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Evaluation of the Antimutagenic Activity of Different Vegetable Extracts Using an *In Vitro* Screening Test

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N-Nitroso compounds (NOC) are considered to be an important group of genotoxic carcinogens, involved in human cancer etiology. Extensive experimental and epidemiological data suggest that humans are susceptible to carcinogenesis by N-nitroso compounds. The presence of these compounds in foods may be regarded as an etiological risk factor involved in certain human cancers including cancers of the esophagus, stomach and nasopharynx [1]. Over 300 NOC have been shown to be carcinogenic in one or more animal species [2,3] and more than 40 animal species, including higher primates, are susceptible to NOC-induced carcinogenesis [4].

Druckrey *et al.* [5] observed that tumors induced by N-nitroso compounds in experimental animal showed similar morphological properties as tumors found in the corresponding human organs. Human exposure results via several sources (e.g. consumer products, foods, occupational exposure and tobacco consumption) at a wide range of concentrations [6]. The dietary exposure to NOC starts early in life and persists over a long period. Thus, continuous exposure to low concentrations of several N-nitroso compounds in the diet would be expected to be an etiological risk factor for certain human cancers.

The formation of NOC carcinogens may result from endogenous nitrosation reaction of nitrite (one of the most widely used food additives) and amines (present in food) producing N-nitrosamines, a large group of chemical carcinogens found in different food products [7,8]. Therefore, humans are exposed not only to preformed NOC but also to a wide range of nitrogen-containing compounds and nitrosating agents that can react *in vivo* to form NOC. Nitrosating agents and NOC can also be synthesized endogenously in reactions mediated by bacteria and activated macrophages. Thus, endogenous formation of NOC can occur at various sites in the body [9].

On the other hand, in recent years, more attention has been dedicated to exploring compounds in foods with anti-mutagenic and anti-carcinogenic potential. Such compounds are found in almost all categories of food, fruits and vegetables being the main source [10]. Studies of dietary components such as green pepper, suggest a direct relationship between their consumption and the decrease of certain types of diseases, including cancer [11,12]. Garlic extract showed a significant antimutagenic and anticarcinogenic effect [13]. Antimutagenic activity has been also reported in cabbage, parsley, spinach, mustard green and broccoli [14]. A recent study indicated that poblano green pepper extract displays antimutagenic properties, due to the fact that some compounds present in it inhibit the endogenous nitrosation process [15]. The nitrosation reaction can be influenced by the presence of inhibitors (redox compounds such as ascorbate and vitamin E) or catalysts (metal ions, carbonyl compounds and nucleophilic anions such as Cl^-, I^-, and SCN^-). Plant-based food compounds can catalyze or inhibit nitrosation process depending on their structure [16]. Recognition of inhibitors of nitrosation reactions is relevant for the primary prevention of cancer. In the present study we applied the Ames test to study the anti-nitrosating properties of different vegetables commonly used in human diet, such as pumpkin, pea, string bean, purslane and bean.

MATERIAL AND METHODS:

Compounds. Methylurea (MU, CAS 598-50-5), sodium nitrite (SN, CAS 7632-00-0), sulfanilic acid (CAS 121-57-3), *N*-(1-naphthyl)ethylendiamine (CAS 1465-25-4) and ammonium sulfamate (CAS 7773-06-01) were purchased from Sigma.

Preparation of vegetables extracts. The vegetables chosen for this study represented commonly used edible plants. The following were purchased from local markets: pumpkin (*Cucurbita pepo*), pea (*Pisum sativum),* string bean (edible pods of *Phaseolus vulgaris),* purslane (*Portulaca oleoracea)* and bean (dry seeds of *Phaseolus vulgaris)*. All vegetables except bean were carefully washed under cold running water, sliced, and processed by an ordinary juice extractor. The juice was stored in plastic containers in an ultra-low freezer (-70°C) until use. Bean testa were homogenized in 100% methanol, 50% methanol - 50%water, or pure water and freeze-dried.

Nitrosation reaction. Nitrosation of methylurea followed the procedure described by Stich [17] was used. Reaction mixture consisted of methylurea (final concentration, 25 mM) and sodium nitrite (final concentration,100 mM) in standard buffer adjusted to pH 3.6 (citric acid 68 mM and dibasic sodium phosphate 64 mM; final volume 2 ml). This solution was incubated at room temperature (22°C) for 60 min and neutralized to pH 7.4 by adding 0.7 ml sodium bicarbonate (7.5% solution). The final volume was adjusted to 3.0 ml by the addition of 0.3 ml of a 10X phosphate-buffered saline solution (80 g sodium chloride, 2 g potassium chloride, 11.5 g dibasic sodium phosphate, 2 g monobasic

potassium phosphate, pH 7.4). To test the anti-nitrosating properties, the vegetable extracts were added to the methylurea solution just prior to the addition of the sodium nitrite.

Mutagenicity test. The mutagenicity of the nitrosation reaction products was assayed by the method described by Ames [18]. Overnight cultures of *S. typhimurium* TA1535 (1 x 10^8 cells/ml) were removed from Difco nutrient broth by centrifugation and suspended in the resulted nitrosation reaction. Treatment duration was 20 min at 37ºC. The bacteria were then pelleted and washed in phosphate buffer. Bacteria were suspended in phosphate buffer at the original cell concentration and aliquots were diluted with 0.85% NaCl. Bacterial suspension was added to low-histidine top agar and loaded onto minimal agar plates(in triplicate) in order to estimate the number on his+ revertants. Plates were scored after 48 h incubation at 37°C.

Determination of nitroso compound. The concentration of nitroso compounds was measured using the method of Takeda and Kanaya [19] with slight modifications. The test sample (0.2 ml) was treated for 15 min at 4°C with 0.25 ml of ammonium sulfamate (30 mg/ ml) with shaking. A small amount of the mixture (0.025 ml) was reacted with 1 ml of hydrobromic acid (1% in glacial acetic acid) for 10 min at 25ºC. The reaction mixture was then treated with 2 ml of the Griess reagent (0.5% sulfanilic acid and 0.05% *N*-(1-naphthyl)ethylenediamine·2 HCl in 30% acetic acid) for 10 min. The absorbance of the reaction mixture was determined at 550 nm. A standard curve with N-nitrosomethylurea was used to calculate the concentration of the nitroso compound formed.

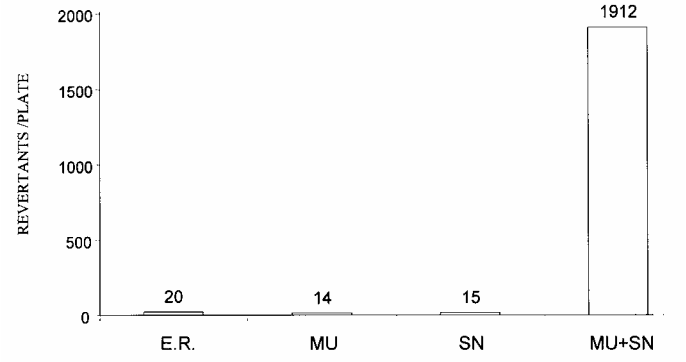

Figure 1. Mutagenic activity of the nitrosation reaction and its precursors. E.R. *S.thyphimurium* TA 1535 spontaneous reversion: MU Methylurea; SN Sodium Nitrite; MU+SN Methylurea + Sodium Nitrite.

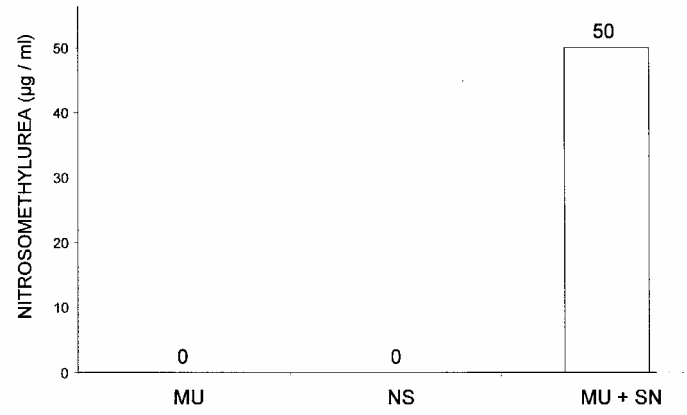

Figure 2. Chemical determination of nitrosmethylurea. MU Methylurea; NS Sodium Nitrite; MU+SN Methylurea + Sodium Nitrite.

RESULTS: Methylurea or sodium nitrite alone did not display mutagenic activity as detected by TA1535 *Salmonella* strain. The number of revertant colonies in plates containing these compounds are similar to that obtained in spontaneous reversion plates (Fig. 1). On the other hand, the mixture resulting from the nitrosation reaction (sodium nitrite plus methylurea) gave a 96-fold increase in the number of revertant colonies over the control (Figs. 1 and 2).

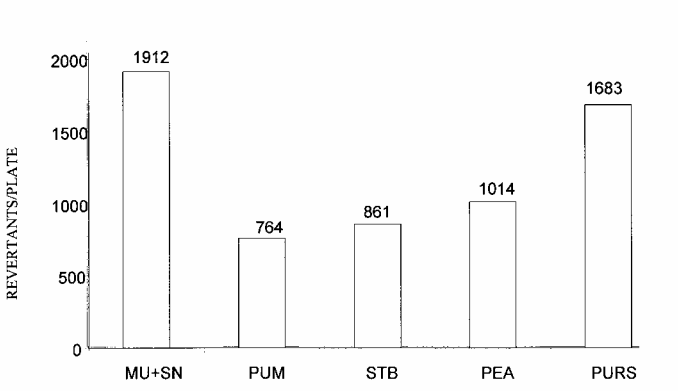

Figure 3. Inhibition of mutagenicity of nitrosation mixture by vegetable extracts. MU+SN Methylurea + sodium nitrite; PUM Pumpkin extract; STB String bean extract; PEA pea extract; PURS Purslane extract.

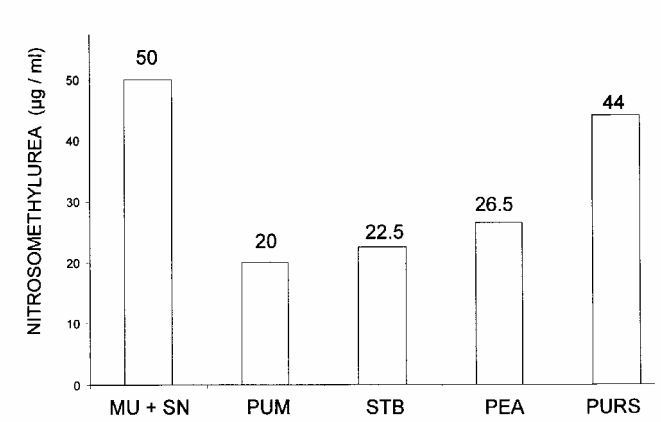

Figure 4. Inhibition of nitrosomethylurea formation by vegetable extracts. MU+SN Methylurea + sodium nitrite; PUM Pumpkin extract; STB String bean extract; PEA pea extract; PURS Purslane extract.

The effects of the different plant extracts on the generation of mutagenic products in the nitrosation mixture are shown in Figure 3. A reduction in mutagenic activity of the nitrosation mixture by 60%, 47%, 55% and 12% was seen with the extracts obtained from pumpkin, pea, string bean and purslane respectively. Reduction in mutagenicity was accompanied by a reduction in the amount of N-nitrosomethylurea formed during the reaction between the precursors, methylurea and sodium nitrite, as shown in Figure 4.

In the case of bean, the organic extracts obtained were also capable of inhibiting the mutagenicity and the concentration of the reaction products of nitrosation at the

two concentrations tested, showing a dose-dependant relationship (Figures 5 & 6).

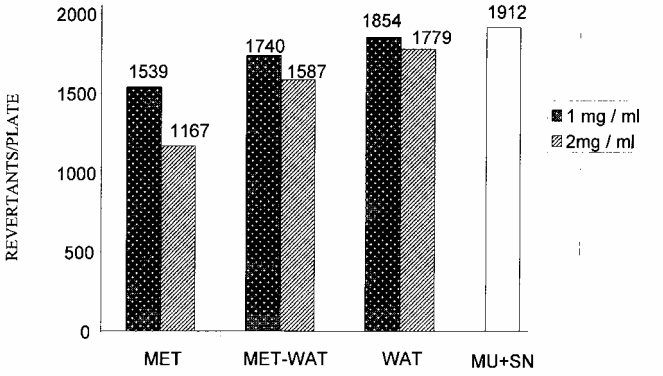

Figure 5. Inhibition of mutagenicity of nitrosation products by bean extracts. Beans were extracted with: MET Methanol; MET-WAT 50% Methanol – 50% water; WAT Water. MU+SN Methylurea + Sodium nitrite.

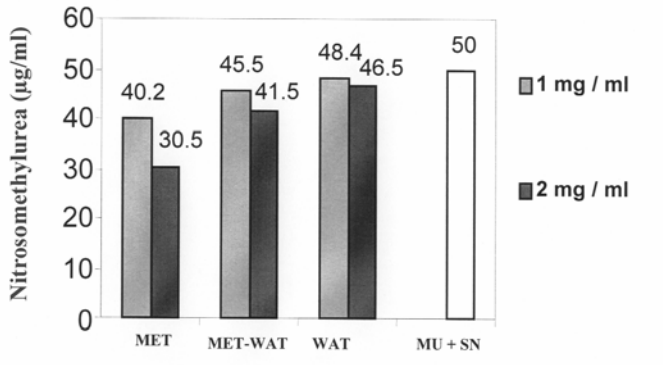

Figure 6. Inhibition of nitrosomethylurea formation by bean extracts. Beans were extracted with: MET Methanol; MET-WAT 50% Methanol – 50% water; WAT Water. MU+SN Methylurea + Sodium nitrite.

DISCUSSION: The *in vitro* nitrosation of methylurea by sodium nitrite leads to the formation of direct-acting mutagens that can be readily detected in the *S. typhimurium* mutagenicity assay. This well-defined system was used as a model to examine the effect of several common vegetables. Pumpkin, pea, string bean, purslane, and bean reduced the formation of mutagenic compounds.

The antimutagenic activity of vegetable extracts may be attributable to one or more molecules (micro nutrients) including anti-oxidant agents like vitamin C, chlorophyll, carotenoids and polyphenols, as has been reported by others.

One of the few things on which nutritionists agree is that the incidence of cardiovascular and neurodegenerative diseases and different types of cancer can be diminished by ingestion of diets rich in fruits, grains and vegetables. Vitamin C [20], a well known inhibitor of endogenous nitrosation, and vitamin E, the major fat-soluble antioxidant in vegetable oils and the most potent lipid peroxyl radical scavenger [21], can be considered as two feasible candidates responsible for the antimutagenic properties found in the vegetable extracts considered in this study. Additionally, carotenoids, which also occur in many vegetables, are potential antioxidants and evidences from epidemiological studies points to a protective effect against cancer [22].

N-nitroso compounds are ubiquitous mutagens that cause cancer in experimental animals and human beings. Nitrite and nitrates in the diet are precursors for the *in vivo* formation of N-nitroso compounds. This reaction occurs under low pH conditions in the stomach. Certain dietary phenolic compounds including resorcinol, kaempferol, quercetin, catechin and naringin have the ability to catalyze this reaction in similar conditions than those found in the human stomach [23].

Deciding which compound is the most important antimutagen may be difficult, because the vegetable extracts used are complex mixtures of many other possible antimutagens. Thus, the antimutagenic activity of the extracts may not depend only on the action of one of its components, but on the interaction of all of them. Therefore, an increase in the consumption of fruits and vegetables could be a better option than the ingestion of isolated vitamins or antioxidant components, in order to prevent the carcinogenic process.

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