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White tea (*Camellia sinensis*) inhibits proliferation of the colon cancer cell line, HT-29, activates caspases and protects DNA of normal cells against oxidative damage



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ABSTRACT

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. The MTT assay was used to test the extract on the effect of the proliferation of the colorectal cancer cell line, HT-29. The extract inhibited the proliferation of HT-29 cells with an IC₅₀ of 87 µg/ml. The extract increased the levels of caspase-3, -8, and -9 activity in the cells. DNA damage in 3T3-L1 normal cells was detected by using the comet assay. The extract protected 3T3-L1 cells against H₂O₂-induced DNA damage. The results from this study show that white tea has antioxidant and antiproliferative effects against cancer cells, but protect normal cells against DNA damage. Regular intake of white tea can help to maintain good health and protect the body against disease.

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1. 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 (Levin et al., 2008). An estimated 96,830 cases of colon and 40,000 cases of rectal cancer are expected to occur in 2014 (American Cancer Society, 2014). 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 (Reddy, 1986).

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 (Gallaher & Trudo, 2013).

The health benefits associated with tea (*Camellia sinensis*) consumption have been attributed in part to the antioxidant and free radical scavenging activity of the relatively high levels of flavonoids, including catechins, and other polyphenols present in tea (Serafini, Del Rio, Yao, Bettuzzi, & Peluso, 2011).

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, *C. sinensis* L, belonging to the Theaceae family, native to south and southeastern Asia. The differences amongst teas arise from processing, growth conditions, and geographical regions. Based on processing and harvesting the leaves, tea types are black, green, oolong and white (Venditti et al., 2010).

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 (Hilal & Engelhardt, 2007). Green, oolong and black teas are processed to a greater extent compared to white tea, though green tea is also unfermented.

Tea contains a number of polyphenolic compounds belonging to the flavan-3-ol (catechin) family, such as (–)-epigallocatechin,

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(–)-epicatechin, (–)-epigallocatechin-3-*o*-gallate, (–)-epicatechin-3-*o*-gallate, (+)-catechins and (+)-gallocatechin (Leung & Foster, 1996). 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 (Almajano, Carbó, Jiménez, & Gordon, 2008; Fassina et al., 2002; McKay & Blumberg, 2002; Yang, Wang, Lu, & Picinich, 2009). Tea also contains volatile oils, vitamins, minerals and purines (McKay & Blumberg, 2002).

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 (Yang et al., 2009).

The aim of this study was to evaluate the antioxidant activities of white tea and its role in protecting against oxidative damage to DNA. The effect of the tea on the inhibition of proliferation of the colorectal cancer cell line, HT-29, and on cellular caspase activities were also investigated.

2. Materials and methods

2.1. Chemicals

All the solvents and reagents used in the study were of analytical grade and used without any further purification. Ultrapure water was used for dilutions and extractions. Catechin, rutin, quercetin, gallic acid, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2-diphenyl-1-picrylhydrazyl, 2-deoxy-D-ribose, Na₂EDTA, ethidium bromide, sodium dodecyl sulphate and agarose were purchased from Sigma Aldrich Chemical Company (USA). Folin–Ciocalteu reagent, aluminium chloride and acids and bases were purchased from Chemical Systems Company. Trichloroacetic acid, nitroblue tetrazolium, phenazine methosulphate, sodium nitroprusside, phosphate buffer saline and sulphanilamide, N-(1-naphthyl)-ethylenediamine dihydrochloride was obtained from Fisher Scientific Company (UK). MTT bromide was purchased from Merck Company (Germany).

2.2. Tea samples and preparation

White tea (Silver needle, KWF Food Industries, China), was purchased from the local market. Tea infusion was prepared by placing 2 g of tea leaves in 100 ml of distilled water at boiling temperature (100 °C) and brewing for 5 min. The sample (Silver needle white tea infusion) was filtered through Whatman filter paper No. 1 and the water extracts were freeze-dried. The concentrated extract was diluted appropriately with distilled water according to each specific assay and stored at –20 °C until further analysis.

2.3. Total phenolic and flavonoid content of tea extracts

The total phenolic content (TPC) of the different tea extracts was measured using the Folin–Ciocalteu assay (Singleton & Rossi, 1965) with some modifications. The volumes were scaled down to accommodate microtiter plate volumes. Briefly, 10 µl of tea sample was added to the well of the plate, followed by 500 µl of Folin–Ciocalteu reagent and mixed. After 5 min, 350 µl of 10% Na₂CO₃ was added. After gentle mixing, the samples were left in the dark for 2 h at room temperature. The absorbance was then read at 765 nm against the reagent blank containing water instead of sample. For estimating the TPC values, standard concentrations of gallic acid were used to construct a calibration curve (absorbance vs. µg/ml gallic acid). Catechin was used as control.

The values for TPC are expressed in mg gallic acid equivalents (GAE) per g dried sample (dry weight, dw).

The total flavonoid content (TFC) was evaluated by the protocol of Chang, Yang, Wen, and Chern (2002). Briefly, 10 µl of 5% sodium nitrate (NaNO₃) was added to 100 µl of the original stock solution (1 mg/ml) of the samples. The mixture was incubated in the dark for 5 min. A 10 µl volume of aluminium chloride (AlCl₃, 10%) was added to the mixture and incubated for a further 5 min in the dark. A 100 µl of NaOH, 1 M, was added to the resulting mixture followed by addition of 30 µl of distilled water. The absorbance was read at 510 nm. The total flavonoid content of the sample was expressed in milligram quercetin equivalents (per g dried weight). All analyses were carried out in triplicate.

2.4. Antioxidant activities of the white tea extract (WTE)

2.4.1. Ferric reducing antioxidant power

The FRAP assay measures the reducing potential of antioxidants by reaction with the ferric tripyridyltriazine (Fe³⁺–TPTZ) complex, producing a blue colour from the ferrous form that can be detected by absorbance at 593 nm. Antioxidant compounds that act as reducing agents exert their effect by donating a hydrogen atom to the ferric complex, thus breaking the radical chain reaction.

The Fe³⁺ reducing power (FRAP) of the extract was determined by the method of Benzie and Strain (1996) with slight modifications. The volumes were scaled down to accommodate microtiter plate volumes. The FRAP reagent was prepared by mixing 10 ml of 300 mM acetate buffer with 1 ml of 10 mM TPTZ (2,4,6 tripyridyl-S-triazine) in 40 mM of HCl and 1 ml of 20 mM of FeCl₃·6H₂O. Then 3 µl of tea extract, standard or positive control and 9 µl of water were added to 90 µl of FRAP reagent. Absorbance readings were measured instantly upon addition of the FRAP reagent at 593 nm every 10 s for 4 min. The ferric reducing activity was determined by plotting a standard curve of FeSO₄·7H₂O (0–1000 µmol/l). Results were expressed as mmol ferric reducing activity of the extracts per g of dried extract.

2.4.2. DPPH radical scavenging activity

Antioxidant activities of the white tea extracts (WTE) were estimated by measuring their scavenging capacity against the DPPH radical (Gerhauser et al., 2003). Tea extract (20 µl) was added to 120 µl of a 0.004% MeOH solution of DPPH. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as standard. Absorbance at 517 nm was determined after 30 min incubation in dark at room temperature, and the percentage of inhibition was calculated as $[(A_0 - A_1)/A_0] \times 100$, where A₀ is the absorbance of the control (dH₂O), and A₁ is the absorbance of the samples. A graph of the DPPH radical scavenged (%) vs. concentration of sample was plotted. The IC₅₀ value denotes the effective concentration of sample used to reduce 50% of available DPPH radicals. In the DPPH radical scavenging assay, the deep violet colour of DPPH is reduced to a light yellow colour due to the abstraction of hydrogen atoms from the antioxidant compound (Molyneux, 2003).

2.4.3. Hydroxyl radical scavenging activity

The effect of hydroxyl radicals was estimated by using the 2-deoxy-D-ribose oxidation method according to the modified method established by Halliwell and Gutteridge (1985). The hydroxyl radical assay is based on the principle that H₂O₂ in the presence of Fe³⁺–EDTA, at pH 7.4, generates free radicals which can be measured by using deoxyribose.

The following reagents were mixed with 200 µl of sample solution of various concentrations in the stated order: 200 µl of FeCl₃ 100 Mm, 200 µl of 1.25 mM H₂O₂, 200 µl of 2-deoxy D-ribose, 2.5 mM, and 200 µl of 100 mM vitamin C. The reaction mixture was incubated at 37 °C for 1 h. Then, 100 µl of a 0.5% TBA

(thiobarbituric acid) diluted with NaOH (0.025 M) and 100 μ l of a 2.8% TCA (trichloroacetic acid) were added and the mixtures were placed into a temperature-controlled water bath at 100 °C for 30 min. This was followed by cooling in ice to room temperature, and then absorbance at 532 nm was measured. All values were determined in triplicate. The percentage of hydroxyl radical scavenging activity was calculated using the following equation: $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control (dH_2O), and A_1 is the absorbance of the samples.

2.4.4. Nitric oxide radical scavenging activity

Nitric oxide radical scavenging activity was determined according to the method described by Sreejayan and Rao (1997). For the estimation, 50 μ l of sodium nitroprusside (SNP, 5 mM) was added to 50 μ l of various concentrations of the samples. Mixtures were incubated under visible polychromatic light for 1 h, and then 100 μ l of Griess reagent was added to the mixtures and incubated for a further 5 min and the absorbance measured at 532 nm. Trolox was used as the standard. To prepare Griess reagent an equal amount of SA (1% sulphanilamide, 5% H_3PO_4) and NED (0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NED)) were mixed. The nitric oxide scavenged (%) was calculated using following formula:

$$\text{Nitric oxide radical scavenged, \%} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the control (dH_2O), and A_1 is the absorbance of the extract/standard. A graph of nitric oxide radical scavenged (%) vs. concentration of sample was plotted.

2.4.5. Superoxide anion radical scavenging activity

The superoxide anion radical scavenging activity of the sample was investigated by the method of Liu, Ooi, and Chang (1997). In this estimation, 50 μ l of NADH (468 μ M), 50 μ l NBT (150 μ M) and 50 μ l phenazine methasulphate (PMS, 60 μ M) were added to the various concentrations of the samples. All the above reagents were diluted in PBS (pH 7.4). The mixtures were incubated for 15 min in the dark and the absorbance measured at 560 nm. Trolox was used as standard and catechin was used as positive control. The superoxide anion radical scavenging capacity was calculated using the following equation:

$$\text{Superoxide anions scavenging activity \%} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the control (dH_2O), and A_1 is the absorbance of the extract/standard.

2.5. Cell culture

Human colorectal adenocarcinoma cells (HT-29), human dermal fibroblasts-adult (HDF-a) and mouse fibroblasts (3T3-L1) were used in this study. HT-29 and 3T3-L1 cells were purchased from the American Type Culture Collection (ATCC, USA). HT-29 (ATCC[®] HTB-38[™]) cells were cultured in RPMI-1640 (Sigma, UK) and 3T3-L1 cells (ATCC[®] CL-173[™]) were grown in DMEM (Lonza, USA). HDF-a was purchased from ScienCell Research Laboratories, USA. HDF-a cells were routinely cultured in fibroblast growth medium (ScienCell Research Laboratories, CA, USA).

The cells were supplemented with 5 or 10% foetal bovine serum (FBS), 100 IU/ml penicillin and 100 μ g/ml streptomycin (iDNA, South America). Cells were grown at 37 °C in a humidified incubator with 5% CO_2 .

2.6. In vitro anti-proliferative effects of WTE

The inhibitory effect of WTE on the proliferation of the colorectal adenocarcinoma cell line, HT-29, was determined by using the

MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983). A normal adult human fibroblast line, HDF-a, was used as a control to identify any cytotoxic effect of the extracts.

In brief, cells were seeded in 96-well plates at 5000 cells/well and allowed to attach overnight. Media was changed and the cells were treated with various concentrations of the extract (0–500 μ g/ml) incubated for an additional 48 h. After 48 h, 20 mM of MTT solution (5 mg/ml MTT bromide in PBS) was added to each well and incubated for 4 h at 37 °C. The supernatant was aspirated and the MTT-formazan crystals formed by metabolically viable cells were dissolved in 100 μ l of dimethyl sulphoxide (DMSO). Finally, the absorbance was monitored by a microplate reader at a wavelength of 590 nm. Growth inhibition of cells was calculated according to the following formula:

$$\text{Inhibition (\%)} = [(OD_{\text{blank}} - OD_{\text{sample}})/(OD_{\text{blank}})] \times 100$$

2.7. Cellular caspase activities (Caspase-3/7, -8 and -9)

HT-29 cells (25,000 cells/well), in RPMI-1640 and 10% FBS, were seeded in the white 96-well plate (SPL, Korea) and incubated overnight at 37 °C with 5% CO_2 and 37 °C. The medium was then replaced with fresh medium and the cells treated with the IC_{50} concentration of WTE for different time periods, i.e., 2, 8, 16, 24 and 48 h. Cells without any treatment and media alone without cells in triplicate for the different times incubated were used as the control and blank. Caspases-3/7, -8 and -9 activities were carried out by using commercial kits purchased from Promega Company (USA) according to the manufacturer's protocol. In brief, after the treatment time, the reagents of Caspase-Glo[™] -3/7, -8 and -9 reagents were prepared and added directly to the cells in 96-well plates and incubated for 30 min before recording luminescence. To reduce nonspecific background activity in cell-based assays, MG-132 inhibitor was added to Caspase-Glo[®] -8 and -9 reagent. The plates were read in a luminometer (GloMax microplate luminescence reader, Promega Company, USA). The raw data were collected from the luminometer and the average calculated from the replicates. Background readings were determined from wells containing culture medium without cells. The "no cell media" blank control value was subtracted from each value.

2.8. Fluorescence microscopic examination

The morphological characterisation of HT-29 cells was performed by using propidium iodide (PI) and acridine orange (AO) double staining according to the method described by Ng et al. (2013) and observed under a fluorescence microscope. Briefly, HT-29 cells were plated at a density of 1×10^6 cells/ml in a 25 ml culture flask and treated with the IC_{50} concentration of WTE determined from MTT assay at varying time points of incubation. The cells were incubated in an atmosphere of 5% CO_2 at 37 °C for 8, 12 and 24 h. Then the cells were harvested and washed twice using PBS after centrifuging at 1800 rpm for 5 min to remove the remaining media. An equal volume of fluorescent dye (AO/PI) containing AO (10 μ g/ml) and PI (10 μ g/ml) were added to the cellular pellet and freshly stained cells were observed under a UV-fluorescence microscope within 30 min before the fluorescence colour started to fade.

2.9. Analysis of DNA damage

Analysis of DNA damage was carried out by using the comet assay in the murine embryonic fibroblast line, 3T3-L1 (ATCC). The 3T3-L1 cells were cultured in 12-well tissue culture plates

(1×10^5 cells/well) for 24 h at 37 °C, in a humidified atmosphere containing 5% CO₂. Then the cells were pre-treated for 24 h with WTE at concentrations of 5–25 µg/ml. After 24 h of incubation, the cells were treated with 100 µM of H₂O₂ for 60 min in an ice bath to prevent the action of DNA repair mechanisms (Miller, Thomas, & Buschbom, 1995), and then harvested using trypsin–EDTA, centrifuged for 5 min at 1500 rpm and resuspended in 1 ml of PBS. A volume of 25 µl of cell suspension was mixed with 75 µl of 0.6% low melting agarose. The suspension was spread on a frosted microscopic slide pre-coated with 250 µl of 0.8% normal melting agarose, covered with a cover slip, and then allowed to solidify on ice for 10 min. The cover slips were then removed and the slides were immersed in cold lysis solution containing 1% sodium dodecyl sulphate, 2.5 M NaCl, 100 mM Na₂EDTA, 1% Triton X-100, and 10% DMSO, with the DMSO for 1 h at 4 °C in the dark. The slides were arranged in an electrophoresis tank filled with pre-chilled electrophoretic buffer (1 mM Na₂EDTA and 300 mM NaOH) and incubated for 20 min. Electrophoresis was conducted at 25 V (300 mA) for 20 min using a power supply. The slides were washed with 0.4 M Tris–HCl (pH 7.5) and stained with 20 µg/ml ethidium bromide. An Olympus BX50 fluorescence microscope was used for viewing the slides. A total of 50 cells in triplicate per group were used to calculate the DNA damage induced by hydrogen peroxide. The comet tail length was measured using an ocular micrometer and the DNA damage was calculated by the following formula:

Comet tail length = maximum total length – head diameter

2.10. Statistical analysis

Experimental results are presented as means ± SD, and all measurements and analyses were carried out in triplicate. Excel 2007 and SPSS V.18.0 statistical software were used for the statistical and graphical evaluations in this study. Statistical analysis was performed by one-way analysis of variance (ANOVA) with Tukey's multiple comparisons and the Student's *t*-test. All *p*-values <0.05 were considered significant.

3. Results and discussion

3.1. Total phenolic and flavonoid content

The total phenolic content (TPC) was quantified as gallic acid equivalents (GAE). The TPC of Silver needle white tea was

863 ± 5.6 mg GAE/g dried weight of sample in hot water extraction (Fig. 1). In our study, white tea extracted with hot water showed high amounts of total phenols within the extraction time of 5 min. The TPC of the hot water extract was higher than that of the pure flavonoid, rutin. According to (Venditti et al., 2010), the levels of total polyphenols were high in green and white teas under two different extraction methods, hot water for 7 min and cold water for 2 h. However, we decided to steep the tea for only 5 min as this would reflect the actual time commonly used in steeping tea.

The content of total phenols and flavonoids in white tea extract are presented in Fig. 1. The total flavonoid content (TFC) of Silver needle white tea infused in hot water for 5 min was 530 ± 10.6 mg quercetin/g dried weight of sample. The TFC of the controls, catechin and rutin, were 999.2 ± 9.9 and 569.7 ± 12.026 mg quercetin/g dried weight, respectively. These results indicate that Silver needle white tea, extracted for only 5 min, is a rich source of flavonoids, when extracted in hot water, since the TFC is comparable to that of rutin. However, a study by Rusak reported that the TFC of green and white tea increased with extraction time (Rusak, Komes, Likic, Horzic, & Kovac, 2008).

3.2. Ferric reducing antioxidant power (FRAP)

In this assay sample readings were referenced to the standard curve of ferrous sulphate, FeSO₄·H₂O. The FRAP value was expressed as mmol Fe²⁺/g sample. Table 1 shows the reductive capability of WTE. The FRAP value of the sample in hot water extractions was 1.6 ± 0.044 mmol Fe²⁺/g and the FRAP values for the controls, catechin, quercetin and rutin, were 6.5 ± 0.004, 7.5 ± 0.035 and 3.03 ± 0.028 mmol Fe²⁺/g, respectively. Since the positive controls, catechin, quercetin and rutin, used were pure compounds, it is expected that they would have higher FRAP values. Pearson correlation analysis was performed to assess the relationship between the phenolic content of the extract and ferric reducing activity. There was a strong positive correlation between the TPC of WTE and FRAP value (*r* = 0.902).

3.3. Assay of DPPH radical scavenging activity

The complete reduction of the DPPH radical is related to the high scavenging activity shown by a particular sample. A high level of antioxidants in the extract would indicate greater radical scavenging activity.

Other studies on tea extracts showed that the levels of catechin affect the antioxidant capacity. Green and black teas were efficient

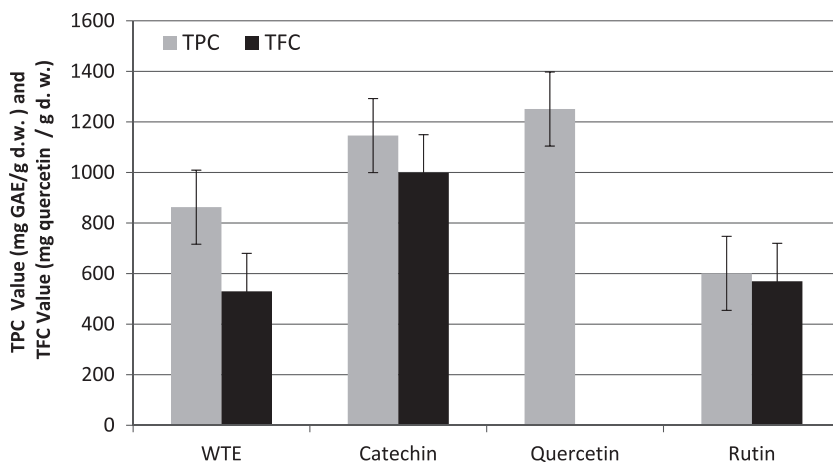


Fig. 1. Total phenolic and total flavonoid content of white tea extracts. Total phenolic content (expressed as mg GAE/g dried weight of sample) and the total flavonoid content (expressed as mg quercetin/g dried weight of sample) of white tea extracted with hot water for 5 min was estimated as described in Section 2.3.

Table 1
Antioxidant activities of extracts of white tea and controls.

	Ferric reducing power (mmol/g Fe ²⁺ /g dried weight)	DPPH radical scavenging activity (IC ₅₀) (μg/ml)	Nitric oxide radical scavenging activity (IC ₅₀) (μg/ml)	Hydroxyl radical scavenging activity (IC ₅₀) (μg/ml)	Superoxide anion radical scavenging activity (IC ₅₀) (μg/ml)
<i>Extracts</i>					
White tea hot water extract	1.6 ± 0.04	99.9 ± 4.9	118.2 ± 0.6	344.3 ± 32.6	94.0 ± 12.9
<i>Controls</i>					
Catechin	6.5 ± 0.004	39.3 ± 4.6	88 ± 5.8	146.7 ± 17.8	58.4 ± 2.2
Quercetin	7.46 ± 0.035	35.5 ± 3.6	83.9 ± 1.1	149.5 ± 10	57.2 ± 3.3
Rutin	3.03 ± 0.03	44.4 ± 5.8	99.5 ± 3.0	199.4 ± 21.5	61.8 ± 5.2

Results are expressed as means ± SD of three parallel measurements (*n* = 3). The ferric reducing antioxidant power is expressed as mmol Fe²⁺/g sample and the hydroxyl (·OH), nitric oxide (·NO) and superoxide anion (O₂⁻) radical scavenging activities are expressed as IC₅₀. The IC₅₀ values were calculated from linear regression analysis. IC₅₀ denotes the effective concentration of samples used to reduce 50% of the reagent colour.

in reducing DPPH due to their high catechin levels (Katalinic, Milos, Kulisic, & Jukic, 2006). The results of our study showed that white tea effectively reduces the oxidant free radical, DPPH. This could be due to the presence of high levels of EGCG (epigallocatechin gallate), a tea polyphenol in white tea (Almajano et al., 2008). Hot water extract of tea showed IC₅₀ values of 99.9 ± 4.9 μg/ml (Table 1). White tea extract was able to reduce the stable radical DPPH to the yellow coloured diphenylpicrylhydrazine. The scavenging effect increased with increasing concentrations of the extract. The results showed that the scavenging activity of catechin, a known antioxidant, was higher than extracts of *C. sinensis*. The IC₅₀ value of catechin, quercetin and rutin was 39.3 ± 4.6, 35.5 ± 3.6 and 44.4 ± 5.8 μg/ml, respectively. Other studies showed the relationship between tea TPC and scavenging of DPPH radical. An increase in TPC resulted in an increase of DPPH radical scavenging activity (Horžić et al., 2009). In the present study, the tea with high TPC exhibited high potential DPPH radical scavenging activity.

3.4. Assay of hydroxyl free radical scavenging activity

In a state of oxidative stress, reactive oxygen molecules (ROS) such as superoxide, hydroxyl and peroxy radicals are generated. A number of studies have shown that ROS play an important role in the pathogenesis of various chronic diseases, such as, neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation (Aruoma, 1998). As shown in Table 1, the samples tested showed IC₅₀ values of hydroxyl ion scavenging activity of 353.4 ± 31.9 μg/ml, in hot water extract. The results indicated the scavenging capacity of the hot water extract of white tea used against hydroxyl radicals.

3.5. Assay of nitric oxide scavenging activity

Nitric oxide is a reactive oxygen species involved in inflammation, cancer and other pathological conditions (Moncada, Palmer, & Higgs, 1991). One of the major causes of nicotine-induced cancer metastasis and DNA damage is nitric oxide formation. Tea prepared using hot water for 5 min showed a protective effect against nitric oxide radicals by scavenging the nitric oxide radicals (Table 1). It has been hypothesized that scavenging of nitric oxide is one of the main mechanisms for the bioactivity of flavonoids.

3.6. Assay of superoxide radical scavenging activity

The superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxygen species (Halliwell & Gutteridge, 1985). Table 1 shows the superoxide

radical (O₂⁻) scavenging activity of the extract. In general the sample tested in this study showed high superoxide scavenging activity. The extract from the hot water infusion exhibited high scavenging activities against superoxide radicals. The IC₅₀ value of tea extracted with hot water was 94 ± 12.9 μg/ml.

3.7. *In vitro* antiproliferative effect of white tea extracts on cancer cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay is an indirect colorimetric assay to assess the number of viable cells which has been adapted to measure the growth modulation of cells *in vitro*.

HT-29 cells were treated with different concentrations of extracts (10–100 μg/ml) for 48 h. The hot water extract demonstrated strong antiproliferative activity against HT-29 cells (IC₅₀ = 86.68 ± 0.73 μg/ml) (Table 2).

Numerous studies have proposed the inhibitory effect of tea against carcinogenesis of lung, skin, esophagus, liver and stomach cancers (Bushman, 1998; Yang, Maliakal, & Meng, 2002). Epidemiological studies suggested that the antiproliferative effects against colonic tumorigenesis (Chen et al., 2003) is due to the presence of tea polyphenols and that the protective activity is related to the strong radical scavenging and antioxidative capacity of tea (Yang & Wang, 1993).

A fibroblast cell line, HDF-a, was used to measure the possible cytotoxicity of white tea extracts on normal cells. The IC₅₀ of the extracts on fibroblasts (IC₅₀ > 160 μg/ml) was significantly higher than that of the cancerous cell line. Thus, the white tea extract showed high anti-proliferative activities against tumourigenic HT-29 cells, without being toxic to normal fibroblast cells.

3.8. Apoptosis and caspase-3/7, -8 and -9 activity

A family of cysteine proteases known as the caspases is believed to play a central role in mediating various apoptotic responses. Activation of the caspase cascade is an integral event in the apoptotic pathway. Apoptotic caspases can be divided into two classes: initiator and executioner caspases. Initiator caspases (caspase-2,

Table 2
Cytotoxicity and antiproliferative activities of Silver Needle white tea extracts on a colorectal cell line (HT-29) and a normal human fibroblast line (HDF-a).

Extracts	HT-29	HDF-a
Hot water extraction for 5 min	86.68 ± 0.73	164.26 ± 2.02

Results are expressed as IC₅₀ which signifies the effective concentration used to inhibit the growth of 50% of the cells (μg/ml). Values are expressed as mean ± SD (*n* = 3).

caspase-8 and caspase-9) are the apical caspases in apoptosis signalling cascades and their activation is normally required for executioner caspase (caspase-3, caspase-6 and caspase-7) activation (Cohen, 1997; Lamkanfi, Festjens, Declercq, Vanden Berghe, & Vandennebeele, 2007).

Human caspase-8 and caspase-9 are involved in initiating apoptosis through two different signalling mechanisms and are known as initiator caspases. Activation of these caspases occurs before activation of caspases-3 and -7. They cleave pro-caspases-3 and -7 to activate them and commit cells to apoptotic death. Caspase-3, -6, and -7 are thought to coordinate the execution phase of apoptosis by cleaving multiple structural and repair proteins (Riedl & Shi, 2004).

Caspases-8 and -9 are considered early indicators of apoptosis. They are each involved in a specific pathway in response to stimuli to the cell that causes programmed cell death. Caspase-9 is a marker for the intrinsic or mitochondrial pathway for apoptosis. Caspase-8 is an indicator that the extrinsic pathway to apoptosis has been activated. Caspase-Glo-3/7 is a general indicator of apoptosis.

In this study, HT-29 cells were treated with $86.68 \pm 0.73 \mu\text{g/ml}$ (IC_{50}) of WTE incubated for different time periods to investigate the caspase activity and the possible mechanism of action.

The Caspase-Glo-3/7 assay targets both caspase-3 and caspase-7, as they recognize the same peptide for cleavage and the substrate in the kit is a peptide that becomes luminescent on cleavage. These caspases are both considered to be markers of when a cell is committed to apoptosis. The Caspase-Glo-8 and -9 assays were used to determine which pathway was triggered by treatment of the tumorigenic cell line, HT-29, with the white tea extract.

The activity of caspases-3/7, -8 and -9 in HT-29 cells treated with the white tea extract (hot water) is shown in Fig. 2. The activity of each of the caspases increased with respect to the number of cells undergoing apoptosis in the experiment. The extracts increased the activities of caspase-3/7, -8 and -9 activities up to 16 h incubation time and then decreased after 24 and 48 h. The expression levels of caspase-3/7, -8, and -9 in treated cells showed the highest activities at 16 h, increasing by 2.8, 2.4 and 6.08-fold,

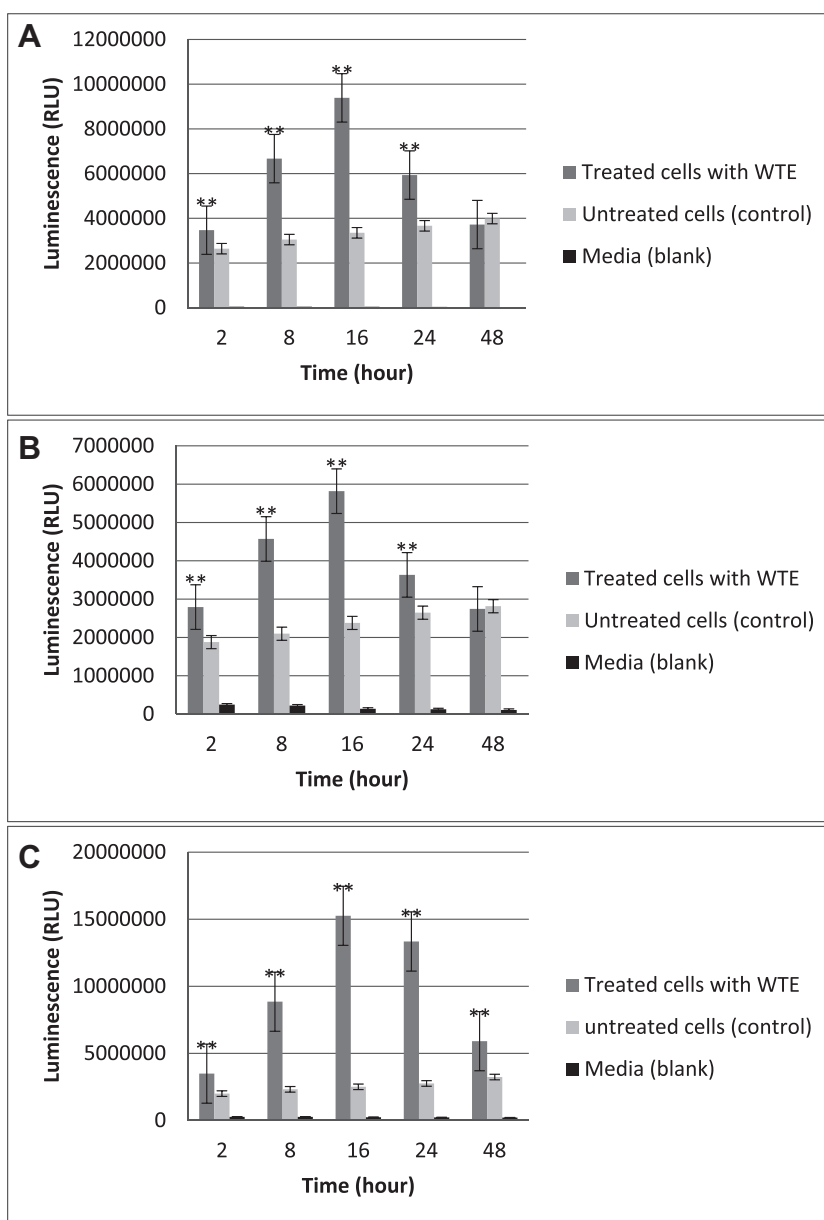


Fig. 2. The effect of white tea extract on caspase activity in HT-29 cells. HT-29 cells were treated with the 5 min in hot water extract of white tea and the activity of caspase-3/7 (A), caspase-8 (B) and caspase-9 (C) was determined. Cells were treated with the IC_{50} concentration of the extract for different time intervals. Data shown are the mean of triplicates; ** $p < 0.01$.

respectively, as compared to levels in untreated cells. These increases were only significant up to 24 h incubation and then decreased after 48 h, except for the activity of caspase 9, which was significantly higher than untreated cells even after 48 h incubation ($p < 0.01$). The diminished activity of initiator caspases-8 and -9 after 16 h treatment is possibly due to the activation of other cascades related to apoptosis.

The activity of caspase-3/7 in treated cells was highly significant when compared to the untreated cells ($p < 0.01$). The results showed that the extract was able to induce apoptosis by activation of caspase-3/7 (Fig. 2A). Caspases-8 (Fig. 2B) and -9 (Fig. 2C) showed significantly higher activity in treated cells when compared with the untreated cells. This suggests that the treatments induced apoptosis via death receptors through activation of caspase-8 and also the mitochondrial pathway by activating caspase-9.

Activated caspase-8 and -9 may then have resulted in the serial initiation and activation of the caspase cascade that eventually led to activation of caspase-3 that in turn cleaved cytoskeletal proteins, activated DNase and caused cell death by apoptosis.

The cytotoxic effects and antiproliferative activity of plant compounds against HT-29 by activating caspases and inducing apoptosis have been shown in previous studies (Seung Yuan, Trishna, Si-Kwan, & Beong Ou, 2013). The activation of caspase-3 and -9 in HT-29 cells treated with EGCG (epigallocatechin-3-gallate), a major component in green tea polyphenols, was detected after 12 h treatment, accompanied by mitochondrial transmembrane potential transition and cytochrome c release (Chen et al., 2003). It also has been reported that black tea induced tumour cell apoptosis by Bax translocation, loss in mitochondrial transmembrane potential, cytochrome c release and caspase activation (Bhattacharyya, Lahiry, Mandal, Sa, & Das, 2005). Our results were in line with other studies; our white tea extracts induced apoptosis initiated via the death receptor and also via the mitochondrial apoptotic pathway as demonstrated by increased expression levels of caspases-3, -8 and -9.

Initiator caspases (caspase-8 and caspase-9) are involved in initiating apoptosis in response to stimuli to the cell that causes programmed cell death. In this study cells treated with WTE after 16 h showed the maximum activities of the caspase-8 and caspase-9 as compared to the 24 h and 48 h treatment. Both the death receptor and the mitochondrial pathways are suggested for the mechanism of action of tea extract to induce apoptosis. It was concluded that WTE induced apoptosis via death receptors and mitochondrial pathway by activation of caspase-8 and caspase-9, respectively.

Activation of the initiator caspases lead to activation of executioner caspases (caspases-3 and -7). Activation of caspases-3 and -7 commit cells to apoptotic death. The reduction of the caspase activity after 16 h is possibly due to the increasing number of apoptotic cells on prolonged treatment (24 and 48 h) and the accumulation of dead cell debris which could interfere with the enzyme action. The results of our study suggest that the activation of the caspase pathway triggered apoptosis in HT-29 cells treated with the Silver needle white tea extract.

3.9. Fluorescence microscopic examination

Acridine orange and propidium iodide staining was used to study the morphological characterisation of the cells. HT-29 cells were incubated, for 8, 16 and 24 h (corresponding to the times of high caspase activity) with the IC_{50} concentration of WTE and viewed under a fluorescent microscope to analyse the viable cells, early apoptosis and late apoptosis (Fig. 3).

Acridine orange (AO) and propidium iodide (PI) stains the DNA within the nuclei. AO can only cross the plasma membrane of viable and early apoptotic cells. AO exhibits viable cells with green nuclei and an intact structure and early apoptotic cells with bright-green nuclei showing condensation of chromatin in the nucleus. PI penetrates the nuclear matter when the cell membrane integrity is disturbed and produces orange fluorescence. Apoptotic cells are stained green with nuclei stained orange, and contain multiple yellow/green dots of condensed nuclei. PI stained necrotic

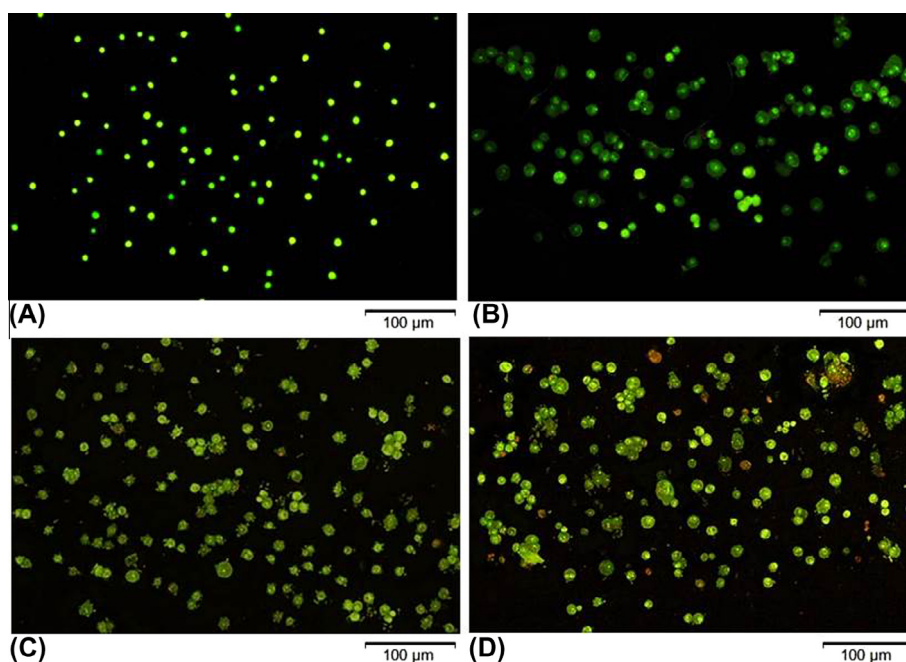


Fig. 3. Fluorescent micrograph of acridine orange and propidium iodide double-stained human colorectal cancer cells (HT-29). (A) Untreated cells showed normal structure without prominent apoptosis. (B) Cells treated with white tea extract after 8 h showed early apoptosis features including membrane blebbing and chromatin condensation. (C) More cells showed apoptotic features at 16 h which represent intercalated acridine orange amongst the fragmented DNA and nuclear fragmentation. (D) Presence of reddish-orange colour at 24 h. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cells appear as bright-red colour and dead cells with red nuclei (Ng et al., 2013).

The results showed that the untreated cells showed normal configuration with green nuclei and an intact structure, without prominent apoptosis (Fig. 3A). As shown in Fig. 3B and C, early apoptosis features, such as cell shrinkage, membrane blebbing and chromatin condensation were observed after 8 h and more cells showed apoptotic features at 16 h which represent intercalated acridine orange (bright green) amongst the fragmented DNA and nuclear fragmentation. In Fig. 3D, the presence of reddish-orange colour was observed after 24 h of incubation. AO/PI staining of HT-29 cells treated with WTE showed that the cells had undergone remarkable morphological changes in apoptotic bodies.

3.10. Analysis of DNA damage by the comet assay

Analysis of DNA damage was measured in mouse fibroblasts (3T3-L1) by using the comet assay. The length of the comet was measured and used as an index of the extent of DNA damage.

DNA damage was induced in 3T3-L1 cells by the addition of 100 μM of hydrogen peroxide. This induced a comet tail length of $11.18 \pm 0.7 \mu\text{M}$. In treated groups, the effect of the tea extract on protection was tested by pre-treating the cells with various concentrations (5–25 μM) of hot water WTE and then treating with 100 μM of H_2O_2 . Another control group with no H_2O_2 treatment served as the normal negative control group.

The results are presented in Fig. 4. The comet length for cells pre-treated with 5, 10, 15, 20 and 25 $\mu\text{g/ml}$ concentration of the extract and then treated with H_2O_2 was 9.3 ± 0.5 , 9.07 ± 0.8 , 7.2 ± 1.06 , 5.3 ± 0.7 and $3.9 \pm 0.3 \mu\text{M}$, respectively. The length of the comet in the normal group (control without H_2O_2) was $1.29 \pm 0.5 \mu\text{g/ml}$. 3T3-L1 cells which were pre-treated with *C. sinensis* extract at 5–25 $\mu\text{g/ml}$ showed a significant dose-dependent decrease in comet tail length compared to the control of H_2O_2 treatment alone ($P < 0.05$). This translates into statistically significant ($p < 0.05$) protection against DNA damage of 16.7, 18.6, 35.1, 52.4, 64.3% with treatment of 5, 10, 15, 20 and 25 $\mu\text{g/ml}$ concentrations of the extract, respectively. At a concentration of 25 $\mu\text{g/ml}$,

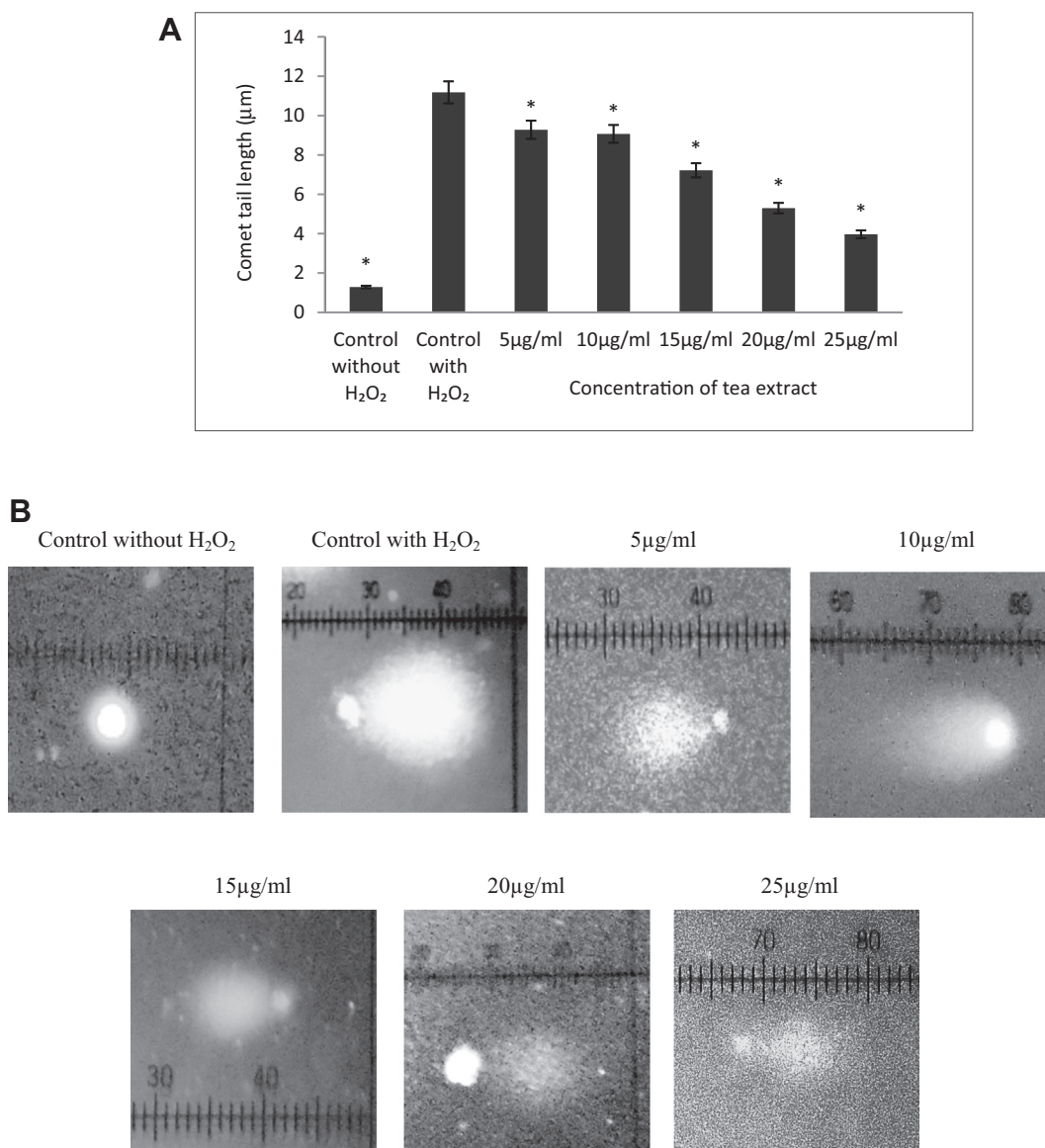


Fig. 4. Analysis of DNA damage in 3T3-L1 cells treated with tea extract using the comet assay. The comet tail length (μM) was estimated and used as an index for the extent of DNA damage induced by H_2O_2 (A). Comet images of 3T3-L1 fibroblasts, control group (control with H_2O_2), normal group (control without H_2O_2) and cells which were pre-treated with 5 $\mu\text{g/ml}$ to 25 $\mu\text{g/ml}$ of white tea extract (B). *Values were significantly different at $P < 0.05$ compared to control (with H_2O_2), as tested by the Student's *t*-test. Results are expressed as means \pm standard deviations.

the white tea extract could protect normal cells against H₂O₂-induced DNA damage up to 64.3%.

Reactive oxygen species (ROS) are involved in a number of degenerative diseases such as atherosclerosis, cancer, liver cirrhosis and diabetes mellitus (Aboutwerat et al., 2003; Aruoma, 1998). ROS can cause oxidative damage and interact with cellular macromolecules such as DNA, proteins, fatty acids and carbohydrates. It is known that the damage to macromolecules, especially DNA, leads to many degenerative diseases like cancer (Halliwell, 1997).

Our results indicate that the white tea extract could significantly protect DNA damage even at low concentrations and this increases with increasing concentrations of the white tea extract. Reactive hydroxyl radicals produced from H₂O₂ by the Fenton reaction, can bind to DNA at metal binding sites and induce strand breaks associated with DNA damage, mutations and genetic instability (Jayakumar & Kanthimathi, 2012), which could lead to carcinogenesis.

4. Conclusion

The present study demonstrates the antioxidant, anticancer and DNA protective effects of white tea (*C. sinensis*). White tea extract exhibited a strong antioxidant activity in the five assays carried out, i.e., the FRAP, DPPH, nitric oxide, superoxide anion and hydroxyl radical scavenging assays. The high antioxidant activities correlated significantly to their phenolic content. Pre-treatment of 3T3-L1 cells with the extract protected against H₂O₂-induced DNA damage.

The tea extracts also showed high anti-proliferative activity against HT-29 cells, without being toxic to normal fibroblasts. The extract inhibited HT-29 colon cancer cells by the death receptor and mitochondrial apoptotic pathways as demonstrated by increased expression levels of caspases-3/7, -8 and -9.

In conclusion, white tea extracts show potential as chemotherapeutic agents. Regular consumption of white tea could maintain good health and protect the body against disease.

Conflict of interest

The authors have nothing to disclose and have no commercial or financial interest in the product described in this paper.

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