



Synergistic effects of tea polyphenols and ascorbic acid on human lung adenocarcinoma SPC-A-1 cells*

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Received Nov. 11, 2009; Revision accepted Mar. 28, 2010; Crosschecked Apr. 28, 2010

Abstract: Tea polyphenols have been shown to have anticancer activity in many studies. In the present study, we investigated effects of theaflavin-3-3'-digallate (TF₃), one of the major theaflavin monomers in black tea, in combination with ascorbic acid (AA), a reducing agent, and (-)-epigallocatechin-3-gallate (EGCG), the main polyphenol presented in green tea, in combination with AA on cellular viability and cell cycles of the human lung adenocarcinoma SPC-A-1 cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay showed that the 50% inhibition concentrations (IC₅₀) of TF₃, EGCG, and AA on SPC-A-1 cells were 4.78, 4.90, and 30.62 μmol/L, respectively. The inhibitory rates of TF₃ combined with AA (TF₃+AA) and EGCG combined with AA (EGCG+AA) at a molar ratio of 1:6 on SPC-A-1 cells were 54.4% and 45.5%, respectively. Flow cytometry analysis showed that TF₃+AA and EGCG+AA obviously increased the cell population in the G₀/G₁ phase of the SPC-A-1 cell cycle from 53.9% to 62.8% and 60.0%, respectively. TF₃-treated cells exhibited 65.3% of the G₀/G₁ phase at the concentration of its IC₅₀. Therefore, TF₃+AA and EGCG+AA had synergistic inhibition effects on the proliferation of SPC-A-1 cells, and significantly held SPC-A-1 cells in G₀/G₁ phase. The results suggest that the combination of TF₃ with AA or EGCG with AA enhances their anticancer activity.

Key words: Theaflavin-3-3'-digallate (TF₃), (-)-epigallocatechin-3-gallate (EGCG), Ascorbic acid (AA), Synergism, SPC-A-1 cells, Cell cycle

doi:10.1631/jzus.B0900355

Document code: A

CLC number: Q813.1⁺1; R734.2

1 Introduction

Epidemiologic studies have suggested that the consumption of tea is linked to a decreased incidence of various cancers (Yang C.S. *et al.*, 2007). The inhibitory action of tea and tea products against carcinogenesis has been demonstrated in animal models, including skin, lung, esophagus, stomach, liver, small intestine, pancreas, prostate, and mammary gland cancers (Conney *et al.*, 1999; Tu *et al.*, 2004).

Both black tea and green tea have monomeric gallated catechins of the flavanol class. The main polyphenol presented in green tea is (-)-epigallocatechin-3-gallate (EGCG) (Fig. 1a), which accounts for about 60%–70% of total catechins (Agarwal and Mukhtar, 1996). EGCG is a precursor of theaflavin-3-3'-digallate (TF₃) (Fig. 1b). Previous studies showed that TF₃ appears to be the most potent compound in tea on inhibition of proliferation and induction of apoptosis of cancer cells (Yang *et al.*, 1998). Black tea, accounting for 78% of the world tea consumption, is the product of tea catechin oxidation and polymerization via fermentation of fresh tea leaves (Yang *et al.*, 2009). The fermentation results in the formation of theaflavins. The major theaflavin

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* Project supported by the Key Program of Science and Technology of Zhejiang Province (No. 2007C12068) of China and the National Natural Science Foundation of China (No. 30901002)

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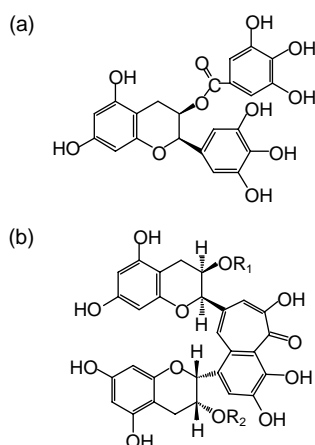


Fig. 1 Chemical structures of EGCG and theaflavins
(a) EGCG; (b) Theaflavins. TF₁ (R₁=R₂=OH), TF₂A (R₁=gallate, R₂=OH), TF₂B (R₁=OH, R₂=gallate), TF₃ (R₁=R₂=gallate)

monomers in black tea are theaflavin (TF₁), theaflavin-3-gallate (TF₂A), theaflavin-3'-gallate (TF₂B), and theaflavin-3-3'-digallate (TF₃). TF₃ has two galloyl groups, TF₂A and TF₂B each have one, and TF₁ has none. More specific investigations concerning with theaflavin structure reported that the antioxidative activities of theaflavin monomers depend on the number and position of hydroxyl groups within their molecules (Yang Z.Y. *et al.*, 2007), e.g., the antioxidation activities following the sequence of TF₃>TF₂>TF₁ (Leung *et al.*, 2001).

The antioxidative activities of black tea theaflavins are also studied for their influence on activation of transcription factors such as nuclear factor kappa B (NFκB). Theaflavins, in particular TF₃, could effectively inhibit the activation of NFκB, preventing the expression of inducible nitric oxide synthase (iNOS) gene or down-regulating the expression of iNOS mRNA, which led to a decrease of nitric oxide synthesis in RAW 264.7 cells (Hong *et al.*, 2001). Ascorbic acid (AA) is an essential nutrient involved in many biochemical reactions and is known to protect some flavonoids against oxidative degradation during processing and storage of juices (Kaack and Austed, 1998). The biochemical roles of AA are related to its ability to act as an electron donor or reducing agent. As a free radical scavenger, AA can protect cellular biopolymers against the initiation and progression of carcinogenesis (Kim *et al.*, 2006).

Lung cancer is one of the most common cancers

worldwide. It has been suggested that there is an inverse association between tea intake and subsequent lung cancer incident (Yang *et al.*, 2009). However, the synergistic effects of TF₃ or EGCG with AA on human epithelial lung adenocarcinoma SPC-A-1 cells have not been examined. In this study, we investigated *in vitro* the combined effects of TF₃ with AA (TF₃+AA) and EGCG with AA (EGCG+AA) on SPC-A-1 cells.

2 Materials and methods

2.1 Cell culture

Human lung adenocarcinoma SPC-A-1 cells were purchased from the Cell Bank of Chinese Academy of Science (Shanghai, China) and cultured in RPMI-1640 medium (GIBCO BRL, Grand Island, NY, USA) supplemented with 10% (w/v) fetal calf serum (GIBCO BRL) in a humidified atmosphere of 37 °C with 5% CO₂.

2.2 Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and AA were purchased from Sigma-Aldrich (St. Louis, MO, USA). EGCG was a generous gift from the Tea Science Department of Zhejiang University, with a purity of above 98% (analyzed by high performance liquid chromatography (HPLC)). Tea polyphenols were provided by Siming Natural Plant Co. (Zhejiang, China). All other chemicals were of analytical grade and were commercially obtained from local chemical suppliers.

2.3 Preparation and analysis of theaflavin monomers

The theaflavins (80% purity) were prepared by the enzymatic oxidation of tea polyphenols using immobilized polyphenol oxidase (PPO) according to the protocol of Tu and Xia (2004). TF₂A, TF₂B, TF₁, and TF₃ were separated using a Sephadex LH-20 gel (Pharmacia Fine Chemicals Inc., NJ, USA) column (Tu *et al.*, 2004).

Theaflavin monomer was determined using our previously established method (Yang *et al.*, 2008). Briefly, theaflavin monomers (0.4 mg) and EGCG (2.0 mg) were dissolved in 1.0 ml methanol, respectively. The concentrations of theaflavin monomer and

EGCG were determined using a Shimadzu LC-2010A (Shimadzu, Kyoto, Japan).

2.4 Inhibition of tea polyphenols on SPC-A-1 cells

Cell viability was measured by quantitative colorimetric assay with MTT, which shows the mitochondrial activity of living cells as described by Mosmann (1983) and Denizot and Lang (1986). SPC-A-1 cells were seeded into 96-well plates 0.1 ml/well at a density of 1×10^5 cells/ml. After the cells attached, EGCG, TF₂B, TF₃, TF₂A, or AA at different doses was added. After an exposure period, 10 μ l MTT (5 mg/ml) was added to each well. The final concentrations for each of AA and TF₂A added to SPC-A-1 were 5, 10, 50, 100, and 200 μ mol/L, and the final concentrations for each of TF₃, EGCG, and the compound of TF₂B and TF₃ were 0.5, 1, 5, 10, and 20 μ mol/L. Six replicates were set for each treatment.

2.5 Analysis of synergistic effects

Using the median-effect method developed by Chou and Talalay (1984), the dose-response curve was plotted for each drug, and the multiple dose of a fixed-ratio combination was plotted by

$$f_a/f_u = (D/D_m)^m, \quad (1)$$

where D is the dose administered, D_m is the dose required for 50% inhibition of growth (IC_{50}), f_a is the fraction affected by dose D , f_u is the unaffected fraction, and m is a coefficient signifying the sigmoidicity of the dose-response curve.

The dose-response curve was plotted using a logarithmic conversion of the equation to determine the m and D_m values. D_x required for x percent effect (f_a) _{x} was then calculated by

$$D_x = D_m [(f_a)_x / (f_u)_x]^{1/m}. \quad (2)$$

Thus, the combination index (CI) can be defined by the isobologram

$$CI = (D)_1 / (D_x)_1 + (D)_2 / (D_x)_2 + \alpha (D)_1 (D)_2 / [(D_x)_1 (D_x)_2], \quad (3)$$

where $(D_x)_1$ is the dose of drug-1 for producing x percent effect and $(D)_1$ for producing the same x percent effect in combination with drug-2; $(D_x)_2$ and $(D)_2$ correspond to drug-2. Consequently, $CI < 1$ indicates

synergism, $CI > 1$ shows antagonism, and $CI = 1$ points to additive effects. The CI values obtained from both the mutually non-exclusive ($\alpha = 1$) and mutually exclusive ($\alpha = 0$) isobologram equations are presented in this work.

2.6 Detection of cell cycle by flow cytometry

Cell cycle analysis for the cell distribution in the sub-G₁ region was determined by flow cytometric analysis after propidium iodide (PI) staining. Cells were treated with: AA (30.62 μ mol/L), TF₃ (4.78 μ mol/L), EGCG (4.90 μ mol/L), TF₃ (4.78 μ mol/L)+AA (30.62 μ mol/L), and EGCG (4.90 μ mol/L)+AA (30.62 μ mol/L) according to their IC_{50} for 48 h. The cells in suspension (5×10^4 cells/ml) with or without drugs were fixed with 80% ethanol at 4 °C for 24 h and then stained with 25 μ g/ml PI. After staining, the population of cells in each cell cycle phase was determined using the FAC Star flow cytometry (Becton-Dickinson, San Jose, California, USA).

2.7 Statistical analysis

All experiments were performed in triplicate. One-way analysis of variance (ANOVA) was used to estimate overall significance, followed by the post-hoc Tukey's tests corrected for multiple comparisons. Data are presented as mean \pm standard deviation (SD). A probability level less than 5% ($P < 0.05$) was considered significant.

3 Results

3.1 Effects of theaflavin monomers, AA, and EGCG on the proliferation of SPC-A-1 cells

Firstly, the effects of tea polyphenols (TF₂A, TF₂B+TF₃, TF₃, and EGCG) and AA on cell viability of SPC-A-1 were measured. The highest inhibition rate of TF₂A on the proliferation of SPC-A-1 cells was 40.39% at 100 μ mol/L, so we were not able to calculate the IC_{50} of TF₂A. Thus, the TF₂A was not considered in the following study.

Cells were continuously exposed to various drug treatments for 48 h and then tested for the cell viability using MTT assay. The lower the IC_{50} value, the higher the inhibition on the SPC-A-1 cells. TF₂B+TF₃, TF₃, and EGCG showed the most potent inhibition on the SPC-A-1 cells, with the IC_{50} values of 6.70, 4.78,

and 4.90 $\mu\text{mol/L}$, respectively. The IC_{50} value of AA was 30.6 $\mu\text{mol/L}$, which was significantly higher than that of tea polyphenols alone.

3.2 Cytotoxicities of EGCG+AA and TF_3 +AA on SPC-A-1 cells

The interactions of EGCG with AA and TF_3 with AA on SPC-A-1 were analyzed by the method of Chou and Talalay (1984). The molar ratios of TF_3 to AA and EGCG to AA were both set at 1:6 based on their IC_{50} values. CI plots were constructed by computer analysis using Eq. (3); and both the mutually non-exclusive ($\alpha=1$) and mutually exclusive ($\alpha=0$) isobologram equations were used.

Biphasic effects of a drug combination were shown for TF_3 :AA and EGCG:AA at the molar ratio of 1:6 against SPC-A-1 cells, with a turning point appearing at 54.4% and 45.5% of f_a values (Fig. 2). The combinations of AA with EGCG and AA with TF_3 both enhanced their inhibition on SPC-A-1 cells at a higher concentration, which is indicated by $\text{CI}<1$.

3.3 Effects of polyphenols and AA on the growth inhibition of SPC-A-1 cells and G_1 -phase arrest

Cell cycle arrest is a characteristic of many anticancer drugs. Cells in G_0/G_1 phase arrest might go into apoptosis, or recover from the G_0/G_1 phase and enter into S phase. The effects of EGCG and TF_3 on distribution of cells in the cell cycle were analyzed by the flow cytometry. The results show that while all treatments led to an increase of cell population in G_0/G_1 phase, TF_3 promoted the biggest increase (Fig. 3c). TF_3 +AA and EGCG+AA obviously increased the cell population in G_0/G_1 phase of the SPC-A-1 cell cycle from 53.9% to 62.8% and 60.0%, respectively (Figs. 3a, 3e, and 3f). TF_3 +AA reduced the cell population in S phase from 46.1% to 23.8% (Figs. 3a and 3e), and increased apoptosis from 1.0% to 22.3%. Interestingly, TF_3 -treated cells exhibited 65.3% of G_0/G_1 phase at the IC_{50} .

4 Discussion

EGCG displayed strong growth inhibitory effects against lung cancer lines H661 and H1299,

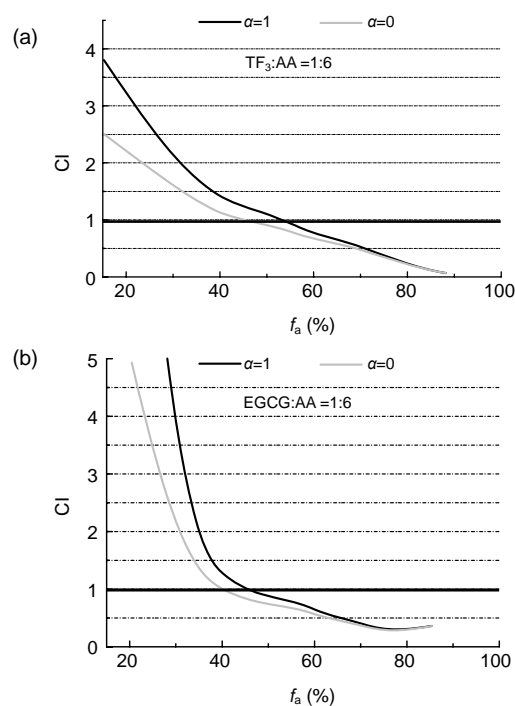


Fig. 2 Combination index (CI) plots of interactions between TF_3 , EGCG, and AA

SPC-A-1 cells were treated with tea polyphenol monomers and AA at a fixed molar ratio: (a) TF_3 :AA = 1:6 and (b) EGCG:AA = 1:6. Using the mutually exclusive or mutually non-exclusive isobologram equation, the affected fraction (f_a)-CI plots for SPC-A-1 cells were constructed by computer analysis of the data generated from the median effect analysis. $\text{CI}<1$ occurred over a wide range of inhibition levels, indicating synergism

with an estimated IC_{50} value of 22 $\mu\text{mol/L}$ (Yang *et al.*, 1998), which is much higher than that of TF_3 or EGCG in lung cancer lines SPC-A-1 in the present study. Moon *et al.* (2007) demonstrated that EGCG was a strong inducer of early growth response gene-1 expression and mediated early growth response gene-1 nuclear translocation via extracellular signal-regulated protein kinase signaling pathway in A549 cells. TF_3 had stronger inhibition on I kappa B ($\text{I}\kappa\text{B}$) kinase (IKK) activity in activated macrophages than other polyphenols. TF_3 strongly inhibited both IKK_1 and IKK_2 activities and prevented the degradation of $\text{I}\kappa\text{B}\alpha$ and $\text{I}\kappa\text{B}\beta$ in activated macrophage cells (Pan *et al.*, 2000). This suggests that the inhibition of IKK activity by TF_3 could occur by a direct effect on IKKs or on upstream events in the signal transduction pathway.

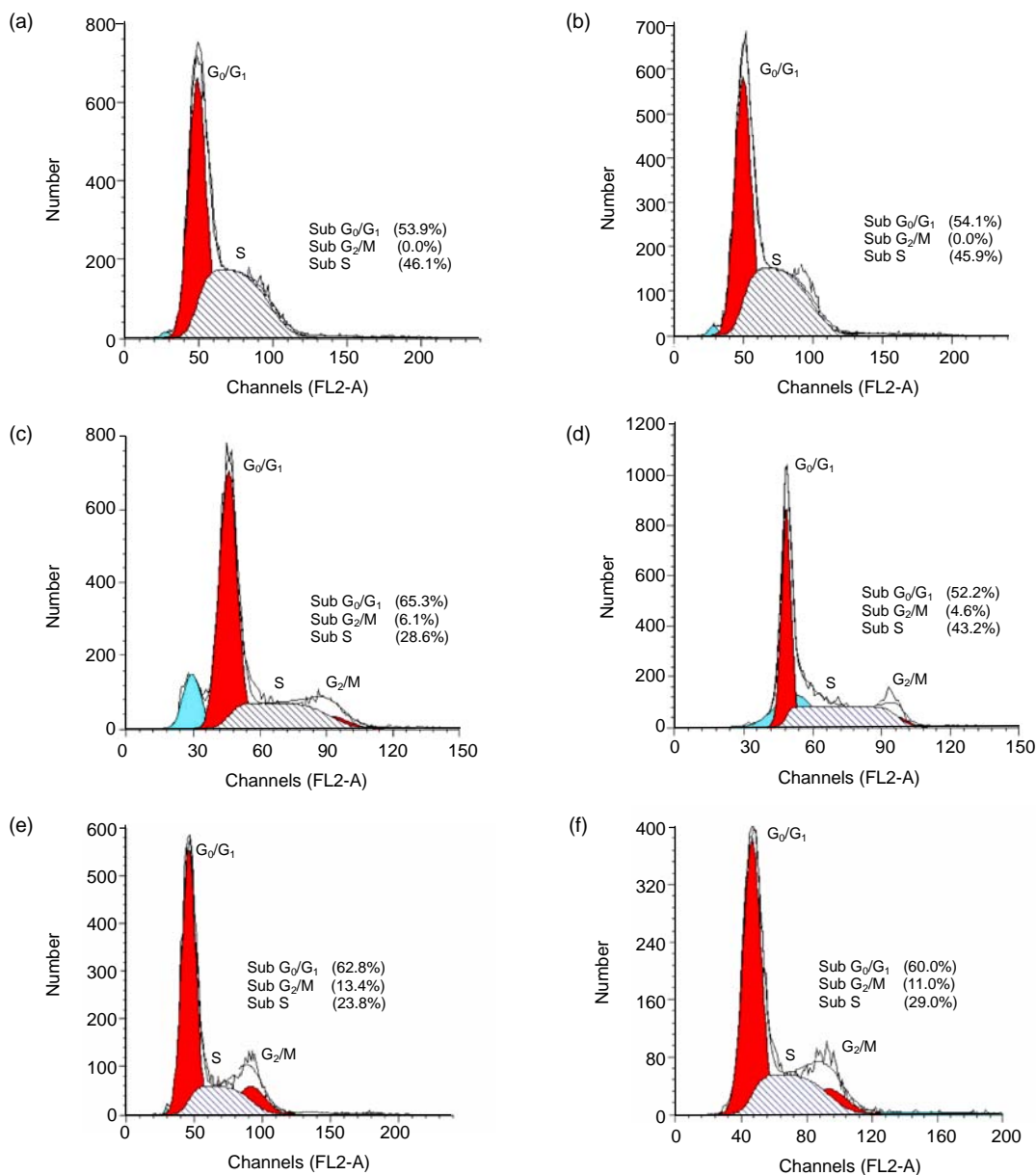


Fig. 3 Cell cycle analysis of SPC-A-1 cells treated with the indicated concentrations of drugs for 48 h by flow cytometry analysis

After cells were fixed and stained with propidium iodide, and the DNA content was measured by flow cytometry. Cell cycle distribution was analyzed using the FAC Star flow cytometry. (a) Control; (b) Ascorbic acid (30.62 μmol/L); (c) TF₃ (4.78 μmol/L); (d) EGCG (4.90 μmol/L); (e) TF₃ (4.78 μmol/L)+AA (30.62 μmol/L); (f) EGCG (4.90 μmol/L)+AA (30.62 μmol/L)

Growth inhibitory activities of EGCG have been demonstrated by Yang *et al.* (2009), which is also confirmed in this study. Whereas green tea contains EGCG, black tea contains theaflavins. In the present study, we showed that TF₃ was as effective as EGCG. We extended our studies to the synergistic inhibition of TF₃ in combination with AA and EGCG with AA on human lung cancer SPC-A-1 cells, and found that

AA enhanced the inhibitory abilities of TF₃ and EGCG on lung cancer cells. When EGCG or TF₃ was combined with AA at a lower concentration, the combination significantly enhanced the antagonism (CI>1). Polyphenols are generally unstable under oxidative conditions, so EGCG levels decrease rapidly, even in solutions of neutral pH (Hatano *et al.*, 2003). The synergism in this study suggests that AA

may protect EGCG from oxidation. The chemical structure of TF₃ is bulkier and has two A-rings linked by a fused seven-member ring (Yang Z.Y. *et al.*, 2007). Theaflavin monomers are transformed from tea catechins through oxidation catalyzed by enzyme and chemical condensation, which makes theaflavin monomers more stable than EGCG. Some experiments demonstrated that the theaflavins presented in black tea have at least the same antioxidant potential as catechins presented in green tea, and that the conversion of catechins to theaflavins during black tea production does not alter significantly their free radical-scavenging activity (Leung *et al.*, 2001). More studies indicated that the synergistic anticancer effects of AA and green tea extract on several cancer cell lines in tissue culture were higher than those of the individual nutrients (Roomi *et al.*, 2006).

AA as a well-known antioxidant can protect tea polyphenols from oxidation. We also found that the synergistic effects of TF₃+AA and EGCG+AA on SPC-A-1 cells were through cell cycle arrest, but the EGCG or TF₃ alone can lead to more apoptosis. In synergistic cultures, the relative number of apoptotic cells did not increase; a maximum of 2.1% of apoptotic cells were observed. TF₃ can increase the cell cycle at the G₀/G₁ phase. When AA is added, it can produce a large amount of H₂O₂ by reducing the concentration of peroxidase (Zheng *et al.*, 2002). TF₃ shows the most potent hydroxyl radicals-scavenging ability (Yang *et al.*, 2008).

Using human lymphoma cells, Saeki *et al.* (1999) revealed that TF₃ and TF₁ had equivalent apoptosis-inducing activities in vitro. Especially, Tu *et al.* (2004) suggested that TF₂B was a more powerful inhibitor to the growth of human liver cancer cells, gastric cancer cells, and leukemia cells than TF₃. Apparently, these findings suggest that multiple mechanisms may be responsible for the anticancer effects of theaflavins on different types of cancers.

In this study, we found that AA with EGCG or TF₃ acts synergistically to suppress cell proliferation of SPC-A-1 in vitro. Presently, it is not clearly defined how black tea theaflavins exert their cytotoxic effects (Lung *et al.*, 2004), cell cycle arrest (Chung *et al.*, 1999), and inducing of apoptosis (Lu *et al.*, 2000). Studies show that cancer cells are more sensitive than normal cells to the in vitro anti-proliferation effects of EGCG (Mittal *et al.*, 2004) and black tea polyphenol

extracts (Weisburg *et al.*, 2004). After entering the cells, EGCG may produce reactive oxygen species (ROS) by an unknown mechanism (Yang *et al.*, 2009).

In conclusion, this paper showed that TF₃, one of the major theaflavin monomers in black tea, in combination with AA, a reducing agent, and EGCG, the main polyphenol presented in green tea, in combination with AA could synergistically inhibited the proliferation of SPC-A-1 cells, and increased its cell population in G₀/G₁ phase of cell cycle. It suggested that the combination of EGCG with AA and TF₃ with AA may be potent anticancer agents for cancer therapy.

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