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Effect of Carotenoids Against Genotoxicity of Diethylnitrosamine on Rat Hepatocytes

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Abstract—Carotenoids have been considered as special nutrients due to their biological activity as provitamin A compounds, and because of their natural antioxidant and anticarcinogenic properties. The main objective of this study was to evaluate the protective effect of carotenoids against the genotoxic cellular damage induced by diethylnitrosamine (DEN), a potent hepatocarcinogen. Normal and freshly isolated hepatocytes were cultured as the biological system. Concentrations of 2.5 and 5 μM DEN caused 1.3 and 2.0 times more DNA T^3H incorporation, respectively, when compared with control cells. Pure carotenoids, β-carotene (50 μM), lutein (1 μM) and a carotenoid extract from green peppers (1 μM eq. lutein) were used as functional nutrients to protect the cells. All the carotenoids studied prevented the genotoxic damage caused by 2.5 μM DEN. When 5 μM DEN was used, only β-carotene and the pepper extract inhibited the damage up to 30–40%. Carotenoids provide a dose-dependent protective effect against DNA damage induced by DEN in isolated hepatocytes.

Keywords: nitrosamines; genotoxicity; carotenoids; chemoprevention.

Abbreviations: DEN = diethylnitrosamine; DMEM = Dulbecco's modified Eagle's medium; DMSO = dimethyl sulfoxide; DPM = desintegrations per minute; FBS = foetal bovine serum; HU = hydroxy urea; LDH = lactate dehydrogenase; PBS = phosphate buffered saline; THF = tetrahydrofuran; T^3H = tritiated thymidine; UDS = unscheduled DNA synthesis.

INTRODUCTION

Nitrosamines and the mechanisms of their toxic action have been the focus of attention for several years (Ames, 1983; Lin and Ho, 1994; Mendoza-Figueroa *et al*., 1983; Tricker and Preussmann, 1991; Yang and Smith, 1996). The presence of nitrosamines in foods is considered a risk factor because or their implication in causing gastrointestinal cancers of the pharynx, oesophagus, stomach, liver, pancreas and colorectum (Chhabra *et al*., 1996; Jen and Yuan, 1994; Magee, 1996). In the last few years, more studies about the use of functional foods as chemopreventive agents against the toxicity of a wide range of substances have been published (Blum, 1996; Draper and Bird, 1984; Krinski, 1993; Miyake and Shibamoto, 1997; Murakami *et al*. 1996; Namiki, 1990; Nir and Hartal, 1995; Waters *et al*., 1990). The focus of several studies has been on the functional nutrients that protect or prevent the damage caused by genotoxic compounds (Odin, 1997). Foods such as fruits and vegetables are rich in these substances, for example, vitamins, selenium, phenolics and carotenoids, among others. In particular, carotenoids have shown special properties as protective agents against human cancer (Peto *et al*., 1981), and as chemopreventive compounds in some cell lines (Davison *et al*., 1993; Krinski, 1993, 1994; Manoharan and Banerjee, 1985; Toma *et al*., 1995; Weitzman *et al*., 1985; Yu *et al*., 1994). However, carotenoids can increase the toxic effects of some compounds in certain organs (Peterson, 1996) or be completely ineffective in cancer chemoprevention (Astrog *et al*., 1996). The majority of these studies were performed using transformed cell lines and few have been done with normal cells to focus on the first steps of cancer. The objective of this study was to evaluate the capacity of pure carotenoids and natural extracts from green peppers against the genotoxic damage caused by diethylnitrosamine (DEN). The study was performed *in vitro*, using primary hepatocyte cultures from normal, healthy rats.

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MATERIALS AND METHODS

Biological materials and reagents

The pepper used was a green variety (*Capsicum* spp.) harvested in Michoacan, Mexico, in December 1994. It was freeze-dried, pulverized, and stored in the dark at −20°C under a nitrogen atmosphere until used.

Male Wistar rats (250–350 g) were fed *ad lib.* with rodent chow and tap water. The animals were fasted for 16 hr before liver perfusion.

Lutein was purified from Chromophyll ORO 20, a commercial product used as a pigment in chicken feeds (Bioquimex Co., Queretaro, Mexico). This product was obtained by extraction of marigold flower (*Tagetes erecta*) and it contained at least 90% lutein. Pure *trans*-β-carotene and *trans*-lutein were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Tetrahydrofuran (THF) (HPLC grade, 99.99% purity) was used as a vehicle for β-carotene (Mallinckrodt, KY, USA). Dimethyl sulfoxide (DMSO) (HPLC grade, 99.9% purity) was used as the lutein vehicle (Omni Solv, NJ, USA).

Diethylnitrosamine (DEN), collagenase type IV, Dulbecco's modified Eagle's medium (DMEM), insulin, penicillin (10,000 U/ml) and streptomycin (10 mg/ml) solutions and foetal bovine serum (FBS) were obtained from Sigma Chemical Co.

Tritiated thymidine (T^3H) (5 mCi/ml) was purchased from Life Sciences (Amersham, Bucks, UK).

Carotenoids extraction

Carotenoids were extracted from green peppers using an official method for dried samples with cold saponification (AOAC, 1990). Lutein was purified from the commercial product following the same method. Characterization and quantification were performed by reverse phase liquid chromatography (HPLC) using a Perkin Elmer Chromatograph, model 400; UV/VIS detector at 460 nm and a Perkin Elmer integrator model LCI-100. The column used was Novapack C-18 and the mobile phase was water–THF–acetonitrile (2.5:20:77.5, by vol) (Bureau and Bushway, 1986). Standard curves of pure *trans*-β-carotene and pure *trans*-lutein were obtained with correlation coefficients of 0.9998 and 0.9969, respectively. After quantification, carotenoid extracts were concentrated at 40°C in a rotoevaporator and redissolved in 100 ml DMSO, stored at −20°C under a nitrogen atmosphere and kept from light until used. Carotenoid concentration was determined in the extract each week, and new extracts were obtained each month.

Hepatocyte culture

A fresh cell suspension was obtained by *in situ* liver perfusion with collagenase (0.05%, p/v) carried out according to the two-step perfusion technique of Seglen (1976). Cell viability (≥80%) was determined by the trypan blue exclusion test and the cells were cultured in 35-mm dishes (Nunc) (500,000 viable cells/dish) or in 96-well plates (Falcon) (20,000 viable cells/dish) in DMEM with added sodium bicarbonate (3.7 g/litre), insulin (100 U/ml), penicillin–streptomycin solution (2 μl/ml) and FBS (10%, v/v). Cells were incubated in a 95% air:5% CO_2 humidified incubator at 37°C. Cell attachment was allowed for at least 2 hr (Swierenga *et al.*, 1991).

Genotoxicity study

Phase 1. Genotoxicity was evaluated as the T^3H incorporation into DNA in relation to control cells (Mitchell *et al.*, 1983; Swierenga *et al.*, 1991). It was necessary to use hydroxyurea (HU), 5 mM as a DNA synthesis inhibitor. A dose–response curve for DEN was performed. Cells were incubated for 2 hr and washed with PBS (5%). Different DEN concentrations were tested (2.5, 5, 10, 50, 10 and 50 mM) using serum-free medium with 1 μl T^3H added (5 μCi/dish) and 5 mM HU. Genotoxic concentrations of DEN were those that caused significantly more incorporation (DPM/mg cell protein) compared with the basal line of control cells. The innocuous response of *trans*-β-carotene 50 μM, lutein 1 μM and pepper extract 1 μM eq. lutein, was verified measuring the lactate dehydrogenase (LDH) enzymatic activity as cell viability indicator using the spectrophotometric method with a Lambda II spectrophotometer at 365 nm and a Merck LDH determination kit. The T^3H incorporation test was used to observe the genotoxic response of the carotenoids. Vehicles used were tested by both methods to verify their innocuity.

Phase 2. Having established the genotoxic DEN concentrations (2.5 and 5 μM), two independent experiments were performed, respectively using each of these concentrations. Fresh cells were cultured for 2 hr, washed with PBS (5%), and serum free medium was added, containing one of the carotenoids and incubated separately for 1 hr. Then cells were washed again and serum-free medium containing the corresponding genotoxic concentration of DEN and T^3H (5 μCi/dish), was added. The cells were incubated for 4 hr and DNA was recovered from cells using 10% trichloroacetic acid. Unscheduled DNA synthesis (UDS) was determined by T^3H incorporation in a Scintillation Counter model LS6000. Total protein was determined in a Lambda II spectrophotometer at 620 nm (Bradford, 1976). Results are reported as DPM/mg cell protein.

RESULTS

Pepper carotenoid extract

Figure 1 shows the chromatographic profile of the green pepper extract showing three different

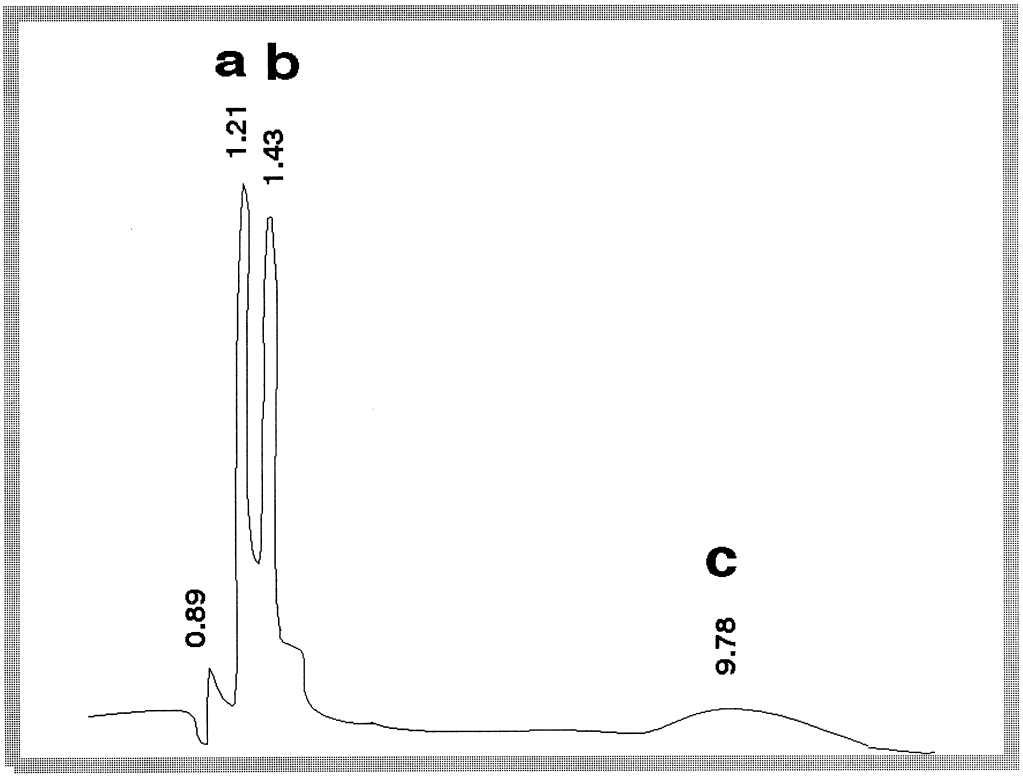

Fig. 1. Chromatographic profiles of pepper carotenoids (*Capsicum* spp.). (a) xantophyll, (b) lutein, (c) β-carotene.

peaks. Peak (a) corresponds to a non-characterized xantophyll; more likely to be neoxanthine or violaxanthine (Camara and Moneger, 1978). Peak (b) corresponds to lutein and peak (c) to β-carotene. The retention times of each carotenoid, as well as their respective concentrations, are shown on Table 1. Lutein was the main carotenoid present in the extract representing approximately 50% of the total sample; β-carotene represented about 12%. Retention times were verified using an internal standard. Results are also summarized on Table 1.

Genotoxicity study

Phase 1. Figure 2 shows the effect of different DEN concentrations tested after 4 hr of exposure. Genotoxic damage was observed with 10, 5 and 2.5 μM DEN concentrations, causing respectively 2.5, 2.0 and 1.3 times more incorporation than the control cells ($P < 0.05$). The genotoxic effect of carotenoids was verified at concentrations of 50 μM β-carotene and 1 μM lutein (Fig. 3). The genotoxic response of both tetrahydrofuran and DMSO was evaluated because carotenoids are not water soluble compounds, and it was necessary to use this kind of vehicle in this biological system. Tetrahydrofuran was considered as the best vehicle for β-carotene (Craft, 1992), and when added to biological systems it is not toxic at a concentration of 0.5% (v/v) (Cooney *et al*., 1993). This was confirmed in this study (data not shown). DMSO was the best vehicle for lutein under the same conditions. Higher concentrations of both solvents may cause a decrease in cell viability (Cooney *et al*., 1993).

Phase 2. Complete treatments using DEN at 2.5 or 5 μM were performed. Carotenoids were able to inhibit DEN genotoxic damage between 30 and

Table 1. Concentration of carotenoids present in pepper (*Capsicum* spp.)

Peak	a	b	c
Carotenoid	Neoxanthine or violaxanthene	*Trans*-Lutein	*Trans*-β-Carotene
Retention time (min)	1.2	1.4	9.7
Concentration (μg/μl)	4.49×10^{-1}	5.35×10^{-1}	1.92×10^{-2}
Molar	7.9×10^{-5}	9.4×10^{-5}	3.58×10^{-6}
Area (%)	38	50	12

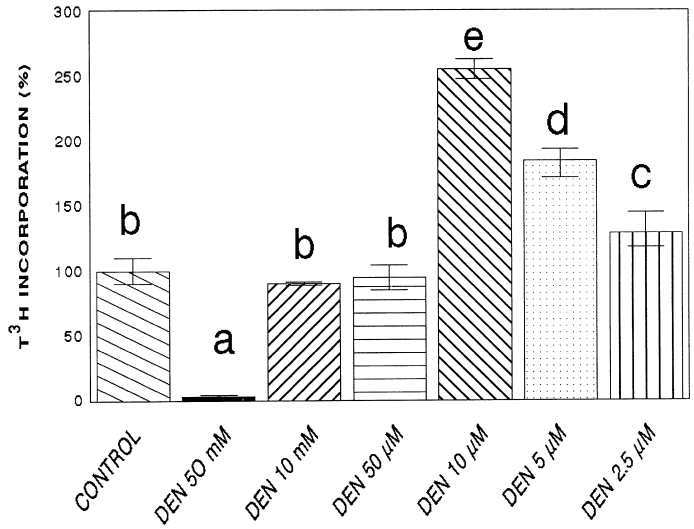

Fig. 2. Dose–response curve of DEN genotoxicity on rat hepatocytes. Cells were exposed to DEN at different concentrations for 4 hr. Lower-case letters mean statistically significant difference ($P \leqslant 0.05$).

40% and 100% when the concentrations were 50 and 2.5 μM, respectively. The results are shown in Fig. 4, and summarized in Table 2. The data are expressed as percent of T^3H incorporation in relation to control cells.

DISCUSSION

Genotoxicity study

Alkylation of DNA by nitrosamines results in a range of methylated bases, and the binding site is mainly the O^6 position of guanine. The formation and persistence of O^6-alkylguanine residues are considered to be important base modifications and result in the incorporation of non-complementary bases (mis-coding); its significance has been clearly demonstrated in carcinogenesis experiments. Such a process is considered as carcinogenesis initiation (Tricker and Preussmann, 1991).

In non-proliferating hepatocytes *in vitro*, two types of DNA damage can be monitored: UDS, or induction of DNA single-strand breaks (Pool *et al.*, 1990). UDS is the enzymatic non-semiconservative repair process which is detected in cells that are not in the S-phase. Quantification of the occurrence of UDS is based on the measurement of the amount

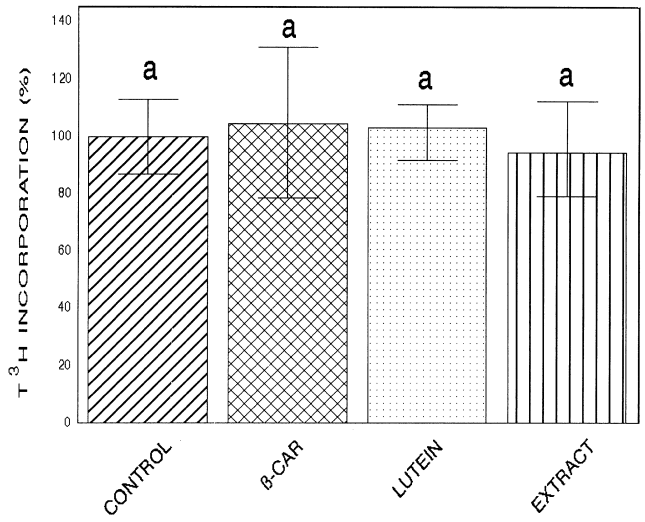

Fig. 3. Effect of carotenoids on UDS. The cells were incubated for 4 hr. Concentration of carotenoids were β-carotene 50 μM, lutein 1 μM and extract 1 μM eq. lutein. Lower-case letters mean statistically significant difference ($P \leqslant 0.05$).

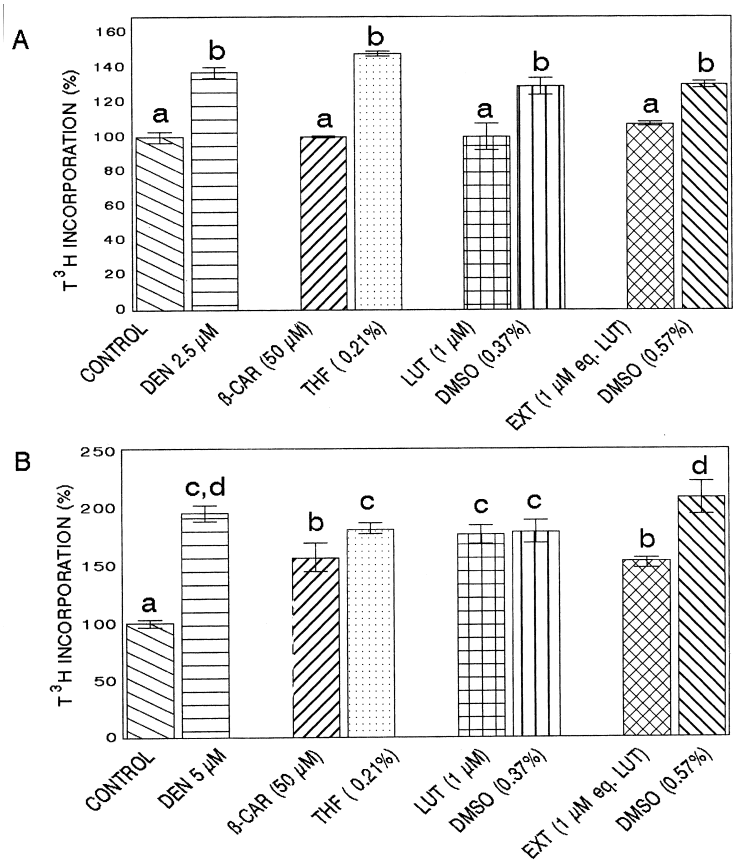

Fig. 4. Chemopreventive effect of carotenoids against the genotoxic damage of DEN on rat hepatocytes. (A) DEN 2.5 μM; (B) DEN 5 μM. Cells were incubated for 4 hr. Vehicle effects are shown. Lower-case letters mean statistically significant difference ($P \leqslant 0.05$).

of radiolabelled thymidine incorporated into DNA when it is damaged by genotoxic substances. UDS is one of many types of short-term tests currently used in the assessment of potential chemical genotoxicity and carcinogenicity (Swierenga *et al.*, 1991). The measurement of DNA repair as an indication of interaction with DNA could be a reliable determinant of carcinogenic potential. Indeed, the appearance of DNA repair has been recommended as a screening technique for carcinogenesis (Williams, 1977).

Phase 1. Figure 2 shows a dose–response curve for DEN genotoxicity. Control cells presented a basal line of T^3H incorporation although they were not exposed to any toxic substance. This baseline may be the result of normal DNA background, such as the one found by Williams (1977) also using nitrosamines. This baseline was considered arbitrary as 100% of incorporation. Higher counts caused by the presence of a specific compound were considered as genotoxic damage. A DEN concentration of 50 mM, which was the LC_{50} for isolated hepatocytes as shown before, caused 2.6% T^3H incorporation. At this point, it is evident that cells already suffered functional or structural damage and that their enzymatic systems could not longer respond. With sublethal doses of 10 mM and 50 μM, incorporation was 100%. It does not mean that those concentrations did not cause any damage. A negative test result can indicate either that the compound is inactive, that it inhibits DNA repair, or that the size of the repair is too small to allow detection (Swierenga *et al.*, 1991). The results suggest that 10 mM and 50 μM DEN caused enough damage to alter enzymatic systems and enzymes could not respond as normally, therefore counts seemed to be low. When concentrations of 10, 5 and 2.5 μM were tested, a real genotoxic effect was observed and there was 2.5, 2.0 and 1.3 times more incorporation in relation to control cells ($P < 0.05$). DEN at 5 and 2.5 μM was used for the chemopreventive study.

Phase 2. Figure 4 shows that both pure carotenoids and the extract were able to protect cells against 2.5 μM DEN genotoxic damage as shown in Fig. 4(A). The nitrosamine caused 37% more incorporation than control cells ($P < 0.05$). Carotenoids had the capacity to inhibit 100% of such damage, that is, cells that were pretreated with either carotenoids or extract did not present any genotoxic

Table 2. Chemopreventive effect of carotenoids against the genotoxic damage of DEN

Treatment		Control	DEN (2.5 μM)	DEN (5 μM)	β-CAR (50 μM)	Lutein (1 μM)	Extract (1 μM eq. lutein)
Control group	Damage (%)	—	36.95	94.05	0	0	0
	DPM/μg prot	10.04	13.75	19.50	10.99	10.78	9.25
Chemoprevention against DEN 2.5 μM	DPM/μg prot	10.04	13.75	—	10.05	10.05	10.80
	Protection (%)	—	—	—	36.85	36.85	29.34
	Inhibition (%)	—	—	—	99.73	99.73	79.40
Chemoprevention against DEN 5 μM	DPM/μg prot	10.04	—	19.50	16.35	18.56	15.17
	Protection (%)	—	—	—	31.47	9.19	41.44
	Inhibition (%)	—	—	—	33.46	9.77	55.06

Genotoxicity was evaluated as T^3H incorporation with respect to control cells.
Results are the mean of at least two independent experiments.

damage. When 5 μM DEN was tested (Fig. 4B), β-carotene and the extract were able to protect cells. Genotoxic damage of the nitrosamine was 94% in comparison to control cells ($P < 0.05$). β-carotene inhibited 34% of such damage, while the extract inhibited 55%. Lutein was not able to prevent genotoxicity at 5 μM DEN. These results suggest that each carotenoid has a different chemopreventive capacity, and the limit depends on the level of damage.

Some authors have found that carotenoids are chemopreventive agents in some cell lines such as CH3/10T1/2 cells (Zhang *et al.*, 1991), mouse mammary cells (Manoharan and Banerjee, 1985) and also antitumour agents of hepatocellular tumours (Matheus-Roth, 1982; Moon, 1989). In the present study, it was demonstrated that carotenoids have the capacity to protect normal cells which did not have any kind of malignant transformation.

Some studies performed *in vivo* showed that β-carotene and canthaxanthin did not have any inhibitory effect on the initiation of liver preneoplastic foci induced by DEN in the rat (Astrog *et al.*, 1996); however, in the present study it was demonstrated that carotenoids have enough inhibitory effect against DEN genotoxicity during the initial phase.

These findings are very important because it is well known that DEN is a potent hepatocarcinogen, principally in the first step of neoplastic transformation (Cortinovis *et al.*, 1991; Lijinsky, 1987; Loquet and Wiebel, 1982; Lotlikar, 1981; Pool *et al.*, 1990). Initiation is the first damage suffered by the cell; if such damage is permanent it could lead to cancer. DEN has a great capacity for causing permanent genotoxic damage to cells, so it is an efficient cancer initiator. Nitrosamines are very common in processed foods such as bacon, dried and canned vegetables, beer and in cigar smoke. Humans are exposed to nitrosamines in several ways, and are these especially effective by the oral route (Tricker and Preussmann, 1991).

On the other hand, fresh vegetables and fruits are an important source of carotenoids. Pepper is a product widely consumed in Mexico, both fresh or cooked. It has an important nutritional contribution, particularly as a source of vitamin C (120 mg), and provitamin A (1000 U) (Lomeli, 1987). β-carotene is the main provitamin A compound, and its concentration in green and fresh peppers is 170–600 μg/100 g dry weight (Mejia *et al.*, 1988). Total carotenoid content is about 229 μg/g dry weight (Cámara and Monéger, 1978). Therefore, it becomes important to know the ability of these kinds of vegetable products to protect the organism against potential carcinogens. This, in turn, can lead to their promotion as part of a healthy diet.

The results of the present study suggest that, using the UDS technique, the carotenoids have the

capacity to inhibit the genotoxic damage caused by DEN at 2.5 and 5 μM, reducing the persistence of T^3H incorporation into DNA from 100 to 40%, respectively. This would allow the prevention of neoplastic initiation on normal and fresh cells, specifically hepatocytes which are important target cells for these compounds. Carotenoids have a different chemopreventive ability that depends on the damage level, the toxic concentration of the carcinogenic substance, and the kind of carotenoid used. Therefore, is very important to perform future studies using carotenoids as genotoxic inhibitors *in vivo*.

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