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Mutation Research 441 (1999) 1–9

Genetic Toxicology and Environmental Mutagenesis

# Antimutagenic effects of natural phenolic compounds in beans

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Received 4 August 1998; received in revised form 18 February 1999; accepted 24 February 1999

**Abstract**

Polyphenols in fruits, vegetables (e.g., flavonols like quercetin) and tea (e.g., catechins such as epigallocatechin gallate) are good antioxidants with antimutagenic and anticarcinogenic properties. In the present study, the *Salmonella typhimurium* tester strain YG1024 was used in the plate-incorporation test to examine the antimutagenic effect of phenolic compounds, extracted from common beans (*Phaseolus vulgaris*), on 1-NP and B[*a*]P mutagenicity. Dose–response curves for 1-NP and B[*a*]P were obtained; the number of net revertants/plate at the peak mutagenic dosage were 880 for 1-NP and 490 for B[*a*]P. For the antimutagenicity studies doses of 0.1 μg/plate and 2 μg/plate for 1-NP and B[*a*]P, respectively, were chosen. We obtained a dose–response curve of ellagic acid (EA) against B[*a*]P and 1-NP mutagenicity. To test the bean extract, a dose of 300 μg/plate of EA was chosen as the antimutagenic control. The EA and bean extracts were not toxic to the bacteria at the concentrations tested. The inhibitory effects of the bean extracts and EA against B[*a*]P mutagenicity were dose-dependent. The percentages of inhibition produced against B[*a*]P (2 μg/plate) using 300 μg/plate of EA and for the extracts 500 μg equivalent catechin/plate were 82%, 83%, 81% and 83% for EA, water extract, water/methanol extract and methanol extract, respectively. However, for 1-NP mutagenicity, only the methanolic extract from beans showed an inhibitory effect. These results suggest that common beans, as other legumes, can function as health-promoting foods.


*Keywords:* Phenolic compound; 1-Nitropyrene; Benzo[*a*]pyrene; Ellagic acid; Mutagenicity; *Salmonella typhimurium*

## 1. Introduction

The diet has been implicated in the prevention or development of certain diseases such as cancer. Recent studies suggest that a low incidence of cancer is associated with the consumption of fresh fruits and vegetables, rich in phenolic compounds, fibre, chlorophyll, β-carotene, and vitamins such as C and E, which have antimutagenic and/or anticarcinogenic properties [1–3]. In Mexico, beans have been cultivated since prehispanic times and their consumption has been part of the tradition and culture for thousands of years. The per capita dietary intake of beans is about 19.5 kg per year [4].

Phenolic compounds are ubiquitous in plant foods, and have been associated with the sensory and nutritional quality of fresh and processed plant foods [2]. Dry bean polyphenols have received considerable attention, largely as a result of their possible influence on the nutritional and aesthetic quality of foods. Phenolic compounds have many biological activities,

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1383-5718/99/$ - see front matter 
PII: S1383-5718(99)00040-6

such as antioxidants, scavengers of active oxygen species and electrophiles, blockers of nitration and chelation of metals. They can undergo autoxidation to produce hydrogen peroxide in the presence of metals and are capable of modulating certain cellular enzyme activities [5]. Hydrolyzable tannins contain either gallotannins or ellagitannins. Upon hydrolysis by acids, bases or certain enzymes, the gallotannins yield glucose and gallic acid. Ellagitannins contain gallic acid and a molecule of hexahydroxydiphenic acid linked to glucose as a diester. Upon hydrolysis, this acid undergoes lactonization to produce ellagic acid (EA). Catechin is a phenolic compound (flavonoid) which is present widely in edible plant material such as soybeans, and especially in tea [2,5,6]. Human intake of all flavonoids has been estimated to be up to 1 g per day [2]. Catechins have been reported to inhibit tumorigenesis and some types of cancers [7–9].

On the other hand, nitroarenes are present in diesel and gasoline emissions, ash particles, cigarette smoke condensates, home heater emissions and in the urban atmosphere. Nitroarenes, such as 2-nitrofluorene (2-NF), 1-nitropyrene (1-NP) and 1,8-dinitropyrene (1,8-DNP), typically are potent mutagens for *Salmonella typhimurium* strains. 1-NP is a direct-acting mutagen, but it requires metabolic activation toward arylhydroxilamines by acetyl-CoA: *N*-hydroxyarylamine *o*-acetyltranferase (OAT) which is present in bacterial cells [10–12].

Benzo[*a*]pyrene is a polycyclic aromatic hydrocarbon (PAH) carcinogen that undergoes metabolic activation to reactive benzo[*a*]pyrene dihydrodiol epoxide, which is an electrophilic species capable of binding to DNA, RNA and some other macromolecules [13].

In the present study, the plate-incorporation test of the *Salmonella* mutagenicity assay [14] was used to examine the effect of phenolic compounds extracted from common beans against 1-NP and B[*a*]P mutagenicity.

## 2. Materials and methods

### 2.1. Chemical Reagents

(+) Catechin, 1-nitropyrene, benzo(*a*)pyrene (B[*a*]P) and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO). Stock solutions of 1-nitropyrene and B[*a*]P were prepared in DMSO at concentrations of 5 μg/ml and 8 mg/ml, respectively.

### 2.2. Quantification of phenolic compounds in common beans (*Phaseolus vulgaris*)

#### 2.2.1. Sampling procedure

The beans (*Phaseolus vulgaris* variety Flor de Mayo), collected from El Bajío, Mexico, were washed with tap water to eliminate external pesticides, soil and other contaminants. Raw whole bean flour and seed coat (which is rich in phenolic compounds) were milled (80 mesh), lyophilized and stored at −70°C and protected from light until their extraction and quantification.

#### 2.2.2. Extraction and quantification of phenolic compounds

Phenolic compounds, expressed as equivalents of catechin (mg/kg), were quantified according to the method of Price and Butler [15]. Two hundred mg of lyophilized raw whole flour of bean seed coat was placed in a 100-ml flask and mixed with 10 ml of water, water/methanol (50/50) or methanol (100%). The flask was placed in a 30°C water bath for 60 min. The samples were centrifuged at $3000 \times g$ for 20 min, the organic solvents were evaporated using a rotavapor and then each sample was lyophilized and stored at −70°C and protected from light and free oxygen until analysis. Phenolic compounds were quantified according to the method of Price and Butler [15]. For the quantification of phenolic compounds, 200 mg of each lyophilized extract (water, water/methanol or methanol) was placed in a 100-ml flask, mixed with 10 ml of methanol and placed in a 30°C water bath for 60 min. Phenolic compounds were quantified by spectrophotometry using a Beckman DU-65 at 436 nm, according to the Price and Butler procedure [15]. The reaction was developed at 30°C using freshly prepared reagents. (+) Catechin (up to 0.2 mg/ml) was used as reference standard, and polyphenols were expressed in mg of catechin equivalent per gram of dry sample (mg cat. eq/g). In order to correct for interference from natural pigments in dry beans, a blank sample was prepared by subjecting the original extract to the same conditions as the reaction, but without vanillin reagent.

*2.2.3. Spectrophotometric analysis*

To identify the absorption spectra of phenolic extracts from beans, the samples were scanned from 200 to 750 nm using a Perkin-Elmer DU-65 spectrophotometer. Appropriate blanks were conducted with each sample. (+) Catechin, tannic acid and ellagic acid were used as controls for phenolic compounds.

*2.3. Mutagenicity and antimutagenicity testing*

Tester strain YG1024 is a derivative of TA98; and contains the gene that overproduces acetyl-CoA: *N*-hydroxyarylamine *o*-acetyltranferase; an enzyme responsible for nitroarene and aromatic metabolism [12]. It was kindly provided by Dr. T. Nohmi, Division of Genetics and Mutagenesis, Biological Safety Research Center, National Institute of Hygienic Sciences, Japan. Strain genetic markers were routinely determined in all mutagenicity experiments. The mutagenic activities of 1-nitropyrene (1-NP), B[*a*]P, and the mutagenic and antimutagenic activity of extracts of phenolic compounds present in the seed coat of common beans (*P. vulgaris*) were determined using the plate-incorporation test of Maron and Ames [14]. Bacteria were grown overnight in Oxoid nutrient broth No. 2 (Oxoid Ltd., Hants, England) up to approximately $1–2 \times 10^9$ cells/ml. The S9 (metabolic enzymes) and S9 mix (enzyme co-factors) were prepared according to the procedure of Ames et al. [16]. The S9 from Aroclor 1254 pretreated Sprague–Dawley male rats, was obtained from Molecular Toxicology (Annapolis, MD), and contained 40 mg protein/ml, as determined by the method of Lowry et al. [17]. The concentration of the S9 in the mix was established at 1.2 mg/ml. The volume of S9 mix was 0.5 ml/plate.

For the plate-incorporation test without metabolic activation (S9), the order of addition, to reach two ml of molten top agar (45°C) containing 90 nmol of histidine and biotin, was 0.1 ml of bacterial culture, 0.1 ml of 1-NP (at 0.01, 0.03, 0.06 or 0.1 μg/plate), 0.1 ml of EA (300 μg/plate) or 0.1 ml of phenolic extracts from common beans added at 50, 125, 250 or 500 catechin equivalents/plate. For the experiments with metabolic activation the order of addition was 0.1 ml of bacterial culture, 0.5 ml of S9 mix, 0.1 ml of B[*a*]P (1, 2, 4 or 8 μg/plate) and 0.1 ml of EA (300 μg/ml) or 0.1 ml of phenolic extracts from common beans at 50, 125, 250 or 500 catechin equivalents/plate. The EA and bean extracts, as B[*a*]P and 1-NP, were also dissolved in DMSO. When the antimutagenic agent (EA or phenolic extracts) was mixed with the toxic compound (1-NP or B[*a*]P) the total volume of DMSO was 0.1 ml. The amount of phenolic extracts expressed as equivalents of EA were calculated based on their respective concentrations in order to adjust such values as the ones used for pure EA. The mixture was poured onto minimal glucose plates, then incubated at 37°C for 48 h, after which the number of histidine-independent ($His^+$) revertants colonies were scored using a MiniCount colony counter (Biotran II, New Brunswick Scientific, Edison, New Jersey). Strain markers and bacterial survival were routinely monitored for each experiment.

Triplicate plates were set up for at least two independent experiments with each assay. Antimutagenicity was expressed as percentage of mutagenicity inhibition following the formula:

$$\%\,\text{Inhibition} = 100 - \left[\left(X_1/X_2(100)\right)\right]$$

where $X_1$ = number of revertants per plate in the presence of extract, expressed as equivalents of catechin; $X_2$ = number of revertants per plate in the absence of extract. The slope values were used to calculate the mutagenic potency.

*2.4. Statistical analysis*

The results are presented as the average and standard deviation of two experiments with triplicate plates per dose per experiment.

The statistical significance of the differences among the bean extracts from three different solvents was analyzed by t intervals. The efficiency of phenolic extracts to inhibit 50% of the mutagenic activity against B[*a*]P was estimated by polynomial adjustment of a second grade equation in the experimental range.

## 3. Results and discussion

*3.1. Quantification of phenolic compounds*

The amount of phenolic compounds from common beans (*P. vulgaris*) is shown in Table 1. The

Table 1
Amount of phenolic compounds from common beans (*Phaseolus vulgaris*)[1]

| Extract | Bean flour (Eq. Catechin) (mg/g) | Seed coat (Eq. Catechin) (mg/g) | Extract from seed coat (Eq. Catechin) (mg/g) |
|---|---|---|---|
| Water | $2.5^{a} \pm 0.05$ | $27.5^{b} \pm 0.49$ | $110.0^{c} \pm 2.0$ |
| Water/methanol | $6.4^{d} \pm 0.05$ | $71.4^{e} \pm 0.51$ | $420.0^{f} \pm 3.0$ |
| Methanol | $18.3^{g} \pm 0.06$ | $203.3^{h} \pm 0.67$ | $813.0^{i} \pm 2.6$ |

[1]Results represents the average of two independent experiments ± S.D. Quantification of phenolic compounds from common beans (*P. vulgaris*) extracts were determined according to Price and Butler, as described in Section 2.
Eq. = Equivalent.
Letters a–i refer to statistical differences with $p < 0.05$.

seed coat, where major amounts of bean polyphenols were located, represented 11% of the total seed, with low or negligible amounts in the cotyledons. The concentration of catechin was dependent on the type of sample. For example, the methanolic extract from the seed coat was the highest (813 mg/g), forty and

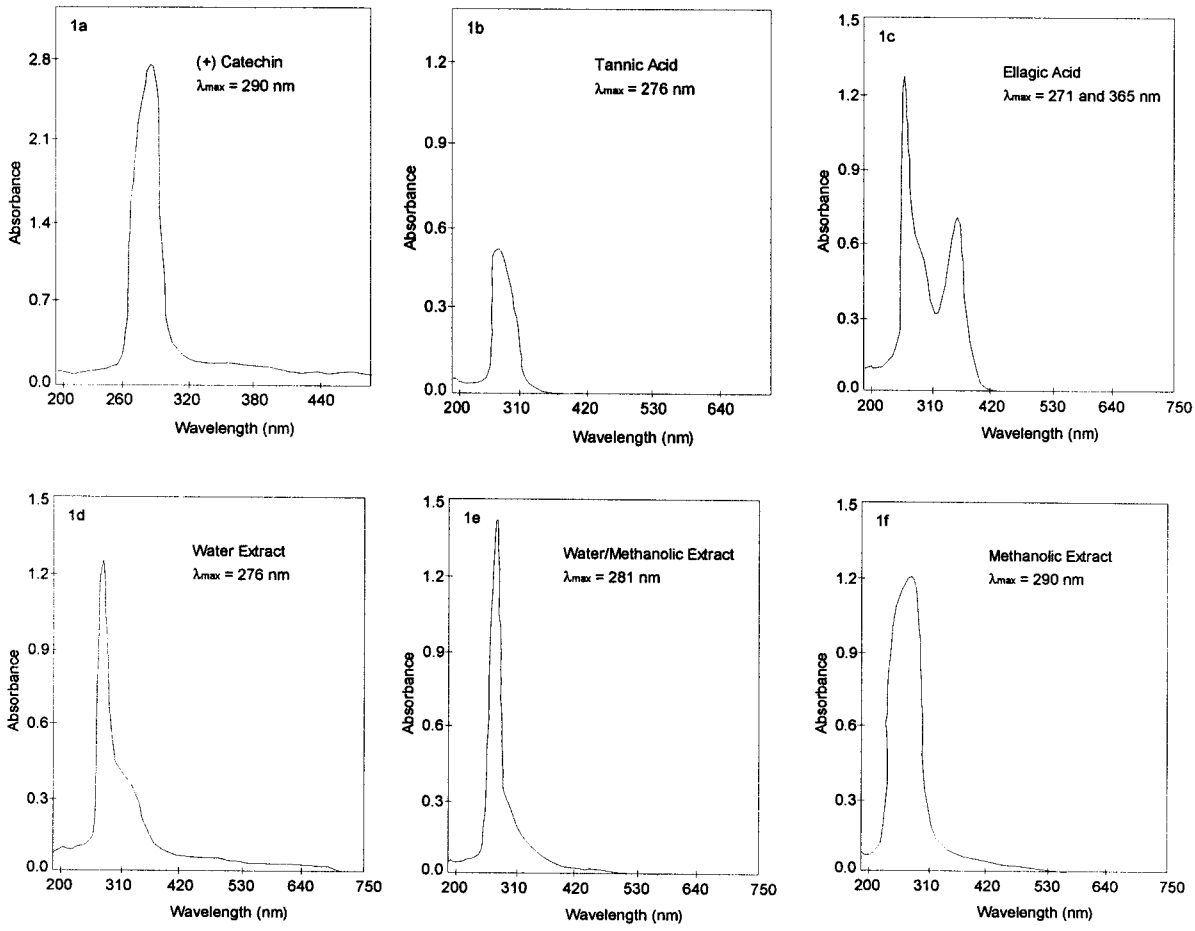


Fig. 1. Absorption spectra of (+) catechin, tannic acid, ellagic acid, bean methanolic extract, bean water/methanol extract and bean water extract. The absorption spectra were recorded from 750 to 200 nm as described in Section 2.

four times higher than the whole bean flour (18.3 mg/g) and seed coat (203.3 mg/g), respectively, without previous methanolic extraction. The results, for the same variety of beans, are in agreement with other authors [18,19]. Among other components of the diet, tea, which is one of the most widely consumed beverages in the world, is rich in polyphenolic compounds. The amount of catechin from green tea was 0.1% of dry weight [20]. Lin et al. [21] reported that the amount of catechin from various fresh young tea leaves was in the range 0.06 to 0.23% per 100 g of leaves.

### 3.2. Spectrophotometric analysis

Fig. 1a shows the absorption spectra of (+) catechin, the peak of maximal absorbance was at 290 nm. The absorption spectra of tannic acid and ellagic acid (EA) are shown in Fig. 1b and c, their peaks of maximal absorbance were 276, and 271 and 365, respectively. The two peaks absorption spectra is typical for EA due to its chemical structure. The bean extracts (water, water/methanol and methanolic) are shown in Fig. 1d–f. The results suggest that the antimutagenic compounds, present in common beans var. Flor de Mayo, were phenols, easily extractable with methanol (with a main peak of absorbance at 290 nm), and hydrolyzable phenols of low molecular weight (with a main peak at 276 nm and a shoulder at 330 nm) present in the aqueous extract.

### 3.3. Mutagenicity and antimutagenicity testing

Dose–response curves of B[*a*]P and 1-NP mutagenicity were obtained as illustrated in Figs. 2 and 3, respectively. 1-NP was not toxic to the bacteria at the concentrations tested (up to 0.1 μg/plate), while B[*a*]P, was toxic to the bacteria at concentrations higher than 2 μg/plate. Non-toxic doses were selected for further testing. Watanabe et al. [12], reported similar revertants/plate induced with 1-NP at the concentrations tested. On the other hand, it is clear that B[*a*]P also induced mutagenicity in the strain YG1024, which was created to improve the detection of environmental nitroarenes and aromatic amines [12]. Doses of 0.1 μg/plate of 1-NP and 2 μg/plate B[*a*]P were chosen for the antimutagenicity studies, since these doses were not toxic. Fig. 4 shows the antimutagenic effect of ellagic acid (EA) on B[*a*]P (2 μg/plate) and 1-NP (0.1 μg/plate) mutagenicity. The dose of 300 μg/plate of EA was chosen as an antimutagenic positive control. The inhibitory effects of the extracts from common beans (*P. vulgaris*) and EA, used as an antimutagenic agent control against B[*a*]P mutagenicity are shown

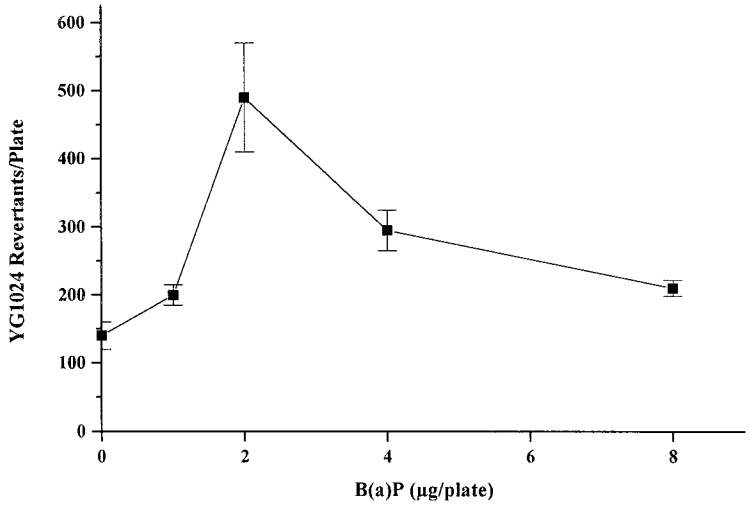


Fig. 2. Dose–response curve of B[*a*]P mutagenicity in YG1024. Each point represents the average of two independent experiments ± S.D. Spontaneous mutation frequency was 95 revertants/plate.

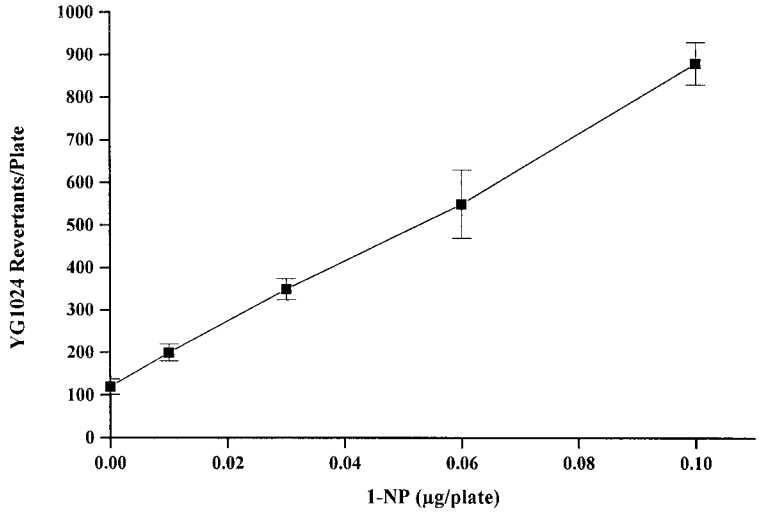


Fig. 3. Dose–response curve of 1-NP mutagenicity in YG1024. Each point represents the average of two independent experiments ± S.D. Spontaneous mutation frequency was 100 revertants/plate.

in Table 2. The water, water/methanol and methanol extracts were neither toxic nor mutagenic to the bacteria at the concentrations tested, and bacterial growth was normal. Doses of 500 μg equivalent catechin/plate produced inhibition of 83, 81 and 83% for water, water/methanol and methanol extracts, respectively. The inhibition was dose-dependent for all the samples tested. However, the methanolic extract was the best at the lowest doses, 50 μg equivalent catechin/plate. Our results are in agreement with the ones found by Mandal et al. [22], with respect to the antimutagenic effect of EA against aflatoxin $B_1$ (premutagenic compound as is B[*a*]P) in TA100, which at concentrations of 1, 5, 30, 300 and

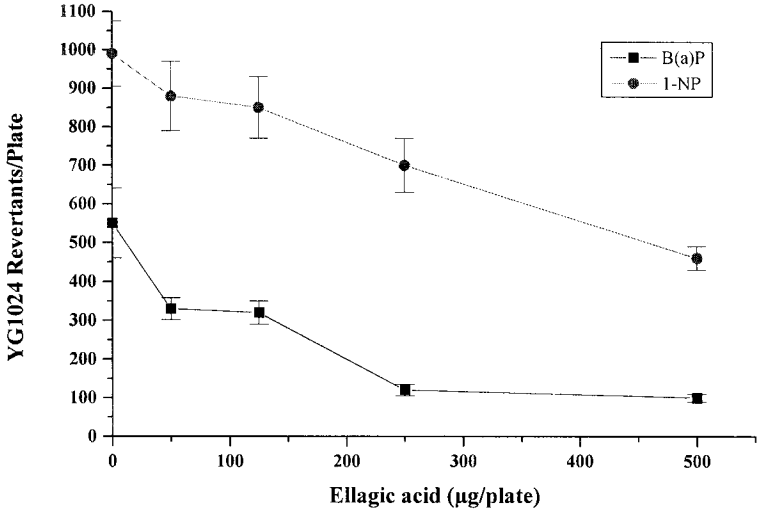


Fig. 4. Antimutagenic effect of ellagic acid against B[*a*]P (2 μg/plate) and 1-NP (0.1 μg/plate) in YG1024. Each point represents the average of two independent experiments ± S.D. Spontaneous mutation frequency was 92 and 98 revertants/plate for B[*a*]P and 1-NP respectively.

Table 2
Antimutagenic activity of phenolic extracts from common beans (*Phaseolus vulgaris*) against B[*a*]P (2 μg/plate) in *S. typhimurium* YG1024[a]

| Extract | Antimutagen/plate (μg/plate)[b] | Revertants/plate (%) | Percent inhibition |
|---|---|---|---|
| Water | 0.0 | 439 ± 50 | 0.0 |
| | 50.0 | 367 ± 15 | 16.0 |
| | 125.0 | 178 ± 28 | 60.0 |
| | 250.0 | 91 ± 10 | 80.0 |
| | 500.0 | 64 ± 12 | 85.0 |
| Ellagic acid[c] | 300.0 | 73 ± 8 | 83.0 |
| Water/methanol | 0.0 | 542 ± 60 | 0.0 |
| | 63.0 | 428 ± 20 | 21.0 |
| | 125.0 | 415 ± 18 | 23.0 |
| | 250.0 | 156 ± 15 | 71.0 |
| | 500.0 | 104 ± 10 | 81.0 |
| Ellagic acid[c] | 300.0 | 144 ± 12 | 73.0 |
| Methanol | 0.0 | 618 ± 25 | 0.0 |
| | 50.0 | 326 ± 12 | 47.0 |
| | 125.0 | 266 ± 18 | 57.0 |
| | 250.0 | 235 ± 20 | 62.0 |
| | 500.0 | 108 ± 10 | 83.0 |
| Ellagic acid[c] | 300.0 | 112 ± 15 | 82.0 |

[a]Results represent the average of two independent experiments ± S.D. Triplicate plates were tested per dose per experiment.
[b]Amount of antimutagen was adjusted at equivalent of catechin.
[c]Ellagic acid as positive control of antimutagenicity.
Spontaneous mutation rate was 95 ± 10 revertants.

Table 3
Antimutagenic activity of phenolic extracts from common beans (*Phaseolus vulgaris*) against 1-NP (0.1 μg/plate) in *S. typhimurium* YG1024[a]

| Extract | Antimutagen/plate (μg/plate)[b] | Revertants/plate (%) | Percent inhibition |
|---|---|---|---|
| Water | 0.0 | 1800 ± 92 | 0.0 |
| | 50.0 | 2041 ± 100 | 0.0 |
| | 125.0 | 1810 ± 85 | 0.0 |
| | 250.0 | 2252 ± 70 | 0.0 |
| | 500.0 | 2221 ± 50 | 0.0 |
| Ellagic acid[c] | 300.0 | 449 ± 35 | 75.0 |
| Water/methanol | 0.0 | 1072 ± 80 | 0.0 |
| | 50.0 | 1382 ± 15 | 0.0 |
| | 125.0 | 1310 ± 10 | 0.0 |
| | 250.0 | 1180 ± 34 | 0.0 |
| | 500.0 | 1393 ± 20 | 0.0 |
| Ellagic acid[c] | 300.0 | 150 ± 20 | 86.0 |
| Methanol | 0.0 | 844 ± 25 | 0.0 |
| | 50.0 | 780 ± 20 | 8.0 |
| | 125.0 | 643 ± 20 | 24.0 |
| | 250.0 | 590 ± 18 | 30.0 |
| | 500.0 | 550 ± 12 | 35.0 |
| Ellagic acid[c] | 300.0 | 140 ± 8 | 83.0 |

[a]Results represent the average of two independent experiments ± S.D. Triplicate plates were tested per dose per experiment.
[b]Amount of antimutagen was adjusted at equivalent of catechin.
[c]Ellagic acid as positive control of antimutagenicity.
Spontaneous mutation rate was 102 ± 16 revertants.

1000 μg EA/plate inhibited, respectively, 50, 55, 80 and 90% of mutagenicity. Loarca-Piña et al. [23], using the microsuspension assay reported that EA (20 μg/tube) inhibited mutagenicity of aflatoxin $B_1$ over 90% in TA100 and TA98 strains. Yen and Chen [24] reported that tea extracts (green tea, oolong tea, pouchong tea and black tea) showed strong antimutagenic action against five indirect mutagens ($AFB_1$, Trp-P-1, Glu-P-1, B[*a*]P and IQ) in TA98 and TA100. Using TA98 the parental strain of YG1024, tea extracts from *Camellia sinensis* showed very potent antimutagenic effects against heterocyclic aromatic amines (HAA), the highest inhibition was obtained with 50 mg of tea leaves/plate [25].

Table 3 shows the inhibition by extracts from common beans (*P. vulgaris*) and ellagic acid (EA) against 1-NP mutagenicity. Only the methanolic extracts showed any antimutagenic effect. More research is needed in order to suggest that the phenolic compounds present in beans are more active against indirect mutagens than direct mutagens. Besides, when the efficiency of phenolic bean extracts to inhibit 50% mutagenic activity against B[*a*]P was calculated, the methanolic extract required 72

Table 4
Efficiency of phenolic beans extracts to inhibit 50% mutagenic activity against B[*a*]P ($IC_{50}$)

| Phenolic extract[a] | B[*a*]P[b] |
|---|---|
| Methanol | 72 |
| Water | 119 |
| Water/methanol | 170 |

[a]Phenolic extract = extract expressed as catechin equivalent (μg/plate).
[b]B[*a*]P = Benzo[*a*]pyrene (2 μg/plate).

μg/plate expressed as catechin equivalent, while water and water/methanol extracts required 119 and 170 μg/plate, respectively (Table 4). The data shows that the compounds present in the methanolic extract from beans are more potent against an indirect mutagen than the other extracts. Edenharder et al. [26] using the tester strain *S. typhimurium* TA 98 showed that the solvents are crucial to extract antimutagenic agents from some fruits and vegetables against two potent food mutagens. The antimutagenic activities were detected in 96% of the *n*-hexane extracts, 64% of the dichloromethane extracts, 44% of the acetone extracts and 36% of the 2-propanol extracts, suggesting that the solvent partition is the major factor to these differences. The antimutagenic potencies also depend on antimutagenic factors, on the mutagenic agent and the tester strain used [27]. The correlation of antimutagenicity of natural compounds of plant origin was positive with polarity of flavonols; also phenolic compounds resulted potent antimutagens [28].

Based on the results presented on Table 3, it was not possible to calculate $IC_{50}$ for 1-NP. The bean extracts may be inhibiting the mutagenic metabolism to the ultimate mutagen/carcinogen; or scavenging the electrophilic metabolite(s). Likewise, the phenolic compounds could be interacting directly and non-enzymatically with the proximate and/or ultimate mutagen(s); or forming a complex between the phenolic compounds and B[*a*]P, thereby reducing the bioavailability of B[*a*]P.

Although more research is needed to define their mechanisms of action, many positive health attributes have been documented in people eating diets that are high in fruit and vegetables [29]. These data further indicated that seed legumes, such as common beans, may also be health-promoting foods.

## Acknowledgements

The authors wish to express their appreciation for the technical help of Mr. Eduardo Pizaña, who used this material as part of his Masters dissertation. Dr. Javier Espinoza Aguirre also offered advice and was an important guide to this research. The authors thank him sincerely for his assistance.

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