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Tea Polyphenols Modulate Antioxidant Redox System on Cisplatin-induced Reactive Oxygen Species Generation in a Human Breast Cancer Cell

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Abstract: Tea polyphenols (TPP) have potent antioxidant and anticancer properties, particularly in patients undergoing radiation or chemotherapy. However, few studies have been conducted on treatments using a combination of TPP and the conventional chemical anticancer drug cisplatin (CP). This study was designed to investigate the mechanism of the cytotoxicity of total TPP and CP, which may synergistically induce cell death in cancer cells. Here, breast cancer cells (MCF-7) were treated with various concentrations of TPP alone or in combination with the chemotherapeutic drug CP. The effect of TPP on cell growth, intracellular reactive oxygen species (ROS) level, apoptosis and gene expression of caspase-3, caspase-8 and caspase-9 and p53 was investigated. The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay revealed that the MCF-7 cells were less sensitive to growth inhibition by TPP treatment than either CP or the combination therapy. Propidium iodide nuclear staining indicated that exposure to this combination increased the proportion of apoptotic nuclei compared with a single-agent treatment. Flow cytometry analysis was used to quantify changes in intracellular ROS. Detection of activated caspases by fluorescently labelled inhibitors of caspases (FLICA) combined with the plasma membrane permeability assay demonstrated that the percentage of early and late apoptotic/secondary necrotic cells was higher in the cells treated with the combination than in those treated with either TPP or CP alone. The combined TPP and CP treatment synergistically induced apoptosis through both caspase-8 and caspase-9 activation and p53 over-expression. This suggests that TPP plus CP may be used as an efficient antioxidant-based combination therapy for estrogen receptor (ER)-positive and p53-positive breast cancer.

Breast cancer is the most prevalent malignancy in all Gulf Cooperation Council (GCC) countries. Despite the low cancer incidence (12.9%) in the Kingdom of Saudi Arabia (KSA) among GCC countries, the World Health Organization (WHO) predicts that future cancer rates could considerably increase in GCC countries [1–3]. Therefore, the healthcare sector must be ready to face the challenge of the foreseeable increase in cancer burden and improve treatment strategies, perhaps using multifaceted combination therapies with food supplements.

Current breast cancer treatment strategies, primarily chemotherapy, result in only minimal improvement in patient survival, and treatments lack specific targeting, thus causing toxicity [4–6]. Platinum-based drugs are commonly used to treat breast tumours [7]. However, the clinical value of cisplatin (CP) is largely compromised by serious adverse reactions, such as neurotoxicity and nephrotoxicity [5]. Hence, efforts have been expended to develop more efficacious treatments and prevent organ toxicity [8].

The molecular biology of breast cancer is very complex. Several genes are involved in the initiation and progression of breast cancer, the most important of which is the tumour suppressor gene p53 [9]. In the majority of tumours, p53 activity is disrupted by mutations and epigenetic changes in the *p53* gene [10]. The molecular mechanism of CP-induced p53 activation is believed to be oxidative stress-mediated DNA damage [11,12]. It is well known that killing tumour cells through apoptosis and the activation of p53 by CP treatment trigger oxidative stress in the ER and mitochondria [13].The major downstream event mediated by p53 is intrinsic caspase-9-mediated apoptosis; this process, more than the extrinsic caspase-8-mediated apoptosis pathway, is constitutively activated with CP chemotherapy in breast cancer [14]. A mitochondrial-based redox-buffering network has been developed to adapt and protect cells against the dangerous effects of oxidative stress. However, pathways involved in reactive oxygen species (ROS)-adaptive responses also play a critical role in protecting cells against the cytotoxic effects of CP treatment [15]. CP-induced oxidative stress in the intracellular environment confers resistance to CP-mediated cell death and chemosensitivity of normal cells, which leads to a poor clinical response. To avoid chemoresistance and to improve therapeutic efficacy, combination treatments with multiple targeted drugs are now considered the standard [16].

Patients with breast cancer often use dietary supplements [e.g. micronutrients, antioxidants, vitamins, minerals, tea polyphenols (TPP)] after diagnosis, which helps prolong the survival time of patients and decrease side effects associated with the treatments [17,18]. Several molecular biological and epidemiological studies have suggested that the consumption of tea

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compounds inhibits growth of many tumour types [19]. Recent research has focused on the ability of dietary supplements, such as tea compounds, to increase the concentration of chemotherapeutics in tumour cells. TPP are well-known antioxidants that can also inhibit the growth of cancer cells through apoptotic mechanisms [20]. Moreover, catechin compounds including (−)- epigallocatechin-3-gallate (EGCG), (−)- epigallocatechin (EGC), epicatechin-3-gallate (ECG) and catechin have a variety of biological activities, including anti-allergic, anti-inflammatory and anti-mutagenic activities [21]. Catechin compounds have also been shown to exhibit cytostatic properties by induction of apoptosis in many tumour models [22,23]. The combination of epigallocatechingallate (EGCG) at suboptimal doses with CP have shown synergistic effects on cell cycle arrest, modulation of ROS- and apoptotic-related gene expression and potent antioxidant activity compared with single-agent treatments [24,25]. This kind of multifaceted inhibition of tumourigenesis is attributed to a unique combination of antioxidant, anti-proliferative and pro-apoptotic effects.

The overall therapeutic properties of green tea observed thus far have focused on the combined activities of several compounds rather than that of a single compound. Supplementary antioxidants such as TPP may act as central coordinators of different homoeostatic mechanisms that balance oxidative stress by p53-mediated caspase activation. The best way to increase the efficacy and reduce the toxicity of CP is to combine treatment with TPP. However, the current molecular-based evidence is inadequate to determine clinical and patient guidelines on the use of TPP as a supplement during breast cancer therapy. Therefore, the ultimate aim of this study was to examine the synergistic effects of CP and TPP combinations in caspase-mediated apoptosis induction, which is the common goal of breast cancer therapy.

Materials and Methods

Total TPP content measurement by HPLC.

Standard preparation. Standards of four catechins (EC, EGC, ECG and EGCG), gallic acid (GC) and caffeine (CA) were prepared in a small volume of 5% (v/v) acetonitrile containing 0.05% (v/v) phosphoric acid (85%). The standards were filtered through a 0.45-μm filter.

Chromatographic conditions. HPLC analysis was performed according to the method of Gotoa *et al.* [26] in a Shimadzu model LC-10 (Shimadzu Co, Tokyo, Japan) equipped with a binary pump, an autosampler and a photodiode array detector (PDA). The standards and TPP (Sigma Aldrich, St. Louis, MO, USA) were injected (10 μL) into a reversed-phase analytical Vydac 201TP54 C_{18} column (250 mm × 4.6 mm) (W.R. Grace & Co., Grace Davison, Bannockburn, IL, USA). All solvents were filtered with 0.45-μm filters. The following gradient elution was carried out mobile phase A, water–acetonitrile–phosphoric acid (85%) (95.45:4.5:0.05, v/v/v) and mobile phase B, water–acetonitriles–phosphoric acid (85%) (49.95:50.0:0.05, v/v/v). The mobile phase composition started at 90% mobile phase A and 10% mobile phase B, was maintained for 12 min and then linearly increased to 15% mobile phase B from 12 to 18 min. This condition was maintained for 2 min followed by a linear increase in mobile phase B to 70% for 20–30 min. The final conditions were held for an additional 10 min. The mobile phase flow rate was 1.0 mL/min, and the temperature of the column oven was set at 35°C. The quantification of GC, catechin and CA compounds by the photodiode array (PDA) detector was performed at 278 nm. Identification of the compounds was carried out by comparing retention times and UV spectra of the unknown peaks to those of the standards.

Cell culture. Breast cancer cell MCF-7 was obtained from National Centre for Cell Science (NCCS), Pune, India. The cell line was provided by Prof. M.A. Akbarsha of the Mahatma Gandhi Doerenkamp Center for Alternatives to Use of Animals in Life Science Education, Bharathidasan University, India. The cells were cultured in DMEM (BIOCHROM AG, Berlin, Germany) supplemented with 10% foetal bovine serum (HyClone; Thermo Scientific, South Logan, UT, USA) and 100 U/mL penicillin and 100 μg/mL streptomycin as antibiotics (HyClone; Thermo Scientific), in T25-cm2, T75-cm2 flasks and 6-well, 12-well, 24-well or 96-well culture plates (TPP, Switzerland), depending upon the context, at 37°C, in a humidified atmosphere of 5% CO_2 in a CO_2 incubator (Thermo Scientific). All experiments were conducted using cells from passage 15 or less. The culture conditions of MCF-7 cells were consistent for all the below mentioned experiments.

Cell viability assay. The TPP and CP were prepared as stock solutions at different concentrations in the microgram (TPP) or micromolar range (CP) and dissolved in dimethyl sulfoxide (DMSO) (Invitrogen, Grand Island, NY, USA). Further working solutions were prepared in the DMEM growth media. The final concentration of DMSO used was below 0.1% in all wells. Working solutions were added to the wells 24 hr after seeding of 2.5×10^4 MCF-7 breast cancer cells per well in 1000 μL of fresh culture media. DMSO (0.1%) was used as vehicle control. Cytotoxicity of the cells was monitored and photographed after exposure to different concentrations of the compounds for 24 hr. After TPP and CP incubation, 100 μL of MTT solution (5 mg/mL in phosphate-buffered saline; Sigma Aldrich) was added to each well, and the plates were wrapped with aluminium foil and incubated for 4 hr at 37°C. The plate was centrifuged at 2900 ***g*** for 5 min., and the purple formazan product was dissolved by the addition of 1000 μL of 100% DMSO to each well. The absorbances were monitored at 570 nm (measurement) and 630 nm (reference) using a multiwell plate reader (Bio Rad, Hercules, CA, USA). The data were collected for four replicates for each dose and used to calculate the median-effect dose or concentration (i.e. IC_{50}Dm value), to measure sigmoidicity of the dose–effect curve (*m* value), to determine the linear correlation coefficient of the median-effect plot (*r* value) and the drug combinational analysis using the CalcuSyn software (Biosoft, Great Shelford, CB, UK).

Experimental design for drug combinations. The cytotoxic assay was carried out for TPP and CP alone to obtain the dose–effect parameters such as median dose $(Dm)_1$, the sigmoidity (m_1), the correlation coefficient (r_1) of TPP and the median dose $(Dm)_2$, the sigmoidity (m_2), the correlation coefficient (r_2) of CP alone for the dose–effect curves and median-effect plot using CalcuSyn software. MCF-7 cells were exposed to various concentrations (control, 25, 50, 75, 100, 125 μg/mL) of TPP alone and (control, 5, 10, 15, 20, 25 μM) of CP alone. After 24-hr exposure, MTT assay was performed. Data were subjected to calculation of the percentage cell death, (IC_{25}, IC_{50}, IC_{75}) and dose–effective parameters using Calcusyn software. For the combination studies, in which MTT assay was carried out for combinations of TPP and CP consisted of three data points of a combination mixture to determine the combination index (CI) value and Isobologram analysis. For combination analysis, MCF-7 cells were treated with TPP at various concentrations (control, 25, 50, 75, 100, 125 μg/mL) and combination with CP at fixed concentration at sthree different points (IC_{25} dose

8.5 μM, IC_{50} dose 12.4 μM and IC_{75} dose 14.8 μM) for 24 hr, and cell viability was determined using the MTT assay, and data were subjected to combination analysis. CI was calculated by the CI equation of Chou and Talalay [27]. CI < 1, CI = 1 and CI > 1 indicate synergism, additive effect and antagonism, respectively. Normalized isobologram analysis was conducted to illustrate additivity, synergism or antagonism of the combinations of various doses. If the combination data point for f_a = 0.5 falls on the diagonal, an additive effect is indicated; if it falls on the lower left, synergism is indicated; and if it falls on the upper right, antagonism is indicated. The synergistic combination dose was selected based on the lowest CI value and isobologram analysis and was applied for all the below mentioned experiments.

Propidium iodide staining. For visualization of the effects of TPP, CP and its combination on the morphological features, MCF-7 cells were seeded on six-well plates on the day prior to the assay. Cells were incubated with TPP (IC_{50} dose 86 μg/mL), CP (IC_{50} dose 12.4 μM) and synergistic combination dose (TPP 50 μg/mL + CP 12.4 μM) in DMEM medium for 24 hr at 37°C. The cell nuclear morphologies were detected by staining the DNA content of trypsinized cells (4.0×10^4/mL) with 10 μL of propidium iodide (1 mg/mL, aqueous; BD Biosciences, San Jose, CA, USA) for 10 min. at 37°C. A drop of cell suspension was placed on a glass slide, and a coverslip was placed over the slide to reduce light diffraction. The cells were photographed at random with a fluorescent microscope at 400× magnification (Carl Zeiss, Jena, Germany) fitted with a 530-/620-nm filter.

Measurement of reactive oxygen species. Intracellular ROS was determined using the fluorescent probe 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester) as per manufacturer's instructions (Invitrogen). It readily diffuses through the cell membrane and is enzymatically hydrolysed by intracellular esterases to form non-fluorescent 2′,7′-dichlorofluorescein (DCFH), which is then rapidly oxidized to form highly fluorescent 2′,7′-dichlorofluorescin (DCF) in the presence of ROS. The DCF fluorescence intensity is indicative of the amount of ROS formed intracellularly. To determine intracellular total ROS levels of untreated (control) and treated with TPP, CP and their combination in MCF-7 cells, cells were seeded on 12-well plates on the day prior to the assay. Cells were incubated with TPP (IC_{50} dose 86 μg/mL), CP (IC_{50} dose 12.4 μM) and synergistic combination dose (TPP 50 μg/mL + CP 12.4 μM) in DMEM medium for 24 hr at 37°C. After 24-hr treatment, the medium was removed, and the cells were washed with PBS and then incubated using 10-μM probe in the loading medium. After probe was removed, the cells were washed and incubated with PBS. The intracellular fluorescence intensity of 10,000 events was measured using BD FACScalibur (BD Biosciences) at FL-1 channel. The acquired data were analysed using the BDCellQuest Prosoftware (BD Biosciences).

Polycaspase apoptosis assay. To determine polycaspase activity of untreated (control) and treated with TPP, CP and their combination in MCF-7 cells, cells were seeded on 12-well plates on the day prior to the assay. Cells were incubated with TPP (IC_{50} dose 86 μg/mL), CP (IC_{50} dose 12.4 μM) and synergistic combination dose (TPP 50 μg/mL + CP 12.4 μM) in DMEM medium for 24 hr at 37°C. After 24-hr treatment, the cells were harvested, resuspended and stained with the fluorescently labelled inhibitors of caspases (FLICA) reagent for polycaspases and propidium iodide using the Vybrant FAM PolyCaspases Assay Kit (Invitrogen; Life Technologies, Grand Island, NY, USA). The 10,000 events were acquired on a FACScanto II flow cytometer at a 488-nm excitation wavelength using 530-nm bandpass and 670-nm longpass emission filters. The acquired data were analysed using the BD FACSDiva software and BDCellQuest Pro software (BD Biosciences, San Jose, CA, USA).

Gene expression analysis. To determine mRNA levels of untreated (control) and treated with TPP, CP and their combination in MCF-7 cells, cells were seeded on 24-well plates on the day prior to the assay. Cells were incubated with TPP (IC_{50} dose 86 μg/mL), CP (IC_{50} dose 12.4 μM) and synergistic combination dose (TPP 50 μg/mL + CP 12.4 μM) in DMEM medium for 24 hr at 37°C. Total RNA from cells was extracted, and cDNA was synthesized using the FastLane Cell cDNA Kit (Qiagen, Valencia, CA, USA). The cycle parameters for the RT reaction were 42°C for 5 min, 42°C for 30 min, 85°C for 5 min, and held at 4°C. Quantitative PCR was performed using a RT-PCR kit (Qiagen) and analysed on a AB 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Quantitect Primer Assays of genes of interest (caspase-3, caspase-8 and caspase-9, p53 and GAPDH) were chosen and purchased from Qiagen. The PCR mix consisted of the RT product, primer and probe for the gene of interest and was cycled in accordance with the manufacturer's instructions for the QuantiFast SYBR Green PCR Kit (Qiagen). The cycle parameters for the PCR were 95°C for 5 min, followed by 40 cycles of a denaturing step at 95°C for 10 sec. and an annealing/extension step at 60°C for 30 sec. The relative mRNA levels in each sample were normalized to the GAPDH content. Quantitative values were obtained from the threshold PCR cycle number (C_t), at which the increase in signal associated with an exponential increase in the PCR product was detected. In each sample, we calculated a ΔC_t (target-reference). The fold change between control (untreated) and TPP, CP or combination-treated samples for p53, caspase-3, caspase-8 and caspase-9 were calculated with the $2^{-\Delta\Delta C_t}$ method, in which $\Delta\Delta C_T = \Delta C_T$ (target-reference) (in TPP, CP or combination-treated samples) – ΔC_T (target–reference) (in untreated samples). Real-time PCR was repeated in triplicate for each sample; an average $2^{-\Delta\Delta C_t}$ value along with its S.D. was calculated for each sample relative to the normal control for expression of p53, caspase-3, caspase-8 and caspase-9. GAPDH was used as reference gene. The expression of p53, caspase-3, caspase-8 and caspase-9 was normalized to reference gene (GAPDH).

Statistical analysis. The results are expressed as the mean ± standard deviation (S.D.). All data were derived from at least three independent experiments with a similar pattern. The statistical analyses of all experimental data were performed using the Microsoft Excel software (Microsoft Corp., KY, USA) and CalcuSyn software. For all comparisons, differences were considered statistically significant at $p < 0.05$.

Results

Tea polyphenols analysis by HPLC.

Tea polyphenols were analysed for the contents of the individual catechins, GC and CA by HPLC. The results obtained are presented in fig. 1 and table 1. Our results show that large amounts of catechins (EC, ECG, EGC and EGCG) were present. The TPP were then dissolved in DMSO, a non-toxic solvent (below 1%) and tested using *in vitro* combination studies.

Cytotoxicity with combination of cisplatin and tea polyphenols treatments.

To evaluate the dose-dependent effects of TPP and CP on human breast cancer cells (MCF-7), the cells were treated with semi-log doses of TPP and CP alone. After 24-hr treatment, cell viability was determined, and inhibitory concentrations were calculated as follows: TPP, IC_{25} = 68.5 μg/mL, IC_{50} = 86 μg/mL and IC_{75} = 122.8 μg/mL and CP,

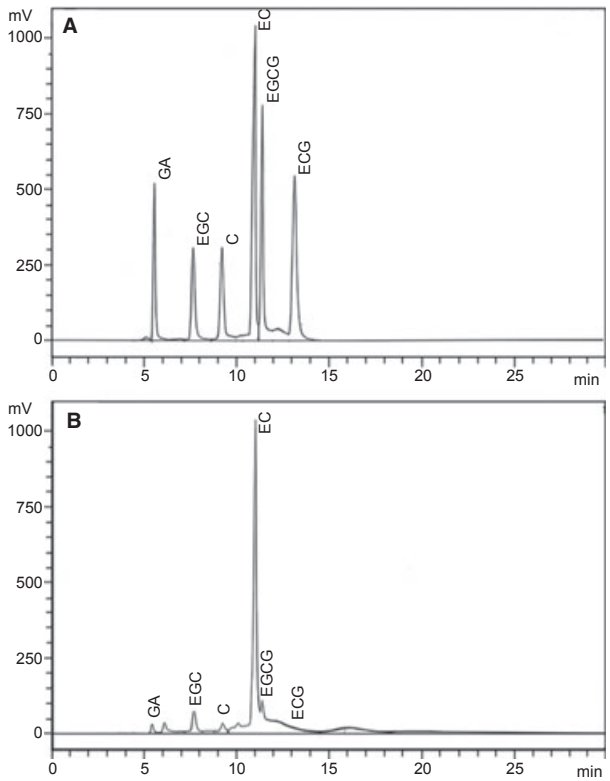

Fig. 1. HPLC chromatogram of tea polyphenols (TPP) standards (A) and TPP samples (B). Panel A shows a chromatogram of standard TPP (GA, EGC, C, EC, EGCG and ECG peaks). (B) Profile of a HPLC chromatogram obtained after injection of 20 μL of TPP (GA, EGC, C, EC, EGGG) retention time at 5.4, 7.4, 8.9, 10.6, 11.09, 12.81 min, respectively, which were matched to the standard chromatogram peaks of TPP shown in panel A. Chromatographic conditions are described in Materials and Methods.

Table 1.

HPLC analysis of the resulting compounds of gallic acids and catechins of tea polyphenols (TPP). Commonly occurring tea polyphenol constituents present in TPP. The quantitative measurement of each polyphenol was calculated based on the standard chromatogram.

S. no	Components	Availability (%)
1	Gallic acid (GA)	0.54
2	Epigallocatechin (EGC)	17.4
3	Catechin (C)	1.5
4	Epicatechin (EC)	10.25
5	Epigallocatechingallate (EGCG)	54.85
6	Epicatechingallate (ECG)	14.65

IC_{25} = 8.5 μM, IC_{50} = 12.4 μM and IC_{75} = 14.8 μM. Then, the cells were treated with combinations of TPP (control, 25, 50, 75, 100, 125 μg/mL) and CP at fixed concentration at three different points (IC_{25} dose 8.5 μM, IC_{50} dose 12.4 μM and IC_{75} dose 14.8 μM) for 24 hr. Combinations index (CI) and isobologram analysis were performed using the CalcuSyn software to analyse the synergistic effects of various TPP and CP combinations. The results show that TPP and CP inhibited the viability of the MCF-7 cancer cell in a dose-dependent manner (fig. 2A,B). The median dose–effect plots of TPP with

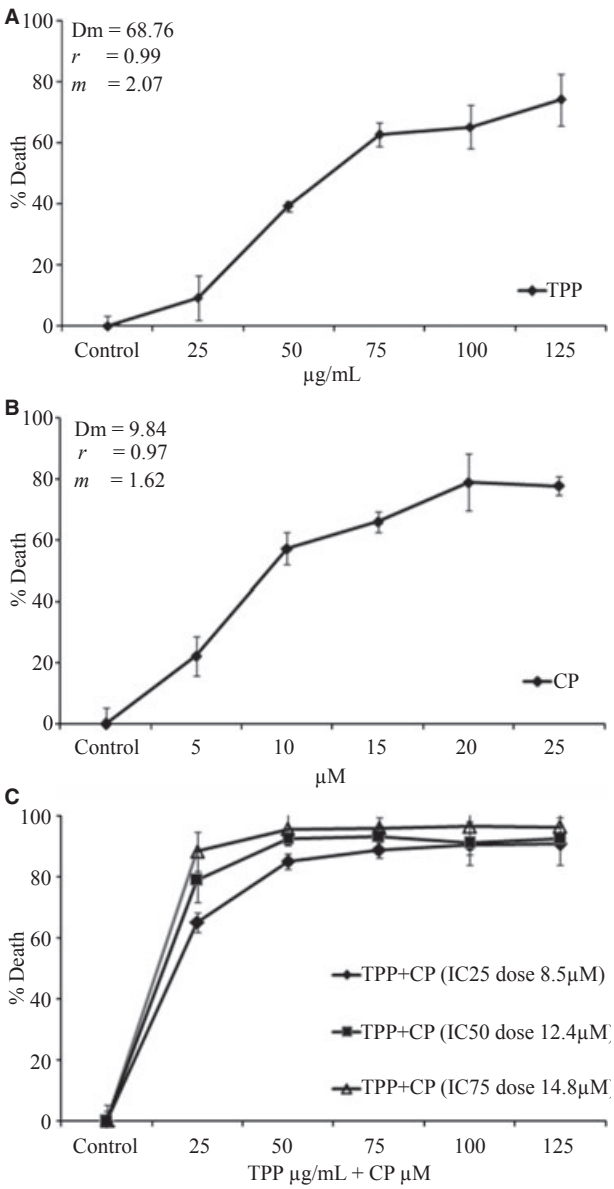

Fig. 2. *In vitro* cytotoxicity assays for tea polyphenols (TPP), cisplatin (CP) and its combination (TPP + CP) treatment in MCF-7 cells. (A) MCF-7 cells were exposed at various concentrations (control, 25, 50, 75, 100, 125 μg/mL of TPP alone. (B) MCF-7 cells were exposed at various concentrations (control, 5, 10, 15, 20, 25 μM) of CP alone. After 24 hr of exposure, the percentage cell death was determined by MTT assay in order to determine the IC_{25}, IC_{50}, IC_{75}, *m* value (a measurement of the sigmoidity of the dose–effect curve), *r* value (the linear correlation coefficient of the median-effect plot) were calculated using Calcusyn software. (C) MCF-7 cells were treated with TPP at various concentrations (control, 25, 50, 75, 100, 125 μg/mL) and combination with CP at fixed concentration at three points (IC_{25}, IC_{50} and IC_{75} dose) for 24 hr. Cell viability was determined with the MTT assay and data were subjected to combination analysis. The data represent the mean ± S.D. of three determinations, each performed in triplicate.

the three different concentrations of CP were the most effective combination treatment than TPP and CP alone in MCF-7 cells (fig. 3A–C). Overall, the CI values of the three different combinations were <1.0, which clearly indicates a synergistic

effect (fig. 3D–F). CI values of (TPP + CP 8.5 μM), (TPP + CP 12.4 μM) and (TPP + 14.8 μM) showed clear synergism in the range from 0.6 to 0.85, from 0.48 to 0.81 and from 0.39 to 0.58, respectively, in MCF-7 cells. Moreover, a normalized isobolographic analysis clearly showed a synergistic pattern, as the data point for $f_a = 0.5$ fell in the lower left of the graph (fig. 3G–I). The synergistic combination dose was selected based on the lowest CI value (0.39 at 50 μg/mL of TPP and 12.4 μM of CP) and isobolorgram analysis and was applied for all the below mentioned experiments.

Propidium iodide staining.

The cells were treated with TPP, CP alone and synergistic combination dose (TPP 50 μg/mL + CP 12.4 μM) for 24 hr and observed for any change in propidium iodide (PI) staining. The observations revealed that treatments with TPP and CP alone and a combination of TPP and CP caused marginalization and/or fragmentation of chromatin, binucleation, cytoplasmic vacuolation, nuclear shrinkage, cytoplasmic blebbing and late apoptosis, as indicated by dot-like chromatin and condensation in the MCF-7 cells (fig. 4). These cytological changes indicated that the cells were committed to cell death, with the majority showing evidence of apoptosis rather than necrosis. Qualitative microscopic analysis showed that the percentage of cells with morphologies characteristic of apoptosis increased significantly when cells were treated with the combination of TPP and CP compared with either TPP or CP alone.

Flow cytometry (changes in intracellular reactive oxygen species level).

MCF-7 cells treated with TPP alone (IC_{25} dose 68.5 μg/mL, IC_{50} dose 86 μg/mL and IC_{75} dose 122.8 μg/mL), CP alone (IC_{25} dose 8.5 μM, IC_{50} dose 12.4 μM and IC_{75} dose 14.8 μM) and synergistic combination dose (TPP 50 μg/mL + CP 12.4 μM) for 24 hr were subjected to analysis of cellular ROS levels. Different histogram patterns of ROS level were obtained in the flowcytometer-based experiment. The treatment caused the MCF-7 cells to generate an elevated amount of ROS during the CP treatment, whereas TPP suppressed ROS generation dose dependently. The intracellular ROS level of the TPP, CP and combination treatment clearly indicated that a significant peak shift in the histogram overlay between CP-induced high ROS production and antioxidants effects by TPP (fig. 5).

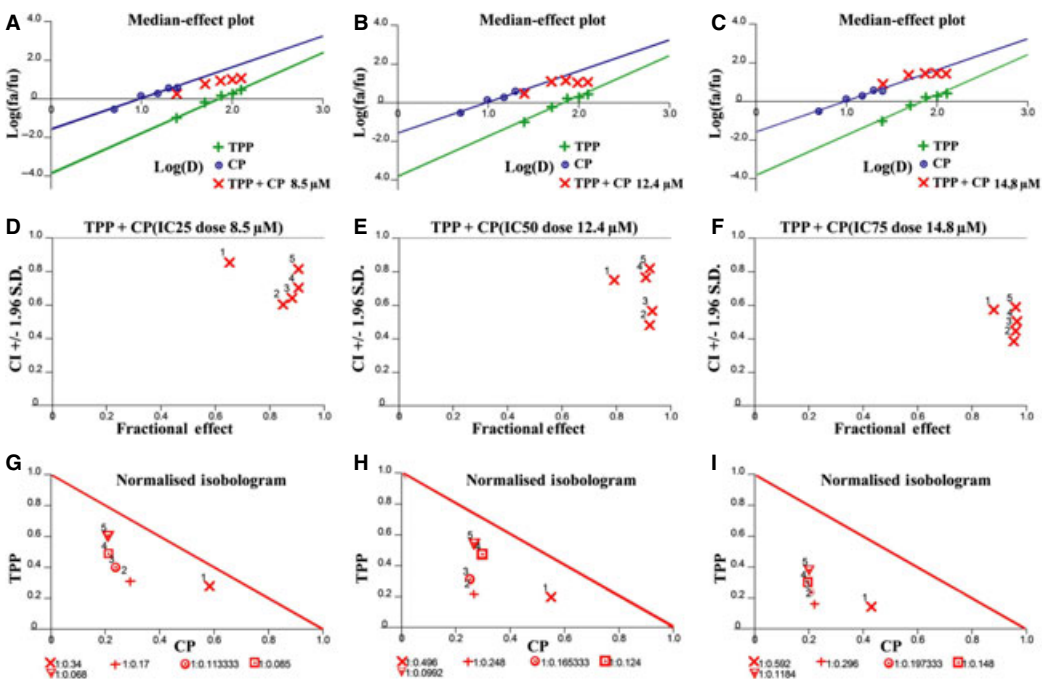

Fig. 3. Drug combination analyses of the tea polyphenols (TPP) and cisplatin (CP) and their combinations in tested MCF-7 cells. TPP and CP combination plots were obtained using Calcysyn software. (A–C) Median-effect plot of TPP, CP and its combination representing the analysis of the goodness of fit for the data to the median-effect equation. Dm value – median-effect dose (concentration which inhibits cell growth by 50%, TPP –μg/mL, CP – mM). *m* value – shape of the dose–effect curve, where $m = 1$, $m > 1$, and $m < 1$ indicates hyperbolic, sigmoidal, and flat sigmoidal curves, respectively. *r* value – linear correlation coefficient of the median-effect plot (indicates conformity of data). (D–F). Combination index (CI) was calculated by the CI equation of Chou and Talalay [27]. CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively. TPP significantly enhanced the CP cytotoxic effects on MCF-7, as indicated by CI values of <1 in all three dose points. CI of (TPP + CP 8.5 μM), (TPP + CP 12.4 μM) and (TPP + 14.8 μM) showed clear synergism in the range from 0.6 to 0.85, from 0.48 to 0.81 and from 0.39 to 0.58 respectively in MCF-7 cells. (F–H) Graphs of normalized isobologram of (TPP + CP 8.5 μM), (TPP + CP 12.4 μM) and (TPP + CP 14.8 μM) combinations to illustrate additivity, synergism or antagonism. If the combination data point for $f_a = 0.5$ falls on the diagonal, an additive effect is indicated; if it falls on the lower left, synergism is indicated; and if it falls on the upper right, antagonism is indicated. Data of three different combinations were felled on the lower left in the normalized isobologram.

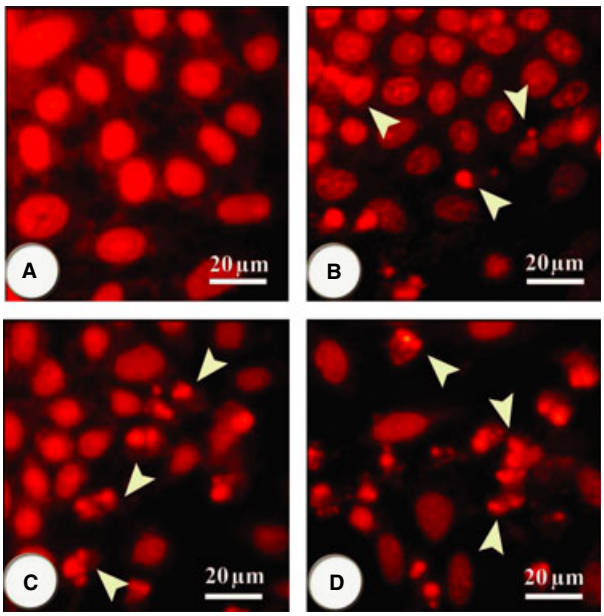

Fig. 4. Fluorescent microscopy images of cells treated with tea polyphenols (TPP), cisplatin (CP) and TPP + CP and the untreated control cells. MCF-7 cells were incubated with TPP (86 µg/mL), CP (12.4 µM) and synergistic combination dose-(TPP 50 µg/mL + CP 12.4 µM) for 24 hr. Propidium iodide-stained MCF-7 cancer cells that were untreated (A), treated with TPP (B), CP (C) or a combination (D) for 24 hr (400× magnification). The white arrowheads point to cells with abnormal nuclei, indicating fragmentation of nuclei/chromatin.

Flow cytometry (caspase activation).

A distinctive feature of the early stages of apoptosis is the activation of caspase enzymes. These enzymes participate in a series of reactions that are triggered in response to pro-apoptotic signals and result in the cleavage of protein substrates and the subsequent disassembly of the cellular system. Here, we found different effects of TPP, CP and the combination treatment on MCF-7 cells after a 24-hr exposure. Based on the differences in binding of the fluorochromes, four different populations were distinguished on the bivariate PI *versus* FAM scatter plots. As shown in fig. 6, the cells were classified into four different types: normal cells ($FLICA^-/PI^-$), early apoptotic cells ($FLICA^+/PI^-$), late apoptotic cells ($FLICA^+/PI^+$) and secondary necrotic cells and necrotic cells ($FLICA^-/PI^+$). A significant increase in the early apoptotic and late apoptotic populations (fig. 6D) was observed in the cells treated with the synergistic combination dose (TPP 50 µg/mL + CP 12.4 µM) compared with the single TPP or CP treatments (fig. 6B,C).

Gene expression analysis by qPCR.

The cells were treated with TPP alone (IC_{50} dose 86 µg/mL), CP alone (IC_{50} dose 12.4 µM) or with a synergistic combination dose (TPP 50 µg/mL + CP 12.4 µM), and a SYBR green-based quantification method was used to evaluate changes in mRNA expression levels. The expression level of each gene was first normalized to the average level of a housekeeping gene (GAPDH) to generate the ΔC_t of each gene, where C_t represents the cycle threshold. Then, the $\Delta\Delta C_t$ was calculated with the formula $\Delta\Delta C_t = \Delta C_t$ (treated group) $- \Delta C_t$ (control group), where the control group was *C*, and the treated groups were TPP alone (IC_{50} dose 86 µg/mL), CP alone (IC_{50} dose 12.4 µM) or with a synergistic combination dose (TPP 50 µg/mL + CP 12.4 µM). As shown in fig. 7, an average $2^{-\Delta\Delta C_t}$ value along with its S.D. was calculated for each sample relative to the normal control for expression of p53, caspase-3, caspase-8 and caspase-9. The results are presented as the mean ± S.D., and $*p < 0.05$ is considered significant when individual TPP and CP treatments are compared with synergistic combination dose (TPP 50 µg/mL + CP 12.4 µM) after 24-hr incubation. The values represent the fold induction in the mRNA levels of p53 and caspase-3, caspase-8 and caspase-9 in the MCF-7 cells treated with TPP, CP and synergistic combination dose (TPP 50 µg/mL + CP 12.4 µM) (fig. 7). With the combination treatment, the levels of p53 transcripts in MCF-7 cells significantly increased by approximately four to sevenfold at 24-hr incubation compared with individual TPP or CP treatments ($p < 0.05$) (fig. 7). According to Livak's calculation, the mRNA expression of the effector caspase-3 and initiator caspase-8 was up-regulated with the synergistic combination dose (TPP 50 µg/mL + CP 12.4 µM) by twofold and 1.7-fold, respectively, while caspase-9 levels did not appear to change (fig. 7).

Discussion

Cisplatin suppresses the proliferation of malignant cells by enhancing apoptosis, which is an efficient approach to chemotherapy. However, unfavourable side effects and resistance to CP represent serious problems [28,29]. CP-induced excess oxidative stress can cause a number of adverse effects including fatigue, nausea, vomiting as well as more serious effects [5,30]. Excess oxidative stress and its mediators (ROS/RNS) have been linked with the promotion and progression of breast cancer malignancy (metastasis) and chemoresistance [31,32].

The 'master watchman' p53 is a vital sensor of cellular stress, including DNA damage, hypoxia, survival factor deprivation, mitogenic oncogenes and telomere shortening [33–35]. ROS accumulation by p53 induction and a number of genes induced by p53 are associated with the metabolism of ROS [36]. Several reports have shown that p53 activation in response to CP causes reversible or permanent growth arrest and apoptosis. Cells undergoing senescence in response to p53 at physiological expression levels have been shown to have a reproducible two- to fivefold increase in ROS accumulation [37]. Macip *et al.* [15] found that both the levels of p53 protein and the p53-induced elevation of intracellular ROS influence the decision between senescence and apoptosis in a cell. By reducing and/or balancing oxidative stress, antioxidants counteract the effects of chemotherapy-induced oxidative stress on the cell cycle and enhance the cytotoxicity of antineoplastic agents [15,38].

Vitamin C and vitamin E are some of the most important natural antioxidants, which are redox modifiers and are capable of

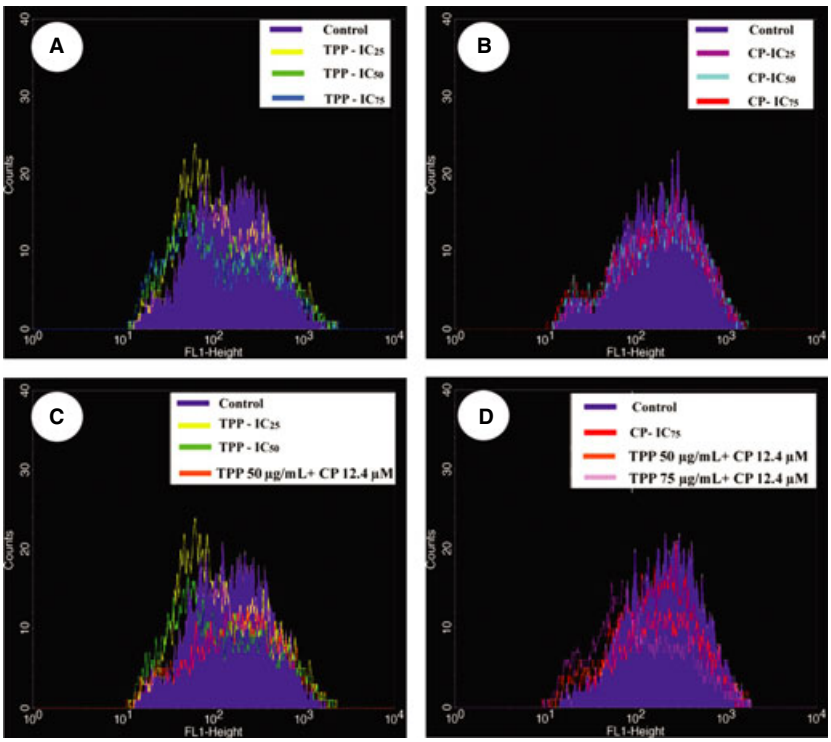

Fig. 5. Measurement of intracellular reactive oxygen species (ROS) by flow cytometry using fluorescent probe (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)). Comparison of the changes in the intracellular ROS level expressed as the mean fluorescent intensity in the MCF-7 cells when treated with tea polyphenols (TPP) (86 µg/mL), cisplatin (CP) (12.4 µM) and synergistic combination (TPP 50 µg/mL + CP 12.4 µM) for 24 hr. The cells were trypsinized and intracellular ROS was stained with using fluorescent probe (6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate, di(acetoxymethyl ester)). The fluorescent intensity of intracellular ROS was measured for 10,000 events with flow cytometry. The experiments were repeated at least three times with results similar. Overlaid histogram representing the intracellular ROS of TPP, CP and combination (TPP 50 µg/mL + CP 12.4 µM) treated and untreated cells (control). (A) Control, TPP-IC_{25}, TPP-IC_{50} and TPP-IC_{75}; (B) control, CP-IC_{25}, CP-IC_{50} and CP-IC_{75}; (C) control, TPP-IC_{25}, TPP-IC_{50} and combination (TPP 50 µg/mL + CP 12.4 µM); (D) control, CP-IC75, (TPP 50 µg/mL + CP 12.4 µM) and (TPP 75 µg/mL + CP 12.4 µM).

directly inducing apoptosis in cancer cells [39,40]. Pace *et al.* [41] conducted a clinical study in which they found that the incidence and severity of neurotoxicity of CP chemotherapy alone was higher than in combination with vitamin E. The supplementation of vitamins C and E protects against some of the harmful effects of anticancer drugs (fluorouracil, doxorubicin and cyclophosphamide) in patients with breast cancer [42–44].

Dietary polyphenols have attracted a great deal of interest because of their perceived ability to act as highly effective chemopreventive and chemotherapeutic agents. Additionally, the side effects of tea compounds are modest and well tolerated [45]. Increasing attention has been paid to the application of TPP for cancer prevention and as adjuvants in cancer chemotherapy. Indeed, TPP can inhibit carcinogenesis by suppressing the expression of pro-oxidant enzymes, inhibiting target genes involved in cell proliferation and inducing apoptosis.

Apoptosis is triggered by p53, which has been reported to be dependent on an increase in ROS and the release of pro-apoptotic factors resulting from mitochondrial damage [38]. The mitochondrial membrane-based Bcl-2 oncoprotein and other related proteins may play an important role in determining whether cells undergo apoptosis through caspase-9 and caspase-3. The effector caspase, caspase-3, and the initiator caspase-8 and caspase-9 are the main executors of apoptosis [46]. Caspase-8 is part of the death receptor pathway, whereas caspase-9 is part of the mitochondrial pathway; both pathways utilize caspase-3 [47].

Nutritional supplements have been used in a variety of chemotherapeutic strategies as antioxidants or combination therapies to provide patients with natural protection from the side effects of platinum-based drugs [48–50]. Several components of green tea, including different types of catechins, are known to inhibit cell growth and induce apoptosis in a variety of cultured cells.

In the present study, the results of combination therapy of TPP plus CP suggest that these combinations may act synergistically for therapeutic benefits. The synergy of TPP plus CP may minimize or slow down the development of drug resistance or allow the use of lower dosages, which would decrease toxicity while increasing or maintaining efficacy. Our results also suggest that the combination of CP and TPP may synergistically inhibit growth of MCF-7 cells compared with individual TPP or CP treatments. The CI and isobologram for the combination of TPP and CP revealed cytotoxic effects that were less than additive. The distinction between cytotoxicity and apoptosis is delineated by a series of typical morphological features, such as shrinkage of the cell and fragmentation

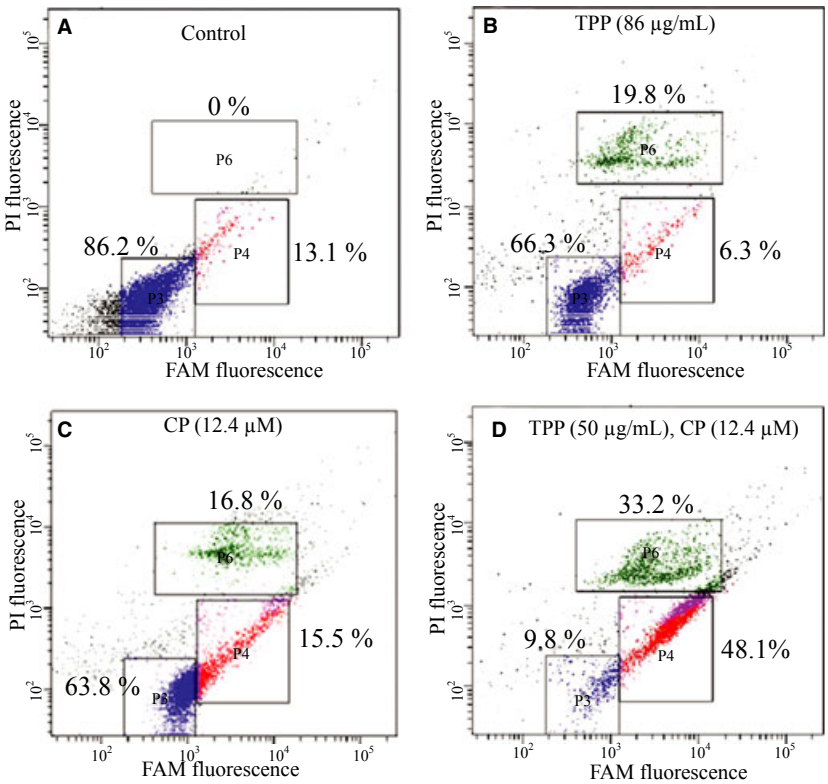

Fig. 6. Detection of activated caspases by flow cytometry using fluorescently labelled inhibitors of caspases (z) combined with the plasma membrane permeability assay [propidium iodide (PI)]. MCF-7 cells were incubated with tea polyphenols (TPP) (86 µg/mL), cisplatin (CP) (12.4 µM) and synergistic combination dose-(TPP 50 µg/mL + CP 12.4 µM) for 24 hr. Treated MCF-7 cells were then processed according to the kit manufacturer's instruction (Polycaspase kit) at 37°C in the dark. Fluorescence intensity was measured for 10,000 events with flow cytometry. MCF-7 breast cancer cells were untreated (A); treated with TPP (B); treated with CP (C); a combination (TPP 50 µg/mL + CP 12.4 µM) (D). Live cells (blue-coloured populations) are negative for both FAM and PI. Early apoptotic cells (red-coloured populations) are FAM-positive and PI-negative. Late apoptotic/secondary necrotic cells (green-coloured populations) are positive for both FAM and PI. The experiments have been repeated at least three times with results similar.

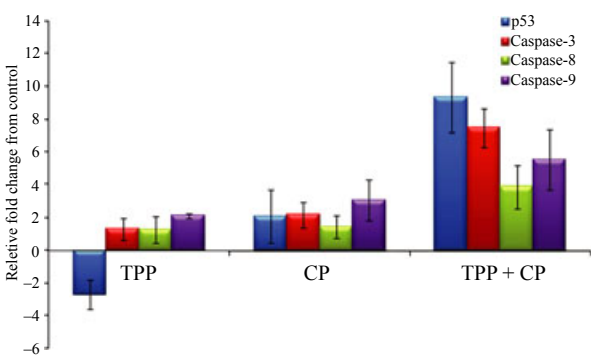

Fig. 7. Quantitative RT-PCR analysis of apoptotic marker genes in human breast cancer MCF-7 cells. Comparison of the change in mRNA expression level, expressed as the mean fold change (as the ratio of the target gene to the reference gene [GAPDH]) in MCF-7 cells after 24 hr exposure of tea polyphenols (TPP) (86 µg/mL), cisplatin (CP) (12.4 µM) and a synergistic combination (TPP 50 µg/mL + CP 12.4 µM). The data represent the mean ± S.D. of three determinations, each performed in triplicate. Statistical analysis was performed using the Student's *t*-test ($p < 0.05$).

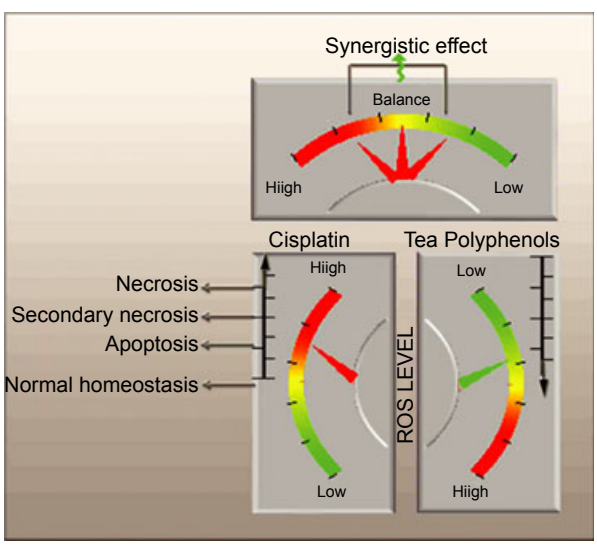

Fig. 8. The scheme represents the balance between cisplatin (CP)-induced reactive oxygen species and antioxidants tea polyphenols (TPP). Synergistic levels of apoptosis are induced through a homoeostatic mechanism by TPP if intracellular oxidation reaches above a certain threshold.

into membrane-bound apoptotic bodies [51]. As shown in fig. 4, PI-stained cells showed nuclear morphological features including fragmentation of chromatin, bi-/multinucleation, dot-like chromatin and apoptotic-body formation in cells treated with TPP plus CP, suggesting that this synergistic combination caused more cell death by apoptosis.

Activation of caspase enzymes, or cysteine-aspartic acid specific proteases, induces a series of reactions that are triggered in response to pro-apoptotic signals and results in the cleavage of protein substrates and the subsequent disassembly of the cell [52].We analysed activation of caspases (including caspase-1, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8 and caspase-9) by fluorescently labelled inhibitors of caspases (FLICA), and plasma membrane permeability was assessed by propidium iodide staining. In the cells treated with the synergistic combination dose (TPP 50 μg/mL + CP 12.4 μM), 48% of cells displayed early apoptosis and 33% late apoptosis (fig. 6D), which were significantly higher than the percentages obtained with control (untreated), and individual TPP (IC_{50} dose 86 μg/mL) and CP (IC_{50} dose 12.4 μM) treatments (fig. 6A–C). As depicted in fig. 6, the TPP plus CP combination treatment induced activation of all caspases at higher levels than in the single-agent treatments.

Overall, we found that p53 up-regulation by the TPP 50 μg/mL + CP 12.4 μM synergistic combination treatment resulted in the activation of caspase-1, caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8 and caspase-9. To confirm the activation of the caspases, we investigated whether this combination significantly altered transcription of caspase-3, caspase-8 or caspase-9 at 24 hr. The possibility that p53-mediated apoptosis may be associated with the activation of caspase-3 through caspase-8 and caspase-9 is suggested by the ability of p53 to activate both the extrinsic and intrinsic apoptotic pathways [47,53]. In fact, the majority of studies have revealed that the mechanism of action of CP mainly involves the disruption of mitochondria, causing the release of cytochrome-*c*, which in turn triggers activation of caspase-9 and caspase-3. CP treatment increases the formation of ROS (fig. 5) and could alter mitochondrial membrane potential (MMP). Our results suggest that TPP and CP synergistically activate caspase-9 in MCF-7 cells, inducing ROS-mediated-mitochondrial apoptosis. However, significant activation of caspase-8 was seen with treatments of TPP, CP and the combination of both TPP and CP, indicating that receptor-mediated activation of apoptosis may also be induced.

In our study, TPP increased CP-induced apoptosis and cytotoxicity. Enhanced up-regulation of p53 was observed with treatment using a combination of TPP and CP compared with individual TPP or CP. The up-regulation of p53 plays a role in the induction of apoptosis through the mitochondrial- and caspase-8-mediated pathways [34]. TPP may cooperate with CP-induced p53 through ROS regulation, which would likely have a synergistic effect on apoptosis [13,14]. These findings suggest the existence of a threshold of cellular oxidation above which the apoptotic programme is initiated. This threshold may vary depending on the dose level. However, the balance between CP-induced ROS and antioxidants (TPP) present in the cell at any given moment is likely crucial for determining cell-fate decisions (figs 5 and 8). Therefore, the combination of TPP and CP might have potential therapeutic benefits.

Generally, many patients with cancer consume tea with or without the knowledge of their oncologist. The TPP in it would circumvent the adverse effects of ROS and therapy, enhance the efficacy and reduce the adverse effects caused by chemotherapeutic drugs [13,14,17–20]. Although synergistic effect of CP and TPP has been demonstrated in various *in vitro* models and *in vivo* studies [7,12,20,22,25], combination of CP with TPP has been, until now, rarely investigated in the clinic. Our observations have given strong evidence that supplemental antioxidant TPP enhance antitumour effects of chemotherapy in breast cancer cells. Thus, clinical trials for patients with breast cancer are needed to address the safety and efficacy of antioxidant TPP with CP with special reference to ROS-mediated apoptosis.

In summary, our data demonstrate that TPP cooperate with the chemotherapeutic agent CP to inhibit viability of breast cancer cells by decreasing proliferation and/or by increasing apoptosis. These data indicate that TPP may be an effective agent in combination with chemotherapy for treating breast cancer patients. Elucidation of the molecular mechanisms by which TPP modulate CP-induced apoptosis is a topic that should be addressed. Further investigations on the screening of several synergistic combinations doses of TPP and CP useful as a cancer preventive or therapy are also needed.

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