX had no farnesyl transferase inhibitor activity (Supplementary Fig. s2).

Our results suggest that TX could be helpful in human pathologies associated with oxidative stress and skeletal muscle wasting, such as aging, Duchenne muscular dystrophy, FacioScapuloHumeral dystrophy, laminopathies, dystrophies caused by mutations in the collagen VI and dysferlin genes (Choi et al., 2016; Passerieux et al., 2015). TX could also improve the efficacy of therapeutic approaches in skeletal muscle diseases by reducing the strong oxidative stress associated with these conditions.

3.5. Taxodione limits lipid and protein oxidation in minced meat.

In processed meat, lipids and proteins undergo oxidation over time, but this can be delayed by addition of antioxidants (Shah et al., 2014). We have developed pilot experiments on mouse post-mortem muscles to characterize the antioxidant potential of TX. Despite the physical and physiological differences between species, mouse post-mortem muscle exhibits similar patterns of protein degradation (Ehrenfellner, Zissler, Steinbacher, Monticelli, & Pittner, 2017). In addition, rodents are inexpensive, available in the majority of laboratories thus allowing better controls of post-mortem muscle experiments. As described in meat for food, the lipid oxidation quantified by TBARS gradually increases in mouse post-mortem muscles from the second day of storage at 4 °C while the thiol levels decrease sharply indicating a high level of protein oxidation (data not shown). To determine TX antioxidant potential, we compared the efficacy in decreasing lipid and protein oxidation of TX, CA and of the synthetic phenolic antioxidant BHT (Fig. 5). In minced mouse muscles (CTRL), lipid oxidation, quantified by TBARS analysis, strongly increased after 7 days of storage at 4 °C. Conversely, thiol levels dropped markedly, indicating a high level of protein oxidation

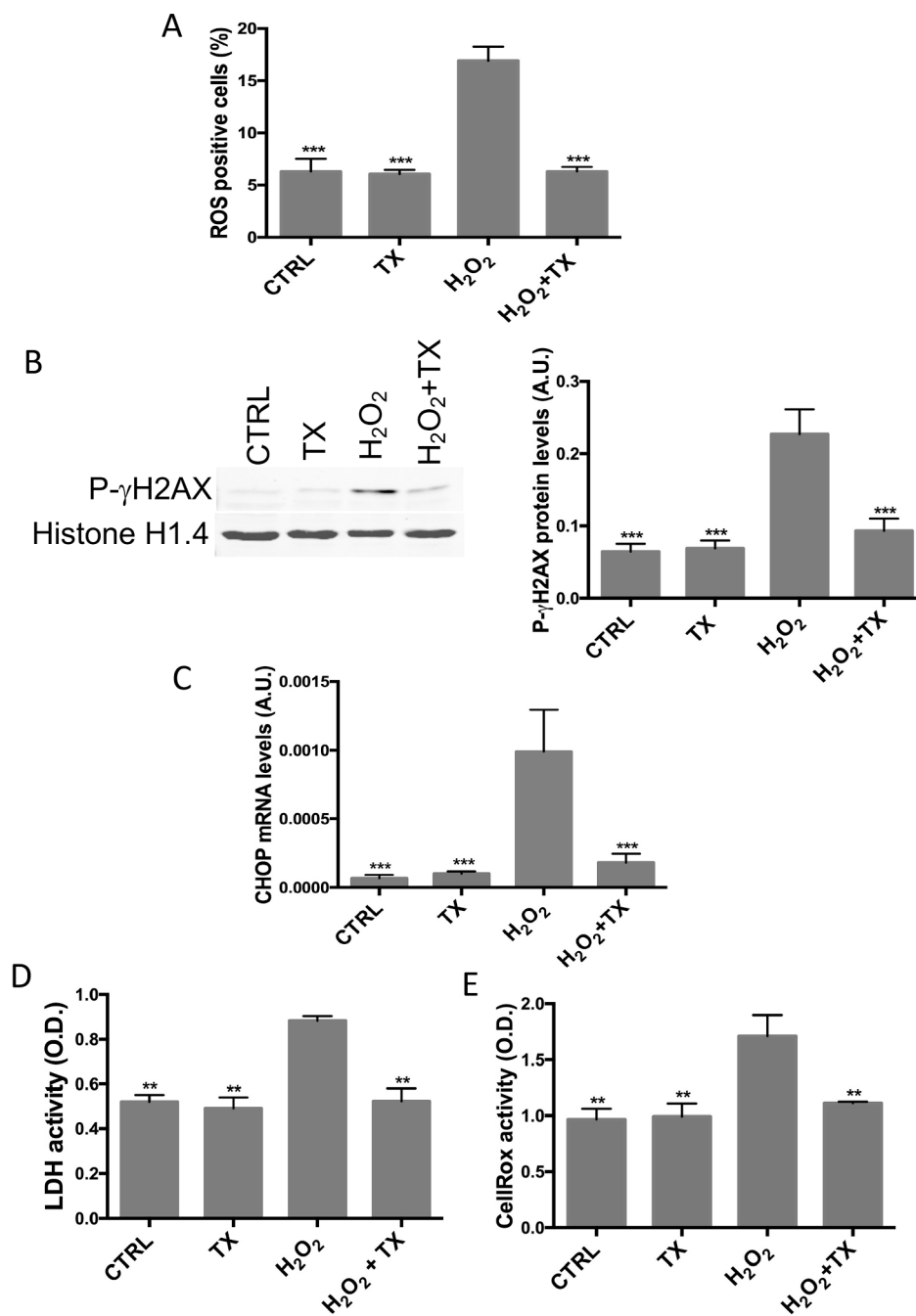


Fig. 4. Taxodione decreases oxidative damage in human muscle cells. Myoblasts were incubated with taxodione (TX) (0.5 µg/mL) for 24 h prior to exposure to H₂O₂. (A) Reactive oxygen species (ROS) production was quantified with the “Muse oxidative stress Kit” and Fluorescence Activated Cell Sorting (FACS). (B) Western blot analysis of phosphorylated γH2AX protein level; histone H1.4 was used as loading control (left panel). Quantification of the Western blot data using the Odyssey software (right panel). (C) RT-qPCR analysis showing the relative expression levels (compared with untreated control) of the *CHOP* gene; *RPLPO* was used as reference gene. (D, E) Confluent human primary myoblasts were switched to differentiation medium for 4 days. At day 2, cells were incubated with TX (0.5 µg/mL) for 24 h and then exposed to H₂O₂ for 24 h. (D) H₂O₂ toxicity was determined by quantifying lactate dehydrogenase (LDH) activity; (E) CellRox (ROS activity probe) was loaded in myotubes and fluorescence was quantified using a TECAN spectrophotometer; $p < 0.01$ (**) and $p < 0.001$ (***) compared with H₂O₂ (one-way ANOVA).

(Fig. 5A and B). In post-mortem muscle samples containing BHT, CA or TX, TBARS values were already significantly lower at day 0 (Fig. 5A) and remained lower than in control (CTRL; non-treated samples) even at day 7 (Fig. 5A). At day 0, thiol levels were comparable in control and samples with BHT, CA or TX, but not for the sample with the highest TX concentration (0.015%) where total thiol level was significantly lower (Fig. 5B). This could be explained by a potential TX pro-oxidant effect on proteins, or a consequence of its sulfhydryl activity (Hanson et al., 1970). After 7 days of storage, thiol level in muscles was significantly lower in control than in the samples with antioxidants, but not for 0.01% BHT (Fig. 5B). To validate these results on meat for human consumption, minced beef meat was treated with BHT, CA and TX for 7 days at 4 °C (Fig. 6). As expected, lipid oxidation greatly increased after 7 days of storage (Fig. 6A). As demonstrated in mouse muscle, lipid oxidation remained low in BHT, CA and TX treated minced beef

(Fig. 6A). Similarly, thiol concentrations in beef meat were significantly lower in the BHT, CA and TX samples than in the controls (Fig. 6B). Our results confirm a protective effect of TX on the oxidation of lipids and proteins during storage of meat. These results from beef meat assays are similar with what we observed from post-mortem mice muscles. Thus, our experiments validate rodent as an animal model useful for predicting skeletal muscle post-mortem changes and establishing biological tests to preserve the integrity of the meat.

4. Conclusion

There is growing interest for cheap and abundant source of natural antioxidants, thus the large-scale availability of agricultural and industrial plant waste materials and their low-cost makes them attractive sources of these bioactive compounds. Rosemary is marketed as dried

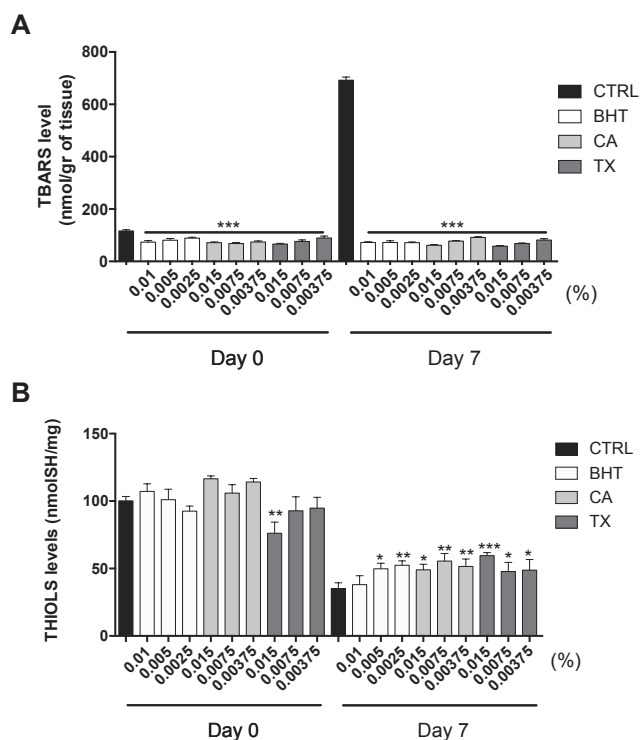


Fig. 5. Taxodione protects mice minced meat from lipid and protein oxidation during refrigerated storage. Minced gastrocnemius muscles from six-month-old C57BL/6 male mice were mixed with ethanol (CTRL) or BHT (0.010%, 0.005%, 0.0025% w/w minced muscle), carnosic acid (CA) (0.015%, 0.0075%, 0.00375% w/w minced muscle) or taxodione (TX) (0.015%, 0.0075%, 0.00375% w/w minced muscle) dissolved in ethanol. At day 0 and day 7 of refrigerated storage (4 °C), (A) lipid oxidation was evaluated by TBARS quantification, and (B) protein oxidation by total thiol quantification; $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) compared with CTRL at day 0 or at day 7 (one way ANOVA).

leaves for cooking, and as extracts for food and cosmetic industries. Research on rosemary antioxidant activity is primarily focused on rosemary leaves and its bioactive compounds carnosic acid and carnosol. Therefore, leaves are collected for essential oil production or compound extraction, whereas stems are not valorised. Our findings indicate that rosemary stems are a cheap source of the antioxidant taxodione. In this manuscript, we showed that taxodione efficiently protects immature (myoblasts) and mature (myotubes) skeletal muscle cells from H_2O_2 -induced oxidative stress damage. Furthermore, addition of taxodione to post-mortem muscles delayed lipid and protein oxidation during refrigerated storage. Thus, taxodione or rosemary stem extracts, in addition to carnosic acid and carnosol, could prevent or limit skeletal muscle oxidation in human health and food applications.

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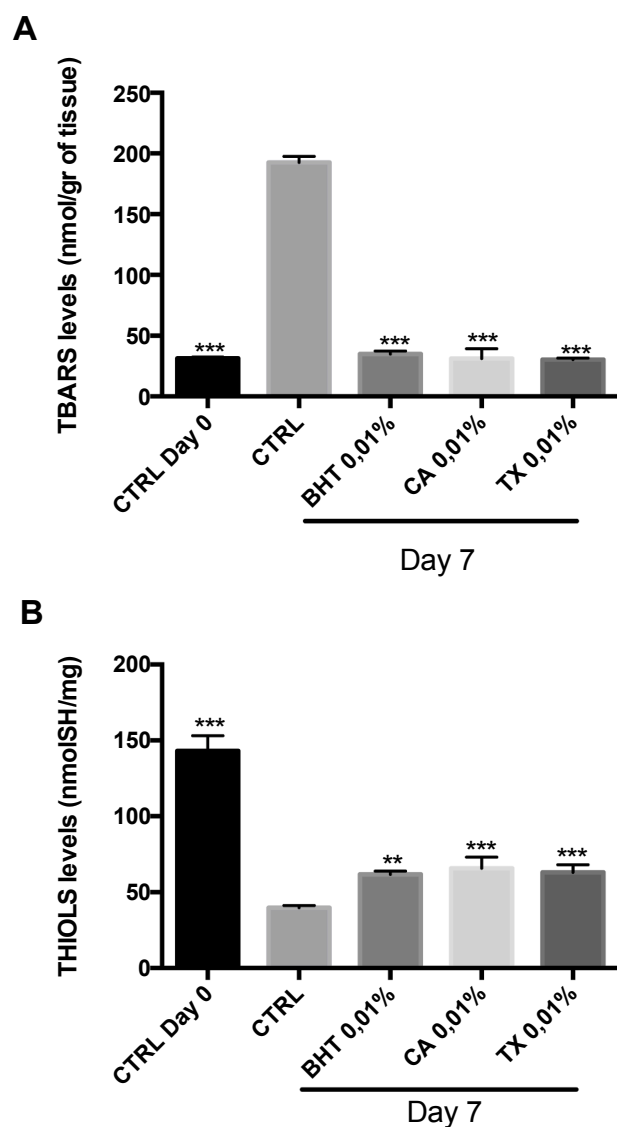


Fig. 6. Taxodione protects beef minced meat from lipid and protein oxidation during refrigerated storage. Minced beef muscles mice were mixed with ethanol (CTRL) or BHT (0.01% w/w minced muscle), carnosic acid (CA) (0.01% w/w minced muscle) or taxodione (TX) (0.01% w/w minced muscle) dissolved in ethanol. At day 0 and day 7 of refrigerated storage (4 °C), (A) lipid oxidation was evaluated by TBARS quantification, and (B) protein oxidation by total thiol quantification; $p < 0.05$ (*), $p < 0.001$ (***) compared with CTRL at day 7 (one way ANOVA).

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jff.2019.103565>.

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