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Protection of Extract from Leaves of *Ardisia compressa* against Benomyl-induced Cytotoxicity and Genotoxicity in Cultured Rat Hepatocytes

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Abstract—The potential of the *Ardisia compressa* extract (EA) was examined regarding its capacity to reduce the cytotoxic effect of benomyl on rat hepatocytes. The protective effect was evaluated by Janus Green dye exclusion method. An approximate 50% cytotoxic effect of benomyl on hepatocytes was observed at 35 μg/ml after 2 hr of incubation. (–)Epigallocatechin 3-gallato (EGCG) and EA decreased the viability of hepatocytes at concentrations above 3 μg/ml and 2.52 μg, equivalent to (+)catechin/ml, respectively. A protective effect against benomyl was observed when hepatocytes were previously exposed to EGCG (3 μg/ml) or EA (2.52 μg, equivalent to (+)catechin/ml) followed by incubation with benomyl (35 μg/ml) alone. When EGCG or EA were in contact with cells, either simultaneously or after pretreatment with benomyl, did not protect hepatocytes. EGCG (1.3×10^{-2} μg/ml) or EA (9.8×10^{-2} μg, equivalent to (+)catechin/ml) inhibited 57% and 34%, respectively, the unscheduled DNA synthesis (UDS) induced by benomyl at a concentration of 23×10^{-2} μM, when both were incubated with hepatocytes prior to benomyl. The simultaneous incubation of benomyl with EGCG or EA did not protect the cell against the genotoxic effect of benomyl. These results indicate that the dried leaves extract of *Ardisia compressa* protect rat hepatocytes from benomyl-induced cytotoxicity and genotoxicity.

Keywords: *Ardisia*; benomyl; genotoxicity and cytotoxicity.

Abbreviations: DMSO = dimethyl sulfoxide; DPM = desintegrations per minute; EA = extract of *Ardisia compressa*; EGCG = (–)epigallocatechin 3-gallato; GSH = glutathione; HU = hydroxy urea; PBS = phosphate buffered saline; T^3H = tritiated thymidine; UDS = unscheduled DNA synthesis.

INTRODUCTION

In recent years there has been a growing interest in identifying naturally occurring minor dietary constituents against the development of several diseases. Plant-derived foodstuffs, particularly fruits and vegetables, are generally considered to be highly beneficial components of the human diet. They provide a wide range of nutrients, vitamins and other compounds that exert a protective effect against diseases (Burr, 1994; Kohlmeier *et al*., 1995; Willett, 1994). Antioxidant compounds are important in reducing the risk of free radical-related oxidative damage, which is associated with a number of clinical conditions and degenerative diseases, such as hyperoxia, reperfusion injury, arthritis, atherosclerosis, ageing and cataractogenesis (Dreosti, 1996), and many plant constituents have been shown to protect against cancer (Ohigashi *et al*., 1996). In Western societies there is a trend towards increasing the variety of plant products consumed by the population by introduction of herbal 'remedies' or health foods (Phillips, 1996). Hundreds of different herbal teas are sold in health-food stores as mixtures of roots, leaves, seeds, barks, or other parts of shrubs, vines or trees (Manteiga *et al*., 1997). In Mexico, the dried leaf of aqueous extract of *Ardisia compressa* has been used effectively in folk medicine against liver disorders and cancer. However, no scientific information is available about the pharmacological, biological and

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toxicological effects of *Ardisia compressa* dry leaves extract.

Primary cultures of rat hepatocytes have been found to provide valuable experimental tools to detect a range of effects, including metabolic disturbances, membrane damage and genetic alterations (Gebhardt, 1997; Phillips, 1996; Tseng *et al.*, 1997).

Benomyl (methyl 1-(butylcarbamoyl) benzimidazole-2-carbamate), is a fungicide registered for fruits, vegetables, flowers, ornamental crops and others. Its fungicidal property is attributed to its ability to interfere with mitosis (Hammerschlag and Sisler, 1973), specifically by binding to tubulin (Ireland *et al.*, 1979) and preventing tubulin polymerization. It has also been reported that benomyl induces hepatocellular carcinomas and neoplasms in both male and female mice (Extension Toxicology Network, 1993). Additionally, benomyl and its metabolites reduce DNA synthesis since these compounds have been reported to inhibit incorporation of thymidine into DNA molecules (Hellman and Laryea, 1990). Rat primary hepatocyte cultures have been used to study the effect of benomyl, and the results demonstrated that benomyl is responsible for the microtubular disorganization of the cytoskeleton (Urani *et al.*, 1995), is a potent glutathione-depleting agent (Urani *et al.*, 1995) and increases the micronucleus frequency (Piatti *et al.*,1994).

The overall objective of the present work was to evaluate the protective effects of the aqueous extract of dry leaves of *Ardisia compressa* on the benomyl-induced cytotoxicity and genotoxicity in rat primary hepatocyte culture.

MATERIALS AND METHODS

Assays

To understand the protective action of the dried leaf aqueous extract of *Ardisia compressa* (EA) in intact cells, the model of benomyl-induced damage in the primary cultures of rat hepatocytes was used.

To evaluate the concentration of benomyl that causes a 50% cytotoxic effect and the maximum concentration of EGCG and EA that avoids cellular injury, the following assays were done in triplicate. (A) Dose–response relationship was determined from cultured hepatocytes at a density of 62500 cell/cm^2 treated with seven levels of benomyl (10, 20, 30, 35, 40, 50 and 60 μg/ml) or DMSO ($\leqslant$1%) incubated at 37°C in 5% CO_2 in air for 2 hr. Cell membrane integrity was evaluated by Janus Green dye exclusion method (Rieck *et al.*, 1993). (B) The cytotoxic effect of EGCG (1, 3, 5, 10, 15, 20, 30, 40, 50 and 60 μg/ml) and EA (0.63, 1.26, 2.52, 3.78, 5.04, 6.30, 7.55, 8.81 and 10.07 μg, equivalent to (+)catechin/ml) was evaluated separately in cultured rat hepatocytes incubated at 37°C in 5% CO_2 in air for 2 hr. Cell membrane integrity was evaluated by Janus Green dye exclusion method (Rieck *et al.*, 1993).

In order to evaluate the cytotoxicity associated with the time of incubation, the following assays were done in triplicate. Benomyl (35 μg/ml), DMSO (0.58%), EGCG (3 μg/ml) and EA (2.52 μg, equivalent to (+)catechin/ml) were incubated separately at 37°C in 5% CO_2 in air for 0, 1, 2, 3 and 4 hr.

To evaluate the protective ability of EGCG and EA against benomyl-induced cytotoxicity, cultured hepatocytes were exposed to three replicates. (A) Simultaneous exposure to benomyl (35 μg/ml) and EGCG (3 μg/ml) or to benomyl (35 μg/ml) and EA (2.52 μg, equivalent to (+)catechin/ml) during 0, 1, 2, 3 and 4 hr. (B) Exposure to benomyl for 2 hr (37°C) then incubated with EGCG (0, 0.5, 1, 1.5 and 2 hr) or EA (0, 0.5, 1, 1.5 and 2 hr) at 37°C. (C) Exposure to EGCG (0, 0.5, 1, 1.5 and 2 hr) or EA (0, 0.5, 1, 1.5 and 2 hr) at 37°C, then incubated with benomyl for 2 hr. For each treatment, cell membrane integrity was evaluated by Janus Green dye exclusion (Rieck *et al.*, 1993).In all the cases mentioned above, the stained cells were counted in 10 randomly chosen areas. The percentage of cytotoxicity was calculated taking the control as 100% of viability.

Genotoxicity was evaluated as the T^3H incorporation into DNA in relation to control cells (Hsia *et al.*, 1983; Mitchell *et al.*, 1983; Swierenga *et al.*, 1991). It was necessary to use hydroxyurea (HU), 10 mM, to prevent semiconservative DNA synthesis. Dose–response curves for benomyl, EGCG and EA were performed. Cells were incubated for 2 hr and washed with phosphate buffered saline (PBS) (5%). Different concentrations of benomyl (60, 30, 15, 7.5, 3.75, 1.88, 0.94, 0.23, 0.06, 0.03 and 0.015 μM), EGCG (5×10^{-2}, 2.50×10^{-2}, 1.3×10^{-2}, 6.3×10^{-3}, 15.6×10^{-4}, 78.1×10^{-5} and 97.7×10^{-6} μg/ml) and EA (15.7×10^{-2}, 78.7×10^{-3}, 39.3×10^{-3}, 19.7×10^{-3}, 98.3×10^{-4}, 49.2×10^{-4} and 24.6×10^{-4} μg, equivalent to (+)catechin/ml) were tested using serum free medium added with T^3H (3.75 μCi/ml) and HU, 10 mM. Genotoxic concentrations were those that caused significantly more incorporation (dpm per μg DNA) compared to the basal line of control cells. DMSO (0.02%) was tested to verify its innocuousness.

Having established the genotoxic concentration of benomyl (23×10^{-2} μM) and the innocuous concentrations of EGCG (1.3×10^{-2} μg/ml) and EA (9.8×10^{-9} μg, equivalent to (+)catechin/ml), the following experiments were performed in order to evaluate the protective ability of EGCG and EA against benomyl-induced genotoxicity. (A) Hepatocytes were cultured at a density of 5×10^5 cell/35-mm dish (62500 cell/cm^2) for 2 hr, washed with PBS (5%) and treated with fresh serum-free medium containing 10 mM hydroxyurea, T^3H (3.75 μCi/ml) and benomyl plus EGCG or benomyl plus

EA. The cells were incubated for an additional 4 hr, and DNA was recovered from cells using 10% trichloroacetic acid. Unscheduled DNA synthesis (UDS) was determined by T^3H incorporation in a Beckman Liquid Scintillation Counter model LS6000 and DNA was quantified according to the diphenylamine assay (Leyva and Kelley, 1974). (B) Fresh cells were cultured as mentioned above, added with serum-free medium containing EGCG or EA and incubated for 2 hr. Then cells were washed again and serum-free medium containing 10 mM hydroxyurea, T^3H (3.75 μCi/ml) and benomyl, was added. The cells were incubated for an additional 4 hr and DNA was recovered from cells and quantified as mentioned before. Unscheduled DNA synthesis (UDS) was determined by T^3H incorporation in a Beckham Liquid Scintillation Counter.

Biological materials and reagents

The leaves of *A. compressa* were collected on the Pacific coast of Mexico (Michoacan State) during the month of July, 1997.

Cell culture medium was obtained from GIBCO BRL Life Technologies (Grand Island NY, USA); 24-well tissue culture plates were obtained from Corning Glass Works (Corning). Dishes for cell culture were 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-gallato (min 80% purity), (+)catechin, Janus Green B (69% purity), penicillin and streptomycin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Tritiated thymidine [T^3H] (1 mCi/ml) from Amersham International plc (Bucks, UK) and dimethyl sulfoxide (DMSO) HPLC grade 99.9% purity from Omni Solv (NJ, USA).

Preparation of herb tea

Fresh leaves of *A. compressa* were dried and 2.7 g were added to deionized water at boiling point (94°C) for 10 min. The mixture was cooled to room temperature (25°C), then it was filtered with a 0.45 μm filter and the filtrate was freeze-dried. The material was kept at −20°C, protected from light in a glass container sealed with parafilm.

Total phenolic content of the extract

A certain amount of freeze-dried extract was dissolved in deionized water, and the total phenolic content of the extract was measured by a modification of the Prussian blue assay of Price and Butler (Graham, 1992). For the standard curve, (+)catechin was used and the equation obtained was $y = 0.02602 \times + 0.01305$; $R = 0.9994$, where y = absorbance at 700 nm and x = concentration expressed as μg, equivalents of (+)catechin. In this work when a quantity was referred for EA, the units used were equivalents to (+)catechin.

Condensed tannins determination

A certain amount of freeze-dried extract was dissolved in 94% (v/v) acetic acid and 6% (v/v) methanol, and the condensed tannin content of the extract was measured by the vanillin assay of Butler *et al*. (1982b). For the standard curve, (+)catechin was used and the equation obtained was $y = 0.0009562 \times + 0.06065$; $R = 0.9947$, where y = absorbance at 510 nm and x = concentration expressed as μg, equivalents of (+)catechin.

Relative degree of polymerization

Anthocyanidin formation was measured by the method of Butler (1982a). Under the conditions of the assay, the flavan-3-ol oligomers are converted to anthocyanidins which are quantified by their absorbance at 550 nm. The relative degree of polymerization was calculated as the ratio of the vanillin absorbance (due to terminal flavan-3-ol residues) to the absorbance of the anthocyanidins formed (due to total oligomeric flavan-3-ol residues).

Preparation of rat hepatocytes

Hepatocytes were isolated from male Wistar rats (200–250 g) from the animal facility from the University of Michoacan, by the two-stage collagenase perfusion (Seglen, 1976). The rats were fed *ad lib*. with rodent chow and tap water. The animals were acclimatized for a minimum of 4 days and fasted for 18 hr before liver perfusion. Viability of the cells ($\geqslant$80%) was routinely checked by staining with trypan blue (DelRaso, 1992).

The cells were seeded at a density of 62,500 cell/cm^2 into 24-well tissue culture plates or a 35 mm diameter petri dish in Dulbecco's modified Eagle's medium with 10% calf serum, 5 μg insulin/ml, 100 U penicillin G/ml and 100 μg streptomycin/ml. The cultures were maintained at 37°C in 5% CO_2 in air. After 2–3 hr they were rinsed with PBS and unattached cells discarded. Fresh medium was added and incubation continued for a further 21–22 hr. Then fresh serum-free medium, containing the test material at appropriate concentrations, was added to triplicate cultures and incubation continued for a period of time, as mentioned before.

Statistical analysis

The results were reported as means of triplicate analysis. Statistical differences were analysed according to Dunnett's test wherein the differences were considered to be significant at $P < 0.05$.

RESULTS

Polyphenols in Ardisia compressa

Table 1 shows the results obtained on the determination of total phenolic substances in the aqueous and freeze-dried extract of *A. compressa* . The aqueous extract contained 16.79 ± 1.22 mg/g dry

Table 1. Polyphenols in *Ardisia compressa*

Polyphenols	mg/g dry leaves*
Total polyphenols (aqueous extract)	16.79 ± 1.22
Total polyphenols (freeze-dried extract)	12.59 ± 1.38
Condensed tannins (freeze-dried extract)	7.97 ± 0.48

*Values were obtained as the average of triplicate determinations.

leaves and the freeze-dried extract 12.59 ± 1.38 mg/g dry leaves. The condensed tannins on the freeze-dried extract were 7.97 ± 0.48 mg/g dry leaves. The relative degree of polymerization obtained was 0.712 ± 0.097 ($P < 0.05$).

Cytotoxicity

The cytotoxicity test revealed that benomyl led to a significant dose- and time-dependent inhibition of cell viability (Tables 2 and 3). The concentration of benomyl required to produce an approximate 50% cytotoxic effect of hepatocytes after 2 hr of incubation was 35 μg/ml. The effect of EGCG on the decrease of viability in comparison with the control occurred at concentrations above 3 μg/ml, and the EA had an effect on viability at concentrations above 2.52 μg, equivalent to (+)catechin/ml in relation to the control. The DMSO (0.58%) was used as negative control and no cytotoxicity was observed ($P < 0.05$). In all these cases the incubation period was 2 hr.

The cells incubated for 1, 2, 3 or 4 hr with 35 μg benomyl/ml, 3 μg EGCG/ml or 2.5 μg, equivalent to (+)catechin/ml EA, show variations on the viability of hepatocytes (Table 3). The addition of the agents significantly reduced the viability of the cells with an effect related to the time of incubation ($P < 0.05$). With DMSO (0.58%) no cytotoxicity was observed ($P < 0.05$) with respect to incubation time.

When hepatocytes were simultaneously exposed to benomyl (35 μg/ml) plus EGCG (3 μg/ml) or benomyl (35 μg/ml) plus EA (2.52 μg, equivalent to (+)catechin/ml) for 0, 1, 2, 3 and 4 hr, no inhibition of the cytotoxic effect induced by benomyl was observed neither with EGCG nor with EA ($P < 0.05$) (Fig. 1).

After pretreatment with the toxic concentration of 35 μg/ml benomyl for 2 hr, hepatocytes were incubated with 3 μg/ml EGCG or 2.52 μg, equivalent to (+)catechin /ml EA for 0, 0.5, 1, 1.5 or 2 hr; no inhibition of the cytotoxic effect induced by benomyl was observed neither with EGCG nor with EA ($P < 0.05$) (Fig. 2).

When the cells were first incubated for 0.5, 1, 1.5 or 2 hr with 3 μg/ml EGCG or 2.5 μg, equivalent to (+)catechin/ml EA, followed by a 2-hr incubation with 35 μg/ml benomyl alone (Fig. 3), it was observed that only after pretreatment for 1, 1.5 or 2 hr with EGCG or EA, the hepatic cytotoxicity was significantly decreased ($P < 0.05$).

Genotoxicity

The results showed that when hepatocytes were simultaneously exposed to benomyl (23×10^{-2} μM) plus EGCG (1.3×10^{-2} μg/ml) or EA (9.8×10^{-9} μg, equivalent to (+)catechin/ml) (Table 4), no inhibition of benomyl-induced DNA repair synthesis ($P < 0.05$) was observed. DMSO was not genotoxic ($P < 0.05$).

When the cells were first incubated for 2 hr with 1.3×10^{-2} μg EGCG/ml or 9.8×10^{-9} μg, equivalent to (+)catechin/ml EA , followed by incubation with 23×10^{-2} μM benomyl alone (Table 4), it was observed that EGCG or EA significantly inhibited the benomyl-induced DNA repair synthesis ($P < 0.05$).

DISCUSSION

The objective of this work was to explore the potential beneficial effect of the dried leaves of *Ardisia compressa*. In the first instance, however, determining the basic cytotoxicity of the plant extract was considered to be a necessary step in establishing the antitoxic effect. This is of particular importance because complex mixtures such as EA might contain a wide variety of known as well as unknown constituents that could be cytotoxic. The preliminary data (Table 1) indicated that EA contains phenolic constituents , which according with the degree of polymerization (0.712 ± 0.097) and the quantifi-

Table 2. Effect of concentration on cytotoxicity of benomyl, EGCG and EA measured with the dye Janus Green

Treatment (μg/ml)	Cell viability (%)*	EGCG (μg/ml)	Cell viability (%)*	EA (μg)†	EA (μg equivalent to (+)catechin/ml)	Cell viability (%)*
Control	100	1	102 ± 1.0	50	0.63	99 ± 0.3
DMSO	99 ± 1.4	3	106 ± 1.0‡	100	1.26	96 ± 1.0‡
Benomyl		5	93 ± 1.8‡	200	2.52	91 ± 0.7‡
10	94 ± 2.0‡	10	82 ± 1.8‡	300	3.78	82 ± 1.2‡
20	86 ± 1.3‡	15	58 ± 1.7‡	400	5.04	64 ± 1.0‡
30	80 ± 1.5‡	20	40 ± 2.3‡	500	6.30	37 ± 0.7‡
35	49 ± 0.8‡	30	35 ± 1.3‡	600	7.55	28 ± 1.2‡
40	30 ± 2.3‡	40	29 ± 0.6‡	700	8.81	12 ± 1.1‡
50	17 ± 2.0‡	50	10 ± 1.2‡	800	10.07	0‡
60	0	60	4 ± 0.9‡			

*Values were obtained as the averages of triplicate determinations. †μg of freeze-dried extract. ‡$P < 0.05$, compared with the control treatment.

Table 3. Effect of incubation time on cytotoxicity of benomyl, EGCG and EA measured with the dye Janus Green

Treatment	Cell viability (%)*			
	1 hr	2 hr	3 hr	4 hr
Control	100	100	100	100
DMSO (0.58%)	100 ± 0.8	98 ± 0.6	98 ± 0.3	98 ± 0.5
Benomyl (35 μg/ml)	95 ± 1.0†	49 ± 0.8†	34 ± 0.9†	0†
EGCG (3 μg/ml)	103 ± 0.6†	107 ± 0.7†	96 ± 0.5†	93 ± 0.5†
EA (2.52 μg equivalent to (+) catechin/ml)	100 ± 1.3	92 ± 0.8†	85 ± 0.7†	79 ± 1.0†

*Values were obtained as the average of triplicate determinations. †$P < 0.05$, compared with the control treatment.

cation of condensed tannins are mainly flavan-3-ol monomers such as catechin and epicatechin. In general, polyphenolic compounds are considered as candidates for its antioxidative effect. For this, EGCG was chosen as positive control in this work. EGCG has many biological functions, including antioxidative activity (Yen and Chen, 1995; Yoshino *et al*., 1994), antimutagenic effects (Shiraki *et al*., 1994), anticarcinogenic effects (Katiyar *et al*., 1993) and inhibitory action on the growth of immortalized and tumour cells (Lin *et al*., 1996).

It is obviously very difficult to choose suitable standards for total phenolic determinations in plant extracts due to the chemical heterogeneity of plant products and the unspecificity of phenolic reagents. Thus, it is only possible to obtain relative equivalents with the standard used (+)catechin.

According to the results of selective staining of damaged cells, shown in Table 2, the concentration of benomyl required to produce an approximate 50% cytotoxic effect of hepatocytes was 35 μg/ml; this means that at this dose benomyl had a median toxic effect. The EGCG was cytotoxic only at concentrations higher than 3 μg/ml (Table 2), this cytotoxic effect has been studied in rat hepatocytes (Nakagawa and Tayama, 1995); this means that is safe to use a dose of 3 μg/ml EGCG in cultured rat hepatocytes to probe its protective effect against the cytotoxicity induced by benomyl. A reasonable cell viability (91%) was found at 2.52 μg, equivalent to (+)catechin/ml of the freeze-dried extract and it was decided to use this concentration to test the protective effect.

Using *in vitro* methods, it is usually possible to achieve toxic concentrations with even the most innocuous materials (Phillips, 1996). Compounds that are toxic at high concentrations may be beneficial at lower concentrations. Thus, the results reported here do not imply that EGCG or EA would have toxic effects in humans but simply constitute a baseline data for further studies.

A second prerequisite for properly evaluating the antitoxic effect of individual molecules or complex mixtures is the exact determination of cytotoxicity associated with the time of incubation of the cells with these materials (Gebhardt, 1997). As shown in Table 3, the addition of benomyl, EGCG or EA significantly reduced the viability of the cells with an effect related to the time of incubation ($P < 0.05$). According to the results, the most ade-

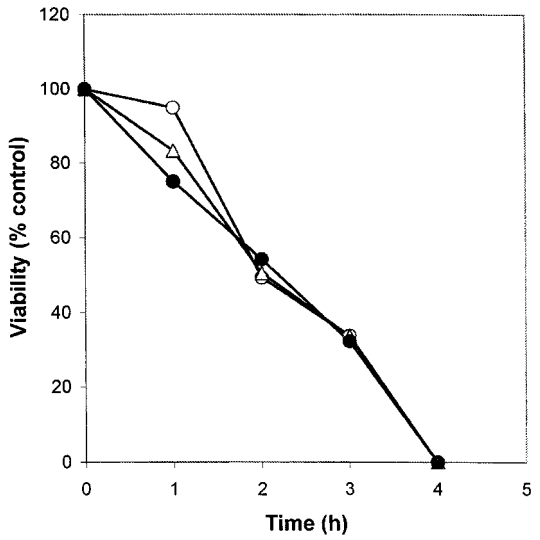

Fig. 1. Cytotoxic effect of benomyl alone (○) and simultaneously with EGCG (●) or EA (△). Viability is expressed as a percentage of control cultures. Each point is the mean value for three cultures.

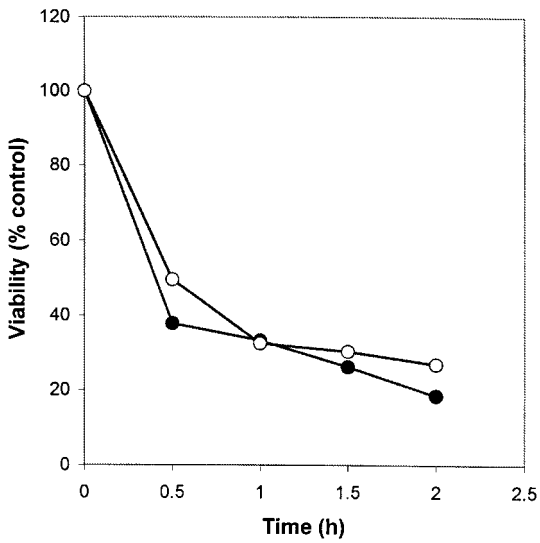

Fig. 2. Cytotoxic effect of benomyl exposured for 2 hr before EGCG (○) or EA (●) treatment for 0, 0.5, 1, 1.5 and 2 hr. Viability is expressed as a percentage of control cultures. Each point is the mean value for three cultures.

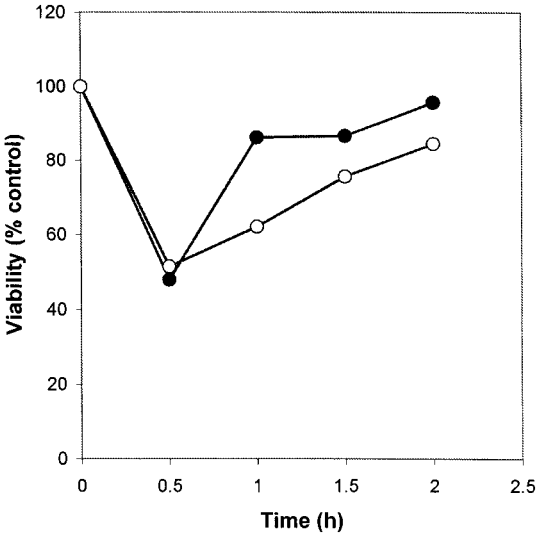

Fig. 3. Cytotoxic effect on rat hepatocytes exposed to EGCG (○) or EA (●) for 0, 0.5, 1, 1.5 and 2 hr before exposure to benomyl for 2 hr. Viability is expressed as a percentage of control cultures. Each point is the mean value for three cultures.

quate time of incubation to be tested with the potential antitoxic material (EGCG and EA) was 2 hr, because at this time cell viability remained high for both compounds. The exposure of cells to EA for more than 3 hr reduced ($P < 0.05$) the cellular viability of hepatocytes.

Once the basic cytotoxicity of EGCG and EA had been established, it was possible to study their beneficial effects against the benomyl-induced damage. The protection was investigated by adding EGCG or EA together with benomyl to hepatocytes cultures. As shown in Fig. 1, no inhibition of the benomyl-induced cytotoxicity was observed when they were added simultaneously. Nevertheless, a complex was formed between benomyl and EGCG and EA, as demonstrated spectrophotometrically (data not shown), the concentration ratios in μM units were 18:1 and 14:1 (toxin:protector), respectively. Probably the protection of the phenol and the aqueous extract decreased the bioavailability of benomyl, but there was enough of it to produce toxicity.

After pretreatment with benomyl for 2 hr, hepatocytes were incubated for 0, 0.5, 1, 1.5 or 2 hr with EGCG or EA, and no decrease of the damage caused by benomyl was observed, as is shown in Fig. 2. These results indicate that there was no induction of repair mechanisms.

When the hepatocytes were first incubated for 0, 0.5, 1, 1.5 or 2 hr with EGCG or EA, followed by a 2-hr incubation with benomyl alone, a significant inhibition of the benomyl-induced damage was avoided (Fig. 3). This means that it could be possible that biochemical changes have occurred, such as enzyme induction or inhibition, but further work is currently in process to investigate this. For instance, the addition of EGCG to microsomes prepared from control, phenobarbital or 3-methylcholanthrene-treated rats resulted in a dose-dependent inhibition of cytochrome P-450-dependent aryl hydrocarbon hydroxylase activity (Wang *et al.*, 1988). Benomyl is a potent glutathione (GSH)-depleting agent (Urani *et al.*, 1995), and it is well known that GSH is important for the maintenance of sulfhydryl groups involved in metabolic and membrane processes.

Choice of appropriate endpoints is a major consideration in all *in vitro* studies of toxicity. It is well known that the cytotoxicity induced by compounds can be tested by different methods such as lactate dehydrogenase leakage, MTT, protein synthesis inhibition, kenacid blue, Janus Green and trypan blue assays. No single endpoint can be expected to detect all types of toxicity or demonstrate all forms of protective effect. Nevertheless, in our laboratory (Leal *et al.*, 1998) we have found that viability determined by selective staining of damaged cells (Janus Green or trypan blue assays) and lactate dehydrogenase release assay shows good correlation.

In non-proliferating hepatocytes *in vitro,* two types of DNA damage can be monitored: UDS, or induction of DNA single-strand breaks (Pool *et al.*, 1990). UDS is the enzymatic non-semiconservative repair process which is detected in cells that are not in the S-phase. Quantification of the occurrence of UDS is based on the measurement of the amount of radiolabelled thymidine incorporated into DNA when it is damaged by genotoxic substances. UDS

Table 4. Effect of EGCG and EA on the benomyl-induced DNA damage in rat hepatocytes cultures

Treatment*	dpm DNA/μg	Inhibition (%)‡
Control	171 ± 40	–
DMSO (0.02%)	165 ± 45	–
Benomyl (23×10^{-2} μM)	206 ± 29	–
EGCG (1.3×10^{-2} μg/ml)	160 ± 42	–
EA (9.8×10^{-9} μg equivalent to (+)catechin/ml)	173 ± 34	0
Benomyl (23×10^{-2} μM) plus EGCG (1.3×10^{-2} μg/ml)	206 ± 23	0
Benomyl (23×10^{-2} μM) plus EA (9.8×10^{-9} μg equivalent to (+)catechin/ml)	208 ± 35	0
Pretreatment EGCG (1.3×10^{-2} μg/ml) followed by benomyl (23×10^{-2} μM)	89 ± 25†	57
Pretreatment EA (9.8×10^{-9} μg equivalent to (+) catechin/ml) followed by benomyl (23×10^{-2}) μM)	137 ± 45†	34

*Values were obtained as the average of triplicate determinations. †$P < 0.05$, compared with the treatment with benomyl alone. ‡% of inhibition = 100% − (treatment/benomyl alone × 100%).

is one of many types of short-term tests currently used in the assessment of potential chemical genotoxicity and carcinogenicity (Swierenga *et al*., 1991). The measurement of DNA repair as an indication of interaction with DNA could be a reliable determinant of carcinogenic potential. Indeed, the appearance of DNA repair has been recommended as a screening technique for carcinogenesis (Williams, 1977).

In the present investigation, EGCG or EA were observed no inhibition the unscheduled DNA repair synthesis (Table 4) induced by benomyl in the rat hepatocyte cultures when the agents and benomyl were incubated simultaneously. When the cells were first incubated with EGCG or EA, followed by benomyl, the incorporation of T^3H into DNA of hepatocytes was inhibited by 57% and 34%, respectively, compared to the toxin alone (Table 4).

It was interesting to know that EGCG and EA exhibited an inhibition effect on the benomyl-induced genotoxicity. The mechanisms by which benomyl induce genotoxicity remains unclear; Hellman and Laryea (1990) believe that the toxic ingredient could be *n*-butyl isocyanate, an intermediate metabolite of benomyl, which has been implicated in the inactivation of enzymes, inhibition of macromolecule synthesis and interference with DNA repair.

At present, the molecular mechanism involved in the antigenotoxic effect of EGCG is unknown. We will be able to provide more detailed explanation on the protective effects of EA against benomyl-induced hepatic cytotoxicity and genotoxicity when the analysis of the constituents of EA is done and the work on enzyme induction or inhibition be completed.

In conclusion, EA showed efficiently protective action against benomyl-induced hepatic cytotoxicity and genotoxicity, possibly by unknown mechanisms associated with the different constituents existing in the aqueous extraction from dried *Ardisia compressa* leaves. The exact mechanisms and the active constituents need to be further investigated.

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