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Protective effect of procyanidin B_2 on hydrogen peroxide (H_2O_2)-induced oxidative damage in MCF-7 cells

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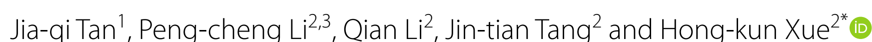

Abstract

The aim of this study is to assess the cytoprotection and potential molecular mechanisms of procyanidin B_2 (PCB_2) on hydrogen peroxide (H_2O_2)-induced oxidative damage in MCF-7 cells. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to determine the viability of MCF-7 cell exposure to H_2O_2 or PCB_2. We measured the antioxidant properties of PCB_2 by determining the activities of SOD, GSH-Px, LDH and MDA levels, and evaluated apoptosis and intracellular reactive oxygen species (ROS) levels. The related proteins expression levels were monitored by Western blot. MCF-7 cells induced with H_2O_2 had a remarkable decrease in cell viability that was suppressed when it was interfered with PCB_2 (0.1–10.0 μM). PCB_2 interference memorably and dose-dependently inhibited H_2O_2-induced LDH leakage, ROS and MDA overproduction, while PCB_2 markedly increased H_2O_2-induced the activities of SOD and GSH-Px. Eventually, H_2O_2 prominently down-regulated the ratio of Bcl-2/Bax and the relative proteins expression levels of Nrf2, GCLC, NQO1 and HO-1, and up-regulated the relative proteins expression levels of cytochrome c, caspase-3 and Keap1. However, the relative expression levels of these proteins were reversed in PCB_2-interfered MCF-7 cells. This study implied that protective effect of PCB_2 on H_2O_2-induced oxidative damage in MCF-7 cells might be related to inhibition of mitochondria-dependent apoptosis, activation of Keap1/Nrf2/HO-1 signaling pathway and improvement of the antioxidant enzymes activities.

Keywords: Procyanidins B_2, MCF-7 cells, Oxidative damage, Signalling pathway

Introduction

Oxidative damage is caused by excessive generation of reactive oxygen species (ROS) in terms of hydroxyl radicals, singlet oxygen and superoxide anions [1]. Growing researches have confirmed that oxidative damage is correlated with the pathological development of cancer, atherosclerosis, diabetes, neurological diseases and other diseases [2, 3]. Moreover, oxidative damage leads to the disorder the balance between oxidation and antioxidant defense system, destruction DNA, protein and lipid functions [4]. Breast cancer is a common clinical malignant tumor, which is a serious threat to women's health. Increasing evidences have implicated oxidative damage and inflammation in the etiology of breast diseases [5]. Moreover, previous researches have indicated that antioxidants prevented or delayed ROS-triggered apoptosis might be a reasonable way to treat a variety of breast related diseases [6, 7]. Among all kinds of antioxidants, natural substances usually obtained by chromatographic separation technology from natural plants showed merits than synthetic chemicals because synthetic chemicals have acute by-effects though strong radical scavenging abilities [8]. Besides, natural antioxidants can prevent the body injury through removing excessive ROS, decreasing MDA and enhancing activities of antioxidant enzymes [9]. Presently, substantial researches had been concentrated on hunting for natural active ingredients with

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breast protective potential, which could scavenge excess free radicals and prevent cell oxidative damage.

Procyanidins, as a natural flavonoid compound and typical antioxidant, are widely found in vegetables, fruits and green plants. Procyanidins are composed of monomers (mainly catechins, epicatechins, etc.), oligomeric procyanidins (two, three, four polymerized monomers) and highly polymerized procyanidins (more than pentamers) [10]. One of the main procyanidins, dimers procyanidins B_2 [epicatechin-(4b-8)-epicatechin] (PCB_2) (Fig. 1) have attracted great interest in nutrition and medicine owing to its anti-aging, anti-oxidative, anti-inflammation and vascular relaxation and so on [11]. Recently, many researches have authenticated that PCB_2 could regulate the redox state of cells and the protect of antioxidant enzymes in colon cells against oxidative damage and exogenous substances [12, 13]. Ildefonso et al. found that PCB_2 protected Caco-2 cells from oxidative damage by activation pathways of JNK and ERKs-p38-MAPK, respectively [14, 15]. PCB_2 can be a potential drug for the treatment of breast related diseases. Nevertheless, up to date, there is not enough information about the effect of PCB_2 on breast cells (MCF-7 cells), and the underlying molecular mechanism of the protective effect of PCB_2 on hydrogen peroxide (H_2O_2)-induced oxidative damage remains to be elucidated.

Therefore, the aim of this paper explored the protective effects of PCB_2 on H_2O_2-induced oxidative damage and cells apoptosis of breast cells (MCF-7 cells). Further, we investigated the potential molecular mechanisms involved in this process.

Materials and methods

Materials

PCB_2 was from Sichuan Vicky Biotechnology Co., Ltd (Chengdu, China). The DMEM medium, dimethyl sulfoxide (DMSO), penicillin, streptomycin and H_2O_2 were provided from Beijing Shengmu Biotechnology Co., Ltd (Beijing, China). MCF-7 cell was purchased from Concorde Cell Bank (Beijing, China). Trypsin digestive fluid and DCFH-DA were afforded from Beijing Fubo Biotechnology Co., Ltd (Beijing, China) and GIBCO-BRL (Grand Island, NY), respectively. The test kits of SOD, GSH-Px, MDA, LDH and bicinchoninic acid (BCA) protein were offered from Nanjing Jiancheng Bioengineering Research Institute Co., Ltd (Nanjing, China). The anti-Nrf2, anti-HO-1, anti-Keap1, anti-GCLC, anti-NQO1, anti-Bax, anti-Bcl-2, anti-cytochrome c, anti-caspase-3 and anti-GADPH were obtained from Shenzhen Zike Biotechnology Co., Ltd (1:1000 dilution; Shenzhen, China).

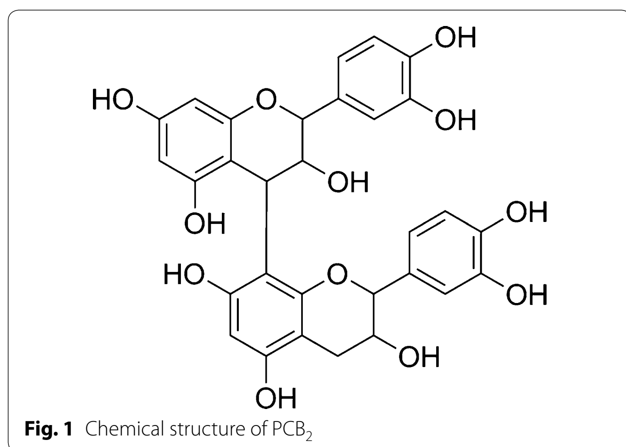

Fig. 1 Chemical structure of PCB_2

Cell culture

DMEM medium, including 10% fetal bovine serum (FBS) and 100 IU/mL penicillin and 100 μg/mL streptomycin, was used to culture MCF-7 cells at 37 °C in 5% CO_2 atmosphere incubator with media replenished every 1 day. The MCF-7 cells were cultured in the culture plate, and the experiment was carried out when the cells entered the logarithmic growth period.

Establishment of MCF-7 cells injury model

The MCF-7 cells (5×10^3 cells/mL) were inoculated in 96-well plates at 37 °C in a humidified 5% CO_2 incubator for 12 h. The medium was subsequently replaced with different final concentrations (0, 25, 50, 100, 200, 300, 400 μM) of H_2O_2 diluted with the DMEM medium for 0, 1, 2, 3, 4, 5 h, and each group has 6 parallel wells. After treatment H_2O_2, each well was added to 10 μL MTT (5 mg/mL) and cultured 4 h. The culture medium was removed, and then added to 150 μL DMSO in each well to dissolve the formed blue formazan crystals. The absorption value of each well was determined at 490 nm by a WD-2102b automatic enzyme labeling instrument (Linyi Yingjia Scientific Instrument Co., Ltd, China). Cell viability is calculated by using the Eq. (1) [16]. The optimal conditions of the oxidative damage model were H_2O_2 concentration and stimulation time when the MCF-7 cell viability was about 50%.

$$\text{Cell viability\%} = \frac{\text{Absorbance of sample group}}{\text{Absorbance of control group}} \times 100\% \tag{1}$$

Determination of the dosage range of PCB_2

MCF-7 cells were seeded in 96 well-plates at the cell concentrations of 5×10^3 cells/mL in a humidified incubator for 12 h, then intervened with PCB_2 (0, 0.1, 1.0, 10.0, 20.0, 40.0 μM) for 24 h. Simultaneously, we utilized a phase contrast microscope to detect MCF-7 cells morphology. Following the experimental steps are described in

establishment of MCF-7 cells injury model. Cell viability is calculated via the Eq. (1).

Effect of PCB_2 on cell viability in H_2O_2-induced MCF-7 cells

The viability of MCF-7 cells was determined by MTT assay as previously reported [17]. Briefly, MCF-7 cells were placed in 96-well plates (5×10^3 cells/mL) and cultured for 12 h at 37 °C in 5% CO_2 incubator, then treated with 300 μM H_2O_2 for 4 h, followed by exposure to 0, 0.1, 1.0, 10.0 μM PCB_2 (According to the cytotoxic results of PCB_2) for another 24 h. The next experimental steps are showed in establishment of MCF-7 cells injury model. Cell viability is calculated by using the Eq. (1).

Hoechst 33342 staining

The laser scanning confocal microscope (Olympus, Japan) was performed to observe apoptosis morphology of MCF-7 cells via Hoechst 33342 staining as previously described [18]. MCF-7 cells at density of 3×10^4 cells/mL were grown in 6-well plates and cultured 12 h at 37 °C in 5% CO_2 incubator. After treatment, MCF-7 cells were stained with Hoechst 33342 (1 mL/well) for 20 min, and then fixed with 4% formaldehyde for 10 min. Finally, we utilized the FV3000 laser scanning confocal microscope to observe morphological changes of the nuclear chromatin at 620 nm.

Determination of ROS production

The effect of PCB_2 on H_2O_2-induced ROS production in MCF-7 cells was monitored by the DCFH-DA. Briefly, MCF-7 cells were cultured in 6-well plates with a density of 3×10^4 cells/mL and incubated overnight. Cells were induced by H_2O_2 (300 μM) for 4 h, and then pretreated with different concentrations (0, 0.1, 1.0, 10.0 μM) of PCB_2 for another 24 h. DCFH-DA (5 μM, 30 min) as a probe was employed to detect ROS indicator. After DCFH-DA incubation, MCF-7 cells were collected and determined by a flow cytometry (Thermo Fisher Scientific Co., Ltd, China).

Measurement of antioxidant parameters

MCF-7 cells (3×10^4 cells/mL) were seeded in 6-well plates and cultured for 12 h. The cells were stimulated with 300 μM H_2O_2 for 4 h, following the cells were exposed to 0, 0.1, 1.0, 10.0 μM PCB_2 for 24 h. The MCF-7 cells were then washed with PBS and centrifuged (6000 r, 15 min) at 4 °C. The supernatant was used to determine antioxidant parameters. The activities of SOD and GSH-Px, LDH leakage and MDA levels were detected by commercial assay kits according to their instructions.

Apoptosis detection by flow cytometry

MCF-7 cells apoptosis were measured by the flow cytometry (Thermo Fisher Scientific Co., Ltd, China), as previously described [19]. Simply, the treated-MCF-7 cells were collected and washed three times with cold PBS, then slowly re-suspended in Annexin V binding buffer, which were incubated with Annexin V-FITC and PI in dark for 10 min, respectively. After incubation, cells were collected and measured using the flow cytometry.

Western blot analysis

MCF-7 cells were grown in 6-well plates with a density of 3×10^4 cells/mL and cultured for 12 h. The cells were treated with 300 μM H_2O_2 for 4 h, and then the cells were intervened to 0, 0.1, 1.0, 10.0 μM PCB_2 for 24 h. Subsequently, the cells were homogenized and dissolved in RIPA buffer of radioimmunoprecipitation test to obtain protein extracts in the presence of protease inhibitor. The BCA protein determination kit was used to determine protein concentration. Samples were loaded onto micro protein TGXTM prefabricated electrophoretic gels. The obtained proteins through separation were transferred to the PVDF membranes. The nonspecific sites were sealed with 5% skimmed milk powders in PBST for 60 min, and then the blots were incubated with anti-Nrf2, anti-HO-1, anti-Keap1, anti-GCLC, anti-NQO1, anti-Bax, anti-Bcl-2, anti-cytochrome c, anti-caspase-3, anti-GADPH and anti-β-action (1:1000 dilution, Shenzhen, China) in PBST 24 h at 4 °C. GAPDH and β-action were performed to confirm equal loading of protein in each lane, respectively. The protein expression was determined by Western blot analysis.

Statistical analysis

All experimental results are shown as the mean ± SD. The one-way analysis of variance (ANOVA) was employed to analyze statistical differences between the groups. $p < 0.05$ represents the experimental results with statistical significance. Origin 9.0 software (OriginLab, USA) is used for drawing in this paper.

Results

Concentration and time-dependent viability losses in MCF-7 cells exposed to H_2O_2

First, the viability in MCF-7 cells exposed to H_2O_2 were measured by MTT assay. MCF-7 cells viability dramatically diminished with H_2O_2 from 25 to 400 μM ($p < 0.05$). The pre-treatment with H_2O_2 showed (63.01 ± 3.27) % in 200 μM, (51.35 ± 1.24) % in 300 μM and (30.58 ± 0.86) % in 400 μM (Fig. 2a). The results indicated that H_2O_2 resulted in remarkable damage to MCF-7 cells in a dose-dependent manner. Figure 2b showed that 300 μM H_2O_2

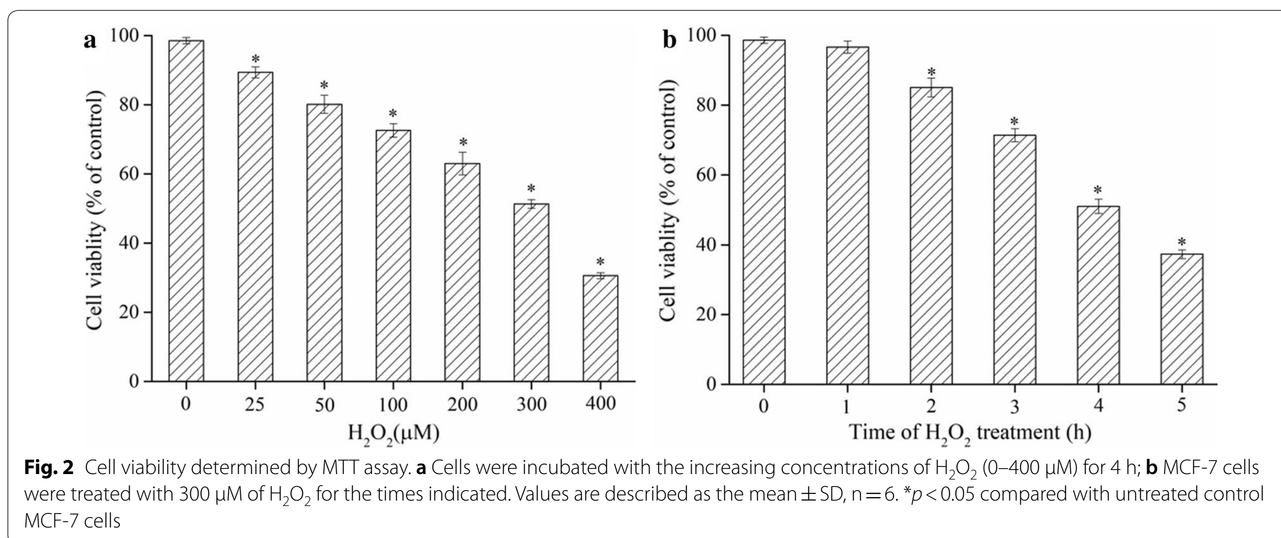

Fig. 2 Cell viability determined by MTT assay. **a** Cells were incubated with the increasing concentrations of H_2O_2 (0–400 μM) for 4 h; **b** MCF-7 cells were treated with 300 μM of H_2O_2 for the times indicated. Values are described as the mean ± SD, n = 6. *$p < 0.05$ compared with untreated control MCF-7 cells

treatment for 1–5 h markedly reduced MCF-7 cell viability in a time-response dependent manner ($p < 0.05$). Previous studies have shown that the corresponding H_2O_2 concentration and treatment time were regarded as the best conditions for oxidative damage when the cell viability was about 50% [20]. Based on the above results, we chose to use a 4 h exposure of 300 μM H_2O_2 for subsequent experiments.

Determination of the dosage range of PCB_2

Cytotoxicity of PCB_2 (0.1–40.0 μM) on MCF-7 cells was determined by MTT assay. PCB_2 did not show any prominent cytotoxic effect when the concentrations of PCB_2 were in the range of 0.1 to 10.0 μM (Fig. 3a). Meanwhile, there were no prominent change in cell number and morphology (Fig. 3b). Subsequently, the cytotoxicity of PCB_2 to MCF-7 cells increased significantly with the increase of PCB_2 concentration ($p < 0.05$). Simultaneously, the number of cells decreased significantly when the PCB_2 concentration was in the range of 20.0–40.0 μM. Consequently, 0.1–10.0 μM of PCB_2 were employed for latter experiments.

The effect of PCB_2 on cell viability in H_2O_2-induced MCF-7 cells

Cell viability is the most direct index to reflect the degree of cell damage caused by the external environment. H_2O_2, a considerable active oxygen molecule with relatively stable properties, is often used as a model drug for oxidative injury in vitro. As shown in Fig. 4, the cell viability of the oxidative damage model group constructed by H_2O_2 decreased significantly compared to the control group ($p < 0.05$), indicating that the oxidative damage model was successfully developed. Nevertheless, the application of PCB_2 (0.1–10.0 μM) treated dose-dependent increase

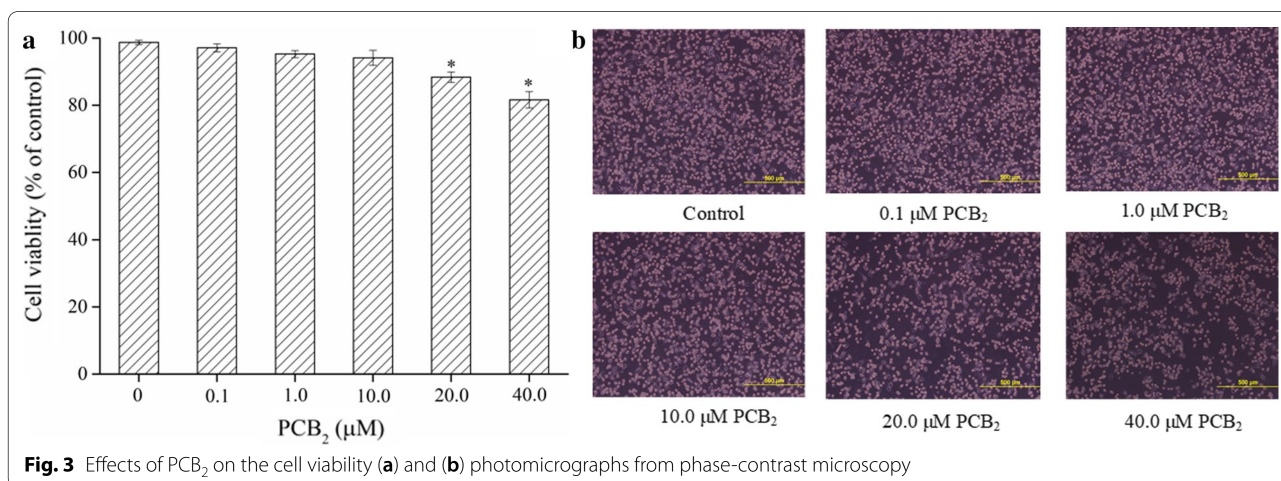

Fig. 3 Effects of PCB_2 on the cell viability (**a**) and (**b**) photomicrographs from phase-contrast microscopy

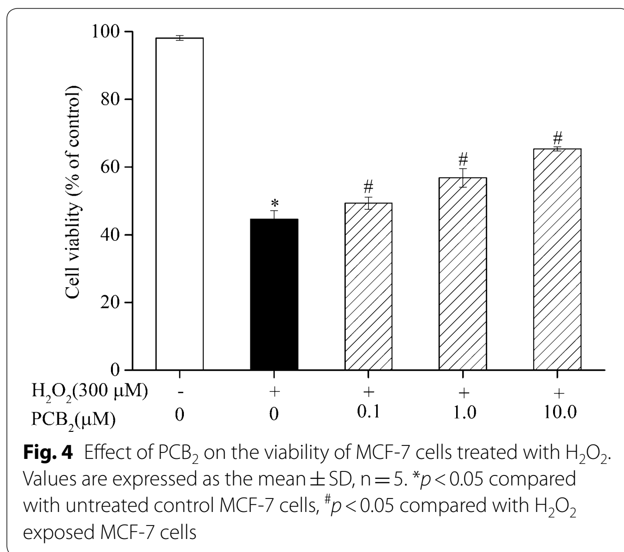

Fig. 4 Effect of PCB_2 on the viability of MCF-7 cells treated with H_2O_2. Values are expressed as the mean ± SD, n = 5. *$p < 0.05$ compared with untreated control MCF-7 cells, #$p < 0.05$ compared with H_2O_2 exposed MCF-7 cells

in cell viability when compared with H_2O_2 alone treated group.

The effect of PCB_2 on H_2O_2-induced ROS production in MCF-7 cells

The production of endogenous ROS causes cell damage. Hence, we investigated the ability of PCB_2 to inhibit the production of ROS by DCFH-DA as a fluorescent probe. As illustrated in Fig. 5, the ROS levels were prominently increased in MCF-7 cells after H_2O_2 induction compared to the vehicle-treated group. However, the increased ROS levels caused by H_2O_2 induction was attenuated in the MCF-7 cells pre-treated with PCB_2. The results hinted that PCB_2 could inhibit the generation of ROS in MCF-7 cells.

Fig. 5 Effect of PCB_2 on the intracellular ROS levels in H_2O_2-induced MCF-7 cells. **a** The fluorescent intensities; **b** Analysis of statistical results of flow cytometry detection. Values are showed as the mean ± SD, n = 3. *$p < 0.05$ compared to the vehicle-treated group, #$p < 0.05$ compared to the H_2O_2 alone treated group

The effects of PCB_2 on the activities of SOD, GSH-Px, LDH leakage and the MDA levels in H_2O_2-induced MCF-7 cells

It is well known that cellular antioxidant systems, including mainly SOD and GSH-Px, can enhance the ability of cells to deal with oxidative damage caused by H_2O_2. LDH is a critical indicator of cell membrane integrity. Furthermore, MDA is acted as a biomarker of oxidative stress. To clarify whether the protective effect of PCB_2 on H_2O_2-triggered MCF-7 cells is owing to antioxidant properties, the activities of SOD and GSH-Px, LDH leakage and MDA levels were determined by commercial kits. As shown in Fig. 6, the activities of SOD and GSH-Px were dramatically decreased in 300 μM H_2O_2 treated group than that the vehicle-treated group ($p < 0.05$), while the LDH leakage and MDA levels were substantially increased in 300 μM H_2O_2 when compared to the vehicle-treated group. Conversely, intervention with the increasing concentrations of PCB_2 (0.1, 1.0, 10.0 μM) markedly improved the activities of SOD and GSH-Px as well as attenuated the LDH leakage and MDA levels in H_2O_2-induced MCF-7 cells ($p < 0.05$). These results indicated that the protective effect of PCB_2 on oxidative damage induced by H_2O_2 was due to an improvement in the cellular antioxidant systems.

The effect of PCB_2 on apoptosis of MCF-7 cells

To elucidate the inhibitory effect of PCB_2 on H_2O_2-treated MCF-7 cell apoptosis, flow cytometry analysis was conducted in this study. Meanwhile, the Hoechst 33342 assay was employed to further explicate the protection of PCB_2 against H_2O_2-treated cytotoxicity and MCF-7 cell nuclear changes. As shown in Fig. 7a, the untreated control MCF-7 cell nuclei was shown dimly blue, and H_2O_2-triggered MCF-7 cells memorably increased fluorescence intensity of cell nuclei. While the effect of nuclear fluorescence was sharply reversed in H_2O_2-induced MCF-7 cells pre-treated with PCB_2 ($p < 0.05$). As illustrated in Fig. 7c, we found that H_2O_2-treated MCF-7 cells markedly increased apoptosis rate ($p < 0.05$). Conversely, intervention with the

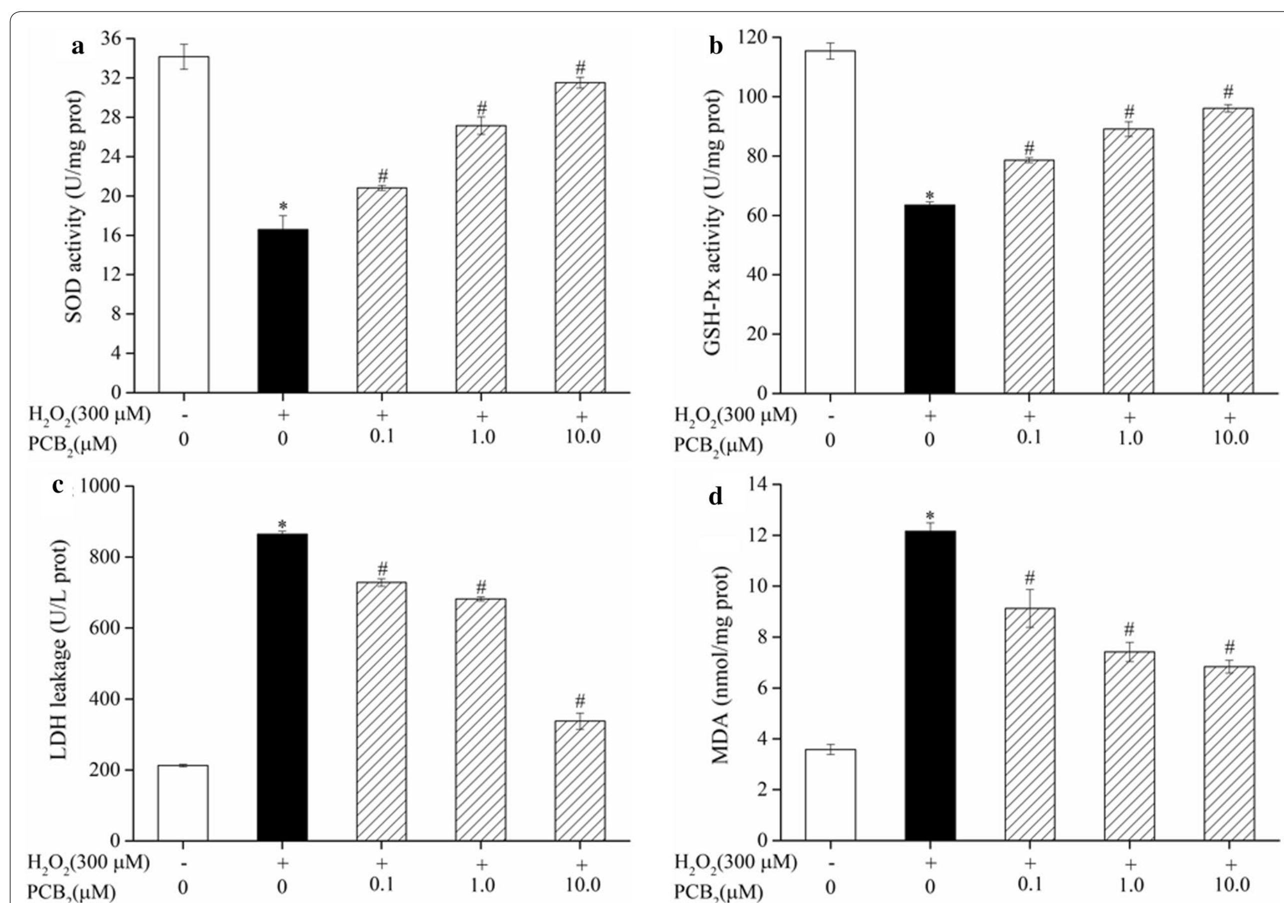

Fig. 6 Effects of PCB_2 on antioxidant enzyme activities, LDH leakage and MDA levels in H_2O_2-induced MCF-7 cells. **a** SOD activity; **b** GSH-Px activity; **c** LDH leakage; **d** MDA levels. Values are described as the mean ± SD, n = 3. *$p < 0.05$ compared with untreated control MCF-7 cells, $^{\#}p < 0.05$ compared with H_2O_2 exposed MCF-7 cells

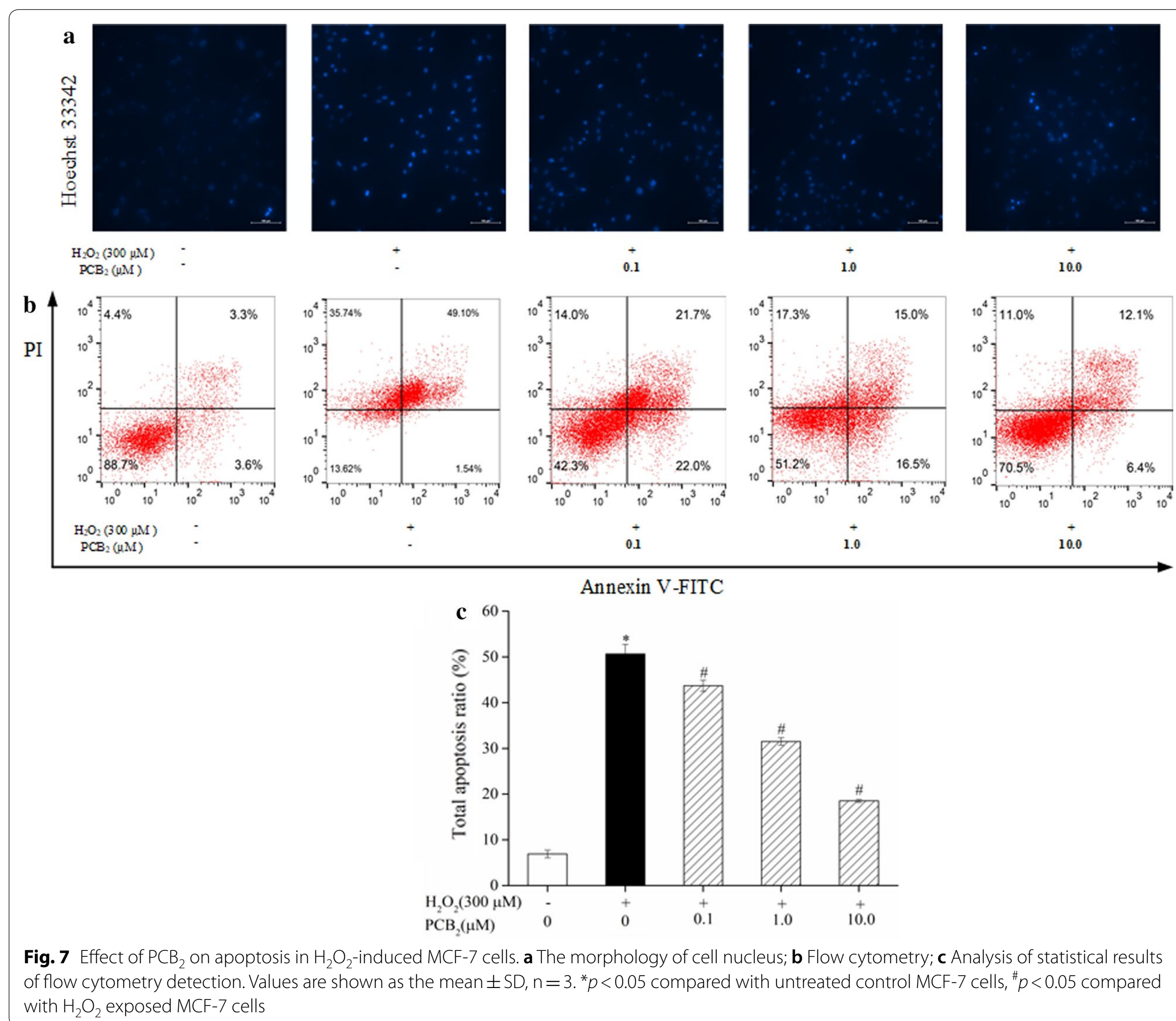

Fig. 7 Effect of PCB_2 on apoptosis in H_2O_2-induced MCF-7 cells. **a** The morphology of cell nucleus; **b** Flow cytometry; **c** Analysis of statistical results of flow cytometry detection. Values are shown as the mean ± SD, n = 3. **p* < 0.05 compared with untreated control MCF-7 cells, #*p* < 0.05 compared with H_2O_2 exposed MCF-7 cells

various concentrations of PCB_2 (0.1–10.0 μM) inhibited dramatically MCF-7 cells apoptosis rate in a concentration-dependent manner ($p<0.05$). The results of flow cytometry were similar to those of fluorescence microscopy. The results suggested that PCB_2 protected MCF-7 cells by suppressing H_2O_2-induced cell apoptosis.

The effect of PCB_2 on the expression levels of related apoptotic protein induced by H_2O_2

Based on the above results, we have confirmed that PCB_2 could protect MCF-7 cells from oxidative damage. We performed further study to investigate its potential mechanism. The ratio of Bcl-2/Bax plays a critical role in the process of apoptosis. Therefore, the ratio of Bcl-2/Bax was detected by Western blot. H_2O_2 down-regulated dramatically Bcl-2/Bax ratio compared to the control group (Fig. 8b). Nevertheless, the groups of intervention with PCB_2 (0.1, 1.0, 10.0 μM) substantially up-regulated the ratio of Bcl-2/Bax as when compared with H_2O_2-mediated MCF-7 cells group ($p < 0.05$).

Cytochrome c and caspase-3 are biomarkers of oxidative damage-induced cell death through mitochondrial-dependent apoptotic pathway [21]. The relative expression levels of these apoptosis related proteins were evaluate by Western blot. H_2O_2 simulation MCF-7 cells markedly increased the relative proteins expression levels of cytochrome c and caspase-3 compared to the control group. Conversely, MCF-7 cells treated with PCB_2 (0.1–10.0 μM) prominently suppressed the relative proteins expression levels of cytochrome c and caspase-3 ($p < 0.05$) (Fig. 8c, d), which illustrated that PCB_2 was able to suppress mitochondrial-dependent apoptosis caused by H_2O_2.

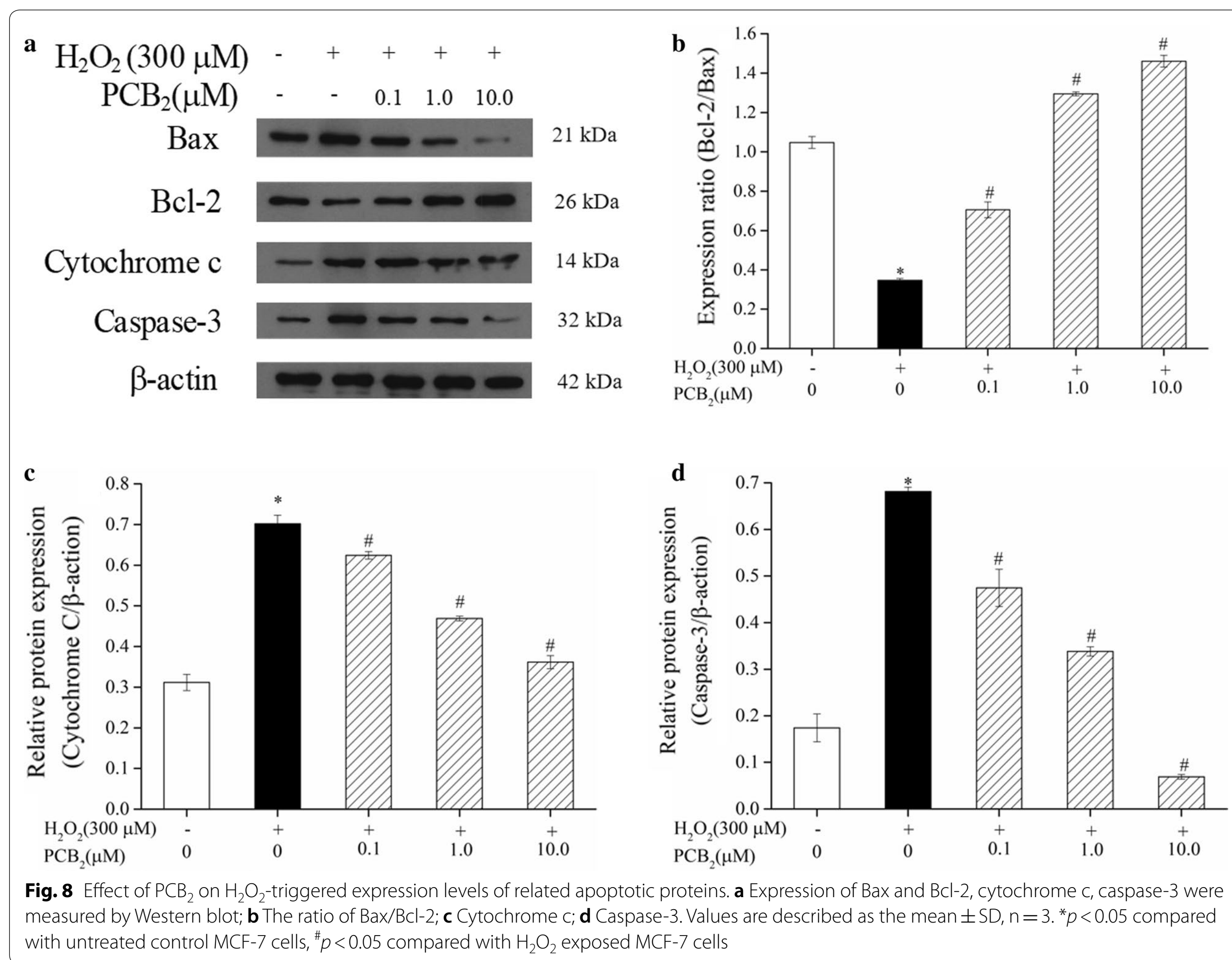

Fig. 8 Effect of PCB_2 on H_2O_2-triggered expression levels of related apoptotic proteins. **a** Expression of Bax and Bcl-2, cytochrome c, caspase-3 were measured by Western blot; **b** The ratio of Bax/Bcl-2; **c** Cytochrome c; **d** Caspase-3. Values are described as the mean ± SD, n = 3. $^{*}p < 0.05$ compared with untreated control MCF-7 cells, $^{\#}p < 0.05$ compared with H_2O_2 exposed MCF-7 cells

The effect of PCB_2 on Keap1/Nrf2/HO-1 pathway related proteins induced by H_2O_2

To determine whether the Keap1/Nrf2/HO-1 pathway is triggered by H_2O_2-induced oxidative damage in MCF-7 cells, Western blot was conducted to examine the relative proteins expression levels of Keap1, Nrf2, GCLC, NQO1 and HO-1 in MCF-7 cells after the treatment with H_2O_2 or PCB_2. Nrf2, as a considerable regulatory transcription factor upstream of antioxidant defense system, regulates the transcription expression of hundreds of antioxidant and detoxification related proteins [22]. Keap1 is a vital negative regulator of Nrf2 that regulates the relative expression levels of antioxidant proteins and phase II detoxification enzyme by interacting with antioxidant response elements [23]. As seen Fig. 9c, the relative protein expression levels of Keap1 was sharply increased in H_2O_2-induced MCF-7 cells ($p < 0.05$). This increase in expression was conspicuously diminished by treating it with the increase concentration of PCB_2 (0.1–10.0 μM). The expression of downstream representative antioxidant proteins (GCLC, NQO1 and HO-1) regulated by Nrf2 was further detected. We found that H_2O_2 exposure notably decreased the relative expression levels of these antioxidant proteins compared to the control group ($p < 0.05$). Conversely, MCF-7 cells treated with PCB_2 (0.1–10.0 μM) prominently increased relative proteins expression levels of Nrf2, GCLC, NQO1 and HO-1 ($p < 0.05$). Our findings suggested that PCB_2 protected MCF-7 cells against oxidative damage through induction of the Keap1/Nrf2/HO-1 pathway.

Discussion

In this present study, we utilized MCF-7 cells as a model to explore the protective properties of PCB_2 against oxidative damage. MCF-7 cells were initially treated with H_2O_2 (300 μM) for 4 h, and then intervented with different concentrations of PCB_2 for 24 h. We found that (1) PCB_2 evidently inhibited the production of ROS in MCF-7 cells; (2) PCB_2 suppressed substantially H_2O_2-triggered MCF-7 cells apoptosis as authenticated

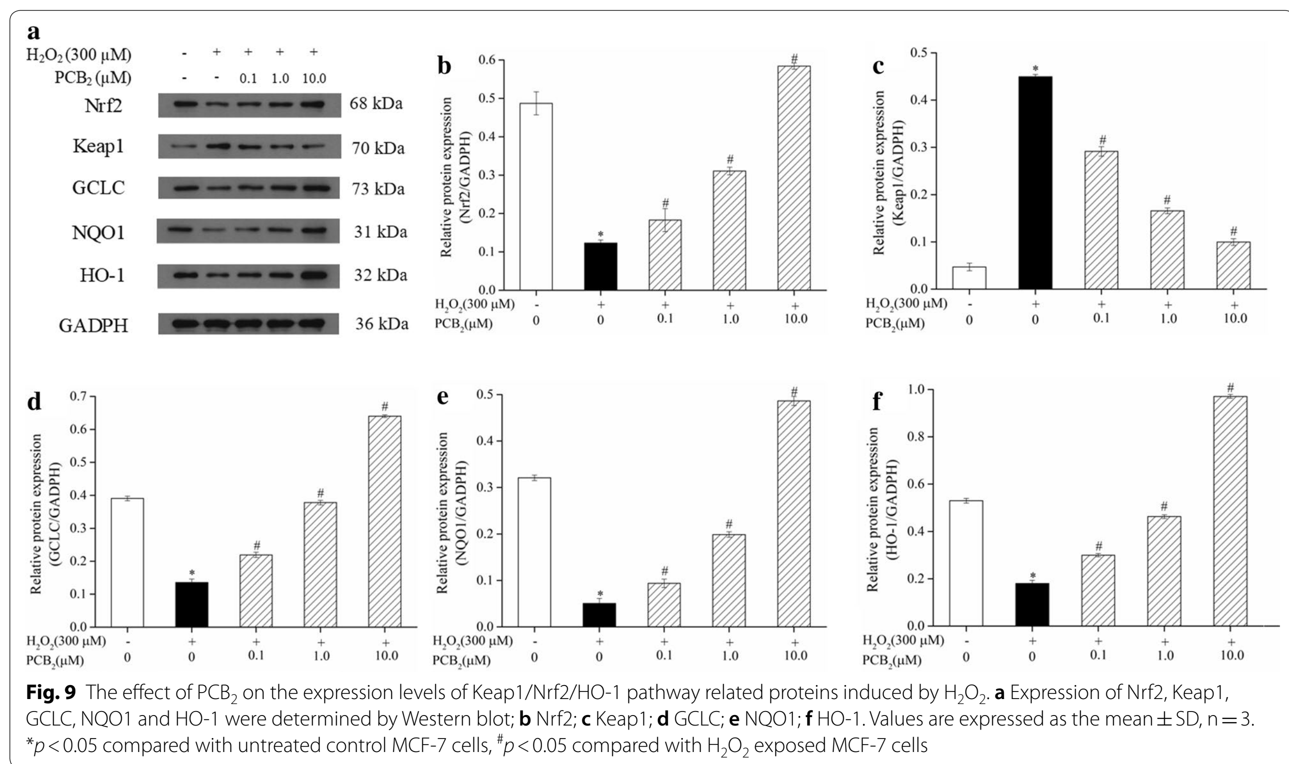

Fig. 9 The effect of PCB_2 on the expression levels of Keap1/Nrf2/HO-1 pathway related proteins induced by H_2O_2. **a** Expression of Nrf2, Keap1, GCLC, NQO1 and HO-1 were determined by Western blot; **b** Nrf2; **c** Keap1; **d** GCLC; **e** NQO1; **f** HO-1. Values are expressed as the mean ± SD, n = 3. $^*p < 0.05$ compared with untreated control MCF-7 cells, $^{\#}p < 0.05$ compared with H_2O_2 exposed MCF-7 cells

through experiments (MTT, Hoechst 33342 staining and flow cytometry); (3) Intervention of MCF-7 cells with PCB_2 (0.1–10.0 μM) could dramatically enhance the activities of SOD and GSH-Px, while the LDH leakage and MDA levels substantially decreased in H_2O_2-induced MCF-7 cells; (4) PCB_2 markedly up-regulated the ratio of Bcl-2/Bax and significantly down-regulated the cytochrome c and caspase-3 relative proteins expression levels, indicating that PCB_2 was able to inhibit mitochondria-dependent apoptosis caused by H_2O_2; (5) PCB_2 led to the down-regulated Keap1 relative protein expression levels, and the up-regulated the expression levels of Nrf2, GCLC, NQO1 and HO-1 relative proteins in MCF-7 cells, suggesting that PCB_2 protected MCF-7 cells from oxidative damage via activation of the Keap1/Nrf2/HO-1 pathway.

H_2O_2, as an important active oxygen molecule with relatively stable properties, is often used as a model drug for oxidative injury in vitro. Thus, we chose H_2O_2 to establish the MCF-7 cells oxidative damage model in this study. The results found that treatment of MCF-7 cells with H_2O_2 concentration from 25 to 400 μM resulted in conspicuous decrease MCF-7 cells viability (Fig. 2a). The viability of MCF-7 cells decreased to (51.04 ± 2.03) % when MCF-7 cells were treated with 300 μM H_2O_2 for 4 h (Fig. 2b). Previous studies have shown that the optimal conditions of oxidative damage model were the concentration of H_2O_2 and the stimulation time when the cell viability was about 50% [20]. Hence, we chose to use a 4 h exposure of 300 μM H_2O_2 for follow-up experiments. The MTT assay was initially used to determine the toxicity of PCB_2 in MCF-7 cells. Results indicated that PCB_2 (from 0.1 to 10.0 μM) was non-toxic in MCF-7 cells (Fig. 3a). Furthermore, those results showed a dose-dependent protective effect of PCB_2 against H_2O_2 treated loss of MCF-7 cells viability (Fig. 4). Moreover, according to the analysis results of flow cytometry, it can be seen that the apoptosis rate of MCF-7 cells increased from (6.90 ± 0.83) % to (50.64 ± 2.11) % after exposure to 300 μM H_2O_2 for 4 h (Fig. 7c). This result illustrated that the programmed cell death of MCF-7 cells caused by H_2O_2 treatment [24, 25]. However, a remarkable decrease in the number of apoptotic MCF-7 cells was observed when MCF-7 cells were treated with PCB_2 (0.1–10.0 μM). Taken together, these results implied that PCB_2 protected against H_2O_2-mediated MCF-7 cells apoptosis.

ROS is one of the vital factors in the formation and development of various diseases. Cell oxidative damage can produce excessive ROS, which damages human endothelial function as well as promotes cell death and apoptosis [26]. Meanwhile, ROS and MAD levels are the most typical indicators of oxidative damage [27]. Accordingly, we utilized the fluorescent probe and MAD kit to determine the production of ROS and MAD levels in

MCF-7 cells, respectively. These results presented that the H_2O_2 treated MCF-7 cells, resulting in overproduction of intracellular ROS and MAD. Yet intervention with PCB_2 observably suppressed the production of intracellular ROS and MAD when compared with H_2O_2 treated group (Figs. 5b, 6d). This hinted that the ability of PCB_2 to restrain the generation of ROS and MAD might be related to its anti-apoptotic activities. Moreover, antioxidant enzyme defense system plays an indispensable role in scavenging ROS and preventing cell from oxidative damage. Increasing studies have indicated that the overexpression of SOD and GSH-Px could provide cytoprotective effects against ROS in HepG2 cells, HUVECs and BRL-3A cells [28–30]. The oxidative damage induced by H_2O_2 decreased memorably the activities of SOD and GSH-Px (Fig. 6a, 6b), while ROS levels increased markedly. This phenomenon could be efficiently reversed by intervention with PCB_2. These results suggested that PCB_2 diminished the oxidative damage via improving antioxidant enzymes activities in H_2O_2-mediated MCF-7 cells. LDH is a critical indicator of cell membrane integrity. Cells will release LDH into the culture medium during the oxidative damage process [31]. Thus, the LDH leakage in the culture medium can reflect the degree of cell death or damage. LDH leakage was notably increased in H_2O_2 induced MCF-7 cells when compared with control group. Nevertheless, 0.1–10.0 μM PCB_2 treatment remarkably decreased LDH leakage when compared with H_2O_2 alone treated group (Fig. 6c).

A series of apoptosis related proteins in terms of Bax, Bcl-2 and caspase-3 are major regulators of cell death and cell survival [32]. In this paper, we conducted if PCB_2 could restrain the apoptotic signalling pathway through H_2O_2-induced. The experimental results illustrated that H_2O_2 decreased markedly the Bcl-2/Bax ratio compare to the control group. However, the H_2O_2-induced effect was significantly and dose-dependently suppressed by pretreatment of PCB_2 (Fig. 8b). Increasing events have confirmed that ROS enhanced cytosolic caspases activity by activating Bax and cytochrome c dissociation from mitochondrial inner membrane [33, 34]. Bcl-2 family proteins, including anti-apoptosis (Bcl-2) and pro-apoptosis (Bax) members, play a critical role in the early stages of the apoptotic pathway [35]. Results demonstrated that H_2O_2 up-regulated pro-apoptotic (Bax) and cytochrome c, and down-regulated anti-apoptotic (Bcl-2) in MCF-7 cells, and these effects were suppressed by intervention with PCB_2. In conclusion, PCB_2 initially regulated Bcl-2 and Bax proteins. This, in turn, might regulate mitochondrial membrane permeability and released cytochrome c, following-up restrained caspase-3 activation.

Nrf2 is a highly conserved basic leucine zipper transcription factor, which is mainly expressed in intestine, lung, liver and kidney [36]. It is considered to be a critical transcription factor regulating cells against foreign bodies and oxidative damage [37]. Keap1 is an important negative regulator of Nrf2 in vivo. Under normal physiological conditions, the most of Nrf2 is chelated with Keap1 in the cytoplasm, which makes Nrf2 unable to enter the nucleus to play its biological activity [38]. When the body is subjected to oxidative stress, the cysteine residues of Keap1 is modified to change the conformation of Keap1, resulting in decoupling of Keap1 and Nrf2. Following the activated Nrf2 is transferred into the nucleus and specifically combined with the antioxidant responsive elements (ARE). A series of downstream antioxidant enzymes proteins (GCLC, NQO1 and HO-1) are expressed to enhance the antioxidant activity of the body to resist the damage caused by oxidative stress [39, 40]. A hesperetin, as a polyphenol compound, is found in citrus that can memorably augment the antioxidant HO-1 by the up-regulation Nrf2 and decrease the stability of Keap1 [41]. A large number of researches have indicated that natural polyphenol antioxidant could protect cells from oxidative damage through p38 and Keap1/Nrf2-dependent signaling pathway [42, 43]. The mechanism of antioxidant action for PCB_2 might be related to the activation of Keap1/Nrf2/HO-1 signaling pathway. To further determine the underlying mechanism, the effect of PCB_2 on the relative proteins expression levels of Keap1, Nrf2, GCLC, NQO1 and HO-1 in MCF-7 cells was investigated via Western blot. The results suggested that PCB_2 (0.1–10.0 μM) could remarkably increase relative proteins expression levels of Nrf2, GCLC, NQO1 and HO-1 (Fig. 9b, 9d–f), and decreased the relative protein expression levels of Keap1 (Fig. 9c). In a word, our findings hinted that PCB_2 protected MCF-7 cells from H_2O_2-triggered oxidative damage, which might be related to activation of Keap1/Nrf2/HO-1 signaling pathway, and enhancement of the activities of antioxidant enzymes.

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Authors' contributions
HKX conceived and designed research, and revised manuscript. JQT and PCL conducted experiments. QL and JTT analyzed data. JQT wrote the manuscript. All authors read and approved the manuscript.

Availability of data and materials
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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