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The Role of Green Tea Extract on the Proliferation of Human Ovarian Cancer Cells (*in vitro*) Study

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Abstract: This study investigated the role of green tea extracts as a cytotoxic agent against human ovarian cancer cells *in vitro* using different concentrations of green tea extracts (20, 50, 100 and 250 $\mu\text{g mL}^{-1}$) for 24 h. *In vitro* proliferation assays were performed to compare the growth rate of all groups of cells. The numbers of viable cells were counted using Hemacytometer and Trypan blue staining (0.4%). The effect of green tea extracts was also studied for long period (24, 48 and 72 h) using the $\text{IC}_{50} = 100 \mu\text{g mL}^{-1}$. The results of statistical analysis revealed significant decrease in the number of treated human ovarian cancer cells inversely proportion with increasing the concentration of green tea extracts, this experiments determined that the $\text{IC}_{50} = 100 \mu\text{g mL}^{-1}$. In long term study, the results approved that there was significant reduction in the number of tumor cells by time in contrast with control none treated one. Morphologically, the images of the fixed and stained human ovarian cancer cells with Coomassie stain revealed that the Green tea extracts causing cells shrinkage, blabbing, chromatin condensation and loss of cell-cell contacts which were known as sign of apoptosis. In conclusion, green tea extract has been shown antiproliferative activity against the growth of HOCC which might be considered in clinical situation.

Key words: Green Tea Extracts (GTEs), polyphenol, tissue culture, ovarian cancer cells, IC_{50}

INTRODUCTION

Cancer is one of the major causes of death worldwide. It is estimated that 12.8% of the world population die due to cancer (WHO, 2004; Hanachi *et al.*, 2006). The number of new cases has been increasing every year from the year 1990 to 2000 alone, there has been increase of 22% in incidence and mortality (Parkin, 2001). Green tea has shown promise effect in the prevention of several cancers (Lee *et al.*, 2005). It contains several compounds, including polyphenols that have been reported as chemoprotective agents (Graham, 1992). Ioannides and Yoxall (2003) reported that tea intake has been linked to many beneficial effects in human health. There is ample evidence from *in vitro* and *in vivo* studies indicating that components of tea are associated with decreased risk or progression of several cancers (Chung *et al.*, 2003), cancer and cardiovascular disease (McKay and Blumberg, 2002). Currently, the epidemiologic evidence is strongest with organs of the gastro- intestinal tract, possibly because of their direct contact with tea constituents

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(Setiawan *et al.*, 2001; Su and Arab, 2002; Sun *et al.*, 2002; Sasazuki *et al.*, 2004). Evidence has also emerged from human observational studies on cancer of the skin (Hakim *et al.*, 2000) prostate, breast (Nakachi *et al.*, 1998; Inoue *et al.*, 2001; Chung *et al.*, 2003; Wu *et al.*, 2003; Crespy and Williamson, 2004) pancreas, esophagus and lung (Nagano *et al.*, 2001) and from over 80 published studies in animal models (Matsumoto and Yamana, 2000; Chung *et al.*, 2003). Green tea components can be distributed to a wide variety of target organs in rodents after ingestion (Suganuma *et al.*, 1999). Interestingly, the majority of studies reporting protective effects were conducted in Asian countries where, green tea is predominantly consumed (Chung *et al.*, 2003). In studies conducted in rodents, tea has exhibited strong anticarcinogenic activity against a number of carcinogens of human relevance including heterocyclic amines and polycyclic aromatic hydrocarbons that are important food contaminants, being formed during the normal cooking process (Yang *et al.*, 2002; Chung *et al.*, 2003). The potent chemopreventive activity of tea has been observed on a number of target organs and has been attributed to the fact that it influences favorably all stages of carcinogenesis process, namely initiation, promotion and progression as well as metastasis (Fujiki and Suganuma, 2002). Tea can modulate the initiation stage by preventing the bending of genotoxic carcinogens to DNA to induce mutations, that is on its antimutagenic activity (Matsumoto and Yamana, 2000; Ioannides and Yoxall, 2003).

Findings from *in vitro* and *in vivo* studies need to be supported by human studies that take into account the absorption and uptake of green tea compounds *in vivo*. Many *in vitro* and animal studies used very high concentrations of one component of green tea polyphenols called catechins to demonstrate a protective effect (Chung *et al.*, 2003; Crespy and Williamson, 2004). However, green tea polyphenols undergo several processes after ingestion so that the high catechin concentrations do not reflect the actual levels found in the human body (Chung *et al.*, 2003). Furthermore, animal studies utilize a variety of preparation methods for green tea that can influence the content of green tea components such as catechins, leading to unstable levels (Crespy and Williamson, 2004). It is thus, difficult to evaluate the relationship between the amount of green tea ingestion and the biologic effect that can be applied to humans (Gupta *et al.*, 2001; Crespy and Williamson, 2004; Baliga *et al.*, 2005; Nihal *et al.*, 2005).

The anticancer properties of green tea and of the bioactive polyphenol, (-)-epigallocatechin-3-gallate (EGCG), are a result of induction of G1 arrest and apoptosis as well as regulation of cell cycle-related proteins in cancer cell lines (Huh *et al.*, 2004).

Yihai and Renhai (1999) reported that, a study by Swedish researchers, suggested that green tea's cancer fighting benefits may be due to a component of the drink that prevents angiogenesis, the process of blood vessels growth. Tumors are depended on the continual development of new blood vessels to grow and multiply, they believed that a EGCG may be active in preventing the growth of new blood vessels in tumors and could be the green tea's association with lower cancer incidence, they found 70% less blood vessel growth in mice that were given green tea than in water consuming controls.

There is ample evidence from *in vitro* and *in vivo* studies indicating that components of tea are associated with decreased risk or progression of several cancers, relatively few studies have specifically investigated ovarian cancer. This stimulated the present investigation to study the effect of GTEs on the human ovarian cancer, using human ovarian cancer on *in vitro* study.

MATERIALS AND METHODS

This study was conducted between 2007-2009 in Tissue Culture Unit King Fahd Medical Research Center, King Abdul-Aziz University, Jeddah, Saudi Arabia.

Cell Line

Human ovarian tumor was kindly obtained through the courtesy of Abdul-Jabbar, Professor of Gynecology, King Abdul-Aziz University Hospital (KAUH), Jeddah. It was immediately transported in sterilized transporting media to Tissue Culture Unit, King Fahd Medical Research Center (KFMRC), to prepare the tissue for *in vitro* study.

Green Tea Extracts Preparation

Green tea was locally purchased from Green tea center Jeddah-KSA and stored at 4°C in sealed bag (2.5% w/v). It was prepared by adding boiling water (100 mL) to the tea (2.5 g) in flask, leaving to stand for 10-15 min inverting every 30 sec and then filtering through cotton wool, according to the method of Nicolas *et al.* (1999).

Human Ovarian Cancer Cells

Human ovarian tumor cells were prepared for *in vitro* study in growth media (MCDB 105+M199 in a 1:1) supplemented with 10% Fetal Calf Serum (FCS) and 1% penicillin-streptomycin at 37°C under 5% CO₂ and 95% air (Dunfield *et al.*, 2002). The cells were dispensed in 24 wells plate 1×10⁵ mL in each well.

Assay of Cytotoxic Activity

Part of cells were treated with tea extract for 24 h and IC₅₀ was determined by Trypan blue dye exclusion test according to the method of Pollard and Walker (1989), Khorshid *et al.* (2005) and Khorshid and Moshref (2006). Other part were treated with concentration equal to IC₅₀ and the treatment was continued for (24, 48 and 72 h). Third part of the cells was used as control.

For morphological effect of the tea extract, cells of each group were fixed in 4% formaldehyde for 5 min at room temperature after double washing with 1×PBS each for 5 min. Cell was then stained with Coomassie blue for 5-10 min. Followed by repeated washing with tap water (Khorshid and Moshref, 2006; Khorshid *et al.*, 2005) assays were performed in duplicate.

Statistical Analysis

Data in the present investigation were given as Mean±SD. The results were analyzed statistically using One-way Analysis of Variance (ANOVA) and Two-way ANOVA and Statistical Package for Social Science (SPSS 16.0 for window), at p-value<0.05 indicating statistical significant difference.

RESULTS AND DISCUSSION

The effect of GTEs at different concentrations was examined on the number of viable cells of Human Ovarian Cancer Cells (HOCC) by the Trypan blue method (Pollard and Walker, 1989; Khorshid and Moshref, 2006; Khorshid *et al.*, 2005). The results were compared with control (non-treated HOCC), where the cancer cells were incubated in ordinary media.

Cytotoxic Assays (IC₅₀ Estimation)

Cytotoxic activity was defined as the number of cells in tea treated group compared with untreated cells using Trypan blue dye exclusion test revealed that, the treated groups of HOCC cells incubated in GTEs media in different concentration exhibited conspicuous and

Table 1: Represents cytotoxic activity of green tea extract on the proliferation of HOCC incubated for 24 h

GTEs ($\mu\text{g mL}^{-1}$)	Mean $\times 10^5 \pm \text{SD}$
0 (control)	1.700 ± 0.14142
20	1.160 $\pm 0.01414^*$
50	1.045 $\pm 0.00707^*$
100	0.640 $\pm 0.02828^*$
250	0.590 $\pm 0.01414^*$

Values represents as (Mean \pm SD). *Significant at $p < 0.05$

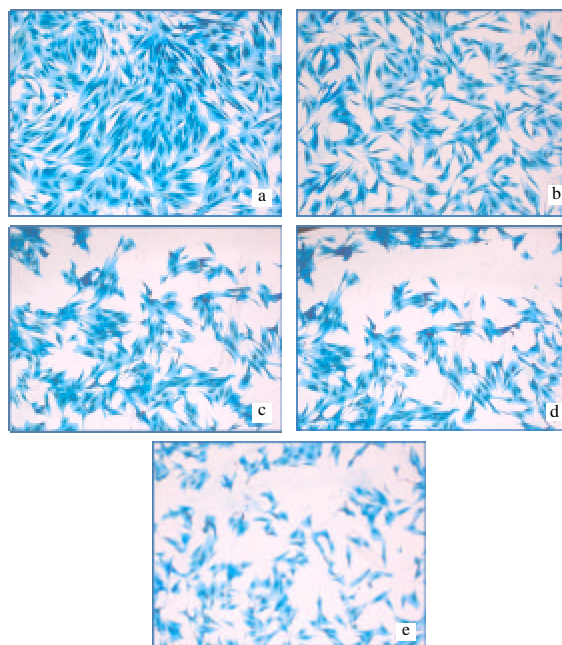


Fig. 1: (a-e) Represent HOCC incubated in ordinary and GTEs medium for 24 h. ($\times 4$) Scale par 50 μm showing: (a) HOCC incubated in ordinary medium as control showed the crowded and contact between cancer cells. (b-e) HOCC incubated in 20, 50, 100 and 250 μg of GTEs, respectively, showing reduction in the number of the cancer cell

significant decrease in the number of the cells by several concentrations. At the control (0 concentrations of GTEs) the mean number of the cancer cells was (1.7×10^5) and, by incubated in different concentrations of GTEs (20, 50, 100 and 250% $\mu\text{g mL}^{-1}$ for 24 h) the mean number of the cancer cells was (1.1600, 1.0450, 0.6400 and 0.5900×10^5) as shown in the Table 1. Whereas, statistical analysis revealed that the number of treated cells significantly reduced. Table 1 showed that the ideal concentration of GTEs affect the proliferation of cancer cells was 100 $\mu\text{g mL}^{-1}$.

On long term exposure of HOCC to green tea extract showed there were about (0.61500, 0.56000, 0.43500×10^5 cells) decrease in the number of cells in comparison with control after (24, 48 and 72) h incubation, respectively (Fig. 4).

Morphological Study

The images of the fixed and stained HOCC with Coomassie stain revealed that the cancerous cells appear very crowded in control group, large in its size, contain no vacuoles, clear distinguished, its nuclei appear very large and pleomorphism (Fig. 1a-e, 2a and 3a).

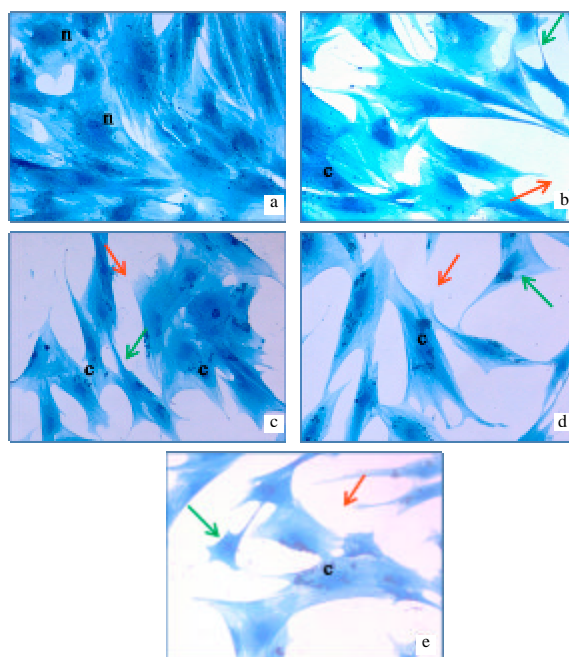


Fig. 2: (a-e) Represent HOCC incubated in ordinary and GTEs medium for 24 h (x20), Scale par 200 μm showing: (a) HOCC incubated in ordinary as control showing pleomorphism nuclei (n) and large number of cells. (b, c, d and e) Represent HOCC incubated in 20, 50, 100 and 250 μg GTEs showing chromatin condensation (C) loose cell-cell contacts (red arrows) shrinkage of cells (green arrows) signs of apoptosis

Whereas, in the tea treated groups, the cells show a degree of injury and discomfort. Moreover, the cells shrank and started to degenerated at various concentrations (20, 50, 100 and 250 $\mu\text{g mL}^{-1}$) up to 24 h (Fig. 1 a-e, 2b-e and 3b-e).

This indicates that the examined substrate attacks cancer cells and may causing loss of cell-cell contacts (Fig. 2b-e and 3b-e), cells shrinkage, blabbing and specific chromatin condensation (Fig. 2, 3b-e), which leads to cell death by apoptosis not by necrosis.

The present study proved that the green tea extract inhibited the proliferation rate of human ovarian cancer cells *in vitro* in a dose dependent manner.

Chemoprevention therapy by the use of green tea or green tea polyphenols has offered new approaches to block tumor growth and progression. Green tea extract and especially its major polyphenolic component EGCG, is capable of inhibiting the growth of a variety of human cancer cells, via induction of apoptosis *in vitro* (Masuda *et al.*, 2001, 2003; Huh *et al.*, 2004; Chan *et al.*, 2006), our work proved this approach.

Chang *et al.* (2003) reported that, the role of tea in protection against cancer has been supported by ample evidence from studies in cell culture and animal models.

However, results of epidemiological studies on tea and cancer have been inconsistent, some of which associated with reduced risk of cancer, whereas others found that tea lacks protective activity against certain human cancers.

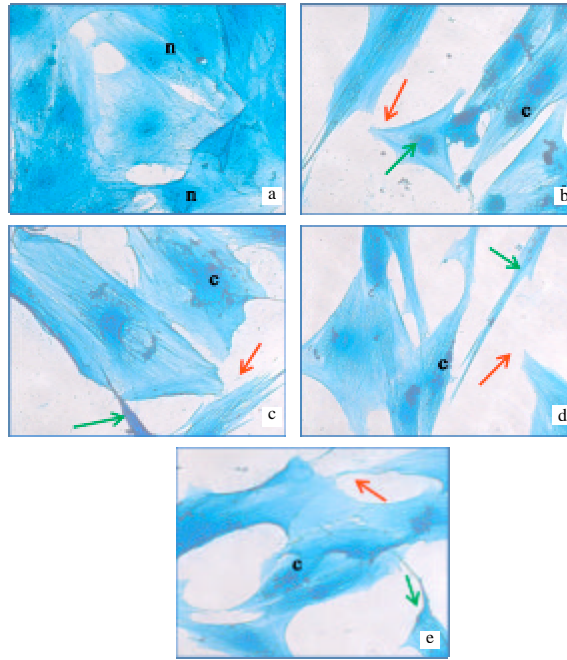


Fig. 3: (a-e) Represent HOCC incubated in ordinary and GTEs medium for 24 (x40), Scale par 500 µm showing: (a) HOCC incubated in ordinary as control showing pleomorphism nuclei (n) and large number of cells. (b, c, d and e) Represent HOCC incubated in 20,50, 100 and 250 µg GTEs showing chromatin condensation (C), loose cell-cell contacts (red arrows) shrinkage of cells (green arrows) signs of apoptosis

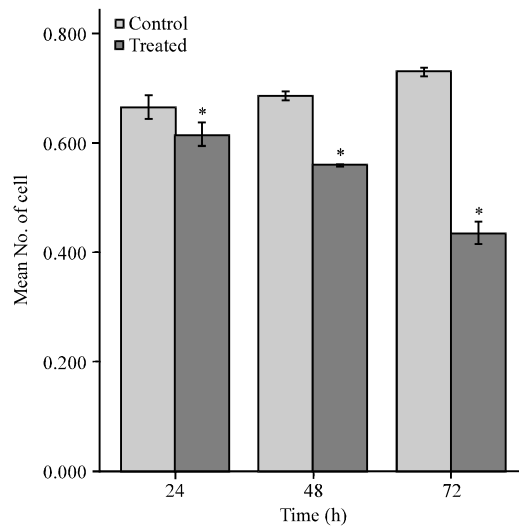


Fig. 4: Represent the difference of proliferation between HOCC cells incubated in IC_{50} of GTEs and HOCC cells incubated in ordinary medium. Values represents as (Mean±SD). *Significant at $p < 0.05$

Aucamp *et al.* (1997) also, has agreeing results with ours, in cultured human leukemia cells, where EGCG from green tea and theaflavins gallates from black tea inhibited xanthine oxidase activity.

Frei and Higdon (2003) investigated that, a great deal of research has evaluated the antioxidant and biological activities of green and black tea as well as their individual catechins and polyphenols *in vitro*. Also, Thangapazham *et al.* (2007) said that, our investigation results showing the effect of GTP and EGCG treatment *in vivo* in human breast cancer MDA-MB321 cells xenograft in nude mice as well as *in vitro* cell culture models inhibits proliferation and induce apoptosis of MDA-MB231 cells *in vitro* and *in vivo*, these data sustain our contention that GTEs have anti-tumor properties.

The anticancer effect of green tea may relate to the phase II detoxification enzymes that promote the excretion of potentially toxic or carcinogenic chemicals. Most phase II enzymes contain *cis*-acting regulatory elements called Antioxidant Response Elements (ARE). Glutathione S-Transferases (GST) are a family of phase II enzymes that catalyze the conjugation of glutathione to electrophiles, thereby reducing their ability to react with and damage nucleic acids and proteins (Parkinson, 1996). Green tea polyphenol extract (Yu *et al.*, 1997) as well as individual green tea catechins (Chen *et al.*, 2000) have been found to increase ARE-mediated reporter gene activity in transected HepG2 cells. Feeding rats green tea leaves significantly increased liver Glutathione S-Transferases (GST) activity (Lin *et al.*, 1998) and providing mice with green tea polyphenols in their drinking water also significantly increased GST activity in the liver and small intestine (Khan *et al.*, 1992) thus, may explained our results in this project.

Epidemiologic studies on tea and ovarian cancer have generated inconsistent results as a recent research reported a protective effect of green tea on ovarian cancer risk (Zhang *et al.*, 2002) and survival rates (Zhang *et al.*, 2004) among Chinese women.

In terms of cancer chemoprevention, tea polyphenols can inhibit several of these transcription factors, such as activator protein-1 and nuclear factor- κ B, thereby blocking mitotic signaling pathways (Lin, 2002).

Lu *et al.* (2000) showed that green tea and caffeine enhance UV-induced apoptosis and P53 p21 waf1-positive skin cells in SK-H1 mice. Administration of green tea as drinking water induces apoptosis in lung adenomas that developed in A/J mice after exposure to NNK and prostate cancer cell in TRAMP mice (Gupta *et al.*, 2001; Liao *et al.*, 2001).

August *et al.* (1999) showed that green tea causes a significant reduction in prostaglandin E synthesis in rectal mucosa of human volunteers suggesting the active compounds in tea inhibit the cyclooxygenases.

The P53 (tumor suppression gene) plays a pivotal role in protecting cells from various stresses including DNA damage, oncogene stimulation and change in cellular redox potential (Vogelstein *et al.*, 2000). It regulates cell cycle arrest and apoptosis in response to certain stresses in its role as tumor suppressor. The EGCG and theaflavins digallate are inhibitors of cell growth and both agents induce a significant antiproliferative and proapoptotic effect on various cell types including human oral epithelial cells (Ahmad *et al.*, 1997, 2000; Liang *et al.*, 1999; Katdare *et al.*, 1998; Yang *et al.*, 1998, 2000). Tea polyphenoles increase P53 levels and induction of apoptosis by stresses such as UVB1, which are known to activate P53, mediated apoptosis (Lu *et al.*, 2000; Ahmad *et al.*, 1997; Yang *et al.*, 1998).

Green tea extract and especially its major polyphenolic component EGCG, are capable of inhibiting the growth of a variety of human cancer cells via induction of apoptosis *in vitro* (Masuda *et al.*, 2001, 2003; Huh *et al.*, 2004; Chan *et al.*, 2006).

Kennedy *et al.* (1999) found that GTEs exhibits cytotoxicity to Ehrlich ascites tumor cells in the cellular thiol-dependent way. The GTEs caused a significant reduction in the

viability of the tumor cells and further analysis revealed that this effect was attributable to one of GTEs EGC caused significant reductions in both non-protein sulfhydryls (GSH) and protein sulfhydryl (PSH) levels.

The effect of EGC on the reductions in cellular thiol levels was found to be both dose and time dependent. The EGCG also had a slight effect on cell viability but like the other polyphenols, which had no effect on cell viability, did not have any effect on GSH or PSH reductions. The unique effect of one catechin over other structurally related catechins is not new in tea polyphenol research. Several studies have reported differential inhibitory or enhancement effect of structurally related catechins. Most recently Watanabe *et al.* (1998) found an IC_{50} value of acetyl CoA carboxylase inhibitory activity of EGCG and EGC to be $\sim 300 \mu\text{g mL}^{-1}$, whereas, (+)-catechins, EC, EGC and gallic acid had no effect. The inhibitory activity was attributable to the presences of the 3-O-gallate group of the catechin structure. Green tea polyphenols have been shown to be efficient antioxidants and the concept that tea components may inhibit carcinogenesis through antioxidative activities is supported by many findings. The H_2 formation induced by 120-tetradecanoylphorbol 13-acetate in HeLa cells was inhibited by EGCG and the oxidation of lard as evaluated by the active oxygen method was suppressed as well (Matsuzaki and Hara, 1985; Bhimani and Frenkel, 1991). Activation of protein tyrosine kinase activity by EGC via decreased cell viability (Kennedy *et al.*, 1998). Green tea and its individual epicatechin derivatives inhibited skin tumor promoter-mediated induction of epidermal ornithine decarboxylase in SENCAR mice.

CONCLUSION

In conclusion, green tea extract has antiproliferative activity against the growth of HOCC which might be considered in clinical situation.

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