

Inhibition of liver carcinogenesis in Wistar rats by consumption of an aqueous extract from leaves of *Ardisia compressa*

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Abstract

This study evaluates the chemopreventive effect of an aqueous extract of dried leaves of *Ardisia compressa* against liver cancer. A rat liver assay that mimics progressive forms of human disease was used as a carcinogenesis model. Forty-five male Wistar rats (180–200 g body weight) were injected intraperitoneally on day 1 with a single dose (100 mg/kg) of diethylnitrosamine (DEN), and also received via gavage 20 mg/kg acetylaminofluorene (2-AAF), on days 7, 8 and 9. The rats were randomly divided into four groups (n = 15). Control groups (Group 1 and Group 2) had free access to water. Group 3 received 0.5% (w/v) of *A. compressa* tea for 10 days before treatment and during the study as the sole source of fluid until the rats were killed. A fourth group of 15 rats received no carcinogen or promoter but did receive 0.5% (w/v) of *A. compressa* tea. All animals had 70% partial hepatectomy at day 10. The incidences of hepatocellular foci, nodules and carcinoma were significantly smaller in Group 3 than in Group 2 ($P < 0.01$). *A. compressa* tea consumption alone (Group 4) did not induce the development of foci, nodules or carcinomas ($P < 0.01$). The striking observation of this study was that consumption of *A. compressa* tea resulted in complete inhibition of the chemically-induced hepatocarcinogenesis in Wistar rats.

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1. Introduction

Over the past decade, several epidemiological and case control studies have linked tea consumption, especially green tea, to a reduced risk of cancer in humans (Su and Arab, 2002; Ke et al., 2002). Evidence obtained from in vitro and in vivo animal studies suggest possible beneficial effects of tea polyphenols at most stages of cancer development (Yang and Wang, 1993; Dreosti, 1996; Katiyar and Mukhtar, 1996; Sartippour et al., 2001; Hirose et al., 2002; Yang et al., 2002). Several reports have confirmed this and have identified many possible sites of action in cancer prevention. There has been much attention paid to the expression of the phase

II detoxifying enzymes (Yu et al., 1997), as well as to involvement of tea polyphenols in apoptosis (Hibasami et al., 1996). The balance between phase I carcinogen-activating enzymes and phase II detoxifying enzymes is important in determining the risk of developing chemically-induced cancer subsequent to tea consumption (Maliakal et al., 2001). More recently, it has been reported that human biliary tract cancer cells showed a significant suppression of cell growth and invasive ability by epigallocatechin gallate (EGCG) treatment in a dose-dependent manner (Takada et al., 2002).

The potential of using tea components as cancer chemoprotective nutraceuticals and functional foods is promising (Dufresne and Farnworth, 2001). Hundreds of different herbal teas are sold in health-food stores (Manteiga et al., 1997). In Mexico, the aqueous extract of the dried leaf of *A. compressa* has been used in folk medicine against liver disorders including liver cancer.

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In our laboratory we have demonstrated that extracts of the dried leaves of *A. compressa* are protective in cultured rat hepatocytes against genotoxicity, cytotoxicity and oxidative damage induced by benomyl and 1-nitropyrene (Ramírez-Mares et al., 1999; González de Mejía and Ramírez-Mares, 2002). The present follow-up study evaluates the anticarcinogenic effect of the aqueous extract of dry leaves of *A. compressa*, using the rat liver preneoplastic foci assay as experimental model.

2. Material and methods

2.1. Animals, biological material and reagents

Male Wistar rats (180–200 g) were obtained from the Production Unit of Experimental Animals of CINVESTAV, Mexico. The leaves of *A. compressa* were collected on the Pacific coast of Mexico (Michoacan State) during the month of July. Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), carboxymethylcellulose (CMC), dimethyl sulfoxide (DMSO), bencidine hydrochloride, hematoxylin, eosin and safranin were purchased from Sigma Chemical Co. (St. Louis, MO). The placental form of anti-glutathione S-transferase (GST-P, 1:250) used in this study was obtained by the method of Marché-Cova et al. (1995).

2.2. Preparation of *A. compressa* tea

Fresh leaves of *A. compressa* were first air dried without exposure to sunlight, kept in large plastic bags and stored in a cool and dry place. The dry material (5 g) was then added to 1 l of tap water at boiling point (94 °C) and allowed to stand for 10 min (the tea preparation used in this investigation simulates the domestic brewing conditions). The mixture was cooled to room temperature (25 °C), and filtered (0.45- μ m nylon filter). This extract was prepared freshly every day and the total phenolic content was 12.59 ± 1.38 mg/g dry leaves, measured by a modification of the Prussian blue assay (Graham, 1992).

2.3. Chemical hepatocarcinogenesis scheme in Wistar rats

Hepatocarcinogenesis was evaluated using the rat liver preneoplastic foci assay (Semple et al., 1987; Ito et al., 1989; Williams, 1982). Sixty male weaning Wistar rats (180–200 g weight, 2 months of age) were randomly allocated into four groups (Fig. 1) of 15 animals each and were housed in a well-ventilated environment. They were fed with rodent chow and given tap water (G1 and G2) or *A. compressa* tea (0.5%, w/v) in tap water (G3 and G4), ad libitum. For the induction of preneoplastic foci, rats were treated according to the chemical

hepatocarcinogenesis scheme modified by Semple et al. (1987). Rats in the positive control group (G2) were injected intraperitoneally with a single dose of diethylnitrosamine (DEN) dissolved in water (100 mg/kg body weight). They also received via gavage a solution of 2-acetylaminofluorene (2-AAF) (20 mg/kg body weight) in dimethyl sulfoxide (DMSO) and carboxymethylcellulose (CMC) on days 7, 8 and 9, and 70% partial hepatectomy on day 10. G3, with the same chemical hepatocarcinogenesis scheme as in the positive group (G2), received *A. compressa* tea (0.5%, w/v) instead of drinking water 10 days before the treatment and throughout the experimental period. Rats in the negative control group (G1) were given an i.p. injection of the same volume of water and gavage administration of DMSO and CMC as those in the positive group (G2), and 70% partial hepatectomy on day 10. In order to screen the tea preparation for carcinogenic potential, the consumption of the aqueous extract of dry leaves of *A. compressa* followed by partial hepatectomy was tested (G4). Group 4 animals were pretreated with 0.5% (w/v) *A. compressa* tea ad libitum 20 days before 70% partial hepatectomy and throughout the experimental period until they were killed.

Three rats from each group were killed at 25, 60, 90, 120 and 270 days after the treatment. Livers were removed and a transversally cut segment of the central lobe was taken, frozen, processed, sectioned and subjected to immunostaining with anti-glutathione S-transferase placental form (GST-P) antibody in order to evaluate the expression of GST-P (Taylor, 1976). Another fragment from a different lobule was stained after freezing, processing and sectioning, with hematoxylin–eosin (Melby and Altman, 1974). Both fragments were fixed in 4% neutral buffered formalin solution for 24 h prior to processing and staining. The histologically

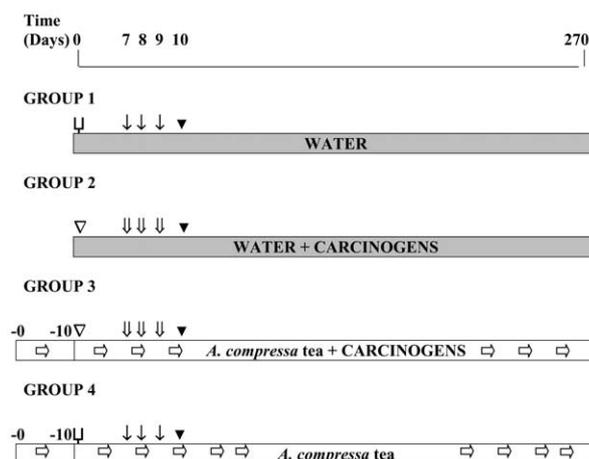


Fig. 1. Regimen for experimental hepatocarcinogenesis. \Downarrow = Water, I.P.; \Downarrow =DMSO and CMC, gavage; \blacktriangledown =partial hepatectomy; ∇ =DEN (100 mg/kg body weight in water, i.p.); \Downarrow =2-AAF (20 mg/kg body weight in DMSO and CMC, gavage); \Rightarrow =0.5% (w/v) *A. compressa* tea.

stained sections were observed and classified morphologically in order to diagnose the hepatocellular lesions.

2.4. Immunohistochemistry with anti-glutathione S-transferase placental form

Transverse histological sections (6 μm thick) were obtained from paraffin embedded samples of liver, and slides were delipidated and rehydrated by passing through graded alcohols. Specimens were stained immunohistochemically according to Taylor (1976). The primary antibody was obtained from rabbits against the placental form of glutathione S-transferase (GST-P, 1:250). The secondary antibody was an anti-rabbit IgG to which was bound horseradish peroxidase (1:500). Benzidine hydrochloride was used as a chromophore, and hematoxylin as a counterstain. The interpretation of the proliferative lesions was based on the report by Squire and Levitt (1975).

2.5. Statistical analysis

The Dunnett test was used to compare each treatment mean (G2, G3 and G4) with the negative control group (G1). The critical value of the Dunnett statistic for a two-sided test was determined using an error rate of $\alpha_E = 0.01$. The grade of hepatocellular lesions (negative = 0, foci = 1,

foci and nodules = 1.5, nodules = 2, nodules and carcinoma = 2.5 and carcinoma = 3) was computed as a measure of progression through the multiple stages of cancer and used as the response variable.

3. Results

Table 1 presents the assessment of hepatocellular lesions on rat liver stained with hematoxylin and eosin. In the negative control group (G1), only one of the rats killed at day 270 had changes indicative of initiation (micrograph not shown). This represents 6.6% of the 15 rats tested in this group. In G2, all the rats treated with the carcinogens had visible liver tumors or microscopic evidence of hepatocellular lesions by the end of the 17th week. On average, the number of tumors per liver were 3 ± 1 and 5 ± 1 in the rats killed at 120 and 270 days, respectively. None of the animals in G1, G3 or G4, developed liver tumors. An example of the lesions found is presented in Fig. 2, an acidophilic nodule in a positive control rat exposed to carcinogen with no tea treatment (Group 2). Histological examination indicated that all of the rats in the positive group develop pre-neoplastic lesions or tumors (Fig. 3). In G3, none of the rats had foci, and only one rat in each group, at days 25, 60 and 270, had pericholangiolar inflammatory cell infiltration

Table 1
Incidence of hepatocellular lesions observed in rat liver histological sections stained with hematoxylin and eosin^a

Day of sacrifice	Group 1 (Negative control, no carcinogens, no tea)	Group 2 (Positive control, carcinogens, no tea) ^b	Group 3 (carcinogens and tea) ^c	Group 4 (no carcinogens, and tea) ^c
25	Negative Negative Negative	Foci Foci Foci	Negative Negative Negative ^d	Negative Negative Negative
60	Negative Negative Negative	Foci Foci Foci	Negative Negative Negative ^d	Negative Negative Negative
90	Negative Negative Negative	Foci + nodules Foci + nodules Foci + nodules	Negative Negative Negative	Negative Negative Negative
120	Negative Negative Negative	Nodules + carcinoma Nodules + carcinoma Nodules + carcinoma	Negative Negative Negative	Negative Negative Negative
270	Negative Negative Foci	Carcinoma Carcinoma Carcinoma	Negative Negative Negative ^d	Negative Negative Negative ^d

Group 1 = Negative control: no carcinogens, no tea. Group 2 = Positive control (rats were treated according to the chemical hepatocarcinogenesis scheme): carcinogens, no tea. Group 3 = Rats were pretreated with herbal tea 10 days before be treated with the chemical hepatocarcinogenesis scheme: carcinogens and tea. Group 4 = Rats were pretreated with herbal tea 20 days before be partially hepatectomized: no carcinogens, and tea.

^a The data in the table are the results of each of three animals killed at the time indicated.

^b Treatment significantly different from the control with a significant level $\alpha = 0.01$ (Dunnett method).

^c Treatment not different from the control (Dunnett method).

^d Pericholangiolar inflammatory cell infiltration.

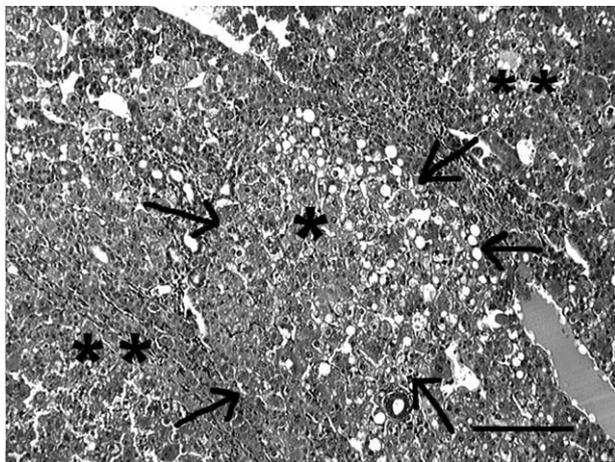


Fig. 2. Photomicrograph of an acidophilic nodule in a positive control rat exposed to carcinogen with no tea treatment (Group 2). Hepatocytes in the nodule (*) are paler, slightly larger and vacuolated compared to unaffected hepatocytes (**). Arrows delineate the borders of the nodule. (Bar = 100 μ m, Hematoxylin and Eosin).

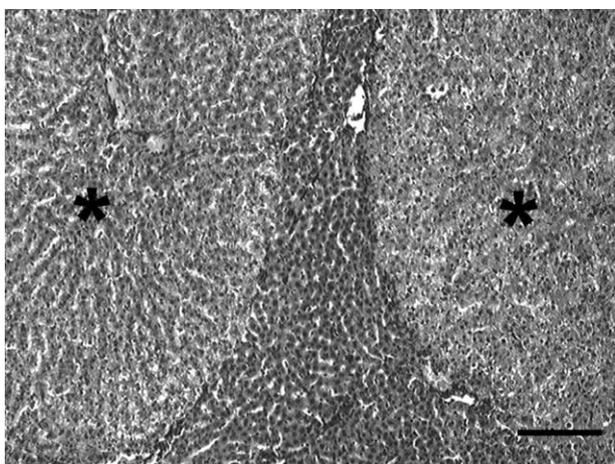


Fig. 3. Photomicrograph of two preneoplastic nodules (*) compressing normal liver between them in a rat exposed to carcinogen with no tea treatment (Group 2). These nodules are expansile and composed of slightly enlarged, pale hepatocytes (Bar = 150 μ m, Hematoxylin and Eosin).

as well as bile stasis (Table 1). Histological examination of livers from G4 (0.5% tea extract, no carcinogens) showed that none of the rats developed foci (Table 1) and only one, killed at day 270, had bile duct proliferation with inflammatory cell infiltration.

Fig. 4 shows a hepatocellular carcinoma in a rat (Group 2) at 270 days exposed to carcinogen but receiving no tea treatment. The neoplastic hepatocytes are much larger and irregular compared with the smaller, darker normal hepatocytes. A close-up photomicrograph (Fig. 5) of along the border between a hepatocellular carcinoma and normal, slightly compressed hepatic parenchyma in a rat from Group 2 at 270 days exposed to carcinogen but receiving no tea

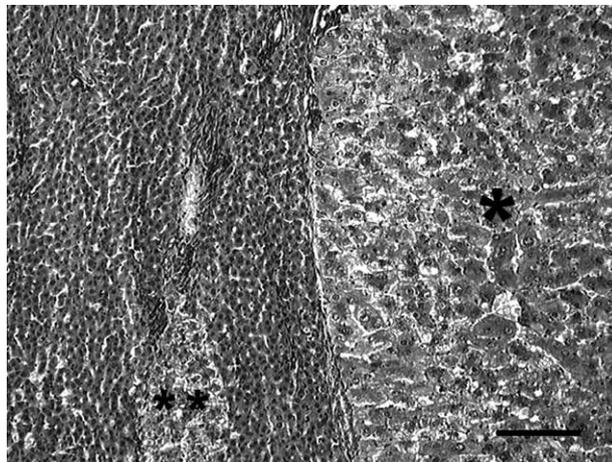


Fig. 4. Photomicrograph of a hepatocellular carcinoma (*) in a rat from Group 2 at 270 days exposed to carcinogen but receiving no tea treatment. Note that the neoplastic hepatocytes are much larger and irregular in shape compared to the smaller, darker normal hepatocytes. Invasion (**) into normal hepatocellular parenchyma is also apparent. (Bar = 100 μ m, Hematoxylin and Eosin).

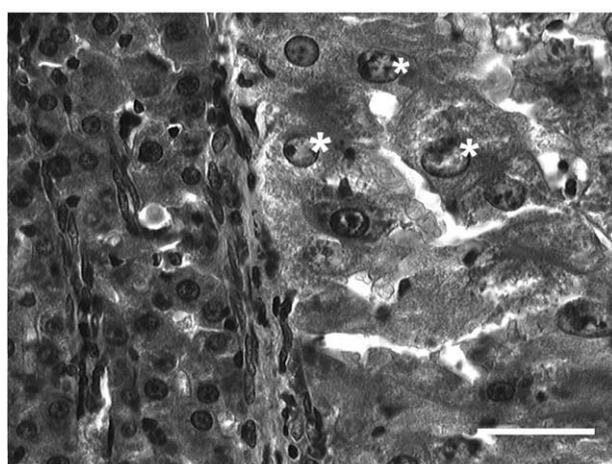


Fig. 5. Close-up photomicrograph of along the border between a hepatocellular carcinoma (right) and normal, slightly compressed hepatic parenchyma (left) from a rat from Group 2 at 270 days exposed to carcinogen but receiving no tea treatment. The neoplastic hepatocytes are quite large, are variable in size and contain enlarged nuclei (*). (Bar = 50 μ m, Hematoxylin and Eosin).

treatment. The neoplastic hepatocytes are quite large, are variable in size and contain enlarged nuclei.

Table 2 presents the expression of GST-P activity in histological sections of the liver. The results of the GST-P staining are congruent with the hepatocellular lesions shown in Table 1. One rat in the G1 group had only one positive expression of the GST-P after 270 days. In G2, all rats had GST-P positive foci in their livers.

Fig. 6 shows a small expansive GST-P positive preneoplastic nodule composed of large, pale brown staining hepatocytes, while normal hepatocytes are blue. This photomicrograph corresponds to one of the three rats killed at day 270 (G2). In G3, all the rats had negative GST-P and only one rat in each group, at days

Table 2
Incidence of hepatocellular lesions and expression of GST-P in rat liver histological sections stained immunohistochemically^a

Day of sacrifice	Group 1 (Negative control, no carcinogens, no tea)	Group 2 (Positive control, carcinogens, no tea) ^b	Group 3 (carcinogens and tea) ^c	Group 4 (no carcinogens, and only tea) ^c
25	Negative Negative Negative	Foci (+) Foci (+) Foci (+)	Negative Negative Negative ^d	Negative Negative Negative
60	Negative Negative Negative	Foci (+) Foci (+) Foci (+)	Negative Negative Negative	Negative Negative Negative
90	Negative Negative Negative	Foci + nodules (+) Foci + nodules (+) Foci + nodules (+)	Negative Negative Negative	Negative Negative Negative
120	Negative Negative Negative	Nodules + carcinoma (+) Nodules + carcinoma (+) Nodules + carcinoma (+)	Negative Negative Negative	Negative Negative Negative
270	Negative Negative Foci (+)	Carcinoma (+) Carcinoma (+) Carcinoma (+)	Negative Negative Negative ^d	Negative Negative Negative

Group 1 = Negative control: no carcinogens, no tea. Group 2 = Positive control (rats were treated according to the chemical hepatocarcinogenesis scheme): carcinogens, no tea. Group 3 = Rats were pretreated with herbal tea 10 days before be treated with the chemical hepatocarcinogenesis scheme: carcinogens and tea. Group 4 = Rats were pretreated with herbal tea 20 days before be partially hepatectomized: no carcinogens, and tea.
(+) GST-P positive hepatocellular lesions.

^a The data in the table are the results of each of three animals killed at the time indicated.

^b Treatment significantly different from the control with a significant level $\alpha = 0.01$ (Dunnett method).

^c Treatment not different from the control (Dunnett method).

^d Periportal hepatocytes for GST-P. This lesion was not a preneoplastic GST-P positive focus.

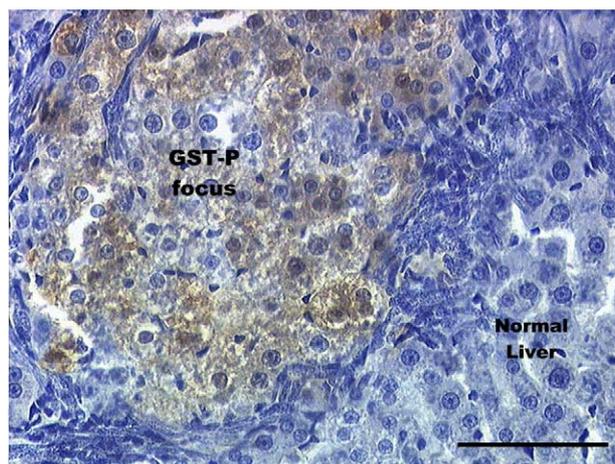


Fig. 6. Photomicrograph of a GST-P positive preneoplastic nodule in the liver of a Group 2 rat receiving carcinogen but no tea treatment. The GST-P positive hepatocytes are stained brown while normal hepatocytes are blue. (Bar = 100 μ m, Hematoxylin counterstain).

25, 60 and 270, had weakly positive periportal hepatocytes for GST-P (not shown). Finally, in G4, only one rat (Table 2) had also weakly positive periportal hepatocytes for GST-P, however, this lesion was not a preneoplastic GST-P positive focus.

4. Discussion

The results of the present investigation suggest that there is a chemopreventive effect of *A. compressa* tea extract against hepatocarcinogenesis in rats. The major advantage of the hepatocarcinogenesis model used in this study to investigate *Ardisia compressa* tea effect is that in this model, cancer arises in the natural tissue microenvironment of the animal and progresses through multiple stages much as human cancer does (Enzmann et al., 1998).

All 15 rats under the positive control group (G2) developed foci, nodules and carcinomas (Tables 1 and 2), while none in the tea group (G3) developed foci. Stated in another way, pretreatment with *A. compressa* tea resulted in significant ($P < 0.01$) protection induction of foci (0%), neoplastic nodules (0%) and hepatocellular carcinoma (0%).

The consumption of the aqueous extract of *A. compressa* by Wistar rats over a 9-month period did not elicit any signs of toxicity or apparent sign of illness. The incidence of spontaneous tumor induction in this study, using the GST-P expression as a marker, was 6.6% (G1). This finding is in line with the incidence of spontaneous neoplasms in these animals. Schulte-Hermann et al. (1983) reported that the Wistar strain

has a low background of spontaneous hepatomas (2%); therefore, this could explain the presence of a putative preneoplastic GST-P positive focus on one of the G1 animals. Several authors have also reported that a background incidence of $0.5\text{--}1.0 \times 10^3$ single GST-P positive hepatocytes may be found in livers of untreated rats (Tsuji et al., 1992; Yokota et al., 1990).

The placental form of glutathione sulfhydryl transferase is possibly the best marker for early detection of preneoplastic cells in the chemical hepatocarcinogenesis models (Sato et al., 1984). GST-P expression appears very early in initiated hepatocytes and persists in foci, nodules, and cancer but not in surrounding normal hepatocytes (Farber, 1979).

Takada et al. (2002) showed a significant suppression of the human biliary tract cancer cell growth (TGBC-2, SK-ChA-1, and NOZC-1) by 200 mM of epigallocatechin gallate (EGCG) treatment and a significant suppression of invasive ability of the same human cells at a dose of 100 mM. EGCG is a highly active principle of green tea, acts as an antioxidant, induces various phase II enzymes, inactivates DNA-reactive metabolites of genetic carcinogens and inhibits angiogenesis (Knasmüller et al., 2002). Earlier *in vitro* studies conducted in our laboratory have shown that the aqueous extract of *A. compressa* possesses antioxidant, antigenotoxic, and anticarcinogenic activities (Ramírez-Mares et al., 1999; González de Mejía and Ramírez-Mares, 2002). This protective action of the herbal tea was demonstrated at micromolar concentrations (2.52 µg eq [+] catechin/ml) in cultured rat hepatocytes.

The anticarcinogenic activities of tea polyphenols may not be due entirely to their antioxidative properties but rather to their prooxidant features (McKay and Blumberg, 2002). The prooxidant activity of tea polyphenols has been shown to be associated with induction of apoptosis in cultured cancer cells (Yang et al., 1998). In recent years, apoptosis has gained much attention as a preferential way of eliminating initiated cells (Gupta et al., 2001). Zhang et al. (2000) found that tea extracts significantly inhibited the proliferation of a rat hepatoma cell line (AH109A) through induction of apoptosis and cell cycle arrest.

Uesato et al. (2001) observed that (–)-epigallocatechin (EGC) and EGCG inhibit the growth of human HepG2 hepatocellular carcinoma cells. It has also been found that green tea drinking inhibits aberrant cryptic foci colonic tumor formation due to suppression of cell proliferation and induction of apoptosis in the intestinal crypts (Jia and Han, 2000). Jung and Ellis (2001) reported that EGCG inhibits tumor invasion and angiogenesis, processes that are essential for tumor growth and metastasis. The intestine, besides the liver, is also an important site for metabolism of food polyphenols (Piskula and Terao, 1998). Feng et al. (2001) has clearly demonstrated that tea polyphenols are

inhibitors of mitochondrial ROS production. The antigenotoxic effect of the aqueous extract of *A. compressa* demonstrated *in vitro* (Ramírez-Mares et al., 1999) may be another mechanism of chemopreventive activity of *A. compressa* tea.

Our results are in agreement with Das et al. (2002) who found that both black and green tea inhibited tumor growth and prevented metastasis as well as reduced malignancy. Our results agree also with Qin et al. (2000) and Gong et al. (2000) who found that green tea inhibits initiation and promotion of hepatocarcinogenesis in rats. The polyphenolic content of *A. compressa* tea may inhibit liver carcinogenesis by carcinogen blocking activities such as inhibition of carcinogen uptake, inhibition of formation or activation of carcinogen, prevention of carcinogen binding to DNA and enhancement of the level or fidelity of DNA repair.

The protective effect of the aqueous extract of *A. compressa* against rat liver cancer observed in this investigation may be due not only to the polyphenolic compounds but also to other tea ingredients present in the aqueous extract. In comparison to green tea, *A. compressa* has a low total polyphenol content referred as equivalents of [+] catechin. This indicates that catechins are not the main phenolic compounds in *Ardisia* tea. However, in a separate study, we have detected the presence of low concentrations of epicatechin gallate and gallic acid (unpublished results). The catechin content of *A. compressa*, in the present investigation, was 12 mg eq. [+] catechin/g dry leaves. This concentration is significantly lower than that found in the green tea from Thomas J. Lipton Co. (46 mg eq [+] catechin/g dry leaves). Therefore, the anticarcinogenic effect observed in this study is most probably related to ardisin type of compounds also found in *A. compressa* (González de Mejía et al., 2002). The role of the phenolic content of *A. compressa* tea on the observed effects is unclear. Therefore, research is currently underway in our laboratory in order to gain a better understanding of the effect of phenolic compounds from *A. compressa* on liver carcinogenesis.

As a whole, the abilities of antioxidation, antigenotoxicity and cancer prevention of *A. compressa* may depend on the combined effects of several kinds of active ingredients including ardisin, catechins, and others still undetermined compounds. This is a key topic to be studied in order to understand the mechanisms of action of *A. compressa* tea and its possible protection against liver cancer in humans. In comparison with green tea, unpublished observations in our laboratory suggest that *A. compressa* has a higher cytotoxic effect ($IC_{50} = 47$ µg eq [+] catechin/ml), on human cancer cells HepG2, than green tea ($IC_{50} = 70$ µg eq [+] catechin/ml). On the other hand, *A. compressa* tea (34 µg eq [+] catechin/ml) showed a higher anti-topoisomerase II activity (65%) than green tea (126 µg eq. catechin/ml,

15%). These results suggest the probability of a higher chemopreventive potential of *A. compressa* versus green tea.

The most notable implication of our work is that the oral ingestion of a human-achievable dose of *A. compressa* tea, results in significant inhibition of the development and progression of liver cancer in an animal model that emulates human disease. *A. compressa* tea, though effective in inhibiting rat liver carcinogenesis, needs to be tested in human cancers.

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