JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Topoisomerase I and II Enzyme Inhibitory Aqueous Extract of *Ardisia compressa* and Ardisin Protect against Benomyl Oxidation of Hepatocytes

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Tea preparations of *Ardisia compressa* (AC) have been used in folk medicine against liver disorders. The objective of this study was to evaluate the in vitro topoisomerase I and II enzyme inhibition and the antioxidant effect of an aqueous extract from dry leaves of AC and a pure component (ardisin) purified from AC on benomyl (Be)-induced cytotoxicity in primary culture rat hepatocytes. Lipid peroxidation (malondialdehyde), antioxidant enzyme activities of glutathione reductase, glutathione peroxidase, and superoxide dismutase, and glutathione levels were studied. Topoisomerase I and II enzyme inhibition was used to guide purification of ardisin, which was purified using TLC, MPLC, and preparative and analytical HPLC methods. Benomyl increased malondialdehyde (58% change in comparison to the control) and glutathione peroxidase (10%), producing a significant consumption of endogenous antioxidant glutathione (65%, $P < 0.05$). A 94% hepatocyte protection was observed when cells were first exposed to ardisin (0.27 μg/mL), followed by Be (35 μg/mL). Cell protection by the tea extract of AC (AE) was greater than that by (−)-epigallocatechin 3-gallate (EGCG). Ardisin showed a clear inhibition of topoisomerases I and II catalytic activity in *Saccharomyces cerevisiae* mutant cells JN 394, JN394t$_{-1}$, and JN394t-$_{2-5}$. The potency of ardisin was superior to that of AE and EGCG as an antioxidant, protecting rat hepatocytes when exposed to Be. On the basis of the effective concentrations of equivalents to {+}catechin found in the present study, it can be estimated that, in order to gain antioxidative protection, a person would need to ingest approximately 1 L of AC tea per day, with a total content of 10.8 g of plant material.

KEYWORDS: ***Ardisia compressa*; ardisin; epigallocatechin gallate; benomyl; oxidative stress; antioxidant protection**

INTRODUCTION

Several reports have attributed to green and black tea chemopreventive and therapeutic properties (*1*, *2*). Tea contains several antioxidants, including polyphenols of the catechin (green tea) and theaflavin (black tea) groups (*3*). Green tea extract may improve muscle health by reducing or delaying necrosis in mice by an antioxidant mechanism (*4*). The protective effects of black tea against CCl_4-induced lipid peroxidation in liver, kidneys, and testes were considered due to, at least in part, its ability to scavenge CCl_4-associated free radicals (*5*). Antioxidants such as quercetin and tea polyphenols are proposed as inhibitors against mitochondrial ROS production (*6*). On the other hand, one study suggests that, in humans, polyphenolic antioxidants derived from green or black tea do not inhibit in vivo lipid peroxidation (*7*).

Benomyl, a benzimidazole fungicide, is metabolized via butylisocyanate, *S*-(*N*-butylcarbamoyl)glutathione, and *S*-(*N*-butylcarbamoyl)cysteine to *S*-methyl *N*-butylthiocarbamate (MBT). Benomyl acts as a mitochondrial aldehyde dehydrogenase inhibitor to ip-treated mice (*8*). Banks and Soliman (*9*) found that benomyl induces lipid peroxidation and glutathione depletion when fed to rats. The in vivo toxicity of benomyl may be associated with oxidative stress to cellular membranes, and some degree of protection against this toxicity could be afforded by antioxidants. A reduction in the activities of the hepatic microsomal mixed-function oxidases was noted 24 h following oral

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10.1021/jf0258757 CCC: $22.00 © 2002 American Chemical Society
Published on Web 11/15/2002

benomyl administration at a dose of 500 mg/kg (*10*). Benomyl was also responsible for a time- and dose-dependent microtubular disorganization in rat primary hepatocyte cultures (*11*). The effect was reversible, after benomyl was removed, and benomyl acted as a potent glutathione-depleting agent (*11*). Benomyl caused morphological damage to the rat organs, especially the liver, resulting in an increase in several serum enzymes (*12*).

Ardisia compressa is a plant of the Myrsinaceae family that grows on the Pacific coast of Mexico. The leaf extract of this plant, in the form of herbal tea, has been used effectively in folk medicine against liver disorders and other disease conditions (*13*). The aqueous extract of *A. compressa* dry leaves contained phenolic constituents, mainly flavan-3-ol monomers such as catechin, which were found to provide protection against 1-nitropyrene-induced cytotoxicity (*14*).

The main objective of this study was to evaluate the protective effect of an aqueous extract (AE) and of the pure compound, ardisin, isolated from the dry leaves of *A. compressa* on the benomyl-induced toxicity in rat hepatocytes. Malondialdehyde (MDA) and glutathione (GSH) concentrations and antioxidant defense activities (superoxide dismutase, SOD; glutathione peroxidase, GPx; and glutathione reductase, GR) were measured to determine potential alterations of antioxidant status in rat hepatocytes exposed to benomyl alone, simultaneously pretreated, and posttreated with ardisin, AE, or (−)-epigallocatechin 3-gallate (EGCG) as a control.

MATERIALS AND METHODS

Biological Materials and Reagents. The cell culture medium was obtained from Gibco BRL Life Technologies (Grand Island, NY). Twenty-four well tissue culture plates were purchased from Corning Glass Works (Corning, NY). Dishes for cell culture were obtained from Nunc (Denmark). Collagenase type IV (clotridiopeptidase A; EC 3.4.24.3) from *Clostridium histolyticum*, with a collagen digestion activity of 426 units/mg, insulin, (−)-epigallocatechin 3-gallate (minimum 80% purity), (+)-catechin, Janus green B (69% purity), benomyl (Be), penicillin, and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO, HPLC grade and 99.9% purity) came from Omni Solv (Lyndhurst, NJ).

Preparation of *A. compressa* Extract. Fresh leaves of *A. compressa* collected on the Pacific coast of Mexico (Michoacan State) were first air-dried without exposure to sunlight, kept in large plastic bags, and stored in a cool and dry place. The dry material (500 g) was then added to deionized boiling water (2000 mL × 1, 1500 mL × 2, and 1000 mL × 1) and allowed to stand for 10 min. The mixture was cooled to room temperature and filtered (0.45-μm nylon filter). The resulting filtrate was freeze-dried using an FTS System, Inc. tray lyophilizer. The dried and powdered crude extract (93.37 g) was kept at −20 °C and protected from light and moisture in a glass container.

Partial Purification of *Ardisia compressa* Components. Purification of the lyophilized crude extract was carried out using the topoisomerase I and II enzyme inhibitory assay-guided chromatographic methods. The crude extract (20 g) was stirred for 30 min with MeOH (3 × 200 mL) at room temperature and then centrifuged, and the solvent was removed under reduced pressure. The insoluble residue was kept aside. The fraction resulting, after the removal of MeOH (2 g), was further purified using C-18 medium-pressure liquid chromatography (MPLC) (55 × 2.5 cm glass column, 50 psi, and a 3.3 mL/min flow rate using a Sanki Engineering Ltd. model LBP-V pump). The column was eluted with a gradient of MeOH/H_2O (280 mL, 50:50), MeOH/H_2O (425 mL, 70:30), and MeOH (350 mL, 100%). Seventeen fractions were collected and analyzed by thin-layer chromatography (TLC) using silica gel (GF Uniplate, Analtech, Inc., Newark, DE) with MeOH/$CHCl_3$ (60:40, v/v). On the basis of TLC R_f values, fractions were pooled together and six fractions (**I**, 1.233 g; **II**, 0.556 g; **III**, 0.065 g; **IV**, 0.048 g; **V**, 0.014 g; and **VI**, 0.040 g) were obtained from them and dried under vacuum at 40 °C. All fractions were evaluated for topoisomerase I and II inhibition. Fractions **V** and **VI** displayed the highest topoisomerase activity and were chosen to continue the purification. Fractions **V** (30.5 mg) and **VI** (30.5 mg) were separately dissolved in 2 mL of the mobile phase, MeOH/H_2O (30:70), and purified by preparative HPLC (LC-20, Japan Analytical Industry, Co., Tokyo, Japan) using two columns (Jaigel-ODS, A 343-10; 250 × 20 mm, 10 μm, Dychrom, Santa Clara, CA) connected in series. The flow rate of the mobile phase was 3.3 mL/min, and peaks were detected at 215 nm. Fractions 1a (t_R 20−30 min, 1.8 mg), 2a (t_R 30−47 min, 9.1 mg), 3a (t_R 47−67 min, 3.4 mg), 4a (t_R 67−82 min, 3.0 mg), 5a (t_R 82−92 min, 3.8 mg), and 6a (t_R 92−160 min, 6.8 mg) were collected and assayed for topoisomerase inhibitory activity. The same procedure was repeated for fraction **VI**, and six fractions were obtained: fractions 1b (t_R 23−33 min, 4.4 mg), 2b (t_R 33−47 min, 12.8 mg), 3b (t_R 47−80 min, 7.5 mg), 4b (t_R 80−91 min, 7.4 mg), 5b (t_R 91−103 min, 2.5 mg), and 6b (t_R 103−120 min, 4.7 mg). On the basis of UV spectra (Shimadzu, Kyoto, Japan), ^{1}H NMR spectra (Varian VXR 300 MHz), and a high antitopoisomerase activity, fractions 5a and 4b were mixed and vacuum concentrated. This combined fraction was exposed to successive fractionations as before, except that the solvent system was twice MeOH/H_2O 30:70 and twice MeOH/H_2O 70:30. In the last stage, the majority compound was concentrated. ^{1}H NMR spectral analyses of this compound revealed that its structure was identical to that of ardisin, which was previously isolated from *A. japonica* (*15*).

Topoisomerase I and II Enzyme Inhibitory Assay. The topoisomerase I and II enzyme inhibitory assay was performed by using 5 × 10^6 UFC/mL *Sacharomyces cerevisiae* mutant cells JN 394, JN394t$_{-1}$, and JN394t-$_{2-5}$ (*16, 17*). The mutant cultures of *S. cerevisiae* were provided by Dr. John Nitiss (St. Jude Children's Hospital, Memphis, TN) and contained recombinant forms of topoisomerase I and II enzymes. The strain JN394 is resistant to both topoisomerase inhibitors, while JN394t$_{-1}$ and JN394t-$_{2-5}$ are resistant to topoisomerase inhibitors I and II, respectively. The assay was conducted according to the method published by Roth et al. (*18*). Crude fractions and purified compound ardisin were tested. Twenty-microliter aliquots containing 100, 250, and 500 μg of the pure compound or crude extract dissolved in DMSO were used. Water and DMSO were used as controls. Petri dishes were incubated at 27 °C for 72 h. The inhibition zone was measured in millimeters, and the activity was assigned (+) if the number of colonies inside the zone was ≥3, (++) if the number of colonies inside the inhibition zone was 1 or 2, and (+++) if there were no colonies inside the inhibition zone after 72 h.

Preparation of Rat Hepatocytes. Hepatocytes were isolated from male Wistar rats (200−250 g) from the animal facility of the University of Michoacan (Mexico) by the two-stage collagenase perfusion method (*19*). The rats were fed ad libitum with a rodent Purina (1050/13) chow diet and sterilized tap water. The animals were acclimatized for a minimum of 4 days and fasted 18 h before liver perfusion. Cell viability (≥90%) was routinely checked by staining with the Janus Green dye exclusion method (*20*). The cells were seeded at a density of 62 500 viable cells/cm^2 into 24-well tissue culture plates or 35-mm-diameter Petri dishes in Dulbecco's modified Eagle's medium containing 10% calf serum, 5 μg/mL insulin, 100 U/mL penicillin G, and 100 μg/mL streptomycin. The cultures were maintained at 37 °C in 5% CO_2 in air. After 2−3 h, they were rinsed with phosphate-buffered saline solution (PBS), and the unattached cells were discarded. Fresh medium was added, and incubation continued for an additional 21−22 h. Fresh serum-free medium, containing the test material at appropriate concentrations, was then added to triplicate cultures, and incubation continued for the desired period of time.

Preparation of Cultured Rat Hepatocytes. The viable cells were collected and suspended in phosphate buffer pH 7, and were then added with EDTA (5 mM) for the determinations of GSH, GR, and SOD, with 2-mercaptoethanol (1 mM) for the determination of GPx, and with BHT (5 mM) for the determination of MDA. The cellular suspension was kept in a vial at −70 °C until further use. Cells were centrifuged at 3000*g* for 10 min at 4 °C, thoroughly washed by centrifugation with phosphate buffer pH 7, and then resuspended, disrupted, and homogenized using a sonicator. Washing of the hepatocytes with the buffer

solution assured the elimination of any potential interfering substances, including EDTA, in the subsequent assays.

Total Phenolic Content of the *A. compressa* Extract Used in the in Vitro Study. The procedure for preparation of the extract was the same as that previously indicated for *A. compressa* extract. In this case, however, the dry material (2.7 g) was added to 250 mL of deionized HPLC-quality boiling water and allowed to stand for 10 min. The total phenolic content of the freeze-dried extract dissolved in deionized water was measured as described by Ramirez-Mares et al. (*13*).

Assays Using Cultured Rat Hepatocytes. The model of benomyl-induced damage in the primary cultures of rat hepatocytes was used to assess the protective action of the *A. compressa* extract, ardisin, and EGCG (*13*). Cell membrane integrity was evaluated throughout the experimental period using the Janus Green dye exclusion method (*20*). The stained cells were counted in 10 randomly chosen areas. The percentage of cytotoxicity was calculated considering the control as 100% cell viability. To evaluate the protective ability of ardisin, AE, and EGCG against Be-induced oxidation, cultured hepatocytes were exposed as follows: (A) simultaneously to Be (35 μg/mL) and ardisin (0.27 μg/mL) or to Be (35 μg/mL) and AE (2.52 μg, equivalent to {+} catechin/mL) or to Be (35 μg/mL) and EGCG (3 μg/mL) for 2 h; (B) to Be (35 μg/mL) for 2 h (37 °C) and then incubated with ardisin (0.27 μg/mL), AE (2.52 μg, equivalent to {+} catechin/mL), or EGCG (3 μg/mL) for 1 h at 37 °C; (C) to ardisin (0.27 μg/mL), AE (2.52 μg, equivalent to {+} catechin/mL), or EGCG (3 μg/mL) for 1 h at 37 °C and then incubated with Be (35 μg/mL) for 2 h. For each treatment, the hepatocytes were always thoroughly washed with fresh serum-free medium to eliminate any residual Be, ardisin, AE, or EGCG before each subsequent procedural step. Rat hepatocytes were also incubated separately with benomyl, AE, ardisin, and EGCG to be used as controls.

Determination of Glutathione Content. The concentration of reduced glutathione in cell lysates was carried out using a Biotech GSH-400 commercial kit (Oxis International, Portland, OR) and a Perkin-Elmer Lambda 2 spectrophotometer. A standard curve of GSH was established using an original GSH concentration of 0.5 mmol/L (purity > 98%).

Determination of GPx and GR Activities. The GPx and GR activities were determined in cell lysates using Biotech GPx-340 and GR-340 commercial kits (Oxis International), which record the disappearance of NADPH at 340 nm utilizing a Perkin-Elmer Lambda 2 spectrophotometer. Activities are reported as mU/mg of protein, where U corresponds to international units.

Determination of SOD Activity. The total SOD activity was determined in cell lysates using a Bioxytech SOD-525 commercial spectrophotometric assay kit (Oxis International).

Determination of MDA Concentration. Lipid peroxidation was evaluated in cell lysates using the Bioxytech LPO-586 commercial colorimetric assay kit (Oxis International).

Determination of Total Protein Content. Total protein content in cultured rat hepatocytes was measured by the Bradford method (*21*).

Statistical Analysis. The results are reported as means ± SD and were analyzed by one-way ANOVA. Triplicate measurements were carried out from each of three different animals. Statistical differences were analyzed according to Dunnett's test, wherein the differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

The hot water extract of *A. compressa* gave good topoisomerase I and II enzyme inhibitory activity, although it was not specific. Antibiotic activity of the extract was ruled out since it was not active in the antimicrobial assays with *Candida* spp. and various fungi and bacteria tested in our laboratory. Therefore, the fractionation and purification of the *A. compressa* aqueous extract were guided by topoisomerase enzyme inhibitory assays. The most active fraction, after repeated purification by HPLC, yielded a spectroscopically pure compound. Detailed NMR studies revealed that the compound was ardisin, a known compound from *A. japonica*. The spectral data of the pure compound and published data for ardisin were identical (*15*).

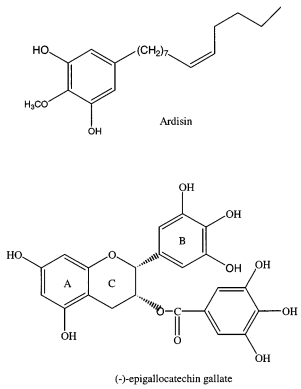

Figure 1. Structures of ardisin extracted from *Ardisia compressa* and (–)-epigallocatechin 3-gallate used as a control.

At 250 μg, ardisin displayed strong topoisomerase I and II inhibitory activities. Additional research is needed to determine its efficacy, dose response, and toxicity compared to known topoisomerase I and II inhibitors. **Figure 1** shows the structures of ardisin purified from *A. compressa* and (−)-epigallocatechin 3-gallate used as a control. It has been shown that the dried leaves extract of *A. compressa* protects rat hepatocytes from benomyl-induced genotoxicity (*13*). It has also been shown that inhibitors of the catalytic activity of topoisomerase II are often effective chemopreventive agents (*22*). It is well known that phenols/catechols are one of three chemopreventive agents that are potent topoisomerases II inhibitors and also are antioxidants. These were the main reasons why topoisomerase I and II were used in this study to guide the fractionation, isolation, and purification of ardisin.

As shown in **Table 1**, for the control cells, cell viability was always higher than 90%. Cell viability decreased depending on the treatment in comparison with the control, as shown in **Table 2**. We have previously found that cell viability, as determined by this selective staining method (*20*), and the lactate dehydrogenase release assay show good correlation (*23*).

For proper evaluation of the oxidant and/or antioxidant effects of the individual molecules (Be, ardisin, EGCG) or the complex mixture (AE), the effects of each one of them were determined (**Table 1**) for comparison with the different treatments (**Table 2**).

Table 1 shows that, in comparison to the control treatment, there were significant differences in all the variables measured when the hepatocytes were exposed to benomyl, except for SOD. The GSH levels (12.3 ± 1.4 nmol/mg protein) decreased 65% ($P < 0.05$), GPx activities (322 ± 31 mU/mg protein) increased 10% ($P < 0.05$), and the activity of GR (14 ± 1.8 mU/mg protein) in hepatocytes lysates decreased 24% ($P < 0.05$). **Table 1** also shows MDA concentrations (pmol/mg protein) in cell lysates. A statistically significant elevation (58% change in comparison to the control) in MDA production was seen when cells were exposed to benomyl, which is indicative of a severe lipoperoxidation process. When hepatocytes were incubated with only AE, increases in GR (36.6% change in comparison to the control) and SOD (25% change in comparison to the control) were observed (**Table 1**). Ardisin caused only an increase in MDA (37%). In relation to the effect of EGCG (3 μg/mL), **Table 1** also ilustrates that MDA, GR, and SOD increased 17, 25, and 35%, respectively.

There were statistically significant differences due to treatment, the type of incubation (simultaneous, pre-, and postincubation), and the interaction treatment−incubation for GSH, MDA, GPx, GR, and SOD. **Table 2** shows the results of the

Table 1. Concentrations of GSH and MDA, and Activities of Glutathione Peroxidase, Glutathione Reductase, and Superoxide Dismutase, in Rat Hepatocytes Incubated Separately with Benomyl, AE, Ardisin, or EGCG[a]

		concentration		activity		
treatment	cell viability (%)[b]	GSH (nmol/ mg protein)	MDA (pmol/ mg protein)	GPx (mU/ mg protein)	GR (mU/ mg protein)	SOD (U/ mg protein)
control	100	35.0 ± 1.8	418 ± 20	292 ± 10	18.3 ± 1.4	20.0 ± 1.8
benomyl	49 ± 2.0	12.3 ± 1.4[c]	659 ± 39[c]	322 ± 31[c]	14.0 ± 1.8[c]	17.6 ± 1.4
AE (2.52 μg equiv (+) catechin/mL)	91 ± 2.0	31.0 ± 2.8	433 ± 27	233 ± 58	25.0 ± 1.8[c]	25.0 ± 1.8[c]
ardisin (0.27 μg/mL)	102 ± 4.5	36.6 ± 2.8	574 ± 36[c]	312 ± 33	22.3 ± 1.9	24.0 ± 2.8
EGCG (3 μg/mL)	106 ± 1.5	36.6 ± 2.3	491 ± 33[c]	262 ± 42	23.0 ± 1.6[c]	27.0 ± 2.4[c]

[a] $X \pm$ SD is the average of triplicate determinations from three different animals. [b] Measured by the Janus Green dye method. [c] $P < 0.05$, in comparison with the control

Table 2. Concentrations of GSH and MDA, and Activities of Glutathione Peroxidase, Glutathione Reductase, and Superoxide Dismutase, in Rat Hepatocytes Incubated under Different Treatment Conditions[a]

		concentration		activity		
treatment[b]	cell viability (%)[c]	GSH (nmol/ mg protein)	MDA (pmol/ mg protein)	GPx (mU/ mg protein)	GR (mU/ mg protein)	SOD (U/ mg protein)
benomyl	49 ± 2.0	12.3 ± 1.4	659 ± 39	322 ± 31	14.0 ± 1.8	17.6 ± 1.4
		Simultaneous Incubation				
benomyl + AE	50 ± 2.0	23.6 ± 2.3[d]	801 ± 35[d]	316 ± 35	7.6 ± 1.9[d]	15.3 ± 2.8
benomyl + ardisin	82 ± 2.0	42.3 ± 2.3[d]	641 ± 40	304 ± 25	5.0 ± 1.8[d]	22.6 ± 2.3
benomyl + EGCG	50 ± 2.0	25.0 ± 1.8[d]	915 ± 29[d]	362 ± 43	5.6 ± 2.8[d]	8.3 ± 1.4[d]
		AE, Ardisin, or EGCG Preincubation + Benomyl				
AE + benomyl	87 ± 3.0	43.3 ± 3.7[d]	430 ± 25[d]	132 ± 30[d]	7.0 ± 1.8[d]	21.6 ± 2.3
ardisin + benomyl	95 ± 3.0	47.0 ± 3.3[d]	612 ± 24	144 ± 13[d]	13.0 ± 2.8	18.6 ± 3.5
EGCG + benomyl	62 ± 4.0	44.3 ± 2.8[d]	505 ± 27[d]	158 ± 37[d]	9.6 ± 2.3	20.6 ± 1.4[d]
		Benomyl + AE or Ardisin, or EGCG Postincubation				
benomyl + AE	32 ± 3.0	21.3 ± 1.4[d]	696 ± 40	367 ± 29	23.6 ± 1.4[d]	6.0 ± 1.8[d]
benomyl + ardisin	40 ± 2.5	25.3 ± 3.2[d]	870 ± 28[d]	339 ± 34	12.3 ± 2.3	10.6 ± 1.4[d]
benomyl + EGCG	31 ± 3.0	20.0 ± 1.8[d]	687 ± 43	350 ± 37	11.3 ± 1.9	9.6 ± 1.9[d]

[a] $X \pm$ SD is the average of triplicate determinations from three different animals. [b] Benomyl, 35 μg/mL; AE, 2.52 μg equiv de (+) catechin/mL; ardisin, 0.27 μg/mL; EGCG, 3 μg/mL. [c] Measured by the Janus Green dye method. [d] $P < 0.05$, compared with Be treatment from **Table 1**.

simultaneous, pre-, and postincubation of benomyl and AE, benomyl and ardisin, or benomyl and EGCG in comparison to the incubation of benomyl. The presence of AE or ardisin during the simultaneous incubation with benomyl gave better values than for EGCG for all the antioxidant parameters measured. The concentration of GSH was 12.3 ± 1.4 nmol/mg protein in the presence of benomyl alone and increased to 42.3 ± 2.3 nmol/mg protein during simultaneous incubation with ardisin. Furthermore, MDA values were no different (641 ± 40 pmol/mg protein) during preincubation with ardisin. On the other hand, in comparison to the cells exposed only to benomyl, the concentration of GSH improved 250, 282, and 260% when the cells were preincubated with AE, ardisin, or EGCG, respectively, following benomyl exposure. The results obtained for GPx were in accord with GSH only during preincubation with AE, ardisin, or EGCG. GR values decreased independently of the incubation approach, with the exception of postincubation with AE. SOD did not show significant changes, except during postincubation with AE, ardisin, and EGCG. **Table 3** shows a summary of the relative changes in the values of GSH, MDA, GPx, GR, and SOD due to the different treatment procedures used for AE, ardisin, and EGCG. The increase or decrease of each value has been compared to the control benomyl. Postincubation, GSH was the only parameter that showed a significant increase, regardless of the treatment procedure. SOD did not present significant differences, except for a decrease during simultaneous incubation with EGCG and postincubation with AE, ardisin, and EGCG.

Table 3. Percentage Change in Parameters of Glutathione Metabolism Produced by Different Treatments As Compared to Benomyl[a]

	change in parameters (%)				
treatment[b]	GSH	MDA	GPx	GR	SOD
1,a	*↑ 92	*↑ –22	NS↓ 2	*↓ –46	NS↓ –3
1, b	*↑ 244	NS↓ 3	NS↓ 6	*↓ –64	NS↑ 28
1,c	*↑ 103	*↑ –39	NS↑ –12	*↓ –60	*↓ –53
2,a	*↑ 252	*↓ 35	*↓ 59	*↓ –50	NS↑ 23
2, b	*↑ 282	NS↓ 7	*↓ 55	NS↓ –9	NS↑ 7
2,c	*↑ 260	*↓23	*↓ 51	NS↓ –32	NS↑ 17
3,a	*↑ 73	NS↑ –6	NS↑ –14	*↑ 69	*↓ –66
3, b	*↑ 106	*↑ –32	NS↑ –5	NS↓ –12	*↓ –40
3,c	*↑ 63	NS↑ –4	NS↑ –9	NS↓ 19	*↓ –45

[a] An asterisk indicates statistical differences in comparison to the control Be alone ($P < 0.05$), using Dunnett's test. NS, no significant difference ($P > 0.05$). ↓, decreased versus Be. ↑, increased versus Be. [b] Incubation: 1, simultaneous; 2, preincubation; 3, postincubation. Treatment: a, Be + AE, b, Be + ardisin; c, Be + EGCG.

The results suggest that *A. compressa* leaves possess a high antioxidant capacity, which can protect cells against the adverse effects of benomyl. *A. compressa* leaves contain a large amount of catechins, a group of very active flavonoids (*14*). Flavonoids occur naturally in fruit, vegetables, and beverages such as tea and wine and have beneficial health effects (*24*). Benomyl is metabolized by the hepatocytes to carbendazym and *n*-butylisocyanate (*25*). This latter metabolite binds to GSH without enzyme activation, in vivo. Ardisin, purified from AE, gave

the highest protection against benomyl-induced oxidation to hepatocytes. AE appears to be more effective than a larger dose of EGCG, probably because AE is an extract that contains several flavonoids and other unidentified compounds that may have an additive or synergistic effect, while EGCG is only a single compound. However, it has to be kept in mind that in mixtures, activity results from the presence of multiple active principles, which can be synergistic or antagonistic. The biological activity of any natural compound can change in both ways. One active compound could affect differently absorption, distribution, and elimination of another natural compound present in a complex matrix, and therefore a desirable or undesirable response will be obtained.

EGCG was used as a positive control because it is one of the phenolic compounds with the highest antioxidant capacity present in tea. EGCG has many biological functions, including antioxidant activity (*26*, *27*), antimutagenic (*28*), and anticarcinogenic effects (*29*), plus inhibitory action on the growth of immortalized and tumor cells (*30*). Several fractions isolated from *A. compressa* showed topoisomerase inhibitory activity, which means that there is the potential that several compounds present in it are chemoprotectors.

The decrease in GSH in the presence of Be, a membrane perturbent, could reflect the formation of the complex *n*-butylisocyanate-GSH. It has also been shown that *n*-butylisocyanate reacts with amino, sulfydryl, hydroxy, carboxyl, and imidazol groups of the proteins (*31*). The decrease in GR may be due to the GSSG increase, which inhibits GR activity when it reacts with SH groups of proteins. It has been shown that *n*-butylisocyanate-GSH inhibits GR (*32*). The decrease of SOD means that lipid peroxides are the main substrates of GPx, because SOD has a permutation reaction between two peroxide radicals and forms H_2O_2. The increase in MDA confirms the increase in the activity of GPx and decrease in GSH (**Table 1**). MDA has been found to be the principal byproduct of the oxidation of polyunsaturated fatty acids present in the cell membrane, with cytotoxic and genotoxic properties (*33*, *34*). Benomyl-induced depletion of GSH levels may be associated with increased free radicals and elevated lipid peroxidation and may be responsible for its observed hepatotoxicity. It was also clear in this investigation that preincubation with the protective compounds reduced the oxidative stress, probably due to the induction of protective mechanisms. This effect was seen with the increase in GSH. For SOD, only postincubation with the three compounds caused a decrease in its activity (34%). In general, it can be considered that there was not a significant increase in superoxide radical, so SOD was not induced. MDA increased when the cells were in the presence of AE and EGCG during simultaneous incubation. It is well known that antioxidants also show a pro-oxidant activity, because of their capacity to reduce metals to certain chemical forms that react with oxygen or with H_2O_2 (*35*).

Due to the complexity of the interpretation of the different treatments, the values obtained after the exposure of the cells to benomyl alone were considered as 100% damage. Values above or below were respectively considered as toxicity or protection, depending on the variable measured. For instance, an increase above 12.3 nmol/mg protein for GSH represents a protective effect. On the other hand, a decrease in the activity of GPx below 322 mU/mg protein represents likewise a protective effect because substrates (ROOH and H_2O_2) are not abundant. An increase in the activity of GR above 14 mU/mg protein also represents a protective effect because GSH is regenerating. Even though an increase in SOD could represent an increase in radical superoxide, at physiological pH, its reactivity is severely limited. Therefore, an increase in SOD above 17.6 U/mg protein was considered as a protective effect. Finally, a decrease below 659 pmol/mg protein for MDA indicates not only a decrease in lipoperoxidation but also less cytotoxic damage to hepatocytes. On the basis of these parameters, preincubation with ardisin (342%), AE (319%), or EGCG (319%) gave the best protection against benomyl, followed by simultaneous incubation with ardisin (217%) and finally a very poor postincubation protection with AE (56%), ardisin (17%), and none with EGCG due to the lack of induction of repair mechanisms. It was also observed in this study that there was a good correlation between oxidative damage and cellular viability (data not shown). However, oxidative stress is likely not the only mechanism responsible for the cytotoxicity induced by benomyl. All these data indicate that, in vitro, *A. compressa* has an effective preventive action against benomyl-induced hepatic oxidation, possibly by enzymatic antioxidative mechanisms associated with the different constituents existing in the aqueous extract. This may, in part, explain the apparent medicinal benefits observed by indigenous people in Mexico who consumed this plant extract as herbal tea (*13*). In the context of the present findings, under normal preparation conditions, the concentration of phenolic compounds in *A. compressa* tea would be about 16.79 ± 1.22 mg/g dry leaves. This level is similar to those found in the literature for other tea types (*36*). In practical terms, and based on the effective concentrations of equivalents to {+} catechin found in the present study, to gain a significant antioxidant protective benefit, a person will have to ingest an approximate amount of 1 L of tea per day. This level of intake could be achieved by consuming this tea as part of the customary diet.

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Received for review August 6, 2002. Revised manuscript received October 10, 2002. Accepted October 16, 2002.

JF0258757