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GEOSCIENCES

Rosmarinus officinalis extract-loaded emulgel prevents UVB irradiation damage to the skin

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Abstract: UVB-irradiation increases the risk of various skin disorders, therefore leading to inflammation and oxidative stress. In this sense, antioxidant-rich herbs such as *Rosmarinus officinalis* may be useful in minimizing the damage promoted by reactive oxygen species. In this work, we report the efficacy of a *R. officinalis* hydroethanolic extract (ROe)-loaded emulgel in preventing UVB-related skin damage. Total phenols were determined using Folin-Ciocalteu assay, and the main phytocomponents in the extract were identified by UHPLC-HRMS. Moreover, *in vitro* sun protection factor (SPF) value of ROe was also assessed, and we investigated the *in vivo* protective effect of an emulgel containing ROe against UVB-induced damage in an animal model. The ROe exhibited commercially viable SPF activity (7.56 ± 0.16) and remarkable polyphenolic content (24.15 ± 0.11 mg (Eq.GA)/g). HPLC-MS and UHPLC-HRMS results showcased that the main compounds in ROe were: rosmarinic acid, carnosic acid and carnosol. The evaluation of the *in vitro* antioxidant activity demonstrated a dose-dependent effect of ROe against several radicals and the capacity to reduce iron. Therefore, we demonstrated that topical application of the formulation containing ROe inhibited edema formation, myeloperoxidase activity, GSH depletion and maintained ferric reducing (FRAP) and ABTS scavenging abilities of the skin after UVB exposure.

Key words: Antioxidants, cosmeceuticals, dermatology, phytochemicals, sunscreens.

INTRODUCTION

Photoaging is a complex and multifactorial process caused by both intrinsic and extrinsic factors among which is UVB radiation. This high frequency radiation is also considered the most energetic and was reported to promote photocarcinogenesis (Strozyk & Kulms 2013). Furthermore, excessive UVB skin exposure can cause sunburn; edema; erythema; hyperplasia; melanoma and even carcinogenesis (Ye et al. 2017).

Regarding radiation-induced oxidative stress, UVB participates in indirect damage to biomolecules through the production of reactive oxygen and nitrogen species (RONS), which are capable of peroxiding lipids, as well as oxidizing proteins and DNA. The accumulation of oxidized products, such as lipid hydroperoxides, protein carbonyls and 8- hydroxydeoxyguanosine, has been linked to the onset of skin cancers (Vayalil et al. 2004). Moreover, UVB exposure also affects the level antioxidants in the skin, therefore impairing the skin's ability to protect itself against RONS, henceforth aggravating inflammatory response and increasing infiltration of inflammatory blood leucocytes to inner tissue layers (Ishida & Sakaguchi 2007).

The chemical and physical filters which are present in most commercial sunscreens lack action over the biochemical and physio pathological mechanisms triggered by UV. In this context, the investigation of new dermal formulae capable of tackling the molecular effects of RONS is of upmost relevance to minimize photoaging and prevent UVB-related ailments (Batista et al. 2018). Concerning innovative strategies to promote photoprotection, the use of natural compounds capable of absorbing UV rays and thereby protect the skin against UVB and UVA radiation is of great interest. In this sense, polyphenolic compounds have been cited in literature as potential photo protecting agents due to their structural similarities and absorbance spectrum profiles to commercial organic UV filters (Velasco et al. 2008). Besides these attributes, polyphenolic compounds exhibit a wide range of biological activities, such as: antioxidant capacity; radical scavenging activity; anti-inflammatory; immunomodulatory and antitumoral activities (Dinkova-Kostova 2008), which are also remarkable in the context of minimizing UV-related skin damage.

Among the several administration pathways to deliver antioxidants into inner layers of the skin, topical application has been the focus of most studies. The non-invasive transdermal delivery of RONS scavengers has been reported to prevent or delay UVB-induced skin damage, and also provides photo-chemoprotective effects which fortify the endogenous protection system and ultimately reduce the oxidative damage in the skin (Abla & Banga 2013, de Souza et al. 2017, Salucci et al. 2014), In this context, many studies have focused on the topical application of natural product-containing formulae such as: marigold, green tea, *Pimenta pseudocaryophyllus* and wild chrysanthemum extracts, all of which showcased biological effects by protecting the skin against UV-related damage (Afaq & Katiyar 2012, Campanini et al. 2013, Fonseca et al. 2011, Sun et al. 2016).

Concerning antioxidant-rich herbs which may be used for photoprotection, some species such as *Rosmarinus officinalis* L. (Lamiaceae) are promising due to their rich polyphenolic profile. Popularly known in Europe as rosemary and in Brazil as alecrim (Mohamed et al. 2016), this herb is widely used in different parts of the world as a flavoring agent in drinks and cosmetics (NGO et al. 2011). Notwithstanding, many studies have shown the pharmacological potential of *R. officinalis* against cancer and oxidative stress (Kontogianni et al. 2013, Parmar et al. 2011), as well as the hepatoprotective (El-Hadary et al. 2019), hypoglycemic-hypolipidemic (Bustanji et al. 2010), antibacterial (Amaral et al. 2019, Bozin & Mimica-Dukić 2007), and anti-inflammatory activities (Altinier et al. 2007). Nonetheless, it is noteworthy that the therapeutic potential of *R. officinalis* may be tapped without many concerns regarding toxicity, given the broad therapeutic window if most of its polar constituents (Derwich et al. 2011, Okoh et al. 2010).

Rosemary has been reported to be of potential therapeutic benefit in the treatment and/or prevention of several illnesses, such as: asthma; spasmogenic disorders; peptic ulcer; inflammatory diseases; hepatotoxicity; atherosclerosis; ischemic heart disease; cataract; and poor sperm motility (De Oliveira et al. 2019, Ulbricht et al. 2010). These different biological properties can be related to the rosemary's appreciable content of polyphenolic compounds, especially rosmarinic acid (Couto et al. 2012, Erkan et al. 2008), carnosic acid and carnosol (Arranz et al. 2015) which are considered chemical markers of this species.

Although *R. officinalis* is known to present remarkable antioxidant and anti-inflammatory activities, its use in topical formulation against oxidative damage caused by UVB radiation

has not been reported so far. In this sense, we investigated *R. officinalis* hydroethanolic extract (ROe) chemical composition and antioxidant capacity by different *in vitro* methods and tested the efficacy of an emulgel comprising ROe in the *in vivo* protection against oxidative stress caused by UVB irradiation in hairless mice model.

MATERIALS AND METHODS

Materials

Gallic acid and Folin-Ciocalteau were obtained from Fluka Chemical Co. (Buchs, Switzerland) and propylene glycol from Chemco LTDA. 2,2-diphenyl-1- picrylhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline- 6-sulfonic acid) (ABTS), 2,4,6-tris(2-pyridyl-s-triazine (TPTZ), o-dianisidine dihydrochloride, ethylene glycol bis (-aminoethyl ether)-N,N,N0,N0-tetraacetic acid (EGTA), reduced glutathione (GSH), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), hexadecyltrimethylammonium bromide (HTAB), luminol, horseradish peroxidase (HRP), xanthine, xanthine oxidase (XOD) and Trolox were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Raw materials for formulations were obtained from Galena (Campinas, SP, Brazil). All other reagents used were of pharmaceutical grade.

R. officinalis L. hydroethanolic extract (ROe)

Rosemary leaves were bought in the Shangri-là folk market in Londrina, Paraná State, Brazil. The leaves were dried at room temperature (25 ± 2°C) and ground using a knife mill. Powdered material was stored protected from light and moisture exposure for subsequent use during the extraction procedures. The ROe was obtained by exhaustive maceration (10% m/v) using a hydroethanolic solution (80% v/v) at room temperature for 24 h. Afterwards, the extract was filtered and concentrated under vacuum using a rotatory evaporator.

Determination of total polyphenolic contents

The concentration of total polyphenols in the ROe was determined by spectrophotometry as described elsewhere (Georgetti et al. 2006). The reaction mixture was prepared by mixing 0.5 mL of test solution, 0.5 mL of Folin-Ciocalteu's reagent and 0.5 mL 10% Na_2CO_3. Blank was concomitantly prepared, containing 0.5 mL of purified water, 0.5 mL Folin-Ciocalteu's reagent and 0.5 mL of 10% m/v of Na_2CO_3. The samples were sheltered from light and maintained at room temperature for 1 h thence the absorbance was measured at 760 nm. Total polyphenolic content was expressed as mg/g (gallic acid equivalents, Eq.GA).

Sun protection factor (SPF)

The ROe was dissolved in ethanol to reach the concentration of 0.2 µg/mL. The SPF model used in this study was according to the methodology described by Mansur et al (Mansur et al. 1986). The sample absorbances were measured in UVB wavelength range (290-320 nm), with 5 nm increase and three determinations were made at each point. The SPF was calculated by the equation: $SPF = CF \times {}_{290}\Sigma^{320}{}_{290}\, EE(\lambda) \times I(\lambda) \times abs(\lambda)$, where: CF (correction factor) = 10; EE (λ) is the erythemal efficiency spectrum; I (λ) is the solar intensity spectrum; abs (λ) is the absorbance of the solution. The values of EE (λ) x I (λ) are in accordance with Sayre et al. (1979).

HPLC-MS analysis

The ROe was analyzed according to a previously described method (Couto et al. 2011), in which a HPLC Prominence Shimadzu® coupled to a mass spectrometer amaZon SL Bruker Daltonics® was used. The HPLC system comprised a LC-20AD pump, DGU 20-A3R online degassing unit, SPD-M20A diode array detector, CTO-20A

column oven, SIL-20AHT automatic injector and CBM-20 communication module. The dried extracts were solubilized in ACN:H_2O (8:2, v/v) at a concentration of 1.0 mg/mL, centrifuged for 3 min at 10000 *g* in an Eppendorf® Minispin centrifuge. Thereafter, the supernatant was analyzed. Chromatographic separations were performed in a Phenomenex® C_{18}-Luna column (250 x 4.6 mm i.d., 5 µm). The injection volume was 2 µL. The eluent system consisted in H_2O (A) and ACN (B), both acidified with 0.1% v/v formic acid, and we used a linear gradient from 5 to 100% B in 40 min at a flow rate of 1.0 mL/min. The oven temperature was set at 25 ˚C. The mass spectra were obtained separately in both positive and negative mode, in a mass range of 50-1200 Da and applying auto-MSn (n = 3) mode. The mass spectrometer source parameters were set as follows: capillary voltage at 4.5 V, nitrogen used as the nebulizing and drying gas (50 psi, 10 L min^{-1}, 300 °C). The data was processed through Bruker Compass Data Analysis 4.3® software.

UHPLC-HRMS analysis

The high-resolution mass spectrometry analysis was performed on a Waters Acquity UPLC H-Class system equipped with a quaternary pump, degassing system, auto sampler, column oven, PDA detector and a Xevo G2-XS QToF Mass Spectrometer (Waters, MA, USA). The ROe was analyzed using an Acquity UPLC HSS T3 (High Strength Silica C_{18}, 1.8 µm, 100 x 2.1 mm), at 40˚C and injection volume of 0.2 µL. The quaternary pump system consisted of acetonitrile (+ 0.1% formic acid v/v, A) and H_2O (+ 0.1% formic acid v/v, D), and a linear gradient was employed from 5 to 100% of organic solvent in 15 min. PDA detection wavelengths were acquired at 200-400 nm. The mass spectra were acquired in negative mode, in a mass range of 50 - 1200 Da using data-dependent acquisition mode (DDA) to obtain MS and MS/MS data, and the parameters used for data acquisition were as follows: capillary voltage of 3.0 kV, cone and desolvation gas flow of 50 and 800 L h^{-1}, respectively, source temperature of 100˚C and desolvation temperature of 650˚C with sampling cone and source offset at 30 and 80, respectively. A ramp collision energy (CE) was employed for MS/MS fragmentation: low CE from 6 to 9 eV and a high CE of 60 to 80 eV. The data were processed through MassLynx® 4.1 software.

Determination of antioxidant capacity

The reduction of $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ radicals were determined by the hypochromic shift in the absorbance at 517 nm (Georgetti et al. 2006) and 730 nm (Campanini et al. 2014), respectively. The ferric reducing antioxidant power of ROe was determined by FRAP assay at 595 nm. The FRAP value was expressed as micromoles trolox standard equivalents per micrograms of extract (Campanini et al. 2014). The antioxidant activity of the extract was analyzed by inhibition of chemiluminescence produced in the H_2O_2/luminol/horseradish peroxide (HRP) system and in inhibiting chemiluminescence produced in the xanthine/luminol/xanthine oxidase (XOD) system (Marquele et al. 2005). In the free radical scavenging methodologies which were herein employed, the percentage of inhibition was plotted against different concentrations of Roe, and the concentration that caused 50% inhibition of the system was reported as the IC_{50} value. The percentage of inhibition was calculated using the Equation (1):

$$Inhibition\ (\%) = 100 - \left[\frac{(100\ x\ As)}{A0}\right] \quad (1)$$

In which as is the absorbance (spectrophotometric methods) or luminescence (chemiluminescent method) observed when the experimental sample was added, and A0 is the absorbance (spectrophotometric methods) or

luminescence (chemiluminescent method) of the positive control (ROe absence).

Emulgel production

A topical emulgel was prepared using a self-emulsifying wax Polawax® (ketostearyl alcohol + polyoxyethylene derived of a fatty acid ester of sorbitan 20E) and non-ionic hydrophilic colloid (hydroxyethylcelulose, Natrosol®) as gelling agent. Caprylic/capric triglycerides was used as emollient, and propylene glycol as moisturizer. In addition, Phenonip® was used to protect the formulation against microbial contamination. Deionized water was used for the preparation of all formulations (Table I). After 24 h, the ROe (5% m/m) was incorporated to the emulgel at room temperature. Control emulgel differed from the active formulation only in the absence of ROe.

Evaluation of *in vivo* antioxidant efficacy of ROe loaded emulgel

Animals

To perform the *in vivo* experiments, sex matched hairless mice (HRS/J) weighing 20 - 30 g were used, obtained from the University Hospital of Londrina State University. Mice were maintained at a temperature of 23°C ± 2 for a 12 h light and 12 h dark cycles, with free access to water and food. The experimental protocol for this study was approved by The Animal Ethics Committee of the Londrina State University (CEUA) nº 44/2017, process nº 2974.2017.14.

Emulgel administration

Hairless mice were randomly separated into groups of 5 mice each, as follows: non-irradiated control; irradiated control, irradiated and treated with control emulgel (absence of ROe); irradiated and treated with ROe 5% enriched emulgel. Treated groups received topically 0.5 g of emulgel (with or without ROe 5%), applied with a brush, every 6 h, starting 12 h before the beginning of the irradiation and ending right after, hence, a total of 4 treatments, three of which before UVB radiation and one after. This protocol followed previous treatment standardizations of our research group (Martinez et al. 2016).

Irradiation

The UVB source used in this work was a Philips TL/12 RS 40W (Medical-Holland) lamp emitting a continuous spectrum between 270 and 400 nm with a peak emission at 313 nm cm^2 (Campanini et al. 2014, Ivan et al. 2014). There was 20 cm distance between the lamp and mice position

Table I. Composition of the emulgels used in the investigation of the *in vivo* efficacy of ROe in preventing UVB irradiation damage to the skin.

Components	Control (ROe free)	Active (plus ROe)
Polawax®	2.0% m/m	2.0% m/m
Propylene glycol	5.0% m/m	5.0% m/m
Caprylic/capric triglyceride	3.0% m/m	3.0% m/m
Natrosol®	1.5% m/m	1.5% m/m
Phenonip®	0.8% m/m	0.8% m/m
ROe	-	5.0% m/m
Deionized water qsp	100.0% m/m	100.0% m/m

with a radiation of 0.209 mW/cm^2. An IL 1700 radiometer (Newburyport, MA, USA) equipped with the sensor for UV (SED005) and UVB (SED240) was used to determine the radiation intensity. UVB dose used to induce skin inflammation was 4.14 J/cm^2 (Campanini et al. 2013). All groups were radiated simultaneously. Mice were terminally anesthetized with 5% isoflurane (Abbott [Abbott Park, IL, USA]) 12 h after radiation exposure, and all dorsal skin was removed for edema, GSH, FRAP, ABTS and myeloperoxidase (MPO) tests.

Skin edema

To evaluate the increase in skin weight after exposure to UVB radiation, samples were collected with the same area cut by using a mold and then weighed. Afterwards the comparison between the unexposed and the exposed controls groups was compared with the results of the mice treated with extract (Campanini et al. 2014, Martinez et al. 2015).

Myeloperoxidase activity (MPO)

Neutrophil infiltration was indirectly determined by MPO activity. MPO catalyzes the oxidation of *o*-dianisidine to a colored compound (Campanini et al. 2013). The skin samples from different groups were homogenized with 400 µL of 0.05 M (pH 6.0) phosphate buffer added of 0.5% hexadecyl trimethyl ammonium bromide (HTAB) with the aid a Tissue-Tearor (Biospec®). The resulting homogenate was centrifuged at 16,100 *g* for 2 min at 4°C. The reaction was performed adding 200 µL of 0.0167 mg/mL *o*-dianisidine in 0.05 M phosphate buffer (pH 6.0) and 0.015% hydrogen peroxide to a 30 µL aliquot of the sample supernatant. Thereafter, the absorbance was measured at 450 nm and compared to a neutrophil standard curve to yield the results, which were expressed as number of neutrophils/mg of skin (Campanini et al. 2014, Martinez et al. 2015).

Reduced glutathione (GSH) assay

Literature reports that UVB irradiation induced a decrease in GSH levels (Casagrande et al. 2006, Fonseca et al. 2011, Martinez et al. 2016). In order to investigate this effect in our work, each skin sample was homogenized in 0.02 M EDTA and whole homogenates were treated with 50% trichloroacetic. The samples were then centrifuged twice (2,700 *g*, 10 min, 4°C). The reaction mixture contained 50 µL of sample supernatant, 100 µL of 0.4 M Tris and 5 µL of a 1.9 mg/mL solution of DTNB in methanol. The absorbance was measured at 405 nm. Data was analyzed using a standard curve of GSH (5-150 µM) and the results were presented as µM GSH per mg of skin(Martinez et al. 2016).

ABTS assay

The ability to scavenge ABTS radical through the skin was measured by the decrease in absorbance at 730 nm. Each skin sample was homogenized with 400 µL of KCl (1.15%), using a Tissue-Tearor (Biospec), and centrifuged (1000 *g*, 10 min, 4°C). A 7 µL aliquot of the supernatant was mixed with ABTS reagent (7 mM of ABTS and 2.45 mM of potassium persulfate) diluted with phosphate buffer pH 7.4 to obtain absorbance of 0.7 at 730 nm (Campanini et al. 2014, Martinez et al. 2015). Samples absorbance were determined after 6 min at 730 nm. The results were compared to a Trolox curve (0.01-20 nmol) and presented as nmol Trolox equivalent per mg of skin.

FRAP assay

Evaluation of the ferric-reducing antioxidant power of skin after exposure to UVB radiation was analyzed by the FRAP assay (Ivan et al. 2014, Katalinic et al. 2005). Skin samples from different groups were homogenized with 400 µL of KCl (1.15%) and centrifuged (1,000 *g*, 10 min. 4°C). A 30 µL aliquot of the supernatant was mixed with the FRAP reagent (0.3 mM acetate buffer

pH 3.6; 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine in 40 mM hydrochloride acid; and 20 mM ferric chloride). The absorbance was determined at 595 nm. The results were compared to a Trolox curve (0.01 - 20 nmol) and presented as nmol Trolox equivalent per mg of skin(Campanini et al. 2014, Martinez et al. 2015).

Data analysis

For all the *in vitro* experiments, data was expressed as average ± standard deviation (SD) of at least five replicates. ROe *in vitro* antioxidant activity in the various methods was expressed by the half maximal inhibitory concentrations (IC_{50}), being determined using hyperbolic curve equation from GraphPad Prism 7 software (GraphPad Software Inc., San Diego, EUA). Statistical analysis of the data from the *in vivo* experiments was performed using the same software package. Data was presented as average ± standard error (SEM) of measurements made with 5 animals in each group per experiment, and analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons test. The results were representative of 2 separate experiments and were considered significantly different at $p<0.05$ (95% confidence interval).

RESULTS AND DISCUSSION

Total polyphenol content, SPF value and antioxidant capacity

Total phenolic content is an important index for evaluating the antioxidant features of herbal extracts because phenols are the major antioxidant compounds that are able to stabilize free radicals (Fu et al. 2011). The phenolic compounds in the ROe were quantified using the Folin-Ciocalteu colorimetric method. Figure 1 showcased ROe phenolic content as well as

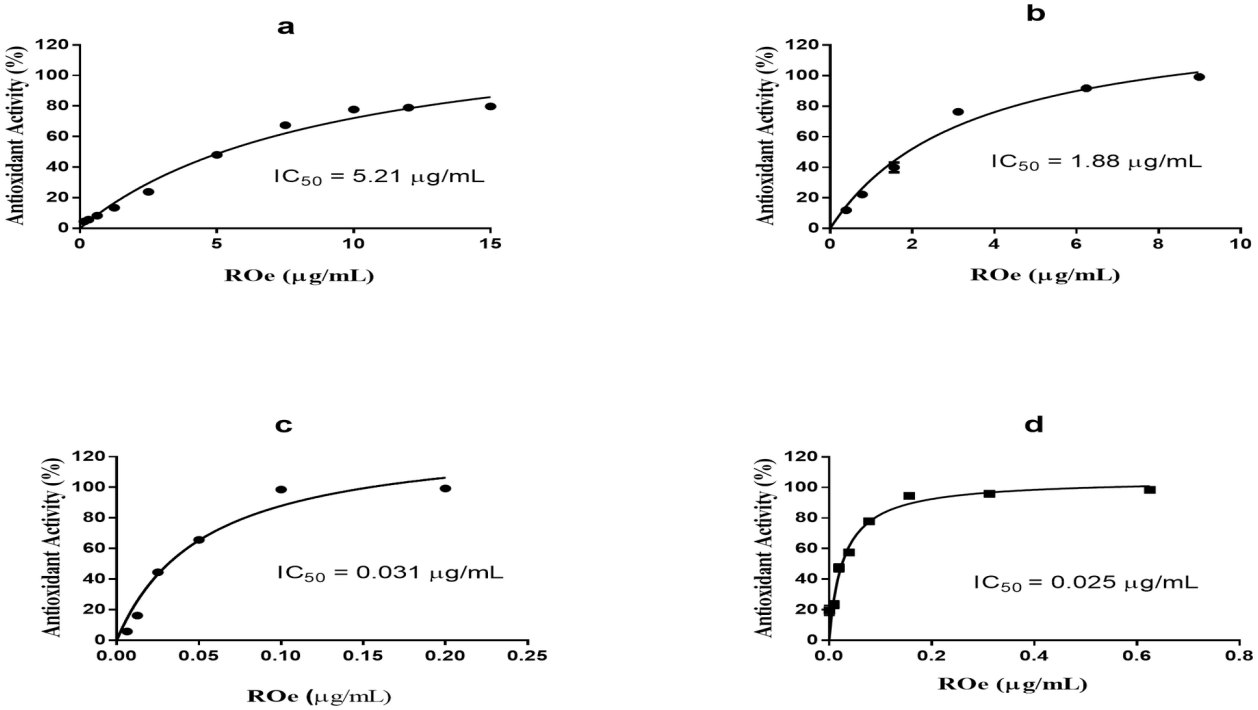

Figure 1. ***In vitro*** **antioxidant activity of ROe. The extract was added at indicated concentrations and assayed for scavenging the radical DPPH (a), radical ABTS (b), inhibition of light emission from H_2O_2/luminol/HRP (c) and xanthine/luminol/XOD (d) luminescent reactions with luminol.**

antioxidant capacity through different *in vitro* tests.

The polyphenolic content of ROe showcased Eq.GA of 24.15 ± 0.11 mg/g and 30.2 ± 0.24% (dry basis) of polyphenolic content. ROe was prepared using percolation with ethanol:water solution (80:20 v/v) as extracting method (Couto et al. 2012). An extract obtained by subsequent extraction of the dry mass with solvents of increasing the polarity, such as ethyl acetate and hexane obtained 54.6 ± 2.2 mg/g extract of polyphenolic content (Kontogianni et al. 2013). Although the polyphenolic content was lower in the present study than previous studies (Couto et al. 2012, Kontogianni et al. 2013), this was not a limiting factor regarding the biological activity of ROe, as will be further discussed in this manuscript. This finding can be explained since it is known that the applied extraction method and the solvent system used to obtain an extract can vary its yield and composition (Moure et al. 2001). In addition, the solvent type and polarity may affect the electron transfer and availability of hydrogen atoms, which is a key aspect of antioxidant capacity (Pérez-Jiménez & Saura-Calixto 2006).

SPF of the ROe was determined using UV-visible spectrophotometer and by applying Mansur equation. The absorbance was determined at a wavelength range of UVB radiation (290-320nm) and the calculated SPF value found for the extract was 7.56. The extract also presented absorption peaks in the UVA and B regions (data not shown). According to the Brazilian law, RDC 30 from June 1, 2012 (Brasil 2012), only SPF equal or greater than 6 is suitable for use in cosmetic products with photoprotective activity. In the concentration of 0.2µg/mL, ROe showcased satisfactory sunscreen activity, greater than the minimum required by Brazilian National Health Surveillance Agency (Anvisa).

Sunscreens are recommended for the protection against UV light-induced skin damage due to their ability to absorb, reflect or scatter UV light. Sunscreens block most of UV rays, although the fraction which crosses them will most likely promote deleterious effects in the skin (Molyneux et al. 2007, Mukherjee et al. 2011). Considering the activity gap of current sunscreens and the accumulating evidence pointing towards the use of antioxidants against UV-induced skin damage, natural products have emerged as candidates to promote simple and effective photo-chemoprotection (Bonina et al. 1996, Zheng et al. 2019).

R. officinalis contains bioactive compounds that have desirable properties for industrial and herbal medicine applications, *e.g.* essential oils (1.5-2.5%); tannins; flavonoids; triterpenes; saponins; resins; phytosterols; rosmarinic acid and many others (Wang et al. 2018). Previous publications list a wide range of rosemary's properties resulting from its content of bioactive compounds, with most reports focusing on the antioxidative properties of this vegetal (Huang et al. 2017, Wang et al. 2018).

In order to investigate the antioxidant capacity of a substance, two or more methods are optimally employed since oxidative stress depends on the type of RONS which is generated, as well as how and where it is generated. Moreover, the oxidative target should also be evaluated. In this sense, plant extracts present a diverse phytochemical composition whose antioxidant potential may be potentialized by synergistic action between different chemical markers (Georgetti et al. 2006).

The hydrogen-donating ability of ROe was evaluated using the stable radical DPPH assay as presented in Figure 1a. The maximum antioxidant activity was of 79.73% at the concentration of 10 µg/mL of ROe, and the IC_{50} was of 5.21 µg/mL. Regarding ABTS method, results showed

that ROe exhibited effective and concentration-dependent scavenging activity (Figure 1b). The IC_{50} was of 1.88 µg/mL and the maximum activity (8.99 µg/mL) reached 100%, in which a plateau was observed. In FRAP assay, ROe-reducing power was 1.28 mM trolox equivalent/µg/mL of extract. In both chemiluminescence methodologies used, the extract showed concentration-dependent activity (Figure 1c and d). The IC_{50} calculated in the H_2O_2/luminol/HRP system and xanthine/luminol/XOD system was of 0.031 µL/mL (Figure 1c) and 0.025 µL/mL (Figure 1d), respectively, showing that the strongest antioxidant capacity for ROe was found when scavenging superoxide radical.

The ROe showed a remarkable antioxidant activity in the different *in vitro* methods herein employed. It was also possible to build a dose-response curve for ROe using all the methodologies, demonstrating that these methods were suitable to evaluate the antioxidant capacity of ROe. In addition, ROe also demonstrated ferric-reducing antioxidant power similar to other results regarding *Calendula officinalis* (Fonseca et al. 2010), *Pimenta pseudocaryophyllus* (Campanini et al. 2014), and *Tephrosia toxicaria* extracts, which also showcased good antioxidant capacity in both *in vitro* and *in vivo* investigations.

Identification of the antioxidant phytochemicals

The antioxidant properties of rosemary are often reported to correlate with the presence of volatile (*e.g.* essential oil) and non-volatile (*e.g.* phenolic compounds) active compounds found in the leaves. In both fractions, these compounds contribute directly to the added-value of the product (Andrade et al. 2018, Bellumori et al. 2015). Figure 2 and Table II showcase the phenolic profile of ROe.

The analysis performed by HPLC-MS for the ROe enabled the detection of the polyphenols previously described for the *R. officinalis*, *e.g.*

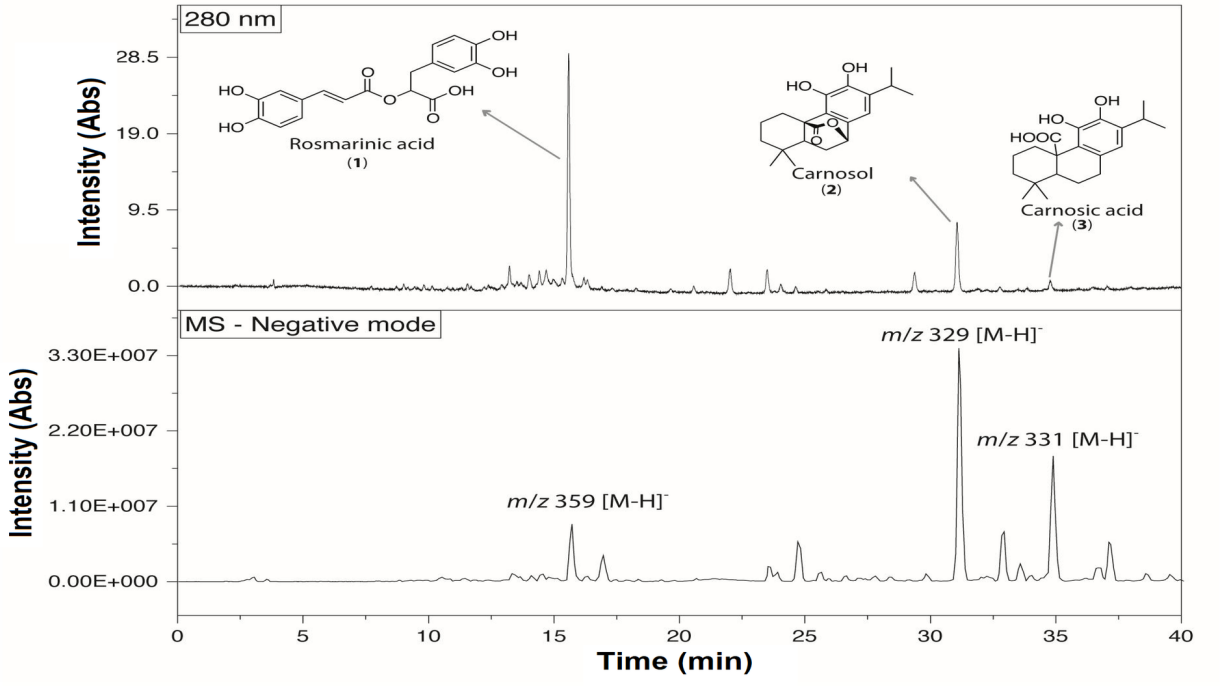

Figure 2. Chromatogram (280 nm) and Total Ion Chromatogram (TIC) obtained in the negative ionization mode of the *R. officinalis* hydroethanolic extract.

rosmarinic acid, carnosic acid and carnosol. The identification of these compounds was based on the fragmentation pattern observed for the ions and comparison with previous reports on the literature (Table II). The presence of these compounds was also confirmed by UHPLC-HRMS analysis, in which the error calculated for these compounds were 1.4, 0.3 and 0.9 ppm for rosmarinic acid, carnosic acid and carnosol, respectively. Additionally, the obtained chromatogram (280 nm, Figure 2) revealed that rosmarinic acid is a major compound in this extract.

Regarding the chemical composition of *R. officinalis*, the major phenolic acids that have been reported in rosemary's non-volatile fraction (extract) include rosmarinic, chlorogenic, r-coumaric and caffeic acids (Sytar Oksana 2012). Moreover, the extract from the leaves also contains a high amount of di and triterpenoids, including carnosic acid, oleanolic acid, betulinic acid, ursolic acid, rosmanol and carnosol amongst others (Andrade et al. 2018).

Borrás-Linares et al (2011) characterized the phenolic profile of 20 rosemary leaves and found as main compounds gallocatechin, luteolin-3'-glucuronide, rosmarinic acid, carnosic acid, carnosol and ursolic acid. However, the powerful antioxidant capacity of the extract was mainly attributed to the presence of the rosmarinic acid, carnosol and carnosic acid (Erkan et al. 2008). In fact, the main identified compounds in ROe by HPLC-MS and UHPLC-HRMS analyses were rosmarinic acid, carnosic acid and carnosol. Both carnosic acid (at *m/z* 331 $[M-H]^-$) and carnosol (at *m/z* 329 $[M-H]^-$) showcased only one fragment at MS2 level, corresponding to the decarboxylation of these compounds (loss of 44 Da). For rosmarinic acid (*m/z* 359 $[M-H]^-$), the main fragments at *m/z* 197, 179 and 161 were observed, corresponding to the formation of 2-hydroxy derivative of caffeic acid, caffeic acid and dehydrated caffeic acid, respectively. The observed fragments for these compounds are in agreement with the previously reported in literature for *R. officinalis* (Herrero et al. 2010, Hossain et al. 2010). Additionally, the identified molecular formula of these compounds was confirmed by high-resolution mass spectrometry.

Considering the promising chemical characteristics and antioxidant capacity showcased by ROe, this research also investigated the effects of topical administration of emulsion containing ROe on UV radiation-induced inflammation and oxidative damage in the skin of mice. This study is important because the chemical and physical filters which are present in sunscreens lack action over the biochemical physio pathological mechanisms triggered by UV, thus stimulating the search for novel approaches to control UV deleterious effects (Batista et al. 2018). Thus, local application of antioxidants has become one of the most important measures to prevent skin photoaging injury. Antioxidants from various sources showcase different effects in their protection of cells and tissues against free radicals (Abla & Banga 2013). Therefore, the use of UV photoprotective dermatological

Table II. Peak number according to Figure 2, retention time (tR), $[M-H]^-$ ion, MSn fragments, and compounds identified from *R. officinalis* extract.

Peak	t_R (min)	$[M-H]^-$	MS^2 fragments (% abundance)	Compound
1	15.7	359	MS^2 (359): 359 (100), 223 (15), 197 (30), 179 (40), 161 (70)	rosmarinic acid
2	31.1	329	MS^2 (329): 329 (100), 285 (65)	carnosol
3	34.9	331	MS^2 (331): 331 (100), 287 (90)	carnosic acid

preparations is one of the most recommended and the most common way of preventing solar UV light-caused damage to the skin (de Souza et al. 2017, Velasco et al. 2008).

Efficacy of the ROe-containing emulgel as an in vivo protective agent against UVB irradiation-induced skin damage

UV radiation is one of the major exogenous agents responsible for the generation of free radicals (Thomaz 2020a, b, c, Thomaz et al. 2018), and continuous exposure to UVB spectrum is a major risk factor for skin disorders. This radiation induces a photooxidation reaction that decreases the antioxidant protection of skin cells and increases ROS levels, producing photooxidative damage in the cells and extracellular matrix, skin lesions and photoaging (de Souza et al. 2017). Figure 3, showcase the results of the *in vivo* experiments regarding the photochemoprotective effects of the extract and its respective semisolid formulation.

Martin et al. (De Luis et al. 2006) analyzed the potential of water-soluble *Rosmarinus officinalis* extract in counteracting UV-induced MMP-1. UVB and UVA radiation stimulates MMP-1, that in turn degrades fibrillar collagen (including collagen type I) and is the main enzyme involved in collagen breakdown in the skin (Bárcenas-Cuadros et al. 2004, Tam et al. 2004). Moreover, literature reports that rosmarinic acid, a polyphenol present in *Rosmarisnus officinalis*, provide substantial cytoprotection against the adverse effects of UVB radiation by modulating cellular antioxidant systems (Fernando et al. 2016).

The emulgel herein reported was produced with non-ionic surfactant and gelling agent, which has been prepared to incorporate the ROe in order to enable their cutaneous administration since this kind of surfactant normally presents low skin irritability (Uchegbu & Vyas 1998).

The emulgel was white with a characteristic odor and a smooth sensory aspect. When the ROe was incorporated, it became light green, with an herbal odor and maintained the smooth sensation. The pH of the ROe-loaded

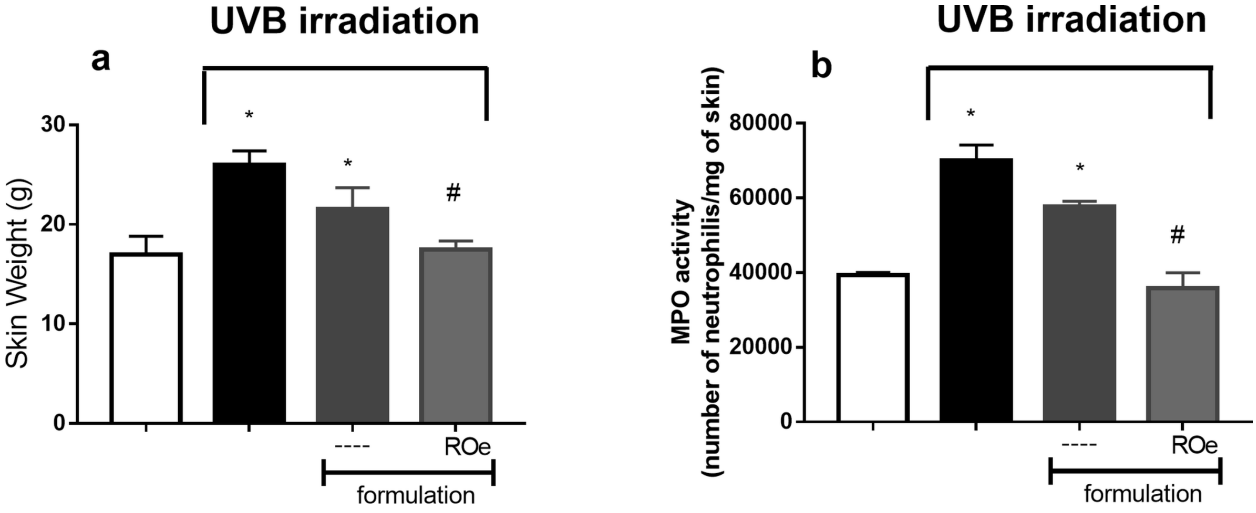

Figure 3. Topical formulation containing ROe reduces skin edema induced by UVB irradiation (a) and inhibit the UV-B irradiation-induced increase of MPO activity (b). Samples of dorsal skin were collected 12 h after the end of irradiation and used to measure the edema. Bars represent means ± SEM of 5 mice per group per experiment and are representative of two separate experiments. *$p < 0.05$ compared to the non-irradiated control groups (white bars); #$p < 0.05$ compared to the irradiated control groups (black bars).

emulgel was 5.3 ± 0.21, which is suitable for skin application (Lambers et al. 2006).

In the present study, the ameliorative effect of the ROe-loaded emulgel on oxidative stress in skin was observed. Results are showcased in figures 3 and 4.

UVB radiation results in the production of reactive oxygen species (ROS) in the skin, which is considered to be the initial inducer for irradiation-triggered tissue damage (Grether-Beck et al. 2014, Martinez et al. 2016). In addition, the inflammatory process is characterized by cells infiltration in the burned tissue, which generates local edema (Bishop et al. 2007). For this reason, the edema and MPO activity parameter were chosen to investigate the topical formulation effects on burned skin (Campanini et al. 2013, Martinez et al. 2016, 2015).

The edema was measured by the increase in tissue weight after the inflammatory stimuli. The UVB dose of 4.14 J/cm^2 induced 36% increase in skin weight compared to non-irradiated control mice. The topical treatment with ROe-loaded emulgel effectively decreased the inflammatory parameters (edema) evaluated in the acute model maintaining a similar level to the non-irradiated control group (Figure 3a).

The anti-inflammatory properties of rosemary extracts using the formaldehyde-induced plantar edema mouse model was reported in a previous publication (Mengoni et al. 2011). The ethanolic *Rosmarinus officinalis* extract was applied to the inflamed area either topically or by injection, or both. It was found that extract injection decreased inflammation by 22% compared to PBS control injection. However, when the extract was applied topically, inflammation was reduced by 80%. In this way, the results suggest that *R. officinalis* extract

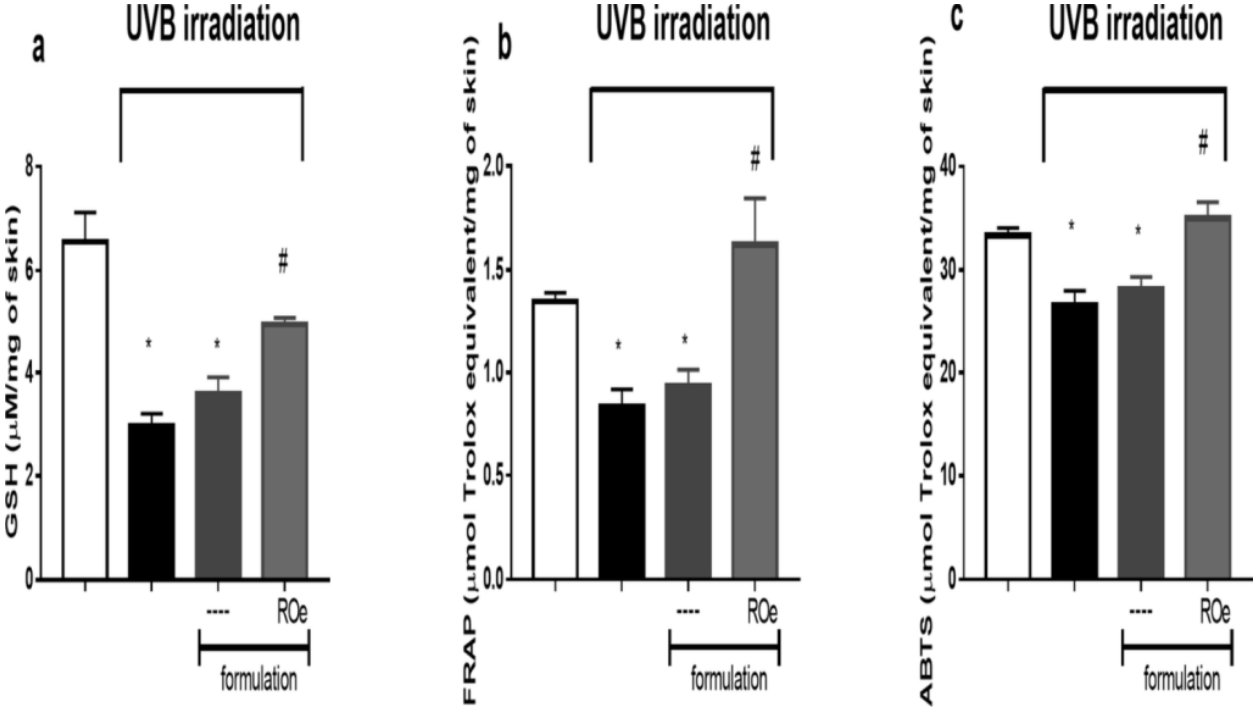

Figure 4. Effect of Topical formulation containing ROe on antioxidant capacity of skin after UVB irradiation. The antioxidant capacity was measured using GSH (a), FRAP (b) and ABTS (c) assays in samples collected 12 h after the end of irradiation. Bars represent means ± SEM of 5 mice per group per experiment and are representative of two separate experiments. *$p < 0.05$ compared to the non-irradiated control groups (white bars); #$p < 0.05$ compared to the irradiated control groups (black bars).

topically applied exhibited anti-edematogenic activity.

UVB radiation can affect several cellular signaling pathways responsible for skin inflammation, e.g. increasing MPO activity induced by the cutaneous infiltration of inflammatory cells. MPO is constitutively produced in neutrophils and has been widely used to quantify inflammation and the influx of neutrophils into tissues (Campanini et al. 2013, Martinez et al. 2015). In irradiated groups, MPO activity was found to be significantly increased when compared to non-irradiated group, while topical formulation treatment with ROe reversed the elevations (Figure 3b).

Corroborating with the results found in the edema, the formulation added with extract was able to decrease the MPO activity to non-irradiated group level. In the evaluation of MPO activity levels, all tested compounds caused a significant decrease in the amount of this enzyme compared to the positive control. In another study, it was shown that *Rosmarinus officinalis* ethanolic extract was able to decrease MPO activity levels induced by ethanol, specifically in the small intestine (Jin et al. 2017). These results confirm that the inhibition of neutrophil migration is an important component of the anti-inflammatory activity of *Rosmarinus officinalis*.

GSH is an important endogenous antioxidant whose nucleophilic and reducing properties plays a central role in metabolic pathways, as well as in the antioxidant system of aerobic cells (Harris et al. 2015). This antioxidant is found in the cytosol of skin cells and it is involved in the maintenance of the intracellular redox balance and in the protection of living cells against oxidative stress and injury (Lu et al. 2020). GSH act as a free radical scavenger and assists other antioxidants to regenerate (vitamins E and C). Furthermore, it is also a cofactor for glutathione peroxidase, which enzymatically reduces hydrogen peroxide, lipid hydroperoxides and other soluble hydroperoxides generated in cells and tissues (de Souza et al. 2017).

UVB irradiation of the mice skin resulted in reduction of the GSH level (45%) after single exposure compared with control (non-UVB exposed) mice as shown in Fig. 6a. Topical application of ROe-loaded emulgel prevented the UVB irradiation-induced depletion of GSH skin levels. The ROe-free emulgel did not inhibit the UVB-induced GSH depletion, and the effect might be attributed to ROe (Figure 4a).

The results showcased that exposure to UVB radiation promoted a significant decrease in GSH levels in the skin of the irradiated group compared to the non-irradiated group. Excessive ROS production after UVB irradiation induces the consumption of GSH, which is the most abundant non-enzymatic antioxidant in the cells (Fonseca et al. 2011). GSH depletion occurs directly by ROS production, but it can also be depleted indirectly because it is a substrate for Gpx during detoxification (Casagrande et al. 2006). Treatment with ROe-loaded emulgel protected the mice's skin from decreasing UVB-induced GSH levels as it inhibited the reduction of GSH by about 1.6-fold compared to the non-irradiated control group.

To verify the protective effect of ROe against the decrease in total antioxidant capacity of the skin exposed to UVB radiation, FRAP and ABTS tests were performed (Katalinic et al. 2005). UVB irradiation decreased the skin ferric reducing ability (FRA) (Figure 4b) and ABTS radical scavenging (Figure 4c) activities compared to non-irradiated mice. In turn, the treatment with ROe-loaded emulgel inhibited UVB irradiation-induced depletion of FRAP and ABTS activities, which were maintained at similar levels to the non-irradiated control group (Figures 4b and c). In line with the FRAP and ABTS results, ROe-loaded emulgel was able to inhibit GSH activity

depletion. Altogether, data arising from this study was sound when compared to semisolid formulations whose intended use is for photochemoprotection (Jadhav et al. 2018, Patil et al. 2014, Priani et al. 2014), and our findings showcase that topical ROe treatment by means a semisolid formulation is a successful strategy to protect the skin from oxidative damage induced by UVB irradiation.

CONCLUSION

This study investigated the *in vitro* antioxidant properties of ROe which were seemingly promoted by its rich phenolic constitution. Moreover, the first semisolid formulation containing *R. officinalis* hydroalcoholic extract was herein reported and its photochemoprotective effects were investigated in an *in vivo* model. The findings suggest that the ROe-loaded emulgel protects the skin against tissue damage caused by UVB radiation, therefore sheding light on a low cost and effective sunscreen product. The photochemoprotection of the formulation may be attributed to the synergic antioxidant and anti-inflammatory actions of the main compounds of the extract, namely: rosmarinic acid, carnosic acid and carnosol.

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S. R. Georgetti (Head) and R. O. Couto conceived the study, designed the formulations and drafted the manuscript. M. M. Baracat, R. Casagrande and W. A. Verri Jr. designed the in vitro and in vivo assays regarding the anti-inflammatory and antioxidant properties and drafted the manuscript. K. S. Takayama, P. Saito, M. C. Monteiro, I. C. Pinto C. T. Nakano and R. M. Martinez obtained the herbal material, prepared the extract, carried out the overall in vitro and in vivo assays and drafted the manuscript. N. S. Arakawa, M. L. Zeraik, H. M. Russo and D. V. Thomaz conceived and performed the qualitative and quantitative phytochemical characterization of the extract and drafted the manuscript. All authors critically revised the manuscript for important intellectual content and approved its final version.