Protective Effect of Broccoli, Onion, Carrot, and Licorice Extracts against Cytotoxicity of *N*-Nitrosamines Evaluated by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide Assay

Adrián Martínez, Isabel Cambero, Youssef Ikken, María L. Marín, Ana Isabel Haza, and Paloma Morales*

Departamento de Nutrición y Bromatología III, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

The protective effect of nine fruit and vegetable aqueous (H_2O) and ethanolic (EtOH) extracts against the cytotoxicity of *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine (NPYR), *N*-nitrosodibutylamine (NDBA), and *N*-nitrosopiperidine (NPIP) was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The fruit extracts under investigation did not show a protective effect against any *N*-nitrosamines tested. Four vegetable extracts exhibited a protective effect (to 100% of survival) and a stimulation of cellular proliferation (>100% of survival) in decreasing order against NDMA and NPYR: $broccoli_{EtOH}$ > $onion_{H2O}$ > $carrot_{EtOH}$ > $onion_{EtOH}$ > $licorice_{H2O}$. Decreasing orders against NDBA and NPIP were, respectively, $broccoli_{EtOH}$ > $licorice_{H2O}$ > $carrot_{EtOH}$ > $onion_{EtOH}$ and $broccoli_{EtOH}$ > $carrot_{EtOH}$ > $licorice_{H2O}$ > $onion_{EtOH}$. Thus, $broccoli_{EtOH}$ extract (19−20 mg/mL) showed greater protective effect and stimulation of cellular proliferation (160% of survival) against all *N*-nitrosamines studied than the other vegetable extracts tested.

Keywords: *Broccoli; onion; carrot; licorice; N-nitrosamines; cytotoxicity; MTT assay*

INTRODUCTION

Since the first demonstration by Sander (1968) that ingested secondary amines and nitrite could react in vivo to produce carcinogenic nitrosamines, increasing attention has been focused on estimating the extent of human exposure to *N*-nitroso compounds.

Under gastric conditions reactions between nitrite and secondary amines can produce *N*-nitrosamines (Mirvish, 1975). Nitrite may be readily derived by bacterial reduction (Ayanaba and Alexander, 1973) and salivary reduction (Ishiwata et al., 1975) from the nitrate present in a wide variety of food products. On the other hand, trace levels of mutagenic and carcinogenic volatile *N*-nitrosamines are present in a wide variety of foods, e.g. cured meat products, smoked fish, dried malt, and beer (Spiegelhalder et al., 1980; Scanlan, 1983; Havery and Fazio, 1985; Österdahl, 1988). However, we do not know whether the extent of exposure to these mutagens and carcinogens is sufficient to result in human cancer. Druckey et al. (1967) showed in experimental animals that the product of daily doses of mutagenic and carcinogenic nitrosamines multiplied by the induction time in days, raised to an exponent in the range of 1.2−4, is constant. This suggests that exposure even to low levels of these compounds throughout life may be significant.

Vitamin C destroys nitrosating agents and thereby inhibits nitrosamine formation (Mirvish et al., 1972). Vitamin E possesses anticancer properties (Chow, 1988), and α-tocopherol, the principal component of vitamin E, is capable of inhibiting N-nitrosation (Bartsch et al., 1988). Many studies have demonstrated that carotenoids from green and yellow vegetables have an anticarcinogenic effect in humans (Doll and Peto, 1981). However, in clinical trials, *β*-carotene has recently been shown not to be an effective agent and, perhaps, to be harmful (Potter, 1997).

Furthermore, it is known that some miscellaneous vegetable and fruit factors have antimutagenic activity. In particular, the juices of broccoli (*Brassica oleracea*), green pepper (*Capsicum annum*), apple (*Malus domestica* Golden Delicious), and pineapple (*Actinidia diasinensis*) present activity as antimutagens against the mutagenicity of tryptophan pyrrolisis products (Kada et al., 1978). Antimutagenic activities have also been found in greengage, kiwi, mangos, and plums against the mutagenic activity induced by 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ). Asparagus, carrots, and radishes were inactive (Edenharder et al., 1994). Onion, leek, garlic, and other vegetables belonging to the *Allium* genus contain a wide variety of specific compounds that act as antimutagens in vitro in laboratory experiments and seem to be anticarcinogens in vivo (Dorant et al., 1994, 1995; Ip and Lisk, 1994). Garlic was found to have a preventive action against $aflatoxin_{B1}$-induced carcinogenesis in the toad *Bufo regularis* (El-Mofty et al., 1994). A water extract of licorice root has been shown to inhibit granuloma angiogenesis in vivo and tube formation in vitro (Kobayashi et al., 1995).

Although the evidence that endogenously formed nitrosamines are involved in human cancers is far from conclusive, it is important to discover naturally occurring or synthetic compounds which can suppress or prevent toxicity, mutagenicity, and carcinogenicity of *N*-nitrosamines.

We report in this paper the protective effect of fruit and vegetable extracts against the cytotoxicity of *N*-nitrosodimethylamine (NDMA), *N*-nitrosopyrrolidine

* Author to whom correspondence should be addressed (fax +34-1-3943743; e-mail pmorales@eucmax.sim.ucm.es).

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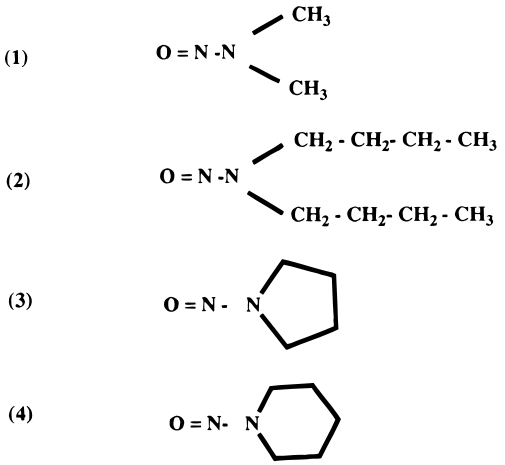

Figure 1. Chemical structures of NDMA (**1**), NDBA (**2**), NPYR (**3**), and NPIP (**4**).

(NPYR), *N*-nitrosodibutylamine (NDBA), and *N*-nitrosopiperidine (NPIP) using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MATERIALS AND METHODS

Materials. Samples of six vegetables and three fruits under investigation, onion (*Allium cepa*), garlic (*Allium sativum*), green pepper (*C. annum*), broccoli (*B. oleracea*), carrot (*Dacus carota*), licorice (*Glycyrrhiza glabra*), apple (*M. domestica* Golden Delicious), kiwi (*Ananas sativus*), and pineapple (*A. diasinensis*), were purchased from a local food market in Madrid, Spain. These fruits and vegetables were selected on the basis of preliminary in vitro assays described by several investigators (Helser et al., 1992; Bronzetti, 1994; Dorant et al., 1995).

Preparation of Aqueous and Ethanolic Extracts of Fruits and Vegetables. Standard amounts of 100 g of fruits and vegetables were sliced and further homogenized at 4 °C in a homogenizer (Sorvall, Norwalk, CO). Homogenization was performed with distilled water or 99% ethanol (1:1 w/v) except for the licorice (*G. glabra*) extract, for which the correspondence was 1:6 (w/v) and before homogenization it was incubated for 72 h. The resulting homogenate was filtered with suction, and the filtrate was centrifuged at 10000*g* for 30 min to remove any fruit and vegetable debris. The supernatant was sterilized by filtration through two Millipore filters (0.45 and 0.22 μm). Aqueous extracts were lyophilized, and ethanolic extracts were evaporated to dryness under reduced pressure. Both aqueous and ethanolic extracts of fruits and vegetables were stored at −20 °C until use (Ikken et al., 1997).

Chemicals. NDMA, NPYR, NDBA, and NPIP (Figure 1) were purchased from Sigma Chemical Co. (St. Louis, MO). Standard solutions of NDMA and NPYR (10 mg/mL) were prepared in Milli Q water (Millipore, Japan) and of NDBA and NPIP (400 mg/mL) in dimethyl sulfoxide (Merck, Darmstadt, Germany). *N*-Nitrosamines are potent carcinogenic agents; safety precautions must be taken for proper handling and disposal of the chemicals.

The *N*-nitrosamines tested were chosen because they are the most frequently occurring volatile nitrosamines in foods. As fruits and vegetables are dietary components, it is more pertinent to evaluate their protective effect against food mutagens which are likely to be consumed simultaneously with fruits and vegetables.

Cell Cultures. Vero cells (African green monkey kidney cells) are defined as a continuous cell line with a fibroblastic-like morphology. This cell line has been used because of its easy maintenance and handling and its sensitivity as target cells in numerous toxicological assays (Gogate, 1996; Núñez et al., 1996; Baudrimont et al., 1997). Vero cells were cultured in Dulbecco's Modified Eagle medium (Gibco Laboratories, Chagrin Falls, IL) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 50 IU of penicillin/mL, 50 μg/mL streptomycin (Gibco), and 1% L-glutamine (Gibco) at 37 °C and 100% humidity in a 5% CO_2 atmosphere.

MTT Cell Culture Assay. Cell proliferation kit I (Böehringer Mannheim, GmbH, Germany) has been used to test the cytoxicity of volatile *N*-nitrosamines and fruit and vegetable extracts. The MTT assay was carried out in 48-well tissue culture microtiter plates (Nunc, Roskilde, Denmark). Vero cells were used to determine the relationship between cell number and amount of MTT formazan generated and time of cell incubation with MTT. Optimal conditions for MTT assay were obtained with a final concentration of 1×10^6 cells/mL and incubation time of 4 h. Cell suspension (200 μL; 10^6 cells/mL) was dispensed in each well, and plates were incubated for 24 h at 37 °C. After incubation, 100 μL of each concentration of H_2O and EtOH extracts of fruits and vegetables (10 wells/concentration) and 100 μL of each *N*-nitrosamine (1 mg/mL) were added to the wells, and plates were incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, 20 μL of stock MTT solution (0.5 mg/mL) was added to each culture well, and plates were incubated for 4 h at 37 °C in a humidified 5% CO_2 atmosphere. The yellow tetrazolium salt MTT is converted in viable cells by the mitochondrial enzyme succinate dehydrogenase (SDH) into a purple formazan substrate. To dissolve the dark formazan crystals, 200 μL of solubilization solution was added into each well and the plates were incubated overnight at 37 °C in a humidified atmosphere. After incubation, the contents of the plates were thoroughly mixed for 5 min on a plate shaker (Heidolph) and the optical density of each well was determined thereafter with an ELISA reader (iEMS Reader MF, Labsystems, Helsinki, Finland) at 620-nm test wavelength and 690-nm reference wavelength.

All fruit and vegetable extracts and negative and positive controls were evaluated in three independent assays. Values presented in this paper are mean ± standard error of the mean. Cells without *N*-nitrosamines and without fruit and vegetable extracts were considered as negative controls, and cells with *N*-nitrosamines as positive controls. Cell survival in exposed cultures relative to unexposured cultures (negative control) was calculated and expressed as percentage of survival (% SDH activity) = $(A_1/A_0) \times 100$, where A_1 is the absorbance of exposed cultures and A_0 is the absorbance of negative control.

Data Analysis. Student's *t* test was used for statistical evaluation of the difference between cell survival in exposed cultures and that in untreated controls (Pagano, 1990).

RESULTS

The protective effect of fruit and vegetable extracts against the cytotoxicity of four *N*-nitrosamines, NDMA, NPYR, NDBA, and NPIP, was evaluated by MTT assay. No cytotoxicity has been previously described with the fruit and vegetable extracts used under the conditions tested (Ikken et al., 1997). At the concentrations used, a protective effect was found for two aqueous (onion and licorice) and three ethanolic (onion, carrot, and broccoli) extracts of the nine fruits and vegetables tested. Data points are means of 30 values from three independent experiments (10 values/experiment). Standard error was in the range of 1−3%.

Results from Figure 2 show the protective effect of onion (*A. cepa*) aqueous ($onion_{H2O}$) extract against cytotoxicity of NDMA and NPYR. The concentrations of onion that increased the cytotoxic activity of NDMA and NPYR were 0.6−3 mg/mL (60−80% of survival). Concentrations >3 mg/mL of $onion_{H2O}$ extract were required to observe a protective effect against NDMA and NPYR (80−100% of survival). Stimulation of cellular proliferation was observed at 4−6 mg/mL (100−120% of survival). However, no protective effect or

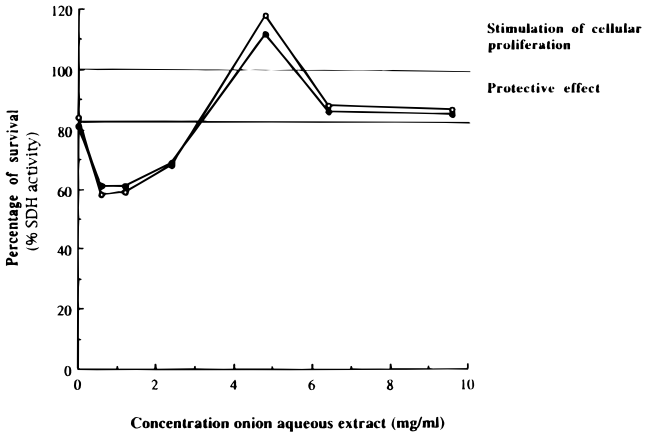

Figure 2. Protective effect of onion (*A. cepa*) aqueous extract against cytotoxicity of NDMA (○) and NPYR (●), using the MTT assay.

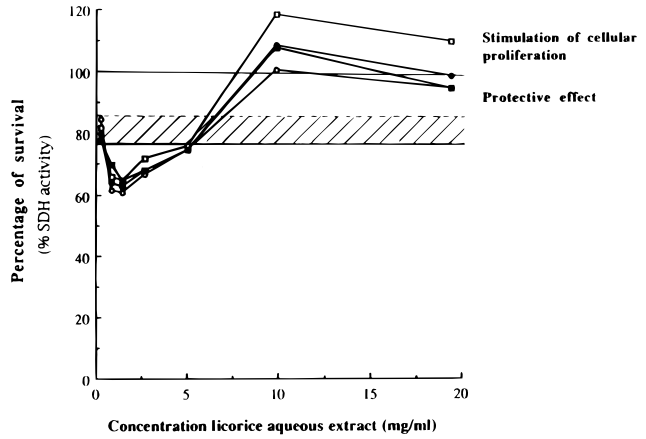

Figure 3. Protective effect of licorice (*G. glabra*) aqueous extract against cytotoxicity of NDMA (○), NPYR (●), NDBA (□), and NPIP (■), using the MTT assay.

stimulation of cellular growth with $onion_{H2O}$ extract against cytotoxicity of NDBA and NPIP was observed (data not shown).

The protective effect of licorice (*G. glabra*) aqueous ($licorice_{H2O}$) extract against the cytotoxicity of NDMA, NPYR, NDBA, and NPIP is shown in Figure 3. At concentrations <5 mg/mL of licorice extract, cytotoxicity was found for NDBA, NPYR, and NPIP, and 6 mg/mL was required for NDBA (60−80% of survival). A protective effect against the four *N*-nitrosamines tested was observed at concentrations of licorice >5−6 mg/mL ($p \leq 0.025$, NDBA, NPYR, and NDMA; $p \leq 0.05$, NPIP). Concentrations from 7.5 to 20 mg/mL of licorice against cytotoxicity of NDBA showed a stimulation of cellular proliferation (100−120%). Similar results were obtained at concentrations of 9−15 mg/mL against NPYR and NPIP (100−110%). However, this effect was not found against NDMA.

Onion ethanolic ($onion_{EtOH}$) extract (Figure 4) showed a protective effect against cytotoxicity of the four *N*-nitrosamines tested [>2.5 mg/mL of onion for NPIP, >4 mg/mL for NPYR ($p \leq 0.05$) and NDBA, and >5 mg/mL for NDMA ($p \leq 0.001$)]. Stimulation of cellular proliferation (100−110% of survival) was observed in the presence of 30−40 mg/mL of $onion_{EtOH}$ extract.

Figure 5 shows the protective effect of carrot ethanolic ($carrot_{EtOH}$) extract against the cytotoxicity of *N*-nitrosamines. The results obtained indicate that a protective effect has been observed against NDBA and NPIP (>10

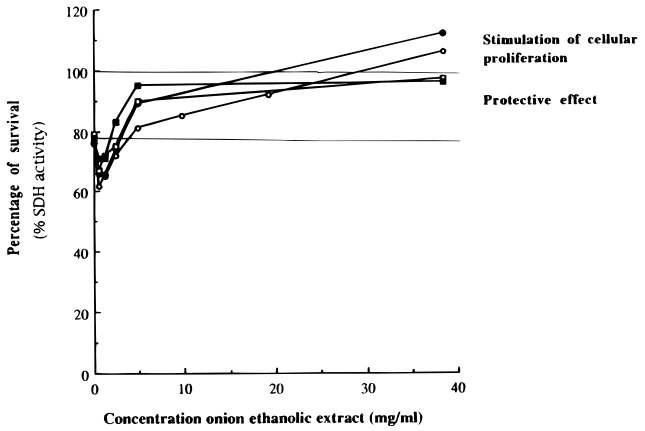

Figure 4. Protective effect of onion (*A. cepa*) ethanolic extract against cytotoxicity of NDMA (○) and NPYR (●), using the MTT assay.

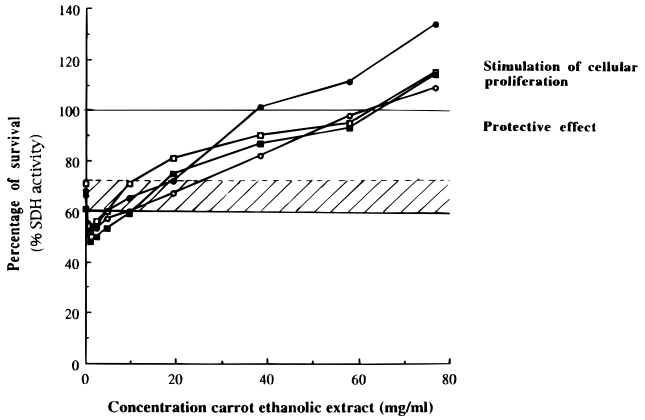

Figure 5. Protective effect of carrot (*D. carota*) ethanolic extract against cytotoxicity of NDMA (○), NPYR (●), NDBA (□), and NPIP (■), using the MTT assay.

mg/mL, $p \leq 0.0001$), NPYR (>15 mg/mL, $p \leq 0.0001$), and NDMA (>20 mg/mL, $p \leq 0.0001$). Concentrations of 65−80 mg/mL of carrot against NDBA, NDMA, and NPIP stimulated cellular proliferation (100−115% of survival) as did concentrations from 40 to 80 mg/mL against NPYR, increasing this percentage to 135% of survival.

Broccoli ethanolic ($broccoli_{EtOH}$) extract was the only one that showed a protective effect at all of the concentrations used against *N*-nitrosamines evaluated (Figure 6). The protective effect increased with increasing concentration of broccoli up to 20 mg/mL ($p \leq 0.01$, NDMA, NPYR, NDBA, and NPIP) and concentrations >7 mg/mL showed stimulation of cellular proliferation (100−160% of survival).

A protective effect against the cytotoxicity of *N*-nitrosamines was not observed with the remaining fruits and vegetables tested under the conditions used (data not shown).

DISCUSSION

The aim of the present study was to test the protective effect of fruit and vegetable extracts against cytotoxicity of *N*-nitrosamines by using the MTT assay. The use of a colorimetric assay for cell growth and survival, performed in microtiter trays and an automatic scanning spectrophotometer, offers major advantages in speed, simplicity, cost, and safety over conventional assays involving functional criteria, such as measure-

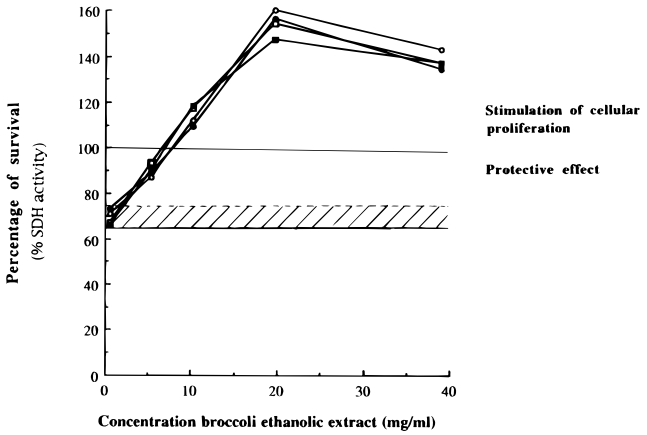

Figure 6. Protective effect of broccoli (*B. oleracea*) ethanolic extract against cytotoxicity of NDMA (○), NPYR (●), NDBA (□), and NPIP (■), using the MTT assay.

ment of protein and DNA synthesis, using radiolabeling techniques. These are limited by the general health hazard caused by radioactive material and the high costs for laboratory equipment and recycling of radioactive refuse (Thompson and Wannemacher, 1984).

MTT assays have been used for various medical, microbiological, and toxicological approaches, particularly in cytotoxical evaluation of chemotherapeutics (Pauwels et al., 1988; Kasugai et al., 1990). Studies in the literature concerning the MTT assay showed that the test system can be applied to almost all cell types (Hanelt et al., 1994).

Fruits are good sources of antioxidants, and considerable attention has been focused on the vitamin C, vitamin E, and β-carotene content of fruits. However, fruits also contain many other substances that have antioxidant activities. It has also been found that vitamin C prevents the conversion of nitrite to the carcinogenic compound nitrosamine, by reacting readily with nitrous acid (Mirvish et al., 1972). Phenols are present in high quantities in human foods derived from plants and fruits, and they also have antioxidant properties and have been shown to reduce the toxicity, mutagenicity, and carcinogenicity of a variety of chemicals other than *N*-nitroso compounds (Bartsch et al., 1988). Fruit extracts used in this investigation have not shown any protective effect against cytotoxicity of the four *N*-nitrosamines evaluated. Further studies on this protective effect of fruits are required and in progress.

Several lines of investigation support the ability of garlic and its constituents to inhibit the synthesis of *N*-nitroso compounds, including *N*-nitrosamines (Mei et al., 1982; Liu et al., 1986). The inhibitory effect of fresh garlic against chemical carcinogenesis induced in experimental animals has been studied by many investigators (Nishino et al., 1989; Choy et al., 1983; El-Mofty et al., 1994). The four allylic compounds of garlic (allyl methyl trisulfide, allyl methyl disulfide, diallyl trisulfide, and diallyl sulfide) were found to have an inhibitory effect on benzo[*a*]pyrene-induced forestomach tumor (Sadhana et al., 1988; Shyu and Meng, 1987). In the present study, garlic extracts did not show a protective effect against *N*-nitrosamines. However, our results indicate that onion (*A. cepa*) exhibits a protective effect and a stimulation of the cellular proliferation. The mechanism whereby onion or its extracts prevent the cytotoxicity of the *N*-nitrosamines remains unclear.

β-Carotene is one of a class of lipid-soluble pigments (the carotenoids) found in foods containing chorophyllin, such as carrots. Carrot (*D. carota*) and broccoli (*B. oleracea*) ethanolic extracts showed a higher protective effect and stimulation of cellular proliferation than other vegetables extracts tested (160%).

Licorice root has been used as an antioxidant inhibiting radiation-induced lipid peroxidation (Palagina et al., 1995) and antitumorigenic activity in granuloma angiogenesis (Kobayashi, 1995). In this regard, our results demonstrate that licorice root aqueous extract showed a protective effect against the nitrosamines evaluated.

Results obtained in this work indicate that all samples except broccoli ethanolic extract show nonlinear dose responses. This is a common situation in work with complex mixtures or combined treatments. The effect of the modifying chemical can be either enhancing or inhibiting depending on its mechanism of action. The modifier agent(s) may act either outside the cell by reaction with the mutagenic compound or inside the cell by interfering with the cellular metabolism (Wallum et al., 1990). Other modifying effects inside as well as outside the cells may act by modifications of the mutagen chemically or enzymatically.

The studies confirm that the MTT assay proved to be a simple bioassay for the testing of a large variety of sample material and may be used primarily for screening purposes. However, one single bioassay and cell type cannot resolve all problems connected with the detection of anticytotoxic factor(s) from fruit and vegetable extracts and cannot replace other biological or chemical methods. Thus, the use of the MTT assay system as described here can only provide information on the protective effect of fruit and vegetable extracts against *N*-nitrosamines on Vero cells. Studies are in progress to understand the mechanism of action of anticytotoxic factor(s) from fruits and vegetables and to understand how they may provide protection against toxic and mutagenic activities of *N*-nitrosamines.

Conclusion. This paper has discussed the protective effect of fruit and vegetable extracts against the cytotoxicity of NDMA, NPYR, NDBA, and NPIP using the MTT assay. Vegetable extracts exhibited a protective effect (100% of survival) and a stimulation of cellular proliferation (>100%) in decreasing order against NDMA and NPYR: $\text{broccoli}_{\text{EtOH}} > \text{onion}_{\text{H2O}} > \text{carrot}_{\text{EtOH}} > \text{onion}_{\text{EtOH}} > \text{licorice}_{\text{H2O}}$. Decreasing orders against NDBA and NPIP were, respectively, $\text{broccoli}_{\text{EtOH}} > \text{licorice}_{\text{H2O}} > \text{carrot}_{\text{EtOH}} > \text{onion}_{\text{EtOH}}$ and $\text{broccoli}_{\text{EtOH}} > \text{carrot}_{\text{EtOH}} > \text{licorice}_{\text{H2O}} > \text{onion}_{\text{EtOH}}$. The results indicate that vegetables could be useful to humans as chemoprotective agents against *N*-nitrosamines.

ABBREVIATIONS USED

NDMA, *N*-nitrosodimethylamine; NPYR, *N*-nitrosopyrrolidine; NDBA, *N*-nitrosodibutylamine; NPIP, *N*-nitrosopiperidine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SDH, succinate−dehydrogenase.

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