

## Evaluation of the Cytotoxicity, Genotoxicity, Mutagenicity, and Antimutagenicity of Propolis from Tucuman, Argentina

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This study evaluates the toxic, genotoxic/mutagenic, and antimutagenic effects of propolis extract from Amaicha del Valle, Tucumán, Argentina. The cytotoxicity assays carried out with the lethality test of *Artemia salina* revealed that the LD<sub>50</sub> was around 100 µg/mL. Propolis extracts showed no toxicity to *Salmonella typhimurium* TA98 and TA100 strains and *Allium cepa* at concentrations that have antibiotic and antioxidant activities. Otherwise, for the testing doses, neither genotoxicity nor mutagenicity was found in any sample. The propolis extracts were able to inhibit the mutagenesis of isoquinoline (IQ) and 4-nitro *o*-phenylenediamine (NPD) with ID<sub>50</sub> values of 40 and 20 µg/plate, respectively. From this result, the studied propolis may be inferred to contain some chemical compounds capable of inhibiting the mutagenicity of direct-acting and indirect-acting mutagens. A compound isolated from Amaicha del Valle propolis, 2',4'-dihydroxychalcone, showed cytotoxic activity (LC<sub>50</sub> values of 0.5 µg/mL) but was not genotoxic or mutagenic. Furthermore, this compound was able to inhibit the mutagenicity of IQ (ID<sub>50</sub> values of 1 µg/plate) but was unable to inhibit the mutagenicity of NPD. Our results suggest a potential anticarcinogenic activity of Amaicha del Valle propolis and the chalcone isolated from it.

**KEYWORDS:** Cytotoxicity; mutagenicity; antimutagenicity; propolis; 2',4'-dihydroxychalcone

## INTRODUCTION

The concept of chemoprevention, that is, the use of natural or synthetic compounds to block, reverse, or prevent the development of cancers, has great appeal. There are at least two major mechanisms for cancer chemoprevention (1, 2). One is anti-mutagenesis. It includes carcinogen uptake inhibition, carcinogen deactivation/detoxification, carcinogen–DNA bindings blocking, and DNA repair fidelity enhancement. Another mechanism is anti-proliferation/anti-progression. Examples of this are hormone/growth factor activity modulation, signal transduction modification, oncogene activity inhibition, cellular differentiation promotion, arachidonic acid metabolism modulation, apoptosis enhancement, etc.

Many kinds of agents, antioxidants, antiinflammatories, antiestrogens, and antiandrogens (1) have shown a promising chemopreventive activity. An example of a cancer chemoprevention strategy is the use of a group of natural products known as flavonoids (3). They are generally nontoxic and show a variety of biological activities (anti-allergic, anti-inflammatory, anti-oxidative, free radical scavenging, antimutagenic activities (4)).

Propolis is a complex composition resinous material collected by honeybees from various plants. It contains a wide variety of phenolic compounds, mainly flavonoids (5–11). Propolis flavonoid patterns have been attributed to the foraging plants preferred by bee colonies. Bee glue has been used as a functional food for a long time (12). It has shown a variety of biological effects such as antiviral activity against human immunodeficiency virus (13), herpes simplex type 1 virus, herpes simplex type 2 virus, adenovirus type 2, vesicular stomatitis virus, and poliovirus type 2 (14). It has also had antimicrobial activity against many Gram positive and Gram negative bacteria, yeast, and fungi associated with varying degrees of pathogenicity in man (15, 16). Anticancer (17), antiinflammatory (18), antioxidative (19–24), anaesthetic, and cytostatic (25) pharmacological properties have been ascribed to the ethanolic extracts of propolis. The biological activities of propolis have been studied extensively in Europe, but only a few reports can be found in Argentina. Analysis of Argentine propolis alcoholic extracts showed evidence of antibacterial and free radical-scavenging activities in addition to a protective action against copper-mediated oxidative modification of lipids (11, 16, 22, 24). We recently demonstrated that the major chemical component responsible for the antibacterial effect against Gram negative bacteria would be 2',4'-dihydroxychalcone (not published).

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In this work, a battery of short-term assays (*Artemia salina* test, Ames test, and *Allium cepa* test) was used to evaluate the cytotoxicity and genotoxicity of different concentrations of propolis ethanolic extracts and antibacterial compound isolates from Argentine propolis extracts. They were also tested for their antimutagenic capacity against the effect of a model mutagen.

## MATERIALS AND METHODS

**Reagents.** All reagents used were of analytical grade; 2',4'-dihydroxychalcone was purchased from Indofine Chemical Co.

**Propolis Origin.** Crude propolis (bee glue) was obtained from colonies of *Apis mellifera*, hand gathered in March 1995 and March 2003 from an apiary located at 2000 m above sea level in Amaicha del Valle, Tucumán, Argentina.

**Separation of Propolis Components.** *Ethanolic Propolis Extracts.* Propolis was frozen at  $-20^{\circ}\text{C}$  and ground in a chilled mortar. It was then extracted with ethanol (15 mL of 80% ethanol/g of propolis) with stirring for 24 h at room temperature. The suspension was decanted by centrifugation at 27 000g for 20 min. The mixture was then frozen at  $-20^{\circ}\text{C}$  for 2 h and centrifuged at 27 000g for 20 min to eliminate ethanol insoluble substances. The supernatant was concentrated in a rotary evaporator under reduced pressure at  $40^{\circ}\text{C}$  until reaching constant weight, redissolved in 96% ethanol, filtered through Whatman N° 4 filter paper, and kept at room temperature in the dark until use.

The preparation obtained was named partially purified propolis extract (PPE).

**Thin-Layer and Preparative Chromatography.** The thin-layer chromatography (TLC) was performed using precoated silica gel plates with a fluorescent indicator as the stationary phase (Kieselgel 60 F254, 0.2 mm, Merck) and toluene:chloroform:acetone (4.5: 2.5: 3.5; v/v) as the mobile phase. Separated components were visualized under ultraviolet light (254 and 360 nm, UV lamp model UV 5L-58 Mineralight Lamp) and sprayed with natural product reagent (1% methanolic 2-aminoethyl diphenylborate) or aluminum chloride for phenolic compounds (26), methanolic potassium hydroxide for coumarins, Dragendorff's reagent for alkaloids, and anisaldehyde/sulfuric acid for steroid and terpenes (27). Bioautographic assays were used to locate the antibacterial compounds (16). Silica gel H plates were used for preparative chromatography (20 × 20 cm, 1 mm thick). PPE were loaded (500  $\mu\text{L}$  per line) and developed in the same solvent system as before. Phenolic compounds fractions were visualized by UV irradiation at 360 nm, and the antibacterial compounds were scraped from the air-dried plates and eluted into spectral grade ethanol. The slurry was centrifuged (5 min at 5000g), and the supernatant was evaporated to dryness. The dried residues of active components were used for further analysis. HPLC was used to identify 2',4'-dihydroxychalcone by comparison with commercially available 2',4'-dihydroxychalcone. UV-visible absorption spectra (200–600 nm) were obtained using a Beckman DU 650 spectrophotometer (28), while NMR spectra were recorded in a Bruker AC 200 (200 MHz) spectrometer in DMSO at room temperature.

**Analysis of Propolis Flavonoids and Phenolic Compounds by Spectrometric Methods.** Total phenol content was quantitatively determined by the method of Folin–Ciocalteu phenol reagent (29). Results were expressed as micrograms of quercetin.

Total flavonoid content was quantitatively analyzed by two complementary colorimetric methods (30). Quercetine was used as a standard for the calculation of flavones and flavonols concentration, and naringenin for the calculation of flavanones concentration in PPE. HPLC was used to quantify 2',4'-dihydroxychalcone.

**Microwell Cell Cytotoxicity Assay Using *Artemia salina* (Brine Shrimp).** Brine shrimp eggs were hatched in a shallow rectangular dish filled with artificial seawater. After 24 h, the phototropic nauplii were collected. A microwell cytotoxicity assay was carried out according to Solis et al. (31). A propolis ethanolic extract or 2',4'-dihydroxychalcone was evaporated and then dissolved in 50  $\mu\text{L}$  of DMSO prior to adding seawater. Serial dilutions were made in the wells of microplates in triplicate in 100  $\mu\text{L}$  of seawater. A suspension of nauplii containing 10–15 organisms (100  $\mu\text{L}$ ) was added to each well, and the covered plate was incubated at  $22\text{--}29^{\circ}\text{C}$  for 24 h. Plates were then examined

under a microscope, and the number of dead (non motile) nauplii in each well was counted. 100  $\mu\text{L}$  of methanol was added to each well, and after 15 min the total number of shrimp in each well was counted.  $\text{LC}_{50}$  values were then calculated by Probit analysis (32). A reference test with caffeic acid phenethyl ester (CAPE) was regularly performed for the control of the sensitivity of the test population. Negative controls (DMSO) were made.

Cellular viability in the presence and the absence of experimental agents was determined using 3-(4,5-dimethyl-2-yl)-2,5-dimethyltetrazolium bromide (MTT). After a solution of MTT was added for 4 h, the amount of formazan formed was measured spectrophotometrically at 590 nm (33).

**Ames Test.** *Toxicity Assay.* To examine the toxic effects on *Salmonella typhimurium* strains TA98 and TA100, a diluted propolis extract (0.003–300  $\mu\text{g}$  of soluble principle/plate) or 2',4'-dihydroxychalcone (0.003–3  $\mu\text{g}$ /plate) were added to overnight-cultured *Salmonella typhimurium* strains TA98 or TA100 (0.1 mL) and S9 mix (0.5 mL) or 0.1 M phosphate buffer, pH 7 (0.5 mL), instead of S9 mix. The mixture was preincubated at  $37^{\circ}\text{C}$  for 5 min before it was diluted with phosphate buffer, and the mixture was then poured onto nutrient agar plates. The plates were incubated at  $37^{\circ}\text{C}$  for 2 days, and the number of colonies was counted (34). The propolis extracts and 2',4'-dihydroxychalcone were then tested for their mutagenic/antimutagenic potency exclusively in the nontoxic concentration range.

**Mutagenicity Assay.** The mutagenic effects of propolis extract and antimicrobial compound were assayed according to the Ames test using *Salmonella typhimurium* strains TA98 and TA100 (35) with and without metabolic activation (S9 mix fraction). The tested strains were cultured overnight in Oxoid Nutrient Broth for 12 h. Different concentrations of propolis extract (0.003–120  $\mu\text{g}$ /plate) or 2',4'-dihydroxychalcone (0.003–3  $\mu\text{g}$ /plate) were added to 2 mL of top agar and 0.1 mL of bacterial culture and then poured onto a plate containing minimum agar. The plates were incubated at  $37^{\circ}\text{C}$  for 48 h, and the  $\text{His}^+$  revertant colonies were manually counted. The influence of metabolic activation was tested by adding 500  $\mu\text{L}$  of S9 mixture prepared with S9 fraction obtained from liver of Sprague–Dawley rats pretreated with a polychlorinated biphenyl mixture (Araclor 1254).

All experiments were analyzed in triplicate with at least two replicates. A sample was considered to be mutagenic when the number of revertant colonies was at least twice the negative control yield ( $\text{MUI} \geq 2$ ) and showed a significant response in analysis of variance. The mutagens used as positive controls were 4-nitro *o*-phenylenediamine (NPD, 5  $\mu\text{g}$ /plate), which is a direct-acting mutagen, and isoquinoline (IQ, 0.1  $\mu\text{g}$ /plate for TA98 and 0.5  $\mu\text{g}$ /plate for TA100), which required S9 mix for metabolic activation.

**Antimutagenicity Test.** The antimutagenic effects of propolis extract and 2',4'-dihydroxychalcone were assayed using the Ames *Salmonella*/mammalian microsome mutagenicity test, but the mutagen was added before preincubation (35). The mutagen (0.1 mL) was added to the mixture of propolis extracts and bacterial culture (0.1 mL) with S9 mix (0.5 mL) for IQ or with phosphate buffer (0.1 M, pH 7.4) for NPD. The mutagenicity of each mutagen in the absence of propolis extract or chalcone is defined as 100%. The number of  $\text{his}^+$  revertants (after subtracting the spontaneous reversions) induced by direct and indirect mutagens tested without any extract or chalcone was considered as 100%. Dose–response curves were constructed from measurements with six different doses of assayed natural product.  $\text{ID}_{50}$  values, the dose of a given compound causing 50% inhibition of the mutagenicity, are means from three independent series.

**Plant Genotoxicity Test (*Allium cepa* Test).** For the *Allium* root anaphase aberration assay (AL-RAA) (36–38) and the *Allium* root-micronuclei assay (AL Root-MCN) (37), equal sized young bulbs of common *Allium cepa* were used. Onions bulbs were kept in distilled water for 48 h and then exposed to propolis extract or 2',4'-dihydroxychalcone for 24 h. A fraction of root sample was immediately fixed in ethanol–acetic acid, while another was left in tap water for another 24 h (recovery time). The roots were then fixed in 1:3 acetic acid–ethanol solution for 24 h, and finally stored in 70% ethanol. Other onion bulbs were kept directly in propolis samples for 72 h. The roots growing in tap water were used as a negative control, while the

treatment with  $1 \times 10^{-4}$  M of maleic hydrazine (MH) represented a positive control.

Macroscopic and microscopic parameters were considered. Length of roots as index of toxicity and modifications in root consistency and root shape (formation of tumors, hook roots, twisted roots) were observed as macroscopic parameters. Microscopic parameters were mitotic index (1000 cells per slide) to evaluate cellular division rate, anaphasic aberrations (bridges, laggard chromosomes, and fragments; 800 anaphasic cells per sample), and micronuclei formation (five slides, 1000 cells per slide), as indicators of DNA damage. Analysis of variance and Dunnett's test were performed for data analysis.

**Chromosome Preparation and Staining.** Root tips were hydrolyzed in 1 M HCl at 60 °C for 10 min before staining in Schiff's reagent for 15 min. After the root caps were removed from well stained root tips, 1 mm of the meristematic or mitotic zones was immersed in a drop of 45% acetic acid on a clean slide and squashed into single cells using the eraser end of a pencil to apply pressure. The staining of the chromosomes was carried out with 2% carmine in 45% acetic acid.

## RESULTS AND DISCUSSION

Propolis contains a wide variety of phenolic compounds, mainly flavonoids. The flavonoid content of propolis is attributed to the different preferred plants collected by honeybees. These substances and other phenolic compounds have been suggested to play a preventive role in the development of cancer and heart disease. Recently, we determined by RPHPLC that Amaicha del Valle propolis contained pinobanksin, quercetin, kaempferol, apigenin, pinocembrin, chrysin, galangin, kaempferide, tectochrysin, 1,1-dimethylallylcaffeic acid, ferulic acid, and cinnamic acid. Previous works on biological activity of Amaicha del Valle propolis showed the presence of free radical-scavenging activities and a protective action against copper-mediated oxidative modification of lipids (22, 24) correlated with total flavonoid content. Otherwise, this propolis showed antibacterial activity against antibiotic resistant human pathogenic bacteria (16) and Gram negative phytopathogenic bacteria.

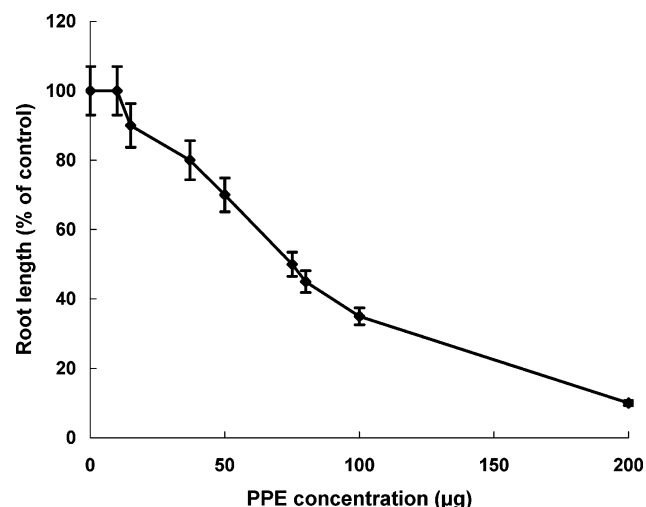
We conducted bioactivity-guided (bioassay in situ) separation of bactericidal compound(s) from propolis extract. The results of bioassay showed that the biggest zone of bactericidal activity against *Staphylococcus aureus* methicillin-resistant and *Pseudomonas aeruginosa* was around 2',4'-dihydroxychalcone, which was confirmed with purchased 2',4'-dihydroxychalcone. We then used preparative chromatography to isolate 2',4'-dihydroxychalcone (13 mg/g of propolis). Spectral features ( $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR) shown by the isolated compound were similar to those previously reported in the literature (39, 40) and agreed with those of a purchased 2',4'-dihydroxychalcone. The flavone and flavonol, flavonone, and polyphenolic compounds content of Argentine propolis extract were 178.5, 111.3, and 720 mg/g, respectively.

In this work, the propolis extract and 2',4'-dihydroxychalcone were assayed to determine the cytotoxic, genotoxic, and mutagenic/antimutagenic activity. With the concept that there are carcinogens that in a metabolically activated system attack the DNA, in contrast with other agents that act by promoting and enhancing processes through totally distinct mechanisms, we used the test for DNA reactivity, in a prokaryotic organism (*S. typhimurium*) and in a eukaryotic system (*Allium cepa*).

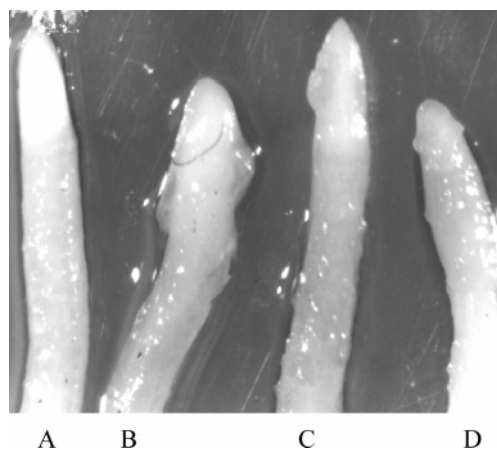
**Artemia salina Test.** The cytotoxicity assays were carried out with the lethality test of *Artemia salina*. The  $\text{LC}_{50}$  of the propolis extract was around 200-fold higher than that of some biologically active compounds isolated from other propolis, such as CAPE, a standard substance with well-known cytotoxicity (41). Otherwise, 2',4'-dihydroxychalcone showed potent cytotoxic activity, with  $\text{LC}_{50}$  values less than 10  $\mu\text{g/mL}$ . The

**Table 1.** Toxicity Assay of Propolis Extracts and Isolated Compounds on Nauplius Larvae of *Artemia salina*

drugs	brine shrimps $\text{LC}_{50}$ ( $\mu\text{g/mL}$ )
CAPE	0.45
propolis	100
2',4'-dihydroxychalcone	3.5



**Figure 1.** Results from *Allium cepa* root inhibition test. Propolis concentration is plotted against the root length as percent of control.

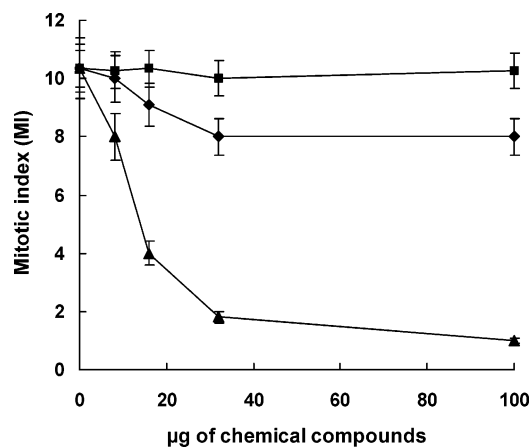


**Figure 2.** *Allium cepa* roots. (A) Negative control. Treated root with propolis: (B) 300  $\mu\text{g}$  of PPE, (C) 100  $\mu\text{g}$  of PPE, (D) 50  $\mu\text{g}$  of PPE.

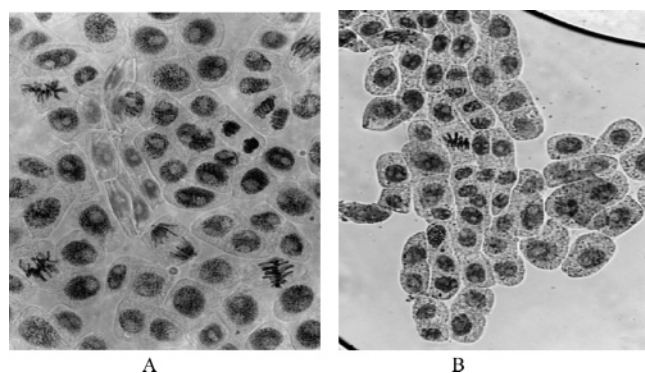
midpoint cytotoxicity value was confirmed by colorimetric tests using MTT (Table 1).

**Allium cepa Test.** According to our results, Amaicha del Valle propolis does not have phytotoxic effects on onions in the concentration range that shows antibacterial and antioxidant activities. Propolis extracts were found to be toxic, causing an inhibition on root growth, at concentrations higher than 50  $\mu\text{g/mL}$ . This effect was dose-dependent with an  $\text{LC}_{50}$  value of 75  $\mu\text{g}$  of soluble principle/mL (Figure 1). Higher concentrations (300  $\mu\text{g}$  of soluble principle/mL) of propolis extracts induced root degeneration (Figure 2), such as modifications in root consistency and root form. The mitotic index (MI) is a parameter that estimates the frequency of cellular division. The analysis of the effect of propolis extracts shows how a decrease of the MI in the exposed roots is dependent on the concentration (Figure 3). At 100  $\mu\text{g/mL}$ , few cells are in prophase, metaphase, anaphase, and telophase. Representative micrographs of control and treated cells are shown in Figure 4.





**Figure 3.** Effect of different concentrations of maleic hydrazine, MH ( $\blacktriangle$ ), and propolis extracts ( $\blacklozenge$ ) on the mitotic index, MI, in root tips of *A. cepa* L. The negative control ( $\blacksquare$ ) was DMSO.



**Figure 4.** Representative light micrographs of cell division in *A. cepa* comparing controls with treated cells with propolis extracts (magnification  $\times 400$ ).

One of the purposes of the toxicity test was to determine the concentration values for the genotoxicity/mutagenicity assays. The  $LC_{50}$  value was chosen as the highest concentration for the genotoxicity test. Propolis extracts were not able to induce chromosomal damage. On the other hand, the positive control (MH) showed high toxicity, with MI below 4 for the highest concentration (100  $\mu\text{g/mL}$ ) and changes in the organization and morphology of the chromosomes (anaphasic bridges accompanied by chromosomal rupture, isolated chromosomes, and micronuclei) in the root tips exposed to the herbicide.

The antibiotic compound isolated from Amaicha del Valle propolis was not toxic nor genotoxic for *Allium cepa* in the

**Table 3.** Mutagenicity of Propolis Extract and 2',4'-Dihydroxychalcone toward *S. typhimurium* TA98 in the Presence and the Absence of S9 Mixture

dose ( $\mu\text{g/plate}$ )	mutagenicity relation: His <sup>+</sup> revertant per plate <sup>a</sup> /His <sup>+</sup> spontaneous revertant <sup>b</sup>			
	propolis extract		2',4'-dihydroxychalcone	
	–S9	+S9	–S9	+S9
120	0.82 $\pm$ 0.02	0.92 $\pm$ 0.02		
60	0.90 $\pm$ 0.04	0.90 $\pm$ 0.03		
30	0.87 $\pm$ 0.04	0.78 $\pm$ 0.02		
15	1.04 $\pm$ 0.03	0.80 $\pm$ 0.04		
7.5	1.27 $\pm$ 0.01	1.07 $\pm$ 0.05	1.08 $\pm$ 0.03	1.08 $\pm$ 0.10
1.5	1.13 $\pm$ 0.04	1.00 $\pm$ 0.10	1.05 $\pm$ 0.05	1.20 $\pm$ 0.05
0.75	1.07 $\pm$ 0.04	0.97 $\pm$ 0.05	1.10 $\pm$ 0.04	1.20 $\pm$ 0.05
0.3			1.27 $\pm$ 0.05	1.23 $\pm$ 0.10
0.03			1.20 $\pm$ 0.05	0.88 $\pm$ 0.10
0.003			0.97 $\pm$ 0.05	0.95 $\pm$ 0.10

<sup>a</sup> Propolis extracts or 2',4'-dihydroxychalcone and TA98 were preincubated at 37 °C for 20 min with or without S9 mix. Data are means  $\pm$  SD of three plates.

<sup>b</sup> The number of spontaneous revertants was determined in assays without propolis extract or 2',4'-dihydroxychalcone. The number of spontaneous revertants obtained was  $41 \pm 5$ . NPD and IQ were used as positive control. Revertants induced by IQ (0.1  $\mu\text{g/plate}$ ) and NPD (5  $\mu\text{g/plate}$ ) were  $2540 \pm 30$  and  $2343 \pm 20$ , respectively.

same values that were bioactive on Gram negative and Gram positive bacteria.

***S. typhimurium* Test.** The propolis extract exhibited non-toxicity on both *S. typhimurium* TA98 and TA100 strains at a dose 300  $\mu\text{g/plate}$  with and without S9 mix (Table 2). Otherwise, for the testing doses, no mutagenicity was found in any samples (Tables 3 and 4; Figure 5).

Most food-derived carcinogens are heterocyclic amines and are classified in three types: the indole type such as 3-amino-1-methyl-5H-pyrido[4,3-b]indole (Trp-P-2), the quinoline type such as 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) used here, and the pyridine type such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). All three types are activated via metabolism by the CYP1A family enzymes, 1A1 and 1A2. Indeed, propolis extract has been found to be antimutagenic against IQ with  $ID_{50}$  values of 40  $\mu\text{g/plate}$ . It has been described that liver expressed both CYP1A1 and 1A2, while other tissues such as pancreas, thymus, prostate, small intestine, and colon expressed mainly 1A1 (42). Propolis extract was able to inhibit the mutagenesis NPD with  $ID_{50}$  values of 20  $\mu\text{g/plate}$ . From these results, the propolis studied may be inferred to contain chemical compounds capable of inhibiting the mutagenicity of direct-acting and indirect-acting mutagens. The mechanism by which the antimutagens in the propolis extracts inhibited the

**Table 2.** Toxicity of Propolis Extract and 2',4'-Dihydroxychalcone toward *S. typhimurium* TA100 and TA98

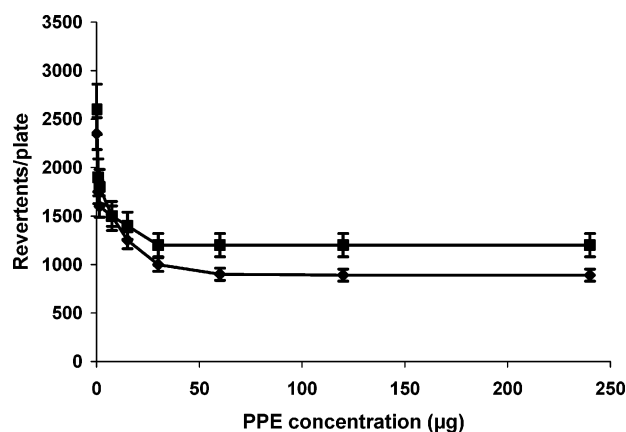
dose ( $\mu\text{g/plate}$ )	toxicity							
	TA98				TA100 (with or without S9)			
	propolis extract		2',4'-dihydroxychalcone		propolis extract		2',4'-dihydroxychalcone	
	colony number (mean $\pm$ SD)				colony number (mean $\pm$ SD)			
	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9
300	922 $\pm$ 50	950 $\pm$ 30			2251 $\pm$ 30	2250 $\pm$ 30		
30	1090 $\pm$ 50	1050 $\pm$ 50	990 $\pm$ 60	1000 $\pm$ 50	2060 $\pm$ 10	2160 $\pm$ 50		
3	1098 $\pm$ 30	1063 $\pm$ 50	1090 $\pm$ 40	1050 $\pm$ 50	2385 $\pm$ 50	2300 $\pm$ 50	2047 $\pm$ 30	2100 $\pm$ 100
0.3	1110 $\pm$ 20	1080 $\pm$ 10	1090 $\pm$ 40	1000 $\pm$ 50	2380 $\pm$ 50	2250 $\pm$ 50	2289 $\pm$ 40	2300 $\pm$ 50
0.03	1100 $\pm$ 10	1000 $\pm$ 50	1140 $\pm$ 10	1000 $\pm$ 50	2384 $\pm$ 40	2300 $\pm$ 50	2289 $\pm$ 30	2370 $\pm$ 30
0.003	1100 $\pm$ 10	1090 $\pm$ 20	1100 $\pm$ 20	1000 $\pm$ 50	2380 $\pm$ 30	2370 $\pm$ 30	2289 $\pm$ 30	2330 $\pm$ 30
negative control	1100 $\pm$ 30	1090 $\pm$ 30	1110 $\pm$ 20	1100 $\pm$ 20	2391 $\pm$ 20	2370 $\pm$ 40	2461 $\pm$ 20	2400 $\pm$ 50

**Table 4.** Mutagenicity of Propolis Extract and 2',4'-Dihydroxychalcone toward *S. typhimurium* TA100 in the Presence and the Absence of S9 Mixture

dose ( $\mu\text{g}/\text{plate}$ )	mutagenicity relation: His <sup>+</sup> revertant per plate <sup>a</sup> /His <sup>+</sup> spontaneous revertant <sup>b</sup>			
	propolis extract		2',4'-dihydroxychalcone	
	–S9	+S9	–S9	+S9
120	0.73 $\pm$ 0.05	0.92 $\pm$ 0.02		
60	0.91 $\pm$ 0.05	0.90 $\pm$ 0.03		
30	0.96 $\pm$ 0.04	0.78 $\pm$ 0.02		
15	0.99 $\pm$ 0.04	1.00 $\pm$ 0.07		
7.5	1.07 $\pm$ 0.03	0.91 $\pm$ 0.05	0.94 $\pm$ 0.04	0.70 $\pm$ 0.05
1.5	1.23 $\pm$ 0.05	1.20 $\pm$ 0.05	0.94 $\pm$ 0.03	0.77 $\pm$ 0.05
0.75	1.23 $\pm$ 0.05	1.35 $\pm$ 0.05	0.84 $\pm$ 0.04	0.87 $\pm$ 0.04
0.3			0.80 $\pm$ 0.05	0.69 $\pm$ 0.10
0.03			0.97 $\pm$ 0.03	1.09 $\pm$ 0.10
0.003			1.12 $\pm$ 0.10	1.04 $\pm$ 0.10

<sup>a</sup> Propolis extracts or 2',4'-dihydroxychalcone and TA98 were preincubated at 37 °C for 20 min with or without S9 mix. Data are means  $\pm$  SD of three plates.

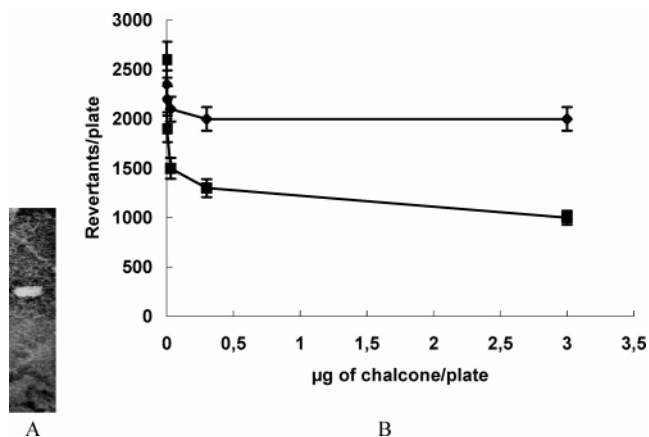
<sup>b</sup> The number of spontaneous revertants was determined in assays without propolis extract or 2',4'-dihydroxychalcone. The number of spontaneous revertants obtained was 41  $\pm$  5. NPD and IQ were used as positive control. Revertants induced by IQ (0.5  $\mu\text{g}/\text{plate}$ ) and NPD (5  $\mu\text{g}/\text{plate}$ ) were 2980  $\pm$  50 and 2343  $\pm$  20, respectively.

**Figure 5.** Effect of propolis extract on the mutagenicities of IQ in the presence of S9 mix (■) and NPD in the absence of S9 (◆) toward *S. typhimurium* TA98. Each point represents the average of three plates; revertants induced by IQ (0.1  $\mu\text{g}/\text{plate}$ ) and NPD (5  $\mu\text{g}/\text{plate}$ ) were 2540  $\pm$  30 and 2343  $\pm$  20, respectively.

mutagenicities of IQ is not known, but it is suggested that these antimutagens may interact with some specific enzymes in the liver homogenates, which are necessary for the activation of chemical mutagens.

The mutagenicity of IQ in *S. typhimurium* TA98 and TA100 is inhibited by chalcone with ID<sub>50</sub> values of 1  $\mu\text{g}/\text{plate}$ . This compound was unable to inhibit the mutagenicities of NPD (Figure 6). Based in our results, the antimutagenic effect of Amaicha del Valle propolis on NPD may be attributed to another chemical compound.

**Conclusions.** De Flora et al. propose a detailed classification of endpoints by which it is possible to prevent mutation and cancer (43). The points including inhibition of genotoxic effects, antioxidant activity, inhibition of cell proliferation, induction of cell differentiation, and interference with signal transduction pathways may lead to protection at more than one point in the pathway. According to our results, the antimutagenic effect of the propolis extract could be related, at least in part, to its chalcone content. Although the antimutagenic mechanism was not well established during this study, the propolis extract was

**Figure 6.** (A) Antimicrobial activity of 2',4'-dihydroxychalcone against *Staphylococcus aureus* by bioautographic assay. (B) Inhibition by chalcone of mutagenic activities induced by IQ (■) and NPD (◆) in *S. typhimurium* TA98. Each point represents the average of three plates; revertants induced by IQ (0.1  $\mu\text{g}/\text{plate}$ ) and NPD (5  $\mu\text{g}/\text{plate}$ ) were 2540 and 2343, respectively.

seen to inhibit the direct and indirect mutagenic action and was not shown to be mutagenic or clastogenic in different assays. This suggests that the interference of the propolis extract must occur with the active group of genotoxic compounds tested and with the metabolic enzymes. Because the Argentine propolis extract is antioxidant, free radical scavenging (11, 22, 24), and antimutagenic, it can be used as a primary chemopreventive agent inhibiting mutation and cancer initiation by triggering protective mechanisms such as inhibition of genotoxic effects and maintaining DNA structure (44–46).

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