Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
23(2): 249-258, Mar./Apr. 2013

Influence of rosmarinic acid and *Salvia officinalis* extracts on melanogenesis of B16F10 cells

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Abstract: Melanin is a photoprotective skin pigment, and pathologies characterized by hypo or hyperpigmentation are common. New compounds that regulate melanogenesis are, therefore, opportune, and many natural products with this property, as polyphenols, have been described. *Salvia officinalis* L., Lamiaceae, is a widely used food spice that contains high amounts of phenol derivates, including rosmarinic acid. The aim of this work was to evaluate the contribution of rosmarinic acid in the melanogenic activity of sage extracts. Fluid and aqueous extracts of sage and purified rosmarinic acid were assayed for B16F10 cytotoxicity and, then, evaluated on melanin production and tyrosinase activity. While sage extracts showed a concentration-dependent ability to significantly increase melanin production without necessarily changing the enzymatic activity, rosmarinic acid showed a dual behavior on melanogenesis, increasing melanin biosynthesis and tyrosinase activity at low concentrations and decreasing it at higher levels. Rosmarinic acid may collaborate with sage extracts activity on melanogenesis, although other compounds may be involved. This is the first time that a dual action of rosmarinic acid on melanogenesis is reported, which may be useful in further studies for therapeutic formulations to treat skin pigmentation disorders.

Article

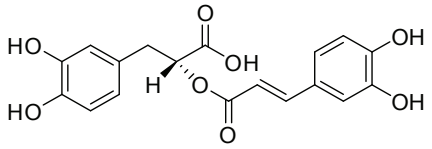
Received 4 Jun 2012
Accepted 24 Sep 2012
Available online 22 Nov 2012

Keywords:
melanin
melanogenesis
rosmarinic acid
Salvia officinalis
tyrosinase

ISSN 0102-695X
DOI 10.1590/S0102-695X2012005000135

Introduction

Salvia officinalis L., Lamiaceae (sage) is a well-known vegetal specie largely used for food spicing. It presents many pharmacological properties, most of them associated with its polyphenol content (Lu & Foo, 2002; Bauer et al., 2012; Ben Taarit et al., 2012; Generalic et al., 2012). Rosmarinic acid (RA – **1**) is the major phenol compound of sage, also found in many other plants used as seasoning (Petersen & Simmonds, 2003). Relevant biological activities such as neuroprotective (Iuvone et al., 2006; De Oliveira et al., 2012), antioxidant (Zheng & Wang, 2001; Perez-Tortosa et al., 2012), anticancer (Karmokar et al., 2012; Sharmila & Manoharan, 2012), anti-inflammatory (Sahu et al., 1999; Al-Musayeib et al., 2011), and immunomodulatory (Kang et al., 2003; Costa et al., 2012) have been attributed to RA. Photoprotection and melanogenic properties have also been described, in which positive effects on human keratinocyte cells after UVA (Psotova et al., 2006) and UVB exposures (Vostalova et al., 2010) were observed, resulting in an increased cell viability and reduced DNA damage. The effects of RA on mouse melanoma cells (Lee et al., 2007) and in the reduction of photo-induced skin alterations in mice (Sanchez-Campillo et al., 2009) have also been shown.



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Melanin pigmentation of the skin has important cosmetic and therapeutic roles, which can be compromised in various dermatological conditions. Hyperpigmentation disorders, such as freckles, lentigines, melasma or age spots, can be the result of a misregulated production or accumulation of melanin by melanocytes (Virador

et al., 1999). Hypomelanosis, on the other hand, is caused by lower number of melanocytes, interference on melanin biosynthesis or by a decrease in tyrosinase availability (Wulf et al., 2004). The treatment of these conditions is based on melanin biosynthesis, which initial steps involve the enzyme tyrosinase, the key element that catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA), and from that to dopaquinone. After several steps melanin is produced as a complex mixture of pigments (Kim & Uyama, 2005; Chang, 2009). Within this context, compounds that interfere with this biosynthetic pathway, increasing or decreasing the amount of melanin formed, may be potential candidates to treat pigmentary disorders.

Some agents used to treat skin hyperpigmentation, like hydroquinone, can cause side effects such as dermatitis and skin irritation (Maeda & Fukuda, 1996). Other disorders, such as vitiligo, have no effective cure and limited treatment options (Huggins et al., 2012). The search for natural products for treatment of skin pigmentation disorders is, therefore, opportune. Many inhibitors and enhancers of melanin biosynthesis have been described and some are obtained from natural sources. Kojic acid, for example, is a well-known tyrosinase inhibitor produced by *Aspergillus* and *Penicillium* fungi (Smit et al., 2009). Some plant polyphenols, on the other hand, have been described as melanin inducers (Kim & Uyama, 2005).

Considering that *S. officinalis* contains high amounts of RA among other polyphenols, the aim of this work was to study the melanogenic activity promoted by RA, and its contribution in the melanogenic effects promoted by a fluid extract and an aqueous extract prepared from dried sage leaves.

Materials and Methods

Plant material

Dried and crushed *S. officinalis* leaves were purchased in a local market (Curitiba, Brazil), and identified by macro and microscopic analysis (Wichtl, 2004), and by comparison with an authentic sample of the Laboratory of Pharmacognosy, Department of Pharmacy, Federal University of Paraná, Brazil. A specimen voucher (300-A) of the plant has been deposited at the Herboteca Carlos Stellfeld, Federal University of Paraná, Brazil. For isolation of RA, a dry extract of aerials parts of sage, containing around 6% (w/w) of RA according to the supplier label and confirmed by chromatographic quantification, was purchased from Apsen Pharmaceuticals.

Instrumentation and chemicals

^{1}H and ^{13}C NMR spectra were acquired on a Bruker Avance 200 spectrometer (Rheinstetten, Germany) using tetramethylsilane (TMS, Sigma Aldrich) as internal standard. A Varian 920 series HPLC system (Palo Alto, CA) with quaternary pump, autosampler, column oven and diode array detector was used for the chromatographic analyses. The absorbances were measured in a Shimadzu Model 1800 UV-VIS spectrophotometer. A Harrison Research Chromatotron Model 7924T (Palo Alto, CA) was used for preparative chromatography. HPLC grade solvents were purchased from Merck (São Paulo, Brazil). All other solvents and reagents used were of analytical grade.

Preparation of extracts

Aqueous extract (AE) of sage was obtained by extraction of crushed dried leaves (10 g, <710 μm) with water at 80 °C (100 mL) for 15 min. The mixture was filtered (Whatman nº 1), and the extract volume completed to 100 mL. Fluid extract (FE) was prepared by successive percolations of crushed dried leaves of sage (20 g, <710 μm) with 56% ethanol (v/v) for three days, in the dark, at room temperature (Farm Bras, 2002). The extracts were combined and concentrated to 20 mL. Samples were sterilized by filtration through a 0.22 μm membrane filter and stored at -25 °C till use.

Rosmarinic acid isolation and characterization

Isolation of RA from sage was based in a previous report with modifications (Christ & Kesselring, 1982). Sample of 10 g of dry extract of sage was dissolved in water (300 mL) at 80 °C with magnetic stirring. The solution was filtered (Whatman nº 1), acidified to pH 2.5 and centrifuged. The supernatant was extracted with diethyl ether (5 x 100 mL), the organic fractions were combined, and the solvent evaporated. The resultant solid was fractionated by Chromatotron, in a silica gel with gypsum (Aldrich, 346446) rotor. Elution was performed with ethyl acetate/hexane/acetic acid (55:35:10 v/v/v) and all obtained fractions were analyzed by TLC. Those containing RA were combined, the solvent evaporated and the residue stored under nitrogen. The isolated solid was characterized as RA by spectroscopic methods (UV, ^{1}H and ^{13}C NMR). For the biological assays, RA stock solution (10^{-2} M in RPMI full medium) was sterilized by filtration through a 0.22 μm membrane filter and stored at -25 °C.

Rosmarinic acid content of FE and AE

The concentration of rosmarinic acid in AE and FE was determined by HPLC as previously described,

with some modifications (Wang et al., 2004). Briefly, samples of AE and FE were diluted to 1:50 in methanol 50% (v/v), and injected into a silica C_{18} column (Agilent Eclipse C18, 150 mm x 4.6 mm x 5 µm) at 25 °C. Elution was performed with methanol (eluent A) and 0.1% phosphoric acid in water (eluent B) in a step gradient mode (45% of A in B, 0-5 min, and 80% of A in B, 5-10 min). The flow rate was 1.0 mL/min and detection at 330 nm. The concentration of RA was determined by external standard calibration using a five-point analytical curve.

Cell cultures and viability

B16F10 and McCoy cell lines were maintained under sterile conditions at 37 °C in RPMI 1640 medium (Himedia Laboratories; Mumbai, India) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/mL penicillin G, and 50 µg/mL streptomycin sulfate (RPMI full medium). The cell lines were subcultured twice a week at 10^5 cells/mL, and the number of viable cells used in the experiments, determined by the trypan blue exclusion test, always exceeded 95%.

Cell proliferation assay and cytotoxicity evaluation

B16F10 and McCoy cells were seeded in 24-well culture plates in RPMI full medium, and allowed to attach overnight at 37 °C in a fully humidified, 5% CO_2 incubator. Test solutions (RA, AE and FE) were then added to semi-confluent cultures to give final RA concentrations ranging from 0.001 to 1,000 µM, and the cultures re-incubated at the same conditions for 24 h. The medium was then removed, and the cells were properly treated with neutral red (NR) dye, following the NR assay (Borenfreund & Puerner, 1985). Absorbance of the treated cells was measured at 540 nm, and viability was quantified as the percentage (%) of living cells relative to the control. Data was expressed as mean (%) ±SD from a triplicate.

Preparation of cell lysates

In order to test RA, FE and AE on melanin synthesis and tyrosinase activity, B16F10 cells were cultured in 25 cm^2 culture flasks in full RPMI medium containing 10 mM of NH_4Cl as lysosomotropic agent (Saeki & Oikawa, 1983), in the presence of FE, AE or RA, with or without 500 µM L-tyrosine, for 24 h at 37 °C, in a fully humidified, 5% CO_2 incubator. Treatment of the cells with 400 mM kojic acid dissolved in DMSO was used as a positive control for melanogenesis inhibition. B16F10 cells kept under the same conditions but without any treatment were used as a control of the experiments. After treatment, the cells were washed twice with cold phosphate buffered saline (PBS), and detached by short incubation with 0.02% EDTA at 37 °C. After centrifugation at 800 *g* for 5 min, the cell pellets were sonicated on ice for 1 h with 0.1 M Tris-HCl pH 7.2 lysis buffer (200 µL). The cell homogenates were used for melanin quantification and protein determination by the Bradford method (Bradford, 1976). Their respective supernatants (cell lysates), obtained after centrifugation of the homogenates at 10,000 *g* for 10 min at 4 °C, were used for tyrosinase activity.

Melanin production in B16F10 cells

Aliquots of 100 µL of the homogenates, obtained from lysed cells pretreated with RA, AE and FE, were added to 900 µL of 1 M NaOH and incubated overnight at 37 °C. Melanin concentration was determined spectrophotometrically at 470 nm, using an external calibration curve (10-100 µg/mL) prepared with melanin from *Sepia officinalis* (Sigma, M-2649). Melanin content was calculated relative to the control, following the formula [(concentration of melanin per µg of protein of treated cells/concentration of melanin per µg of protein of control) x100], and expressed as mean intracellular melanin per ug of total protein (%) ±SD (Tsuboi et al., 1998; Heo et al., 2009).

Cellular tyrosinase activity

Tyrosinase was assayed spectrophotometrically according to a published method (Sarkar et al., 2006) with some modifications. Briefly, the reaction mixture (1 mL), composed of cell lysates (100 µg of protein) from pre-treated B16F10 cells with FE, AE, or RA, added of 0.1% L-DOPA in 0.1 M NaH_2PO_4 pH 6.8, was kept at 37 °C for 1 h. Absorbance was then measured at 470 nm. Tyrosinase activity was calculated as a percentage (%) relative to the control, according to the equation [(absorbance of pre-treated cells lysate/absorbance of control cell lysate) x100], and was expressed as mean (%) ±SD.

Free tyrosinase assay

Solutions (1 mL) were prepared with cell lysates from control B16F10 cells (100 µg of protein) as a source of enzyme, added of RA, FE or AE, and 0.1% L-DOPA in 0.1 M NaH_2PO_4 pH 6.8. The reaction mixture was kept at 37 °C for 1 h, and the absorbance measured at 470 nm. Proper controls were conducted in order to correct for any potential interference. The percentage (%) of tyrosinase activity relative to the control was calculated with the equation {[(Aa-Ab)/(Ac-Ad)] x100}, where Aa is the absorbance of the test sample in the presence of enzyme and substrate; Ab, the absorbance of the test sample in the presence of substrate; Ac, the absorbance of the substrate with enzyme; and Ad, the absorbance of

the substrate alone. Results were expressed as mean (%) ±SD (Nagata et al., 2004; An et al., 2008).

Zymography

Lysates of B16F10 cells (100 µg of protein) pre-treated with RA, FE and AE were mixed with appropriate volume of Blue/Orange 6X Loading Dye (Promega, Madison, WI, USA) and resolved in a 8% SDS-polyacrylamide gel at 200 mV for 50 min. After electrophoresis, the gel was washed in 0.1 M NaH_2PO_4 pH 6.8, transferred to a staining solution containing 5 mM L-DOPA, and kept at 37 °C for 5 h in the dark, after which the dark bands of tyrosinase could be visualized and photo documented (Sato & Toriyama, 2009).

Statistical analyses

Student's *t*-test or analyses of variance (ANOVA) followed by Tukey HSD -α = 0.05 whereas necessary were carried out using the Graph Pad-Prism 5 software. Differences were considered significant at $p \leq 0.05$.

Results and Discussion

Isolated RA

The amount of purified RA obtained from 10 g of sage dry extract was 4.9±0.85 % (w/w). Its spectral profile, and the ^{1}H and ^{13}C NMR data were all consistent with the literature data for RA, confirming the identity of the compound (Kelley et al., 1975; 1976; Wang et al., 2004; Xu et al., 2008).

Rosmarinic acid content in extracts

The concentration of RA in FE (5.88 mg/mL) was about 10 times higher than in AE (0.51 mg/mL) as expected. From these results and considering the dry weights of FE (156.1 mg/mL) and AE (14.3 mg/mL), proper dilutions were made in order to obtain solutions with standardized RA concentrations for bioassays, allowing the comparison of the effects.

Cell phenotype alterations induced by extracts and RA

After treating B16F10 cells with varying concentrations of RA, FE, and AE, the effects on their morphology evaluated by phase-contrast microscopy were evident, leading to profound modifications in the cell pigmentation patterns. They changed from the characteristic spindle-like morphology of fibroblasts, interspersed by spherical-shaped cells (Figure 1A) to irregular, flattened shape, with long dendrites-like protrusions around a cytoplasm darkened by the presence of melanin (Figure 1B). These phenotypic changes were far more evident when L-tyrosine was added to the cultures, and were all suggestive of melanogenesis induction (Fernandes et al., 2004).

The treatment of McCoy cells with non-toxic

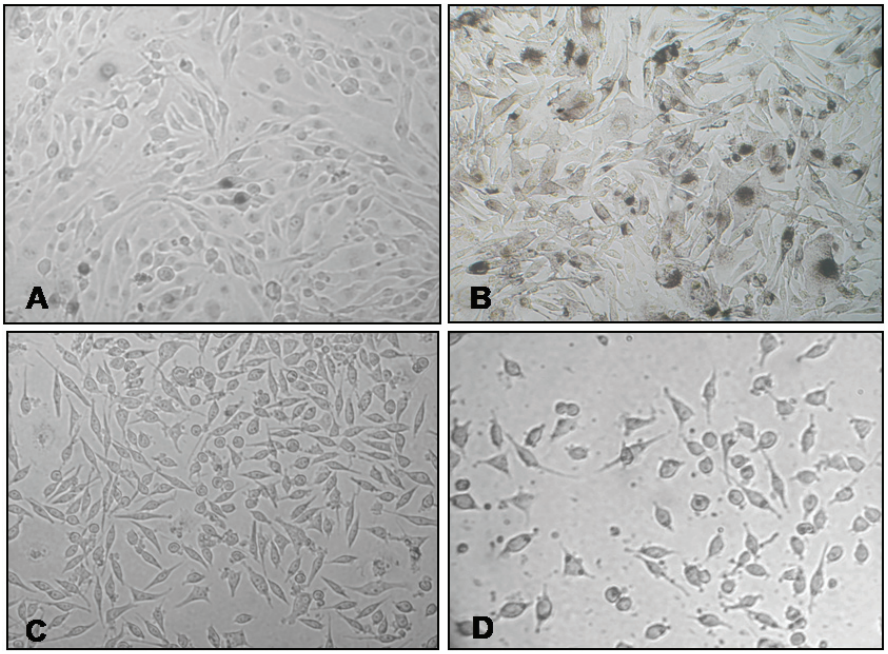

Figure 1. Effects of rosmarinic acid on B16F10 and McCoy cell phenotypes. Phase-contrast microscopy of (A) B16F10 culture showing characteristic fibroblastic and spherical cell morphology; (B) melanocytes appearing as flattened cells, with scattered and darkened cytoplasm surrounded by multipli protrusions after 24 h exposition to rosmarinic acid (10 µM) and L-tyrosine (500 µM); (C) McCoy cells after rosmarinic acid (10 µM) exposition; (D) McCoy cells after rosmarinic acid (1000 µM) exposition.

concentrations of tests did not induce any morphological alterations (Figure 1C). On the other hand, classical toxic effects such as cytoplasm vacuolization, retraction of dendrites, and cell detachment were easily observed for both cell lines, particularly in experiments with the extracts at 1,000 µM of RA (Figure 1D). When the incubation time was longer (>48 h), the culture dishes of B16F10 and McCoy cells were fully confluent and signals of deterioration were evident, as detached and floating cells in the culture medium. These observations led us to perform all experiments within 24 h, in order to avoid misinterpretation of results obtained from stressed, unhealthy cells (Virador et al., 1999).

Cell toxicity of sage extracts and RA

Different from McCoy cells, the B16F10 cell line is very sensitive to environmental changes, easily acquiring characteristics of melanogenic activation under stress. During the screening of prospective bioactive compounds for modulation of skin pigmentation using this cell line, it is therefore important to consider possible adverse effects on their survival and proliferation status, in order to avoid misinterpretation of results that could be compromised by unhappily cultured cells. Therefore, quantitative determination of cell viability after treatment with sage extracts and RA for 24 h was carried out using the NR technique. The results for B16F10 cells (Figure 2) were consistent with the phenotypic observations described earlier. RA did not interfere with their viability up to 1,000 µM (Figure 2A), contrasting with a previous report (Yoshida et al., 2005). However, RA was significantly deleterious for McCoy cells at 100 and 1,000 µM, reducing their viability to 70 and 35%, respectively. FE also reduced significantly the viability of McCoy cells in all concentrations tested (Figure 2B), being more pronounced in the 10-1,000 µM range of RA content. For B16F10 cells, the viability was reduced only at FE concentrations higher than 100 µM of RA. The results of AE showed that only at 1,000 µM of RA the viability of both cell lines was compromised (Figure 2C).

With these results, it become evident that the McCoy fibroblastic cells were more sensitive than B16F10 melanoma cells to the toxic effects of RA treatment, which is expected and in accordance with the characteristic drug resistance of tumor cells in contrast with normal cells (Rumjanek et al., 2001).

Comparing the results obtained for FE, AE and RA, a clear indication that FE has other compounds apart from RA that may be responsible for the toxicity observed raised. Also, the distinct results obtained for FE and AE treatments may be explained by their different production ways, and consequently, by their different chemical composition. In fact, the chromatograms of the extracts showed distinct profiles and less polar substances, such as luteolin, were found in higher concentrations in FE (data not shown). Hence, it is likely that compounds present only in FE may account for at least some of the more pronounced toxic effects observed.

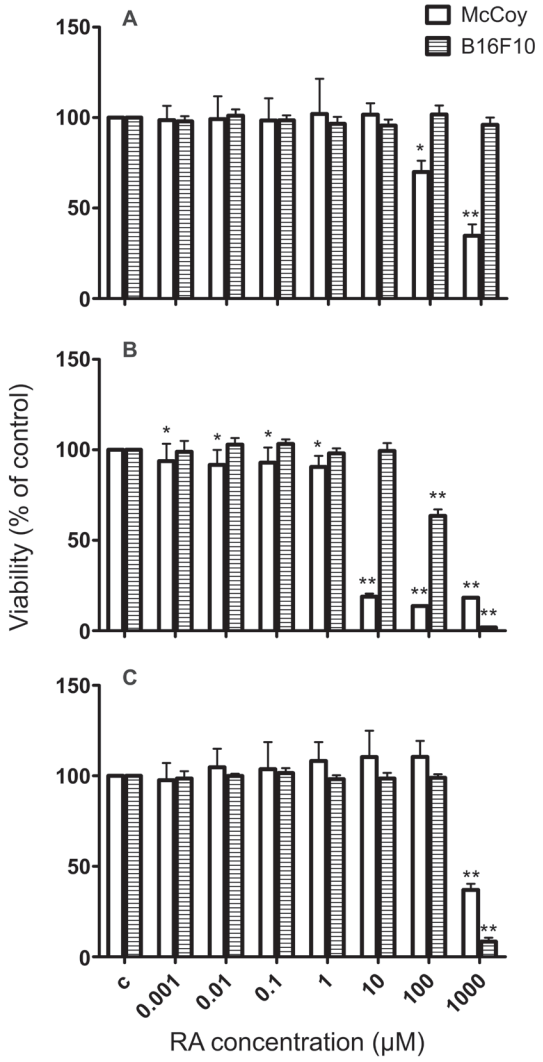

Figure 2. Effects of rosmarinic acid (RA) (A), sage fluid (B) and aqueous extracts (C) on McCoy and B16F10 cell lines viability. For conditions see *Material and Methods*. Each bar represents the percentage of living cells relative to control, expressed as mean±SD from four independent experiments, each one performed in triplicate (*p<0,05; **p<0.0001).

Influence of RA and sage extracts on melanin production

Inhibition of tyrosinase gene expression or maturation and direct inactivation of the enzyme can be used as tools for controlling melanogenesis (Virador et al., 1999). Using one or both approaches, the melanogenic activity will, therefore, reflect on melanin production and tyrosinase activity. Because B16F10 cells share with normal human melanocytes many of

the melanogenic pathways, they were used in this work as a model to evaluate the effects of sage extracts and RA on melanogenesis. In order to avoid interferences on melanogenesis caused by the reduction of cell number influenced by the testing substances, only non-toxic concentrations of FE, AE, and RA, as well as of kojic acid, L-tyrosine, DMSO and NH_4Cl, used alone or in combinations, were used in the experiments.

Since cellular melanin contents reflect turnover, accumulation, and distribution of melanin associated with cellular proliferation, semi-confluent B16F10 cultures were treated with sage extracts and RA for 24 h, with or without L-tyrosine, and both melanin content and tyrosinase activity were determined. FE was the only sample that, in the absence of L-tyrosine, showed little ability to interfere positively with the melanin content of B16F10 cells (Figure 3A), in contrast with L-tyrosine alone, which increased more than three times the melanin content compared to control population. These results for L-tyrosine were expected since it is a substrate for tyrosinase activity, empowering melanin synthesis (Kongshoj et al., 2007). However, the discrepancy of the effects with extracts and RA led us to question whether a low availability of substrate for melanin production could be obscuring the results.

To address this hypothesis, a new set of experiments with addition of L-tyrosine (500 µM) was carried out, having in mind that L-tyrosine not only stimulates melanin production but also can be used as a substrate for tyrosinase action. Under this circumstance, a new spectrum of activities was developed and a strikingly significant influence of FE, AE, and RA on pigment production in the presence of L-tyrosine was observed (Figure 3B). Both sage extracts had a concentration-dependent ability to significantly stimulate melanogenesis in B16F10 cells. It was even more pronounced at 10 µM of RA, when FE and AE increased in 117% and 90%, respectively, the melanin content. Furthermore, the well known melanogenic inhibitory effect of kojic acid (Chang, 2009) could then be revealed, decreasing in more than 50% the melanin content.

While the melanin assay results showed that both *S. officinalis* extracts have significantly stimulated melanin production in B16F10 cells at 1 and 10 µM, the effect of purified RA on pigment production was intriguing, acting significantly as a stimulator of melanin formation at 10 µM, and as an inhibitor at 1,000 µM. Increased melanin production by RA in the 1-100 µM range using a similar cell system has been reported (Wulf et al., 2004; Lee et al., 2007; Sanchez-Campillo et al., 2009). However, to the best of our knowledge, this is the first time that a significant inhibitory melanogenic activity for RA is described.

Interestingly is also the results indicating that FE and AE were even better than RA alone on melanin production when L-tyrosine was present, suggesting that RA is one but not the only one of the compounds present in these mixtures contributing with these effects. Therefore, it is reasonable to keep in mind that FE and AE may have substances that would serve as alternative substrates for tyrosinase activity, leading to dramatic changes in melanin production. Moreover, since FE induced a higher melanin biosynthesis than AE, it is possible that stimulating agents of melanogenesis are in higher concentrations in FE.

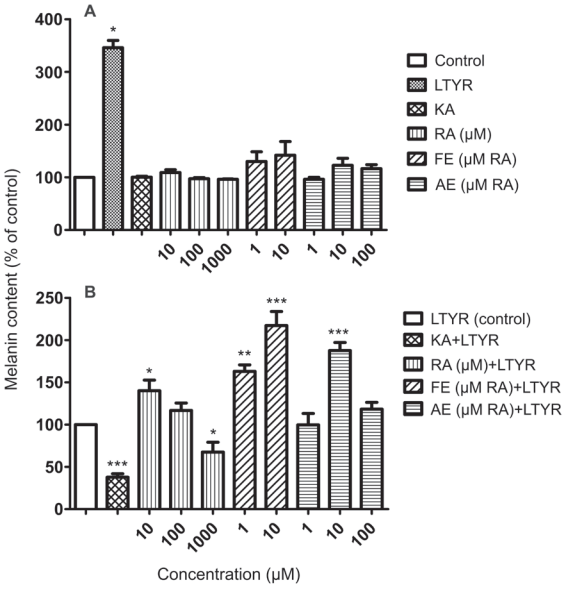

Figure 3. Influence of rosmarinic acid (RA), sage fluid (FE) and aqueous extracts (AE) on melanin production by B16F10 cells. B16F10 cells were treated with the indicated concentrations of the tests for 24 h at 37 °C, without (A) or with (B) L-tyrosine (LTYR). For conditions see *Material and Methods*. Each bar represents the mean percentage±SD of melanin content related to control (n=5-8; **p*<0.05; ***p*<0.01; ****p*<0.0001). KA: kojic acid.

Cellular and free tyrosinase activity

Tyrosinase is an enzyme widely distributed in nature, and considered the key enzyme of melanogenesis in animals (Jimenez-Atienzar et al., 2005; Kim & Uyama, 2005). The influence of substances and extracts in the oxidative steps catalyzed by tyrosinase may be related to the mechanisms of action in the biosynthesis of the enzyme or in its activity. In order to rationalize the pigment inducing ability of FE, AE, and RA on B16F10 cells, their effects on cellular and free tyrosinase activities were assessed. To do so, two series of experiments were performed. First, B16F10 cells were exposed to RA, FE, and AE for 24 h, and, then, the enzyme activity measured in cell lysates (Figure 4A) and by zymography (Figure 5). In a second round, untreated B16F10 cell lysates were

used as a free enzyme source (An et al., 2008) and added of RA, FE, and AE for 60 min in a L-DOPA solution; then, the enzyme activity was measured (Figure 4B).

As expected and in contrast with the stimulant effects observed for L-tyrosine, kojic acid significantly reduced both cellular (Figure 4A) and free (Figure 4B) tyrosinase activities, which is in accordance with the results obtained for melanin content (Figure 3B), and also with its well documented characteristic as a tyrosinase inhibitor. RA stimulated the cellular tyrosinase activity but only at 10 μM, increasing it at 50% when compared to controls. This significant effect was confirmed by the greater intensity of the tyrosinase band observed in the zymogram (Figure 5). However, at that same concentration, RA did not alter the free tyrosinase activity (Figure 4B), suggesting that the main mechanism of action involved is related to the enzyme synthesis instead to its activity. At higher concentrations, RA led to an opposite effect, with a significant dose-related decrease in tyrosinase activity in both systems, recapitulating the trend profile of pigment formation observed for similar concentrations (Figure 3B).

These contrasting results led us to propose that RA, depending on its concentration, has a dual effect on melanogenesis of B16F10 cells. Within this hypothesis, RA, at low concentrations, would act as a positive modulator of melanogenesis, interfering on tyrosinase synthesis that is followed by a rapid increase in its activity and visualized by melanin production. This action would explain the increased melanin content and cellular tyrosinase activity observed in our experiments at 10 μM. Moreover, this suggestion is consistent with the reported data showing that RA increases tyrosinase expression by activating the PKA pathway (Lee et al., 2007).

On the other hand, as a strong enzymatic inhibition has been observed for RA at higher but non-cytotoxic concentrations, it is possible that RA, as other polyphenols, acts as a competitive inhibitor of the tyrosinase binding site, leading to the formation of *o*-quinone derivates and blocking the enzymatic oxidation of L-DOPA to *o*-dopaquinone. Moreover, RA is a potent antioxidant, and as such, at high concentrations, it may also act reducing the *o*-dopaquinone present in the reaction mixture to L-DOPA, limiting the formation of dopachrome and, consequently, of melanin (Chang, 2009). Within this context, the exposure of B16F10 cells to higher concentrations of RA would trigger several processes related to oxidation, simultaneously acting as a negative modulator of melanogenesis.

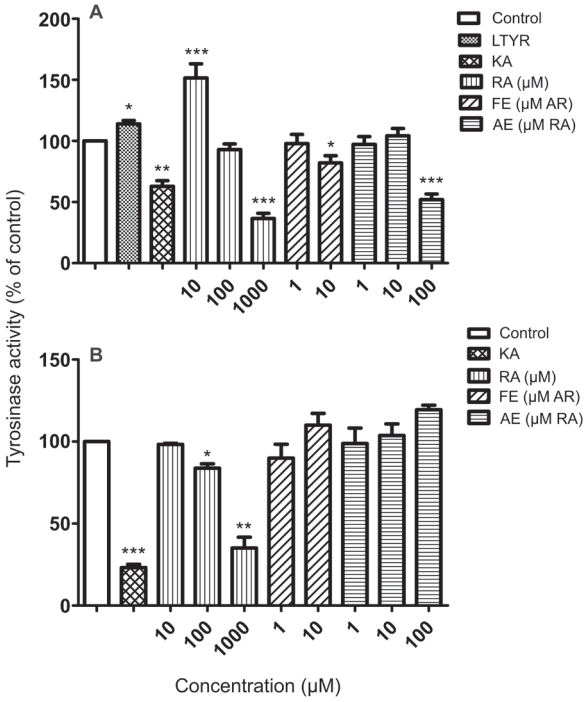

Figure 4. Influence of rosmarinic acid (RA), sage fluid (FE) and aqueous extracts (AE) on cellular (A) and free tyrosinase activities (B). For conditions see *Material and Methods*. Each bar represents the mean percentage ± SD of tyrosinase activity related to control (n= 5-8; **p*<0.05; ***p*<0.01; ****p*<0.0001). KA: kojic acid; LTYR: L-tyrosine.

The interpretation of the results obtained for FE and AE may be even more complex due to the wide variety of substances that are present in those extracts. If they do exist, they may have different ways of acting on melanogenesis, interfering with various/different steps of tyrosinase synthesis and activity, and possibly acting synergistically. FE did not change cellular and

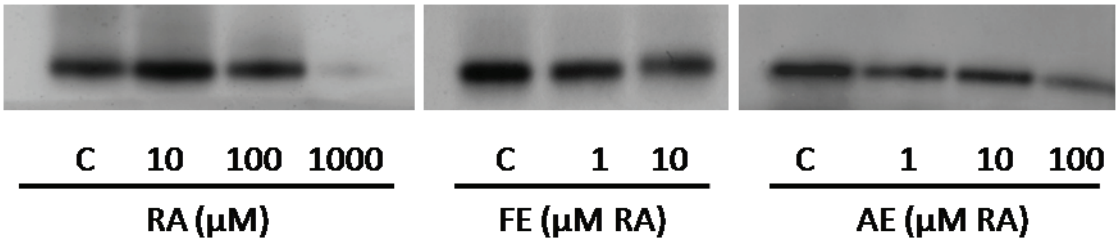

Figure 5. Zymogram presenting the effect of rosmarinic acid (RA), sage fluid (FE) and aqueous extracts (AE) on cellular tyrosinase activity. For conditions see *Material and Methods*.

free tyrosinase activity, except at 10 μM of RA, when a significant decrease in enzyme activity by 20% was observed. AE showed similar behavior, and only at 100 μM of RA it reduced significantly the cellular tyrosinase activity by 60%. Thus, the concentrations of FE and AE that induced an increase in melanin synthesis (Figure 3B) did not affect free or cellular tyrosinase activities in the same way.

Together, the results suggest the involvement of substances other than RA in sage extracts with different mechanisms on the biosynthesis of tyrosinase and/or on its activity. It is known that the *S. officinalis* aqueous and hydroalcoholic extracts have polyphenols as the major chemical constituents, particularly flavonoids, mostly represented by luteolin and its derivate luteolin-7-*O*-glucuronide, and the caffeic acid derivates, including RA (Fecka & Turek, 2007). Previous report has shown that luteolin inhibits cAMP synthesis, an important factor for tyrosinase biosynthesis (Choi et al., 2008). Also, at higher concentrations, this flavonoid was able of inhibiting tyrosinase activity in a reversible and non-competitive manner (Xie et al., 2003; An et al., 2008; Choi et al., 2008). Consequently, compounds like luteolin present in the extracts may indirectly reduce tyrosinase biosynthesis and its activity, eventually blocking the stimuli evoked by other substances such as RA, for example, upon enzyme transcription and action. Furthermore, the higher production of melanin induced by sage extracts could lead to higher amounts of oxidants such as H_2O_2 in the medium. As an inhibitory action of H_2O_2 on melanogenesis, mediated by a reduction of the intracellular concentration of the melanogenic enzymes achieved partially at the transcriptional level, has been described (Jimenez-Cervantes et al., 2001; Wood et al., 2004), it may contribute to amplify the oxidant potential of the system enhancing the tyrosinase inhibition (Jimenez-Cervantes et al., 2001; Wood et al., 2004).

It is worthy of note that FE and AE extracts were produced by different methods, and differences in their chemical composition and biological activities are expected to occur, as shown in this work, emphasizing the need of standardized plant drug processing for therapeutic purposes.

In conclusion, the results of the present study suggest that sage extracts and RA interfere in melanogenesis at cellular melanin levels depending on their concentration in the medium, but not necessarily changing the activity of cellular and free tyrosinase. RA and other phenolic substances may collaborate with the effects of sage extracts in melanin production and tyrosinase activation. RA showed a dual behavior on melanogenesis, acting as stimulator of melanin synthesis and cellular tyrosinase activity at low concentrations, and as an inhibitor at high levels. *Salvia officinalis* and RA can be considered as potential therapeutic agents for treating diseases related to skin pigmentation. Further studies of the mechanisms involved in their action on melanogenesis and about their potency as pigmenting agents are, thus, encouraged.

Acknowledgement

The financial support of CAPES/Ministry of Education, Brazil, to KBO is greatly acknowledged.

Authors contributions

KBO (PhD student) has contributed to the plant material identification, has ran the lab work concerning to the plant extracts' preparation; rosmarinic acid isolation, identification and evaluation along with most of the cellular tests as well as analysing the data, drafting, and reviewing the manuscript. EK contributed to the cytotoxicity tests. AMWS contributed to the cellular tests' design, analysis of the data, supervision of the cellular lab work, and critically reviewing the manuscript. BHO supervised the plant material identification; the isolation, and identification of rosmarinic acid, the data analyses as well as the review of the manuscript. All authors have read the final manuscript and approved its submission.

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