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Rhodiola rosea, a protective antioxidant for intense physical exercise: An *in vitro* study

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ABSTRACT

In physiological conditions, the response to oxidative stress induced by strenuous physical training, is not always efficient. Furthermore, a weak muscle fiber antioxidant system can contribute to muscle wasting during ageing and pathological conditions. Natural antioxidant supplements could counteract the effects of oxidative stress. An *in vitro* model of exercise allowed us to mimic intense or moderate exercise by electrically stimulating cultured mouse myotubes with high- and/or low-frequency pulses; the resulting ROS production was then monitored. The herbal supplement *Rhodiola rosea* aqueous extract was characterized in terms of its polyphenol content and used to test for antioxidant activity against ROS, produced during muscle contraction. Our results showed that *Rhodiola rosea* extract reduced the oxidative stress produced by muscle contraction and brought values of ROS production back to physiological levels. We suggest that *Rhodiola rosea* root extract could exert a protective antioxidant role during intense physical exercise.

1. Introduction

The observation that contracting skeletal muscles produce reactive free radicals was first reported in 1982 (Davies, Quintanilha, Brooks, & Packer, 1982). Since then, many studies (Castrogiovanni & Imbesi, 2012; Finaud, Lac, & Filaire, 2006; Fisher-Wellman & Bloomer, 2009; Nikolaidis et al., 2012; Teixeira, Valente, Casal, Marques, & Moreira, 2009) have confirmed that contracting muscles produce free radicals and other reactive oxygen/nitrogen species. Moreover, it is now established that the levels of reactive species in skeletal muscle play a critical role in regulating force production. Indeed, an optimal redox balance exists in muscle, whereby the contractile apparatus generates the highest force production. The low levels of reactive oxygen species (ROS) present in skeletal muscle during basal conditions are a requirement for normal force production, while a modest increase in ROS in skeletal muscle fibers results in an increase in force production (Powers & Jackson, 2008).

High levels of ROS however, cause functional oxidative damage of proteins, lipids, nucleic acids and cell components; they also induce a significant rise of intracellular Ca^{2+} concentration and promote signaling cascades for apoptosis or autophagy. For these reasons, and in line with hormesis theory, low/moderate levels of ROS are considered beneficial, while high ROS levels are reputed to be involved in muscle atrophy, sarcopenia (loss of muscle mass with ageing), and other chronic/aging- related muscle diseases and myopathies.

It is widely reported that high-intensity prolonged exercise promotes muscle ROS production, resulting in oxidative damage and impaired muscle function (Powers & Jackson, 2008). Such produced radicals are related to fatigue and injury in skeletal muscle, and in recent years, have stimulated studies on the ability of natural antioxidants to prevent both exercise-induced oxidative damage and muscle fatigue (Powers, DeRuisseau, Quindry, & Hamilton, 2004; Powers & Sen, 2000).

Rhodiola rosea (*R. rosea*) is a popular herbal medicine with adaptogenic properties, generally used as a non-toxic natural health product to generate an unspecific resistance to stress, depression, fatigue and improve exercise performance (De Bock, Eijnde, Ramaekers, & Hespel, 2004; Hung Shao Kang, Perry, & Ernst, 2011; Ishaque, Shamseer,

Abbreviations: ROS, reactive oxygen species; NMJ, neuromuscular junction

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Bukutu, & Vohra, 2012; Parisi et al., 2010). Botanists have identified several species of *Rhodiola*, but not all of them have been investigated for their health benefits (Brown, Gerbarg, & Ramazanov, 2002; Cuerrier & Ampong-Nyarko, 2015; Kumar, Tayade, Chaurasia, Sunil & Singh, 2010; Yousef et al., 2006). The beneficial effects *R. rosea* have been related to the powerful adaptogens found in the root extracts (Chiang, Chen, Wu, Wu, & Wen, 2015; Khanum, Bawa, & Singh, 2005; Panossian, Hammb, Wikmana, & Efferth, 2014; Zhou et al., 2015). *R. rosea* is the species considered most safe for human consumption, having no reported adverse effects (Brown et al., 2002; Khanum et al., 2005). Extracts of *R. rosea* roots were found to positively affect a number of physiological aspects: neuro-cardio and hepato-protection, cardioprotective effects, stimulation of the central nervous system, as well as increasing cognitive functions such as attention, memory and learning. Because of its antidepressive, anxiolytic and antioxidant properties, it has become particularly popular in the United States, Europe and Soviet Union (Cuerrier & Ampong-Nyarko, 2015). The therapeutic properties of *R. rosea* are not only attributed to the presence of a complex mixture of active compounds (Panossian, Wilkmana, & Sarris, 2010), but also to their relative composition. High composition variability is observed in relation to the region where the plant is grown as well as to the commercial extraction method (Elameen, Klemsdal, Dragland, Fjellheim, & Rognli, 2008; Ma et al., 2011). Several constituents of *R. rosea* were isolated including salidroside (rhodioloside), p-tyrosol, phenols, flavonoids (rhodiolin, rhodionin, rhodiosin, tricin, rhodalgin, and acetylrhodalgin), and monoterpenes rosiridol and rosiridin. What distinguishes it from the other species of *Rhodiola* are the presence of three water-soluble cinnamyl glycosides: rosavin, rosin, and rosarin, that are collectively known as rosavins. The simultaneous presence of all these components is considered to be responsible of the main active phytochemical properties of *R. rosea* (Kelly, 2001). Moreover, due to the presence of several phenolic compounds, it was also highlighted to be a strong antioxidant and anticarcinogen (Liu, Li, Simoneau, Jafari, & Zi, 2012).

There is a consensus on the beneficial effects of *R. rosea* for the treatment of illness and neurodegenerative pathologies where oxidative stress plays a major role in disease development and progression (for a review see Nabavi et al., 2016). However, there are conflicting reports on the effects of *R. rosea* on physical performance on trained and untrained humans, probably related to R. *rosea* dose, exercise protocols and tests used. In addition, beneficial effects of *R. rosea* supplementation on muscle performance could be related to the time of the adaptogen administration (Noreen, Buckley, Lewis, Brandauer, & Stuempfle, 2013; Shanely et al., 2014).

In rodents, long-term *R. rosea as well as fermented R. rosea* extract supplementation, at different dosages for 4 weeks, improved the swimming performance (Abidov, Crendal, Grachev, Seifulla, & Ziegenfuss, 2003; Huang, Lee, Kuo, Yang, & Chien, 2009). In addition, salidroside was also able to elevate the rat exercise tolerance following exhaustive exercise (Xu & Li, 2012).

Studies on animals and humans have suggested that *R. rosea* is involved in ATP and creatine phosphate metabolism (Abidov et al., 2003). In particular, the effects of salidroside on glucose uptake indicate an important role of *R. rosea* not only in pathological conditions (for example in type 2 diabetes mellitus and cardiovascular disease (Li, Ge, Zheng, & Zhang, 2008), but also in athletes. On the other hand, a study conducted by Walker and co-workers on trained men (Walker, Altobelli, Caprihan, & Robergs, 2007) failed to show any correlation between *R. rosea* consumption and improved ATP turnover.

Acute doses of *R. rosea* have positive effects on endurance exercise performance and on mood and cognitive function; a decrease of heart rate was observed during the warm-up. Subjects were able to finish the time trial faster, although without any significant differences in mood state (Noreen et al., 2013). Randomized chronic supplementation of *R. rosea* to marathon runners failed to show any differences in muscle function or endurance, or in stress molecule and cytokines production (Shanely et al., 2014). In addition, molecular mechanisms responsible for the reduction of recovery time after intense physical training in athletes, is still a matter of investigation.

In line with the novel intriguing hypothesis that low concentrations of ROS might promote muscle regeneration (Barbieri & Sestili, 2012; Powers, Talbert, & Adhihetty, 2011; Steinbacher & Eckl, 2015), natural antioxidant compounds could represent useful therapeutic tools for the treatment of high harmful levels of ROS in aged muscle or over-trained muscle. The aim of our present work was to develop an experimental model to test the muscle cell adaptations *in vitro* under controlled conditions in the presence and absence of *R. rosea* extracts. Extracellular electrical stimulation (ES) of cultured mouse myotubes, mimicked nerve activity in a situation of moderate or intensive physical exercise. The potential effect of aqueous *R. rosea* root extract (Asoltech s.r.l.) in protecting murine skeletal muscle cells from ROS generated by myotube contractions was then examined.

2. Materials and methods

2.1. Plant materials and extraction

Dry powdered Asiatic *R. rosea* roots (1000 mg), kindly provided by Asoltech s.r.l., Trieste, were extracted in 10 min with 10 mL MilliQ water by sonication (Sonica, Soltech) at 20 °C. The sample was centrifuged and filtered through a 0.45 μm Millipore membrane. The pellet was re-suspended and then re-extracted for an extra 10 min under the same conditions as the first water extraction. All aliquots were lyophilized and stored at −80 °C until use.

2.2. Chemical characterization of R. rosea extract/total polyphenol content

Total polyphenol content of *R. rosea* aqueous extract was measured using a Folin–Ciocalteu colorimetric method with slight modification from Gao and others (Gao, Ohlander, Jeppsson, Björk, & Trajkovski, 2000). Briefly, the root extract (100 μL) was mixed with 0.2 mL of Folin–Ciocalteu's reagent and 2 mL of H_2O, and incubated at room temperature for 3 min. Following the addition of 1 mL of 20% (w/v) Na_2CO_3, the sample was mixed and the total polyphenol content was determined after 1 h of incubation at room temperature. The absorbance was measured at 765 nm in a cuvette with 1 cm path length. Quantification, done with respect to the standard curve of gallic acid and polyphenol contents, was expressed as gallic acid equivalent (GAE, mg g^{-1}).

2.3. Total flavonoid content

Total flavonoids were estimated using the method of Ordoñez and co-workers (Ordoñez, Gomez, Vattuone, & lsla, 2006). A 2 mL sample and 2 mL of 2% (w/v) $AlCl_3$ ethanol solution were mixed. After 1 h at room temperature, the absorbance was measured at 420 nm. Total flavonoid content was calculated as the quercetin equivalent (QE, mg/g of extract) from a calibration curve.

2.4. Identification of phenols by Liquid Chromatography/Mass Spectrometry (LC/MS-MS)

The Multiple Reaction Monitoring (MRM) phenols extraction protocol was carried out using a liquid chromatography and mass spectrometry procedure as reported in Vrhovsek and coauthors (Vrhovsek et al., 2012). 100 mg of sample was added in an Eppendorf to 600 μL MeOH/H_2O (2:1). After addition of 400 μL of chloroform and 20 μL of internal standard (gentisic acid & rosmarinic acid, 50 mg/L), the mixture was shaken in an orbital shaker for 15 min. The separation of organic and aqueous layers was obtained by centrifuging at 15000*g* for 5 min at 4° C. The upper aqueous fraction was immediately transferred in a new Eppendorf. 600 μL of MeOH/H_2O (2:1) were added in the first

Eppendorf for a second extraction as just described. Both aqueous fractions were mixed and dried under a nitrogen stream at 35° C. The dried sample was dissolved with 500 µL of MeOH/H_2O (2:1), centrifuged at 15000*g* for 5 min at 4° C and transferred carefully in a HPLC vial with glass insert. Analyses were performed with a Waters Acquity UPLC system (Milford, MA, USA) coupled to a Xevo TQ MS System (Waters, UK), based on a method previously described (Vrhovsek et al., 2012). Samples were kept at 6 °C and injected (2 µL) on a Waters Acquity HSS T3 column (1.8 µm, 100 × 2.1 mm; Milford, MA), thermostated at 40 °C. The flow rate was 0.4 mL/min. The solvents were as follows: solvent A (0.1% formic acid in water), solvent B (0.1% formic acid in acetonitrile). The gradient profile was set as follows: 0 min, 5% B; from 0 to 3 min, linear to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, linear to 45% B; from 9 to 11 min, linear to 100% B; from 11 to 13 min, wash at 100% B and from 13.01 to 15 min, back to the initial conditions of 5% B.

2.5. Determination of antioxidant activity using the DPPH (1,1- diphenyl 2-picrylhydrazyl) method

Free radical-scavenging activity of DPPH was measured by the method of Lu and co-authors (Lu, Cheng, Hub, Zhang, & Zou, 2009). DPPH is a stable free radical that accepts an electron or hydrogen radical producing a stable diamagnetic molecule. The effect of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen donating ability. 50 µL of 1 mg/mL DPPH, dissolved in ethanol, was added to different dilutions of *R. rosea* extract (2 mL). DPPH is a stable free radical with deep purple color in ethanolic solution having a typical absorbance at 517 nm, and becomes pale yellow colored when trapped by an antioxidant. The control consisted of 2 mL of MilliQ water and 50 µL of DPPH solution. The decrease in absorbance of the resulting solution was monitored at 517 nm for 80 min. Using the adsorbance value at 60 min, the antioxidant activity of the *R. rosea* extract was calculated as the DPPH scavenging activity (%) as follows:

$$DPPH_{(rsa,\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \cdot 100 \quad (1)$$

where *rsa* is the radical scavenging activity; $A_{control}$ is the absorbance of DPPH radical and ethanol; A_{sample} is the absorbance of DPPH radical and *R. rosea* extract. The concentration of sample required to halve the DPPH absorbance was calculated (IC_{50}). Measurements were performed in triplicate ($n = 3$).

2.6. Ferric-reducing antioxidant power assay (FRAP)

The FRAP assay implies the use of a ferric salt, $Fe(III)(TPTZ)_2Cl_3$ (TPTZ 2,4,6-tripyridyl-s-triazine), as an oxidant agent (Benzie & Strain, 1999). The assay is carried out under acidic conditions. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl and 20 mM iron (III) chloride solution in proportions of 10:1:1 (v/v). Freshly prepared working FRAP reagent (3.0 mL) was mixed with 100 µL of *R. rosea* sample and vortex stirred. The absorbance was measured on a Jenway 6300 Spectrophotometer after 30 min at 593 nm against a reagent blank (Tayade et al., 2013). The standard curve was obtained using $FeSO_4 \cdot 7H_2O$ solution and ascorbic acid. Results were expressed as µmoles Fe(II)/g of dry weight of plant material.

2.7. Cell culture

Primary murine myotube cultures (Fig. 2A) derived from the differentiation of i28 myoblasts were kindly provided by Dr A. Wernig (Department of Physiology and Medical Policlinic, University of Bonn, Germany). Briefly, satellite cells were isolated from the hindlimb of 7-day old male Balb/c mice and expanded *in vitro* following previous protocols (Irintchev, Langer, Zweyer, Theisen, & Wernig, 1997).

Myoblasts were seeded in 6-well matrigel-coated plates (Fig. 2B) at a density of 70,000 cells and cultured in Growth Medium (GM), composed of HAM'S F-10 (HAM'S/F-10, Euroclone) plus 20% foetal bovine serum (FBS), L-glutamine 4 mM, penicillin (100 units/ml) and streptomycin (100 µg/mL). To induce myoblast fusion into myotubes, 24 h after seeding, GM was replaced with Differentiation Medium (DM), consisting of Dulbecco's modified Eagle's Medium supplemented with 2% horse serum HS, L-glutamine 4 mM, penicillin and streptomycin as above. The culture medium was renewed every 3 days to avoid loss of nutrients and growth factors. Cell cultures were maintained at 37 °C in a humid air with 5% CO_2. Under this condition, myotubes were considered mature at 6–7 days.

2.8. PrestoBlue cell viability assay

In order to evaluate any cytotoxic effect of *R. rosea* on myotubes, we also used the PrestoBlue™ Cell Viability kit (Molecular probe, Invitrogen) following the manufacturer's instructions. Myotubes were seeded in 96-well plates in DM at a cell density of 1×10^3 cells per well. After 7 days of incubation, culture medium was removed and the myotubes were washed with 200 µL of PBS. 100 µL of DM medium alone (untreated) or with increasing concentrations of *R. rosea* aqueous extract (1–1000 µg/mL) (treated) were added to the cells for 1 h or 24 h. 10 µL of PrestoBlue™ Reagent was added directly to the cells in culture medium and incubated for 30 min. Fluorescence was read at an excitation wavelength of 560 nm and emission wavelength of 590 nm. Background fluorescence was corrected by including blank wells containing only cell culture media on each plate. The cytotoxicity was calculated as:

$$Cell\ viability\,(\%) = \frac{FU_{treated} - FU_{blank}}{FU_{untreated} - FU_{blank}} \cdot 100 \quad (2)$$

where *FU* is the fluorescence in arbitrary units.

2.9. Lactate dehydrogenase (LDH) assay

To detect the effect of ES, the amount of LDH activity released into the medium was used as a measure of membrane damage and expressed as a percentage of total cellular activity. LDH activity in the medium was determined using a commercially available kit from Sigma-Aldrich (Tox-7). Myotubes were seeded in 6-well plates in DM at a cell density of 70,000 cells per well. After 7 days of incubation, culture medium was removed and the myotubes were firstly washed with 1 mL PBS, then Hank's Balanced Salt Solution (HBSS) with or without 2.5 µg/mL of *R. rosea* extract was added for 1 h. Cells were incubated for 60 min in 5% CO_2 in a humid atmosphere at 37 °C, while delivering or not delivering the high-frequency stimulation protocols. The medium was removed and centrifuged at 600*g* for 5 min to sediment the cells. Aliquots were transferred to clean tubes for the enzymatic analysis.

Equal volumes of LDH assay substrate solution, dye solution and cofactor preparation were mixed and added to samples medium, according to the product manual, in a 96 well/plate. The plates were protected from light and incubated at room temperature for 20–30 min. The reaction was terminated by the addition of 1/10 vol of 1 N HCl to each well. Absorbance was measured spectrophotometrically at 490 nm in a microplate reader (Synergy H1, Biotek). Background absorbance was measured at 690 nm and subtracted from the primary wavelength measurement (490 nm). Maximum LDH activity was evaluated from total cell lysate; blank absorption was evaluated by adding water as sample and calculated as:

$$LDH\ release\,(\%) = \frac{(A_{490} - A_{690})_{sample} - (A_{490} - A_{690})_{blank}}{(A_{490} - A_{690})_{lysate} - (A_{490} - A_{690})_{blank}} \cdot 100 \quad (3)$$

2.10. Malondialdehyde (MDA)

The quantitative measurement of malondialdehyde (MDA), the end product of lipid peroxidation, was performed according to the method of Agarwal (Agarwal & Chase, 2002) with some modifications. Following the heat derivatization, samples were filtered on a 0.45 µm PTFE membrane and directly injected into a HPLC chromatographic apparatus (HPLC Dionex equipped with a quaternary pump GP50, UV–visible detector AD25 and an in-line GF2000 fluorescence detector, Water Symmetry C18 column: 5 µm, 250 × 4.6 mm). Integration was carried out using the Chromeleon (Dionex Corporation; Sunnyvale, CA, USA) software, version 6.8. Pump flow-rate was 0.5 mL/min with acetonitrile–water (40:60, v/v) as mobile phase. The concentration of MDA was expressed as µmol/L.

2.11. Electrical stimulation of cultured myotubes

A custom-built electronic device was designed to electrically stimulate the cultured myotubes eliciting contractile activity (Fig. 2A and B). Myotubes were seeded in sterile matrigel-coated 6-well plates; in each of them, two platinum-iridium electrodes (0.25 mm diameter) were placed within 2 cm of each other and hanging in the bath solution 1–2 mm above the skeletal myotubes. Two plastic plates were connected by electrical cables to a Grass S88 stimulator (Grass Instruments, Quincy, MA, USA). To generate signals with alternating polarity avoiding electrolysis, capacitors were inserted in series with the circuit. Such a system was maintained in a standard cell incubator and controllable electrical fields were elicited in each well. Two independent protocols were used to induce myotube contractions. The stimulations consisted of 0.7 s trains with 0.3 s pauses between the trains. The single trains consisted of a 1 ms pulse width at 4 Hz (moderate stimulation) or 100 Hz (intense stimulation).

2.12. ROS imaging

Intracellular ROS generation before and after ES of cultured myotubes was investigated using: 5-(and-6)-chloromethyl-2′,7′dichlorodihydrofluorescein diacetate acetylester (CM-H_2DCFDA, Molecular Probes,™ Invitrogen, Eugene, OR). Mouse i28 cells were seeded in 6-well plates in DM (7×10^4 cells/well). After 7 days of incubation, myotubes were loaded with 2 µM CM-H_2DCFDA in Hank's Balanced Salt Solution (HBSS) with or without *R. rosea* aqueous extract (0.25, 2.5 and 25 µg/mL) during 30 min at 37 °C and 5% CO_2. Upon incubation, the cells were washed twice with PBS 1X and 2 mL of HBSS was added in each well (Sriram et al., 2011). Fluorescence was then immediately determined before applying the stimulation protocols at time 0 (t_0). After 20 min incubation in ES conditions (low or high frequency), kinetics of ROS generation was registered every 5 min up to 30 min using a fluorescent multi- label plate reader (Synergy H1 Multi-Mode Reader, BioTek U. S., VT, USA). Fluorescence was detected at 37 °C in the dark, using an excitation and emission wavelength of 495 and 525 nm, respectively. To quantify the cellular antioxidant activity, the *CAA units* (%) were calculated as follows (Ziberna et al., 2010):

$$CAAunit = 100 - \left(\frac{\int A_S}{\int A_C}\right) \times 100 \quad (4)$$

where $\int A_S$ is the integrated area under the sample fluorescence curve and $\int A_C$ is the integrated area under the control curve.

2.13. Catalase enzyme activity

Catalase activity was measured in myotube lysates in PBS 1X with the addition of 1% Triton-X100. Degradation of hydrogen peroxide was measured in 50 mM phosphate buffer (pH 7) by the decrease in absorbance at 240 nm ($\varepsilon = 0.0436\ mM^{-1}\ cm^{-1}$, Beers & Sizer, 1952).

Table 1
Content of major polyphenol classes identified in the *Rhodiola rosea* aqueous extract. Classification is according to Vermerris and Nicholson (2008, chap. I).

		Water extract (mg/g) ·10^{-3}	%
PHENOLIC COMPOUNDS			
Benzoic acid and derivatives	Anthranilic acid	0.21	0.01
	4-aminobenzoic acid	0.01	0.00
	p-hydroxybenzoic acid	111.71	3.02
	Vanillic acid	22.39	0.61
	Gallic acid	938.64	25.38
	Vanillin	20.94	0.57
	Methyl gallate	1.98	0.05
	Syringaldehyde	0.86	0.02
	Ellagic acid	183.65	4.97
Cinnamic acid and derivatives	Cinnamic acid	215.24	5.82
	p-coumaric acid	68.16	1.84
	Caffeic acid	92.44	2.50
	Ferulic acid	22.47	0.61
	Neochlorogenic acid	1.27	0.03
	Chlorogenic acid	10.90	0.29
	Cryptochlorogenic acid	2.70	0.07
	Coniferyl aldehyde	4.44	0.12
FLAVONOIDS			
Dihydrochalcones	Phloretin	0.03	0.00
	Phlorizin	0.59	0.02
Flavones	Luteolin	47.43	1.28
	Luteolin-7-O-Glc	0.57	0.02
	Apigenin-7-Glc	0.21	0.01
	Laricitrin	5.08	0.14
Flavanols	Catechin	19.37	0.52
	Epicatechin	2.03	0.05
	Epigallocatechin	65.87	1.78
	Gallocatechin	18.36	0.50
	Epicatechin gallate	50.21	1.36
	Epigallocatechin gallate	1557.62	42.11
Flavanones	Naringenin	6.62	0.18
Flavonols	Kaempferol	122.79	3.32
	Kaempferol-3-Glc	29.97	0.81
	Isorhamnetin	0.43	0.01
	Quercetin-3-Rha	3.05	0.08
	Quercetin-3-Glc + quercetin-3-gal	11.56	0.31
STILBENES and derivatives			
	t-resveratrol	1.73	0.05
	t-piceide	1.20	0.03
	Piceatannol	1.28	0.03
	Pallidol	26.21	0.71
	Alpha-viniferin	4.45	0.12
	E-cis-miyabenol	3.66	0.10
COUMARINS			
	Daphnetin	3.71	0.10
	Scopoletin	0.30	0.01
	Procyanidin B1	2.71	0.07
PROANTHOCYANIDINS			
	Procyanidin B2 + B4	4.20	0.11
	Procyanidin B3 (as B1)	16.57	0.45
TOTAL		3705.9	

Catalase activity was expressed as U/mg prot.

2.14. Statistical analysis

All data are presented as the means ± SE. All statistical analysis was performed with Prism 4.0 (GraphPad Software, San Diego, CA). Differences between data were evaluated by unpaired Student's *t*-test if normality test was passed and considered significant at $P < 0.05$.

3. Results

3.1. Chemical characterization of polyphenols in R. rosea water extract

The LC/MS-MS analysis of polyphenolic classes of *R. rosea* samples are presented in Table 1. The water extract was found to contain phenols and flavonoids as major phytocomponents. Total polyphenolic compounds account for 3.7 mg/g of water extract. In this mixture, the main components were epigallocatechin gallate (flavonoid, 42%) and gallic acid (phenolic compound, 25%). Minor but relevant percentages (1–6%) of other phenolic compounds (3% p-hydroxybenzoic acid, 5% elargic acid) were found as well as cinnamic acid and derivatives (5.8% cinnamic acid, 2.5%, caffeic acid and 1.8% p-coumaric acid), flavones (1.3% luteolin, flavanols (1.8% epigallocatechin, 1.4% epicatechin gallate) and flavonols (3.3% kaempferol). The content of bioactive compounds of the water extract such as salidroside (2.35%) and rosavin (3.35%) was declared and certified by Asoltech s.r.l. The content of total polyphenolic compounds in the aqueous extract of *R. rosea* was also assessed using Folin-Ciocalteu reagent giving 247.2 ± 35.0 mg/g (n = 27) of powder expressed as gallic acid equivalents (mg GAE/g of extract). Since this method is able to reveal all components possessing phenolic substituents, *e.g.* proteins (accounting generally for almost 25 mg/g of root), a higher value of phenolic constituents with respect to the LC-MS data, was found.

3.2. Antioxidant properties of R. rosea

Significant ROS scavenging activity has been shown for both *R. rosea* alcoholic and water extracts having different fractions of antioxidant compounds (Lee, Lee, & Park, 2000). However, the direct antioxidant capacity of the aqueous extracts of *R. rosea* on cultured muscle cells has not been studied in detail. The antioxidant activity of various concentrations (0.5, 5, 25 and 50 μg/mL) of *R. rosea* was tested using the DPPH assay. Absorbance values measured for 80 min are reported in Fig. 1. In the range of 0.5–50 μg/mL, the free radical scavenging activity increased with increasing *R. rosea* concentrations. A linear fit of the steady-state absorbance values at 60 min *vs*. extract concentration was used to obtain, by interpolation, the IC_{50} (54 μg/mL) for *R. rosea* water extract. The capacity to scavenge the DPPH radicals suggests that the water extract is an electron donor and could react with free radicals to convert them to more stable molecules and terminate radical chain reactions.

The FRAP method confirmed the results obtained using the DPPH assay (Table 2). The correlation coefficient (R^2 = 0.964) between the FRAP value and the total phenolic content of different *R. rosea* concentrations, revealed a marked contribution of phenolic compounds to the overall *R. rosea* antioxidant activity.

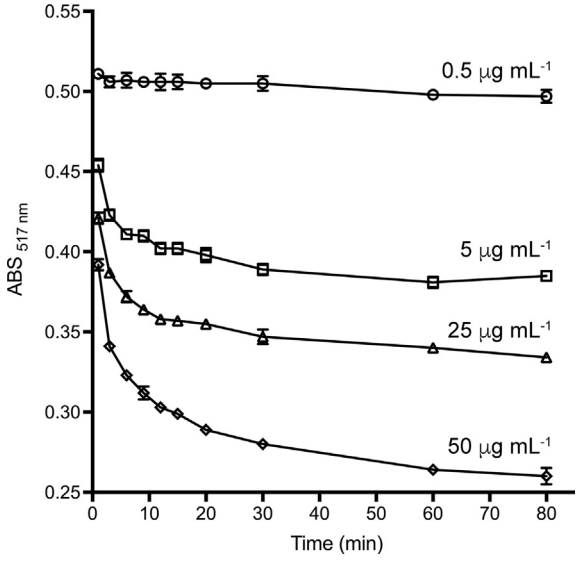

Fig. 1. Time-course of scavenging of DPPH free radicals by *R. rosea* extract.

Table 2
Characterization of the antioxidant activity of the aqueous extract of *R. rosea* (Asoltech).

Parameter	Value
FRAP value (μmoli Fe(II)/g of extract)	4767 (± 541, n = 5)
Antioxidant activity (IC_{50}) (mg/L of extract)	54
Total phenolic substances (mg GAE/g of extract)	247.2 (± 35.0, n = 27)
Flavonoids (mg QT/g of extract)	10.7 (± 1.8, n = 25)

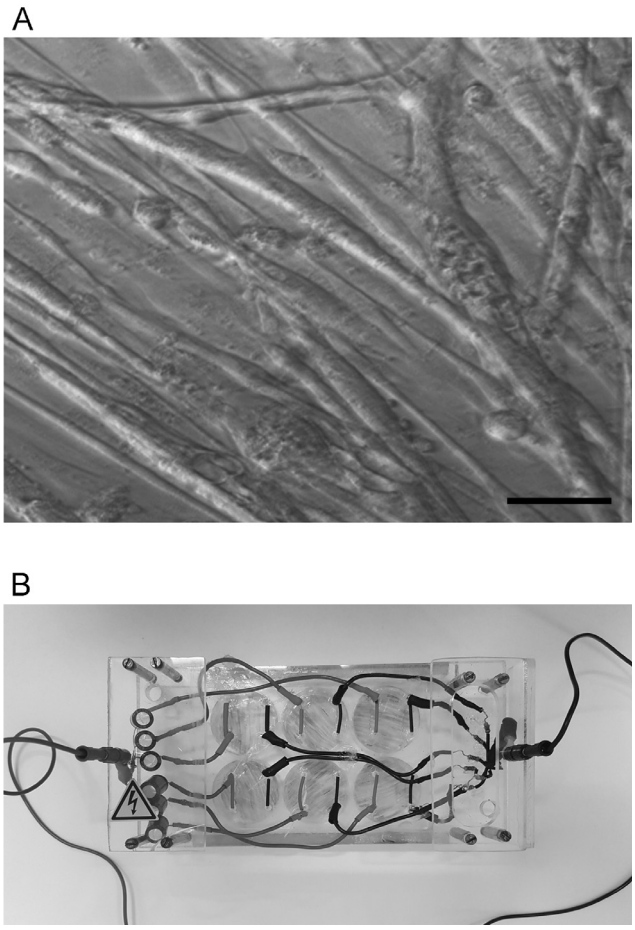

Fig. 2. Bright field representative image of skeletal mouse myotubes at 5 days in culture (A) and the electrical stimulator device designed and developed for 6-well culture plates (B). Bars, 50 μm.

3.3. Evaluation of skeletal myotube viability in R. rosea

Primary cultures of skeletal mouse myotubes (Fig. 2A) allow a characterization of a large number of skeletal muscle cell responses in controlled conditions and were used as a convenient experimental cell model to test the putative antioxidant effects of *R. rosea*. All the experiments were performed at 6–7 days in culture in differentiation medium (DM, for details see in Materials and Methods).

A first set of experiments was carried out to check the viability of skeletal myotubes exposed to R. *rosea*. The PrestoBlue viability assay was used, treating the cells with increasing concentrations of *R. rosea* aqueous extract (0.1–1000 μg/mL) for 1 or 24 h. As shown in Fig. 3, short time treatment (1h) did not affect cell viability at any of the

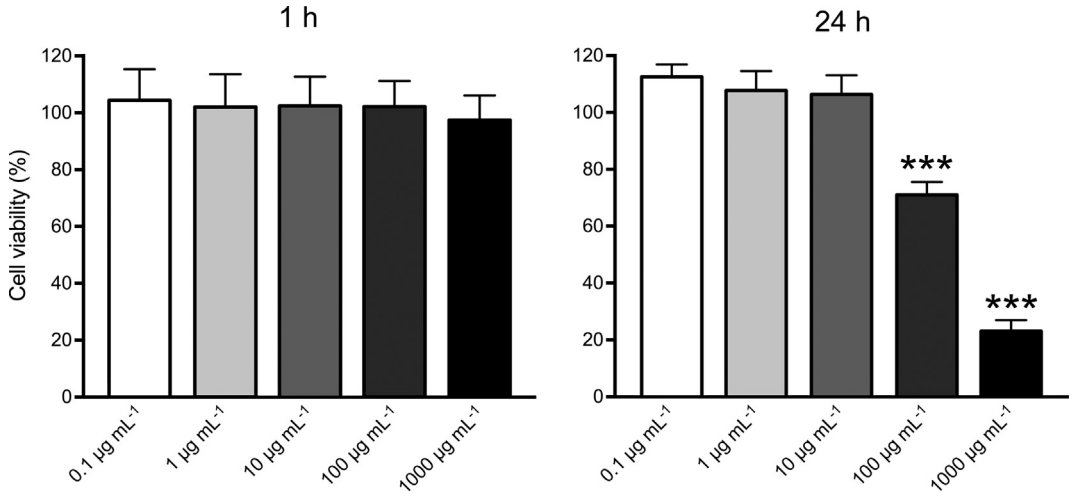

Fig. 3. Viability of mouse myotubes after 1 and 24 h treatment with different concentrations of *R. rosea*. Results are expressed as percentage cell viability relative to the controls. Four independent cultures were used, with at least eight replicates in each. Bars are mean ± SE (*** $P < 0.0001$).

concentrations tested. On prolonging the treatment to 24 h, a significant reduction in cell viability was detected at 100 and 1000 µg/mL, suggesting a cytotoxic effect of the compound at higher concentrations. Therefore, in all the following experiments, the concentrations of *R. rosea* were maintained in the range of 0.25–25 µg/mL to exclude any effect due to the toxicity of the extract.

3.4. Skeletal myotubes' ROS production induced by field electrical stimulation

Field ES was used to mimic the nerve activity able to trigger myotube twitching. Aiming to reproduce the effects elicited by moderate and intense exercise of training in *in vitro* models (Hellsten & Frandsen, 1997; Silveira, Pereira Da Silva, Carsten, & Hellsten, 2003), myotubes were plated in 6-well plates, and electrically stimulated in an incubator for 20 min, with 6 V biphasic rectangular 1 ms pulses, organized in 700 ms trains with a time interval of 300 ms. In particular, intra-train pulses were given at low (4 Hz) and high (100 Hz) frequencies to mimic a session of moderate or intense exercise, respectively. The fluorescent signal, proportional to the amount of ROS produced in the intracellular environment, was detected using the CM-H_2-DCFDA probe in control cells (unstimulated) and after muscle cell ES. Measurements were performed every 5 min for 30 min (Fig. 4). Measuring the Area Under the Curve (AUC, arbitrary units), the mean ROS production, was significantly increased (**** $P < 0.0001$) after 20 min of low (67671 ± 9410, 18 wells, 6 cell cultures) as well as high (164216 ± 28281, 45 wells, 9 cell cultures) frequency stimulations with respect to the control condition (35148 ± 1808, 37 wells, 10 cultures, Fig. 4).

In addition, a significant difference in ROS content was observed between low and high frequency stimulations ($P < 0.0001$).

According to the LDH and MDA releasing assays, ES caused no cell damage *per se* (Table 3).

3.5. The antioxidant effect of R. rosea extracts in skeletal myotubes

At first, the ROS production was measured in 7 day cultured myotubes, incubated for 30 min in 2.5 µg/mL *R. rosea versus* controls (untreated and unstimulated). No significant changes were observed measuring the Area Under the Curve (AUC) in *R. rosea* - treated cells (31.680 ± 2.431, n = 21 wells, 2 cell cultures) *versus* control condition (35.148 ± 1.808, n = 37 wells, 7 cell cultures, $P > 0.05$), revealing no significant *R. rosea* scavenging effect on the basal ROS production (Fig. 4). Then, the effect of *R. rosea* on ROS contents was quantified in

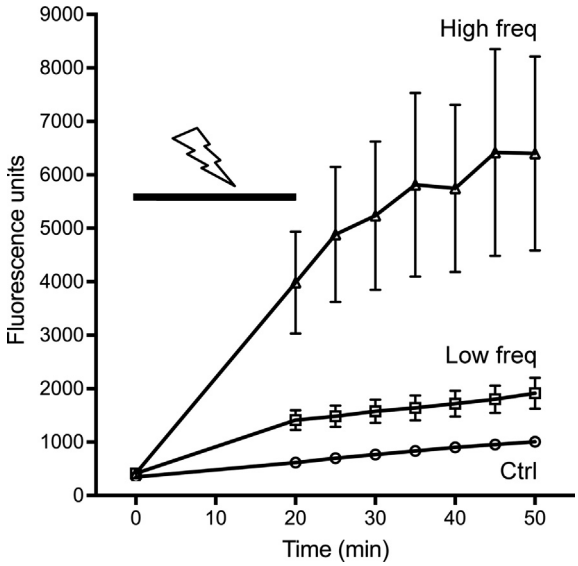

Fig. 4. Plot of intracellular ROS production before and after field electrical stimulation of myotubes. Average fluorescence signals (fluorescence units) are plotted over time before (at time 0) and after delivery of low (open squares) and high (open triangles) frequency electrical stimulations. The time-course of ROS production in non-stimulated cells is also shown (Control: open circles). Symbols represent means ± SE.

skeletal myotubes stimulated at low and high frequency. The cellular antioxidant activity (CAA), was calculated as defined in the Methods section. The addition of 2.5 µg/mL *R. rosea*, 30 min before the ES, attenuated the formation of intracellular fluorescence, both after low and high frequency stimulations, pointing to a marked protection effect against the acute intracellular oxidative stress (Fig. 5).

It is noteworthy that, despite the high variability of results, there was a recognizable trend to improve oxidative conditions when the cells were pre-treated with *R. rosea*. The high and low frequency ES produced indeed a pro-oxidant environment (bars 1, 3) but *R. rosea* as an antioxidant agent was able to modify the oxidative status of the myotubes (bars 2, 4–6). A dose-dependent effect of *R. rosea* treatment on intracellular ROS generation was also tested after high stimulation frequency (Fig. 5, bars 4–6). A significant reduction ($P < 0.05$) of ROS was observed in *R. rosea* used at 0.25–25 µg/mL although no significant difference was seen between 2.5 and 25 µg/mL, probably due to a possible saturation of intracellular transport of the antioxidant active

Table 3
The LDH release and MDA values on cell culture supernatants (n = 2 cell cultures for LDH and n = 3 cell cultures for MDA).

	control	control + 2.5 µg/mL *R. rosea*	high freq	high freq + 2.5 µg/mL *R. rosea*
LDH release (%)	4.526 (± 0.33)	4.545 (± 0.33)	3.675 (± 0.24)	3.510 (± 0.29)
MDA (µmoli/L)	0.13 (± 0.02)	0.22 (± 0.01)	0.16 (± 0.03)	0.16 (± 0.01)

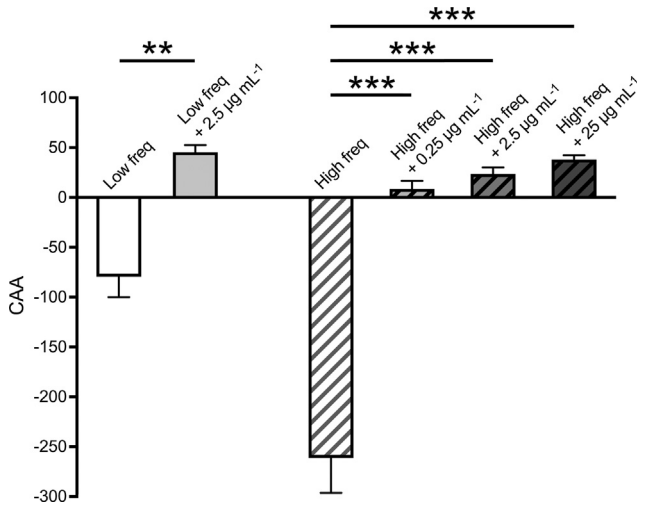

Fig. 5. Percentage of cellular antioxidant activity (CAA), of *R. rosea* during different ES conditions. 1: low ES ($n = 18$ wells, 6 cell cultures); 2: low ES + 2.5 µg/mL *R. rosea* ($n = 18$ wells, 6 cell cultures); 3: high ES ($n = 45$ wells, 9 cell cultures); 4: high ES + 0.25 µg/mL *R. rosea* ($n = 16$ wells, 6 cell cultures); 5: high ES + 2.5 µg/mL *R. rosea* ($n = 23$ wells, 8 cell cultures); high ES + 25 µg/mL *R.* rosea ($n = 10$ wells, 4 cell cultures). Negative CAA values indicate a *pro*-oxidant effect (CM-H2-DCFDA fluorescence levels above that of control cells), whereas positive values indicate an antioxidant effect. $^{**}P < 0.001$; $^{***}P < 0.0001$.

compound.

Catalase is the antioxidant enzyme responsible for the decomposition of hydrogen peroxide to water and oxygen. The potential effect of *R. rosea* extract (2.5 µg/mL) on catalase activity was also tested. The high frequency stimulation, *per se*, did not significantly change the enzymatic activity (stimulated: 4.769 ± 0.729 U/mg prot; unstimulated: 5.623 ± 2.608 U/mg prot; $P > 0.05$). Likewise, R. *rosea* treatment did not significantly change the enzymatic activity both in stimulated (R. *rosea*: 4.309 ± 2.500 U/mg prot; control: 4.769 ± 0.729 U/mg prot; $P > 0.05$) and unstimulated cells (*R. rosea*: 4.558 ± 1.399; control: 5.623 ± 2.608 U/mg prot; $P > 0.05$).

4. Discussion

Despite numerous theories and the extensive literature relating to the natural adaptogen *R. rosea*, its direct effect on single muscle cells has not previously been tested. An aqueous extract of *R. rosea* was used here, which appeared to protect cultured skeletal muscle cells from oxidative damage, an effect particularly evident after subjecting myotubes to high frequency electrical stimulations, responsible for higher ROS production.

4.1. Polyphenols in the R. rosea water extract

Many classes of compounds are present in the *Rhodiola* root and their relative percentages are species-dependent: oils, fats, sterols, glycosides, organic acids, polyphenolics, tannins and proteins (Panossian, et al., 2010; Pooja, Anilakumar, & Bawa, 2006; Tayade, Dhar, Sharma, et al., 2013).

One of the characteristic features of *R. rosea* is the presence of a relatively high content of cynnamic alcohol glucosides like rosavin (phenylpropanoids), and tyrosol glucosides such as salisroside/rhodioloside (phenylethanoids), not detected in other genus of *Rhodiola* species (Cuerrier & Ampong-Nyarko, 2015). In this study, only the polyphenolic classes have been taken into account, due to their known antioxidant properties. Among the many extraction and separation methods of polyphenolic compounds proposed (Kalili & de Villiers, 2011; van Diermen et al., 2009), the water extraction method has been chosen here in order to evaluate soluble compounds released/dissolved during oral administration, obtaining only water soluble fractions, to be used in muscle cell cultures. The complex composition of such *R. rosea* extracts included several polyphenols, in line with the antioxidant activity of *Rhodiola*, highly known for its adaptogenic effects. Given the presence of aromatic rings and hydroxyl groups, plant-derived phenolics exhibit, among others, marked antioxidant properties (Škerget et al., 2005; Zheng & Wang 2001).

4.2. Antioxidant properties of R. rosea

The strong antioxidant activity of our sample was confirmed by the IC_{50} value of 54 µg/mL, lower than that found in other studies (350 µg/mL, *Rhodiola imbricata*, Senthilkumar, Parimelazhagan, Om Prakash Chaurasia, & Srivastava, 2013). Even at low concentrations, many of the phytocomponents found in the *R. rosea* extract have marked antioxidant properties (Zhou et al., 2015). For example, epigallocatechin gallate, kaempferol and quercetin derivatives have a wide range of antioxidant effects, both *in vitro* and *in vivo*. They act as super-oxide scavengers mainly against ROS species (Boots et al., 2008; Calcabrini et al. 2010; Casanova et al., 2014). At high concentrations, they increase the activity or expression of antioxidant enzymes such as SOD and CAT also in experiments *in vivo* (Kanlaja, Khamchun, Kapincharanon, & Thongboonkerd, 2016). In particular, kaempferol prevented the oxidation of low-density lipid proteins showing a protective role against atherosclerosis (Fuhrman & Aviram, 2001; Vaya et al., 2003). A protective antioxidant role of such compounds to detoxify metabolic reactive intermediates during the high muscle activity has therefore been suggested.

4.3. An in vitro model of exercise to reveal the antioxidant properties of R. rosea

At the skeletal neuromuscular junction, acetylcholine (ACh) is released from motor nerve terminals to bind nicotinic acetylcholine receptors (nAChRs) at the endplate membrane and depolarize the skeletal fibers to elicit action potentials and muscle contractions. Such nerve activity can be mimicked by ES of isolated muscle cells in culture. An *in vitro* model of exercise was used here to detect the antioxidant properties of the natural product *R. rosea* on muscle cells, when ES induced muscle cell twitching. Myotube cultures with a developed excitation–contraction coupling were used, as a well-established *in vitro* model (Lorenzon et al., 2002). Even if simplified, with such a system, physiological events such as visible contractions, stimulation of glucose uptake, and up-regulation of the cytokine IL-6, can be induced (Nikolić et al., 2017) giving information on what occurs *in vivo* and mimicking muscle cell activity during exercise (Clausen, 2013; Nikolić et al., 2012; Sciancalepore, Coslovich, Lorenzon, Ziraldo, & Taccola, 2015).

In particular, ROS production has been associated with muscle contraction during physical exercise (Davies et al., 1982) and was found

to be related to the intensity, frequency and duration of exercise (Gomez-Cabrera, Ferrando, Brioche, Sanchis-Gomar, & Via, 2013). ROS induced by oxidative stress mediates cell damage through oxidation of lipids, proteins and DNA (Avery, 2011; Davalli, Mitic, Caporali, Lauriola, & D'Arca, 2016; Ďuračková, 2010; Finaud et al., 2006). In a first attempt, the purpose of our study was not to detect the nature of the oxygen intermediates or the specific damage of high ROS production. Our aim was to examine the potential antioxidant effect of the aqueous extract of *R. rosea* on muscle cells exposed to oxidative insults, in particular during high frequency twitching, when ROS production can be less easily counteracted by endogenous antioxidants. ROS agents could be represented by superoxide radicals, hydrogen peroxide and hydroxyl radicals. Those produced by intense ES in cultured muscle cells appear to be represented by H_2O_2, freely diffusible across cell membranes (Silveira et al., 2003). The adaptogen under examination displayed an overall significant decrease of intracellular ROS generation during intense and moderate ES, when compared to controls. The results on the effect of *R. rosea* on catalase activity excluded the adaptogen antioxidant effect mediated by the positive regulation of such enzymatic activity.

Murine myotubes treated for 1 h with *R. rosea* did not experience any loss of viability at concentrations of 1–1000 μg/mL. On the other hand, when the cells were treated for 24 h, a concentration-dependent increase in cytotoxicity was observed. Since polyphenols have low bioavailability, it is difficult to reach cytotoxic concentrations under physiological conditions (Lee et al., 2002; Shahrzad, Aoyagi, Winter, Koyama, & Bitsch, 2001). Consistent with the results obtained by Silveira and colleagues in similar experiments (Silveira et al., 2003), 20 min of intense ES, increased intracellular DCFH oxidation with respect to the controls, even if the range of intracellular ROS production was not comparable, probably because of the different experimental conditions and stimulation patterns. Such experimental differences could be also responsible for the different results mediated by moderate ES which, in our case, induced low but significant levels of ROS production.

According to the hormesis principle (Calabrese & Baldwin, 2003), low ROS production during moderate exercise can also have beneficial roles on muscle tissue; among them it can contribute to promote the ATP-mediated proliferation of mouse skeletal myoblasts favoring muscle cell regeneration (Sciancalepore et al., 2012). Interestingly, the higher ROS production observed during high frequency stimulation in cultured cells, comparable to repetitive muscle contractions characterizing intensive exercise (Davies et al., 1982; Steinbacher & Eckl, 2015; for a review Powers & Jackson, 2008), was counteracted by the natural antioxidant *R. rosea* in a dose-dependent way. The antioxidant activity of *R. rosea* extracts is widely described in the literature. In all cases, the protective role was assessed against an exogenous trigger such as H_2O_2, tert-butylhydroperoxide, Fenton type reactions, etc. (Calcabrini et al., 2010; Kanupriya et al., 2005; Schriner, Avanesian, Liu, Luesch, & Jafari, 2009). Indeed, it was reported that murine skeletal muscle lines (C2C12 myotubes), treated with a *R. rosea* extract (Rhodiolife), exhibit an increased resistance against H_2O_2-induced oxidative stress after 24 h exposure (Hernández-Santana, Pérez-López, Zubeldia, & Jiménez-del-Rio, 2014). ROS-induced fatigue and injury of skeletal muscles (Powers et al., 2004; Powers & Sen, 2000) can be counteracted by endogenous antioxidants but because their activity is not always sufficient, natural herbal antioxidants are thought to deliver health benefits. Our data evaluated the effect of the water extract on the physiological production of ROS during muscle contraction, showing that *R. rosea* is able to break down the oxygen species produced at the resting cell level. The efficacious tested antioxidant activity of *R. rosea* in high frequency stimulated cultures was at 0.25, 2.5 μg/mL and much more at higher dose (25 μg/mL). These results are in good agreement with the doses found to be efficient on HSP70 modulation in C2C12 cells (10 μg/mL, Hernández-Santana et al., 2014) as well as those used evaluating the anti-adipogenic and lypolitic activity on human visceral adipocytes (5–70 μg/mL, Pomari, Stefanon, & Colitti, 2015).

Our findings are interestingly promising, suggesting that *R. rosea* can be proposed as antioxidant supplementation to prevent the onset of degenerative diseases in less active but also in old active people, more susceptible to oxidative stress, when antioxidant enzymes and regenerative capacity decline (Bobeuf, Labonte, Dionne, & Khalil, 2011). Moreover, the antioxidant effects of *R. rosea* on muscle cells under strenuous physical exercise may restore the physiological ROS level. In this sense, the consumption of *R. rosea* by athletes, as an active principle of drug, may be able to reduce the overtraining side effects. The effects of long-term *R. rosea* intake on physical performance remains to be further clarified, taking into account the possible combined antioxidant effect of *R. rosea* as a radical scavenger and positive regulator of enzymatic activity.

R. rosea and adaptogen effects cannot be explained by a single receptor-based view of drug action (for a review see Panossian, 2017). *R. rosea* root extracts protect skeletal muscle cells against chemically-induced oxidation by free radical scavenging and multi-target action including up-regulation of HSP70, activation of enzymatic systems via signal transduction pathways and enhancement of mitochondrial function (Jówko et al., 2016; Panossian, 2017). New questions can be posed for further investigations about the molecular mechanisms through which *R. rosea* could be exerting its fascinating effects.

5. Conflict of interest

The authors declare no conflict of interest.

6. Ethical statement

The material of our manuscript has not been published in whole or in part elsewhere. The manuscript is not currently being considered for publication in another journal. All authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content. Primary murine myotube cultures were kindly provided by Dr A. Wernig (Department of Physiology and Medical Policlinic, University of Bonn, Germany). Briefly, satellite cells were isolated from the hindlimb of 7-day old male Balb/c mice and expanded *in vitro* following previous protocols. All animal procedures were performed in accordance with the German law for protection of experimental animals (Irintchev et al., 1997).

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