

Antigenotoxic and antimutagenic potential of an annatto pigment (norbixin) against oxidative stress

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ABSTRACT. Carotenoids are 40-carbon molecules with conjugated double bonds, making them particularly effective for quenching free radicals. They have always been believed to possess anticancer properties, which could be due to their antioxidant potential. Norbixin is an unusual dicarboxylic water-soluble carotenoid present as a component in the pericarp of the seeds of *Bixa orellana* L. (from the Bixaceae family), a tropical shrub commonly found in Brazil. The main carotenoids present in these seeds, bixin and norbixin, form a coloring material, known as annatto, which is mainly used in the food industry. As annatto is only used as a coloring material, most studies of annatto pigments have focused on the determination of annatto levels in food. However, little attention has been given to the biological properties of bixin and norbixin. We evaluated the effect of norbixin on the response of *Escherichia coli* cells to DNA damage induced by UV radiation, hydrogen peroxide (H₂O₂) and superoxide anions (O₂⁻) and found that norbixin protects the cells

against these agents. Norbixin enhanced survival at least 10 times. The SOS induction by UVC was inhibited 2.3 times more when cells were grown in the presence of norbixin. We also found that norbixin has antimutagenic properties, with a maximum inhibition of H₂O₂-induced mutagenic activity of 87%, based on the *Salmonella* mutagenicity test.

Key words: Annatto pigments, Antigenotoxicity, Antimutagenicity, Norbixin

INTRODUCTION

The seeds of *Bixa orellana* L., a shrub native to tropical America, are a rich source of orange-red pigments that have been widely used by the food coloring industry. These pigments are commercially known as annatto (E160b), and their main coloring component is bixin (C₂₅H₃₀O₄), an unusual carotenoid having a free carboxyl and an esterified carboxyl as end groups. Currently, the allowable daily intake for annatto is 0-2.5 mg kg body weight⁻¹ day⁻¹ (for a preparation containing 2.6% carotenoids expressed as bixin) and 0-0.065 mg kg body weight⁻¹ day⁻¹, when it is expressed as the pure pigment (JECFA, 1982). Approximately 80% of the pigments present in annatto seeds correspond to bixin (Presto and Rickard, 1980); more recently several other minor carotenoids have been isolated and identified (Mercadante et al., 1997a,b, 1999).

Annatto pigments are considered not to be genotoxic, based mainly on *in vitro* screening data (Sasaki et al., 1980; Haveland-Smith, 1981; Ishidate et al., 1984; Fujita et al., 1988). We recently demonstrated that fibroblasts treated with norbixin *in vitro* were rendered either resistant or susceptible to DNA damage induced by hydrogen peroxide, as measured by the comet assay (Kovary et al, 2001). The *in vivo* genotoxic potential of the annatto pigments was also investigated in our laboratory and we observed that norbixin ingestion did not induce any detectable DNA breakage in liver and kidney (Fernandes et al., 2002).

Naturally occurring antioxidants, such as carotenoids, have been extensively studied for their potential in reducing the risk for cancer and other chronic diseases (Collins, 2001). We evaluated the effect of norbixin on the response of *Escherichia coli* cells to DNA damage that has been induced by UV radiation, hydrogen peroxide (H₂O₂) or superoxide anions (O₂⁻). We also tested, using the *Salmonella* mutagenicity test, whether norbixin has a potential antimutagenic activity against an oxidative mutagen (H₂O₂).

MATERIAL AND METHODS

Isolation of norbixin

Norbixin present in the pericarp of the annatto seeds was extracted with three volumes of CH₃CH₂OH:H₂O(93:7, v/v), at 37°C for several hours, with vigorous shaking following the procedure described by Kovary et al. (2001). Norbixin levels were determined by spectrophotometry (Shimadzu UV-160A spectrophotometer, Japan).

Survival experiments

We used strains derived from *E. coli* K12 (BW535 (*xthA nfo nth*), (AB1157 (wild-type) and PQ65 (*sfIA::lacZ*). Cells were grown overnight at 37°C in LB rich medium (Miller, 1972), containing, or not, 2 mM norbixin. A starting inoculum was taken from these cultures and grown until the exponential phase in the same medium, containing, or not, 2 mM norbixin. The cells were then centrifuged three times, resuspended in saline (0.9% NaCl) and irradiated with different doses of UVC, or treated with 2.5 mM H₂O₂ (30% Perhidrol, Merck-Brazil) for 15 min or with 10 mM menadione for 60 min. Samples (0.1 ml) were collected, appropriately diluted in saline, and spread onto LB medium solidified with 1.5% agar. The colonies were counted after overnight incubation at 37°C. The standard deviations did not exceed 5% for all points.

SOS chromotest and antimutagenicity assay

The induction of β -galactosidase expression by UV radiation was measured in *E. coli* PQ65, as previously described (Asad et al., 1997, reviewed by Quillardet and Hofnung, 1993), and the induction factor was calculated, based on Goerlich et al. (1989). The result was considered positive when the increase in the induction factor was more than 1.5, combined with an increase in the β -galactosidase activity. Antimutagenicity was measured with *Salmonella typhimurium* TA102 cells using Ames test procedures (Kovary et al., 2001). This strain is particularly responsive to oxidative and alkylating mutagens, and it detects active forms of oxygen. After incubation for 72 h at 37°C, the number of reverting *his*⁺ bacteria colonies was scored. The data shown correspond to the mean of two independent determinations. The standard error of the mean did not exceed 15%.

RESULTS AND DISCUSSION

Norbixin (2 mM) gave protection against UVC radiation in wild-type cells (Figure 1A). Besides, we also observed protection against H₂O₂ (Figure 1B) and menadione (an O₂⁻ producer) (Figure 1C) in the *xthA-nfo-nth* mutant. The data obtained with UV are in agreement with those reported by Tuveson and Sandmann (1993).

The typical absorption spectra of norbixin in water displays maximum absorptions at 453 and 482 nm (Kovary et al., 2001) (Figure 2). As norbixin also gave absorption at the shorter wavelengths of UVC radiation (<300 nm), norbixin-induced protection against far-UV could be due to UV absorption by norbixin. However, this effect was dose-dependent, and the protection was only evident at high UV-doses (Figure 1A).

The SOS response plays a central role in the response of *E. coli* to genotoxic agents (Quillardet and Hofnung, 1993). We studied the effect of norbixin on the genotoxicity induced by UVC radiation using an *E. coli* strain harboring a *lacZ::sfIA* fusion (PQ65) in the SOS chromotest assay. We observed that SOS induction by UVC was inhibited when cells were grown in medium containing norbixin (Table 1). These data suggest that SOS induction by UVC radiation is not only due to adducts formed between neighboring pyrimidines (Friedberg et al., 1995), but is also due to oxidative DNA damage.

Norbixin was also found to have antimutagenic properties, with a maximum inhibition of H₂O₂-induced mutagenic activity of 87% (Table 2). The mutagenic activity of norbixin alone

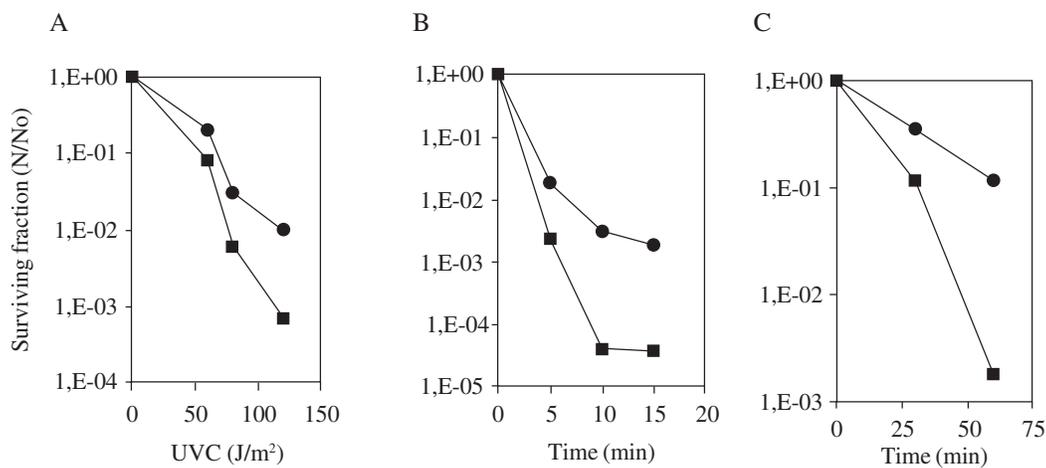


Figure 1. Survival of *Escherichia coli* cell strains. **A**, AB1157 irradiated with UVC; **B**, BW535 treated with H₂O₂; **C**, BW535 treated with menadione. Circles - with norbixin; squares - without norbixin.

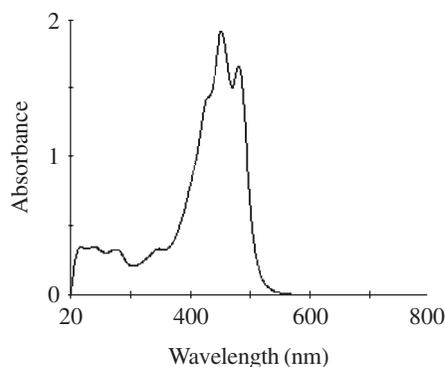


Figure 2. Absorption spectrum of norbixin in water after isolation from *Bixa orellana* seeds.

Table 1. Induction of β -galactosidase in wild-type *Escherichia coli* (PQ65) irradiated with different doses of far-UV (254 nm).

Doses (J/m ²)	2 mM norbixin	Without norbixin
0	1	1
80	5	11.5
120	2.2	7
160	1	1.8

(without H₂O₂) was also tested and no mutagenic activity was detected (data not shown).

The carotenoids, in the form of β -carotene, are known as powerful quenchers/scavengers of singlet O₂, which can be formed during lipid peroxidation (Halliwell and Gutteridge, 1999). Singlet O₂ has been suggested to mediate the toxicity of O₂^{•-} (Halliwell and Gutteridge, 1999). Based on our observations, we suggest that norbixin (an annatto pigment) can be a scavenger of singlet O₂, preventing the generation of O₂^{•-} and H₂O₂.

Table 2. Norbixin inhibition of the H₂O₂ (200 µg) mutagenesis induction in *Salmonella typhimurium* TA102.

Norbixin (5 mg/plate)	No. of revertent colonies
0	930
0.5	540
1.0	460
2.5	345
4.0	300
5.0	60

The mutagenic test was performed as described in Ames et al., 1995.

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