Original Article

www.advmolmed.com
doi:10.2399/amm.07.069

Advances in
Molecular Medicine
International Journal of Molecular Biology, Biochemistry and Gene Technology

The differential anticancer effects of green tea in estrogen receptor-positive and estrogen receptor-negative human breast cancer cell lines

Ferda Özdikicioğlu[1], Engin Ulukaya[2]* , Arzu Yılmaztepe Oral[2], Mustafa Z. Özel[3], Özkan Kutlular[3]

[1]Department of Biochemistry Biology, Faculty of Science and Art, Uludağ University, Bursa, Turkey

[2]Department of Biochemistry, Faculty of Medicine, Uludağ University, Bursa, Turkey

[3]Department Chemistry , Faculty of Science and Arts, The University of Pamukkale, Denizli, Turkey

Green tea components exert many biological effects, including antitumor and cancer preventive activities. In the search for breast cancer, the effects of green tea extract (GTE) were tested on the estrogen receptor-positive (MCF-7) and estrogen receptor-negative (MDA-MB-231) human breast cancer cell lines. The growth inhibition by GTE was tested by the MTT assay in which the number of alive cells is proportional to the amount of formazan produced by the cells, which is measured by ELISA. GTE treatment resulted in a dose-dependent inhibition of cell growth in MDA-MB-231 cell line. However it did not show any inhibition effect in MCF-7 cell line. GTE further increased the cytotoxic activity of classical FEC treatment at the lowest doses in MDA-MB-231 cell line. However, as contrast, it diminished the effect of FEC treatment in MCF-7 cell line. These findings suggest that the effect of green tea depends on the estrogen-receptor status of the breast cancer cell line.

Key words: Green tea, breast cancer, the MTT assay

Adv Mol Med 2007; 3(2): 69-75

Introduction

Tea [*Camellia sinesis* (Theacacea)] is one of the most-consumed beverages worldwide. Green tea is produced by drying fresh leaves from plant Camellia sinensis. Its composition is similar to that of fresh tea leaves, which contains characteristic polyphenolic compounds, epigallocatechin-3-gallate (EGCG), epigallocatechin (EGC), epicatechin-3-gallate (ECG) and epicatechin (EC).[1] Consumption of tea has been associated with many health benefits and their role in cancer chemoprevention has been studied extensively.[2-3] *In vivo* and *in vitro* experimental studies have demonstrated anticancer activity for green tea, including inhibitory effects on tumor formation, growth, invasion and metastasis.[4-7]

Breast cancer is the most common cancer in women and makes up one tenth of all new cancer diagnoses worldwide.[8] Epidemiological studies sug-

*Correspondence to: Engin Ulukaya MD, PhD
Uludağ University Medical School
Biochemistry and Clinical Chemistry Department
16059 Gorukle Bursa, Turkey
Phone: +90 224 442 82 45
e-mail: eulukaya@uludag.edu.tr

Accepted: May 12, 2007, Published online: March 5, 2008

gest that increased consumption of green tea is also related to improved prognosis of human breast cancer.[9] An inverse association between the risk of breast cancer and the intake of green tea has also been reported in Asian- Americans.[10-11] Although green tea and its constituents have been shown to inhibit breast cancer, the mechanism(s) of the inhibition is not completely known.[12-14]

In this study with two different breast cancer cell lines in terms of the estrogen receptor status, we evaluated the growth inhibition of GTE and its combinations with anticancer drugs which are used for the classical of breast cancer therapy. Our results suggest that the effect of GTE depends on the estrogen-receptor status of the breast cancer cell line.

Materials and Methods

Cell culture

A breast cancer cell lines, called MCF-7 (estrogen receptor-positive) and MDA-MB-231 (estrogen receptor-negative), were used in the study. The cells were cultured in RPMI 1640 supplemented with penicillin G (100 U/ml), streptomycin (100 µg/ml), L-glutamine, and 10% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2.

Preparation of green tea extracts: Superheated water extraction

GTE was prepared by Superheated Water Extraction methods. A detailed description of the laboratory-built SWE apparatus has been given elsewhere.[15] The water was purged with nitrogen to remove dissolved oxygen prior to the extraction. Deoxygenated water was used in an HPLC pump programmed for a constant flow of 2 mL.min.$^{-1}$ A Carlo Erba series 4200 GC oven heated the extraction system. A 3 m long pre-heated coil (0.76 mm i.d. x 1.6 mm o.d.) was used to equilibrate the water to the desired temperatures. A 24 mL extraction cell (Keystone Scientific, Bellefonte, Pa., USA) equipped with a 0.5 mm frit both at the inlet and outlet was connected to a 1-m cooling loop (in iced water) outside of the oven. A pressure control valve was placed between the cooling loop and the oven. SWE was performed using 5.0 g of dried green tea leaves, an extraction cell which contained a stainless steel filter and glass wool at both ends, 2 mL min^{-1} flow rate, a temperature of 150°C, a pressure of around 20-50 atm and 10 min of extraction time. 10 microliter of the yielded extract was diluted with 10 mL of culture medium and this dose (0.002 % (w/w)) was defined as 200 TDC (test drug concentration).

Preparation of drugs

All the anticancer drugs were obtained from the Department of Oncology of Uludag University Medical School. The drugs tested were 5-flourouracil (5-FU), 4-HC (4-hydroperoxycyclophosphamide, the active metabolite of cyclophosphamide), epirubicine as well as combinations of these drugs (FEC). They were those normally used for the patients. Stock concentrations of each drug were prepared either in physiological saline or in the dilution buffer provided by the drug company. The working solutions of the drugs were prepared from stock solutions by diluting in the culture medium. Six different concentrations for each drug were used and they were defined as TDC as previously described.[16-17] The concentrations used were 200% TDC, 100% TDC, 50% TDC, 25% TDC, 12.5% TDC, 6.12% TDC.

Experimental design

MCF-7 and MDA-MB-231 cells were seeded at the density of 5000 cells per well of 96-well plates in 200 ml medium. After an overnight incubation, the media were replaced by fresh ones with the test drugs. For the minimal viability, the cells were incubated with 1 M hydrogen peroxide, which resulted in total cell death (positive control). Cells were treated with different doses of GTE with 0.002% (w/w) as the highest dose (200 TDC in the figures) and 0.00001% (w/w) as the lowest dose (6.25 TDC in the figures). Cells were also treated with 0.0002% (w/w) GTE in combination with the increasing doses of the other anticancer drugs. This dose (0.0002%) was found to be equivalent to approximately IC_{50} value in the MDA-MB-231 breast cancer cell line. The untreated cells received only medium without any drug were used for the maximal viability (negative control). All the cells were treated for 72 h.

The MTT assay

The MTT viability assay was performed with slight modifications as previously described.[18] MTT was first prepared as a stock solution of 5 mg/ml in phosphate buffer (PBS, pH 7.2) and filtered. At the end of the treatment period (72 h) with 6 different TDCs in triplicate, 25 µl of MTT solution was added to each well. After incubation for 4 h at 37 °C, 100 µl of solubilizing buffer (10% sodium dodecyl sulfate dissolved in 0.01 N HCl) was added to each well. After overnight incubation, 96-well plate was read by an enzyme-linked immunosorbent assay (ELISA) reader at 570 nm for absorbance density values to determine the cell viability. The viable cells produced a dark blue formazan product, whereas no such staining was formed in the dead cells.

Data analysis

The viability was calculated with regard to the untreated cell control, which was set to 100% viability (maximal viability, MO). The dead cell control (hydrogen peroxide treated cells) was set to 0% viability (minimal viability, MI). The degree of inhibition of drug-treated cells is expressed as a percentage of the untreated cell control. The inhibition formula is the following: % Inhibition = [1–(Test-MI)/(MO-MI)]x100. The TDCs were plotted against the corresponding inhibition values using SPSS 13.0, resulting in the inhibition curves. The significance was calculated using one-way analysis of variance (ANOVA) and Student's t-test. A value of $P<0.05$ was taken as statistically significant. And the results were calculated as mean with standard error (±SE) values.

Results

Dose response curve was established for GTE in MDA-MB-231 cell line. According to the curve, IC_{50} value was found to be about 0.0002% (w,w). In combinations with either FEC or the drugs alone, this combination of GTE was used.

Figure 1 shows the effect of GTE and its combinations with FEC treatment on MCF-7 cell line. GTE alone did not show any growth inhibition effect on this cell line. It even surprisingly supported the proliferation of MCF-7 cell line. When used in combination with FEC regimen, the effect of FEC was diminished by GTE although the difference was not statically significant ($p> 0.05$). When the drugs were analyzed on their own, GTE was found to significantly

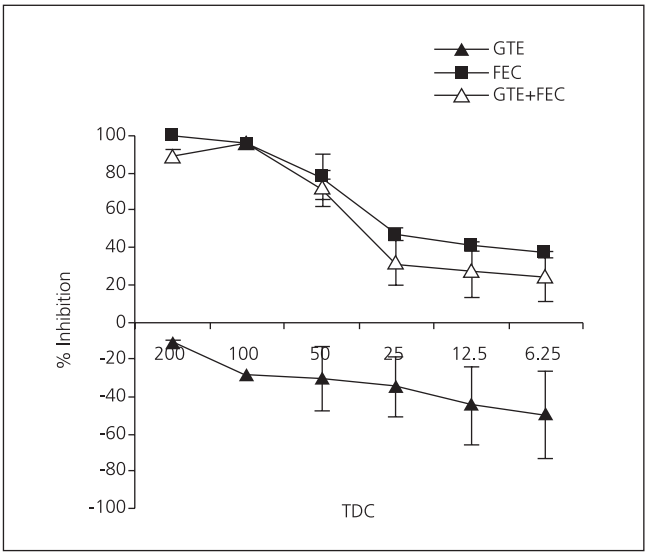

Figure 1
Growth inhibition effects of green tea extract (GTE) and its combination with FEC on MCF-7 cell line. TDC: Test drug concentration

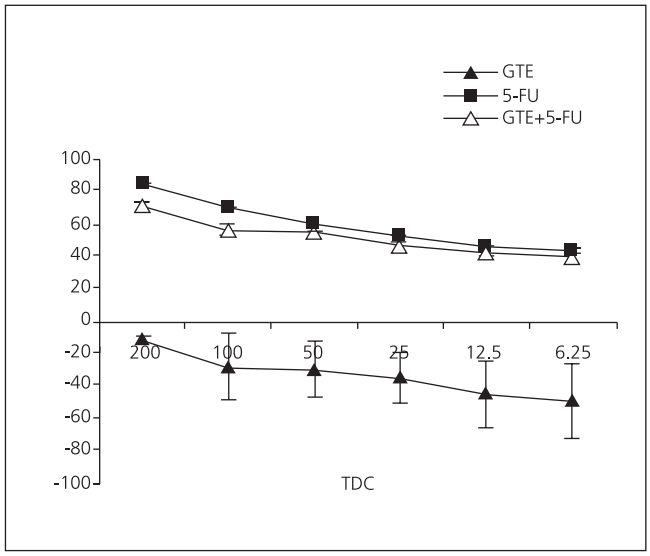

Figure 2
Growth inhibition effects of green tea extract (GTE) and its combination with 5-FU on MCF-7 cell line. TDC: Test drug concentration

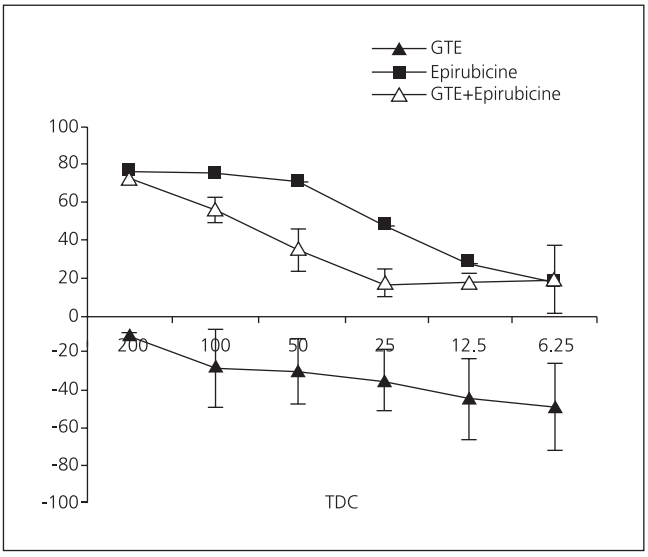

Figure 3
Growth inhibition effects of green tea extract (GTE) and its combination with Epirubicine on MCF-7 cell line. TDC: Test drug concentration

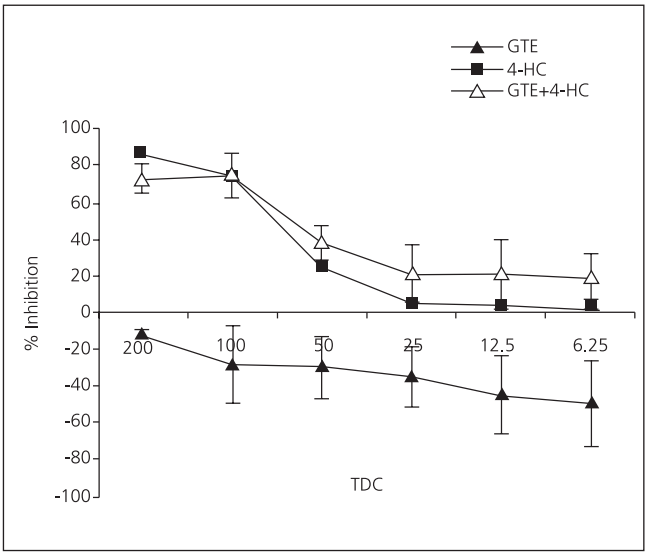

Figure 4
Growth inhibition effects of green tea extract (GTE) and its combination with 4-HC on MCF-7 cell line. TDC: Test drug concentration

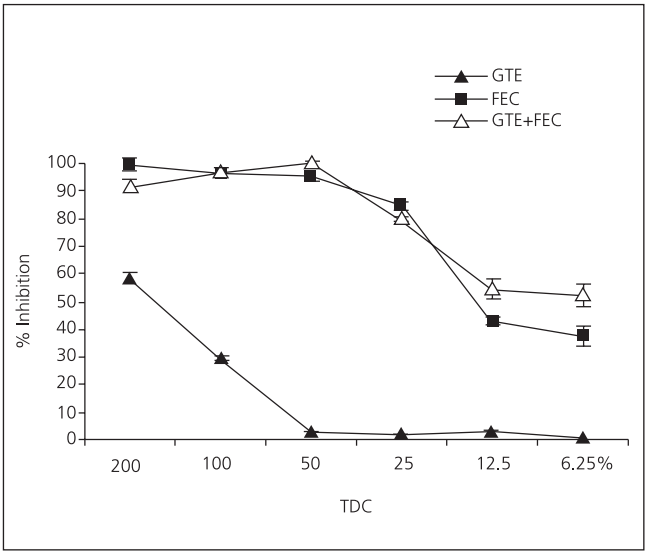

Figure 5
Growth inhibition effects of green tea extract (GTE) and its combination with FEC on MDA-MB-231 cell line. TDC: Test drug concentration

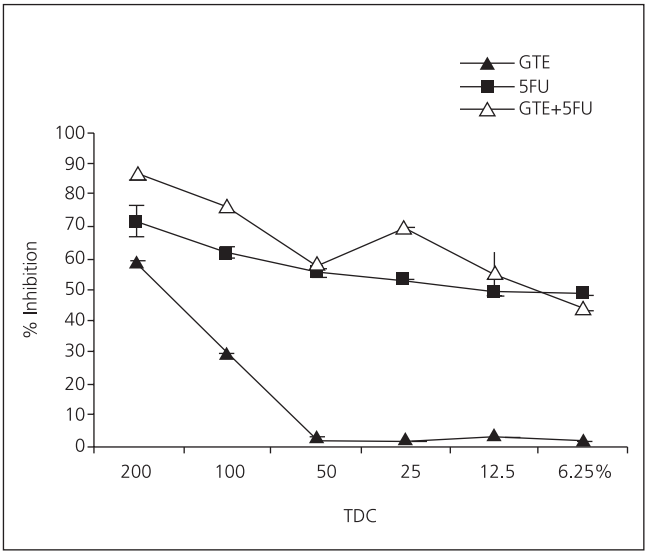

Figure 6
Growth inhibition effects of green tea extract (GTE) and its combination with 5-FU on MDA-MB-231 cell line. TDC: Test drug concentration

reduce the effect of 5-FU at higher concentrations (200 TDC and 100 TDC), ($p<0.05$), (Figure 2). Also, GTE significantly reduced the effect of epirubicine at moderate concentrations (50 TDC, 25 TDC), ($p<0.05$), (Figure 3). However, GTE increased the effect of 4-HC at almost all concentrations used, except for 200 TDC (Figure 4).

Figure 5 shows the effect of GTE and its combinations with FEC treatment on MDA-MB-231 cell line. GTE and other drugs alone showed growth

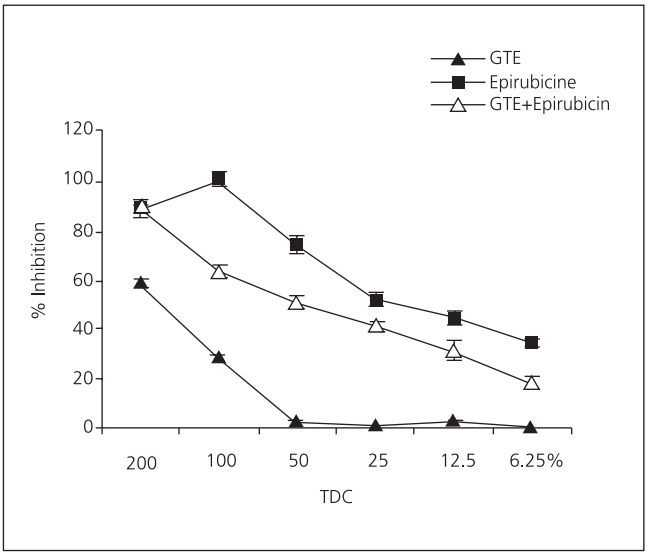

Figure 7
Growth inhibition effects of green tea extract (GTE) and its combination with Epirubicine on MDA-MB-231 cell line. TDC: Test drug concentration

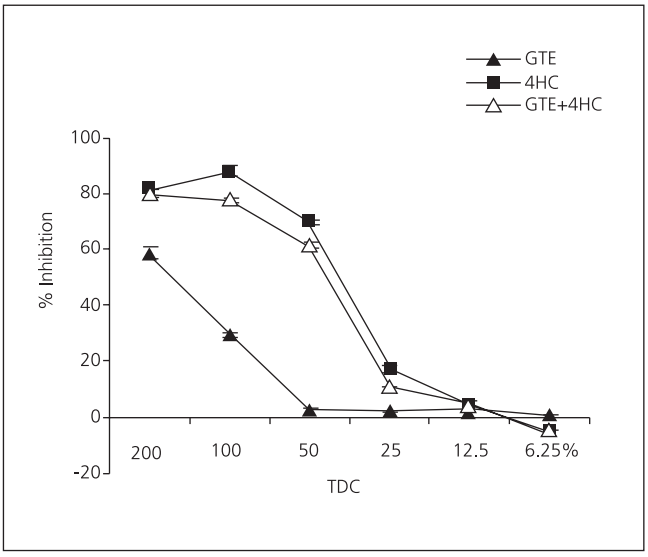

Figure 8
Growth inhibition effects of green tea extract (GTE) and its combination with 4-HC on MDA-MB-231 cell line. TDC: Test drug concentration

inhibition on this cell line at all concentrations. When used in combination with GTE, the effect of FEC was increased at the lowest doses (12.5 and 6.25 TDC) ($p<0.05$). GTE increased the effect of 5-FU at all concentrations in this cell line. Increments were significant at 100 and 25 TDC ($p<0.05$) (Figure 6). On contrary, it decreases the effect of both epirubicine and 4-HC (Figure 5, 7 and 8).

Discussion

Green tea and its polyphenols, potent chemopreventive agents, have been extensively studied as a potential treatment for a variety of diseases, including cancer.[19-22] Breast cancer is the most common cancer in women worldwide.[8] Epidemiological studies suggest that increased consumption of green tea is also related to improved prognosis of human breast cancer.[9] However, the mechanism by which it shows anticancer property needs to be elucidated further, especially in terms of estrogen receptor status. Estrogen receptor action is based on the fact that the regulation of normal growth, development, and function of the breast cancer is intimately associated with estrogen status, playing a significant role in the response to therapy.

To elucidate the mechanism, in this study, we employed two types of human breast cell lines that differ in their responsiveness to the hormonal therapy. We observed that GTE increased the inhibitory effect of FEC on the proliferation of the estrogen receptor negative MDA-MB-231 cell line. Its suggested that green tea polyphenols and its principal constituent EGCG are effective in suppressing the proliferation of MDA-MB-231 as shown by growth inhibition and apoptosis induction both in-vivo and in-vitro.[23] Mechanisms through which green tea-induced apoptosis may be mediated include cell cycle arrest, changes in intracellular signaling, cell adhesion migration (uPA, uPA receptor, vitronectin, integrin receptor) and cell invasion (uPA, uPA receptor) signaling cascade.[24-25] In another study, it was reported that GTE and EGCG (40 mg/L) significantly decreased the RNA levels of VEGF (vascular endothelial growth factor) in MDA-MB231 cells,[26] which implies the effect of GTE on the survival pathways of the cells. In estrogen receptor positive MCF-7 cells, we found that although GTE reduced the inhibitory effect of 5-FU, epirubicine and the combination of FEC regime, the effect of 4-HC was found to be increased.

GTE mainly increased the effect of FEC treatment in MDA-MB-231 cell line, while it decreased the effect of FEC treatment in MCF-7 cell line. This differential action may be resulted from the hormonal status in the cells. A recent work has shown that treatment with GTE and EGCG demonstrated significant changes in various endocrine parameters.[27] Another report suggested synergistic in-vitro cytotoxicity of tamoxifen and EGCG in estrogen receptor-negative MDA-MB-231 cells, but not in estrogen receptor-positive MCF-7 cells.[28] In a study of premenopausal women in Japan, Nagata et al. reported a significant inverse association between green tea intake and follicular phase blood estradiol levels.[29] Wu et al. stated that green tea may have downregulatory effects on estrogen levels.[30] These interesting results imply that the effect of green tea strongly depends on the estrogen-related pathways. Moreover, the effect of green tea also seems to be dose-dependent.[31] Taken together, the cytotoxic potential of green tea may be influenced by several factors (e.g. estrogen receptor status, concentration used).

In conclusion, the focus of future research regarding green tea and its possible role in cancer research should be on the combinations of green tea with some anticancer drugs used for breast cancer chemoprevention and treatment, by taking the estrogen receptor status into account. Further investigation into possible hormonal effects of green tea at a molecular level should provide a better understanding of the role of green tea in the treatment of breast cancer.

References

1. Friedman M, Mackey BE, Kim HJ, et al. Structure-activity relationships of tea compounds against human cancer cells. *J Agric Food Chem* 2007; 55: 243-53.
2. Surh YJ. Cancer chemoprevention with dietary phytochemicals. *Nat Rev Cancer* 2003; 3: 768–80.
3. Moyers SB, Kumar NB. Green tea polyphenols and cancer chemoprevention: multiple mechanisms and endpoints for phase II trials. *Nutr Rev* 2004; 62: 204–11.
4. Jankun J, Selman SH, Swiercz R, Skrzypczak-Jankun E. Why drinking green tea could prevent cancer. *Nature* 1997; 387: 561.
5. Fujiki H, Suganuma M, Okabe S, et al. Mechanistic findings of green tea as cancer preventive for humans. *Proc Soc Exp Biol Med* 1999; 220: 225-8.
6. Fujiki H. Two stages of cancer prevention with green tea. *J Cancer Res Clin Oncol* 1999; 125: 589-97.
7. Komori A, Yatsunami J, Okabe S, et al. Anticarcinogenic activity of green tea polyphenols. *Jpn J Clin Oncol* 1993; 23: 186-90.
8. Bray F, McCarron P, Parkin DM. The changing global patterns of female breast cancer incidence and mortality. *Breast Cancer Res* 2004; 6: 229–39.
9. Nakachi K, Suemasu K, Suga K, Takeo T, Imai K, Higashi Y. Influence of drinking green tea on breast cancer malignancy among Japanese patients. *Jpn J Cancer Res* 1998; 89: 254-61.
10. Wu AH, Tseng CC, Van Den Berg D, Yu MC. Tea intake, COMT genotype, and breast cancer in Asian-American women. *Cancer Res* 2003; 63: 7526–9.
11. Wu AH, Yu MC, Tseng CC, Hankin J, Pike MC. Green tea and risk of breast cancer in Asian Americans. *Int J Cancer* 2003; 106: 574–9.
12. Kavanagh KT, Hafer LJ, Kim DW, et al. Green tea extracts decrease carcinogeninduced mammary tumor burden in rats and rate of breast cancer cell proliferation in culture. *J Cell Biochem* 2001; 82: 387– 98.
13. Sartippour MR, Heber D, Ma J, Lu Q, Go VL, Nguyen M. Green tea and its catechins inhibit breast cancer xenografts. *Nutr Cancer* 2001; 40: 149–56.
14. Zhou JR, Yu L, Mai Z, Blackburn GL. Combined inhibition of estrogen-dependent human breast carcinoma by soy and tea bioactive components in mice. *Int J Cancer* 2004; 108: 8–14.
15. Ozel MZ, Gogus F, Lewis AC. Subcritical water extraction of essential oils from Thymbra spicata. *Food Chem* 2003; 82: 381-6.
16. Hunter EM, Sutherland LA, Cree IA, et al. The influence of storage on cytotoxic drug activity in an ATP-based chemosensitivity assay. *Anticancer Drugs* 1994; 5: 171-6.
17. Andreotti PE, Cree IA, Kurbacher CM, et al. Chemosensitivity testing of human tumors using a microplate adenosine triphosphate luminescence assay: clinical correlation for cisplatin resistance of ovarian cancer. *Cancer Res* 1995; 55: 5276 -82.
18. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983; 65: 55-63.
19. Lee AH, Fraser ML, Meng X, Binns CW. Protective effects of green tea against prostate cancer. *Expert Rev Anticancer Ther* 2006; 6: 507-13.
20. Zhao X, Tian H, Ma X, Li L. Epigallocatechin gallate, the main ingredient of green tea induces apoptosis in breast cancer cells. *Front Biosci* 2006; 11: 2428-33.
21. Paschkaa AG, Butlera R, Younga CY. Induction of apoptosis in prostate cancer cell lines by the green tea component, (-)-epigallocatechin-3-gallate. *Cancer Letters* 1998; 130: 1–7.

22. Mukhtar H, Ahmad N. Tea polyphenols: prevention of cancer and optimizing health. *Am J Clin Nutr* 2000; 71: 1698–702.
23. Thangapazham RL, Singh AK, Sharma A, Warren J, Gaddipati JP, Maheshwari RK. Green tea polyphenols and its constituent epigallocatechin gallate inhibits proliferation of human breast cancer cells in vitro and in vivo. *Cancer Letters* 2007; 245: 232-41.
24. Stuart EC, Scandlyn MJ, Rosengren RJ. Role of epigallocatechin gallate (EGCG) in the treatment of breast and prostate cancer. *Life Sci* 2006; 17: 2329-36.
25. Slivova V, Zaloga G, DeMichele SJ, et al. Green tea polyphenols modulate secretion of urokinase plasminogen activator (uPA) and inhibit invasive behavior of breast cancer cells. *Nutr Cancer* 2005; 52 :66-73.
26. Sartippour MR, Shao ZM, Heber D, et al. Green tea inhibits vascular endothelial growth factor (VEGF) induction in human breast cancer cells. *J Nutr* 2002; 132: 2307-11.
27. Liao S. The medicinal action of androgens and green tea epigallocatechin gallate. *Hong Kong Med J* 2001; 7: 369-74.
28. Chisholm K, Bray BJ Rosengren RJ. Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells. *Anticancer Drug* 2004; 15: 889–97.
29. Nagata C, Kabuto M, Shimizu H. Association of coffee, green tea, and caffeine intakes with serum concentrations of estradiol and sex hormone-binding globulin in premenopausal Japanese women. *Nutr Cancer* 1998; 30: 21-4.
30. Wu AH, Arakawa K, Stanczyk FZ, Van Den Berg D, Koh WP, Yu MC. Tea and circulating estrogen levels in postmenopausal Chinese women in Singapore. *Carcinogenesis* 2005; 26: 976-80.
31. Hsuuw YD, Chan WH. Epigallocatechin gallate dose-dependently induces apoptosis or necrosis in human MCF-7 cells. *Ann N Y Acad Sci* 2007; 1095: 428-40.