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ORIGINAL RESEARCH

Green synthesis palladium nanoparticles mediated by white tea (*Camellia sinensis*) extract with antioxidant, antibacterial, and antiproliferative activities toward the human leukemia (MOLT-4) cell line

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Susan Azizi,[1] Mahnaz Mahdavi Shahri,[2] Heshu Sulaiman Rahman,[3–5] Raha Abdul Rahim,[6] Abdullah Rasedee,[5] Rosfarizan Mohamad[1,7]

[1]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia; [2]Department of Chemistry, Shiraz Branch, Islamic Azad University, Shiraz, Iran; [3]College of Veterinary Medicine, University of Sulaimani, Sulaimani Nwe, [4]College of Health Science, Komar University of Science and Technology (KUST), Chaq-Chaq Qularaise, Sulaimani City, Iraq; [5]Faculty of Veterinary Medicine, [6]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, [7]Laboratory of Biopolymer and Derivatives, Institute of Tropical Forestry and Forest Products, Universiti Putra Malaysia, UPM Serdang, Selangor, Malaysia

Correspondence: Mahnaz Mahdavi
Department of Chemistry, Shiraz Branch, Islamic Azad University, Shiraz 74731-71987, Iran
Email mahnaz.chem@gmail.com

Rosfarizan Mohamad
Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, UPM Serdang, Selangor 43400, Malaysia
Email farizan@upm.edu.my

Abstract: Among nanoparticles used for medical applications, palladium nanoparticles (PdNPs) are among the least investigated. This study was undertaken to develop PdNPs by green synthesis using white tea (W.tea; *Camellia sinensis*) extract to produce the Pd@W.tea NPs. The Pd@W.tea NPs were characterized by UV–vis spectroscopy and X-ray diffractometry, and evaluated with transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The Pd@W.tea NPs were spherical (size 6–18 nm) and contained phenols and flavonoids acquired from the W.tea extract. Pd@W.tea NPs has good 1-diphenyl-2-picrylhydrazyl (DPPH), OH, and NO-scavenging properties as well as antibacterial effects toward *Staphylococcus epidermidis* and *Escherichia coli*. MTT assay showed that Pd@W.tea NPs (IC_{50} =0.006 µM) were more antiproliferative toward the human leukemia (MOLT-4) cells than the W.tea extract (IC_{50} =0.894 µM), doxorubicin (IC_{50} =2.133 µM), or cisplatin (IC_{50} =0.013 µM), whereas they were relatively innocuous for normal human fibroblast (HDF-a) cells. The anticancer cell effects of Pd@W.tea NPs are mediated through the induction of apoptosis and G2/M cell-cycle arrest.

Keywords: green synthesis, palladium nanoparticles, white tea, leukemic cells, cytotoxicity, medical application, nanobiotechnology

Introduction

Nanotechnology has generated new materials for application in medicine. However, inorganic nanoparticles (NPs) produced by various chemical and physical methods are relatively expensive, and the production methods are often hazardous to the environment.[1] The novel green synthetic method for the production of NPs using biological materials such as microorganisms, marine organisms, micro-fluids, and plant extracts have proven to be superior to other processes.[2] These methods of NP synthesis are simple and efficient, and the products are safe for medical applications.[3] In addition, molecular components of plant extracts have great affinity for the surface of nano-structures, and stabilize and prevent aggregation while improving the biological effects of NPs.[4] Thus far, more than 200 plants have been screened for their potential to produce inorganic NPs.[5,6]

With the discovery of the platinum (II)–cisplatin complex, metal-based compounds are touted as potential drug delivery systems for cancers.[7] Recently, Chen et al[8]

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developed palladium nanoparticles (PdNPs) that had remarkable optical, electronic, and chemical properties. These PdNPs are currently being investigated for use as sensors and catalysts. The PdNPs have enormous catalytic potential in various organic transformations, including Suzuki-cross, Mizoroki-Heck, Stille, and Sonogashira coupling reactions.[9–11] However, the application of PdNPs in the biomedical field is yet to be adequately investigated, although the green-synthesized PdNPs possess remarkable antioxidant[12,13] and antimicrobial activities.[12,14] Plant-derived PdNPs seem to have potential for application in cancer therapy because these compounds are also toxic to cancer cells.[15,16]

In this study, we demonstrated a simple one-step green process to synthesize PdNPs using *Camellia sinensis* (white tea [W.tea]) extract from unfermented young tea leaves or unopened buds.[17] The white tea extract has been used as an efficient reducing and capping agent. The extract contains several polyphenolic compounds belonging to the flavan-3-ol (catechin) family (Figure 1) that possess a wide range of biological activities, including antioxidant, anticancer, antiviral, antibacterial, and antifungal effects.[18–20]

To our knowledge, there is no report on the effect of white tea plant extract-derived PdNPs on leukemic cells. Among treatment options for leukemia are chemotherapy, radiation therapy, stem cell transplantation, biological or immune therapy, and targeted therapy. Chemotherapy and radiation are plagued by side effects that include anemia, hair loss, fatigue, gastrointestinal disorders, and susceptibility to bleeding and infections, whereas other treatments are effective in some receptive patients only. For these reasons, more effective alternative cancer therapeutic compounds with minimal side effects are desperately being sought.

Materials and methods

Materials

White tea plant was purchased from a local herbal store in Shiraz, Iran, and washed several times using distilled water to remove impurities. The leaves were sun-dried and then crushed into powder. $PdCl_2$ (99.98%) was used as a palladium precursor and it was supplied from Merck (Darmstadt, Germany). All solutions were prepared with deionized water. The plant was authenticated by Department of Botany, Shahid Chamran University, Iran, and the voucher has been deposited.

Extract preparation

White tea powder sample (1.0 g) was dispersed in 100 mL distilled water with magnetic stirring and heated at 100°C for 20 min. The extract was cooled to room temperature and filtered through a muslin cloth to collect a clear extract.

Synthesis of palladium nanoparticles

An Erlenmeyer flask containing 50 mL of 1 mM $PdCl_2$ solution was made to react with 50 mL of the aqueous white tea extract at 40°C with continuous stirring. The color of the reaction mixture gradually turned to dark brown from transparent yellow after 30 minutes, indicating the formation of Pd NPs. The synthetic reaction was completed in 2 h. The initial pH of the solution was approximately 7.5, but changed to 5.6 by the end of the reaction. The product sample was collected through centrifugation at 6,000 rpm for 10 min and, after several washings with distilled water, dried in an oven at 60°C. The dried sample, palladium nanoparticles using white tea (Pd@W.tea) NPs, was crushed into powder and stored in an airtight container for further analysis.

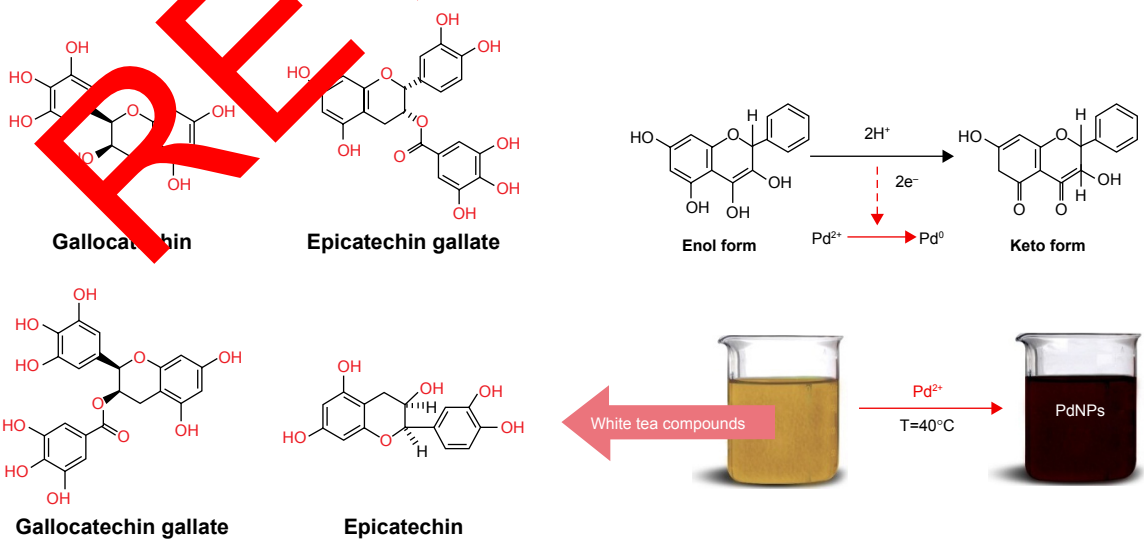

Figure 1 Photograph of formation of Pd NPs in W.tea extract and possible reaction to synthesize PdNPs.
Abbreviations: Pd, NPs, palladium nanoparticles; W.tea, white tea.

Characterization of synthesized Pd@W.tea NPs

The Pd@W.tea NPs was quantitated by UV-Vis spectrophotometry (Lambda 25-Perkin Elmer, Waltham, MA, USA) over wavelength range of 200–800 nm, and the chemical composition was characterized by Fourier-transform infrared (FTIR) spectrometry (Perkin-Elmer 1725X) in the range of 400–4,000 cm^{-1}. The phase purity and particle size of Pd@W.tea NPs were determined using the X-ray diffractometer (XRD-6000; Shimadzu) at 40 kV with nickel-filtered Cu (λ=1.542 Å) in the range of 10° to 80°.[21] Morphological analysis of Pd@W.tea NPs was conducted by using transmission electron microscopy (TEM; HITACHI H-7650, Tokyo, Japan) at voltage 120 kV. The sample suspension was drop-casted on a carbon-coated copper grid and allowed to air-dry at room temperature overnight. The powdered sample was put on the carbon stub using carbon tape and then gold-coated using a sputter coater for ultrastructural examination via scanning electron microscopy (Philips XL-30).[8]

Quantification of phenolic and flavonoid content

The phenolic and flavonoid contents of Pd@W.tea NPs and crude white tea extract were quantified.

Total phenolic content

Phenolic content was determined by the Folin-Ciocalteu assay as described by Singleton and Rossi,[22] with slight modifications. Briefly, 10 µL sample solution and 500 µL Folin-Ciocalteu reagents were added in each well of 96-well plates. Then, 350 µL of 10% of Na_2CO_3 was added to the wells, and the plate was incubated in the dark at room temperature for 2 h. The absorbance was then recorded spectrophotometrically (Agilent 8453 Spectrophotometer, USA) at 765 nm against 10% DMSO as the negative control. Phenolic content was estimated using the gallic acid calibration curve (R^2=0.97) and was expressed as gallic acid equivalent (µg GAE).

Total flavonoid content

Total flavonoid content were determined by an aluminum chloride colorimetric method as described previously.[23] In this test, the reaction mixture was prepared by mixing 100 µL Pd@W.tea NPs or white tea extract, 10 µL 10% aluminum chloride, 10 µL 5% $(CH_3CO_2)K$, and 30 µL distilled water. The mixture was incubated at room temperature for 30 min, and absorbance was read at 415 nm. The calibration curve (R^2=0.99) was obtained using the quercetin solutions at concentrations of 0.0–10 µg/mL and the flavonoid content was determined from the curve and expressed as quercetin equivalent (µg QE).

Antioxidant activity

The antioxidant potential of the Pd@W.tea NPs was determined through 1-diphenyl-2-picrylhydrazyl (DPPH) and radical (–OH and –NO) scavenging activities.

DPPH scavenging activity

The DPPH scavenging activity of Pd@W.tea NPs was determined using the method described by Blois.[24] In brief, approximately 20 µL each of Pd@W.tea NPs and white tea extract at concentrations ranging from 0.156 to 10 µM were added to 100 µL 0.1 mM methanolic DPPH solution. The mixture was incubated for 30 min at room temperature with constant shaking, and the absorbance was recorded at 517 nm. Butylhydroxytoluene was used as the reference. The DPPH radical scavenging activity (RSA) was calculated as percent inhibition using the following equation:

$$RSA\ (\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] \times 100$$

where $A_{control}$ and A_{sample} are the absorbance of the control and sample, respectively.

OH scavenging activity

Hydroxyl radical scavenging activity was determined by the degradation of 2-deoxyribose after condensation with thiobarbituric acid to produce OH radicals.[25] The final reaction mixture containing 100 µM $FeCl_3$, 100 µM ascorbic acid, 1 mM H_2O_2, 20 mM KOH buffer (pH 7.4), 100 µM EDTA, and 2.8 mM 2-deoxyribose was mixed with different concentrations (0.156–10 µM) of the Pd@W.tea NPs or control and incubated at 37°C for 1 h. Then, 0.5 mL reaction mixtures were added to 1 mL of 0.5% thiobarbituric acid diluted with NaOH (0.025 M) and 1 mL of 2.8% trichloroacetic acid. The mixtures were again incubated at 100°C for 30 min to obtain the chromogenic adduct. After cooling to room temperature, the concentration of chromogen was quantified at 532 nm. Gallic acid was used as the reference, and the percent inhibition of radical scavenging activity calculated using Eqn 1.

NO scavenging activity

The NO scavenging activity was determined spectrophotometrically (Agilent 8453 Spectrophotometer, Golden Valley, MN, USA).[25] Approximately 50 µL of 5 mM sodium nitroprusside

was added to various concentrations (0.156–10 µM) of Pd@W.tea NPs or control, and the mixture was incubated at 25°C for 1 h. Then, 1.5 mL of this mixture was obtained and treated with 1.5 mL Griess' reagent (Sigma-Aldrich Co., St Louis, MO, USA) to diazotize and form chromophore. Gallic acid was used as the reference. The concentration of the chromophore was determined at 546 nm, and percent inhibition of scavenging activity was calculated using Eqn 1.

Antibacterial activity

The antibacterial potential of Pd@W.tea NPs was determined by the disc-diffusion method and minimum inhibitory concentration (MIC).[3]

Zone of inhibition

The antibacterial potential of Pd@W.tea NPs was determined using the disc-diffusion method on the Gram-positive *Staphylococcus epidermidis* S273 (*S. epidermidis*) and Gram-negative *Escherichia coli* E266 (*E. coli*) strains. After growing the bacteria overnight in nutrient broth, each bacterial inoculum standardized to 0.5 MF units (~10^8 cfu) was inoculated onto the Muller–Hilton agar (MHA) plate. Discs (6 mm) impregnated with samples at concentrations ranging from 0.156 to 10 µM were placed on the MHA plates and incubated at 37°C for 24 h. White tea extract was used for comparison, with streptomycin as a standard reference. The zone of inhibition was recorded.

Minimum inhibitory concentration

Serial tube dilution method was applied to determine the MIC of the Pd@W.tea NPs on *S. epidermidis* and *E. coli*. Test samples were prepared by mixing Pd@W.tea NPs, white tea, or streptomycin (0.156–10 µM) with 100 µL 10% DMSO. Then, 20 µL of the suspension was mixed with 450 mL LB broth (Sigma-Aldrich Co.) and 50 mL of fresh microbial inoculum (10^8 cfu/mL) incubated at 37°C for 24 h to grow bacteria. The turbidity of the suspension was determined visually before and after incubation.

Antiproliferative activity

The antiproliferative effects of Pd@W.tea NPs and white tea extract was determined on the acute human T-lymphoblastic leukemia (MOLT-4) cell line (American Type Culture Collection [ATCC], Manassas, VA, USA), with cisplatin and doxorubicin as reference drugs.[26] The analyses used the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay kit (Sigma-Aldrich Co.). Briefly, cells were allowed to grow in a 75 m^2 cell culture flask until 95% confluent before seeding at 1×10^5 cells/mL into each well of a 96-well plate. These cells were then treated with various concentrations (0.001–3.5 µM) of Pd@W.tea NPs, white tea extract, cisplatin, and doxorubicin. Normal adult human fibroblast (HDF-a) cell line was used as the control. After incubation for 48 h at 37°C at pH 7.5 and under 5% CO_2, 25 µL 5.5 mg/mL MTT solution was added to each well and the plate was covered with aluminum foil and incubated for a further 3 h in the dark. The medium was immediately aspirated and the purple formazan lysed with MTT solution. The assay was conducted in triplicates. The absorbance was determined at 570 nm using the Spectra Max Plus 384 UV–Vis plate reader. The half maximum inhibitory concentration (IC_{50}) was determined by non-regression analysis using Graph Pad Prism V. 5.03 (Graph Pad Software Inc., USA).

Annexin-V/PI double staining assay

Phosphatidylserine translocation in MOLT-4 cells treated with Pd@W.tea NPs was determined by the Annexin-V-FITC/propidium iodide (PI) apoptosis detection kit (Sigma-Aldrich Co.).[21] In brief, cells treated with 0.006 µM Pd@W.tea NPs for 12, 24, or 48 h were harvested, washed with PBS twice, resuspended in binding buffer, and incubated with Annexin V-FITC and propidium iodide solutions at room temperature in the dark for 30 min. Apoptosis was determined by flow cytometry using FACS Calibur (BD Biosciences, San Jose, CA, USA) with 15,000 ungated cells.

Cell-cycle analysis

MOLT-4 cell-cycle distribution after treatment with Pd@W.tea NPs was determined by flow cytometry according to the method described previously.[27] Briefly, MOLT-4 cells after incubation for 12, 24, or 48 h with 0.006 µM Pd@W.tea NPs were collected and centrifuged at 200× *g* for 5 min before washing with a mixture of PBS and sodium azide. The harvested cells were fixed with chilled 70% ethanol at –20°C for 5 days and then centrifuged at 200× *g* for 5 min. The supernatant was discarded and the cells were again washed twice with PBS and sodium azide; stained with PBS-staining buffer containing 0.1% triton X-100, 10 mM ethylenediamine tetra-acetic acid, 50 µg/mL RNAase A, and 3 µg/mL PI; and incubated on ice in the dark for 30 min. Flow cytometric analysis was conducted using the BD FACS Calibur flow cytometer (BD), and data analysis was conducted using the Cell Quest Pro software with a DNA histogram to express the proportion of cells in cell-cycle phases. Apoptotic cells with hypodiploid DNA content were detected by quantifying the sub-G1 peak.

Protease activity of caspases 3 and 9

The caspase-3 and -9 activities were determined by a colorimetric assay kit (Gene script kit, code: L00289, GenScript, Piscataway, NJ, USA) in MOLT-4 cells.[28] Cells treated with 0.006 µM Pd@W.tea NPs for 12, 24, or 48 h were washed twice with ice-cold PBS, centrifuged at 200× *g* for 5 min, and the medium was discarded before harvesting cells. The cell pellet was suspended in cells lysis buffer and incubated on ice for 1 h. Untreated MOLT-4 cells incubated for 12, 24, or 48 h served as controls. The protein concentrations were determined using the Bradford method. Approximately 1×10^8 MOLT-4 treated cells were transferred to the 96-well plate and 5 µL caspase substrate was added to the cells; the plate was wrapped with aluminum foil and incubated at 37°C for 4 h. After incubation, the plate was read at 405 nm in a microplate reader (Universal Microplate reader, Biotech, Inc., Winooski, VT, USA) to determine caspase activity, and the results are presented as optical density (OD).

Results and discussion

Characterization of PdNPs

White tea contains several polyphenols and flavonoids. The Pd@W.tea NPs suspension prepared from white tea was dark brown (Figure 1), which is due the PdNPs[29] formed through the reduction process. Because hydroxyl groups are abundant in flavonoids, the reduction of palladium from Pd(II) to Pd(0) involved oxidation of the hydroxyl groups with concomitant decrease in pH of the reaction suspension.[30]

UV–Vis spectroscopy is a useful method to validate the presence of metal NPs. Using this technique, aqueous white tea solution showed an intense absorption peak at 250 nm, which is associated with the benzoyl $\pi \rightarrow \pi^*$ transitions – an indication of the presence of flavonoids[31] (Figure 2). The white tea extract with Pd(II) solution exhibited another

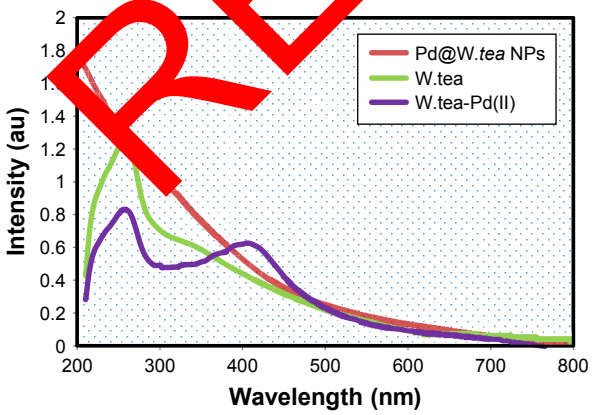

Figure 2 UV–vis spectra of Pd@W.tea NPs, white tea (W.tea) plant extract, and white tea with Pd(II).
Abbreviations: NPs, nanoparticles; Pd, palladium.

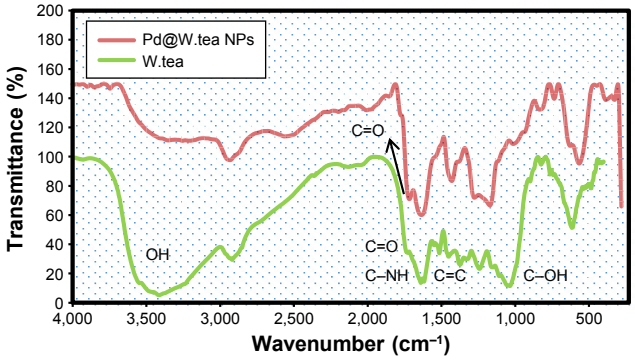

Figure 3 FTIR spectra of Pd@W.tea NPs and white tea (W.tea) extract.
Abbreviations: FTIR, fourier-transform infrared; NPs, nanoparticles; Pd, palladium.

discrete absorption peak at 410 nm, which is attributed to the Pd^{2+} ion content. Pd@W.tea NPs produced a wide, continuous, absorption spectrum without the typical peaks of white tea extract or white tea with Pd(II) solution, indicating formation of PdNPs.

An FTIR analysis was used to identify molecules responsible for the reduction of Pd ions and coating of the PdNPs. The FTIR spectrum of white tea extract showed peaks at 3,345, 2,860, 1,712, 1,667, 1,520, 1,389, 1,124, and 720 cm^{-1}, representing free OH, and stretching of CH group of aldehydes and alkanes, carbonyl group (C=O), (NH)–C=O group, C=C aromatic ring, C–OH, and phenyl (C–H), respectively (Figure 3). These peaks showed that flavonols and other phenolics molecules are present in the white tea extract. With reduction of Pd^{2+} ions, the position and intensity of peaks changed, especially by the decrease in the hydroxyl peak and appearance of the carbonyl peak, indicating the involvement of phytochemical sites in synthesis and binding of PdNPs. Thus, the results confirmed that reduction of Pd(II) to Pd(0) occurs through oxidation of hydroxyl to carbonyl groups.

The phenolic content of Pd@W.tea NPs was significantly ($P<0.05$) low, and the flavonoid content lower in Pd@W.tea NPs than in crude white tea extract (Table 1). Differences in these chemical contents are responsible for some of the variability in biological activities between Pd@W.tea NPs and crude white tea extract.

The Pd@W.tea NPs were examined under X-ray diffraction to determine crystalline structure (Figure 4). There are

Table 1 Total phenolic and flavonoid contents of white tea (W.tea) extract and Pd@W.tea NPs

Sample	Total phenolic content (µg, GAE/mg)	Total flavonoid content (µg, QE/mg)
White tea	232.05±2.23	25.46±0.87
Pd@W.tea NPs	29.6±0.68	9.87±0.43

Abbreviations: NPs, nanoparticles; Pd, palladium.

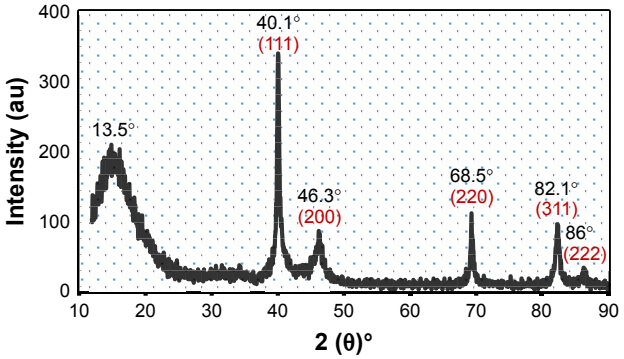

Figure 4 X-ray diffraction pattern of the Pd@W.tea NPs.
Abbreviations: NPs, nanoparticles; Pd, palladium; W.tea, white tea.

five well-defined and characteristic diffraction peaks at 40.1°, 46.3°, 68.5°, 82.1°, and 86.2° representing reflections from (111), (200), (220), (311), and (222) planes, respectively, of face-centered cubic crystal structure of Pd(0). The presence of a broad and intense diffraction peak at 2θ of 13.5° may be assigned to the chemical contents of the white tea extract. Using the Scherrer equation, the average size of Pd@W.tea NPs was calculated at 15 nm.

Under scanning electron microscopy (SEM) and transmission electron microscopy (TEM), Pd@W.tea NPs were shown to be spherical with narrow size distribution (Figure 5). The particles did not appear to aggregate, although some were present in clumps that suggested passive contact. The ultrastructure studies showed that Pd@W.tea NPs are stable and had little tendency to aggregate, which is attributed to the presence of phenols and flavonoids in NPs. It is proposed that, while inhibiting particle aggregation, phenols and flavonoids play a major role in the chelation of NPs to ligands. The size distribution of Pd@W.tea NPS is in the range of 6–18 nm, averaging at 11 nm, which is marginally smaller than the value obtained by X-ray diffraction determination.

Radical scavenging

To assess the antioxidant activity of white tea extract and Pd@W.tea NPs, DPPH radical scavenging activity was determined in MOLT-4 cells (Figure 6). It is assumed that the content of phenolic and flavonoid compounds of the extract and Pd@W.tea NPs would afford them promising radical scavenging activity because these compounds are known to be scavengers of DPPH, OH, and NO.[25,31,32] The radical scavenging activities of white tea extract for DPPH was high even at low doses and was dose-dependent (Figure 7). On the other hand, the DPPH scavenging activity of Pd@W.tea NPs was low at low concentrations and only began to approach antioxidative efficacy of white tea extract only after reaching high doses.

Both white tea extract and Pd@W.tea NPs showed dose-dependent scavenging activities of OH and NO. However, overall, white tea extract is still more efficacious than Pd@W.tea NPs at OH and NO scavenging. This phenomenon can be attributed to the concentration of phenolic and flavonoid compounds, which were much higher in the crude extract than Pd@W.tea NPs.

Antibacterial activity

The fact that white tea extract and Pd@W.tea NPs showed antioxidant activities makes them good candidates as

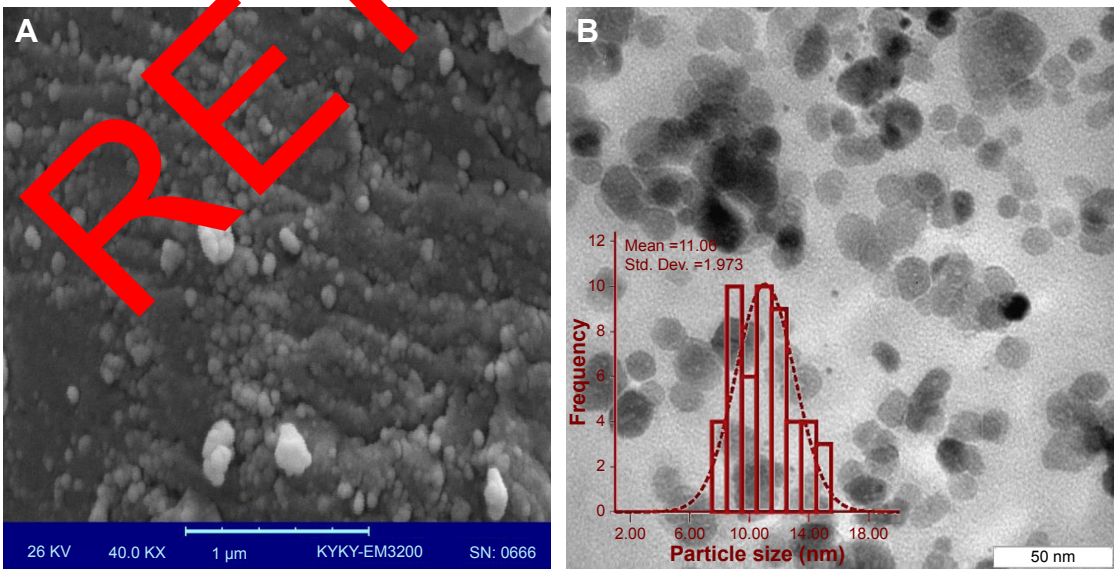

Figure 5 The (**A**) SEM and (**B**) TEM images, and particle size distribution (inset) of Pd@W.tea NPs.
Abbreviations: Pd, palladium; SEM, scanning electron microscopy; Std. Dev., standard deviation; TEM, transmission electron microscopy; W.tea, white tea.

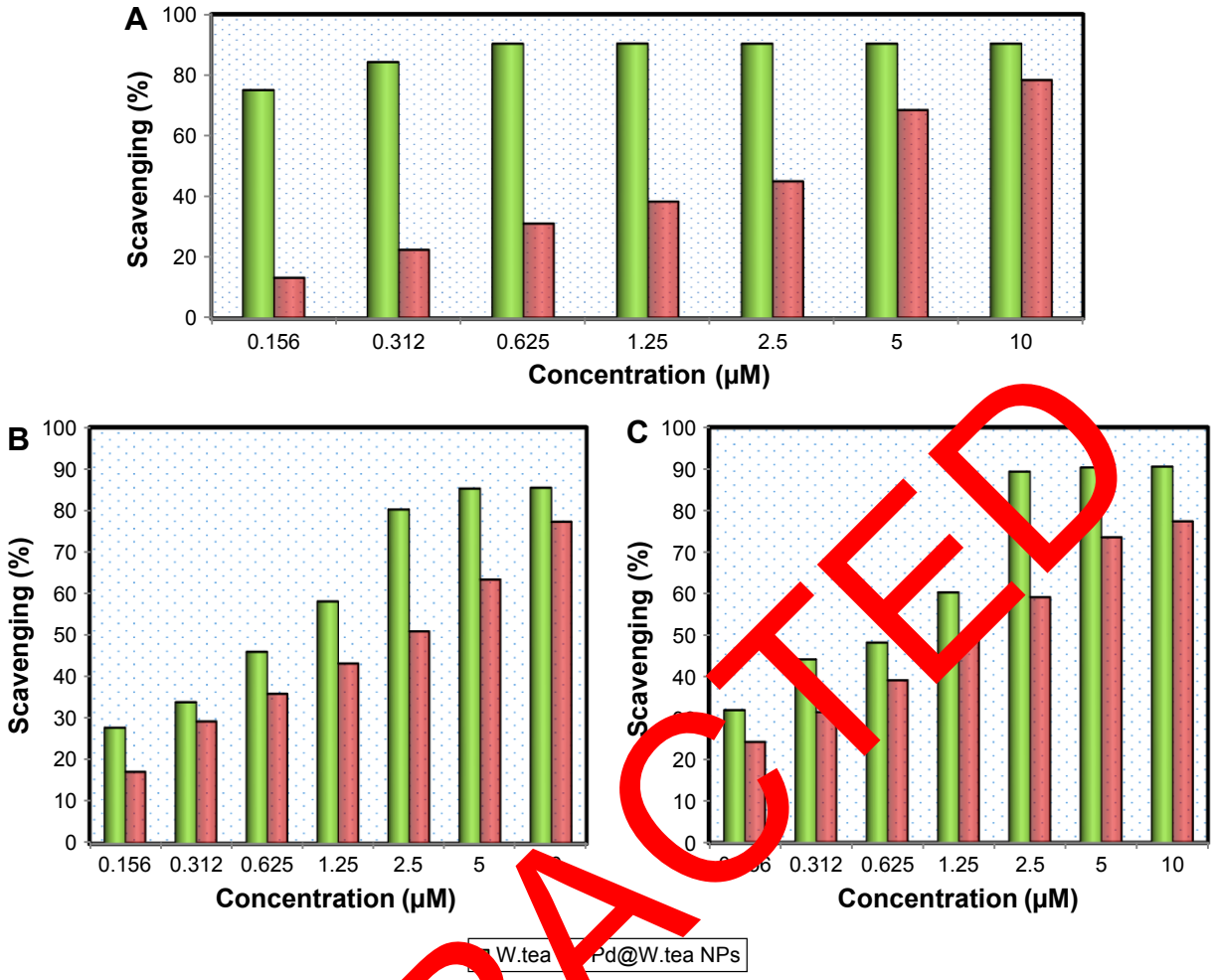

Figure 6 (**A**) DPPH, (**B**) ·OH, and (**C**) ·NO scavenging activity of white tea extract and Pd@W.tea NPs.
Abbreviations: NPs, nanoparticles; Pd, palladium; W.tea, white tea.

antibacterial agents. By the disc diffusion assay, Pd@W.tea NPs and white tea extract variably inhibits growth of *S. epidermidis* and *E. coli*, with the effect being more potent on the *S. epidermidis* than *E. coli* (Table 2 and Figure 8). *E. coli*, a Gram-negative bacterium, possesses an external lipopolysaccharide membrane that protects the peptidoglycan layer and allows the bacteria to survive in harsh environments.[33] Presumably, this protective barrier is one of the contributing factors toward the greater ability of *E. coli* than *S. epidermidis* to survive the toxic effects of white tea extract and Pd@W.tea NPs.

The antibacterial efficacy of white tea extract and Pd@W.tea NPs was further verified via MIC. Pd@W.tea NPs showed higher MIC values toward *S. epidermidis* and *E. coli* than white tea extract (Table 3). These findings indicate that Pd@W.tea NP is a better antibacterial agent than white tea extract, possibly because of the greater ability of Pd@W.tea to specifically interact with the bacteria. Interaction of compounds with the bacterial membrane is dependent on physical and physicochemical characteristics of the ligands and surface of the bacterial membrane. In fact, for nanoparticulated drug delivery systems, particle size and surface area play significant roles in their antibacterial activities.[34] The unique physicochemical properties of the NPs also facilitate interactions of the Pd@W.tea NPs with the bacterial cell membrane.[35] White tea extract lacks formed structures with appropriate size and characteristics to aid specific interactions with bacteria surface effectively, and these account for its lesser antibacterial effects compared to Pd@W.tea NPs.

In vitro cytotoxicity

The antiproliferative activity of the white tea extract and Pd@W.tea NPs was determined on the MOLT-4 cell line with cisplatin and doxorubicin for comparison (Figure 9). Pd@W.tea NPs were highly toxic to MOLT-4 cells, with effect increasing with increase in concentration. In fact, based on IC_{50}, Pd@W.tea NPs are >2, >100, and >3,000 times more toxic to MOLT-4 cells than ciplastin, crude white tea extract, and doxorubicin, respectively (Table 3).

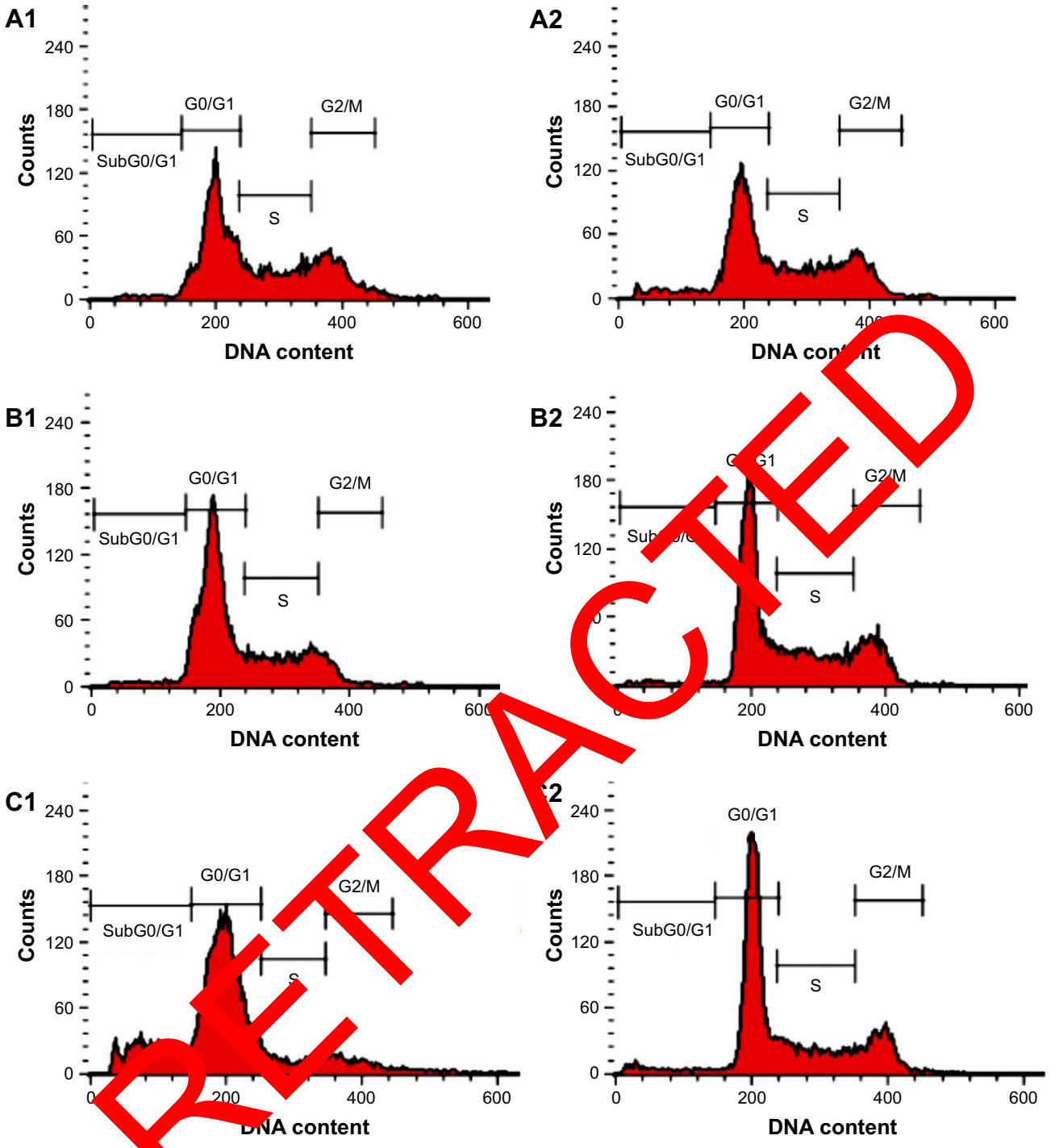

Figure 7 Cell cycle of MOLT-4 cells treated with Pd@W.tea NPs after staining with propidium iodide. (**A1**–**C1**) untreated (control) MOLT-4 cells at 12, 24, and 48 h, respectively. (**A2**–**C2**) MOLT-4 cells treated with Pd@W.tea NPs at 12, 24, and 48 h, respectively. G0/G1, G2/M, and S are cell cycle phases and subG0/G1 is the apoptotic cell population.
Abbreviations: NPs, nanoparticles; Pd, palladium; W.tea, white tea.

Table 2 Antibacterial activities of white tea (W.tea) extract and Pd@W.tea NPs

Bacteria	White tea extract		Pd@W.tea NPs	
	Zone of inhibition (mm)	MIC (μM)	Zone of inhibition (mm)	MIC (μM)
S. epidermidis	11.0±1.2	0.625	17.0±1.4	0.156
E. coli	8.0±1.1	1.25	14.0±1.3	0.313

Abbreviations: *E.coli, Escherichia coli*; MIC, minimum inhibitory concentration; NPs, nanoparticles; Pd, palladium; S. epidermidis, *Staphylococcus epidermidis*.

These findings show that Pd@W.tea NPs have potential as an anticancer compound. Both Pd@W.tea NPs and crude white extract showed low toxicity to the normal HDF-a cell line. It is proposed that the high cytotoxicity of Pd@W.tea NPs to the cancer cell line is due to the presence of Pd, which interacts physicochemically with the functional groups of cellular proteins, nitrogen bases, and phosphate groups of the DNA,[36] thus causing cell death. Moreover, previous studies

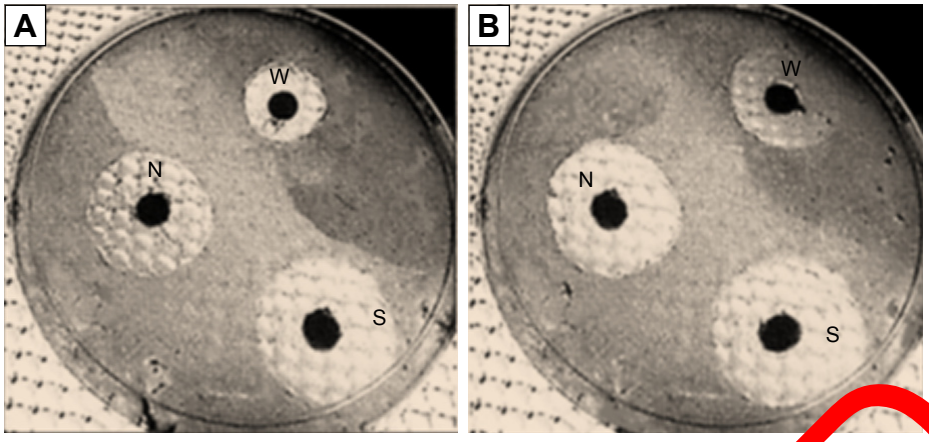

Figure 8 Antibacterial activity of (W) white tea extract (N) Pd@W.tea NPs and (S) streptomycin toward (**A**) *E. coli* and (**B**) *S. epidermidis*.
Abbreviations: *E. coli*, *Escherichia coli*; NPs, nanoparticles; Pd, palladium; *S. epidermidis*, *Staphylococcus epidermidis*; W.tea, white tea.

showed that Pd causes production of free radical,[37] lactate dehydrogenase leakage,[15] and cell-cycle disturbances[38] that could be among mechanisms of the anticancer effects of Pd@W.tea NPs.

Flavonoid compounds can control metabolic activities of cancer cells.[39] The anticancer effects of flavonoids occur through oxidative destruction, inhibition of proliferation, inactivation of carcinogen, promotion of differentiation, induction of cell-cycle arrest and apoptosis, impairment of tumor angiogenesis, and suppression of metastasis.[40–42] Flavonoids can interact with xenobiotic metabolizing enzymes and inhibit involvement of kinases signal transduction, interact with estrogen type II binding sites, and alter gene expression patterns,[43,44] with resultant promotion of antiproliferative activity of Pd@W.tea NPs.

The size of NPs is an important factor in their cytotoxic effects. Small-sized NPs can exert greater cytotoxicity than large ones.[45,46] At an average of 11 or 15 nm, Pd@W.tea NPs can evade the mononuclear phagocytic system whereas easily crossing cell membranes to exert anticancer effects. The antiproliferative effect of Pd@W.tea NPs on the MOLT-4 is selective because these NPs are relatively innocuous to normal fibroblast cells. The IC_{50} values of white tea extract and Pd@W.tea NPs on the HDF-a cells were considerably higher than on the MOLT-4 cells. It is suggested that surface modifications of the PdNPs by the flavonoids acquired from white tea extract are responsible for the toxic effect of Pd@W.tea NPs on cancers without adversely affecting normal cells.[44]

Table 3 The IC_{50} of Pd@W.tea NPs, white tea extract, cisplatin, and doxorubcin after 48 h incubation

Cell line	IC_{50} (μM)			
	Pd@W.tea NPs	White tea extract	Cisplatin	Doxorubicin
MOLT-4	0.006±0.002	0.894±0.01	0.013±0.05	2.133±0.9
HDF-a	3.311±0.75	4.921±1.1	–	–

Note: Values are expressed as mean ± (SD =0.001) of three replicates.
Abbreviations: HDF-a, human fibroblast cell line; MOLT-4, human leukemia cell line; NPs, nanoparticles; Pd, palladium; W.tea, white tea.

Phosphatidylserine translocation

Annexin-V/PI double-staining assay showed that MOLT-4 cells treated with Pd@W.tea NPs became apoptotic gradually with concomitant decrease in viable cells in a time-dependent manner. After 12-h treatment, MOLT-4 cells were primarily in the early phase of apoptosis and began to enter the late phase of apoptosis from 24 h onward (Figure 10 and Table 4). This effect is similar to that seen with other green-synthesized NPs, such as *Sargassum muticum* gold nanoparticles on the human leukemia (K562) cell line induced,[28] egg white-mediated silver NPs on human breast adenocarcinoma (MDA-MB231) cell line,[49] hyaluronan/zinc oxide nanocomposite on acute promyelocytic leukemia cells (HL-60) cells,[27] and selenium NPs on human (MCF-7) breast-cancer cells.[50]

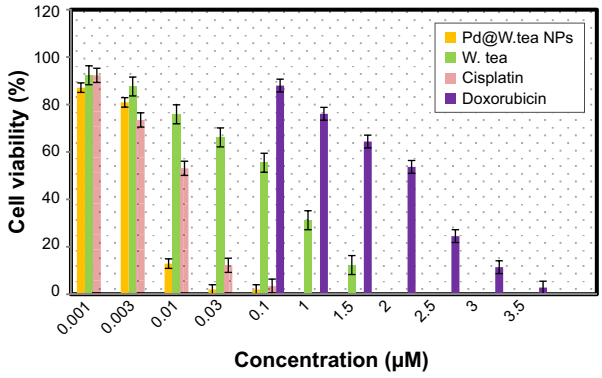

Figure 9 Viability of MOLT-4 cells after treatment with Pd@W.tea NPs, white tea extract (W.tea), cisplatin, and doxorubicin for 48 h, determined by MTT assay.
Abbreviations: NPs, nanoparticles; Pd, palladium.

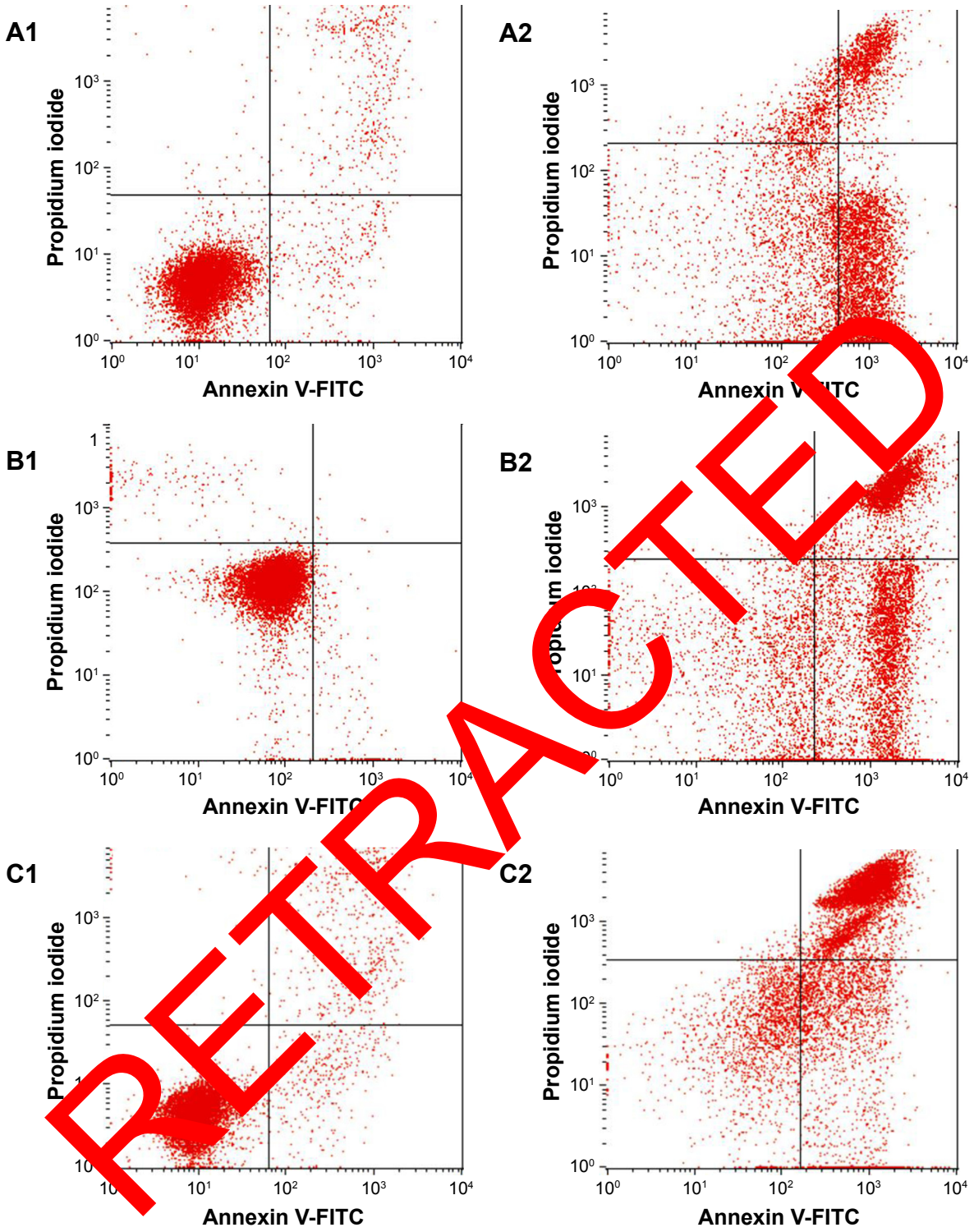

Figure 10 Induction of MOLT-4 cell apoptosis by Pd@W.tea NPs after staining with FITC-conjugated Annexin V-FITC. (**A1**–**C1**) untreated (control) MOLT-4 cells at 12, 24, and 48 h, respectively. (**A2**–**C2**): MOLT-4 cells after treatment with Pd@W.tea NPs at 12, 24, and 48 h, respectively.
Abbreviations: Annexin V-FITC, phosphatidylserine on cell membrane; FITC, green fluorescence channel; MOLT-4, human leukemia cell line; NPs, nanoparticles; Pd, palladium, W.tea, white tea.

MOLT-4 cell-cycle arrest

DNA fragmentation is an important marker of cell death that is reflected as cell-cycle arrest. We used PI-flow cytometric analysis to confirm that the mode of MOLT-4 cell death induced by Pd@W.tea NPs is, in fact, through apoptosis. MOLT-4 cells treated with Pd@W.tea NPs significantly entered the G2/M apoptotic phase after 48 h (Figure 7 and Table 5). The sub-G0/G1 population of Pd@W.tea NPs-treated leukemia cells

Table 4 Flow cytometry analysis of FITC-conjugated Annexin-V/PI-stained MOLT-4 cells treated with Pd@W.tea NPs

Cell stage	Cells (%)					
	12 h		24 h		48 h	
	Control	Treated	Control	Treated	Control	Treated
Viable	95.2±0.38	81.59±0.65	92.13±0.55	79.20±0.15	89.9±0.25	75.9±0.13
Early apoptosis	2.77±0.15	8.55*±0.99	5.51±0.70	9.80*±0.30	6.11±0.44	11.5*±0.32
Late apoptosis/necrosis	1.65±0.35	9.86*±0.95	2.35±0.50	11.0*±0.81	3.99±0.20	12.6*±0.20

Notes: Values are expressed as mean ± SD of triplicate experiments. Data has been analyzed using one-way ANOVA with Tukey's test for post hoc comparison. *For each treatment period is significantly ($P>0.05$) different from control.
Abbreviations: ANOVA, analysis of variance; FITC, green fluorescence channel; MOLT-4, human leukemia cell line; NPs, nanoparticles; Pd, palladium; PI, propidium iodide; W.tea, white tea.

Table 5 Flow cytometry analysis of PI-stained MOLT-4 cells treated with Pd@W.tea NPs

Cell cycle phase	Cells (%)					
	12 h		24 h		4[illegible]	
	Control	Treated	Control	Treated	Cont[illegible]	Treated
G0/G1	55.34±0.06	52.01±0.45	57.20±0.29	58.11±0[illegible]	50.64±0.3[illegible]	48.24±0.68
G2/M	18.85±0.76	24.00*±0.41	14.2±0.26	19.2[illegible]±0.3[illegible]	16.3[illegible].22	26.06*±0.93
S	22.01±0.06	20.03±0.33	20.3±0.06	2[illegible]±0.12	2[illegible]±0.61	20.35±0.18
SubG0/G1	3.8±0.23	4.00±0.28	2.30±0.34	1.[illegible]±0.20	12.20±0.46	5.35*±0.56

Notes: Values are expressed as mean ± SD of triplicate experiments. Data has been analyzed using one-way AN[illegible]VA with Tukey's test for post hoc comparison. *For each treatment period is significantly ($P>0.05$) different from control.
Abbreviations: ANOVA, analysis of variance; MOLT-4, human leukemia cell line; NPs, n[illegible]oparticles; Pd, palladium; P[illegible]ropidium iodide; W.tea, white tea.

Table 6 Caspase activities in MOLT-4 cells treated with Pd@W.tea [illegible]Ps

Caspase	Cells (%)					
	12 h		[illegible]4 h		48 h	
	Control	Treated	[illegible]ntrol	Treated	Control	Treated
Caspase-3	0.064±0.030	0.173±0[illegible]*	0.0[illegible]±0.051	0.29±0.007*	0.11±0.0032	0.41±0.006*
Caspase-9	0.077±0.071	0.17[illegible]0.03*	0.08[illegible]0.001	0.25±0.005*	0.10±0.002	0.33±0.0035*

Notes: Values are expressed as mean ± SD of tri[illegible]icate [illegible]erim[illegible]ts. Data [illegible]en analyzed using one-way ANOVA with Tukey's test for post hoc comparison. *For each treatment period is significantly ($P>0.05$) differ[illegible] from co[illegible]
Abbreviations: ANOVA, analysis of varian[illegible] MOLT-4, huma[illegible]ukemia cell line; NPs, nanoparticles; Pd, palladium; W.tea, white tea.

decreased after 48 h[illegible]nus, the study show[illegible]s that Pd@W.tea NPs are antiprolif[illegible]tive t[illegible]ard leukemia cells by inducing DNA damage and G2/[illegible]arrest. T[illegible]s antiproliferative effect on cancer[illegible]ell lin[illegible] is typ[illegible]l[illegible] many NPs.[27,28,49] Untreated MOLT[illegible] cells sh[illegible]ved typical normal DNA content and cell-cycle distri[illegible]on of viable cells.

Caspases

Caspase-3 and -9 are the primary executioners of apoptosis. In MOLT-4 cells, treatment with Pd@W.tea NPs significantly increased the activities of caspase-3 and -9 (Table 6). Caspase pathway-mediated apoptosis seems to be the common mode of cell death instituted by NPs; for example, with zerumbone-loaded nanostructured lipid carriers and magnetic iron oxide NPs on Jurkat cells,[51] amine-modified polystyrene NPs on astrocytoma cells,[52] and nickel-zinc ferrite NPs on the HepG2, HT29, and MCF-7 cell lines.[53]

Conclusion

We developed an ecofriendly and efficient process for the synthesis of PdNPs with white tea extract as the medium. The mild reaction condition and easy workup procedure as well as the scope of pharmacological and medicinal applications are making our methodology a valuable contribution to the processes for green synthesis of NPs. The Pd@W.tea NPs with size range between 6 and 18 nm are ideal as a drug carrier system, because it avoids clearance by the monocyte–phagocytic system while easily penetrating cell membranes. The Pd@W.tea NPs has potent radical scavenging capability, antibacterial properties, and are antiproliferative to the human leukemia cells without adversely affecting the normal human fibroblast cells. The antiproliferative of Pd@W.tea NPs is via apoptosis and G2/M cell-cycle arrest. Thus, Pd@W.tea NPs has the potential to be developed into an antibacterial and anticancer agent.

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Disclosure

The authors report no conflicts of interest in this work.

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