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Original Research

Cell-based evaluation of a novel *Dictyophora indusiata* polysaccharide against oxidative-induced erythrocyte hemolysis

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Abstract: The protective effectof a polysaccharide from *Dictyophora indusiata*(DP1)against oxidative hemolysis was comprehensively evaluated. The 2, 2-azobis (2-amidino-propane) dihydrochloride (AAPH)-induced erythrocyte hemolysis assay showed that DP1 exhibited excellent anti-hemolytic activity(87.4% hemolysis suppression ratio at 20 nmol/mL). Also, the formation of conjugated diene induced by cupric chloride ($CuCl_2$) in plasma was significantly inhibited by DP1. Besides, DP1 could effectively inhibit AAPH-induced overproduction of reactive oxygen species (81.5 % inhibition at 20 nmol/mL) and alleviated the enhancement of intracellular antioxidant enzymes including superoxide dismutase(SOD), glutathione peroxidase (GPX) andcatalase (CAT) activities. Also, the malondialdehyde (MDA) formation caused by oxidative stress was suppressed by 57.0% at DP1 concentration of 20 nmol/mL. Taken together, the possible intracellular antioxidant detoxifying mechanism of DP1 was probably via preserving the activities of the antioxidant enzymes (SOD, GPx and CAT) as well as inhibiting lipid peroxidation, and thus alleviated erythrocytes oxidation and plasma oxidation.

Key words: Polysaccharide, *Dictyophora indusiata*, oxidative hemolysis, reactive oxygen species, antioxidant enzymes.

Introduction

Dictyophora indusiata is a precious, edible and medicinal fungi belongs to the *Phallaceae Corda* family. It is referred to as the Veiled Lady Mushroom due to its beautiful appearance. The basidioma of *Dictyophora indusiata* forms only after a few hours of growing from the initial “egg stage” (1). Profuse bioactive substances isolated from *Dictyophora indusiata* showed a variety of biological activities, including neuroprotective ability, anti-tyrosinase activity and anti-inflammatory activity (2-4). Moreover, it was well acknowledged that the antioxidant property of methanolic extracts from *Dictyophora indusiata* was better than that from other mushrooms (5,6).

In recent years, the polysaccharides from *Dictyophora indusiata* have attracted much attention due to their excellent beneficial potency and relatively low toxicity. Numerous studies had been focused on the antioxidant activity based on oxygen radical absorbance capacity (ORAC) assay, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay, hydroxyl radical scavenging activity assay and superoxide radical scavenging activity assay (7-9). However, these chemical based methods are carried out *in vitro* and might not reflect the authentic physiological environment such as pH, temperature and metabolism of human body (10). Besides, the partitioning of compounds between the water and lipid phases and the influences of interfacial behavior are also neglected. Also, the multiple mechanisms through which antioxidant functions cannot be revealed. Therefore, bio-relevant methods for antioxidant activity evaluation taking metabolism and uptake into account are urgently needed. Considering this, the cell-based assays including AAPH-induced hemolysis assay and $CuCl_2$-induced plasma oxidation assay, were applied to investigate the antioxidant ability of DP1 in the present study.

Reactive oxygen species (ROS) is one of the most important causes of the oxidation *in vivo* (11,12). In physiological conditions, ROS act as a double-edged sword in aerobic cells. Low levels of ROS can serve as key signal molecules in proliferation and intracellular messaging (13,14). However, when the ROS generation overwhelms the cellular intrinsic antioxidant defense system, oxidative stress occurs (15,16). Overproduction of ROS can cause the development of cardiovascular diseases, neurodegenerative diseases and ophthalmologic problems, as well as impair fetal vascular development (17-23). ROS also play a major role in initiation and progression of tumor (12,24-26). It is universally acknowledged that the ROS-induced oxidative damage of DNA is an essential mutagenic and carcinogenic factor (27-29). Besides, ROS are also involved in several crucial signal transduction pathways in tumor metastasis and metastasis-related genes such as integrins and matrix metallo proteinases (MMPs) (30-33). Thus, the functions of intracellular antioxidant systems are mainly to scavenging the excessive ROS to alleviate the oxidative damage (6,34-36). It has been reported that intrinsic enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and

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catalase (CAT) play pivotal role on ROS inhibition by scavenging the superoxide and peroxide to prevent the generation of ROS (37-40).

Recently, a novel polysaccharide DP1, with an average molecular weight of 1,132 KDa, was purified from the fruiting body of *Dictyophora indusiata* in our lab. The chemical composition of DP1 was proved to be (1→3)-linked-α-L-Man, (1→2, 6)-linked α-D-Glc, (1→6)-linked β-D-Glc, (1→6)-linked β-D-Gal and (1→6)-linked β-D-Man (41).DP1 has been proved to have good immunomodulatory activity by enhancing the macrophage NO, TNF-α and IL-6 secretion in murine RAW264.7 cells. In the present study, we aimed to explore the protective effects of DP1 against free radical induced oxidative damages by AAPH-induced erythrocytes oxidative hemolysis assay and copper-induced plasma oxidation assay. Also, the contribution of DP1 on the intracellular antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) activities was also evaluated (42,43). The underlying mechanism of the intracellular antioxidant detoxifying activity was also discussed and we finally proved that DP1 could be used as an ideal functional food for the treatment of oxidative stress-related diseases.

Materials and Methods

Materials

Dictyophora indusiata was collected from Zhijin, Guizhou, China. The 2,2'-azobis (2-amidinopropane) (AAPH) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma Company (St. Louis, MO, USA). Copper chloride ($CuCl_2$) was acquired from Adamas Reagent Co., Ltd. (Basel, Switzerland). L-glutamine was purchased from Feibo Biotechnology Co., Ltd. (Guangzhou, China). Malondialdehyde (MDA) kit and bicinchoninic acid (BCA) kit were obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China). Quercetin standards were purchased from Sigma Company (St. Louis, MO, USA). The 2',7'-dichlorofluorescein diacetate (DCFH-DA), penicillin-streptomycin solution (100X) and Cellular Glutathione Peroxidase Kit, Total Superoxide Dismutase Assay Kit and Catalase Assay Kit were obtained from Nanjing Jiancheng Institute of Biotechnology (Nanjing, China).Phosphate-buffered saline (PBS, pH 7.4) were obtained from Gibco Life Technologies (Grand Island, NY, USA).

Preparation of DP1

DP1 was extracted and purified as described in our previous publications (41). The DP1 was separated from the fruiting body of *Dictyophora indusiata* using "water extraction and alcohol precipitation" method. The crude polysaccharide was deproteinated by Sevag method and further purified by DEAE-52 anion-exchange chromatography column and Sephadex G-200 column."

AAPH-induced hemolysis assay

The protective effect of DP1 against AAPH-induced hemolysis was measured according to the method described by Cheung et al (44) with some modifications. Blood sample was collected from healthy consenting male adults at ages 20-30. Erythrocytes were separated from the plasma and buffy coat by centrifugation at 1200 g for 10 min at 4°C. The obtain erythrocytes were washed three times using 10 mL of 10 mM PBS and resuspended to the level of 20 %. An aliquot of 100 μL erythrocyte suspension was mingled with 100 μL DP1 at different concentrations (0.66, 1.3, 2.6, 5.2, 10 and 20 nmol/mL, respectively). After incubation with gently stirring at 37 °C for 20 min, 200 μL of AAPH (200 mM) was added as an alkyl radical initiator. The reaction mixture was placed in a stirred-incubation for 2 h at 37 °C. Finally, the mixture was diluted with 8 mL PBS and centrifuged at 1200 g for 10 min at 4 °C. The absorbance of the supernatant was measured at 540 nm by a spectrophotometer (recorded as absorbance A).

To achieve complete hemolysis, 8 mL of distilled water was added to the reaction mixture and the absorbance of the supernatant harvested after centrifugation was measured at 540 nm (recorded as absorbance B). The percentage hemolysis inhibition was calculated using the formula:

% hemolysis inhibition=(1-A/B) × 100%

where A refers to the absorbance A and B refers to absorbance B as mentioned above.

Plasma oxidation assay

The inhibitive effect of DP1 on copper-induced plasma oxidation assay was performed as described before with some modification (45). Blood sample was collected from healthy consenting male adults at ages 20-30. Plasma were separated from the whole blood by centrifugation at 1200 g for 10 min and diluted 40-fold with PBS, and then stored at 4 °C for analysis. After that, 50 μL of plasma was blended with the same volume of DP1 at different concentrations (0.11, 0.22 and 0.44 nmol/mL, respectively) for 15 min at 37 °C. PBS was used as a control. Then, 100 μL of $CuCl_2$ (200 μM) was added to the mixture to trigger lipid oxidation. The formation of conjugated diene was measured using Fluoroskan Ascent Microplate Fluorometer (Thermo Electron Corporation, Vantaa, Finland) at 245 nm for 24 h at 37 °C.

Scanning electron microscope (SEM) of the treated blood samples

A drop of blood sample was spread onto a polished microscope glass slide to form a single layer of the blood film. The film was then fixed with 2.5% glutaraldehyde and the morphology of the blood sample was observed under a scanning electron microscope (Hitachi TM3000, Hitachi Ltd., Tokyo, Japan).

Intracellular reactive oxygen species determination

The fluorometric assay was used to measure the ROS production (46). Cells were collected and incubated with a fluorecence probe 2', 7'-dichlorofluorescein diacetate (DCFH-DA) at 37 °C for 30 min. Then the cells were washed with PBS for three times and preincubated with DP1 prior to AAPH (200 mM) treatment for 2 h. The fluorescence value of oxidized probe was measured by Fluoroskan Ascent Microplate Fluorometer with excitation and emission wavelengths set at 488 and 525 nm, respectively.

Determination of lipid peroxidation and the enzymatic activities of GPx, SOD and CAT

Erythrocytes were washed three times with PBS after treatment with DP1 samples and AAPH, and then lysed in 600 μL of ultrapure water. The protein concentration of erythrocytes was measured by a BCA protein assay kit. Themalondialdehyde (MDA) formation was determined by a Micromount MDA kit based on TBA (thiobarbituric acid) reaction. The activities of GPx, SOD and CAT were analyzed using a Cellular Glutathione Peroxidase Kit, Total Superoxide Dismutase Assay Kit and Catalase Assay Kit, respectively.

Statistical Analysis

All experiments were carried out in triplicate and results were reported as mean (standard deviation. Statistical analysis was performed using SPSS 19.0 software (IBM Corporation, New York, USA). The difference between three ormore groupswere analyzed by one-way ANOVA multiple comparisons. A P value of <0.05 was considered to indicate statistical significance. If there is statistical significance between two groups, different letters (a, b, c, etc.) were used to label these groups in the figures. If no, the same letter was used. If one group is not significantly different with another two groups, but these two groups are significantly different and labeled as for example “a” and “b” respectively, then “ab” was used to label this group in figures.

Results and Discussion

Attenuation of erythrocyte hemolysis and plasma oxidation by DP1

The antioxidant capacity of DP1 was investigated by erythrocyte hemolysis assay and plasma oxidation assay. Erythrocytes are highly susceptible to peroxidation because their membranes are abundant in polyunsaturated fatty acid. The endogenous antioxidants in erythrocytes such as glutathione, tocopherol, ascorbate and enzymes like GPx, SOD, and CAT can protect erythrocytes from oxidative damage by quenching radicals (47). When erythrocytes was incubated with AAPH, the production of peroxyl radicals was so high that has beyond the capacity of intracellular antioxidants and the hemolysis was initiated intensively. An oxidative stress had developed, leading to damage on erythrocyte membrane. As shown in Fig.1, the hemolysis was significantly inhibited with the increment of DP1 concentrations. The hemolysis suppression ratio reached 87.4% at 20 nmol/mL of DP1. Moreover, when the concentration of DP1 was20 nmol/mL without AAPH existing, hemolysis was maintained at background level which nearly equaled to the control sample. This indicated that DP1 have a strong protective effect against hemolysis without antiproliferative activity.

To confirm the protective effect of DP1 on AAPH-induced damage on the erythrocytes, the morphologic changes of erythrocytes were observed by SEM. As shown in Fig.2A, the typical biconcave shape with smooth and rounded surface was observed for normal cell. However, the morphology of the cell changed dramatically after cultivating with AAPH for 2 h. The membrane of erythrocyte was destroyed and became rough and prickly. On the contrary, the pretreatment of

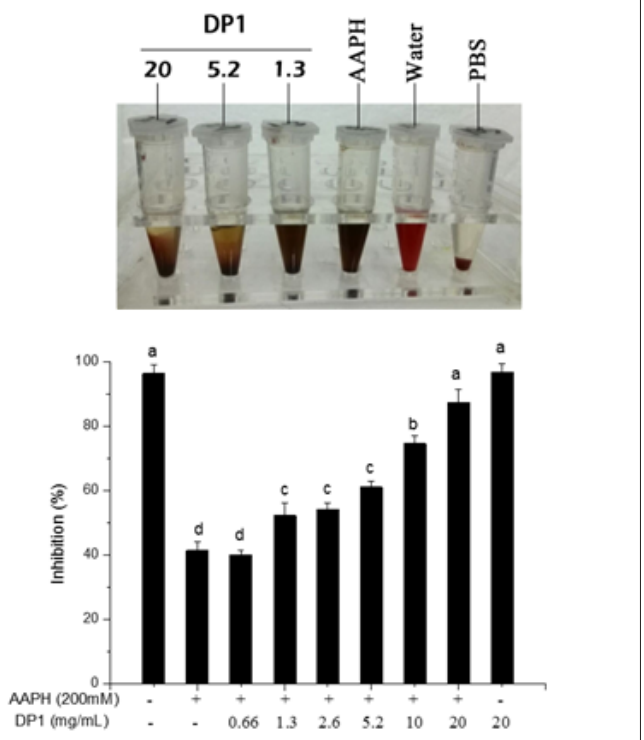

Figure 1. Percent inhibition of erythrocytes hemolysis by DP1. Erythrocytes were pretreated with DP1 (0.66-20 nmol/mL) for 20 min and then co-incubated with 200 μL AAPH for 2 h at 37 °C. The positive control was treated with 200 μL AAPH only.

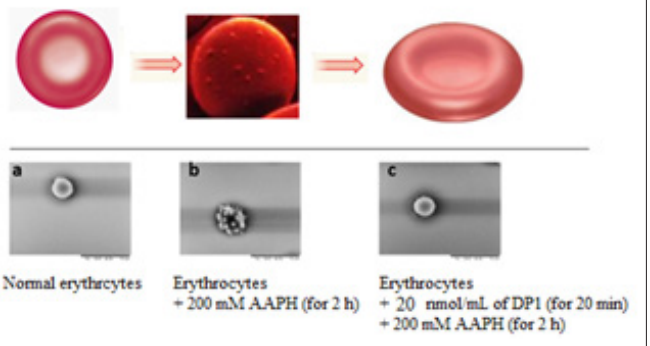

Figure 2. SEM micrographs of erythrocyte sample. (a) Normal erythrocyte. (b) Erythrocytes were treated with 200 mM AAPH for 2 h only. (c)Erythrocytes were pretreated with 20 nmol/mL of DP1 for 20 min and subsequently cultured with 200 mM AAPH for 2 h.

20 nmol/mL DP1 on erythrocytes remarkably moderated the damage of AAPH and the morphology of erythrocyte was quite similar to that of the normal one. Besides, the spines were inconspicuous compared to the cell morphology in Fig.2B, which indicated obvious protective effects of DP1 against the AAPH-induced oxidative damage on erythrocytes.

The knowledge about the antioxidant capacity of DP1 on the inhibition of plasma oxidation has important pathological significance since the oxidative hypothesis of atherosclerosis revealed that the oxidation of low density of lipoprotein (LDL), the most known oxidizable lipoprotein in plasma, plays an essential role in the early development of atherosclerosis (48). Intrinsic factors that have been demonstrated to influence the inclination of LDL to undergo lipid peroxidation are mainly metal ions, matrix components, and high-density lipoprotein (HDL). In the present work, copper was used to induce the oxidation of lipoprotein. The treat-

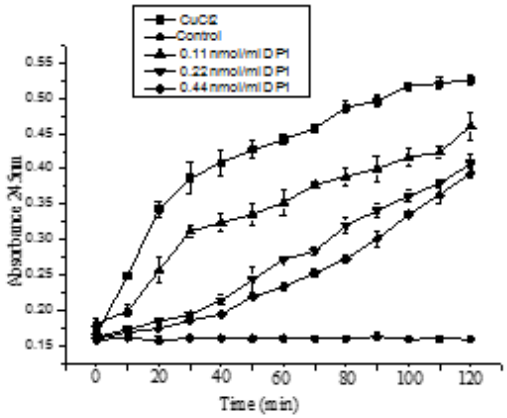

Figure 3. Time dependence of diene accumulation during the copper-induced plasma oxidation. Plasma was pretreated with DP1 (0.11-0.44 nmol/mL) for 15 min, and then co-incubated with 200 μM $CuCl_2$.The positive control group was treated with 200 μM $CuCl_2$ only.

ment of $CuCl_2$ led to conjugated diene accumulation and plasma lipoprotein oxidation. The susceptibility of lipoprotein to lipid peroxidation in the presence of 200 mM $CuCl_2$ was continuously monitored by measuring the increase of the absorbance. Fig.3 shows the kinetics of conjugated diene formation of plasma exposed to $CuCl_2$. As shown in this figure, the absorbance increased drastically with time increase after the addition of $CuCl_2$, which indicated the accelerated generation of conjugated diene. However, it was found that the absorbance value conspicuously dropped after the treatment of DP1. For instance, the absorbance value of the sample treated only with $CuCl_2$ was 0.44 ± 0.01 after 60 min treatment, while those of the samples incubated with different concentrations of DP1 (0.11, 0.22 and 0.44 nmol/mL) decreased by 20.5%, 38.6%, 47.7% respectively. It revealed the protective effect of DP1 on plasma oxidation was remarkable and dose dependent. Besides, within the first 40 min treatment, the absorbance value decreased remarkably with the increase of DP1 concentration, manifesting that the intensity of the plasma oxidation was greatly attenuated by DP1.

Inhibition of AAPH-induced intracellular reactive oxygen species by DP1

Previous study has revealed that ROS generation was closely related to hemolysis. In the present work, a fluorescein-labeled dye, DCFH-DA, was used to determine the cellular ROS. The level of ROS generated by AAPH and the extent of inhibition by DP1 could reflect the antioxidant capacity of DP1 in attenuating the erythrocyte hemolysis. As shown in Fig.4, the ROS level of erythrocytes treated with AAPH (3733.3 ± 60.9) was remarkably increased compared to that of the control group (100.0 ± 3.9), indicating the successful construction of oxidative damage cell model. As a comparison, erythrocytes pretreated with DP1 for 30 min showed significantly decrease in ROS level. The DCF fluorescence of the group treated with DP1 at 20 nmol/mL was 81.5% lower than that of the AAPH group. Furthermore, the DCF fluorescence decreased with the increase of the concentration of DP1, indicating a dose-dependent manner for the inhibition of DP1 on AAPH-induced ROS formation. From the above analysis, it is reasonable to speculate that the antioxidant protective effect of DP1 on AAPH-induced erythrocyte hemolysis was mainly through the inhibition of cellular ROS generation. It was probably due to that the hydrogen atoms can separate from hydrocarbon chain of DP1 and react with hydroxyl radicals to form water molecules.

DP1 prevents AAPH-induced changes in level of lipid peroxidation and enzyme activities of GPx, SOD and CAT

MDA is a low-molecular-weight end product originated from the decomposition of some primary and secondary lipid peroxidation products. It can destroy the structure and the function of cell membrane, damage cellular metabolism and even lead to antiproliferative activity. Researches also revealed that there is a positive correlation between the content of MDA and the extent of the lipid peroxidation. As shown in Fig.5A, erythrocytes treated with AAPH notably caused lipid peroxidation since the MDA concentration (1.14 ± 0.10 nmol/mg protein) increased dramatically as compared with that of the control group (0.39 ± 0.01 nmol/mg protein). However, DP1 treatment significantly inhibited MDA formation. The MDA concentration decreased by 7.9% (from 1.14 ± 0.10 nmol/mg protein to 1.05 nmol/mg protein) after the addition of DP1 (0.66 nmol/mL), while up to 57.0% (from 1.14 ± 0.10 nmol/mg protein to 0.49 nmol/mg protein) when DP1 concentration reached 20 nmol/mL. Obviously, the inhibitive effect of DP1 on MDA generation was significant and in a dose-dependent manner.

To counteract oxidative damage caused by ROS, the defense system of human body equipped with various antioxidant enzymes such as GPx, SOD and CAT will be activated once oxidation is recognized. When oxidative stress arises by a pathologic event, the defense system will enhance the regulation and expression of these antioxidant enzymes (11,49). Thus, the relatively high content of SOD in the cell may be as the result of the response to the increase of ROS. The augment of SOD activity is to catalyze dismutation of highly reactive superoxide anion to O_2 and to the less reactive species H_2O_2 which can lead to the formation of •OH radical followed by MDA (50).GPx and CAT are also

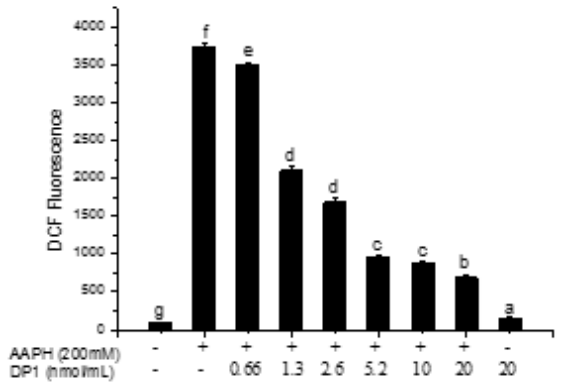

Figure 4. Inhibition of AAPH-induced ROS generation in erythrocytes by DP1. Erythrocytes were treated with AAPH and different concentration of DP1(0.66-20 nmol/mL) and cultivated for 30 min at 37 °C.As positive control, erythrocytes were treated with AAPH for 2 h only.

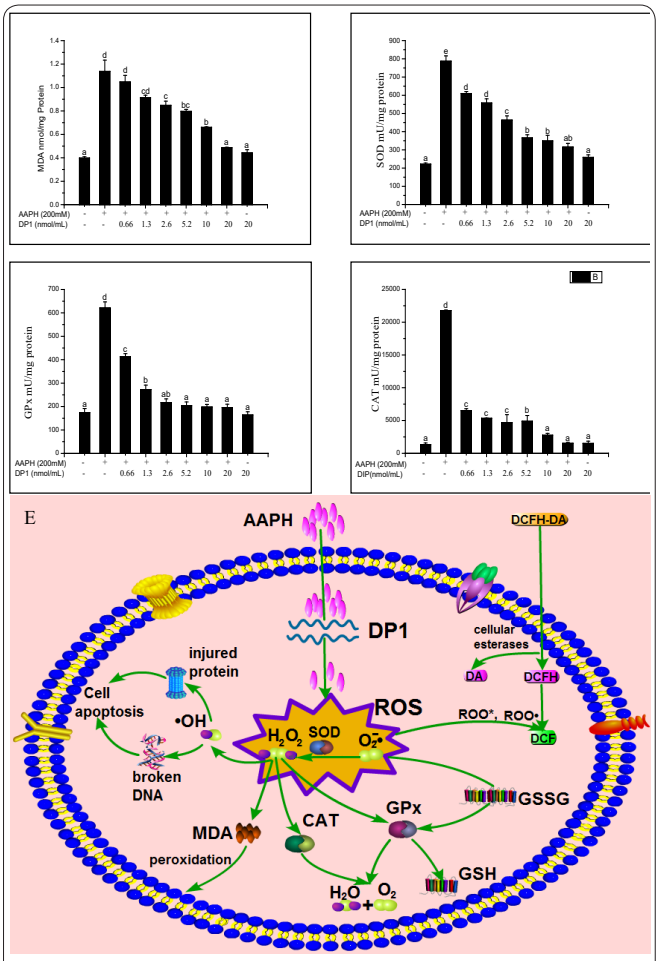

Figure 5. Changes in MDA content (A), enzyme activities of SOD (B), enzyme activities of GPx(C) and CAT (D) in human erythrocyte. (E) intracellular antioxidant mechanism of DP1 toattenuate AAPH-induced oxidative stress by scavenging ROS. Erythrocytes were pre-incubated with DP1 (0.66-20 nmol/mL) for 20 min and then co-incubated with 200 mM AAPH for 2 h at 37 °C. The positive group was treated with 200 mM AAPH only.

very crucial in the detoxified process. They can react efficiently with peroxide to form water and molecular oxygen. As shown in Fig.5B, 5C and 5D, the SOD, GPx and CAT activities were found significantly ($p < 0.05$) rose as compared to their control group, respectively. Especially for CAT activity, it was more sensitive to DP1 treatment since it declined dramatically after DP1 was added. For example, as the erythrocytes incubated with the DP1 at 20 nmol/mL, there are 60.0% reduction in the SOD activity (from 787.01 ± 29.36 to315.28 ± 20.73 mU/mg protein), 68.5% in the GPx activity (from 621.10 ± 25.83 to 195.81 ± 14.15mU/mg protein), while 93.0% in the CAT activity (from21775.35 ± 82.38 to 1529.93 ± 141.30 mU/mg protein) as compared to the group treated with only AAPH. It was also found that the activity of CAT was far higher than that of SOD or GPx, so the hypothesis was that CAT might play a more important role than SOD or GPx in controlling cellular ROS level. This conclusion was also in accordance with the result of previous studies which indicated that the administration of CAT was helpful to reduce ROS levels in breast cancer patients (50). It showed that a high ROS production and decreased CAT activity, but high SOD and GPx activities, was found in the blood of patients (46).Based on the above results, it's safe to draw the conclusion that DP1 could significantly alleviate the AAPH-induced hemolysis via preserving the activities of the antioxidant enzymes, namely SOD, GPx and CAT, as well as inhibiting lipid peroxidation.

The possible intracellular antioxidant mechanism of is clearly illustrated in Fig. 5E. Excessive cellular ROS can cause protein injury, DNA breakage and lipid peroxidation and ultimately lead to cell apoptosis. Cellular antioxidant enzymes such as GPx, SOD and CAT can react with some of ROS such as superoxide anion and peroxide to scavenge a certain amount of ROS. When the AAPH enters into the cell, radical chain reactions will be triggered and a large amount of ROS will generate. However, the presence of DP1 can effectively inhibit the ROS formation and preserve the activity of GPx, SOD and CAT, which will attenuate the oxidative stress induced by AAPH.

Conclusion

In conclusion, the intracellular antioxidant effect of DP1 against oxidative stress was demonstrated by erythrocytes oxidative hemolysis assay and plasma oxidation assay. The results showed that DP1 had distinctly protective effect on AAPH-induced erythrocytes hemolysis (87.3% inhibition at 20 nmol/mL) and copper-induced plasma oxidation by suppressing the intracellular reactive oxygen species production. The underlying mechanism for DP1 as an antioxidant is mainly by decreasing the accumulation of ROS, regulating the activities of antioxidant enzymes and inhibiting the lipid peroxidation of LDL and HDL. The results of this study suggest that DP1is a promising natural product with potential applications in treatment of oxidative stress-induced diseases.

Acknowledgments

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