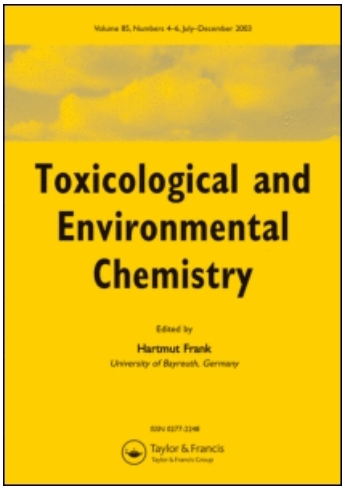

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The antimutagenic capacity of the aqueous extract of *Baccharis articulata* (Lam.) Persoon

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Baccharis articulata (Lam.) Persoon, known in South America as "carqueja or carquejilla", is used as folk medicine infusion for gastrointestinal and liver disease treatments in Southern Brazil, Uruguay, and Argentina. The antimutagenic effect of aqueous extracts from aerial parts of *B. articulata* against the mutagens 2-aminofluorene (2-AF), 2-nitrofluorene, and sodium azide was evaluated by the Ames test, with both the classic plate and pre-incubation assays. Results demonstrated that the antimutagenic effect of the extract depends not only on the mutagen tested but also on the incubation method used. The antimutagenicity curves for the pro-mutagen 2-AF requiring metabolic activation showed clear differences between TA98 and TA100 bacterial test strains. The presence of chlorogenic acid was detected by high performance liquid chromatography. This finding indicates that the antimutagenic capability of *B. articulata* could be related to its antioxidative properties.

Keywords: antimutagenicity; *Baccharis articulata*; *Salmonella*/microsomal assay

Introduction

Xenobiotics-induced cellular damage in DNA is the cause of disability in most living organisms, including humans. The accumulation of mutations is directly associated with diverse types of neoplasias, degenerative disorders, aging, and offspring defects, among other consequences (Soussi 2007). In order to prevent this deleterious risk, environmental genotoxic agents must be identified so as to minimize human exposure to them.

Phytochemicals with antioxidative, antimutagenic, and anticarcinogenic properties found in fruit and cruciferous vegetables (de Oliveira et al. 2003; Pedreschi and Cisneros-Zevallos 2006; Cariño-Cortés et al. 2007) can help reduce 7–31% human cancers (Hung et al. 2004). However, antinutritional and toxic phytochemicals, such as oxalic acid, nitrate, or erucic acid are present in many plants (Ames 1983).

The genus *Baccharis* L. from Astereae tribe comprises more than 400 species that spread from Southern USA to Southern Argentina and Chile. These are shrubs or sub shrubs, occasionally small trees and herbs, nearly dioecious (Nesom 1994). While several

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species are used in traditional medicine, others are toxic (Varaschin and Alessi 2003). Plants of these species are mostly used as infusion for a variety of medicinal purposes, e.g., gastrointestinal treatments, general infections, as antirheumatic, antispasmodic, abortive, or anti-inflammatory agents, among others (Weimann et al. 2002; Abad et al. 2006; Betoni et al. 2006). Ethnobotanical information available shows that approximately 20 *Baccharis* L. species are used as folk medicines in Argentina (Freire, Urtubey, and Giulano 2007). Among these, *Baccharis articulata* (known as "carqueja" or "carquejilla") is widely used as cholegogue.

In this study, we have analyzed the antimutagenic effect of the aqueous extract of *B. articulata* (Lam.) Persoon (AEC) on the two directly acting mutagens 2-nitrofluorene (2-NF) and sodium azide (SAZ), and on 2-aminofluorene (2-AF), which requires metabolic activation. The Ames test was performed with two different incubation methods (classic plate assay (PA) and pre-incubation assay (PIA)) using TA98 and TA100 *Salmonella typhimurium* tester strains as indicator bacteria.

Materials and methods

Chemicals

2-NF (CAS 607-57-8), 2-AF (CAS 153-78-6), SAZ (CAS 26628-22-8), D-biotin (CAS 58-85-5), L-histidine-HCL (CAS 71-00-1), glucose-6-phosphate (G6 P, CAS 56-73-5), β-nicotin-amide-adenine dinucleotide phosphate (β-NADP, CAS 24292-60-2), rutin (CAS 7621-88-7), and kaempferol (CAS 520-18-3) were provided by Sigma Chemical Co. (St. Louis, Mo, USA,) while nutrient broth no. 2 (NBN 2, CM0067) was purchased from Oxoid (Hamshire, England). Ampicillin (Amp, CAS 69-53-4), dimethyl sulfoxide (DMSO, CAS 67-68-5), and bacteriological agar type A (CAS 9002-18-0) were purchased from Merck KGaA (Darmstadt, Germany). S9 fraction was purchased from Moltox Molecular Toxicology Inc. (Booen, NC, USA). Quercetin (CAS 117-39-5) was purchased from ICN Biochemicals (Korea) while chlorogenic acid (CAS 327-97-9.100), caffeic acid (CAS 331-39-5), methanol (CAS 67-56-1), and formic acid (64-18-6) were provided by Mallinckrodt (Paris KY, USA).

Preparation of aqueous extract

B. articulata (Lam.) Persoon dried leaves and stems were purchased from Farmacity S.A. (La Plata, Argentina, supplier: "Herbores" S.A., Buenos Aires, Argentina). Prof. E. Spegazzini (Laboratory of Pharmacobotanics, Faculty of Exact Sciences, National University of La Plata, Argentina) identified the material and deposited the specimens under the number 1107, 09/06 at the Herbarium in the Museum and Pharmacognosia "Carlos Spegazzini" (National University of La Plata, Argentina). Thirty grams of dried leaves and stems of the plant were homogenized in 100 mL sterile distilled water (prior to boiling point) and then centrifuged ($9000 \times g$, 4°C, 30 min, Avanti TM J-25 centrifuge, Beckman Coulter, Fullerton, CA, USA). Afterwards, the supernatant was freeze dried (−80°C) and then stored at −20°C. Lyophilized material (2 g) was diluted in 10 mL sterile distilled water (room temperature), filtered through a 0.45 mm filter, and then stored at −20°C until use as aqueous extract of "carqueja" (AEC).

Chromatographic procedures

Polyphenols were analyzed in a Zorbax 300 SB-C18 column ($250 \times 4.6\,mm^2$, i.d.) packed with 5 μm particles and connected to a guard column. A HP 1100 liquid chromatograph, equipped with a binary pump, thermostatized column compartment, autoinjector, degasser, and diode array detector (DAD) connected to an HP workstation was used. Water was purified with a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). All solutions were filtered through 0.22 μm nylon membranes before injection into the high performance liquid chromatography (HPLC) column. The mobile phase "A" consisted of a mixture of water, methanol, and formic acid (79.7 : 20.0 : 0.3), and mobile phase "B" was a mixture of methanol and formic acid (99.7 : 0.3). The staggered gradient elution program at $0.9\,mL\,min^{-1}$ was as follows: 0% B for 15 min, 10% B for 15 min, 30% B for 10 min, 60% B for 10 min, and 80% B for 2 min. Finally, the mobile phase composition returned to 0% B in 5 min, and this composition was maintained for 10 min to equilibrate the stationary phase with the original solvent composition. Rutin, quercetin, kaempferol, chlorogenic acid, and caffeic acid were used as standards for identification. Their retention times, DAD spectra stored in the library, and the real samples spiked with each standard were used for identification. The AEC sample (30 μL) was injected into the HPLC column in triplicate. Standard solutions of quercetin ($0.103\,mg\,mL^{-1}$), rutin ($0.105\,mg\,mL^{-1}$), chlorogenic acid ($0.101\,mg\,mL^{-1}$), kaempferol ($0.108\,mg\,mL^{-1}$), and caffeic acid ($0.107\,mg\,mL^{-1}$) showed retention times of 44.52, 36.48, 11.92, 45.47, and 12.88 min, respectively. Based on the maximum absorption, the wavelengths selected for the chromatographic runs were 330 nm for the most polar, less retained compounds (chlorogenic acid and caffeic acid), and 360 nm for the most hydrophobic, longest retained compounds (rutin, quercetin, and kaempferol).

Bacterial strains

TA98 and TA100 *S. typhimurium* strains were obtained from Moltox Molecular Toxicology Inc. (Boone, NC, USA). Stock bacteria strains were stored at 80°C in NBN 2 (1 mL) supplemented with DMSO (90 μL). The tester strain genotypes and His^- requirement was confirmed as reported elsewhere (Maron and Ames 1983; Mortelmans and Zeiger 2000).

Mutagenicity and antimutagenicity assays

Plate assay

This assay was performed as described elsewhere (Maron and Ames 1983). Briefly, 0.1 mL overnight culture of the tester strain (TA98 or TA100), 0.1 mL mutagen (2-AF or 2-NF or SAZ) at specified concentrations, and/or AEC (1 or $10\,mg\,plate^{-1}$) were added to 2 mL molten top agar ($0.5\,mmol\,L^{-1}$ histidine $-0.5\,mmol\,L^{-1}$ biotine). In the case of 2-AF, 0.5 mL S9 mix was also added. Then, the mixture was poured onto minimal agar plates. The plates were incubated at 37°C for 48 h and His^+ colonies were then counted.

Pre-incubation assay

Either S9 mix (0.5 mL) or phosphate buffer, pH 7.4, (0.5 mL), was added with 0.1 mL overnight bacterial culture (TA98 or TA100 strain), 0.1 mL mutagen (at specified concentrations), and/or 0.1 mL AEC (up to a final concentration of 1 or 10 mg).

The mixture ($V_f = 700\,\mu L$) was pre-incubated at 37°C for 60 min. After pre-incubation, the mixture was added to 2 mL molten top agar (0.5 mmol L^{-1} histidine −0.5 mmol L^{-1} biotine) and then poured onto plates with minimal medium. After 48 h incubation (37°C), His^+ revertant colonies were counted. Triplicate plates were set up and run in parallel for each experimental point. The complete experiment was repeated twice. Data were expressed as mean number of colonies ±mean SD.

The inhibition of the diagnostic mutagen-induced mutagenicity in the presence of AEC was calculated as suggested by Pedreschi and Cisneros-Zevallos (2006) following the formulae:

$$\text{Inhibition}(\%) = 100 \times \left[1 - \left(\frac{\text{AEC plus mutagen} - \text{negative control}}{\text{mutagen} - \text{negative control}}\right)\right],$$

where results are expressed as revertants $plate^{-1}$: "AEC plus mutagen" corresponds to results obtained in presence of carqueja extract; "mutagen" to results obtained with the mutagen alone (positive control); and "negative control" to spontaneous revertants. Mutagenicity of each agent in the absence of AEC was defined as 100%. A lower percentage of His^+ revertants found in AEC-treated samples relative to positive controls denotes an inhibitory effect on the mutagenic response (Yen, Chen, and Peng 2001).

Toxicity assay

In order to examine the toxicity of the extract, 0.5 mL S9 mix or 0.5 mL of phosphate buffer was added with 0.1 mL AEC (up to a final concentration of either 1 or 10 mg) to 0.1 mL bacteria (overnight culture). The entire mixture ($V_f = 700\,\mu L$) was pre-incubated at 37°C for 60 min. Afterwards, an appropriate dilution was plated onto a nutrient agar plate. After incubation, the colony forming units per milliliter in the incubation mixture was calculated. The same procedure was applied with 2AF (0.25–2.50 μg $plate^{-1}$ concentration range), 2NF (0.25–2.50 μg $plate^{-1}$ concentration range), and SAZ (0.50–5.00 μg $plate^{-1}$ concentration range), with and without AEC. As suggested elsewhere, toxicity was also checked by direct observation of the total bacterial background lawn, grown in the selective plates until histidine traces consumption (Verschaeve and van Staden 2008).

Statistical analysis

The statistical analysis was performed by means of ANOVA and Student's *t*-test, considering a twofold enhancement in the number of revertants $plate^{-1}$ as a positive result. The percentage of inhibition error of mutagenicity was calculated by the propagation of errors according to Himmelblau (1970). The level of significance selected was 0.05, unless otherwise noted.

Results

The antimutagenic potential of AEC on the indirectly acting mutagen 2-AF was assessed with TA98 and TA100 Ames bacterial tester strains. The PA and PIA were used in the presence of S9 mix (see "Materials and methods" section). The dose–response curve observed with the TA98 strain showed that the protective effect depends upon the AEC concentration used, and similar responses were observed regardless of which of the two

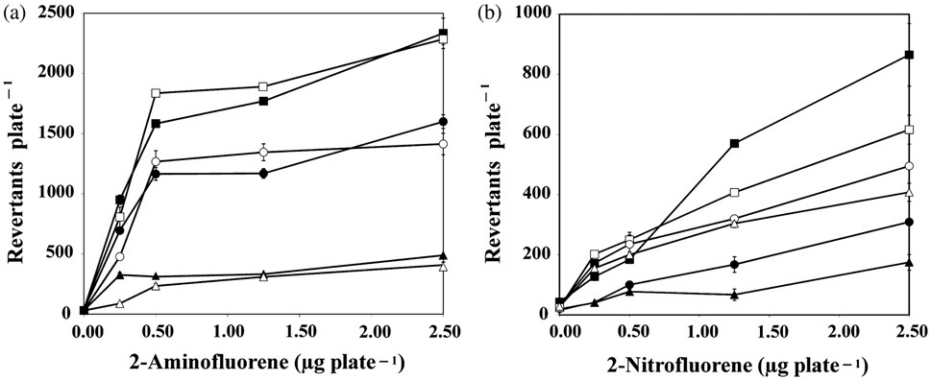

Figure 1. Antimutagenic effect of an aqueous extract of *B. articulata* (AEC) against the mutagenicity induced by 2-AF with metabolic activation (S9) (a), and 2-NF without metabolic activation (b) in a TA98 *S. typhimurium* strain. The numbers of revertants plate^{-1} (y-axis) in each graph are plotted against the concentrations of the mutagen assayed (x-axis). Squares, circles, and triangles denote the number of revertants per plate induced by the mutagen alone, and in the presence of 1 and 10 mg AEC per plate, respectively. Experiments were carried out using both the plate (black squares, circles, and triangles) and the pre-incubation (empty squares, circles, and triangles) assays.

assays were employed (PA or PIA) (Figure 1a, Table 1). A different behavior was observed when TA100 strain was used. The dose–response curves obtained were dependent on the incubation method used, being PIA less sensitive than PA in detecting 2-AF mutagenic activity (Figure 2a). AEC antimutagenic effect showed to be fully active under PIA method at all the mutagen concentrations assayed independent of AEC concentration (inhibition percentage varying between 93% and 100%) (Table 1). On the other hand, the inhibitory effect of AEC was shown to be less effective under PA, fluctuating between 31% and 56%, with both 1 and 10 mg plate^{-1} of AEC (Table 1).

When the directly acting mutagen 2-NF was used with the TA98 strain, PA was more sensitive than PIA in detecting the mutagenicity of the compound assayed (Figure 1b). These findings also demonstrate that AEC was more effective in counteracting 2-NF-induced mutagenicity when PA was used than when PIA was employed. Furthermore, efficiency was higher when 10 mg plate^{-1} AEC were tested, reaching almost 90% inhibition at the highest mutagen concentrations (Table 1). The inhibitory effect of AEC with PIA showed to be less effective and independent of AEC concentration, varying between 20% and 37% (Figure 1b, Table 1).

When the directly acting mutagen SAZ was tested with TA100 strain, the inhibitory effect of AEC was weakly expressed (only expressed by PIA at 5 μg plate^{-1} mutagen concentration (Table 1)).

In both methods, the plating efficiency of the bacterial population exposed was not affected by the presence of AEC and/or the mutagens, at any of the concentrations assayed ($p > 0.05$) (data not shown). Non-mutagenicity was observed for AEC independently of the concentration tested (1 and 10 mg plate^{-1}) or the method assayed ($p > 0.05$) (Figures 1 and 2, point zero).

The detection limits for chlorogenic acid, rutin, kaempferol, caffeic acid, and quercetin were 0.0024, 0.0019, 0.0026, 0.0035, and 0.0047 mg mL^{-1}, respectively. According to the retention time, the corresponding UV–vis spectrum, and a sample spiked with the standard, the presence of chlorogenic acid was identified in the extract (Figure 3).

Table 1. Antimutagenic effect of an aqueous extract of *B. articulata* (Lam.) "carqueja" (AEC) against 2-AF, 2-NF, and SAZ, using the Ames test with TA98 and TA100 *S. typhimurium* (His^- strains, with the classic PA and PIA assays.

	TA98 strain				TA100 strain			
Assay	S9 mix	Mutagen ($\mu g\,plate^{-1}$)	AEC (1 mg) % I^a	AEC (10 mg) % I^a	S9 mix	Mutagen ($\mu g\,plate^{-1}$)	AEC (1 mg) % I^a	AEC (10 mg) % I^a
PA	+	2-AF			+	2-AF		
		0.25	28.1 ± 2.9	67.0 ± 1.9		0.25	37.0 ± 5.8	34.0 ± 4.9
		0.50	28.0 ± 0.7	82.0 ± 0.4		0.50	52.0 ± 2.7	50.0 ± 2.7
		1.25	40.0 ± 1.2	73.0 ± 0.6		1.25	41.0 ± 2.7	56.0 ± 2.5
		2.50	32.0 ± 5.6	80.0 ± 3.0		2.50	31.0 ± 2.6	46.0 ± 2.3
PIA	+	2-AF			+	2-AF		
		0.25	41.0 ± 5.8	92.0 ± 2.1		0.25	93.0 ± 0.7	95.0 ± 0.7
		0.50	31.0 ± 1.0	89.0 ± 0.4		0.50	91.0 ± 0.7	110.0 ± 0.1
		1.25	28.0 ± 0.5	84.0 ± 0.2		1.25	99.0 ± 0.4	98.0 ± 0.3
		2.50	38.0 ± 1.9	84.0 ± 0.9		2.50	100.0 ± 0.0	99.0 ± 0.1
PA	−	2-NF			−	AZS		
		0.25	67.0 ± 0.9	74.0 ± 0.8		0.50	7.0 ± 3.1	10.0 ± 5.1
		0.50	60.0 ± 1.4	59.0 ± 1.1		2.50	1.0 ± 1.9	10.7 ± 1.7
		1.25	71.0 ± 0.3	91.0 ± 0.4		5.00	5.0 ± 2.4	26.0 ± 2.1
		2.50	64.0 ± 0.5	81.0 ± 0.7				
PIA	−	2-NF			−	AZS		
		0.25	20.0 ± 0.9	28.0 ± 0.7		0.50	5.0 ± 4.2	11.0 ± 4.1
		0.50	23.0 ± 2.6	23.0 ± 2.4		2.50	28.0 ± 2.1	26.0 ± 2.1
		1.25	26.0 ± 0.4	26.0 ± 0.4		5.00	40.0 ± 2.0	41.0 ± 2.0
		2.50	37.0 ± 4.4	37.5 ± 4.0				

Note: [a]Percentage of inhibition ± SD (estimated by the propagation of errors according to Himmelblau 1970).

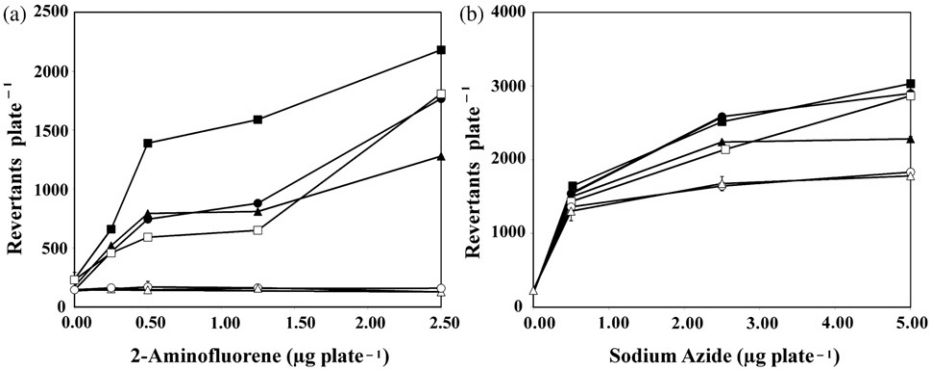

Figure 2. Antimutagenic effect of an aqueous extract of *B. articulata* (AEC) against the mutagenicity induced by 2-AF with metabolic activation (S9) (a), and SAZ without metabolic activation (b) in a TA100 *S. typhimurium* strain. The numbers of revertants per plate (*y*-axis) in each graph are plotted against the concentrations of the mutagen assayed (*x*-axis). Squares, circles, and triangles denote the number of revertants per plate induced by the mutagen alone and in the presence of 1 and 10 mg AEC per plate, respectively. Experiments were carried out using both the plate (black squares, circles, and triangles) and the pre-incubation (empty squares, circles, and triangles) assays.

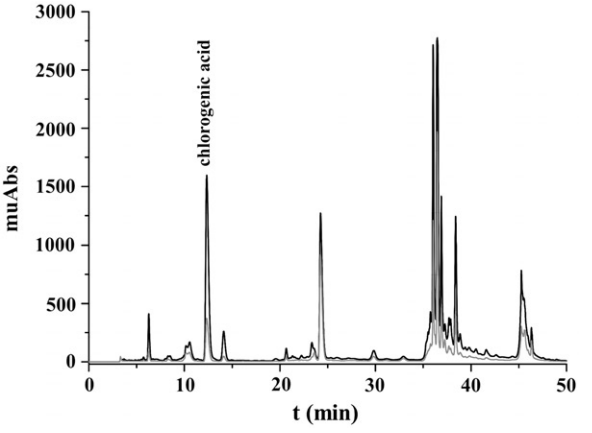

Figure 3. Chromatogram of an aqueous extract from *B. articulata* aerial parts. The run was performed under detection at wavelengths of 330 nm (continuous black line) and 360 nm (continuous gray line).

The amount of chlorogenic acid detected was 2.05 mg mL^{-1}. Taking into account the starting material, this value indicated that chlorogenic acid content was almost 2 mg per 200 mg AEC (1%). None of the other peaks observed matched the retention times or the spectrum for the remaining pure standards used. However, most of the unknown chromatographic peaks showed a UV–Vis spectrum which matched the spectrum of chlorogenic acid, suggesting that they correspond to chlorogenic acid structurally related compounds. Chlorogenic acid constituted 25% of all these compounds.

Discussion

Several researchers have previously used the Ames assay to examine the antimutagenic properties of several herbal infusions (Marnewick, Gelderblom, and Joubert 2000; Santana-Rios et al. 2001; Lee et al. 2005; Horn and Vargas 2008), and plants used in traditional folk medicine (Yen, Chen, and Peng 2001; Horn and Vargas 2003; Reid et al. 2006; Cariño-Cortés et al. 2007; Verschaeve and van Staden 2008). Recently, the antimutagenic properties of *Gochnatia cordata* and *Gregarina polymorpha* medicinal plants belonging to the Asteraceae family, taxon including *B. articulata*, have been reported (Horn and Vargas 2008). These species have been suggested to share properties ascribed to flavonoids and/or tannins present in aqueous extracts and acting as potential inactivators of enzymes involved in mutagen metabolism pathways (Horn and Vargas 2008).

Our results showed that the aqueous extract was neither toxic nor mutagenic at the concentrations assayed. Even then a borderline mutagenic effect has been previously reported at 250 and 500 mg $plate^{-1}$ concentration range for *B. articulat*a extract by PIA (Borgo, Rosa, and Vargas 2004). The high concentration used by these authors might have interfered with the assay, allowing bacterial duplication during the pre-incubation stage (Chu et al. 1981).

Since the Ames test is a reversion mutagenicity assay, the bacterial tester strain response is dependent on the point mutation which generates the histidine auxotrophy. Accordingly, the strain response is conditioned not only by the mutagenic potency of the active compound but also by its mutational specificity.

The mutagenic activity of 2-AF towards the base-pair substitution mutant TA100 (*HisG46*) could depend on the reactive electrophilic species generated by N-oxidation of the compound (Levin, Schaaped, and De Marini 1994). When TA100 strain was used as indicator, the complete inhibition of mutagenicity exerted by AEC against 2-AF may be the result of the antioxidant effect of the extract, which could be more effective under PIA conditions (Table 1). The detection of chlorogenic acid in the extract could support this explanation, since this polyphenol has been reported to have antioxidant properties (Pedreschi and Cisneros-Zevallos 2006).

It has been shown that the (−1) frameshift mutation *HisD3052* in the TA98 strain genetic background can be reverted not only by frameshift mutation but also by complex frameshift mutation (frameshift mutation plus a flanking or nearby base pair substitution) (Levin, Schaaped, and De Marini 1994; De Marini et al. 1998, 2000). The frameshift mutation depends on the planar molecular moiety of the active compound, which enables its intercalation into the DNA. The complex mutation also requires the presence of molecular side groups capable of forming adducts with DNA (mostly C-8-Guanine) (Levin, Schaaped, and De Marini 1994). This effect is induced by aromatic amines in the presence of S9 mix (Levin, Schaaped, and De Marini 1994). These findings suggest that the active metabolites which induce mutagenicity onto TA98 strain are different from those acting on TA100 strain. In fact, by contrast with the TA100 response, when TA98 is used as indicator strain, the antimutagenic effect of AEC depends on the extract concentration, and seems not to be affected by the incubation method employed (Figure 1a).

With regard to the directly acting mutagen 2-NF, the inhibitory mechanism seems to be less dependent on AEC concentration. However, the response observed shows a greater influence of the method used, it being more effective with PA than PIA. These findings could indicate that AEC antimutagenic mechanisms acting against 2-NF are different from those exerted against 2-AF. Further studies will determine the actual impact of such difference.

The protective action of AEC against SAZ mutagenicity (TA100 strain) shown to be less effective, reaching values as high as 40% mutagenicity inhibition even when the highest mutagen concentration was employed with PIA (Table 1). These results suggest that the protective mechanism of AEC against this particular mutagen might be dependent on the interference with the bacterial metabolic activation of the mutagen. In agreement with this assumption, the mutagenicity of inorganic azide (N3–) has been reported to be mediated through a metabolically synthesized organic azide, L–azidoalanine (N_3–CH_2–CH(–NH_2)–COOH) in the bacterial environment (Owais and Kleinhofs 1988).

The results of this study indicate that the antimutagenic effect of AEC could be exerted by different mechanisms which might be affected up to different extents by the mutagen assayed, the indicator bacteria, and/or the incubation method employed.

Acknowledgments

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