

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Redox properties and cytoprotective actions of atranorin, a lichen secondary metabolite

Marcelia Garcez Dória Melo^a, João Paulo Almeida dos Santos^a, Mairim Russo Serafini^a, Fernanda Freitas Caregnato^b, Matheus Augusto de Bittencourt Pasquali^b, Thallita Kelly Rabelo^a, Ricardo Fagundes da Rocha^b, Lucindo Quintans Jr.^a, Adriano Antunes de Souza Araújo^a, Francilene Amaral da Silva^a, José Cláudio Fonseca Moreira^b, Daniel Pens Gelain^{b,*}

^a Departamento de Fisiologia, Universidade Federal de Sergipe, São Cristóvão, Sergipe, Brazil

^b Centro de Estudos em Estresse Oxidativo, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

ARTICLE INFO

Article history:

Received 6 October 2010

Accepted 17 November 2010

Available online 25 November 2010

Keywords:

Atranorin
Lichen metabolites
Free radicals
Cytotoxicity
Oxidative stress

ABSTRACT

Atranorin (ATR) is a lichenic secondary metabolite with potential uses in pharmacology. Antinociceptive and antiinflammatory actions have been reported, and the use of atranorin-enriched lichen extracts in folk medicine is widespread. Nonetheless, very few data on ATR biological actions are available. Here, we evaluated free radical scavenging activities and antioxidant potential of ATR using various *in vitro* assays for scavenging activity against hydroxyl radicals, hydrogen peroxide, superoxide radicals, and nitric oxide. The total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR) indexes and *in vitro* lipoperoxidation were also evaluated. Besides, we determined the cytoprotective effect of ATR on H₂O₂-challenged SH-SY5Y cells by the MTT assay. ATR exerts differential effects towards reactive species production, enhancing hydrogen peroxide and nitric oxide production and acting as a superoxide scavenger; no activity toward hydroxyl radical production/scavenging was observed. Besides, TRAP/TAR analysis indicated that atranorin acts as a general antioxidant, although it demonstrated to enhance peroxy radical-induced lipoperoxidation *in vitro*. ATR was not cytotoxic, and also protected SH-SY5Y cells against H₂O₂-induced cell viability impairment. Our results suggest that ATR has a relevant redox-active action, acting as a pro-oxidant or antioxidant agent depending on the radical. Also, it will exert cytoprotective effects on cells under oxidative stress induced by H₂O₂.

© 2010 Elsevier Ltd. Open access under the [Elsevier OA license](http://www.elsevier.com/locate/elsevier/oa-licence).

1. Introduction

Lichens are a symbiotic association constituted mostly of ascomycetous fungi (mycobiont) and algae or cyanobacterial (photobiont) partners (Hale, 1973). They occur in a wide variety of habitats and natural environmental conditions such as low temperatures, prolonged darkness, drought and continuous light. It has been suggested that in response to these extreme conditions, natural selection has favored species producing high concentrations of characteristic compounds, such as depsides, depsidones, depsones, dibenzofurans, and chromones, among others (Schmitt and Lumbsch, 2004).

The majority of compounds synthesized via the polyketide pathway are unique to lichens (Blanco et al., 2005). These compounds were reported to exhibit antibiotic, anti-mycobacterial, antiviral, anti-inflammatory, analgesic, antipyretic, antiprolifera-

tive or cytotoxic activities (Oksanen, 2006; Stocker-Worgotter, 2008). Lichen extracts have been long used for medicinal applications, probably due to the biological activity of their endogenous secondary metabolites; besides, the strong UV absorption properties of some of these compounds, which are a result of the lichen's adaptation to high solar radiation exposure, have been explored for the development of sunscreens and other cosmetic formulations for skin (Bernard et al., 2003; Muller, 2001).

Atranorin (ATR) is the main compound from the lichen *Cladonia kalbii* Ahti which grows in the arid lands of the Brazilian Northeast. ATR is an important member of the depside group and is found in a variety of lichen species (Kristmundsdottir et al., 2005). The molecular structures of these depsides (Fig. 1) present aromatic esters containing the methyl ester group on the terminal ring (Edwards et al., 2003). Studies on bioactive properties of extracts containing ATR have revealed antimycobacterial/antimicrobial activity (Honda et al., 2010; Ingolfssdottir et al., 1998; Yilmaz et al., 2004), antinociceptive and antiinflammatory properties (Bugni et al., 2009) and photoprotective capacity (Fernandez et al., 1998). Isolated ATR was obtained exhibit antinociceptive effects (Melo

* Corresponding author. Address: Rua Ramiro Barcelos, 2600, anexo, CEP 90035-003, Porto Alegre, RS, Brazil. Tel.: +55 51 3308 5577; fax: +55 51 3308 5535.

E-mail address: dgelain@yahoo.com.br (D.P. Gelain).

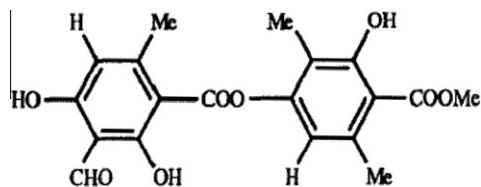


Fig. 1. Structure of atranorin.

et al., 2008) and to inhibit leukotriene B₄ synthesis in leukocytes, which might affect inflammatory processes (Kumar and Muller, 1999). Besides, ATR was reported to exhibit antibiotic action against *M. aurum* (Ingoldsdottir et al., 1998) and exhibited anti-proliferative action against malignant cell lines (Kristmundsdottir et al., 2005). In a study of the mitochondrial uncoupling activity of lichen metabolites, ATR was the only compound which did not exhibit toxic effects, indicating it could substitute other related lichen metabolite, usnic acid, which also presents potential medicinal applications, in the formulation of novel therapeutic compounds (Abo-Khatwa et al., 1996). However, little has been explored on the mechanisms of ATR biological effects.

The role of reactive oxygen species (ROS) in physiological and abnormal processes is the subject of intense studies motivated, in part, by the large and growing number of associations between various pathological conditions and changes in oxidative balance, redox status, and oxidative injury (Fantel, 1996). Antioxidants with different chemical characteristics may act synergistically with each other in a network of coupled oxi-reduction reactions. The actions of antioxidants have been attributed to their ability to scavenge free radicals, thereby reducing oxidative damage of cellular biomolecules such as lipids, proteins, and DNA (Halliwell and Gutteridge, 2007). Besides, antioxidants function as reducing agents, chelators of pro-oxidant metals or as quenchers of singlet oxygen (Gelain et al., 2009).

Many of the biological properties associated to ATR include processes mediated by free radicals and related species, such as mutagenicity, and inflammation (Halliwell and Gutteridge, 2007). Most actions of secondary metabolites in biological systems also have been related to their redox properties; possible health-promoting and beneficial effects of naturally occurring compounds are traditionally ascribed to a general antioxidant action (Aravindaram and Yang, 2010). Nonetheless potential toxicity is also frequent, generally underestimated and also associated to promotion of pro-oxidant processes and induction of oxidative stress in biological systems (Hayes et al., 2005). Few works have studied potential antioxidant effects of ATR, using assays with little specificity or limited evaluation capacity (Carlos et al., 2009; Jayaprakasha and Rao, 2000; Toledo Marante et al., 2003; Valencia-Islas et al., 2007). In the present work, we studied the redox properties of ATR against different reactive species generated *in vitro*, and evaluated its cytoprotective actions in cells challenged with hydrogen peroxide.

2. Materials and methods

2.1. Lichen material

Cladonia kalbii was collected in March, 2007, Itabaiana-Sergipe, Brazil (10°44'S, 37°23'W). Atranorin was isolated as described below (Melo et al., 2008) and stored at −20 °C. Herbarium voucher specimens (registry number SP 393235) were prepared and deposited at the Botanical Institute of São Paulo-SP, Brazil and identified by M.P. Marcelli.

2.2. Extraction and isolation of ATR

Atranorin (C₁₉H₁₈O₈) was isolated from the crude extract of the lichen *C. kalbii*. The air-dried parts (100 g) of *C. kalbii* were extracted with 150 ml of chloroform using a Soxhlet apparatus to isolate ATR. The crude extract was filtered and stored at 4 °C for 24 h to precipitate ATR. The ATR precipitates were collected and subjected to silica gel (70–230 mesh) column chromatography (CC) and eluted with chloroform:hexane (80:20) as the solvent system. At the end of this process, 840 mg of ATR was obtained with a 0.84% (w/w) yield. After isolation, ATR was stored at −20 °C, a temperature at which it presents high stability (Melo et al., 2008). For assays, ATR was dissolved in DMSO (10 mg/ml) and serial dilutions were obtained from this stock solution. Therefore, at the highest concentration of ATR in the assays (100 µg/ml), concentration of the vehicle DMSO corresponds to 0.01%.

2.3. Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR)

The total reactive antioxidant potential (TRAP) is employed to estimate the antioxidant capacity of samples *in vitro*. This method is based on the quenching of luminol-enhanced chemiluminescence (CL) derived from the thermolysis of 2,20-azo-bis(2-amidinopropane)dihydrochloride (AAPH) as the free radical source (Dresch et al., 2009).

The CL was measured by adding 4 ml of AAPH dissolved in glycine buffer to a glass scintillation vial. Then, luminol was added and the CL was measured until reached constant light intensity. After this stabilization time, the Trolox solutions or the sample was added and the CL was measured in a liquid scintillation counter. The last count before the addition of Trolox or samples was considered as 100%. The count time was 10 s, and the CL emission was monitored for 3000 s after the addition of Trolox or samples. Graphs were obtained by plotting percentage of counts per minute (%cpm) versus time (s) of instantaneously generated values of CL inhibition and area under curve (AUC). The total antioxidant reactivity (TAR) was calculated as the ratio of light intensity in absence of samples (I_0)/light intensity right after ATR addition (I) and expressed as percent of inhibition. AUC and radical basal production were acquired by software GraphPad Prism software 5.0.

2.4. Thiobarbituric acid reactive species (TBARS)

TBARS (thiobarbituric acid reactive species) assay was employed to quantify lipid peroxidation (Draper and Hadley, 1990) and an adapted TBARS method was used to measure the antioxidant capacity of ATR using egg yolk homogenate as lipid rich substrate (Silva et al., 2007). Briefly, egg yolk was homogenized (1% w/v) in 20 mM phosphate buffer (pH 7.4), 1 ml of homogenate was sonicated and then homogenized with 0.1 ml of ATR at different concentrations. Lipid peroxidation was induced by addition of 0.1 ml of AAPH solution (0.12 M). Control was incubation medium without AAPH. Reactions were carried out for 30 min at 37 °C. Samples (0.5 ml) were centrifuged with 0.5 ml of trichloroacetic acid (15%) at 1200g for 10 min. An aliquot of 0.5 ml from supernatant was mixed with 0.5 ml TBA (0.67%) and heated at 95 °C for 30 min. After cooling, samples absorbance was measured using a spectrophotometer at 532 nm. The results were expressed as percentage of TBARS formed by AAPH alone (induced control).

2.5. Hydroxyl radical-scavenging activity

The formation of ·OH (hydroxyl radical) from Fenton reaction was quantified using 2-deoxyribose oxidative degradation (Lopes et al., 1999). The principle of the assay is the quantification of

the 2-deoxyribose degradation product, malondialdehyde, by its condensation with 2-thiobarbituric acid (TBA). Briefly, typical reactions were started by the addition of Fe^{2+} (FeSO_4 6 mM final concentration) to solutions containing 5 mM 2-deoxyribose, 100 mM H_2O_2 and 20 mM phosphate buffer (pH 7.2). To measure ATR antioxidant activity against hydroxyl radical, different concentrations of ATR were added to the system before Fe^{2+} addition. Reactions were carried out for 15 min at room temperature and were stopped by the addition of 4% phosphoric acid (v/v) followed by 1% TBA (w/v, in 50 mM NaOH). Solutions were boiled for 15 min at 95 °C, and then cooled at room temperature. The absorbance was measured at 532 nm and results were expressed as MDA equivalents formed by Fe^{2+} and H_2O_2 .

2.6. Nitric oxide (NO^\bullet) scavenging activity

Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside in 20 mM phosphate buffer (pH 7.4). Once generated NO interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (Basu and Hazra, 2006). The reaction mixture (1 ml) containing 10 mM sodium nitroprusside (SNP) in phosphate buffer and ATR at different concentrations were incubated at 37 °C for 1 h. A 0.5 ml aliquot was taken and homogenized with 0.5 ml Griess reagent. The absorbance of chromophore was measured at 540 nm. Percent inhibition of nitric oxide generated was measured by comparing the absorbance values of negative controls (only 10 mM sodium nitroprusside and vehicle) and assay preparations. Results were expressed as percentage of nitrite formed by ATR alone.

2.7. Determination of catalase-like activity (CAT)

The ability of ATR to scavenge H_2O_2 (“catalase-like activity” or “CAT-like activity”) was measured as described previously (Aebi, 1984). Briefly, H_2O_2 diluted in 0.02 M phosphate buffer (pH 7.0) to obtain a 5 mM final concentration was added to microplate wells in which different concentrations was placed. The microplate was immediately placed to monitor the rate of H_2O_2 decomposition in the microplate reader set at 240 nm.

2.8. Determination of superoxide dismutase-like activity (SOD)

The ability of ATR to scavenge superoxide anion (“superoxide dismutase-like activity” or “SOD-like activity”) was measured as previously described. ATR was mixed to native purified catalase (100 U/ml stock solution) in glycine buffer (50 mM, pH 10.2). Superoxide generation was initiated by addition of adrenaline 2 mM and adrenochrome formation was monitored at 480 nm for 5 min at 32 °C. Superoxide production was determined by monitoring the reaction curves of samples and measured as percentage of the rate of adrenaline auto-oxidation into adrenochrome (Banister and Calabrese, 1987).

2.9. Cell culture and cytotoxicity assay

SH-SY5Y cells were cultured in 10% FBS DMEM/F12 medium. Cells were used for cytotoxicity measurements when reached 70–90% confluence. Cells were treated with different concentrations of ATR alone or in the presence of H_2O_2 400 μM for 3 h, and cell viability was assessed by the MTT assay. This method is based on the ability of viable cells to reduce MTT (3-(4,5-dimethyl)-2,5-diphenyl tetrazolium bromide) and form a blue formazan product. MTT solution (sterile stock solution of 5 mg/ml) was added to the incubation medium in the wells at a final concentration of 0.2 mg/ml. The cells were left for 45 min at 37 °C in a humidified 5% CO_2 atmosphere. The medium was then removed and plates

were shaken with DMSO for 30 min. The optical density of each well was measured at 550 nm (test) and 690 nm (reference).

2.10. Statistical analysis

Data are expressed as mean \pm SEM. The obtained data was evaluated by one-way analysis of variance (ANOVA) followed by Tukey's test. All tests were performed in triplicate. Data analyses were performed using the GraphPad Prism 5.0 software. In all cases differences were considered significant if $p < 0.05$.

3. Results

The TRAP and TAR methods are widely employed to estimate the general antioxidant capacity of samples *in vitro*. We observed that the chemiluminescence induced by the peroxy radical generation initiated by AAPH decreased following addition of ATR to the system. At the TRAP assay, ATR concentrations of 1–100 $\mu\text{g}/\text{ml}$ showed significant antioxidant effects in a dose-dependent manner (Fig. 2A). Atranorin at 100 $\mu\text{g}/\text{ml}$ also showed significant anti-

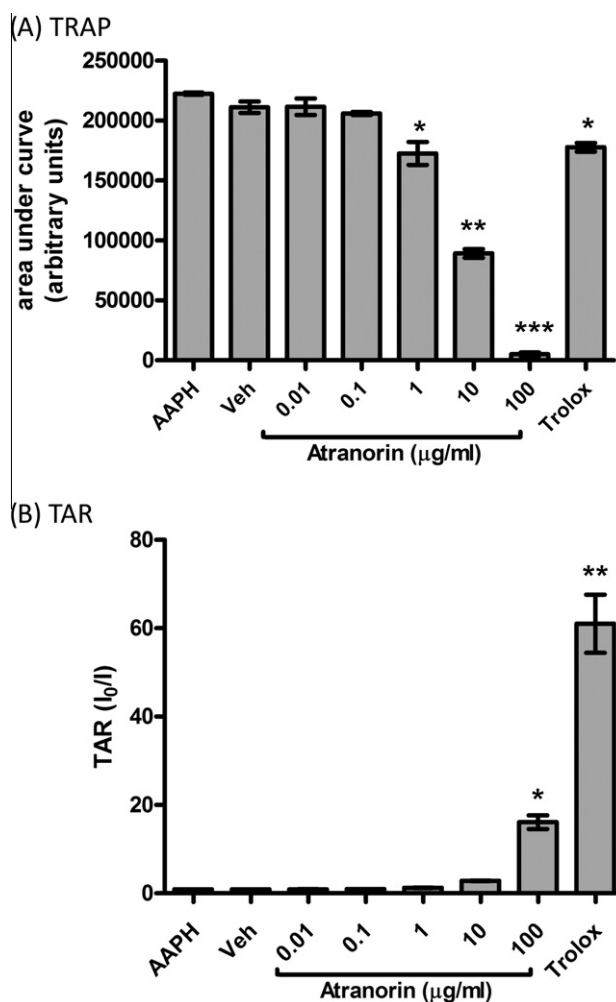


Fig. 2. Total reactive antioxidant potential (TRAP) and total antioxidant reactivity (TAR). (A) TRAP analysis. A free radical source (AAPH) generates peroxy radical at a constant rate, and the effect of different concentrations of ATR on free radical-induced chemiluminescence is measured as area under curve during 60 min. (B) TAR values are calculated as the ratio of light intensity in absence of samples (I_0)/light intensity right after ATR addition (I) and expressed as percent of inhibition. All groups denote samples in the presence of AAPH. Trolox (75 $\mu\text{g}/\text{ml}$) was used as standard antioxidant. Bars represent mean \pm SEM. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$ (1-way ANOVA followed by Tukey's multiple comparison post hoc test).

oxidant capacity in TAR measurement (Fig. 2B). Trolox (75 $\mu\text{g/ml}$) was used as a reference antioxidant for the assays.

The ability of ATR to prevent lipid peroxidation was measured by quantifying thiobarbituric acid-reactive substances (TBARS) generated by AAPH in a lipid-rich incubation medium. The effect of different concentrations on lipid peroxidation is shown in (Fig. 3). Apparently, concentrations of ATR from 0.1 to 100 $\mu\text{g/ml}$ enhanced the AAPH-induced lipoperoxidation.

The ability of ATR to scavenge NO was measured by quantifying the production of nitrite derived from sodium nitroprusside (SNP) by the method of Griess. ATR did not present any scavenging effect upon SNP-induced NO production. On the other hand the highest dose tested enhanced nitrite formation (Fig. 4A). We also tested the ability of ATR to scavenge hydroxyl radicals generated *in vitro*. All doses of ATR tested had no effect on 2-deoxyribose degradation induced by the Fenton reaction induction system (Fig. 4B).

The capacity of ATR to interact with and/or scavenge/quench H_2O_2 and superoxide radicals and *in vitro* was evaluated, respectively, by the catalase-like and the superoxide dismutase-like reaction assays. We observed that ATR caused a significant increase in H_2O_2 formation *in vitro* (Fig 5A). On the other hand, the rate of superoxide degradation was significantly enhanced by ATR in all doses tested (Fig. 5B).

To assess if ATR exerts antioxidant properties in a cell system challenged with a pro-oxidant agent, we tested the effect of ATR in SH-SY5Y cultures, a neuroblastoma-derived catecholaminergic cell line. Different concentrations of ATR alone had no effect on cell viability, as assessed by MTT assay. When cells are treated with H_2O_2 400 μM for 3 h, there is a significant decrease in cell viability to 40% of control levels (Fig. 6). Co-incubation with ATR protects SH-SY5Y cells against the cytotoxic effects of H_2O_2 . All concentrations of ATR reversed the effect of H_2O_2 on cell viability to control levels. These results indicate that ATR exerts antioxidant properties in cells under oxidative stress.

4. Discussion

Antioxidants comprise a broad and heterogeneous family of compounds that share the common task of interfering with (stopping, retarding, or preventing) the oxidation (or autoxidation) of an oxidizable substrate (Halliwell and Gutteridge, 2007). Numerous physiological and biochemical processes in the human body may produce oxygen-centered free radicals and other reactive oxygen

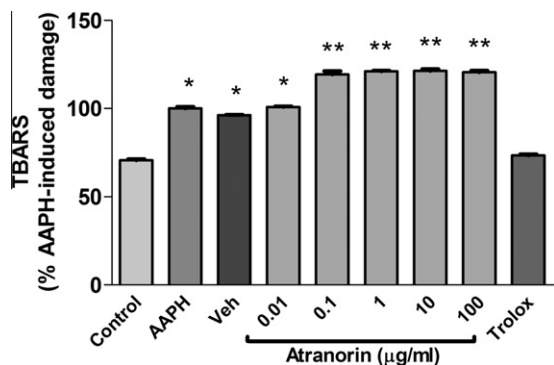


Fig. 3. Thiobarbituric acid-reactive substances (TBARS) *in vitro*. A lipid-rich system was incubated with a free radical source (AAPH) and the effect of different concentrations of ATR on the lipoperoxidation was measured by quantifying TBARS. Control is incubation medium without AAPH; other groups contained AAPH alone or in the presence of different concentrations of ATR or its vehicle (Veh, DMSO 10%) alone. Trolox 75 $\mu\text{g/ml}$ was used as standard antioxidant. Bars represent mean \pm SEM. * $p < 0.05$, ** $p < 0.0001$ (1-way ANOVA followed by Tukey's multiple comparison post hoc test).

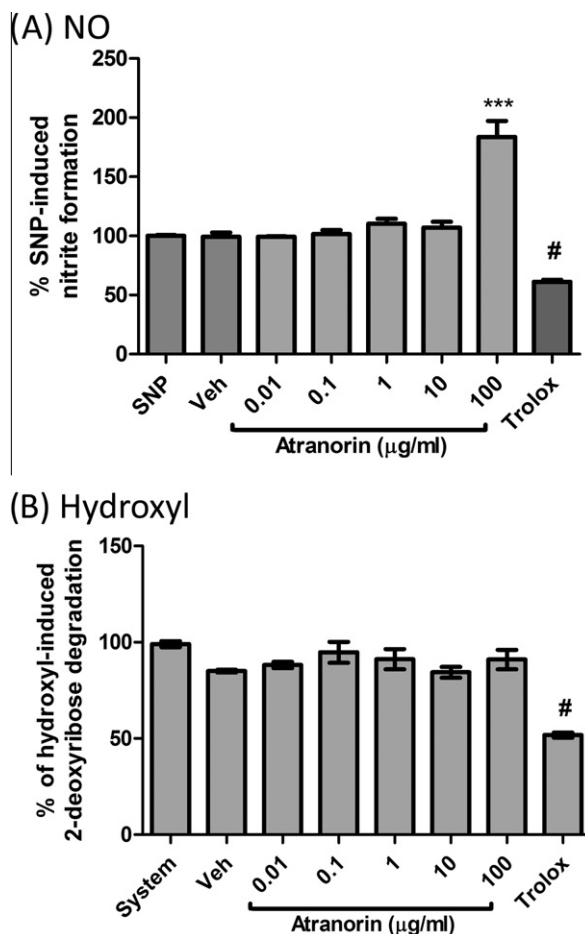


Fig. 4. Nitric oxide (NO) and hydroxyl scavenging activities. (A) NO scavenging assay. Nitric oxide was generated from spontaneous decomposition of sodium nitroprusside (SNP) and interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction. SNP group is sodium nitroprusside alone, other groups denote nitrite production by SNP in the presence of different concentrations of ATR or its vehicle (Veh, DMSO 10%). Bars represent mean \pm SEM. *** $p < 0.0001$; #different from SNP. (B) Hydroxyl radical-scavenging activity was quantified using 2-deoxyribose oxidative degradation *in vitro*, which produces malondialdehyde by condensation with 2-thiobarbituric acid (TBA). System is MDA production from 2-deoxyribose degradation with FeSO_4 and H_2O_2 alone. Other groups denote MDA production by FeSO_4 and H_2O_2 in the presence of different concentrations of ATR or vehicle. Trolox was used as standard antioxidant in both assays. Bars represent mean \pm SEM. #Different from system. One-way ANOVA followed by Tukey's multiple comparison post hoc test was applied to all data.

or nitrogen species as byproducts. Overproduction of such radicals can cause oxidative damage to biomolecules, eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans (Cai et al., 2004). The relative importance of antioxidants *in vivo* depends on which species is generated, how it is generated, where it is generated, and the possible interactions among different antioxidants and reactive species in the system. Hence it is perfectly possible for an antioxidant to protect against damage induced by reactive species in a given system but to fail to protect, or even sometimes to enhance damage, in others, acting thus as a 'redox-active' molecule (Halliwell, 2006; Halliwell and Gutteridge, 2007).

The antioxidant potential of a compound may vary according to different antioxidant assays, and even vary in the same types of assay according to changes in medium polarity, since the interaction of the antioxidant with other compounds plays an important role in the activity (Pekkarinen et al., 1999). Dramatic differences in the relative antioxidant potential of model compounds were

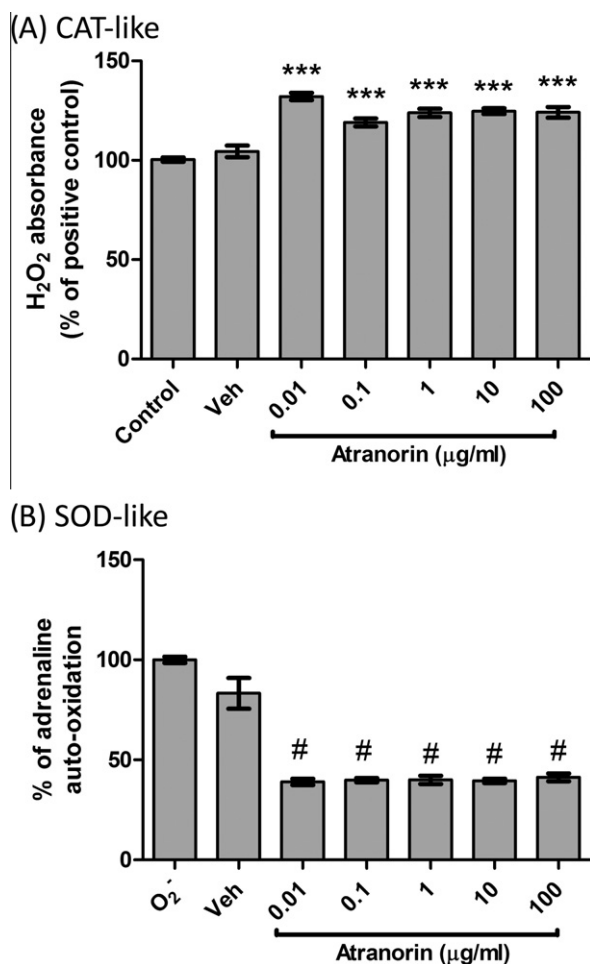


Fig. 5. Catalase-like (CAT-like) and superoxide dismutase-like (SOD-like) activities. (A) CAT-like activity was measured in a catalase reaction buffer with H₂O₂. Bars represent mean ± SEM. ****p* < 0.0001 in relation to control. (B) SOD-like activity was determined by following formation of adrenochrome in a SOD reaction buffer containing native purified catalase and adrenaline (O₂⁻ generator group). Bars represent mean ± SEM, #*p* < 0.0001 in relation to control. One-way ANOVA followed by Tukey's multiple comparison post hoc test was applied to all data.

observed when one model compound is strongly antioxidant with one method and pro-oxidant with another (Moure et al., 2001). For such reason, the antioxidant activity of a compound must always be evaluated with different tests, in order to identify different mechanisms. Tests measuring the scavenging activity with different challengers, such as superoxide radical (O₂⁻), hydroxyl (·OH) and nitric oxide (·NO) are useful to establish in which degree a given compound interacts with the different reactive species. Here, we assessed the redox properties of ATR using different approaches to understand the possible interactions of this compound with different types of reactive species.

Several studies have shown that the redox activity associated with natural antioxidants is attributed to the total content of phenolic compounds (Halliwell, 2008; Rice-Evans et al., 1995; Scalbert et al., 2005). Values of scavenging activity of peroxy radicals by ATR on TRAP/TAR assays confirmed a general antioxidant capacity by this molecule. The antioxidant potential of ATR was significant in the concentration of 100 µg/ml. ATR also presented a significant superoxide dismutase-like activity, evidencing an antioxidant potential against superoxide radicals. TRAP and TAR are different indexes; at the TRAP graph, the bars represent the area under the curve of a kinetic measurement of AAPH-induced luminescence during 60 min; at TAR, the immediate effect of the addition of an

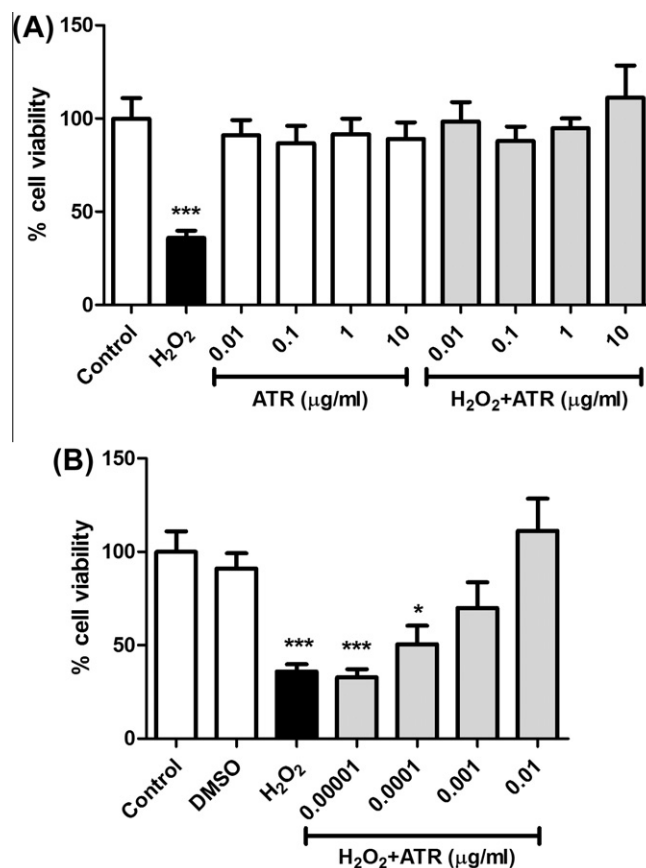


Fig. 6. Effect of atranorin on cell viability. (A) SH-SY5Y cells were incubated with varying concentrations of atranorin (ATR) as indicated and cell viability was assessed by MTT assay. Cells treated with H₂O₂ 400 µM had a significant decrease in viability (***) *p* < 0.0001, ANOVA followed by Tukey's post hoc), and the effect of different concentrations of ATR on H₂O₂-induced cytotoxicity was evaluated. (B) DMSO 0.1% (vehicle) had no effect on cell viability, and decreasing concentrations of ATR had no cytoprotective effect (**p* < 0.05, ANOVA followed by Tukey's post hoc).

antioxidant compound in the free radical-induced chemiluminescence is measured. Thus, even if a sample has the ability of inhibit the AAPH-induced free radical production when immediately added to a system (which is quantified by TAR), that does not mean this same sample is able to maintain the AAPH-induced free radical production inhibited during 60 min (the time-period monitored by TRAP). Thus, the combination of both assays is necessary for a better characterization of the antioxidant activity of a given sample.

On the other hand, ATR presented a pro-oxidant capacity in a lipid-rich system, enhancing TBARS formation induced by AAPH incubation. In assays to evaluate the antioxidant potential against NO and H₂O₂, ATR also demonstrated to enhance the production of such species, acting as a pro-oxidant molecule. Nonetheless, ATR increased NO production only at the higher concentration tested, while other concentrations demonstrated to be innocuous. On the other hand, concentrations as low as 0.01 µg/ml were able to increase H₂O₂ production *in vitro*. We also observed that ATR presented no activity towards hydroxyl radical production or scavenging.

NO exerts important physiological effects, such as vasoconstriction regulation and modulation of pro-inflammatory processes (Mollace et al., 2005; Salvemini et al., 2006, 1996). In elevated concentrations, NO may interact with superoxide radicals to generate the strong oxidizing agent peroxynitrite (ONOO⁻). Peroxynitrite diffuses through membranes and interacts with methionine side chains in proteins, sulphhydryl groups, aromatic rings from tyrosine and guanine and generates nitrogen dioxide, which is an initiator

of lipoperoxidation (Halliwell and Gutteridge, 2007). Thus, it is generally believed that an increase in superoxide radical formation both destroys the biological action of NO by promoting its removal and intensifies the formation of peroxynitrite (Salvemini et al., 2006). We observed here that ATR can act as a superoxide scavenger, and thus limit the action of this reactive species. Besides, it is postulated that during acute and chronic inflammation, superoxide production is enhanced to levels above the cleaning capacity of endogenous SOD enzymes, resulting in endothelial cell damage and increased microvascular permeability, up-regulation of adhesion molecules such as ICAM-1 (intercellular adhesion molecule 1) and P-selectin (through mechanisms not yet defined) that recruit neutrophils to sites of inflammation, autocatalytic destruction of neurotransmitters and hormones such as noradrenaline and adrenaline, lipid peroxidation and oxidation, DNA damage and activation of PARP [poly(ADP-ribose) polymerase] (Salvemini et al., 2006). Superoxide removal by endogenous SOD and ATR would avoid such effects and also allow endogenous and ATR-induced NO to promote the activation of cyclooxygenase and subsequent release of beneficial prostaglandins (Mollace et al., 2005; Salvemini et al., 2006).

The potential of ATR as an antiinflammatory and antinociceptive agent has been investigated based on reports of the utilization of lichen preparations for this purpose (Bugni et al., 2009). Besides, the antimicrobial and antifungal activities of ATR have also been studied for different authors, since secondary metabolites in lichens exert a very important role in preventing the infection of nonspecific microorganisms in the symbiosis (Oksanen, 2006). In this regard, novel natural compounds isolated from lichens present a source of potential new substances with selective biological action, which can be used for the development of novel drugs. Nonetheless, biological actions of ATR have been poorly investigated. Free radicals and related species are involved in the mechanisms of diverse conditions, and the redox properties of novel compounds must be properly determined in order to better estimate and understand its potential usefulness.

Our results suggested that ATR may exert differential types of interactions with various reactive species *in vivo*, and for such reason we tested the effect of ATR on SH-SY5Y cells challenged with an oxidative stress generator, H₂O₂. Redox interactions observed *in vitro* may not be reproduced in the cellular environment, due to the presence of endogenous antioxidants systems composed by non-enzymatic agents (vitamin E, reduced glutathione, uric acid, metal chelators) and specialized enzymes such as CAT, SOD and glutathione peroxidase. We observed here that, alone, ATR had no cytotoxic effect on SH-SY5Y cells, and that it conferred cytoprotection in the presence of toxic concentrations of H₂O₂. Hydrogen peroxide is known to induce cell death by oxidative stress-dependent necrosis and apoptosis, which results from severe oxidative damage to DNA, lipids and proteins. It is very likely that, at the concentration range tested here, ATR acts as an antioxidant inside cells, and many of its claimed biological effects are related to a redox modulation mechanism. We used the SH-SY5Y line because these cells have a well-established 24 h cell division cycle and do not present the malignant characteristics of the neuroblastoma cells they are originally obtained from, thus constituting a suitable model for neurotoxicity assays. SH-SY5Y cells are widely used for *in vitro* assays of cytotoxicity related to the dopaminergic and catecholaminergic systems (see, for instance, Navarra et al., 2010), and for this reason we used a cell line in which the MTT-based assay is extensively utilized and known.

Potent antioxidants can auto-oxidize and generate reactive substances and thus also act as pro-oxidants, depending on the system composition (Moure et al., 2001). Many natural compounds have been first postulated to act solely as antioxidants, with later works demonstrating potential pro-oxidant actions in biological systems

at specific conditions. Carotenoids constitute one such example. Vitamin A was observed to exert a general antioxidant action in biological and *in vitro* systems, and its administration as supplement was even suggested to prevent lung cancer (Fields et al., 2007). Clinical trials, however, revealed that vitamin A administration enhanced lung cancer incidence and death to risk populations (Goodman and Omenn, 1992; Goodman et al., 1993; Omenn et al., 1994; The ABC-Cancer Prevention Study Group, 1994). Later works revealed a pro-oxidant effect of vitamin A and related carotenoids *in vitro* and *in vivo* at specific conditions (Dal-Pizzol et al., 2000; Gelain et al., 2006, 2008; Klamt et al., 2003). Thus, more complete screenings of redox properties of novel compounds are needed to avoid tragic consequences at clinical level, and for this reason we must perform detailed investigations on the chemical properties of such compounds. We found here that ATR is a redox active molecule *in vitro*, acting as a general antioxidant in TRAP/TAR assays and as a superoxide scavenger or enhancing the formation of specific reactive species, such as H₂O₂ and NO, depending on its concentration. When studying the biological effects of ATR as well as determining its concentration range for administration, a careful approach must be taken to avoid more severe consequences related to excessive reactive species formation and oxidative/nitrosative stress, especially if working with concentrations above the antioxidant range observed here in the cytotoxicity assay.

Acknowledgment

This work was funded by the Brazilian agencies/programs CNPq, FAPITEC-SE, and IBN-Net #01.06.0842-00.

References

- Abo-Khatwa, A.N., al-Robai, A.A., al-Jawhari, D.A., 1996. Lichen acids as uncouplers of oxidative phosphorylation of mouse-liver mitochondria. *Nat. Toxins* 4, 96–102.
- Aebi, H., 1984. Catalase *in vitro*. *Meth. Enzymol.* 105, 121–126.
- Aravindaram, K., Yang, N.S., 2010. Anti-inflammatory plant natural products for cancer therapy. *Planta Med.*
- Bannister, J.V., Calabrese, L., 1987. Assays for superoxide dismutase. *Meth. Biochem. Anal.* 32, 279–312.
- Basu, S., Hazra, B., 2006. Evaluation of nitric oxide scavenging activity, *in vitro* and *ex vivo*, of selected medicinal plants traditionally used in inflammatory diseases. *Phytother. Res.* 20, 896–900.
- Bernard, G., Gimenez-Arnau, E., Rastogi, S.C., Heydorn, S., Johansen, J.D., Menne, T., et al., 2003. Contact allergy to oak moss: search for sensitizing molecules using combined bioassay-guided chemical fractionation, GC-MS, and structure-activity relationship analysis. *Arch. Dermatol. Res.* 295, 229–235.
- Blanco, O., Crespol, A., Divakar, P.K., Elix, J.A., Lumbsch, H.T., 2005. Molecular phylogeny of parmotreoid lichens (Ascomycota, Parmeliaceae). *Mycologia* 97, 150–159.
- Bugni, T.S., Andjelic, C.D., Pole, A.R., Rai, P., Ireland, C.M., Barrows, L.R., 2009. Biologically active components of a Papua New Guinea analgesic and anti-inflammatory lichen preparation. *Fitoterapia* 80, 270–273.
- Cai, Y., Luo, Q., Sun, M., Corke, H., 2004. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. *Life Sci.* 74, 2157–2184.
- Carlos, I.Z., Quilles, M.B., Carli, C.B., Maia, D.C., Benzatti, F.P., Lopes, T.I., et al., 2009. Lichen metabolites modulate hydrogen peroxide and nitric oxide in mouse macrophages. *Z. Naturforsch.*, C 64, 664–672.
- Dal-Pizzol, F., Klamt, F., Frota Jr., M.L., Moraes, L.F., Moreira, J.C., Benfato, M.S., 2000. Retinol supplementation induces DNA damage and modulates iron turnover in rat Sertoli cells. *Free Radical Res.* 33, 677–687.
- Draper, H.H., Hadley, M., 1990. Malondialdehyde determination as index of lipid peroxidation. *Meth. Enzymol.* 186, 421–431.
- Dresch, M.T., Rossato, S.B., Kappel, V.D., Biegelmeier, R., Hoff, M.L., Mayorga, P., et al., 2009. Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. *Anal. Biochem.* 385, 107–114.
- Edwards, H.G.M., Newton, E.M., Wynn-Williams, D.D., 2003. Molecular structural studies of lichen substances II: atranorin, gyrophoric acid, fumarprotocetraric acid, rhizocarpic acid, calycin, pulvinic dilactone and usnic acid. *J. Mol. Struct.* 651, 27–37.
- Fantel, A.G., 1996. Reactive oxygen species in developmental toxicity: review and hypothesis. *Teratology* 53, 196–217.
- Fernandes, E., Reyes, A., Hidalgo, M.E., Quilhot, W., 1998. Photoprotector capacity of lichen metabolites assessed through the inhibition of the 8-methoxypsoralen photobinding to protein. *J. Photochem. Photobiol. B* 42, 195–201.

- Fields, A.L., Soprano, D.R., Soprano, K.J., 2007. Retinoids in biological control and cancer. *J. Cell. Biochem.* 102, 886–898.
- Gelain, D.P., Cammarota, M., Zanotto-Filho, A., de Oliveira, R.B., Dal-Pizzol, F., Izquierdo, I., et al., 2006. Retinol induces the ERK1/2-dependent phosphorylation of CREB through a pathway involving the generation of reactive oxygen species in cultured Sertoli cells. *Cell Signal.* 18, 1685–1694.
- Gelain, D.P., Dalmolin, R.J., Belau, V.L., Moreira, J.C., Klamt, F., Castro, M.A., 2009. A systematic review of human antioxidant genes. *Front. Biosci.* 14, 4457–4463.
- Gelain, D.P., de Bittencourt Pasquali, M.A., Zanotto-Filho, A., de Souza, L.F., de Oliveira, R.B., Klamt, F., et al., 2008. Retinol increases catalase activity and protein content by a reactive species-dependent mechanism in Sertoli cells. *Chem. Biol. Interact.* 174, 38–43.
- Goodman, G.E., Omenn, G.S., 1992. Carotene and retinol efficacy trial: lung cancer chemoprevention trial in heavy cigarette smokers and asbestos-exposed workers. CARET Coinvestigators and Staff. *Adv. Exp. Med. Biol.* 320, 137–140.
- Goodman, G.E., Omenn, G.S., Thornquist, M.D., Lund, B., Metch, B., Gyls-Colwell, I., 1993. The Carotene and Retinol Efficacy Trial (CARET) to prevent lung cancer in high-risk populations: pilot study with cigarette smokers. *Cancer Epidemiol. Biomarkers Prev.* 2, 389–396.
- Hale, L.J., 1973. The pattern of growth of *Clytia johnstoni*. *J. Embryol. Exp. Morphol.* 29, 283–309.
- Halliwell, B., 2006. Oxidative stress and neurodegeneration: where are we now? *J. Neurochem.* 97, 1634–1658.
- Halliwell, B., 2008. Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? *Arch. Biochem. Biophys.* 476, 107–112.
- Halliwell, B., Gutteridge, J.M.C., 2007. *Free Radicals in Biology and Medicine*, fourth ed. Oxford University Press, Oxford.
- Hayes, J.D., Flanagan, J.U., Jowsey, I.R., 2005. Glutathione transferases. *Annu. Rev. Pharmacol. Toxicol.* 45, 51–88.
- Honda, N.K., Pavan, F.R., Coelho, R.G., Leite, S.R.D., Micheletti, A.C., Lopes, T.I.B., et al., 2010. Antimycobacterial activity of lichen substances. *Phytomedicine* 17, 328–332.
- Ingolfssdottir, K., Chung, G.A., Skulason, V.G., Gissurarson, S.R., Vilhelmsdottir, M., 1998. Antimycobacterial activity of lichen metabolites in vitro. *Eur. J. Pharm. Sci.* 6, 141–144.
- Jayaprakasha, G.K., Rao, L.J., 2000. Phenolic constituents from the lichen *Parmotrema stuppeum* (Nyl.) Hale and their antioxidant activity. *Z. Naturforsch., C* 55, 1018–1022.
- Klamt, F., Dal-Pizzol, F., Bernard, E.A., Moreira, J.C., 2003. Enhanced UV-mediated free radical generation: DNA and mitochondrial damage caused by retinol supplementation. *Photochem. Photobiol. Sci.* 2, 856–860.
- Kristmundsdottir, T., Jonsdottir, E., Ogmundsdottir, H.M., Ingolfssdottir, K., 2005. Solubilization of poorly soluble lichen metabolites for biological testing on cell lines. *Eur. J. Pharm. Sci.* 24, 539–543.
- Kumar, K.C., Muller, K., 1999. Lichen metabolites. 1. Inhibitory action against leukotriene B₄ biosynthesis by a non-redox mechanism. *J. Nat. Prod.* 62, 817–820.
- Lopes, G.K., Schulman, H.M., Hermes-Lima, M., 1999. Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. *Biochim. Biophys. Acta* 1472, 142–152.
- Melo, M.G.D., Araujo, A.A.S., Rocha, C.P.L., Almeida, E.M.S.A., Siqueira, R.D., Bonjardim, L.R., et al., 2008. Purification, physicochemical properties, thermal analysis and antinociceptive effect of atranorin extracted from *Cladonia kalbii*. *Biol. Pharm. Bull.* 31, 1977–1980.
- Mollace, V., Muscoli, C., Masini, E., Cuzzocrea, S., Salvemini, D., 2005. Modulation of prostaglandin biosynthesis by nitric oxide and nitric oxide donors. *Pharmacol. Rev.* 57, 217–252.
- Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Sineiro, J., Dominguez, H., et al., 2001. Natural antioxidants from residual sources. *Food Chem.* 72, 145–171.
- Muller, K., 2001. Pharmaceutically relevant metabolites from lichens. *Appl. Microbiol. Biotechnol.* 56, 9–16.
- Navarra, M., Celano, M., Maiuolo, J., Schenone, S., Botta, M., Angelucci, A., et al., 2010. Antiproliferative and pro-apoptotic effects afforded by novel Src-kinase inhibitors in human neuroblastoma cells. *BMC Cancer* 10, 602.
- Oksanen, I., 2006. Ecological and biotechnological aspects of lichens. *Appl. Microbiol. Biotechnol.* 73, 723–734.
- Omenn, G.S., Goodman, G., Thornquist, M., Grizzle, J., Rosenstock, L., Barnhart, S., et al., 1994. The beta-carotene and retinol efficacy trial (CARET) for chemoprevention of lung cancer in high risk populations: smokers and asbestos-exposed workers. *Cancer Res.* 54, 2038s–2043s.
- Pekkarinen, S.S., Stockmann, H., Schwarz, K., Heinonen, I.M., Hopia, A.I., 1999. Antioxidant activity and partitioning of phenolic acids in bulk and emulsified methyl linoleate. *J. Agric. Food Chem.* 47, 3036–3043.
- Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., Pridham, J.B., 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* 22, 375–383.
- Salvemini, D., Doyle, T.M., Cuzzocrea, S., 2006. Superoxide, peroxynitrite and oxidative/nitrative stress in inflammation. *Biochem. Soc. Trans.* 34, 965–970.
- Salvemini, D., Wang, Z.Q., Wyatt, P.S., Bourdon, D.M., Marino, M.H., Manning, P.T., et al., 1996. Nitric oxide: a key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.* 118, 829–838.
- Scalbert, A., Johnson, I.T., Saltmarsh, M., 2005. Polyphenols: antioxidants and beyond. *Am. J. Clin. Nutr.* 81, 215S–217S.
- Schmitt, I., Lumbsch, H.T., 2004. Molecular phylogeny of the Pertusariaceae supports secondary chemistry as an important systematic character set in lichen-forming ascomycetes. *Mol. Phylogenet. Evol.* 33, 43–55.
- Silva, E.G., Behr, G.A., Zanotto-Filho, A., Lorenzi, R., Pasquali, M.A., Ravazolo, L.G., et al., 2007. Antioxidant activities and free radical scavenging potential of *Bauhinia microstachya* (RADDI) MACBR (Caesalpinaceae) extracts linked to their polyphenol content. *Biol. Pharm. Bull.* 30, 1488–1496.
- Stocker-Worgotter, E., 2008. Metabolic diversity of lichen-forming ascomycetous fungi: culturing, polyketide and shikimate metabolite production, and PKS genes. *Nat. Prod. Rep.* 25, 188–200.
- The ABC-Cancer Prevention Study Group, 1994. The effect of vitamin E and beta carotene on the incidence of lung cancer and other cancers in male smokers. The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group. *N. Engl. J. Med.* 330, 1029–1035.
- Toledo Marante, F.J., Garcia Castellano, A., Estevez Rosas, F., Quintana Aguiar, J., Bermejo Barrera, J., 2003. Identification and quantitation of allelochemicals from the lichen *Lethariella canariensis*: phytotoxicity and antioxidative activity. *J. Chem. Ecol.* 29, 2049–2071.
- Valencia-Islas, N., Zambrano, A., Rojas, J.L., 2007. Ozone reactivity and free radical scavenging behavior of phenolic secondary metabolites in lichens exposed to chronic oxidant air pollution from Mexico City. *J. Chem. Ecol.* 33, 1619–1634.
- Yilmaz, M., Turk, A.O., Tay, T., Kivanc, M., 2004. The antimicrobial activity of extracts of the lichen *Cladonia foliacea* and its (–)-usnic acid, atranorin, and fumarprotocetraric acid constituents. *Z. Naturforsch., C: Biosci.* 59, 249–254.