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Research Article

The efficacy of protective effects of tannic acid, gallic acid, ellagic acid, and propyl gallate against hydrogen peroxide-induced oxidative stress and DNA damages in IMR-90 cells

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There is increasing evidence that reactive oxygen species (ROS) are intimately involved in the oxidative damage of tissues for a wide variety of pulmonary diseases. Thus, it is desirable to search for chemopreventive agents that can counteract ROS-mediated injury to the pulmonary tissues. Using a human lung fibroblast IMR-90 cells as the experimental model, we first demonstrated that nearly 90% of intracellular ROS could be removed when H_2O_2-treated cells (200 μM) simultaneously incubated with 10 μg/mL of tannic acid (TA), gallic acid (GA), ellagic acid (EA), and propyl gallate (PA). Using C_{11}-BODIPY$^{581/591}$ as a lipid peroxidation probe, we also attested that all these compounds examined (10 μg/mL) could alleviate H_2O_2-evoked lipid peroxidation phenomena. Next, we examined the protective effects of these compounds on the depletion of intracellular glutathione (iGSH) in H_2O_2-treated cells using CMF-DA probe. Interestingly, PA was demonstrated to be the only compound that could effectively protect the integrity of iGSH from being depleted by this system. Finally, the protective effects of these compounds against oxidative DNA damage were evaluated using 8-oxoguanine formation as a marker. Our data indicated that all four compounds suppressed the formation of 8-oxoguanine effectively. Taken together, our data suggested that TA, GA, EA, and PA can protect cells from oxidative stress.

Keywords: DNA damage / Hydrogen peroxide / Lipid peroxidation / Oxidative stress / Tannins

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1 Introduction

Tannins (also referred to as tannic acid (TA)) are a type of water-soluble polyphenol that are present in plant food, particularly in bananas, grapes, raisins, sorghum, spinach, red wine, persimmons, coffee, chocolate, and tea [1, 2]. Upon hydrolysis, gallotannins or ellaginnins can be obtained [3]. Further hydrolysis of gallotannins or ellagitannins by acid, bases, or certain enzymes, glucose and gallic acid (GA) or ellagic acid (EA) can be released from both compounds [3]. In addition, tannins are always used as a food additive and its

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Abbreviations: CMF-DA, chloromethyl-fluorescein-diacetate; **DCF,** dichlorofluorescein; **EA,** ellagic acid; **FBS,** fetal bovine serum; **GA,** gallic acid; **GSH,** glutathione; **iGSH,** intracellular glutathione; **8-OxodG,** 8-oxo-2′-deoxyguanosine; **PA,** propyl gallate; **ROS,** reactive oxygen species; **TA,** tannic acid

* Both the authors contributed equally to this work.

safe dosage ranges from 10 to 400 ppm, depending on the type of food to which it is added [3]. Propyl gallate (PA) is also a certified food additive and is often added as an antioxidant [3].

TA and its related compounds have also been shown to possess antimutagenicity. For example, we have demonstrated previously that tannins reduce the revertant numbers of *Salmonella* tester strains TA 98 in the presence of rat liver supernatant (S9 mix) when benzidine, 3,3′-4,4′-tetraaminobiphenyl, 4-aminobiphenyl, and *N*,*N*-*N*′,*N*′-tetramethyl benzidine were used as the mutagens [3]. Furthermore, numerous reports have demonstrated that tannins and its related components can inhibit the mutagenicity of certain mutagens such as nitropyrenes [4], bay region diol exposides of polycyclic aromatic hydrocarbons [5], aflatoxin B1 [6–8], *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) [9], and benzo[*a*]pyrene (B[*a*]P) [10]. In addition, TA gives a significant reduction of frequencies of sister-chromatid exchange (SCEs) in Chinese hamster ovary cells induced by UV-light and mitomycin C (MMC) [11]. MMC-induced chromosome aberrations in mouse bone marrow cells were also reduced by the tannin components extracted from green tea and black tea [11]. Recently, we have shown that TA and its related compounds can also inhibit the oxidative DNA damage in hydrogen peroxide (H_2O_2)-treated human lymphocytes [12].

The impact of reactive oxygen species (ROS) on the lung may be especially important because this organ is exposed to greater oxygen tension than other tissues over a much larger surface area. The lung is a major target of air pollutants, several of which cause oxidative stress. As observed for other tissues, exposure of lung to elevated oxygen results in increased intracellular generation of superoxide (and hence of H_2O_2). H_2O_2 can also be generated by oxidases in the lung including uricases, monoamine oxidases, and xanthine oxidase. Another source of ROS within lung is the activation of phagocytes. ROS has been implicated to be the causative factor involved in many human degenerated diseases including pulmonary disorders and antioxidants have been found to have some preventive and therapeutic effects on these diseases [13, 14]. For these reasons, it is of interest to search for chemopreventive agents existing in the natural products that can counteract the deleterious effects of ROS. In this study, we set out to evaluate whether or not TA along with its related compounds could offer protection against H_2O_2-evoked oxidative damage to a human lung fibroblast IMR-90 cell line.

2 Materials and methods

2.1 Cell culture, chemical procurement, and treatments

TA (purity 90%), GA (purity 97%), (EA (purity 95%), and PA (purity >98%) were all purchased from Sigma Chemical (St. Louis, MO, USA). Unless stated otherwise, all chemicals used were of analytical quality, and doubly distilled water was used throughout. The basal medium for IMR-90 cell culture was DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin G, 100 μg/mL streptomycin, and 250 μg/mL amphotericin B. All the compounds tested (10 μg/mL) were fresh dissolved in FBS-free DMEM medium. The times of treatment with H_2O_2 were 1 or 2 h for ROS generation studies and 3 h for lipid peroxidation, iGSH, and 8-oxoguanine. IMR-90 cells were pretreated with all the compounds tested for 1 h prior to the addition of H_2O_2.

2.2 Measurement of intracellular ROS by flow cytometry

Production of intracellular ROS was detected by flow cytometry using dichlorofluorescein-diacetate (DCFH-DA) [15]. The IMR-90 cells were cultured in 60-mm tissue-culture dishes. The culture medium was replaced with new medium when the cells were 80% confluent. Following drugs incubation, cells were treated with 10 μM DCFH-DA for 30 min in the dark, washed once with phosphate buffered saline (PBS), detached by trypsinization, collected by centrifugation, and suspended in PBS containing 5 μg/mL of propidium iodide (PI) for 10 min prior to flow cytometry. PI treatment differentiates between integrated and nonintegrated cell membranes, since the latter permit the entrance of this dye into the cells, and the former do not. The intracellular ROS, as indicated by the fluorescence of dichlorofluorescein (DCF), was measured with a Becton-Dickinson FACS-Calibur flow cytometry.

2.3 Measurement of lipid peroxidation by flow cytometry

C_{11}-BODIPY$^{581/591}$, a lipophilic fluorescent probe, was measured to estimate the extent of lipid peroxidation in IMR-90 cells [16]. Peroxidation of C_{11}-BODIPY$^{581/591}$ was accompanied by a shift in fluorescence from red to green, and the relative value of nonoxidized probe was decided as the ratio of red: (red + green) fluorescence as detected by flow cytometry [17]. Briefly, IMR-90 cells were trypsinized, washed with PBS, and resuspended with FBS-free DMEM medium to a concentration of 8×10^5 cells/mL. The resuspended cells were loaded with 10 μM C_{11}-BODIPY$^{581/591}$ in DMSO (final concentration 0.1%) for 30 min at 37°C. Unbound probe was removed by centrifugation, and the probe binding cells were resuspended to the fresh FBS-free DMEM medium. After loading with C_{11}-BODIPY$^{581/591}$, IMR-90 cells were incubated with TA, EA, GA, and PA for 60 min. Samples were then incubated with 100 μM H_2O_2. Red and green fluorescence were determined at 15-min intervals for 120 min by Becton-Dickinson FACS-Calibur flow cytometry. Between times, cells were maintained at 37°C in 5% CO_2.

2.4 Measurement of iGSH content by flow cytometry

The level of iGSH *per* cell was determined by flow cytometry after staining with chloromethyl-fluorescein-diacetate (CMF-DA) [18]. CMF-DA, containing a mild thiol reactive chloromethyl reactive group, is colorless and nonfluorescent. This probe is primarily conjugated to the abundant tripeptide glutathione by glutathione S-transferase. Once inside the cell, cytosolic esterases cleave off their acetates and then the chloromethyl group reacts with intracellular thiols, transforming the probe into a cell-fluorescent dye-thioether adduct. In our experiments, CMF-DA was prepared as a 25 mM solution in DMSO and stored at –20°C. It was added at 25 μM in cell suspensions adjusted at $1–2 \times 10^6$ cells *per* mL. After 30 min of incubation at 37°C, cells were washed twice in PBS, resuspended at a concentration of 10^6 cells/mL in PBS, and analyzed on a Becton-Dickinson FACS-Calibur flow cytometry. The fluorescent dye-thioether adduct was excited at 488 nm and the fluorescence was collected with a 525 nm band pass filter. Analyses were performed on 10 000 cells and fluorescence intensities were measured on a logarithmic scale of fluorescence of four decades of log. The data were collected, stored, and analyzed with the CellQuest software.

2.5 Measurement of intracellular 8-oxoguanine by flow cytometry

The levels of intracellular 8-oxoguanine were performed using the Biotrin OxyDNA Assay kits [19]. Both floating and adherent cells were harvested and fixed using 1% paraformaldehyde for 15 min on ice. After fixation, cells were permeated with 70% ethanol for 30 min at –20°C, followed by one wash with PBS and one wash with wash solution (Tris buffered saline/Tween-20). Cells ($1–2 \times 10^6$) were added blocking solution and incubated for 1 h at 37°C. Cells were washed twice with wash solution, incubated with binding protein–FITC conjugate for 1 h in the dark at room temperature. Cells were washed twice with wash solution, once with PBS, suspended in PBS and read in a Becton-Dickinson FACS-Calibur flow cytometry.

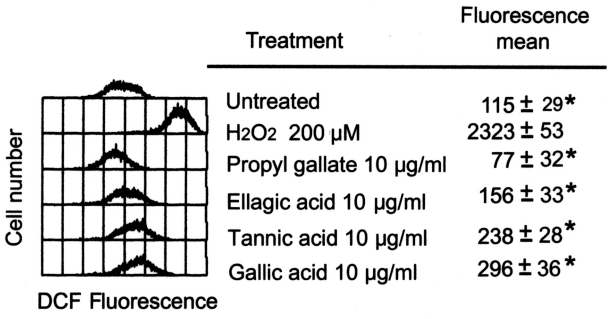

Figure 1. The ROS-scavenging activities of all the four compounds tested in IMR-90 cells challenged with 200 μM of H_2O_2 as determined by the drop of mean DCF fluorescence using flow cytometry. The data of fluorescence mean were derived from at least three independent experiments. The values shown are mean ± standard errors. Significant differences from the H_2O_2-treated group are $p < 0.05$ (*).

2.6 Statistical analysis

Data were presented as means ± standard errors from at least three independent experiments and analyzed using Student's *t*-test. A *p* value of less than 0.05 was considered as statistically significant.

3 Results and discussion

3.1 Comparison of the inhibitory effect of TA, EA, GA, and PA on intracellular ROS level in H_2O_2-treated cells

A common denominator in the pathogenesis of most chronic disease is the involvement of oxidative stress, related to the production by all aerobic organisms of reactive oxygen and nitrogen species, including free radicals. In addition to having a role in intra- and extracellular signaling, these reactive molecular species may initiate damaging biochemical reactions [20–23]. In response to such damages, a complex antioxidant defense has developed, and natural antioxidants comprise an important role in this defense. Therefore, it is desirable to search for potential antioxidants existing in natural foodstuffs and herbal preparations for therapeutic applications.

Notwithstanding its various desirable applications, TA has been reported to cause breakage of calf thymus DNA and supercoiled DNA in the presence of Cu(II) [24]. The contributing factor for DNA cleavages was attributable to the free radicals produced from the Fenton pathway [25]. It was proved that structure features of TA, such as its digalloyl moiety and free hydroxyl groups, are important for DNA degradation in the presence of Cu(II) [26, 27]. In contrast, we showed that TA, EA, GA, and PA can enhance the resistance of human lymphocytes toward single DNA strand breaks induced by food mutagens 3-amino-1-methyl-5H-pyrido(4,3-b)indole (Try-P-2) and 2-amino-1-methyl-6-phenylimadazo(4,5-b)pyridine (PhIP) or H_2O_2 *in vitro* [12]. In this study, H_2O_2, a cell membrane permeable and a precursor of various free radicals, was chosen as an oxidant model. Using DCF fluorescence as an indicator [15], we found that the contents of ROS in IMR-90 cells were elevated significantly when these cells were challenged with 200 M H_2O_2 (Fig. 1), suggesting that H_2O_2 was permeable to the cell membrane. As to the question to what extent H_2O_2 is subsequently converted to other species of intracellular free radicals is awaited for further clarification. Next, the ROS-scavenging ability of TA, EA, GA, and PA was determined by measuring the drop of DCF fluorescence intensity in IMR-90 cells. Our results demonstrated nearly

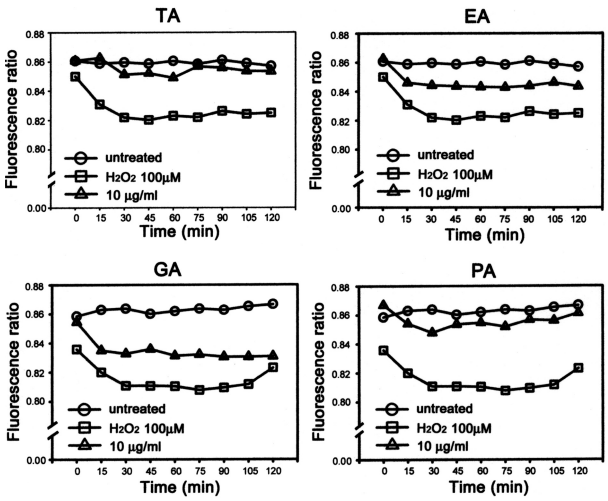

Figure 2. Effect of all the four compounds tested on lipid peroxidation of IMR-90 cells with 10 μM C_{11}-BODIPY$^{581/591}$ in the presence of 100 μM H_2O_2. The suspended cells were treated with 10 μM C_{11}-BODIPY$^{581/591}$ for 30 min at 37°C. After washing with PBS, IMR-90 cells were incubated with 10 μg/mL of each of the four compounds for 60 min. The cells were then incubated with 100 μM H_2O_2. Red and green fluorescence were determined at 15-min intervals for 120 min by flow cytometry analysis. These experiments were performed at least three times and a representative experiment is presented.

90% of intracellular ROS could be removed when H_2O_2-treated cells were simultaneously incubated with 10 μg/mL of each of the compound tested. Among all four compounds tested, PA was found to be the most efficient ROS scavenger. This is probably attributable to the fact that PA is a nonpolar compound and is capable of penetrating across the cell membrane to interact with intracellular ROS.

3.2 Effect of TA, EA, GA, and PA on H_2O_2-induced lipid peroxidation

To further assess if H_2O_2 can induce lipid peroxidation, we use a fluorescent fatty acid probe, C_{11}-BODIPY$^{581/591}$ [16]. This probe can change from red to green fluorescence upon oxidation, and the ratio of red to green fluorescence has been used as a measurement of lipid peroxidation in living cells [17]. As shown in Fig. 2, when IMR-90 cells were challenged with 100 mM of H_2O_2, the increased level of the oxidation of the probe was observed as reflected by the drop of the ratios of red-to-green fluorescence during the 2-h incubation time, as compared to the untreated group. Interestingly, when these cells were pretreated with all four compounds with a dose of 10 μg/mL, the oxidation of the probe could be effectively prevented. These results clearly demonstrated that all four compounds tested can act as the protective agents for lipid peroxidation.

3.3 Effect of TA, EA, GA, and PA on H_2O_2-induced iGSH depletion

Glutathione (GSH) is the principal intracellular nonprotein thiol [28]. It is present in concentrations up to 10 mM in many cells and provides a primary defense against oxidation stress by its ability to scavenge free radicals or participate in the reduction of H_2O_2 catalyzed by GSH peroxidase, a selenium-dependent enzyme [29–32]. For these reasons, we investigated whether exogenously administered H_2O_2 could induce depletion of intracellular GSH contents in IMR-90 cells and whether all four compounds tested could alleviate this depletion process. Our results revealed that when IMR-90 cells were challenged with 200 μM of H_2O_2 for an extended period of 3-h, nearly 80% of GSH-negative cells could be demonstrated (Fig. 3). However, when cells were pretreated with 10 μg/mL of each of the four compounds, followed by challenging with 200 μM H_2O_2, 14, 59, 65, and 75% of GSH-negative cells were observed in cells pretreated with PA, TA, EA, and GA, respectively. Comparatively, PA can offer the maximum protection of GSH against H_2O_2-evoked oxidation reaction. Again, the nonpolar nature of PA may be responsible for its ability to protect the oxidation of iGSH to a greater extent as compared to the polarized GA.

3.4 Effect of TA, EA, GA, and PA on H_2O_2-induced oxidative DNA damage

Oxidative injury to macromolecules, such as DNA has been shown to be associated with the occurrence of carcinogene-

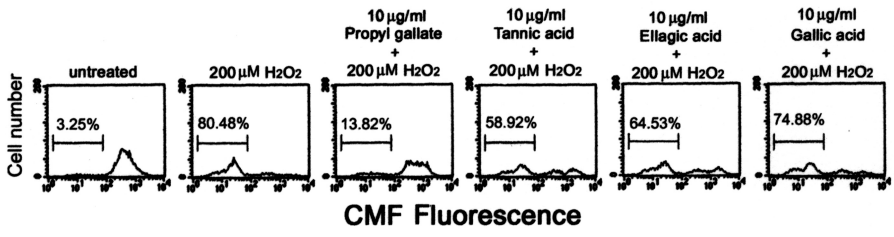

Figure 3. Effect of each of the four compounds on iGSH content in H_2O_2-treated IMR-90 cells. IMR-90 cells were pretreated with 10 μg/mL for 1 h, exposed to 200 μM H_2O_2 for 3 h followed by the addition of 25 μM CMF-DA for the further 30 min. The fluorescence mean intensity was measured by flow cytometry analysis. Data represent the percentage of cell numbers displaying iGSH negative cells. These experiments were performed at least three times and a representative experiment is presented.

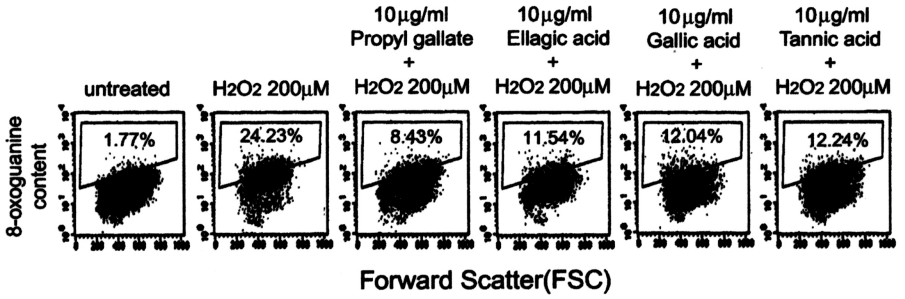

Figure 4. Effect of each of the four compounds on oxidative DNA damage in H_2O_2-treated IMR-90 cells. IMR-90 cells were pretreated with 10 μg/mL of each of the four compounds for 1 h, exposed to 200 μM H_2O_2 for 3 h, and proceeded to 8-oxoguanine assay. Data represent the percentage of cells in the upper box of 8-oxoguanine positive cells. These experiments were performed at least three times and a representative experiment is presented.

sis, teratogenesis, and cell death [33–36]. To investigate whether H_2O_2-treatment could also induce oxidative damage to DNA of IMR-90 cells, we used 8-oxoguanine as a marker. As shown in Fig. 4, in the absence of H_2O_2, 8-oxoguanine-containing IMR-90 cells were shown to be minimal (<1.8%). However, when these cells were challenged with 200 μM H_2O_2, the percentage of 8-oxoguanine-positive cells rose to 24%. Conversely, pretreatment of these cells with 10 μM of each of the four compounds prior to challenging with 200 μM H_2O_2, the formation of 8-oxoguanine-positive cells drastically decreased to 8.4, 11.5, 12, and 12% in PA, EA, GA, and TA pretreated cells, respectively. This indicated that TA, EA, GA, and PA could offer protection against H_2O_2-evoked oxidative damage to DNA with a rank of efficiency being PA > EA > GA = TA, respectively.

TA and other polyphenols have been shown to process antimutagenic and anticarcinogenic activities, which might be related to antioxidant properties of these compounds [37]. It was reported that TA inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions [38]. Previously, we also found that inhibition of Fenton reaction by the four compounds tested is, at least in part, due to the complexation of Fe^{2+} ions necessary for hydroxyl radical formation [39]. TA was shown to inhibit the cleavage of plasmid DNA mediated with the action of hydroxyl radicals and single oxygen [26]. EA can also be acted as the scavenger of oxygen species produced from H_2O_2-treated Chinese hamster ovary cells [40]. In our previous study, we also suggested that TA and its related compound modulate H_2O_2-mediated DNA strand breaks in human lymphocytes by scavenging free radicals as well as ions such as Fe^{2+} or Cu^{1+} [12]. Also, EA offers the maximal (>80%) inhibition of 8-oxo-2′-deoxyguanosine (8-oxodG) formation in Cu^{2+}-mediated Fenton reaction as compared to the abilities of other known antioxidants in terms of inhibition of 8-oxodG formation [41]. In this study, the ability of the four compounds in inhibiting 8-oxodG formation in H_2O_2-treated IMR-90 cells was first compared. As for inhibition of 8-oxodG by PA, this observation was not presented elsewhere. Collectively, all these four compounds tested can be acted as the antioxidants against oxidative DNA damage.

3.5 Possible mechanisms underlying the protective effects of TA, EA, GA, and PA on H_2O_2-induced oxidative stress in IMR-90 cells

It has been documented that H_2O_2 is permeable to the cell membrane and thus can be inferred that a more ROS will possibly be produced, such as hydroxyl radicals ($^{\bullet}OH$). The production of $^{\bullet}OH$ radicals can be generated rapidly *via* either Fenton reaction [42] or Harber–Weiss reaction [43]. Hydroxyl radicals, once generated, will react with the molecules in their immediate vicinity. Consequently, macromolecules in IMR-90 cells, such as lipids in the membrane as well as intracellular DNA can be randomly attacked by $^{\bullet}OH$. The plethoric elevation of 8-oxoguanine concentrations in IMR-90 cells after H_2O_2 challenge in our experiments is a direct evidence of $^{\bullet}OH$ formation.

Previously, we showed that GA, EA, and PA were demonstrated to be effective in scavenging $^{\bullet}OH$ [39]. In this study, the pretreatment of these four compounds prior to H_2O_2 challenge in IMR-90 cells substantially reduced the availability of $^{\bullet}OH$ for hydroxylating guanine bases on the DNA molecule and lessened lipid peroxidation. This is probably due to the reaction between $^{\bullet}OH$ and the four compounds tested through a hydrogen abstraction mechanism or *via* complexing ferrous ions from Fenton reaction as reported elsewhere [38].

iGSH have been shown to be effective in scavenging $O_2^{\bullet-}$ and $^{\bullet}OH$ [28]. In our experiments, we found that H_2O_2 challenge could also elicit depletion of iGSH in IMR-90 as evident by a drastic increase in GSH-negative cell population. However, pretreatment of IMR-90 cells with these compounds could render these cells to harvest lesser quantity of GSH-negative fraction. Obviously, all these compounds had the capability of active participation in the scavenging reactions involving lipid peroxidation and oxidative DNA damage at the expense of GSH-depleting pathway.

4 Concluding remarks

Oxygen radical research has led to a new paradigm of human health, with a shift toward a greater emphasis on disease prevention. If oxygen-derived free radicals are involved in the etiology of degenerative diseases including pulmonary disorders, then antioxidants should be effective in preventing their occurrence. In this study, we have presented experimental evidence that all four compounds tested could offer protective capability against H_2O_2-mediated oxidative damage *via* scavenging intracellular ROS, inhibiting lipid peroxidation, GSH depletion, and oxidative modification of DNA. Along the same vein, PA, a propylated GA, was found to be a superb protective agent against H_2O_2-evoked oxidative damage of IMR-90 cells. Since PA has already advocated as a dietary addition, another application of using this agent as a chemopreventive agent against oxidative stress-derived diseases would likely to be beneficial to the human health. This possibility warrants further investigation.

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