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Absence of carcinogenic and anticarcinogenic effects of annatto in the rat liver medium-term assay

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Abstract

Annatto (*Bixa orellana* L.) is a natural food colorant extensively used in many processed foods, especially dairy products. The lower cost of production and the low toxicity, make annatto a very attractive and convenient pigment in substitution to the many synthetic colorants. In the present study we investigate the carcinogenic and anticarcinogenic effects of dietary annatto in Wistar rat liver using the preneoplastic glutathione S-transferase (GST-P) foci and DNA damage biomarkers. Annatto, containing 5% bixin, was administered in the diet at concentrations of 20, 200, and 1000 ppm (0.07; 0.80 and 4.23 bixin/kg body wt/day, respectively), continuously during 2 weeks before, or 8 weeks after DEN treatment (200 mg/kg body wt, i.p.), to evaluate its effect on the liver-carcinogenesis medium-term bioassay. The comet assay was used to investigate the modifying potential of annatto on DEN (20 mg/kg body wt)-induced DNA damage. The results showed that annatto was neither genotoxic nor carcinogenic at the highest concentration tested (1000 ppm). No protective effects were also observed in both GST-P foci development and comet assays. In conclusion, in such experimental conditions, annatto shows no hepatocarcinogenic effect or modifying potential against DEN-induced DNA damage and preneoplastic foci in the rat liver.

Keywords: Annatto; Carcinogenesis; Chemoprevention; Diethylnitrosamine; Genotoxicity; GST-P positive foci; Comet assay

1. Introduction

Nowadays, many different approaches are being tried aiming the cancer prevention. Attention has been focused on whether naturally occurring compounds can modify the mutagenic and carcinogenic effects of environmental contaminants. Consequently, many dietary constituents were found to have market influence on the adverse effects of carcinogens and on cancer development (De Marini, 1998; Ribeiro and Salvadori, 2003). Evidence from both epidemiological and experimental studies has suggested that high consumption of fruits and vegetables rich in carotenoids may help prevent cancer and other degenerative diseases in humans (Ben-Amotz and Fishler, 1998).

Over the past decades hundreds of DNA-protective and anticarcinogenic effects have been detected in plant-derived foods (Knasmuller et al., 2002). Annatto (*Bixa orellana* L.) is a natural colorant widely used in many processed foods, especially dairy products. This yellow–red pigment is a mixture of some carotenoids such as bixin, norbixin, phytoene, and δ-carotene (Mercadante et al., 1996). Once bixin is one of the most

Abbreviations: GST-P, Glutathione S-transferase placental form; DEN, diethylnitrosamine

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effective biological singlet molecular-oxygen quenchers (Di Mascio et al., 1990), and the protective activity of some carotenoids against chemically-induced DNA damage (Salvadori et al., 1992, 1994) have been demonstrated, we investigated the putative chemopreventive potential of annatto on rat hepatocarcinogenesis. However, although some studies have reported no mutagenicity and carcinogenicity induced by this colorant (Haveland-Smith, 1981; JECFA, 1982; Alves de Lima et al., 2003), they are still few data to conclude for the absence of carcinogenic effect.

The lower cost of production and the low toxicity, make the annatto a very attractive and convenient pigment for the food industry in substitution to the many synthetic colorants that can be mutagenic or carcinogenic (Hallagan et al., 1995; Sasaki et al., 2002). Using the GST-P foci development and DNA damage as end-points, the present investigation was designed to evaluate a possible carcinogenic or anticarcinogenic effect of annatto on liver of rats.

2. Material and methods

Male Wistar rats, 7 weeks old, weighing approximately 230 g, were obtained from Centro Multidisciplinar de Investigação Biológica (CEMIB—UNICAMP, Campinas—São Paulo—Brazil). During the acclimation (2 weeks) and experimental periods, animals (5 rats per cage) were maintained in an experimental room under controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 10%), and 12 h light/dark cycle, with ad libitum access to commercial diet (NUVILAB—CR1 from Nuvital—Curitiba—Brazil) and water. Body weight, and water and food consumption were measured three times a week during the experimental period. The University Ethical Committee for Animal Research approved the protocols used in this study.

2.1. Chemicals

N-diethylnitrosamine (DEN, CAS 7756 Sigma—USA) was used to initiate hepatocarcinogenesis. The carcinogen was diluted in saline solution and i.p. administered at single dose of 20 (comet assay) and 200 mg/kg body weight (GST-P foci assay).

Immunohistochemical staining for detecting the GST-P foci, was performed using the anti-rat GST-P primary antibody purchased from Medical Biological Laboratories Co. (Tokyo—Japan); biotinylated universal IgG secondary antibody and avidin–streptoavidin peroxidase complex Tissugnost®, from Merck (Germany); 3-3′-diaminobenzidine tetrahydrocloride (DAB), bovine serum albumin (BSA) and poly-D-lysine from Sigma (USA).

2.2. Annatto extract

Annatto color, extracts from seeds of *B. orellana* L., and containing 5% bixin, was purchased from Christian Hansen (Valinhos—São Paulo—Brazil) as an oil extract in soybean oil. The extract was mixed and homogenized into a powdered commercial diet (NUVILAB—CR1) at concentrations of 20, 200 or 1000 ppm, based on the acceptable daily intake (ADI) of 0.065 mg/kg body wt bixin (JECFA, 1982). After pelletization, the diets were given to the animals in the different groups. Soybean oil (vehicle of the extract) mixed to commercial diet was given to the animals in the control group.

2.3. Experimental designs

2.3.1. Rat liver medium-term bioassay (DEN-partial hepatectomy model; Ito et al., 1988)

The animals were randomly distributed into 11 groups (14 rats in each) (Fig. 1): Group 1, negative control, received the commercial diet during the experimental period (10 weeks), and an i.p. injection of NaCl 0.9%, at the 2nd week; Group 2, was fed with 1000 ppm annatto during 10 weeks and i.p. injected with NaCl 0.9%, at the 2nd week; Group 3, positive control, was fed with commercial diet during 10 weeks and, at the 2nd week, received DEN at single dose of 200 mg/kg; Groups 4, 5, and 6 received annatto at 20, 200 and 1000 ppm respectively, for 2 weeks before the single dose of DEN (pre-initiation protocol), and commercial diet for the remainder 8 weeks until the end of the experimental period; Groups 7, 8, and 9, initially fed with commercial diet (during the first 2 weeks), received a single dose of DEN at the 2nd week, and were fed with annatto at 20, 200, and 1000 ppm of annatto, respectively, for 8 weeks after DEN treatment (post-initiation protocol); Groups 10 and 11 received commercial diet mixed with soybean oil for 2 weeks before, or 8 weeks after DEN treatment (200 mg/kg), respectively. At the 5th week, all the animals were submitted to 70% partial hepatectomy. All the animals were sacrificed by pentobarbital injection (40 mg/kg, body wt) at the end of the experimental period (10 weeks).

2.3.2. DNA damage assay (comet assay)

Animals were distributed into seven groups of 10 animals each (Fig. 2): Group 1 (negative control) received commercial diet during all the experimental period (2 weeks) and an i.p. injection of NaCl 0.9% at the end of the 2nd week; Group 2, was fed with 1000 ppm annatto during the experimental period; Group 3 (positive control) received commercial diet during 2 weeks and, 4 h before the sacrifice, an i.p. injection of DEN (20 mg/kg); Group 4 received soybean oil (1000 ppm) mixed in the diet during all the experimental period and, 4 h before the sacrifice, an i.p. injection of DEN

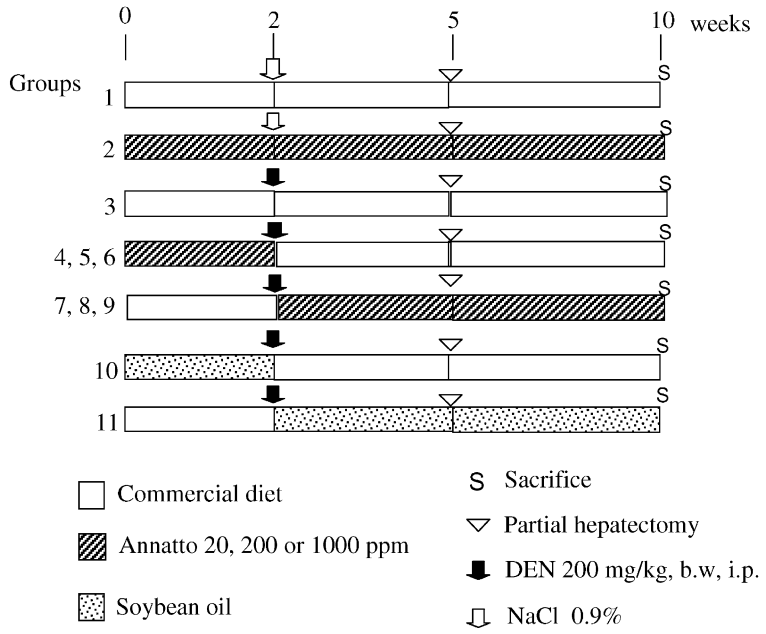

Fig. 1. Experimental protocol to evaluate the effect of annatto on diethylnitrosamine (DEN)-induced-GST-P foci in the rat liver.

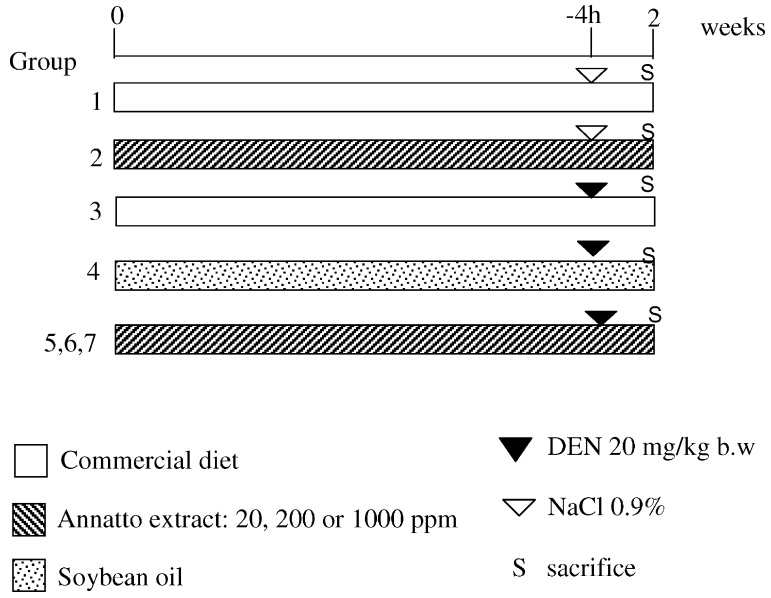

Fig. 2. Experimental protocol to evaluate the effect of annatto on diethylnitrosamine (DEN)-induced DNA damage in the rat liver and peripheral blood cells.

(20 mg/kg); Groups 5, 6, and 7 were fed with 20, 200 and 1000 ppm annatto, respectively, during 2 weeks and, 4 h before the sacrifice, they were i.p. injected with DEN.

2.3.3. Tissue processing, immunohistochemical staining and quantification of GST-P positive foci

At autopsy, the liver was immediately excised and weighted. Sagital sections of each lobe were collected and fixed in 10% phosphate-buffered formalin (24 h). Paraffin-embedded liver samples were cut into 5 μm thick sections for subsequent immunohistochemical staining of GST-P positive foci using the avidin–biotin complex (ABC) method as described previously (Hsu et al., 1981). The number and areas of GST-P positive foci greater than 0.15 mm in diameter were measured using a color video image processor (KS-300, Carl Zeiss, Germany). Data were expressed as number (N/cm^2) and area (mm^2/cm^2) of GST-P positive foci *per* liver section.

2.3.4. Liver and peripheral blood cells collection for the comet assay

The liver was excised, washed in saline solution, and a small fragment of the left lobule was transferred to a *Petri* dish kept on ice. The fragment was washed, minced, and then suspended in 1 ml of Hanks' balanced salt solution (HBSS), supplemented with 20 mM EDTA and 10% DMSO. The liver tissue was minced again and the suspension containing isolated cells was transferred to a tube that was maintained on ice until the preparation of the slides for the comet assay. Blood samples were collected from the periorbital vein plexus immediately before the rats were killed.

2.3.5. Comet assay

DNA damage was measured using the comet assay under alkaline conditions and dim indirect light (Singh et al., 2001). A volume of 10 and 5 μl of the hepatic and peripheral blood cell suspension, respectively, were mixed with 120 μl of 0.5% low melting point agarose (37 °C), layered on pre-coated slides with normal melting point agarose, covered with a coverslip and placed at 4 °C, for 5 min, in order to solidify the agarose. The coverslip was gently removed and the slides were submersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100) at 4 °C for 24 h. After lysis, the slides were briefly washed in PBS to remove the excess of lysis solution, and placed on horizontal electrophoresis unit filled with fresh electrophoresis alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C. Electrophoresis was conducted at 4 °C for 20 min at 25 V and 300 mA. The slides were neutralized in a buffer (0.4 M Tris at pH 7.5), dehydrated in absolute ethanol and dried at room temperature. Before analysis, the slides were stained with 50 μl ethidium bromide (20 μg/ml). Fifty randomly selected cells per animal were examined at 200× magnification in a fluorescence microscope, using an automated image analysis system (Comet Assay II, Perceptive Instruments, UK). Two metric parameters were selected as indicators of DNA damage: tail intensity (% tail DNA, in % of pixels), and tail moment (product of tail DNA/total DNA by the tail center of gravity, in arbitrary units).

2.3.6. Statistical analysis

The statistical analysis was done using Jandel Sigma Stat software, Jandel Corporation (San Rafael, CA, USA). The different parameters were compared between groups using one-way ANOVA (body and liver weights and food consumption), or Kruskal–Wallis tests (GST-P foci and the comet assay data). The contrast between the groups was analyzed by Students–Newman–Keuls test. A significant difference between groups was assumed when $P < 0.05$.

3. Results

Mean final body weights, body weight gains, relative liver weights, food consumption and ingestion of the annatto and bixin are presented in Table 1. The results show no statistically significant difference among the groups for all the parameters evaluated. Based on the food consumption, groups treated with 20, 200 or 1000 ppm annatto ingested, respectively, 1.5, 16.45, and 84.77 mg/kg body wt/day annatto, and 0.07, 0.80, and 4.23 mg/kg body wt/day bixin.

Table 1
Data for body and relative liver weights, food consumption, and annatto and bixin ingestion

Treatment	Final body weight (g)	Body weight gain (g)	Relative liver weight (%)	Food consumption (g/rat/day)	Annatto ingestion (mg/kg body wt/day)	Bixin ingestion (mg/kg body wt/day)
Negative control[a]	364.3 ± 43.5	118.0 ± 31.1	2.5 ± 0.4	25.1 ± 1.8	–	–
A3[b] + NaCl 0.9%	369.0 ± 28.5	125.2 ± 24.6	2.8 ± 0.5	27.2 ± 2.2	87.3 ± 5. 7	4.4 ± 0.2
DEN[c]	346.7 ± 22.3	101.9 ± 15.1	2.6 ± 0.3	24.2 ± 1.6	–	–
Pre-initiation						
Sb[d] + DEN	366.5 ± 36.9	119.1 ± 32.3	3.0 ± 0.5	25.1 ± 1.8	–	–
A1 + DEN	346.0 ± 32.5	115.0 ± 27.7	3.0 ± 0.8	23.8 ± 2.8	1.5 ± 0.1	0.07 ± 0.0
A2 + DEN	358.8 ± 30.7	107.6 ± 22.5	2.7 ± 0.4	25.3 ± 1.8	16.9 ± 0.3	0.8 ± 0.02
A3 + DEN	340.2 ± 22.5	110.3 ± 21.9	2.7 ± 0.4	24.3 ± 1.7	87.5 ± 4.0	4.4 ± 0.2
Post-initiation						
DEN + Sb	353.8 ± 18.6	124.5 ± 23.8	2.8 ± 0.5	24.8 ± 2.2	–	–
DEN + A1	343.9 ± 34.5	112.1 ± 19.0	2.8 ± 0.4	23.5 ± 1.2	1.5 ± 0.1	0.07 ± 0.0
DEN + A2	340.6 ± 21.5	102.6 ± 11.9	2.8 ± 0.4	23.7 ± 1.6	16.0 ± 0.1	0.8 ± 0.0
DEN + A3	347.8 ± 30.9	108.8 ± 22.8	3.0 ± 0.5	24.0 ± 1.5	79.5 ± 3.1	3.9 ± 0.2

[a] NaCl 0.9%.
[b] A1, A2, A3: annatto extract (A1: 20 ppm; A2: 200 ppm; A3: 1000 ppm).
[c] DEN: diethylnitrosamine (200 mg/kg, body wt, i.p.).
[d] Sb: soybean oil (annatto vehicle). Values are mean ± SD. Statistical analysis: one-way ANOVA.

Table 2
Number and area of hepatic GST-P positive foci in rats treated with annatto and diethylnitrosamine (DEN)

Treatment	Effective number of animals	GST-P positive foci	
		Number (N/cm^2)	Area (mm^2/cm^2)
Negative control[a]	14	0	0
A3[b] + NaCl	13	0	0
DEN[c]	13	10.87 ± 4.16*	0.35 ± 0.22*
Pre-initiation			
Sb[d] + DEN	13	11.70 ± 4.03	0.42 ± 0.24
A1 + DEN	12	12.30 ± 6.45	0.47 ± 0.33
A2 + DEN	13	11.94 ± 4.76	0.35 ± 0.20
A3 + DEN	13	12.34 ± 4.99	0.41 ± 0.23
Post-initiation			
DEN + Sb	13	13.47 ± 6.48	0.47 ± 0.26
DEN + A1	13	10.08 ± 4.95	0.32 ± 0.18
DEN + A2	13	11.07 ± 5.08	0.41 ± 0.33
DEN + A3	12	11.44 ± 3.19	0.35 ± 0.16

* $P < 0.01$ (compared to negative control and A3 + NaCl). Statistical analysis: Kruskal–Wallis test.
[a] NaCl 0.9% (DEN vehicle).
[b] A1, A2, A3: annatto extract (A1: 20 ppm; A2: 200 ppm; A3: 1000 ppm).
[c] DEN: diethylnitrosamine (200 mg/kg body wt, i.p.).
[d] Sb, soybean oil (annatto vehicle). Values are mean ± SD.

Table 2 shows quantitative results for the number and area of preneoplastic GST-P positive foci per cm^2 of liver area. No GST-P positive foci were observed in the negative control and in the group treated with diet at the highest annatto concentration (1000 ppm). There was no statistically significant difference observed among the positive control group and those groups treated with DEN and annatto, at the three different concentrations, in both pre- and post-initiation steps. Statistically significant difference was only observed between positive and negative control groups and between positive control and annatto plus saline (A3 + NaCl) groups.

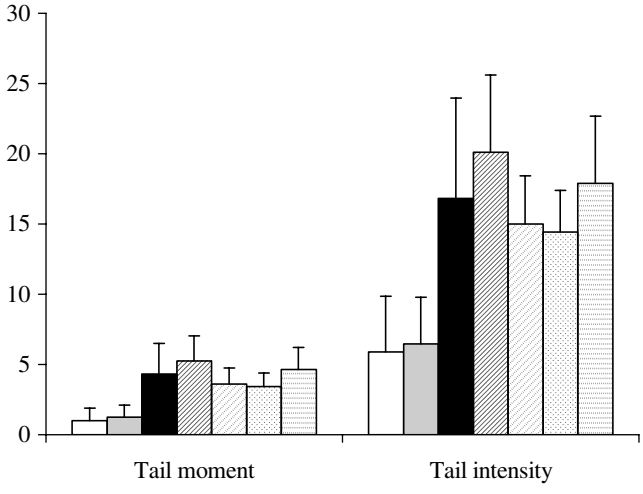

Fig. 3. DNA damage (tail moment and tail intensity) evaluated by the comet assay in liver of rats treated with annatto and diethylnitrosamine (DEN): (□) negative control; (▒) anatto 1000 ppm; (■) diethylnitrosamine; (▨) soybean oil + DEN; (▨) annatto 20 ppm + DEN; (▨) annatto 200 ppm + DEN; (▥) annatto 1000 ppm + DEN. Statistical analysis: Kruskal–Wallis test.

Fig. 3 summarizes the data obtained in the comet assay. Under the present experimental conditions, statistically significant difference was observed only in liver cells, and between the positive and negative control (non-initiated group vs. only DEN-initiated group). Annatto had no significant effect on DEN-induced DNA damage (*tail moment* and *tail intensity data*) in liver and peripheral blood cells. The analysis of the liver cell suspensions showed 91% of hepatocytes.

4. Discussion

Although widely used as food colorant, and rich in antioxidant substance (bixin), there are limited studies about the effect of annatto in carcinogenesis. In this study, the protective role of a fat-soluble annatto extract on liver carcinogenesis was evaluated by the liver-based medium-term bioassay and by the comet assay, in rats exposed to diethylnitrosamine, a well-known hepatocarcinogen.

Once diet is the main route by which humans are exposed to synthetic and natural colorant, annatto containing 5% bixin was incorporated to the basal commercial diet for rats. Based on the average daily food consumption, the three different doses of annatto and bixin ingested corresponded to 1.5, 16.45, and 84.77, and 0.07, 0.80, and 4.23 mg/kg body wt/day, respectively. The highest dose of bixin was approximately 50 times the value of ADI. However, this dose did not cause any evident toxicological effect as demonstrated by the mean body weight and relative liver weights. Similar results have been observed in mice when annatto, containing 30% bixin, was administered at concentrations of 1330, 5330, and 10 670 ppm (Alves de Lima et al., 2003). No changes in plasma chemistry, and liver and kidney weights were noted in rats treated with annatto containing 50% norbixin or only norbixin, during 21 days (Fernandes et al., 2002); no toxic effect was also detected in rats orally administered with 250 mg/kg, body wt annatto containing 28% of bixin or only bixin for five consecutive days (De Oliveira et al., 2003). Nevertheless, extensive damage in mitochondria and endoplasmatic reticulum of liver and pancreas cells has been observed in Mongrel dogs, when pure bixin, dissolved into 40% ethanol, was orally administered at single dose of 0.06 g/kg/body wt (Morrison et al., 1991).

When evaluated in the medium-term liver-carcinogenesis assay and in the comet assay, our data firstly

showed that the highest dose of annatto, 84.77 mg/kg body wt/day (corresponding to 1000 ppm in the diet), did not induce preneoplastic GST-P positive foci formation and DNA damage in the male Wistar rats liver. According to the data provided by the *Joint FAO/WHO Expert Committee on Food* Additives (JECFA, 1982), no increase in the tumor incidence was observed in rats treated with 125 mg/kg fat-soluble annatto, three times a week, for 9 months, and in rats receiving daily dose of 26 mg/kg body wt, for 26 months. However, higher incidence of pulmonary adenomas in female mice that received, subcutaneous, 0.1 ml of butter-color annatto, once a week, for 8 weeks, has been also reported (JECFA, 1982). Regarding to the mutagenicity, no increased DNA damage has been detected in liver, kidney, and bone marrow cells of mice treated with annatto extracts (Fernandes et al., 2002; Alves de Lima et al., 2003), in *Saccharomycces cerevisae* (gene conversion) and in *Salmonella typhimurium* (Ames test) (Haveland-Smith, 1981). Contrarily, increase of chromosome aberration in Chinese hamster fibroblast (Ishidate et al., 1984), and DNA damage in *Bacillus subtilis*, has been described (Nonaka, 1991).

On the other hand, our results also showed that annatto, at the three concentrations tested, did not inhibit DEN-induced GST-P positive liver foci when administered before or after the hepatocarcinogen. This result indicates that, under the conditions tested, dietary annatto up to 4.5 mg bixin/kg body wt/day did not prevent DEN-induced liver preneoplastic lesions. In a previous study we have observed a protective effect of the mushroom *Agaricus blazei* on DEN-induced GST-P positive foci when the hepatocarcinogen was administered at single dose of 100 mg/kg, but not at 200 mg/kg body wt (Barbisan et al., 2002). Other studies have reported that β-carotene, and the natural additive curcumine, caused substantial reduction in the incidence of hepatocyte nodules induced by 200 mg/kg DEN, and restored the normal levels of the GST-P positive foci in rat livers, respectively (Moreno et al., 1991; Shukla and Arora, 2003).

In a previous study conducted in our laboratory we have observed no protective effect of an annatto extract containing 30% bixin on cyclophosphamide-induced micronucleus in mouse bone marrow cells (Alves de Lima et al., 2003). Now, the absence of antigenotoxic activity detected in liver cells indicates that this natural colorant does not prevent chemically-induced primary DNA damage. It must be emphasized that the dose of DEN used in the DNA damage assay was 10 times lower than that used in the medium-term liver assay. Negative results in *S. typhimurium* (Ames test) and *Drosophila melanogaster* assays were also reported (Rauscher et al., 1988; Takahashi et al., 2001). However, our results do not allow a conclusion about the effect of the annatto on peripheral blood cells, since DEN did not induce comet assay-detectable DNA damage in such cells. Similar result has been already described, as DEN (20 mg/kg, body wt)-induced DNA damage was observed in lung, stomach, liver and kidney, but not in bone marrow cells of mice (Tsuda et al., 2000).

In vivo studies have provided evidence of the bixin modifying potential against chromosomal damage induced by radiation in mouse bone marrow cells (Thresiamma et al., 1998), and by cisplatin in rats (Silva et al., 2001). Protective effect of nor-bixin on DNA breakage induced by reactive oxygen species, particularly by hydroxil radicals, in *Escherichia coli* K12 plasmid and *S. typhimurium*, was also demonstrated (Kovary et al., 2001). Since bixin is effective *singlet* molecular-oxygen quencher (Di Mascio et al., 1990), and inhibitor of lipid peroxidation (Zhang et al., 1991; Zhao et al., 1998; Martínez-Tomé et al., 2001), it seems that annatto pigments have a chemopreventive action especially against DNA damage induced by oxygen free radicals or against compounds able to generate such molecules.

Concluding, our results indicate that, in such experimental conditions, the annatto extract containing 5% bixin is not genotoxic or hepatocarcinogenic, and has no modifying potential against DEN-induced DNA damage and preneoplastic foci in the rat liver.

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