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# In vitro chemopreventive activity of *Camellia sinensis*, *Ilex paraguariensis* and *Ardisia compressa* tea extracts and selected polyphenols

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**Abstract**

Several herbal teas contain bioactive compounds that have been associated with a lower risk of chronic diseases including cancer. The aim of this study was to evaluate the chemopreventive activity of tea aqueous extracts and selected constituent pure polyphenols using a battery of in vitro marker systems relevant for the prevention of cancer. The effects of (−) epigallocatechin gallate (EGCG), quercetin (Q), gallic acid (GA), green tea (GT, *Camellia sinensis*), ardisia tea (AT, *Ardisia compressa*) and mate tea (MT, *Ilex paraguariensis*) extracts were tested. Cytotoxicity, TPA-induced ornithine decarboxylase (ODC) and quinone reductase (QR) activities were evaluated in vitro using HepG2 cells. The topoisomerase inhibitory activity was also tested, using the *Saccharomyces cerevisiae* yeast system. Results suggest that MT, AT and GT are cytotoxic to the HepG2 cells, with MT demonstrating dominant cytotoxicity. EGCG showed greater cytotoxicity than Q and GA against HepG2 cells. The greatest inhibition (82%) of TPA-induced ODC activity was shown by Q, with 25 μM ($IC_{50}$ = 11.90 μM). Topoisomerase II, but not topoisomerase I, was the cellular target of MT, AT, EGCG, Q and GA, which acted mainly as true catalytic inhibitors. The cytotoxic activity and the inhibition of topoisomerase II may contribute to the overall chemopreventive activity of AT and MT extracts. Ardisia and mate teas may thus share a public health potential as chemopreventive agents.


*Keywords:* Tea; Ornithine decarboxylase; Topoisomerase; Quinone reductase; Cytotoxicity; Chemoprevention

## 1. Introduction

Tea contains a high amount of flavonoids, the regular consumption of which is linked to a reduced risk for chronic diseases, including cancer, cardiovascular disease and aging [1]. Flavonoids are phenols that are widely distributed in plants; more than 4000 have been identified [2]. Several reports have attributed to green tea (GT, *Camellia sinensis*) chemopreventive and therapeutic properties [2,3]. It has also been shown that tea possesses antimutagenic, anticarcinogenic and anticlastogenic effects [4,5]. Tea contains several antioxidants, including polyphenols of the catechin (green tea) and theaflavin (black tea) groups [6]. Over the past decade, several epidemiological and case control

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studies have linked tea consumption, especially that of green tea, to a reduced risk of cancer in humans [7,8]. Evidence obtained in vitro and from in vivo animal studies suggests potential beneficial effects of tea polyphenols at most stages of cancer development [9,10].

Mate, a tea-like infusion of *Ilex paraguariensis* consumed regularly in many parts of South America, contains caffeine, minerals (P, Fe and Ca) and flavonoids, as well as Vitamin C, B1, and B2 [11]. Mate is sold in commercial herbal preparations as a central nervous system stimulant, diuretic, and antirheumatic [12]. Herbal stores in Europe, and more recently in the United States, market mate under the claim that it helps in weight management [13]. Mate tea (MT) has antimutagenic [14] and antioxidant properties [13,15]. In contrast, based on clinical and epidemiological studies, mate consumption has also been associated with the development of cancer of the oral cavity, pharynx and esophagus [16]. However, there are no data on the carcinogenicity of mate in experimental animals and the mechanism is unknown. Furthermore, the fact that the populations studied also consumed alcohol and tobacco products confounds the influence of mate as an independent factor.

*Ardisia compressa* is a tropical plant of the Myrsinaceae family that grows in the Pacific cost of Mexico. In folk medicine, ardisia tea (AT) preparations are used to treat liver cancer. In previous work in our laboratory we have shown, using rat hepatocytes, that ardisia tea has antigenotoxic, anticytotoxic and antioxidant capacity [17,18]. Furthermore, it has been found to contain the polyphenol ardisin, a potent antitopoisomerase inhibitor [19].

The widespread consumption and availability of teas throughout the world creates the possibility of exploiting their properties as chemopreventive agents. Thus, it has become necessary to define the actual magnitude of their health benefits, and elucidate the potential mechanisms of action of tea and its constituents, particularly those of polyphenols. Several assays have been developed to evaluate the ability of a compound to modulate biochemical events presumed to be mechanistically linked to carcinogenesis [20]. Examples of such assays include: (a) ornithine decarboxylase (ODC) inhibition, which is based on the fact that the induction of ODC is involved in tumor promotion and cell transformation. This approach is considered an attractive targeting strategy in both chemotherapy and chemoprevention [21]; (b) quinone reductase (QR) induction, which occurs coordinately with other phase II metabolizing enzymes by means of a variety of compounds that protect the cells from the toxic, mutagenic and neoplastic effects of carcinogens [22]; (c) topoisomerase inhibitors, which constitute a class of chemopreventive agents that inhibit carcinogenesis via their antiproliferative or cell-differentiating action [23].

The objective of this investigation was to compare the in vitro antitumor capacity of green, mate and ardisia teas and of selected polyphenols against human hepatoma (HepG2) cancer cells, by assessing their cytotoxicity, QR induction, ODC and topoisomerase inhibition.

## 2. Materials and methods

### 2.1. Chemicals

Eagle's modified minimum essential (EMEM) medium, penicillin, streptomycin, fetal bovine serum (FBS), camptothecin (CPT), amsacrine (mAMSA), dimethylsulfoxide (DMSO), adenine, EDTA, dithiothreitol, pyridoxal 5′-phosphate, L-ornithine hydrochloride, chloramine T, 12-tetradecanoyl-13-phorbol-acetate (TPA), α-difluromethyl ornithine (DFMO), Folin Ciocalteu's phenol reagent, sodium bicarbonate, (+) catechin, β-naphtoflavone, digitonin, bovine serum albumin (BSA), tween-20, glucose-6-phosphate, NADP, FAD, tris buffer, glucose-6-phosphate dehydrogenase, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT), menadione and acetonitrile were purchased from Sigma Chemical Co. (St. Louis, MO). L-[1−$^{14}$C] ornithine (100 μCi/ml) was purchased from Moravek Biochemical Inc. (Brea, CA). Peptone bacto, yeast extract, agar bacto and dextrose were purchased from Difco (Sparks, MD).

### 2.2. Teas and selected polyphenols

Green tea (*C. sinensis*) was from Lipton (Englewood Cliffs, NJ). Fresh ardisia (*A. compressa*) leaves were collected from the Pacific coast of Mexico (Michoacan State), and were first air dried without

exposure to sunlight, kept in large plastic bags and stored in a cool and dry place. Mate tea (*I. paraguariensis*) was from Gerula SA (San Jose, Misiones, Argentina). (−) Epigallocatechin gallate (EGCG), gallic acid (GA), and quercetin (Q) were chosen as common representatives of polyphenol components in teas. The pure compounds were obtained from Sigma Chemical Co. (St. Louis, MO).

### 2.3. Preparation of tea extracts

Dry leaves (2.7 g) of GT, AT or MT were separately soaked in 250 ml boiling water (94 °C) and allowed to stand for 10 min. The mixture of each tea was cooled to room temperature and filtered (0.45 μm nylon filter), freeze-dried and kept at −20 °C in plastic containers.

### 2.4. Total polyphenols of tea extracts

The total polyphenol content of the freeze-dried materials was measured as described by Nurmi et al. [24]. This method is based on the reduction of Folin-Ciocalteu reagent by the electrons from the polyphenols. Briefly, 1 ml 1 N Folin-Ciocalteu reagent and 1 ml sample were mixed and allowed to stand for 2–5 min, and then 2 ml of 20% $Na_2CO_3$ solution were added and allowed to stand for 10 min before measuring the absorbance at 730 nm using a Beckman DU® 640 spectrophotometer. The total polyphenolic content was expressed as μg equivalents of (+) catechin per ml of aqueous extract. The equation of the standard curve used was: $y = 0.0269x - 0.50$, $r^2 = 0.983$ (where, $y$: absorbance at 730 nm; $x$: polyphenol concentration; $r^2$: correlation coefficient).

### 2.5. HepG2 culture and incubation conditions

Human hepatoma carcinoma cells were purchased from American Type Culture Collection (ATCC, Rockville, MD); the cells were maintained in Eagle's modified minimum essential medium supplemented with 10% fetal bovine serum and 100 IU penicillin/100 μg streptomycin at 37 °C in a humidified atmosphere containing 5% $CO_2$. Cells were cultured in T75 flasks and were seeded once a week. Culture medium was changed three times a week.

### 2.6. Cytotoxicity assay

HepG2 cells undergoing exponential growth (70–80% of confluents) were trypsinized, suspended in fresh medium at a density of $5 \times 10^4$ cells/ml and inoculated in a 96-well flat-bottomed plate in a volume of 100 μl per well ($5 \times 10^3$ cells) and stabilized by incubation for 24 h at 37 °C in a humidified atmosphere containing 5% $CO_2$. The growth media was then removed and 100 μl of serum-free media containing various concentrations of either GT (100, 90, 80, 70, 60, 50, 40, 30, 20, 10 and 5 μg eq. (+) catechin/ml), MT (51.9, 47.6, 43.2, 38.9, 34.5, 30.3, 25.9, 21.6, 17.3, 12.9, 8.6 μg eq. (+) catechin/ml), AT (164, 82, 41, 20.5, 10.25, 5.1, 2.5, 1.27, 0.63, 0.31 and 0.15 μg eq. (+) catechin/ml), EGCG (400, 350, 300, 250, 200, 150, 100, 75, 50, 25, 5 μM), GA (500, 450, 400, 350, 300, 250, 200, 150, 100, 50, 25 μM), or Q (500, 450, 400, 350, 300, 250, 200, 150, 100, 50, 25 μM) were added directly into the cells, each one in six replications. Control cultures received the same amount of PBS and blank wells contained 100 μl of serum-free media with no cells. After the samples were added, the plates were incubated under the same conditions for 24 h. At the end of the incubation, the medium containing the various concentrations of the tea extracts or polyphenols were discarded, and the cells were washed with PBS. The viability of the cells was determined by a cell counting kit-8 (Dojindo Molecular Technologies Inc.) according to the manufacturer's protocol. The cytotoxicity of the tested crude extracts was determined by comparing the response of the cells with that of the PBS control. $IC_{50}$ values were calculated by analysis of the percent inhibition of each tea extract or polyphenol at eleven concentrations.

### 2.7. Ornithine decarboxylase (ODC) assay

Ornithine decarboxylase catalyses the decarboxylation of ornithine to putrescine, which is further converted to higher polyamines essential for the duplication of DNA. Induction of ODC is involved in tumor promotion and cell transformation, and cultured tumor cells often contain high levels of ODC [25].

Eighty percent confluent cells were washed twice with PBS, trypsinized, suspended in fresh medium and then plated at $5 \times 10^4$ cells/ml per well in 24-well

plates. Plates were placed in an incubator (37 °C, 5% $CO_2$) for 24 h. The growth media was removed and 1 ml of serum-free media containing either 5 μl of GT (4, 2 or 1 μg eq. (+) catechin/ml), MT (5, 3.1 or 1.2 μg eq. (+) catechin/ml), AT (0.6, 0.3 or 0.15 μg eq. (+) catechin/ml), EGCG (5, 2.5 or 1.25 μM), GA (50, 25 or 12.5 μM), or Q (50, 25 or 12.5 μM), dissolved in DMSO, were added. DFMO (43.7 μl) was used as a positive control and it was dissolved in serum free media (final concentration 5 mM). All of them were added with 5 μl of TPA solution (final concentration of 50 nM, dissolved in 2.5% DMSO). Additional wells received only an equivalent amount of DMSO (0.5%), tea extracts, polyphenols or TPA, and served as the experimental controls. Cells were incubated for an additional 8 h, washed twice with cold PBS, placed in −80 °C freezer until the ODC assay was performed, usually within three days. ODC activity was determined by measuring the release of $^{14}CO_2$ from L-[1-$^{14}$C] ornithine essentially according to the procedure of Lichti and Gottesman [26]. The protein content of each of the 24 wells used for the ODC assay was determined following the addition of chloramine T (50 μl, 8 mg/ml) to destroy dithiothreitol (30 min), and of NaOH (50 μl, 5.7 N) to solubilize protein [27]. The protein concentration was determined using the modified Lowry protein assay reagent kit (Pierce 23240). TPA-induced ODC activity was expressed as picomoles of $^{14}CO_2$ released per mg protein/h. The percent inhibition was calculated as follows:

$$\text{inhibition (\%)} = 1 - \frac{\text{sample pmol } ^{14}CO_2 \text{ per mg protein/h} - \text{control pmol } ^{14}CO_2 \text{ per mg protein/h}}{\text{TPA pmol } ^{14}CO_2 \text{ per mg protein/h} - \text{control pmol } ^{14}CO_2 \text{ per mg protein/h}} \times 100$$

### 2.8. Quinone reductase (QR) assay

Quinone reductase is a cytosolic FAD-dependent flavoprotein that is induced coordinately with the glutathione transferases, and which has served as a surrogate marker of phase II enzymes responsiveness in vivo and in vitro; it is thus a convenient screening method for anti-carcinogenic activity [28].

The QR induction activities of either GT, MT or AT were determined using Rochaska and Santamaria assay [29]. The cells were grown in 96-well plates (Costar 3595, Corning Inc., Corning, NY), $2.5 \times 10^4$ cells per well for 24 h and then exposed to the different samples (GT, 0.5–8.0 μg eq. (+) catechin/ml; MT, 0.5–30 μg eq. (+) catechin/ml and AT, 5.0–80.0 μg eq. (+) catechin/ml) for 24 h. Growth media and 5 μM β-naphtoflavone were used as negative and positive controls, respectively. Treated cells were rinsed with PBS pH 7.4, lysed with 50 μl 0.8% digitonin in 2 mM EDTA, incubated and agitated for 10 min. Two hundred microliters mixed solutions [74 ml, 25 mM tris buffer; 50 mg BSA; 0.5 ml, 1.5% tween-20 solution; 0.5 ml thawed cofactor solution (92.7%, 150 mM glucose-6-phosphate; 6.15%, 4.5 mM NADP; 1.14%, 0.75 mM FAD in Tris buffer), 150 units of glucose-6-phosphate dehydrogenase, 22.5 mg MTT (3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide), and 75 μl, 50 mM menadione in acetonitrile] were added into lysed cells. Reading was done at five time points, 50 s apart using a μQuant microplate reader (Bio-Tek Instruments Inc., Winooski, VT) at 610 nm. Immediately after completion of the readings, 50 μl of 0.3 mM dicumarol in 25 mM tris buffer was added into each well and the plate was read again (five time points, 50 s apart). Total protein content was measured by BioRad assay [30]. QR induction activity was expressed as the specific activity (nmol MTT reduced/mg/min) ratio of treated to control cells.

### 2.9. Antitopoisomerase assay

#### 2.9.1. Yeast strains

The yeast strains used in this study are listed in Table 1. *Saccharomyces cerevisiae* strains JN362a, JN394, JN394$t_{-1}$, JN394$t_{2-4}$, JN394$t_{2-5}$, were kindly provided by Dr. John Nitiss of St. Jude Children's Research Hospital, Memphis, Tennessee.

#### 2.9.2. Assay

The requirement of topoisomerase II (Topo II) for the completion of mitosis makes this enzyme essential for cell division and cell proliferation. Differentiated cells express very low levels of Topo II, while highly proliferative and tumor cells often express 25–300 times the levels of quiescent cells [31].

Tea extracts, polyphenols, m-AMSA, CPT or DMSO (vehicle control) sensitivity measurements were carried out in YPDA medium (yeast extract, peptone, dextrose and agar) as previously described

Table 1
Genetically modified *Saccharomyces cerevisiae* strains used in this study

| Strain | Description | Genotype[a] |
|---|---|---|
| JN362a | DNA repair proficient | *Matα ura3–52 leu2 trp1 his7 ade1–2 ISE2* |
| JN394 | DNA repair deficient | *Matα ura3–52 leu2 trp1 his7 ade1–2 ISE2 rad52::LEU2* |
| JN394$t_{-1}$ | JN394 with *TOP1* disruption | *Matα ura3–52 leu2 trp1 his7 ade1–2 ISE2 rad52:TRP1 Δ top1::LEU2* |
| JN394$t_{2-4}$ | JN394 with temperature-sensitive allele of *TOP2* | *Matα ISE2 ura3–52 top2–4 rad52::LEU2* |
| JN394$t_{2-5}$ | JN394 with temperature-sensitive allele of *TOP2* | *Matα ura3–52 leu2 trp1 his7 ade1–2 ISE2 top2–5 rad52::LEU2* |

[a] For the complete genotype and details of construction of these strains, see references [31,49,50].

[32]. Logarithmically growing cells (JN362a, JN394, JN394$t_{-1}$, JN394$t_{2-4}$ or JN394$t_{2-5}$) were adjusted to an initial cell titer of $2 \times 10^6$ cells/ml. In all cases, cells were pre-grown at the same temperature that was used to measure drug sensitivity. Either GT (126 μg eq. (+) catechin/ml), MT (57.5 μg eq. (+) catechin/ml), AT (34 μg eq. (+) catechin/ml), EGCG (150 μM), GA (300 μM), Q (300 μM), m-AMSA (100 μg/ml), CPT (50 μg/ml) or DMSO (1.66%) were added to the cultures and cells were incubated for 24 h. Viable counts were determined by duplicate plating to YPDA medium solidified with 1.75% agar Bacto. Plates were incubated at the optimal temperature for growth of the cells to determine viable titer (25 °C for temperature sensitive top2 mutants, 30 °C otherwise). The percent survival was determined by comparison of the number of colonies counted in the no-drug control culture with those in the drug-treated culture. The $IC_{50}$ is the drug concentration that reduces the number of colonies (in this case in the strain JN394$t_{2-4}$) by 50% as compared with cells grown in the absence of the drugs; this was determined using dilutions of each sample: MT (57.5, 28.7, 5.75 or 0.575 μg eq. (+) catechin/ml), AT (34, 17, 3.4 or 0.34 μg eq. (+) catechin/ml), EGCG (150, 75, 15 or 1.5 μM), GA (300, 150, 30 or 3 μM), Q (300, 150, 30 or 3 μM). All experiments were repeated at least three times, and the means and population standard deviations were calculated for each of the data.

### 2.10. Statistical analysis

Results are expressed as the mean ± S.D.of values obtained in at least triplicate from three different experiments. Differences between groups were compared by Students's *t*-test; *P* values less than 0.05 were considered significant. When more than two means were compared, significance was determined by one-way analysis of variance followed by pairwise comparisons using the Tukey test.

## 3. Results and discussion

### 3.1. Cytotoxicity of the tea extracts and polyphenols

Cytotoxicity is a parameter consistent with anti-tumor activity [33]. Fig. 1 shows the dose response curves of HepG2 cells treated with AT, GT, MT, Q, EGCG and GA. The results from the in vitro anti-hepatoma evaluation of the selected tea extracts and polyphenols are summarized in Table 2, which lists the mean $IC_{10}$, $IC_{50}$ and $IC_{90}$ values against the cells. The six samples tested exhibited a certain degree of cytotoxicity against HepG2 cell. Among the tea extracts, MT demonstrated dominant cytotoxicity, with an $IC_{50}$ value of 12.01 μg eq. (+) catechin/ml. AT and GT had $IC_{50}$ values of 46.91 and 72.05 μg eq. (+) catechin/ml, respectively. Among the pure polyphenols, EGCG showed the greatest cytotoxicity, with an $IC_{50}$ value of 210.7 μM. This result is in agreement with the value ($IC_{50} = 196.4$ μM) obtained by Uesato et al. [34]. Q and GA had $IC_{50}$ values of 391.7 and 422.7 μM, respectively. Other polyphenols present in teas were also tested such as theobromine ($IC_{50} = 7676$ μM), chlorogenic acid ($IC_{50} = 2613$ μM), flavianic acid ($IC_{50} = 474$ μM) and epicatechin gallate ($IC_{50} = 144$ μM). The short-term assays used, such as the cell counting kit-8 (CCK-8), assess the killing due mainly to apoptosis, particularly that of early-stage apoptosis [35]. HepG2 cells have the wild-type p53 gene; the dead cells presented morphological changes

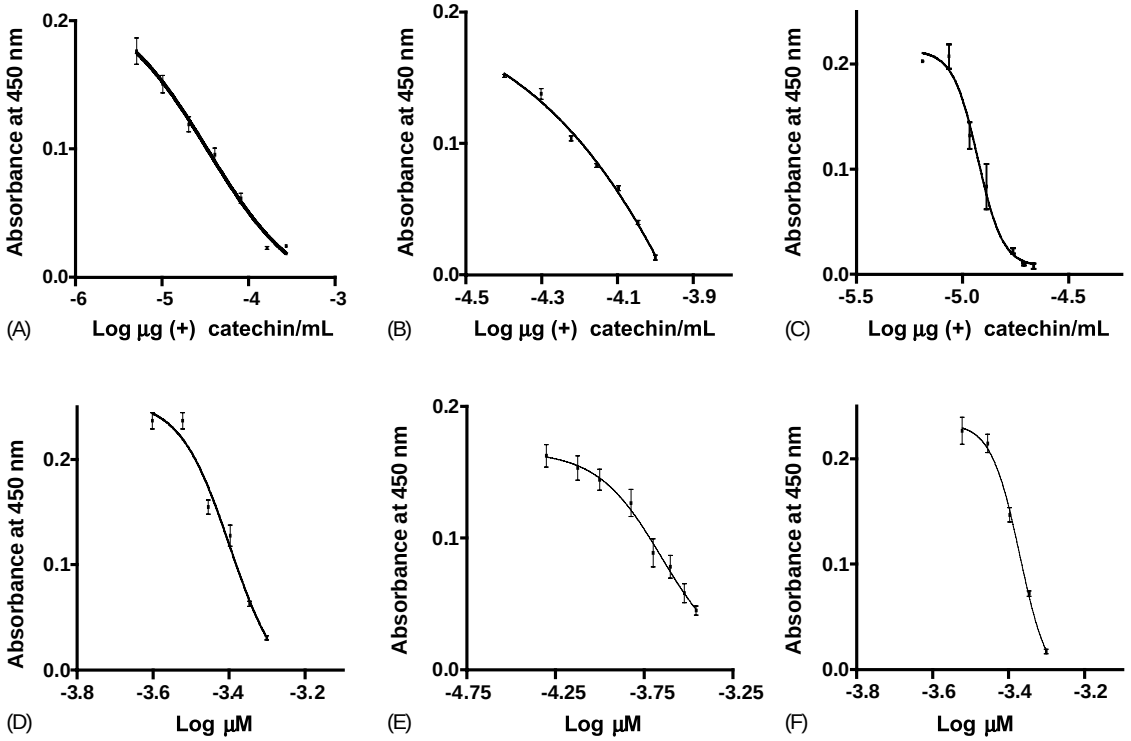


Fig. 1. Cytotoxicity assay. Cells were treated with various concentrations of *Ardisia compressa* (A), *Camellia sinensis* (B), *Ilex paraguariensis* (C), quercetin (D), EGCG (E) or gallic acid (F) for 24 h. Cell viability was determined by CCK-8 assay as described under the materials and methods section. Each curve represents the average of three independent experiments. $IC_{50}$ was calculated from the antilog of the *x*-axis value at the inflection point of each sigmoid-curve fit.

such as membrane blebbing, aggregation of chromatin, and formation of membrane bound vesicles, all of which are well-characterized features of cell death due to apoptosis. These results suggest that the cytotoxicity of the teas and polyphenols tested may confer antihepatoma activity, but the mechanisms remain to be studied. However, in order to explore the possible cytotoxic potential of MT and AT (the less studied samples), further studies will have to be carried out using animal models, and the mechanisms of action as well as the active compounds involved in the pharmacological process also need to be investigated.

### 3.2. Ornithine decarboxylase (ODC) activity

The characterization of TPA-induced ODC activity in HepG2 cells showed a substantial ODC

Table 2
Inhibitory effect of mate tea, ardisia tea, green tea and tea polyphenols against growth of HepG2 cancer cells

| | µg eq. (+) catechin/ml ± S.D. | | | µM ± S.D. | | |
|---|---|---|---|---|---|---|
| | MT | AT | GT | EGCG | Q | GA |
| $IC_{10}$ | 9.3 ± 0.6 | 4.9 ± 1.4 | 50.7 ± 2.5 | 9.0 ± 2.1 | 56.2 ± 4.9 | 51.3 ± 2.5 |
| $IC_{50}$ | 12.0 ± 0.2 | 46.9 ± 3.3 | 72.0 ± 1.8 | 216.4 ± 20.4 | 391.7 ± 3.0 | 422.7 ± 6.7 |
| $IC_{90}$ | 17.6 ± 0.8 | 177.2 ± 33.4 | 113.6 ± 5.5 | 448.4 ± 12.3 | 518.8 ± 8.4 | 495.5 ± 8.0 |

$IC_{50}$ was calculated from the antilog of the *x*-axis value at the inflection point of each sigmoid-curve fit generated for each treatment (as shown in Fig. 1). The $IC_{10}$ and $IC_{90}$ were calculated using the log $IC_{50}$ and the Hill slope (GraphPad software Prism version 4).

induction after 8 h of treatment with 50 nM TPA (data not shown). ODC levels increased about 13-fold, from 126.21 ± 14.27 to 1644.57 ± 198.97 pmol/mg. The amounts of tea extracts and polyphenols used to test inhibition in the ODC assay were selected from the results obtained in the cytotoxicity assay (Table 2) in order to prevent ODC inhibition from being associated with cytotoxicity. The cytotoxic concentrations used in this study were minimized with the intention of finding specific interactions between the inhibitor and the ODC enzyme, as a good inhibitor of ODC activity requires less substance. Five mM DFMO, an irreversible inhibitor of ODC activity, completely inhibited induction of ODC by 50 nM TPA. Fig. 2 shows that none of the tea extracts inhibited the TPA-induced ODC activity at the concentrations tested; surprisingly, all of them increased the levels of ODC. AT (0.6 μg eq. (+) catechin/ml) increased the level of ODC about 3700% in comparison to DMSO treated cells and 285% relative to the TPA treated cells. GT (1.0 μg eq. (+) catechin/ml) and MT (5.0 μg eq. (+) catechin/ml) gave an increased value of 252 and 187%, respectively, in comparison to the TPA control cells. AT (0.6 μg eq. (+) catechin/ml) and GT (4 and 2 μg eq. (+) catechin/ml) significantly increased the value of ODC activity about 3.0, 2.5 and 2.5-fold, respectively, in not TPA treated HepG2 cells.

As shown in Fig. 3, among the polyphenols tested, Q provided significant inhibition of the ODC activity. The greatest inhibition (82%) was obtained with 25 μM Q ($IC_{50}$ = 11.90 μM). EGCG and GA did not inhibit the TPA-induced ODC activity at the tested concentrations; instead, both of them actually increased the value, ranging from 1.3- to 2.3-fold. No inhibition of ODC induction mediated by TPA was obtained by Gerhäuser et al. [25] using 10 μM EGCG in mouse 308 cells. It is interesting that MT, AT, GT, EGCG and GA increased the value of TPA-induced ODC activity in HepG2 cells. It has been demonstrated that incubation of human lung adenocarcinoma cells in cell growth media containing tea polyphenols (EGC and EGCG) induced the dose-dependent formation of $H_2O_2$ [36]. It has also been observed that the incubation of cell culture media with EGCG, EGC and GA leads to the production of substantial amounts of $H_2O_2$ [37,38]. Low levels of $H_2O_2$ can have the effect of increasing the proliferation and expression of growth-related genes in some cell types

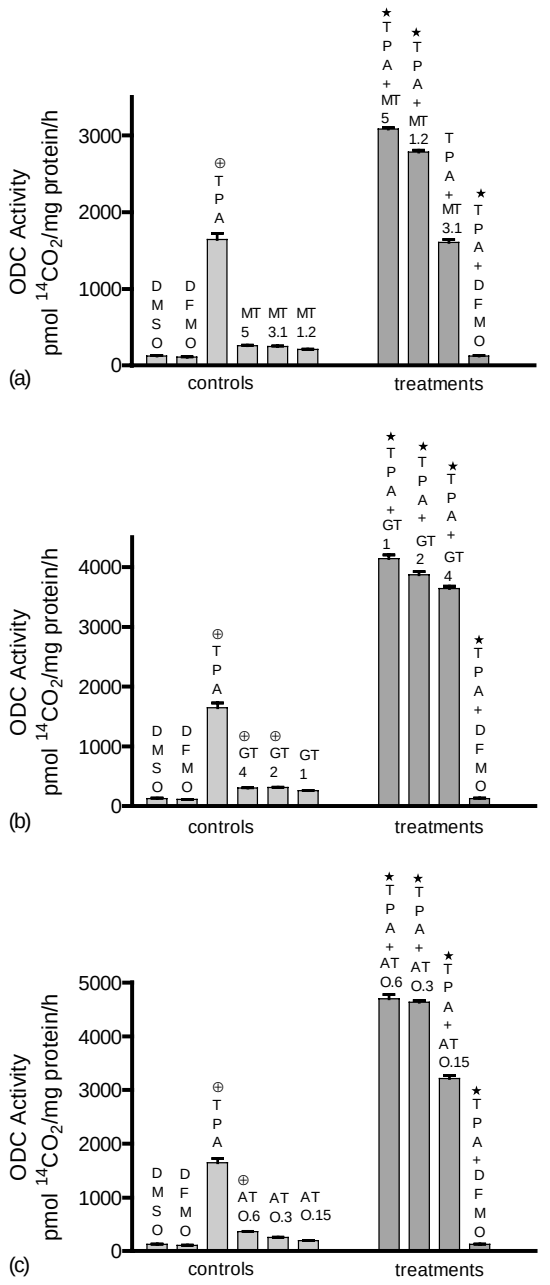


Fig. 2. Effect of (a) mate tea (MT), (b) green tea (GT) and (c) ardisia tea (AT), treatment on TPA-induced ODC activity in HepG2 cells. The numbers indicate the μg equivalents to (+) catechin/ml tested of each aqueous extract. DMSO (0.5%), DFMO (5 mM) and TPA (50 nM) were used as controls. Each value is the mean ± S.D. of three experiments. ⊕ Significantly different from DMSO ($P < 0.05$). ★ Significantly different from TPA ($P < 0.001$).

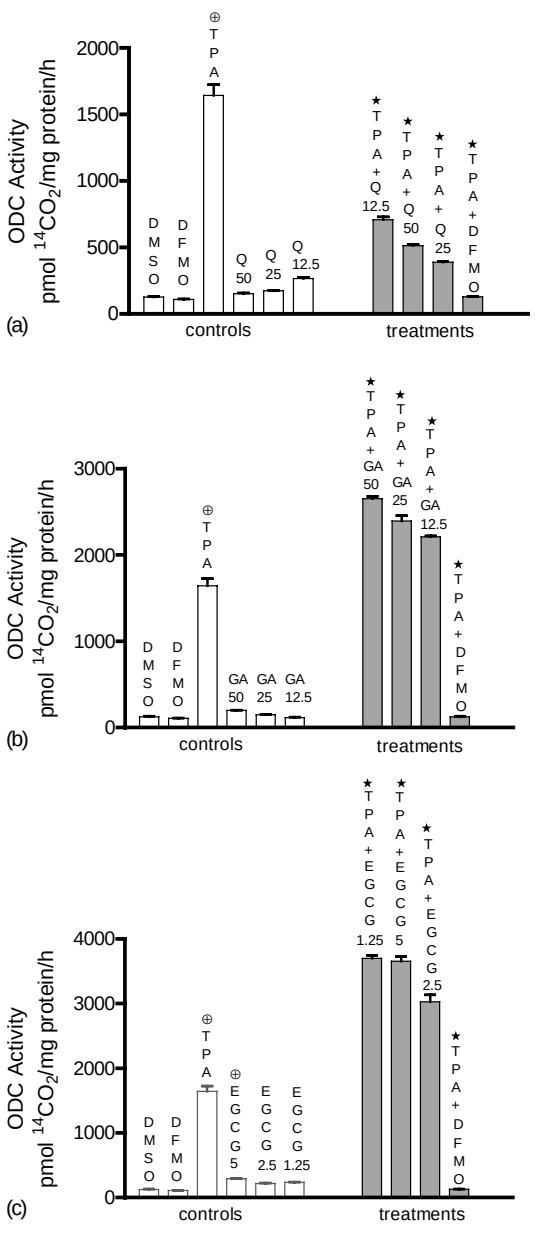


Fig. 3. Effect of (a) quercetin (Q), (b) gallic acid (GA) and (c) (−) epigallocatechin gallate (EGCG), treatment on TPA-induced ODC activity in HepG2 cells. The numbers indicate the μM concentration tested of each polyphenol. DMSO (0.5%), DFMO (5 mM) and TPA (50 nM) were used as controls. Each value is the mean ± S.D. of three experiments. ⊕ Significantly different from DMSO ($P < 0.05$). ★ Significantly different from TPA ($P < 0.001$).

[39]; this may be the case for the samples used in this work. Moreover, ODC induction was suppressed by superoxide dismutase and catalase in murine mammary tumor cells [40], suggesting the intermediacy of reactive oxygen species in tumor promotion. This may explain the ineffectiveness of MT, AT, GT, EGCG and GA to inhibit the TPA-induced ODC activity. In HepG2 cells exposed to antioxidants, AP-1-dependent gene expression, mitogen-activated protein kinases, and ODC expression were all induced [41–43]. It is possible that the antioxidants per se also exert an effect on ODC induction when TPA is present, but further work should be conducted to prove this. Alternatively, the effects of antioxidants may be due to secondary effects on intracellular redox potential, rather than on ROS per se; i.e. through an effector signal that induces apoptosis, or augments transduction of signals that induce apoptosis [44]. This possibility suggests that the results we obtained for the teas and polyphenols are dependent on the sample concentration used, the antioxidant capacity (GT > MT > AT and Q > EGCG > GA), differences in tea composition, and on the intracellular redox potential they induce; the most important factor, however, remains the type of cells used. It is known that GT reduced the activity of ODC in rat tracheal epithelial cells [28]. EGCG reduced the level of ODC in transformed NIH-pATMras fibroblasts [45], but it was ineffective in rat tracheal epithelial cells [28] and in mouse 308 cells [25]. Polyphenols in tea are able to inhibit ODC even in the presence of TPA in other types of cells such as mouse skin and NIH 3T3 cells [46]. Despite the results obtained in this study (i.e. in liver cells), ODC has proven to be a very powerful marker with which to detect the proliferation of different types of cancer cells [47]. This test is sensitive and fast, and it could also be used to test solid tumors of patients.

### 3.3. Quinone reductase (QR) activity

We evaluated whether AT, MT and GT, implicated as potential chemopreventive agents, could influence phase II enzymatic activity in HepG2 cells. Surprisingly, at the concentrations used, the teas failed to induce quinone reductase altogether (data not shown). The ability of a compound to act as a phase II enzyme-inducing agent may depend on the unique profile of gene expression of each cell line. Phase II

enzyme response in HepG2 cells differs significantly [48] from that reported for the murine hepatoma cell line Hepa1c1c7 (the cell line most commonly used to screen for phase II enzyme-inducing compounds). These differences may be partly attributable to their species of origin, particularly since rodent cells are more labile in their phase II-enzyme response than human cells [21].

### 3.4. Antitopoisomerase activity

The concentrations of the teas (GT, AT and MT) used in this assay were based on the solubility factor of each freeze-dried tea extract in DMSO. As shown in Table 3, the strain JN394 was hypersensitive to CPT (99%), which is a Topo I poison, and to m-AMSA (99%) which is a Topo II poison. MT and AT showed 73 and 65% inhibition, respectively. The green tea used showed low topoisomerase inhibition (15%) in comparison to the other teas. We are currently performing additional assays to determine the reason for this observation, as this result stands in contrast with using pure EGCG, a main green tea component, as shown in the present investigation. The growth of JN394 was also inhibited by EGCG (75%), Q (79%) and GA (75%). The strain JN394 is DNA repair-deficient and drug-permeable (carry *ise2* and *rad52* mutations) [49]. These mutations increase the sensitivity of these cells to drugs.

The yeast JN362a, a DNA repair-proficient strain [49], was not affected by GT (126 μg eq. (+) catechin/ml), MT (57.5 μg eq. (+) catechin/ml), AT (34 μg eq. (+) catechin/ml), EGCG (150 μM), GA (300 μM) or Q (300 μM) (data not shown).The strain JN394$t_{-1}$ is isogenic to JN394 and contains a disrupted top1 gene [49]; the absence of the gene resulted in diminished cytotoxicity of antitopoisomerase I drugs. Table 3 shows that CPT failed to reduce the growth of these mutant cells (JN394$t_{-1}$). In contrast, no resistance was observed when top1 cells were treated with m-AMSA (99%), MT (83%), AT (88%) or all of the polyphenols studied (76–87%). Sensitivity in the top1 cells was higher than in the parent cells (JN394). This finding provided evidence that Topo I was not the cellular target of MT, AT, EGCG, Q or GA.

MT and AT teas, and polyphenols (EGCG, GA and Q) displayed weak cytotoxicity (0–22%) toward JN394$t_{2–4}$ yeast at 25 °C even though the inhibition of the wild type strain JN394 was strong. This can be explained by the fact that the number of copies of the $top_{2–4}$ allele present in strain 394$t_{2–4}$ is higher than the number of copies of the top II gene in the wild type.

The strain 394$t_{2–5}$ carries a top2 allele that is resistant to multiple classes of topoisomerase II poisons at its permissive temperature (25 °C) [50]. Table 3 shows that cells with the top2–5 mutation are able to grow in m-AMSA (48%). The sensitivity of the top2–5 strain to CPT (99%), teas (0–20%) and polyphenols (0–5%) is also shown in Table 3. The strain has essentially the same sensitivity to CPT as JN394 (*rad52 top2+* cells), indicating that the observed resistance is specific to anti-topoisomerase II agents.

Eliminating the possibility that Topo I is the target of the teas and polyphenols leaves two additional possibilities for the physiological mechanism of

Table 3
Percent[a] inhibition of mate tea, ardisia tea and tea polyphenols treatments on the survival of different mutants strains of *Saccharomyces cerevisiae*

| | Concentration (μg/ml) | JN394 | JN394$t_{-1}$ | JN394$t_{2–5}$ (25 °C) | JN394$t_{2–4}$ (25 °C) | JN394$t_{2–4}$ (30 °C) |
|---|---|---|---|---|---|---|
| MT | 57[b] | 73 ± 4.3 | 83 ± 3.9 | 14 ± 2.1 | 13 ± 2.3 | 99 ± 0.2 |
| AT | 34[b] | 65 ± 4.0 | 88 ± 4.1 | 20 ± 2.9 | 0 | 99 ± 0.2 |
| GT | 126[b] | 15 ± 4.0 | ND | ND | ND | ND |
| EGCG | 69 | 75 ± 2.0 | 76 ± 3.0 | 5 ± 2.2 | 4 ± 2.4 | 99 ± 0.2 |
| Q | 101 | 79 ± 2.2 | 87 ± 2.3 | 0 | 22 ± 3.3 | 99 ± 0.2 |
| GA | 56 | 75 ± 2.6 | 86 ± 2.6 | 0 | 0 | 99 ± 0.2 |
| AMSA | 100 | 99 ± 0.2 | 99 ± 0.2 | 48 ± 4.2 | 99 ± 0.2 | 93 ± 3.1 |
| CPT | 50 | 99 ± 0.2 | 0 | 99 ± 0.2 | 80 ± 4.0 | 99 ± 0.2 |

DMSO (1.66%) was used as a control and all the results were referred to this value. ND: not determined.

[a] Values are mean ± S.D.

[b] Microgram equivalents of (+) catechin.

cytotoxicity. The first is that Topo II is the primary target responsible for killing cells by trapping the enzyme-mediated DNA cleavage. The second is that the cytotoxicity is correlated with the ability to block the overall catalytic activity of the enzyme. The possibilities described above can be scrutinized by utilizing a yeast strain JN394$t_{2-4}$. This yeast strain expresses the temperature-sensitive top2–4 mutant in place of the wild type top2 gene [49]. The top2–4 protein shows wild type activity at 25 °C, while its activity is reduced to about 5–10% of that of the wild type at the semi-permissive temperature of 30 °C. Therefore, if the polyphenols or teas function as Topo II "poison", a reduction in enzyme activity should greatly diminish the induced cell death. Conversely, if the cytotoxicity is correlated to the ability to impair the catalytic function of the enzyme, cells with decreased levels of Topo II activity should become hypersensitive. m-AMSA was a potent toxic agent toward JN394$t_{2-4}$ yeast cells at 25 °C (Table 3). A smaller value (Table 3) was obtained when the cytotoxicity of m-AMSA was examined at 30 °C. The sensitivity to CPT at 30 °C (99%) was greater than at 25 °C (80%) (Table 3). The teas (MT and AT) and polyphenols (EGCG, GA and Q) displayed weak cytotoxicity (0–22%) toward JN394 $t_{2-4}$ yeast at 25 °C, even though the inhibition of the wild type strain JN394 was strong. This can be explained by the fact that the number of copies of the $top_{2-4}$ allele present in the strain 394$t_{2-4}$ has been increased. Top 2–4 cells were hypersensitive at the semi-permissive temperature (30 °C). The teas (MT and AT) and polyphenols (EGCG, GA and Q) further reduced the level of Topo II activity and the cell killing was enhanced to 99% (Table 3). The increased toxicity toward cells that contain decreased levels of Topo II activity strongly suggests that the catalytic activity of Topo II is the primary physiological target of ardisia and mate teas, as well as of EGCG, GA and Q.The 50% inhibitory concentration ($IC_{50}$) was determined for MT, AT and the polyphenols using the JN394 $t_{2-4}$ yeast strain at 30 °C (Fig. 4). Ardisia tea showed a greater activity against the JN394 $t_{2-4}$ strain with $IC_{50}$ of $13.8 \pm 3$ μg eq. (+) catechin/ml in comparison with mate ($26.3 \pm 3$ μg eq. (+) catechin/ml). Among the polyphenols, EGCG showed the greatest inhibition with an $IC_{50}$ value of $75.8 \pm 4$ μM. Q and GA had $IC_{50}$ values of $87.0 \pm 4$ and $112.2 \pm 5$ μM, respectively. Cho et al. [23] assessed quercetin by the unknotting assay; this assay can identify catalytic inhibitors of Topo II. The $IC_{50}$ obtained in the mentioned study was 6.9 μg/ml, which is lower than the value we observed with JN394$t_{2-4}$ (87.0 μM = 29.5 μg/ml). This apparent discrepancy can be explained by the differences between both bioassays. The most important result is that Q, independently of the type of assay used, has antitopoisomerase II activity, like a true catalytic inhibitor. It has been shown that EGCG inhibits topoisomerase I ($IC_{50}$ = 5 mM) from calf thymus gland, and topoisomerase II ($IC_{50}$ = 3 mM) from human placenta [51], but the in vitro assays used revealed that the mechanism of inhibition is by the polyphenol acting as a Topo I and II poison. A significant inhibition of topoisomerase I in SW 480 human colon carcinoma cells was observed with EGCG treatment at concentrations between 110 and 550 μM, but EGCG had no effect on topoisomerase II activity [52]. Our data shows that EGCG has antitopoisomerase II activity like a catalytic inhibitor; this is the first report that has identified such a property. Cho et al. [23] assessed GA, and no antitopoisomerase activity was observed up to100 μg/ml. The results of the present work show that GA has antitopoisomerase II activity (112.2 μM = 21.2 μg/ml) like a catalytic inhibitor; this inconsistency should be the motive of further research. We have demonstrated that MT and AT inhibited the catalytic activity of Topo II; this observation could provide useful information for the rational design of future research.

In summary, the results of the present study indicate that MT, AT and GT are cytotoxic for the HepG2 cells and that, among them, MT demonstrated dominant cytotoxicity. EGCG showed greater cytotoxicity than Q and GA against HepG2 cells. The data revealed a significant increase in the TPA-induced ODC activity by MT, AT, GT, EGCG and GA, but further research with HepG2 cells will be required to clarify the importance of this observation. The greatest inhibition of TPA-induced ODC activity by Q (82%) was obtained with 25 μM ($IC_{50}$ = 11.90 μM). Topoisomerase II, but not topoisomerase I, was the cellular target of MT, AT, EGCG, Q and GA, which acted mainly as true catalytic inhibitors. The cytotoxic activity and the inhibition of topoisomerase II may contribute to the overall chemopreventive activity of MT and

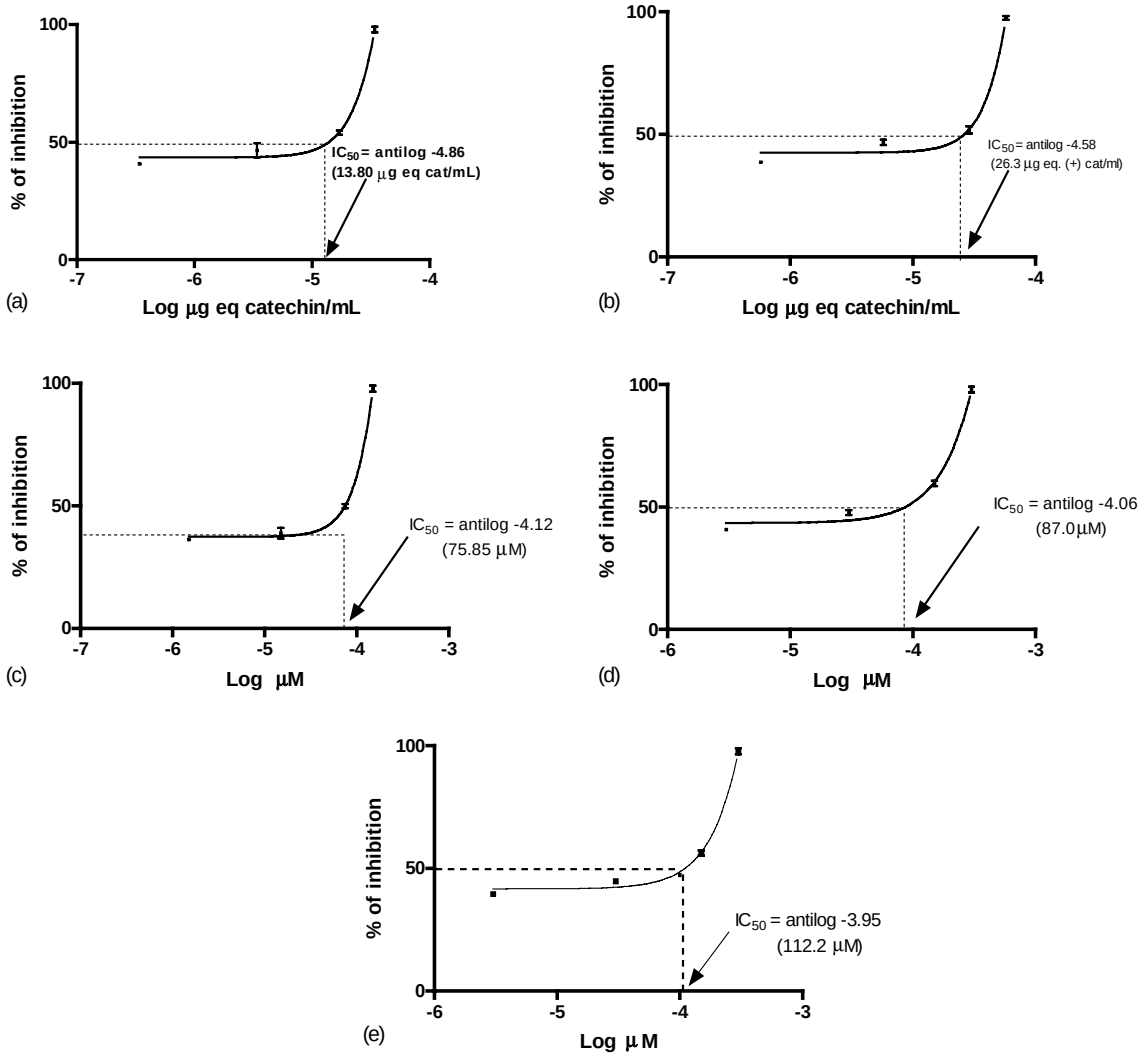


Fig. 4. Strain JN394$t_{2–4}$ was treated at 30 °C with increasing concentrations of either AT (a), MT (b), EGCG (c), Q (d) or GA (e). After 24 h, two aliquots of cells were taken, plated in YPDA and grown for 3–4 days to determine the titer. The percent survival was determined by comparison with the DMSO control. Plots represent an average of two independent experiments. $IC_{50}$ was calculated from the antilog of the *x*-axis value.

AT extracts, making of them promising candidates responsible for the chemopreventive properties of tea.

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