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Research Article

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Characterization and antioxidant activity of carotenoid mixtures present in *Rhodococcus* sp. and *Gordonia* sp.

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ABSTRACT

Structure elucidation of carotenoids pigments present in Rhodococcus sp. and Gordonia sp. was performed with Fourier-transformed infrared spectrometry (FT-IR), high performance liquid chromatography (HPLC), ultravioletvisible spectrometry (UV-Vis), electrospray ion-mass spectrometry (ESI-MS) and proton and carbon nuclear magnetic resonance (¹H and ¹³C NMR). A HPLC carotenoid quantification method was developed, where antioxidant activity with 2,2-diphenyl-1-picrylhidrazyl (DPPH) radical was performed to calculate the inhibitory concentration 50 (IC₅₀) of each carotenoid mixture; contribution of each carotenoid present in mixtures and possible interactions were determined. Rhodococcus sp. and Gordonia sp. presented two and three carotenoids respectively; analytical techniques allowed to suggest that carotenoids present in these strains might have structures similar to the reduced form of 4-oxo retinaldehyde, 8'-apoastaxanthinal and astaxanthin dirhamnoside. Rhodococcus sp., Gordonia sp. between the reduced form of 4-oxoretinaldehyde like carotenoid and astaxanthin dirhamnoside like carotenoid was assessed.

Keywords: Carotenoid characterization, DPPH antioxidant activity, carotenoid interactions.

INTRODUCTION

Carotenoids are organic pigments classified mainly in carotenes (e.g β -carotene, lycopene and phytoene) and xanthophylls (e.g. astaxanthin, canthaxanthin, zeaxanthin) [1]. Such pigments are important because they suppress reactive oxygen species mainly because of their long polyene chain, thus having an active role in electron transfer processes [2-5]. Where such activity depends on the length of such chain and the presence of polar groups within the structure [6-8].

Most of the bacteria that produce pigments have been isolated from aquatic environments, comprising carotenoids as the major constituents of these pigments [9], including actinobacteria [10]. Where the biodiversity of marine environments along with their unique chemical features, allow the discovery of either new bioactive compounds or new sources of already known compounds [11]; where in this regard biotechnological production of marine carotenoids is an alternative technique for large scale production with the advantage of being eco-friendly and self-sustainable [7, 12, 13].

2,2-diphenyl-1-picrylhidrazyl (DPPH) is a stable radical because of the unpaired electron on the molecule compared to other radicals making it capable of accepting electrons [14]. It is one of the most used methods to measure antioxidant activity because it is simple, fast [15] and still is a current colorimetric method that can be used as a first approach to assess biological activity of new compounds [16-23]. However, the presence of pigmented compounds can interfere with colorimetric estimation of this radical [24]. The use of high performance liquid chromatography (HPLC) with DPPH radical with carotenoid pigments has not been researched, solely tested in herbal extracts containing mostly polyphenols [24-26]; where it is considered that this analytical technique can allow the measuring of DPPH in the presence of pigmented compounds, thus making it applicable for carotenoids.

When studying antioxidant activity of natural products, such study becomes complicated because the isolation and study of individual molecules is expensive and ineffective without mentioning the possible synergistic interactions between antioxidants [27]. Nevertheless with the right data treatment it can be compared the contribution of each compound present in extracts as reported by Quiu et al 2012 [28] for peanut shell extracts within a mixture and therefore we can assess if antioxidants are different from each other.

In this research, structure elucidation of carotenoids pigments present in *Rhodococcus* sp. and *Gordonia* sp. was performed with Fourier transformed infrared spectrometry (FT-IR), HPLC, ultraviolet-visible spectrometry (UV-Vis), electrospray ion-mass spectrometry (ESI-MS) and proton and carbon nuclear magnetic resonance (¹H and ¹³C NMR). Furthermore a HPLC carotenoid quantification method was developed, where antioxidant activity with DPPH radical was performed to calculate the inhibitory concentration 50 (IC₅₀) of each carotenoid mixture; the contribution of each carotenoid present in mixtures and possible interactions were determined.

EXPERIMENTAL SECTION

Gordonia sp. and Rhodococcus sp. were isolated from sediments from the Gulf of México obtained during the oceanographic campaign BIOREPES-2005 [29].

Strain Growth and carotenoid extraction of Rhodococcus sp. and Gordonia sp.

Gordonia sp. and *Rhodococcus* sp. were grown in glucose, yeast extract, agar (GYEA) at 28°C, 10 days; these cultures were used to inoculate flasks containing 250 mL of liquid medium. The flask was incubated at 28°C at 150 rpm for 6 days. Biomass was separated at 4500 rpm 15 minutes, and freeze-dried at -70°C, 0.2 bars and the extraction was performed according to Romero et al. 2012 [30] with a mixture of dichloromethane, methanol and acetone (1:1:2).

Carotenoids mixtures were pre-purified with semipreparative high performance liquid chromatography (HPLC) using a General Electric Tricorn 10/150 column packed with silica Hypersep C18 (40-63 μ m, 60 Å) with a mixture of methanol: acetonitrile: ethyl acetate: water (65:10:20:5) (A) and a mixture of methanol: ethyl acetate (50:50) (B) with the following gradient elution: 55-60 minutes 75% B, 70-85 minutes 100% B, 85-90 minutes 100% A, 2 mL/min, detection at 450 nm.

FT-IR analysis of carotenoid mixtures

Pre-purified carotenoid mixtures were dissolved in chloroform, and the detection was performed from 4000 to 600 cm^{-1} .

HPLC and UV-Vis analysis of carotenoid mixtures

Mixtures were observed with HPLC under the same conditions reported by Rivera et al. 2011 [31] in a Waters Symmetry C18 (75x4.6mm, 3.5 μ m) column using as mobile a mixture of acetonitrile, methanol and water, with gradient elution, 2 μ L, UV-Vis spectra was recorded (190-700 nm) during the analysis with the diode arrangement

detector (DAD) and chromatograms were acquired at 450 nm. The maximum absorption wavelength (λ) was assessed and if the UV-Vis spectra presented three λ , proportion of λ III in λ II (% III/II) was calculated.

MS analysis of extracts of carotenoid mixtures

A scanning of compounds present in extract was performed by direct injection with ESI-MS with the following conditions: ion trap in positive mode, needle at 5000 volts 2.6 µAmps, nitrogen was used as drying gas at 350°C, 15 psi, housing at 50°C, nebulizer 25 psi, capillary 70 volts, spray shield 600 volts, data adquisition was recorded for 10 minutes in each sample. Molecular weights were compared with Lipid bank and Carotenoid DB databases.

NMR analysis of carotenoid mixtures

Approximately 20 mg of extract were dissolved in deuterated chloroform at 600 MHz using tetramethyl silane as internal standard.

HPLC carotenoids quantification in mixtures

A standard of β -carotene was used for this quantification; solutions from 5-30 µg/mL were elaborated and filtered in a Millipore of 0.45 µm. With chromatographic conditions previously described; each curve was elaborated in triplicate different days to determine, linearity, variation coefficient, detection and quantification limits according to the Colegio Nacional de Químicos Farmacéuticos Biólogos [32].

An extract curve was elaborated in triplicate ranging from 0.5 to 4 mg/mL, each carotenoid was quantified according to the curve obtained previously and concentrations were added to assess total carotenoid content (TCC) of each strain.

Antioxidant activity of Rhodococcus sp. and Gordonia sp. pigments with DPPH method

In a HPLC vial 350 μ L of pigment and 350 μ L of DPPH solution (20 μ g/mL) were added, agitation for 20 seconds, 37°C for 30 minutes, a blank consisting of 350 μ L of DPPH solution and 350 μ L of ethyl acetate was used to determine the peak area of DPPH before antioxidant reaction. The HPLC analysis was done as previously described where chromatograms were also acquired at 517 nm, antioxidant activity was calculated according to literature [24-26], and each measure was performed in triplicate. A probit regression was done to calculate the IC₅₀ for each pigment. Antioxidant activity of mixtures were compared to that of β -carotene in concentrations from 5-30 μ g/mL.

Percentage in peak area reduction of carotenoids present was used to calculate the contribution of each carotenoid in the pigments according to Qiu et al. 2012 [28] followed by a statistical comparison (an independent T-test and a one-way ANOVA) to determine significant differences in their contributions.

RESULTS AND DISCUSSION

Characterization of carotenoids of Gordonia sp. and Rhodococcus sp. carotenoid mixtures

In Figure 1, FT-IR spectrum of pre-purified carotenoid mixtures of *Rhodococcus* sp. and *Gordonia* sp. are observed; where signals corresponding to hydroxyl groups ($3400-3362 \text{ cm}^{-1}$), carbon-hydrogen bonds corresponding to sp² and sp³ hybridizations ($2922-2852 \text{ cm}^{-1}$) and carbonyl groups ($1731 \text{ and } 1714 \text{ cm}^{-1}$) were observed. Thus inferring that the main components of the carotenoid mixtures are xanthophylls.



Figure 1. FT-IR spectra of Rhodococcus sp. and Gordonia sp. carotenoid mixtures

The carotenoid profile obtained with HPLC shows that *Rhodococcus* sp. extract presents two carotenoids at 10.7 (1) and 11.3 (2) minutes. While *Gordonia* sp. three carotenoids at 9.1 (3), 10.7 (1) and 11.3 (2) minutes are observed (see Figure 2).



Figure 2. Carotenoid HPLC profile of Rhodococcus sp. and Gordonia sp.

Where the carotenoid with retention time (Rt) of 9.1 minutes (3) present in *Gordonia* sp. mixture has 3λ at 427, 450 and 480 nm with a % III/II of 25%; based on the UV-Vis spectra we can conclude that this carotenoid has hydroxy groups and a cyclic ending on the molecule [33]. The ESI-MS show a molecular ion of 301.0 m/z, where comparison with molecular weight reported in Lipid bank [34] and Carotenoid DB [35] databases are in good agreement with a structure as the reduced form of 4-oxo retinaldehyde (Figure 3).



Figure 3. Characterization of carotenoid with Rt of 9.1 minutes (3), where A is HPLC Rt, B is UV-Vis spectra, C is ESI-MS analysis and the structure of the reduced form of 4-oxo retinaldehyde

The carotenoid with Rt of 10.7 minutes (1) present in *Gordonia* sp. and *Rhodococcus* sp. mixture has a λ at 480 nm, indicating the presence of carbonyl groups within their structure [33]. The ESI-MS was of 469.4 m/z, where comparison with previous databases [34, 35], as well as literature [36] are in good agreement with a structure as 8'-apoastaxanthinal (Figure 4).



Figure 4. Characterization of carotenoid with Rt of 10.7 minutes (1), where A is HPLC Rt, B is UV-Vis spectra, C is ESI-MS analysis and the structure of 8'-apoastaxanthinal

Regarding carotenoid with Rt of 11.3 minutes (2) present in both strains, presents a λ at 470 nm, which indicates that its molecule has carbonyl groups [33]. The ESI-MS was of 915.1 m/z, where compared with previous databases [34, 35] are in good agreement with a structure as astaxanthin dirhamnoside (Figure 5).



Figure 5. Characterization of carotenoid with Rt of 11.37 minutes (2), where A is HPLC Rt, B is UV-Vis spectra, C is ESI-MS analysis and the structure of astaxanthin dirhamnoside

Proton and ¹³C NMR spectra were recorded as a mixture of the pre-purified carotenoids obtained from freeze-dried cells of *Gordonia* sp., ¹H NMR chemical shifts in the region of 5.4-6.8 ppm corresponding to the characteristic isoprenoid skeleton of carotenoids, which is also observed as more definite signals in ¹³C NMR from 120-140 ppm. (Figure 6), similar results were observed on *Rhodococcus* sp. ¹H and ¹³C RMN spectra (figure not shown).



Figure 6. ¹H (left) and ¹³C (right) RMN spectra of Gordonia sp. carotenoids mixture

It is to be noticed that ¹H signals are weak due to the presence of other compounds, which can also be observed in the mass spectra, thus causing loss of signal resolution, making hard to predict the carotenoids chemical shifts when they are within a biological matrix, since the spectra is saturated making difficult their identification [37].

HPLC carotenoids quantification in mixtures

A reference curve with β -carotene (5-30 µg/mL) was developed to quantify carotenoids present in mixtures. With determination coefficient of 0.9914 with an intercept of -25.40 and a slope of 28.10 with a variation coefficient of 5.06% with detection limit of 0.11 µg/mL and quantification limit of 0.35 µg/mL. TCC was calculated from individual carotenoid quantification (Table 1).

Extract dilution (mg/mL)	TCC Rhodococcus sp.	TCC Gordonia sp.
0.5	0.97±0.05	0.78±0.34
1.0	2.66±0.32	2.32±0.12
2.0	5.09±0.21	3.38±0.28
4.0	11.70±0.42	8.13±2.12

Antioxidant activity of Rhodococcus sp. and Gordonia sp. mixtures

DPPH radical presented a retention time (Rt) of 3.46 minutes compared to carotenoids present in *Rhodococcus* sp. and *Gordonia* sp. (see Figure 2) and β -carotene (Rt of 14.04 minutes), thus making possible to calculate the reduction percentage of DPPH radical. A concentration-response curve was obtained for each carotenoids mixture and β -carotene against DPPH (Figure 7). Where *Rhodococcus* sp., *Gordonia* sp. and β -carotene presented an IC₅₀ of 1.07, 0.09 and 19.49 µg/mL respectively; thus concluding that *Gordonia* sp. exerts a better antioxidant activity than *Rhodococcus* sp. and β -carotene based on IC₅₀. It was also determined antioxidant activity to present a dose-dependent relationship as reported by Gharibzahedi et al. 2013 [38].



Figure 7. Concentration-response curves of β-carotene, *Rhodococcus* sp. and *Gordonia* sp. mixtures

Observing IC_{50} values of carotenoids mixture of each strain, it is to be noticed that antioxidant activity of carotenoids obtained from actinobacteria, as it is in this study, might be comparable to carotenoids extracted from some plant sources; where Quesada et al. 2011 [39] report the IC_{50} based on TCC in two varieties of peach palm (11.6±0.2 µg/mL for Yurimaguas and 9.1±0.3 µg/mL for Ecuador varieties), presenting a better antioxidant activity *Rhodococcus* sp. and *Gordonia* sp.

HPLC antioxidant activity analysis allowed obtaining of complementary data that made possible the assessment of which carotenoid presented the best antioxidant activity within a mixture, the peak area of the carotenoid before and

after the antioxidant reaction at different initial concentrations; the peak area decrease was defined as the contribution percentage, where the absence of peak after the antioxidant reaction was considered as 100% contribution (Table 2). Regarding peak 3 (reduced form of 4-oxo retinaldehyde like carotenoid) present in *Gordonia* sp. a 100% contribution was observed attributed to its hydroxyl groups [40].

Rhodococcus sp.			Gordonia sp.						
8'-apoastaxanthinal like carotenoid (peak 1)Astaxanthin dirhamnoside like carotenoid (peak 2)		Reduced 4-oxo retinaldehyde like carotenoid (peak 3)		8'-apoastaxanthinal like carotenoid (peak 1)		Astaxanthin dirhamnoside like carotenoid (peak 2)			
[]	Cont. %	[]	Cont. %	[]	Cont. %	[]	Cont. %	[]	Cont. %
0.79	71.36	0.18	44.96	0.07	100.00	0.52	67.13	0.17	51.03
2.09	54.80	0.56	49.89	0.21	100.00	1.55	68.65	0.54	61.22
4.08	44.69	1.07	47.48	0.31	100.00	2.20	53.45	0.86	55.15
8.15	47.19	2.51	55.98	0.69	92.36	4.96	54.53	2.13	57.10

Table 2. Contribution % of carotenoids present in Rhodococcus sp. and Gordonia sp. carotenoids mixtures at different concentrations

[] concentration in µg/mL; Cont. %, contribution %

Carotenoids present in the mixtures of both strains presented a different behavior in their contribution because of the presence of the reduced form of 4-oxoretinaldehyde carotenoid like of *Gordonia* sp. [41]. Observing Figure 8A and 8B each point represents a different initial concentration, where in the case of *Rhodococcus* sp (Figure 8A), contribution of each carotenoid reaches a maximum at approximately 40% of the antioxidant activity followed by a decrease. *Gordonia* sp. (Figure 8B) contribution increases linearly reaching maximum contribution at approximately 80% of the antioxidant activity followed by a decrease.



Figure 8. Contribution of carotenoids present in *Rhodococcus* sp. (A) and *Gordonia* sp. (B)

The statistical comparison between the carotenoids present in both mixtures (8'-apoastaxanthinal and astaxanthin dirhamnoside like carotenoids) demonstrate that antioxidant activity of 8'-apoastaxanthinal like carotenoid could be

equivalent to astaxanthin dirhamnoside like carotenoid, where Del-Toro et al. 2015 [42] demonstrated that hetheranthin, an astaxanthin derivative presents an antioxidant activity comparable to astaxanthin. The presence of the reduced form of 4-oxoretinaldehyde like carotenoid significantly enhances the contribution of astaxanthin dirhamnoside like carotenoid (T value of -2.06, α 0.05), which suggests a possible synergism between both carotenoids, thus explaining why *Gordonia* sp. mixture presented a better antioxidant activity than *Rhodococcus* sp. mixture.

Structure characterization with FT-IR, HPLC, UV-Vis spectrometry, ESI-MS, databases and NMR was possible to propose that carotenoids present in both strains could have structures similar to the reduced form of 4-oxo retinaldehyde, 8'-apoastaxantinal and astaxanthin dirhamnoside; without isolating compounds present in carotenoids mixtures.

The analysis of DPPH radical scavenging activity of carotenoids with HPLC allowed the assessment of IC_{50} of strains and β -carotene, where *Gordonia* sp. was the pigment that presented the best antioxidant activity; based on the follow-up of the carotenoids contributions with the statistical comparison, carotenoid similar to 4-oxoretinaldehyde was the carotenoid that presented the best antioxidant activity. Likewise the statistical comparison of astaxanthin dirhamnoside like carotenoid present in both strains, allowed suggesting a possible interaction between both carotenoids.

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