# White Tea (*Camellia sinensis*) Extract Role as AntiProliferative of Colon Cancer Cell Line, Caco-2

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***Abstract*- OBJECTIVES:** Tea (Camellia sinensis) is one of the most consumed beverages in the world. White tea is made from the buds and young leaves of the tea plant which are steamed and dried, whilst undergoing minimal oxidation.

**METHODS:** Caco-2, cells were incubated with white tea extract at concentrations, 300µM, 500µM, 700µM and 900µM for 72h at 37°C and 5% $CO_2$. MTT assay DNA fragmentation assay, cytological and immunocytochemical investigations were investigated.

**RESULTS:** MTT assay was used to test the extract on the effect of the proliferation of the colorectal cancer cell line, Caco-2. IC50 extract inhibited the proliferation of Caco-2 cells and induced Caco-2 cell death in a dose-dependent manner. Treated cells showed typical characteristics of apoptosis including inhibited the viability and proliferation of treated Caco-2 cells *in vitro* even by DNA fragmentation, cytological alterations and increased of P53 activity.

**CONCLUSIONS:** Results from this study show that white tea extract has antiproliferative effects against cancer cells. Regular intake of white tea can help to maintain good health and protect the body against disease. Future study will may deal with further investigations of white tea extract possible usages as a new alternative or complementary chemotherapeutic agent for human cancer types specially colon cancer type.

***Index Terms*-** white tea extract, Caco-2 Cells, p53, antiproliferative effects.

## I. INTRODUCTION

Colorectal cancer is the third most common form of cancer and the second leading cause of cancer deaths in both men and women around the world. Alarmingly, increasing numbers of reported cases of colon cancer in recent years has made this form of cancer a major health concern[1]. The current treatment for colorectal cancer is generally surgical resection combined with chemotherapy by cytotoxic drugs and radiation. However, this therapy is just moderately successful especially for late stage cancers; therefore new approaches to the treatment of colorectal cancer are required. In recent years, interest has increased in using natural products for pharmacological purposes, as a form of complementary or replacement therapy. It is known that the risk of colorectal cancer increases with dietary habits like high animal fat intake[2]. Epidemiological and prospective studies have reported several beneficial effects of bioactive compounds on human health, particularly in protecting against chronic degenerative diseases, such as cardiovascular disease, diabetes mellitus and cancer. Phenolic compounds, present in fruits and vegetables, show antioxidant and antiproliferative properties. A number of studies have suggested that high consumption of fruit and vegetables decreases the risk of colon cancer[3].

Second only to water, tea is one of the most consumed beverages in the world. Most of the commercial varieties of teas come from the dried leaves of a shrub is *C. sinensis.* Based on processing and harvesting the leaves, tea types are black, green, oolong and white[4]. White tea is an unfermented tea made from young tea leaves or unopened buds covered with tiny, silvery hairs, and the leaves are harvested once a year in the early spring. The leaves are then steamed rapidly and dried, with a minimum amount of processing to prevent oxidation[5]. Green, oolong and black teas are processed to a greater extent compared to white tea, though green tea is also unfermented.

The mechanisms that have been suggested for the health benefits of tea include scavenging of reactive oxygen species (ROS), modification of signal transduction pathways, cell cycle checkpoints, and apoptosis, and the induction of various enzyme activities involved with drug metabolism and carcinogen activation/detoxification[6].

P53 has been shown to be involved in the induction of apoptosis, cell-cycle arrest and differentiation responses that prevent further proliferation of stressed or damaged cells and so protect from the outgrowth of cells harboring malignant alterations[7]. P53 role in the repair of DNA damage has also been described and the ability of p53 to induce reversible cell-cycle arrest may contribute to the ability of cells to repair and recover from damage before reentering a normal proliferative state[8]. The aim of this study was to evaluate the effect of white tea extract on the inhibition of proliferation of the colorectal cancer cell line, Caco-2.

## II. MATERIALS AND METHODS

**Chemical reagents:** White tea extract, MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide, dimethylsulfoxide (DMSO), commercial methanol, commercial ethanol, commercial acetone, Tris-HCl, edetic acid, Triton-X100, RNase A, proteinase K, NaCl, 2-propanol, phosphate-buffered saline (PBS), ethidium bromide, agarose gel, Peroxidase, trypsin, Hematoxylin and eosin (Hx & E) stain, primary monoclonal antibody against p53 and biotinylated immunoglobulin secondary antibody were purchased from Sigma-Aldrich, Egypt.

**Cell line and cell culture:** Caco-2 cell line, was obtained from American Type Culture Collection (ATCC, USA). They were sub-cultured as mono-layer according to the instructions provided by ATCC in Dulbecco modified Eagle medium (DMEM) supplemented with 10% heat inactivated (56°C,

30min) fetal bovine serum, 2mmol/L L-glutamine, 100U/mL Penicillin-Streptomycin and 100U/mL Amphotericin B at 37°C in a humidified atmosphere of 5% $CO_2$. Cells were used when monolayer reached 80% confluence in all experiments. Cell propagation media was purchased from Invitrogen (Carlsbad, CA).

**Methods: 1.Cell Viability Assay: *In vitro* evaluation of antiproliferation effect:** growth inhibition was evaluated by MTT assay. MTT salt or 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was reduced by mitochondrial dehydrogenases to water blue insoluble formazans[9]. Viable cell number/well is directly proportional to formazans production. $8.25\times10^3$ cells were seeded into each well of 96-well plate, incubated with culture medium overnight (12h), replaced with fresh medium containing white tea extract at concentrations: 300µM/L, 500µM/L, 700µM/L and 900µM/L for 72h at 37ºC in an incubator with 5% $CO_2$. After incubation, white tea extract modified medium was replaced by 100µL of MTT (0.5mg/mL) medium for incubation (3h at 37°C and 5% $CO_2$). MTT medium was then replaced with 100µL of DMSO and left for 10min on a platform shaker to solubilize converted formazan. The absorbance values were determined at 570nm test wavelength and 630nm reference wavelength (Spekol 1200 spectrophotometer). Untreated cells were as a positive control cells and all values were correlated with this set of data. The experiment was performed in triplicates. Inhibition Percentage=[1–(net Absorbance of treated well/net Absorbance of control well)]x100%, then was plotted against white tea extract concentrations.

**2.Determination of DNA fragmentation by DNA laddering assay:** cells were seeded in 60-mm petri dishes at density $4x10^5$ cells/plate (treated cells by $IC_{50}$ concentration of white tea extract or positive control cells). Adherent and floating cells were collected by centrifugation at 1000×g/5min. Cell pellet was suspended in cell lysis buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 10mmol/L pH8.0, Triton-X100 0.5%) and kept at 4ºC/10min then, lysate was centrifuged at 25.000×g/20min. Supernatant was incubated with RNase A 40µg/L/1h (37ºC), incubated with proteinase K 40µg/L/1h (37ºC), mixed with NaCl 0.5mol/L and 50% 2-propanol overnight (-20ºC), then centrifuged at 25.000×g/15min. After drying, DNA was dissolved in buffer (Tris-HCl 10mmol/L pH7.4, edetic acid 1mmol/L pH 8.0) and separated by 2%agarose gel electrophoresis at 100V for 50min. DNA was visualized under ultraviolet light after staining with ethidium bromide[10].

**3.Cytological changes investigation**: detached and trypsinized cells ($IC_{50}$ concentration of white tea extract treated cells and positive control cells) were collected and centrifuged at 2000 rpm for 5min. Cell pellet was re-suspended with 100µL of PBS (pH7.3). 10µL of the suspension were smeared on a glass slide, allowed to air-dry, fixed with cool methanol for 5min before proceeding by Hx&E stain and examined under light microscope[11].

**4.Immunocytochemical investigations:** by detection of p53 by immunocytochemistry staining kit. The procedure was done according to the manufacturer's instructions, simplified as follows: 1-2 drops of Peroxidase was applied to cells ($IC_{50}$ concentration of white tea extract treated cells and positive control cells) on the slide (10min), followed by blocking solution (10min). Cells were fixed in ethanol:acetone (9:1) for 30min at -20°C and then rinsed again with cold PBS at room temperature. Cells were incubated overnight with primary monoclonal antibody against against p53 at dilution of 1:75 at 4°C, then in Tris buffer and biotinylated immunoglobulin secondary antibody was used[12]. The slides were then mounted and examined under light microscope.

**5.Statistical analysis**: results were presented as mean±standard deviations (SD). Analysis of variance (ANOVA) for two variables (Two Way-ANOVA) was used together with student t-test. Significant analysis of variance results were subjected to post hoc. Statistical significance was set at $P<0.05$ and high significance was set at $P\leq 0.01$[13].

## III. RESULTS

**1.Cell viability assay: *In vitro* evaluation of antiproliferation effect.**

Cytotoxic effect of different concentrations of white tea extract (300µM, 500µM, 700µM and 900µM) for 72h on Caco-2 cell line was determined by MTT assay (Figure 1). Cells number started to reduce immediately after treatment with white tea extract concentrations in a dose dependent manner. All concentrations were found to be high significantly different ($P\leq0.01$) in respect to their antiproliferative and apoptotic effects when compared with positive control cells. Cell inhibition percentage was gradually increased with white tea extract concentration increasing and 95% of cell inhibition was observed when cells were treated with 900µM/72h. Cell proliferation reduced about 25% and 30% when cells were treated with 300µM and 500µM for 72h, respectively. Cells proliferation decreased to 50% when treated with concentration of 700µM/72h.

**2.Determination of DNA fragmentation by DNA laddering assay.**

DNA degradation into multiple internucleosomal fragments is a distinct biochemical hallmark for apoptosis. Nuclear DNA isolated from Coca-2 cancer cells was separated by agarose gel electrophoresis and stained with ethidium bromide, and a typical ladder formation was observed upon 72h when treated with white tea extract concentration at 700µM whereas untreated cells did not show typical ladder (Figure 2). Results indicated that white tea extract induced DNA fragmentation which was caused by apoptosis.

**3.Cytological changes investigation.**

Positive control cells group had round nuclei, distinct small nucleoli and homogeneous chromatin with an accentuated nuclear membrane (Figure 3a). After Coca-2 cells treatment by white tea extract concentration at 700µM/72h, apoptotic cells were identified by a series morphological changes as an important experimental proof of underlying processes alterations appeared as: bleb plasma membrane, cellular shrinkage, chromatin condensation granules, vacuolated cytoplasm, degrading nucleus and apoptotic bodies formation were observed (Figure 3b, 3c and 3d).

**4.Immunocytochemical investigation.**

After Coca-2 cells treatment by white tea extract concentration at 700µM/72h, when applying p53 stain, p53 protein reaction in the positive control Coca-2 cells, was showed

negative reaction (no brown stain) (Figure 3f). After Coca-2 cells treatment by white tea extract concentration at 700µM/72h, those fields had necrotic or apoptotic nucleus for white tea extract effect showed p53 positive reaction (over expression of p53 protein) when over 55% of cells had nuclear brown staining, with slight intensity degrading in the same field (Figures 3e).

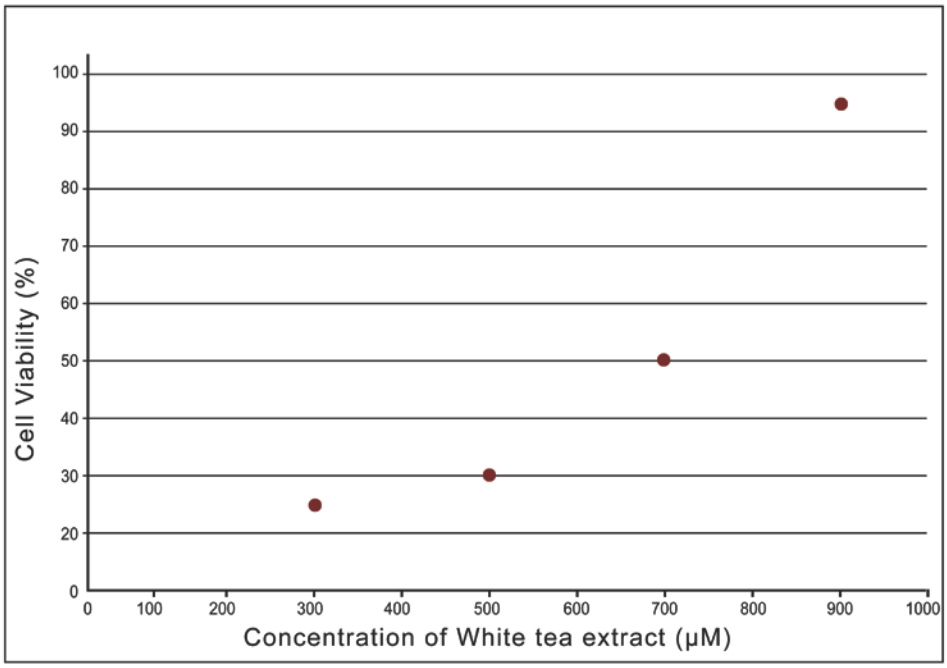


**Figure 1: Effect of white tea extract with different concentrations on the cells viability of Caco-2 cells. The experiment was performed in triplicates and values means were calculated [mean±SD, n (for each concentration)=4].**

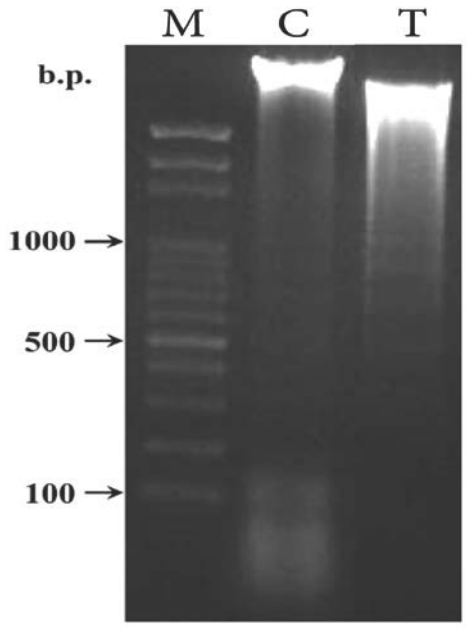


**Figure 2: DNA fragmentation by DNA laddering assay of extracted DNA from white tea extract treated cells and positive control cells. DNA laddering, typical for apoptotic cells, which were visible in treated Coca-2 cells (T), and there was no any apoptotic features in the positive untreated cells (C) where M indicating to marker.**

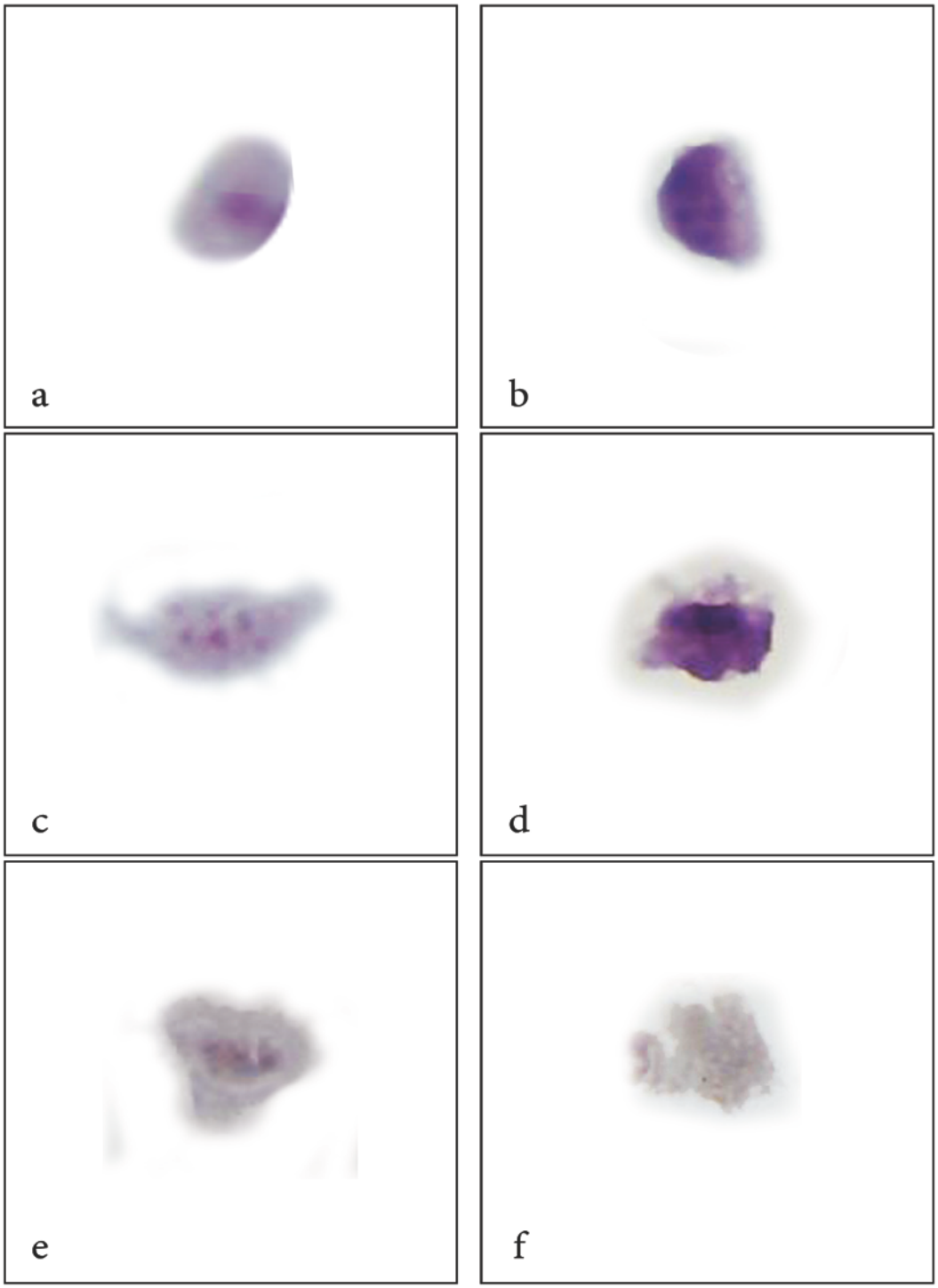


**Figure 3: Cells in different stages of apoptosis in treated cells are easily distinguishable. Cell with normal morphology (a). Complete apoptotic cell (b). Degradation of nucleus, vacuolated cytoplasm with apoptotic bodies (c). Nuclear condensation is evident in cells (dark, condensed and irregular rounded nucleus), bleb membrane and cell shrinkage (d). Immunocytochemistry of treated cell showing p53 protein nuclear positive reaction indicating cell apoptosis incidence (e). Control positive cell showing nuclear negative reaction (f).**

## IV. DISSCUTION

Tea contains a number of polyphenolic compounds belonging to the flavan-3-ol (catechin) family, such as (−)-epigallocatechin, (−)-epicatechin, (−)-epigallocatechin-3-o-gallate, (−)-epicatechin-3-o-gallate, (+)-catechins and (+)-gallocatechin[14]. These compounds are known to have a wide spectrum of biological activities such as antioxidant, antiviral, anticancer, antibacterial, antifungal, antitoxoplasmal,

antitrypanosomal, anticoccidial, antinematodal and antihelminthic[15].

Apoptosis, as programmed cell death, is a highly organized cell death process characterized by an early obvious condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of nucleases, enzymatic cleavage of DNA into oligonucleosomal fragments and segmentation of the cells into membrane-bound apoptotic bodies[16]. DNA fragmentation, a hallmark of apoptosis, is regulated by a specific nuclease called caspase-activated DNase and its inhibitor[17]. Apoptosis has specific signals instructing the cells with specific morphological change as plasma and nuclear membrane blebbings, chromatin condensation, proteases activation and DNA fragmentation that are considered as landmarks of the apoptotic process[18].

That was agreed with the results of recent study after treatment by white tea extract. White tea extract decreased the viable percentage of cell number (dose dependent effect) and induced apoptosis of Caco-2 cells. Therefore, we may presume that as primary mechanism involved in white tea extract growth-inhibitory effects as it considered main apoptotic signals.

P53 is a tumor suppressor gene product which is very important for cells in multicellar organisms to suppress cancer[19]. P53 has been described as 'The Guardian of the genome', referring to its role in conserving stability by preventing genome mutation[20]. Upon genotoxic and other stress, p53 protein levels increase. Activated p53 releases signal to cells to undergo growth arrest, cell differentiation or apoptosis[21]. Caco-2 cells which were treated with white tea extract exhibited increased levels of p53 expression at concentration of 700µM/72h, which suggested that p53 involved in white tea extract-induced Caco-2 cell death.

## V. CONCLUSION

In this study, we have demonstrated that white tea extract inhibited proliferation and induced apoptosis in colon cancer (Caco-2) cells which depended on up-regulation of p53 protein. Future *in vitro* and *in vivo* study will may deal with further investigations of the possible usages of white tea extract as a new alternative chemotherapeutic agent but in limit doses for human colon cancer suggested treatment and other types of cancer.

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