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Antigenotoxic, antimutagenic and ROS scavenging activities of a *Rhoeo discolor* ethanolic crude extract

M. González-Avila[a], M. Arriaga-Alba[b], M. de la Garza[a], M. del Carmen HernándezPretelín[c], M.A. Domínguez-Ortíz[d], S. Fattel-Fazenda[a], S. Villa-Treviño[a,*]

[a]*Departamento de Biología Celular, Centro de Investigación y de Estudios Avanzados del IPN, Apartado postal 14-740, México D.F. 07000, Mexico*

[b]*Laboratorio de Investigación en Microbiología, Dirección de Investigación y Enseñanza, Hospital Juárez de México, Av. Instituto Politécnico Nacional 5160, México D.F. 07760, Mexico*

[c]*Facultad de Medicina, Universidad Veracruzana, Atenas y Managua s/n Minatitlán Ver., 96780, Mexico*

[d]*Instituto de Ciencias Básicas, Universidad Veracruzana, Km 3.5 carretera federal Jalapa-Las Trancas, Jalapa Ver., 96520, Mexico*

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Abstract

Rhoeo discolor is a legendary plant used for treatment of superficial mycoses in Mexican traditional medicine. Despite its extended use, it is not known whether it has side-effects. An ethanolic crude extract from *Rhoeo discolor* was prepared, its mutagenic capacity was investigated by the Ames test, and its genotoxic activity in primary liver cell cultures using the unscheduled DNA synthesis assay. This extract was not mutagenic when tested with *Salmonella typhimurium* strains TA97, TA98 and TA100, and it did not elicit unscheduled DNA synthesis in hepatocyte cultures. In addition, we explored the antimutagenic and antigenotoxic activities of the extract and its ROS scavenger behaviour. Our results show that *Rhoeo* extract is antimutagenic for *S. typhimurium* strain TA102 pretreated with ROS-generating mutagen norfloxacin in the Ames test, and protects liver cell cultures against diethylnitrosamine induction of unscheduled DNA synthesis even at 1.9 ng per dish, which was the lowest dose tested. A free radical scavenging test was used in order to explore the antioxidant capacity of *Rhoeo* extract, as compared with three commercial well-known antioxidants quercetin, ascorbic acid and tocopherol. *Rhoeo* extract showed less radical scavenging effect than quercetin, but similar to that of α-tocopherol and more than ascorbic acid. It is important to note that this extract was neither mutagenic in *S. typhimurium* nor genotoxic in liver cell culture, even at concentrations as high as four- and 166-fold of those needed for maximal antimutagenic or chemoprotective activities, respectively.

Keywords: Antimutagenic; Antigenotoxic; *Rhoeo discolor* extract; Antioxidant; Chemoprotection

1. Introduction

Since the dawn of the human race, medicinal herbs have been used to treat diseases and this practice continues today worldwide. The plant species used medicinally are counted by the hundreds, and they are consumed without regarding to foods. Some natural products are present in fruits, beverages and vegetables, and they protect or even revert several types of human cancer and degenerative diseases (Kohlmeier et al., 1995; Yen and Chen, 1995). This is the case of *Rhoeo discolor,* an endemic plant of south-eastern Mexico commonly used to treat cancer, venereal diseases and mycoses (Martínez, 1969; Mendieta, 1981; Aguilar et al., 1994). *R. discolor* belongs to the family *Commelinaceae,* and there are practically no phytochemistry studies of this family. It is known that *R. spathacea* ethanolic extracts block the antiadrenergic action of bretylium (García et al., 1971) and that similar extracts have contraceptive effects (Weniger et al., 1982). *R. discolor* cellular tissue

Abbreviations: 2AA, 2-aminoantracene; B[a]P, benzo[*a*]pyrene; CP, cyclophosphamide; DEN, diethylnitrosamine; DMSO, dimethyl sulfoxide; DPPH, diphenyl-picryl-hydrazyl free radical scavenging method; ECE, ethanolic crude extract from *Rhoeo discolor* ; ENNG, *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine; G6P, glucose-6-phosphate; ^{3}HdThy, tritiated deoxythymidine; HU, hydroxyurea; β-NADP, β-nicotinamide-adenine dinucleotide phosphate; NF, Norfloxacin; PA, picrolonic acid; PCA, perchloric acid; SALANAL, version 1.0, Salmonella Assay Analysis.

* Corresponding author. Tel.: +52-55-5747-39-93; fax: +52-55-5747-70-81.

E-mail address: svilla@cell.cinvestav.mx (S. Villa-Treviño).

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has been broadly used as a tool for studies on cytogenetics (Frolich and Nagl, 1979), and recently we reported the use of an ethanolic crude extract from *R. discolor* (ECE) to treat superficial mycoses. These treatments, either oral or topical, were very efficient when evaluated clinically, and toxic side-effects were not found (Hernández, 1999).

Studies that analyse the medicinal capacity of natural products use crude plant extracts and subsequently the active compounds. In both cases, products are generally tested for mutagenicity using the Ames assay (Hyun et al., 1987). Non-mutagens and antimutagens that complement DNA repair systems or that have antioxidant properties (chlorophyllin, phenolic compounds, catechin, ascorbic acid and β-carotene) are frequently found in all plants (including medicinal plants). Oxygen free radicals are well known to be the major cause of some genetic damage as well as several degenerative diseases such as cancer, Parkinson's disease and heart ischemia (Chanarat, 1992; Feig et al., 1994). In order to reduce the genotoxic damage caused by exposure to free radicals due to chemical compounds, air pollutants or metabolic procedures, the use of some antimutagens found in products of normal consumption represents a good alternative (Espinosa-Aguirre et al., 1993; Abdelali et al., 1995; Constable et al., 1996; Arriaga et al., 2000). Antioxidant actitvity has been reported using the diphenyl-picryl-hydrazyl radical (DPPH) method by determination of an anti-free-radical scavenging action. This system is useful for evaluation of phytomedicinal compounds and vegetal extracts (Joyeux et al., 1995; Hirano et al., 2001; Kim et al., 2002; Son and Lewis, 2002). Liver cell cultures have been used in several assays to detect toxic, genotoxic and antigenotoxic effects of synthetic or natural products since it is known that hepatocytes are capable of converting xenobiotics (Berry et al., 1991; Nakayama, 1994).

Our results obtained from the Ames assay, the DPPH free radical scavenging method and from liver cell cultures strongly suggest that the ethanolic crude extract of *R. discolor* has antioxidant, antimutagenic and genoprotective properties, which could be eventually utilised as a potential agent in phytomedicine.

2. Materials and methods

2.1. Reagents

Norfloxacin (NF) and picrolonic acid (PA), both 100% pure drug preparations, were kindly donated by Applications Pharmaceutics, S.A. (Mexico City, Mexico). Histidine was from E. Merck Darmstadt (Frankfurt, Germany). D-biotin, thiamine hydrochloride, thymine, 2-aminoantracene (2AA), perchloric acid (PCA), *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG), β-nicotinamide-adenine dinucleotide phosphate (β-NADP), glucose-6-phosphate (G6P), dimethyl sulfoxide (DMSO), diethylnitrosamine (DEN), benzo[*a*]pyrene (B[a]P), diphenyl-picryl hydrazyne (DPPH), ascorbic acid, α-tocopherol, quercetin, hydroxyurea (HU) and cyclophosphamide (CP) were from Sigma Chemical Co. (St Louis, MO, USA). Tritiated deoxythymidine (^{3}HdThy) was from Amersham Life Sciences (Buckinghamshire, UK).

2.2. ECE preparation

The blooming plant was collected in the State of Veracruz, Mexico, and identified at the Universidad Veracruzana Herbarium. The ethanolic extract was elaborated in the same manner as the extracts that are prepared for medicinal purposes. Fresh leaves cut in fragments (100 g) were extracted with 175 ml of ethanol (96°) for 3 h at 25 °C. The extract was filtered through Whatman paper No. 4, and concentrated with a vacuum evaporator (Büchi model R 3000) at 60 °C. The ethanolic extract was evaporated at 37 °C for 12 h and maintained in a dessicator with carbonate for 12 h until it reached constant weight. The dry residue was dissolved in DMSO and its concentration expressed as amount of ECE per dry weight. In a preliminary phytochemical study of ECE, polyphenolic compounds, saturated and unsaturated hydrocarbons, carotenes, sterols and coumarinic compounds were detected (Domínguez-Ortíz, unpublished data).

2.3. Determination of free radical scavenging activity

The antioxidant effect of ECE was compared with quercetin, α-tocopherol and ascorbic acid. This effect was measured by a modified method of Joyeux et al. (1995), who evaluated the free radical scavenging potential of the compounds by electronic neutralization of DPPH. The degree of decoloration indicates the scavenging efficiency of the compound. For the assay, 100 μg, 10 μg or 1 μg of each compound were added to 3 ml of DPPH solution (20 μg/ml methanol) and incubated for 5 min at 25 °C. The absorbance was measured al 517 nm. A 100% decoloration was established using methanol–water 2:1 and the percentage of DPPH decoloration was calculated.

2.4. Detection of mutagenicity and antimutagenicity

Plate-incorporation tests and S9 mix were performed according to Maron and Ames (1983). Male Wistar rats and Aroclor 1254 (Analabs, Inc., UK) were used for induction of rat liver enzymes. S9 mixture activity was tested with ENNG and showed results comparable with those reported. *S. typhimurium* strains TA97, TA98, TA100 and TA102 were kindly provided by Dr B.N.

Ames. For preincubation, 100 μl of an 18-h culture of the tester strains were poured into screwed sterile tubes with addition of increasing amounts of ECE or appropriate quantities of different positive controls. S9 mix (500 μl) was either added or not to the tubes to a final volume of 1 ml. For positive control in TA98 and TA97 strains, PA was used in the absence of S9 mix, but in its presence, B(a)P for TA98, and 2AA for TA97. In the case of the TA100 strain, ENNG was the positive control in the absence of S9 mix and CP in its presence. Samples were incubated for 60 min at 37 °C in a shaking water-bath at 120 rpm, then 2 ml of 5% agar solution were added. Tube contents were mixed on a vortex and poured on Voguel-Bonner plates. They were incubated for 48 h at 37 °C and histidine revertants were registered in a Fisher colony counter. A positive result was considered when the tested chemical induced twice as much as the spontaneous histidine revertants. A dose–response curve was elaborated and the curve slope was evaluated with the computer software SALANAL version 1.0 (Salmonella Assay Analysis). To determine antimutagenicity of ECE, the same preincubation method was employed, using *S. Typhimurium* strain TA102 and NF as mutagen.

2.5. Unschedule DNA synthesis (UDS)

2.5.1. Genotoxicity assay in primary hepatocyte culture

2.5.1.1. Cell culture. Hepatocytes were isolated from male Wistar rats (180–200 g) by the collagenase perfusion method (Mendoza-Figueroa et al., 1979) and four replicates of 8×10^5 cells were seeded onto 35-mm culture dishes. Cells were cultured in Eagle's medium modified by Dulbecco-Vogt, supplemented with 7% bovine fetal serum and 5 μg insulin per ml, and then placed in a 37 °C humidified incubator in an atmosphere of 90% air/10% CO_2. After 2 h of incubation, detached cells were washed out, dishes refilled with fresh medium containing 10 μM HU and incubated for 1 h. The medium was removed, a serum-free medium containing 10 μM HU, 5 μCi of ^{3}HdThy, 1.25 μM DEN, and ECE from 1.9 to 250 ng were dispensed per dish. Control dishes did not receive ECE. Hepatocytes were cultured for 4 h. The maximal DEN-induced incorporation of ^{3}HdThy was obtained by a dose–response curve.

2.6. Determination of ^{3}HdThy incorporation

After incubation, hepatocytes were harvested and processed as described by Leyva and Kelley (1974), washed three times with saline solution, detached with 0.8 ml of saline solution using a rubber policeman, collected, and centrifuged for 5 min at 2500 *g*. Supernatants were discarded and pellets resuspended in 0.35 ml of Tris–HCl 0.01 M, pH 7.4 on an ice-bath, and frozen and thawed twice. The lysate was centrifuged at 2500 *g* for 10 min. Supernatant was removed and the pellet resuspended in 0.35 ml of the same buffer and 0.05 ml of albumin 1 mg/ml. After 30 min on ice, an equal volume of 0.4 M PCA was added and maintained on ice for 30 min. Tubes were centrifuged for 5 min at 2500 *g*, the supernatant was discarded and the pellet finally resuspended in 0.33 ml of 1 M PCA. Suspension was kept at 70 °C for 30 min and centrifuged 5 min at 2500 *g*. The supernatant was divided, one aliquot (0.06 ml) was taken to determine DNA concentration as described by Burton (1956), and the other 0.25-ml aliquot was placed in a vial with 3 ml of Insta-gel (Packard Instrument Co. Inc., Downers Grove, IL, USA). To account for variation in the hepatocyte number between dishes, the results were normalized as a function of DNA concentration, and then as percentage of radioactivity with respect to that incorporated in non-treated cultures.

3. Results

3.1. ECE has an antioxidant effect

The free radical scavenging activity of ECE was tested by the DPPH method. Fig. 1 shows the scavenging activity of three known antioxidant compounds and ECE. All of them were effective at 100 μg/ml, including ECE. This extract was also effective at 10 or 1 μg/ml (68 and 67%, respectively), comparatively similar to α-tocopherol (67% in both concentrations) and more

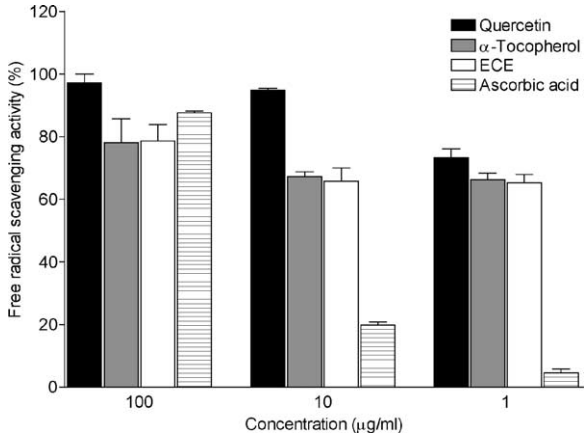

Fig. 1. Free radical scavenging activity of ECE. Free radical scavenging activity was measured by the degree of decoloration of a 20 mg/ml methanolic solution of DPPH. Three different concentrations of each antioxidant (quercetin, α-tocopherol and ascorbic acid) added to the methanolic solution of DPPH. Decoloration percentage was measured after 5 min incubation at 25 °C. Each value represents the average obtained in three different experiments performed in triplicate. Respect to control without antioxidant $P<0.002$. ECE with respect to ascorbic acid at 10 μg/ml and 1 μg/ml were $P<5.2\times10^{-5}$.

effective than ascorbic acid (20 and 9% with respect to 10 and 1 μg/ml).

3.2. ECE was not mutagenic, in fact, it was antimutagenic for S. typhimurium

When tested in the plate-incorporation method, ECE was not mutagenic to *S. typhimurium* (Table 1). This was evaluated with TA97, TA98 and TA100 strains, either alone or in the presence of S9 mix. Concentrations of 4–16 mg dry weight of ECE per plate did not increase the revertant number two-fold above that. On the contrary, in some cases ECE decreased the number of revertants. DMSO alone did not increase the number of revertants. The positive control plates with mutagens showed an increase of six- and 16-, 11- and 52-, and 16- and 32-fold the number of revertants for TA97, TA98 and TA100, without or with S9 mix, respectively.

When ECE was tested by the preincubation method in the presence of S9 mix, it did not induce revertants at all doses tested (Table 2). The number of revertants in positive control plates with mutagen were 18-, 18- and eight-fold, respectively, above those in plates of spontaneous revertion. Again, DMSO did not modify the number of revertants.

The antimutagenic effect of ECE for TA102 strain was tested by the preincubation method in the presence of S9 mix (Fig. 2). ECE (5, 10 or 20 mg dry weight per plate) was not mutagenic. NF at doses of 0.7, 7 and 70 ng per plate induced 684, 1370 and 2500 revertants and the addition of ECE either at 5, 10 or 20 mg per plate diminished *S. typhimurium* revertant number. When 70 ng NF were used, a dramatic decrease of revertants due to ECE was found (45, 60 and 74%, respectively).

3.3. Chemoprotection by ECE

UDS is used as an indicator of DNA genotoxicity. As a prelude to the genotoxic assays in hepatocytes, the sensitivity to different DEN concentrations was tested, being 1.25 μM DEN the concentration that showed the highest value of toxicity (data not shown), thus this condition was used to test ECE. DMSO showed genotoxic effect in this assay (60% in comparison with the 100% established for DEN dissolved in DMSO), that was almost completely reversed by ECE (data not shown). The results in Fig. 3 demonstrated that ECE was not genotoxic and reverted in 75% the genotoxicity induced by DEN.

4. Discussion

Traditional use of plants in alternative medicine frequently provides the basis to select which plant extract it is worth studying. Extract of *R. discolor* has been used extensively in the Gulf Coast of Mexico to treat superficial mycoses, ulcers and cancer, and as anti-inflammatory agent (Hernández, 1999). This characteristic makes it a good therapeutic prospect of study.

Until now, the ECE has been used in the context of traditional therapeutics and no toxicological tests have been published. Our purpose was to assess in vitro, with the Ames and UDS assays, the risk of using ECE in

Table 1
Salmonella typhimurium His^+ revertants obtained by the plate-incorporation method

ECE mg/plate[c]	TA97 110[a]		TA98 34[a]		TA100 251[a]	
	−S9	+S9[b]	−S9	+S9	−S9	+S9
0	102±26	131±24	29±4	31±2	241±11	381±35
4	177±9	150±12	35±5	49±6	294±27	387±6
8	148±33	149±30	34±8	37±6	312±31	304±85
12	155±19	132±29	35±3	38±11	290±30	273±25
16	79±18	128±7	39±2	37±13	245±23	324±29
20[d]	57±8	153±27	0	39±9	238±22	235±16
40[d]	nd	nd	0	0	256±26	228±1
60[d]	nd	nd	0	0	38±33	242±12
Mutagen[e]	590	2065	328	1600	3900	1246

Data represent the mean of three experiments, each experiment in triplicate.

[a] Spontaneous revertants.

[b] Samples were added with 500 μl of S9 mix.

[c] Control plates without ECE were tested with 500 μl DMSO. For the other ECE concentrations different quantity of DMSO (100 μl for 4 mg ECE; 300 μl for 8 or 12 mg ECE; 500 μl for 16 mg ECE or more).

[d] Lethal doses.

[e] Positive controls: for strains TA97 and TA98 without S9 mix, 500 μg/plate of PA were used, for the same strains but with S9 mix 10 μg/plate of 2AA and 10 μg/plate of B(a)P were used, respectively. For strain TA100, in the absence of S9 mix, 10 μg/plate ENNG were used and 500 μg/plate of cyclophosphamide when assayed with the S9 mix.

Table 2
Salmonella typhimurium His$^+$ revertants obtained by the preincubation method

ECE mg/plate	TA97[a]	Ta98[a]	TA100[a]
0	154±10	66±2	236±11
4	118±18	57±2	225±3
8	120±30	53±7	200±10
12	116±6	56±1	235±16
16	124±31	54±4	222±4
20	145±17	58±7	186±6
Mutagen[b]	1976±13	600±6	1862±7

Data represent the mean of three experiments. All samples were assayed with S9 mix, and they were tested in the presence of 20 µl DMSO.

[a] Spontaneous revertants were 110, 34 and 226 for TA97, TA98 and TA100, respectively.

[b] Positive controls: TA97 2 AA 10 µg/plate, TA98 B(a)P 10 µg/plate TA100 CF 500 µg/plate.

human treatment. Results obtained with TA97 and TA98 for frameshift mutations, and TA100 for base-pair substituents, show that ECE is not mutagenic, since even at high concentration it did not induce revertants, verified by both standard and preincubation methods. The absence of mutagenicity is not a characteristic of all natural products in use, since other medicinal plants assayed with the Ames test, with or without S9 mix, have resulted positive for mutagenicity (Hyun et al., 1987; Sohni et al., 1995; Anderson et al., 1997).

Numerous questions still remain about the precise composition of ECE and its biological function. Concerning the chemical nature of ECE, carotenes, polyphenols and coumarinic compounds have been identified (Domínguez-Ortíz, unpublished results). Some of these are antioxidants, a fact congruent with the ability of ECE to inhibit the *S. typhimurium* TA102 revertants induced by the ROS-generating norfloxacin. This antibiotic is converted to a mutagenic metabolite by a cytochrome P450-dependent reaction which leads to the formation of free radicals (Levin et al., 1984). Some mutagenic evaluations have shown that norfloxacin did not induce base pair substitutions in *S. typhimurium* TA100, or frameshift mutations in TA98. However, TA102 is susceptible to mutation due to free radicals generated by NF, which acts as direct mutagen damaging the GC base pair (Chesis et al., 1984; Levin et al., 1984; Arriaga et al., 2000).

The antioxidant effect of ECE was confirmed by our results in the free radical scavenging test using DPPH. The antioxidant ability of this extract is comparable with that of α-tocopherol and better than ascorbic acid.

The antimutagenic ability of ECE is paralleled by its antigenotoxic effect, shown here as the prevention of DEN-induced UDS in primary hepatocyte cultures. It is notable that low concentrations of ECE showed a significant protective effect against DEN or DMSO damage. This property of *R. discolor* is an attribute present in other natural products, such as extracts of *Ardisia compressa* and the carotenoids of *Capsicum* spp. (García-Gasca et al., 1998; Ramírez-Mares et al., 1999). The presence of different compounds in ECE do not rule out the possibility that the observed protective

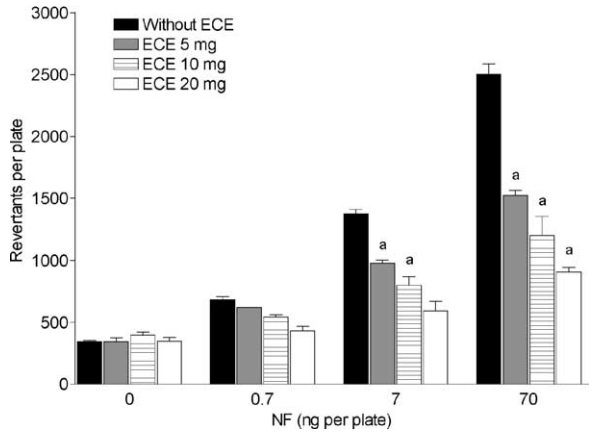

Fig. 2. Antimutagenic effect of ECE on NF-induced mutagenicity to *S. typhimurium* TA102, assayed in the preincubation test. ECE was dissolved in DMSO, mixed with an 18-h culture of *S. typhimurium* TA102 and S9 mix, preincubated for 1 h, mixed with agar, plated and incubated for 48 h. Samples without ECE were made with DMSO only. Bars represent the average±S.D. Spontaneous revertion was 324. Each value represents the average obtained in three different experiments performed with three plates per experiment. [a]Significant values ($P<0.001$). The values of NF-induced mutagenicity are significant ($P<0.001$).

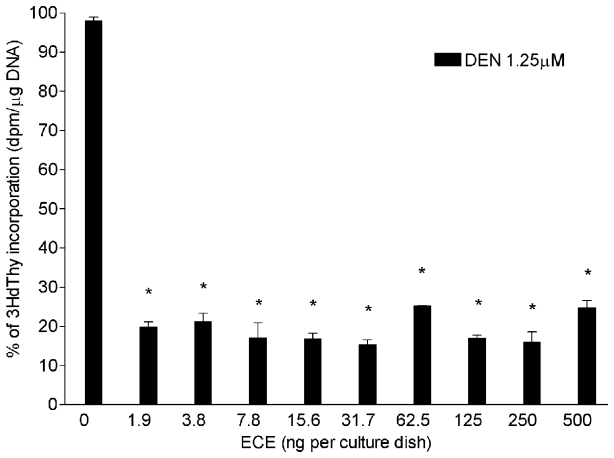

Fig. 3. Revertion by ECE of the DEN genotoxic effect. Four replicates of 8×10^5 liver cells were incubated during 2 h for adhesion, washed, preincubated 1 h with HU, rinsed and incubated with ^{3}HdThy plus HU and 1.25 µM DEN concentration. ECE was added at the concentrations indicated, after preincubation with HU. A set of samples were additionally treated with 50 µl DMSO and incubated for 4 h. After incubation, cells were thoroughly washed and PCA hydrolysates were obtained for DNA determination and ^{3}HdThy incorporation. Results are expressed as percent of dpm of ^{3}HdThy incorporated per µg of DNA, with respect to control. *Values were statistically significant from the controls ($P<0.001$).

effect corresponds to a synergic participation of several of them.

The mechanisms of the protective action of ECE are unknown. Its chemical composition allows the assumption of the expression of: (1) inhibitory polyphenols acting against mutagenic compounds as happens for ellagic acid (Sayer et al., 1982); (2) flavonoids activity that decreases DNA alterations produced by certain chemical carcinogens in human sperm and lymphocytes (Anderson et al., 1997); or (3) the inhibition of aflatoxin adduct formation by vitamins A and E, not related to its antioxidant ability (Yu et al., 1994), and the antimutagenicity through the formation of complexes with xantofiles or with their metabolites (González de Mejía et al., 1997). The antimutagenic effect on *S. typhimurium* strains and the antigenotoxic effect on liver cells strongly contrasts with the fungicide property which is also unexplored.

Chemoprevention protocols have recently been proposed as tools with immediate impact in high-risk populations for cancer (Yuspa, 2000). The source of many human chemoprotective agents, mostly polyphenolic compounds, are plants that also inhibit carcinogenesis in animals (Wattenberg, 1992). This assertion is supported by epidemiological observations of a protective effect of vegetables and fruits against the occurrence of various types of cancers (Rogers et al., 1993; Guengerich, 1995).

In summary, we show that an ethanolic extract from *R. discolor* is antimutagenic and antigenotoxic. These features make ECE a promising candidate for further studies designed to obtain more evidence on its components with potential cancer-preventive activity.

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