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Inhibitory effect of main phenolic acid components of *Jacobaea cannabifolia* (Less.) on inflammation caused by PM_{2.5}

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PM_{2.5} is an important environmental problem threatening human health at present, which poses serious harm to human body after inhalation. *J. cannabifolia* is a traditional Chinese medicine which exhibits anti-inflammatory effect. This study aimed to investigate the inhibitory effect of main phenolic acid components of *J. cannabifolia* on inflammation caused by PM_{2.5}. Effect of PM_{2.5} on cell activity and apoptosis were determined by MTT, flow cytometry and calcein AM/PI staining. PHBA, PHPAA, and mixture of PHBA and PHPAA of different concentrations were given to RAW264.7 cells pretreated with PM_{2.5}. The effect of drugs on cellular inflammatory factors was detected by ELISA. The expressions of TLRs related signal pathway at protein and gene levels were detected by western blot and qRT-PCR. The results showed that PM_{2.5} had no effect on cell activity and apoptosis within the determined concentration range. PHBA and PHPAA could markedly inhibit the level of IL-1 β , IL-6, and TNF- α in RAW264.7 cells. Furthermore, the expressions of TLR2, TLR4, MyD88, IRAK1, TRAF6, TAK1, IKK β , and NF- κ B induced by PM_{2.5} were markedly inhibited by PHBA and PHPAA at protein and gene levels. This study demonstrated that PHBA and PHPAA could attenuated inflammation caused by PM_{2.5} through suppressing TLRs related signal pathway.

KEYWORDS

PM_{2.5}, *Jacobaea cannabifolia* (Less.), PHBA, PHPAA, toll-like receptors

1 Introduction

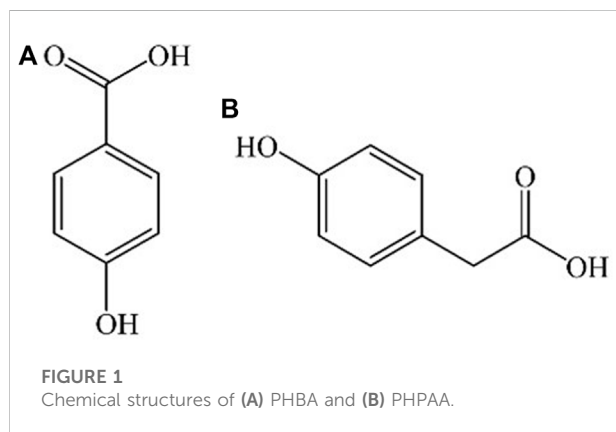
In recent years, some cities in China have experienced haze weather with particulate matter as the main pollution feature, and the ambient air quality is facing a severe test, which is extremely adverse to the national health and socio-economic development (Lin et al., 2018). PM₁₀ (diameter of inhalable particles which are less than 10 μ m) and PM_{2.5} (diameter of inhalable particles which are less than

2.5 μm and can enter the lung) are the main pollutant (Huang et al., 2014). The particle size of PM_{2.5} is smaller, resulting in a larger surface area, which makes it easy to be absorbed. At the same time, PM_{2.5} can stay in the atmosphere for a long time, and can travel a long distance (Han et al., 2015; Ji and Zhao., 2015). Therefore, PM_{2.5} has a great impact on people's health and air quality.

The sources of PM_{2.5} are very complex, mainly including emissions of combustibles, particles generated in chemical processes, human activities, equipment operation, cleaning and cooking (Ji and Zhao., 2015; Li et al., 2016; Zhou et al., 2016). PM_{2.5} is a major threat to human health and can affect many systems of the body, including the central nervous system, blood system, metabolic immune system, urogenital system, digestive system and skin (Boothe et al., 2014; Park and Wang., 2014; Piao et al., 2018; Geng et al., 2019; Jeong et al., 2019; Shaffer et al., 2019). Therefore, it is very important for human health to develop new drugs to treat PM_{2.5}.

Jacobaea cannabinifolia (Less.) E.Wiebe is a plant of *Senecio* genus, which is a genuine medicinal material in Northeast China. *J. cannabinifolia* has anti-inflammatory, bacteriostatic, anti-virus, immune regulation, anti-tumor, and other effects (Li et al., 2005; Chen et al., 2015). It is clinically used to treat acute and chronic bronchitis, asthmatic bronchitis and acute respiratory tract infection. The main metabolites of the herb are flavonoids, phenolic acids, alkaloids, volatile oils, glycosides, and tannins. P-hydroxybenzoic acid (PHBA) and p-hydroxyphenylacetic acid (PHPAA) (Figure 1) are the main phenolic acids in *J. cannabinifolia* and they have anti-inflammatory and other activities (Zhao et al., 2013; Sun et al., 2014).

This study aimed to explore the effect of PHBA and PHPAA on inflammation induced by PM_{2.5} and its possible mechanism, to provide an experimental basis for PHBA and PHPAA to become clinical therapeutic drugs for lung injury caused by PM_{2.5}.



2 Materials and methods

2.1 Chemicals and reagents

PHBA (≥99%) and PHPAA (98%) were provided by Sigma Chemical Co. (St. Louis, MO, United States). PM_{2.5} particles were provided by Hong-Hai Yu, senior engineer of Huadian Electric Power Research Institute Co., Ltd. Northeast Branch. Mouse tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and interleukin-1β (IL-1β) ELISA kits were purchased from Biyuntian Biotechnology Research Institute. TLR4, TLR2, TRAF6, NF-κB, MyD88, IRAK1, TAK1, and IKKβ polyclonal antibody IgG were obtained from Proteintech Group Inc (Chicago, IL, United States).

2.2 Cell culture

RAW264.7 murine macrophage cells were purchased from cell bank of Chinese Academy of Science, Shanghai. Cells were maintained in Dulbecco's modified Eagle's medium (Hyclone,

TABLE 1 Sequences of primers used for qRT-PCR.

Gene	Strand	The sequence of the primer (5'-3')	bp
TLR2	Forword	GAGCATCCGAATTGCATCACC	174
	Reverse	CCCAGAAGCATCACATGACAGAG	
TLR4	Forword	CATGGATCAGAAACTCAGCAA AGTC	179
	Reverse	CATGCCATGCCTTGTCTTCA	
NF-κB	Forword	GAAGCCGCTGACCATGGAA	103
	Reverse	GATCACAGCCAAGTGAGTGGA	
MyD88	Forword	TACAGGTGGCCAGAGTGGAA	119
	Reverse	GCAGTAGCAGATAAAGGC ATCGAA	
IRAK1	Forword	CGGACTTCCACAGTTCGAGGTA	125
	Reverse	TGACCAGCAAGGGTCTCCAG	
TRAF6	Forword	TCATTATGATCTGGACTGCCCAAC	150
	Reverse	TTATGAACAGCCTGGGCCAAC	
TAK1	Forword	AGCAGAGTAGCTGCGGT	134
	Reverse	GAGGAGCTTGCTGCAGAT	
IKKβ	Forword	CAGAATCATCCATCGAGA CCTGAA	122
	Reverse	TGCACAGACTGCCCTGATCC	
GAPDH	Forword	TGTGTCCGTCGTGGATCTGA	150
	Reverse	TTGCTGTTGAAGTCGCAGGAG	

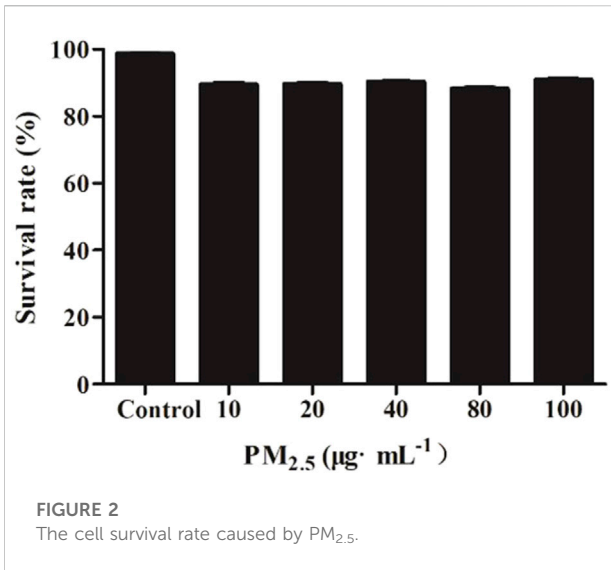


FIGURE 2
The cell survival rate caused by PM_{2.5}.

2.3 Determination of cell viability

The cell viability was determined by MTT method after treating with PM_{2.5}. Collect RAW264.7 cells growing in logarithmic phase, add culture medium to make cell suspension, adjust cell concentration, and add 100 µl cell suspension to each well, and adjust the cell density to 1.0 × 10⁴/well. The cells were incubated in an environment of 5% CO₂ and 37°C. When the cells covered the bottom of 96 wells, a series of concentrations of PM_{2.5} were added. After incubation for 48 h, add 10 µl MTT to each hole, culture the cells for 4 h, discard the culture medium, and carefully rinse it out with PBS for 2–3 times, then add 100 µl DMSO to each hole. Vibrate the shaking table at a low speed for 10 min, set the OD value to 490 nm on the microplate reader and conduct a light absorption measurement for each test hole, and calculate the cell survival rate. Survival % = (A_{490 nm} for treated cells/A_{490 nm} for control cells) × 100%, where A_{490 nm} represents the absorbance value.

Logan, UT) supplemented with 5% FBS (Gibco, United States), 1% streptomycin and penicillin (Hyclone) at 37°C in 5% CO₂ in a humidified atmosphere incubator.

2.4 Annexin V-FITC/PI apoptosis assay

RAW264.7 cells were pretreated with PM_{2.5} (0, 10, 20, 40, 80, 100 µg/ml) for 48 h. Cells were digest with trypsin,

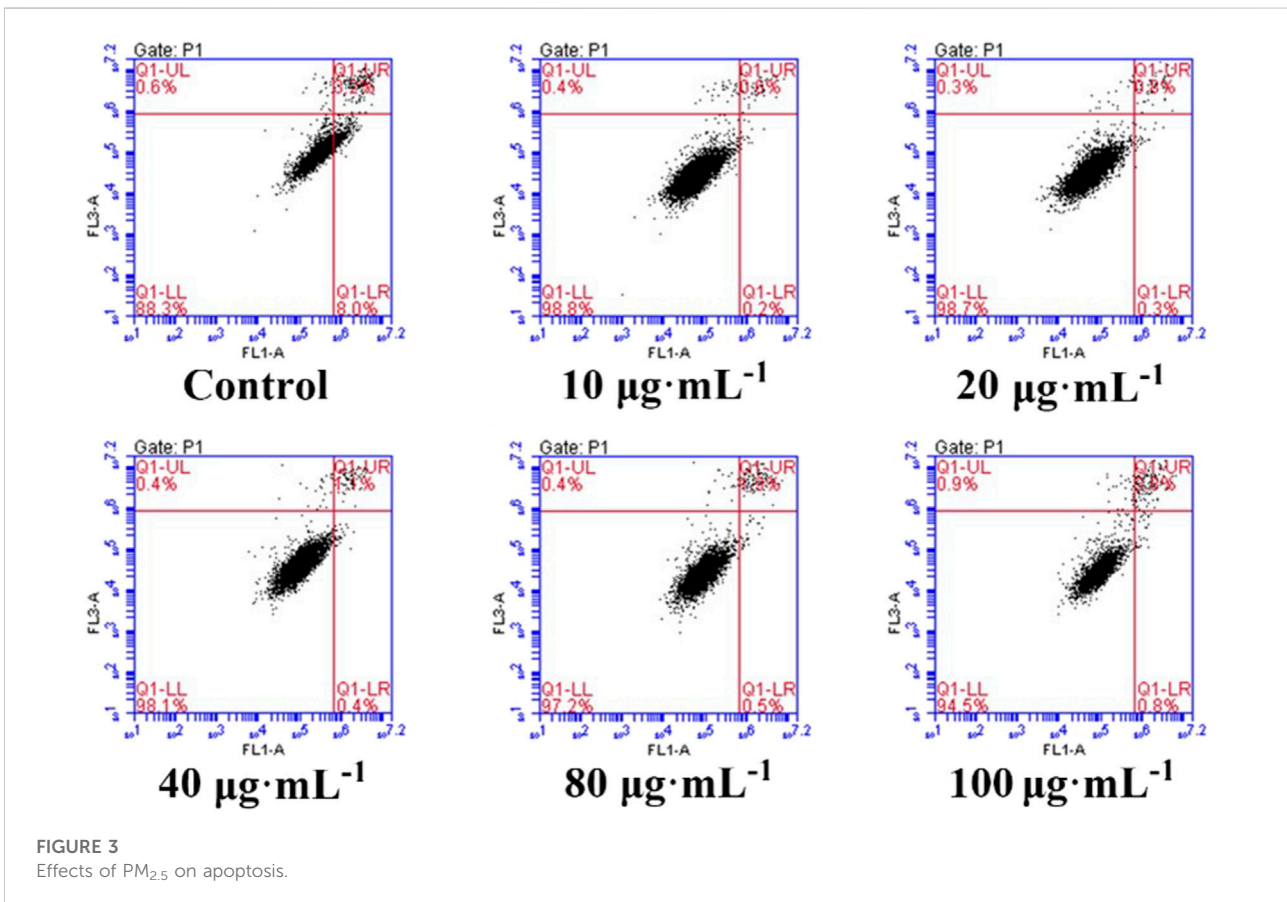
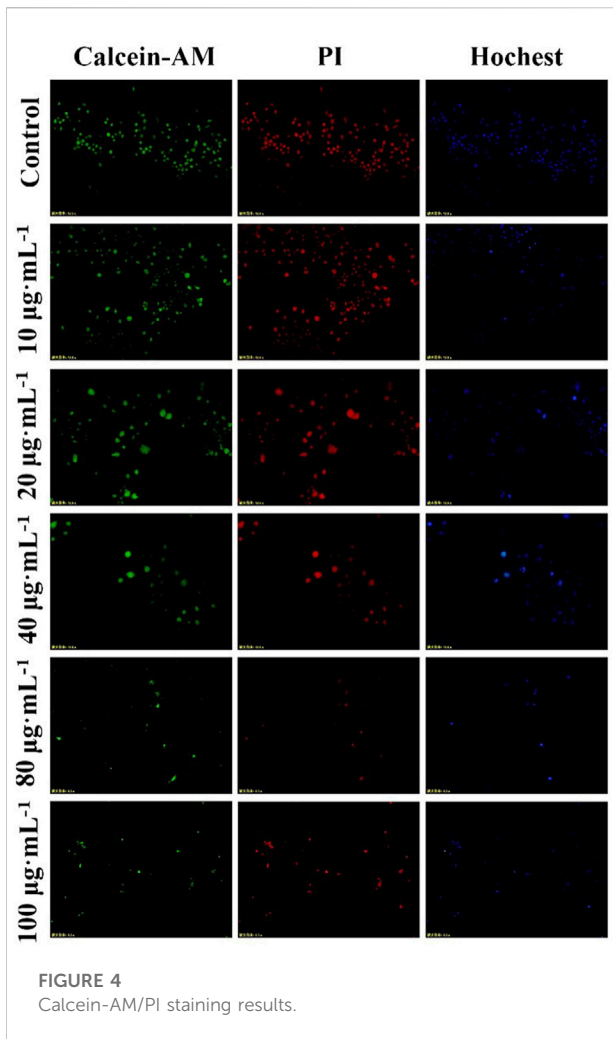


FIGURE 3
Effects of PM_{2.5} on apoptosis.



collected by centrifugation and washed twice with PBS. 1×10^6 cells were suspended in 500 μ l binding buffer followed by the addition of 10 μ l Annexin V-FITC. After incubation at room

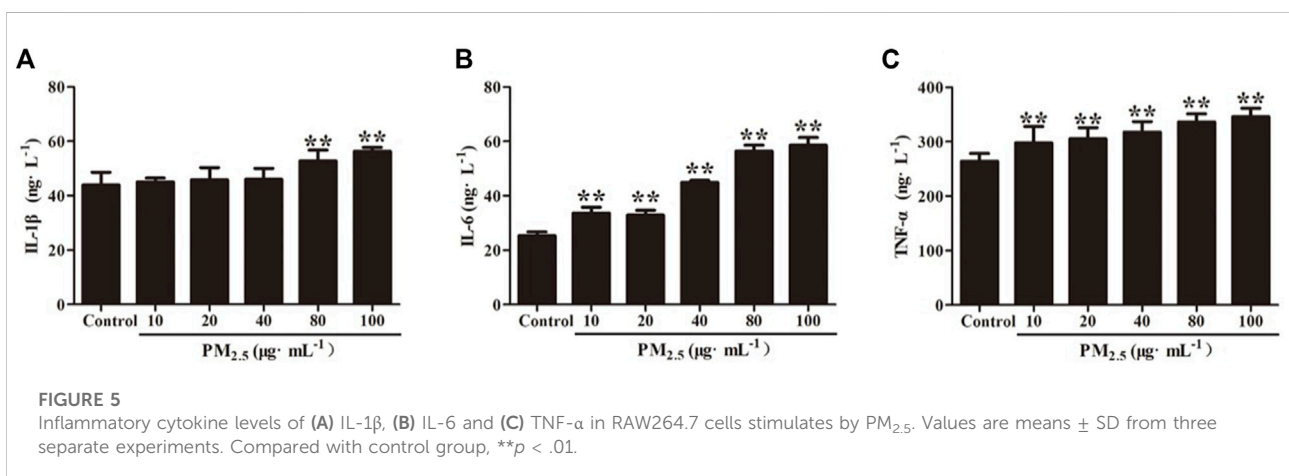
temperature in dark for 15 min, add 5 μ l PI and the cells were then analyzed using a flow cytometer (BD Accuri, US).

2.5 Calcein AM/PI staining

RAW264.7 cells were seeded in a 6-well plate at a density of 1×10^6 cells/well and cultured overnight in a 5% CO₂ incubator at 37°C to adhere. The cells were incubated with different concentrations of PM_{2.5} for 48 h. According to the instructions, the cells were gently washed with PBS twice. A small amount of about 200 μ l suspension was added to the test tube, and then an appropriate amount of 100 μ l dye solution was added. Incubate the cell suspension at 37°C for 15 min and then the fluorescence microscope was used to observe the cell staining.

2.6 Determination of cytokines by ELISA

Determination of the effect of PM_{2.5} on the production of inflammatory factors in RAW264.7 cells by ELISA. RAW264.7 cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and cultured overnight in a 5% CO₂ incubator at 37°C. After cell adhesion, different concentrations of PM_{2.5} (0, 10, 20, 40, 80, and 100 μ g/ml) were added into the culture medium and the cells were continued to culture for 48 h. Then, the concentration of IL-1 β , IL-6, and TNF- α in each was measured according to the ELISA instruction with a microplate reader at 450 nm. The effects of PHBA and PHPAA on inflammatory cytokines induced by PM_{2.5} were also determined using ELISA method and the duration of drugs was 24 h. The concentrations of PHBA or PHPAA were 10, 100, and 1,000 μ M. In the mixture of PHBA and PHPAA, the molar ratio was 1:1.



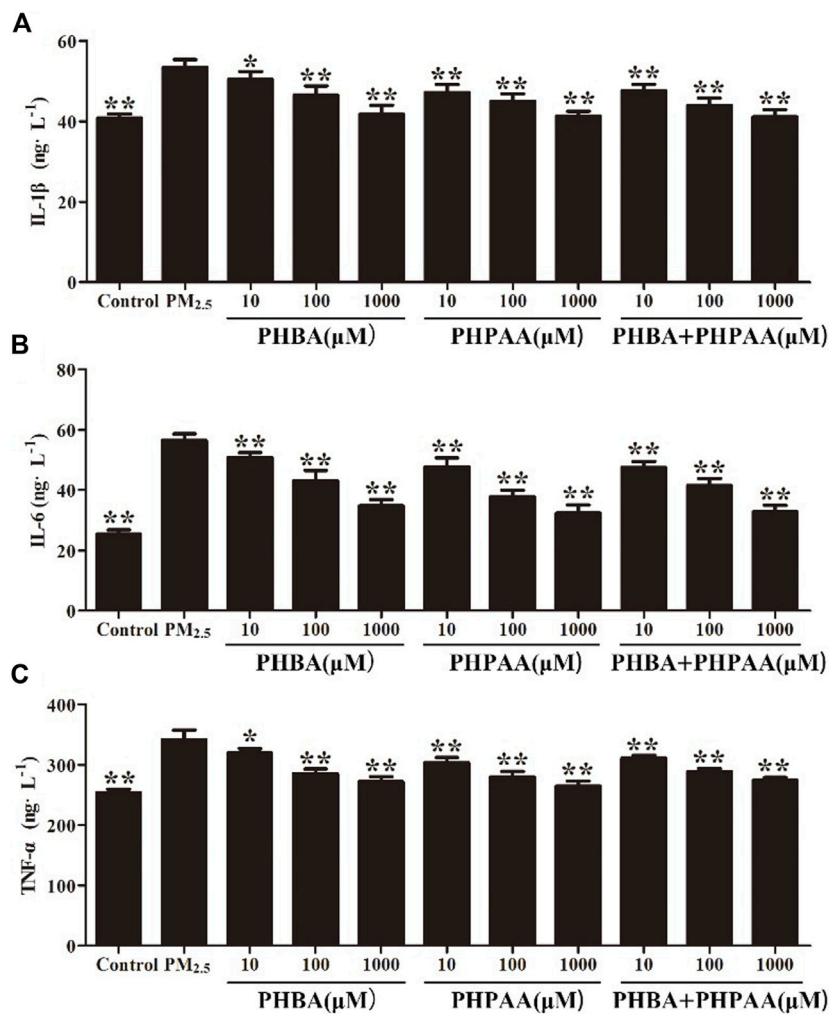


FIGURE 6
Inhibition of PHBA and PHPAA on inflammatory cytokines produced by PM_{2.5}. (A–C), Relative levels of IL-1β, IL-6, and TNF-α levels at 48 h assessed by ELISA. Values are means ± SD from three separate experiments. Compared with PM_{2.5} stimulation group, **p* < .05, ***p* < .01.

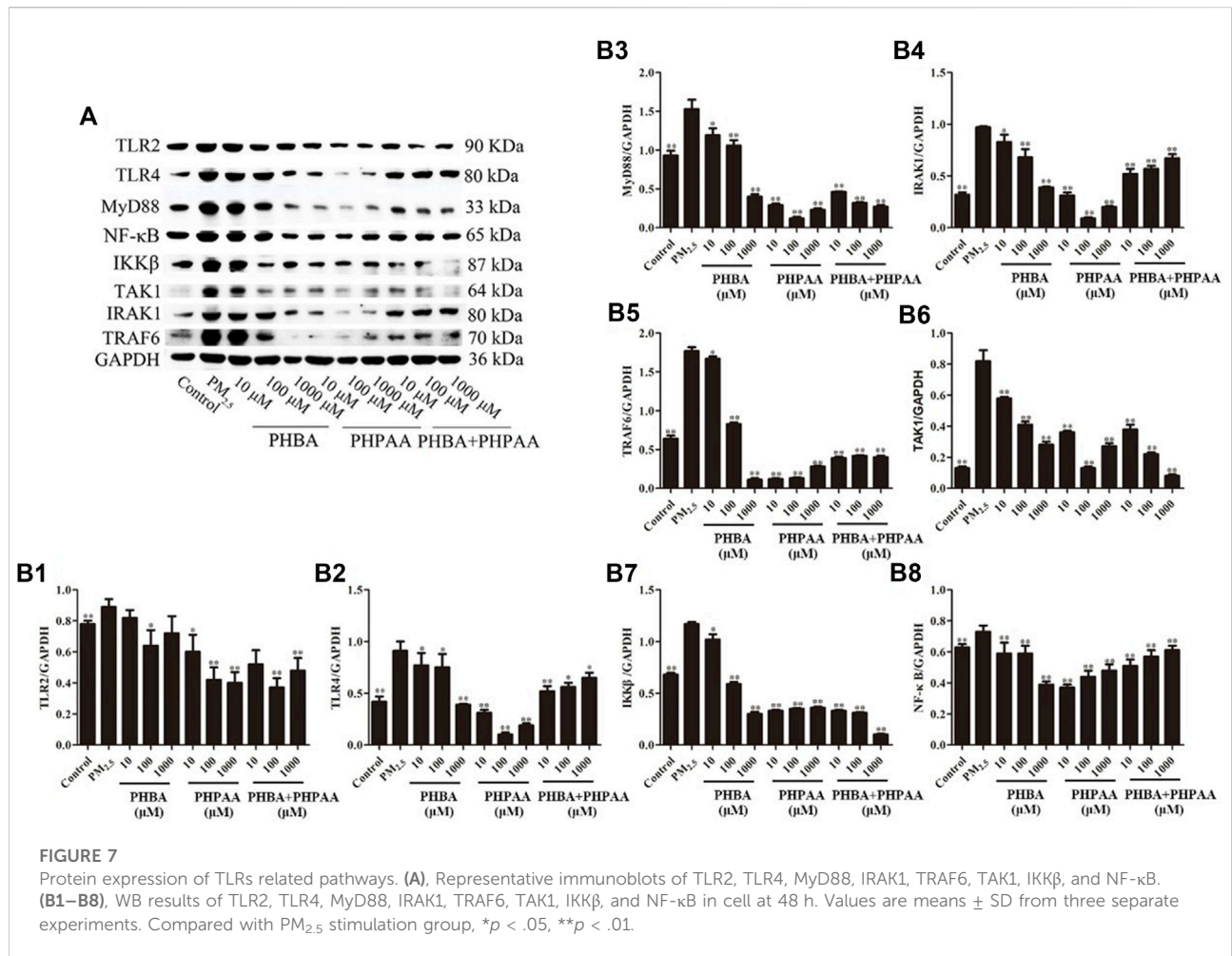
2.7 Quantitative real-time PCR

RAW264.7 cells (2×10^6 cells/well) were seeded in a 6-well plate, pretreated with PM_{2.5} (80 μg/L) for 48 h, and then treated with PHBA, PHPAA or mixture of PHBA and PHPAA for 24 h. Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, United States) was used to extract the total RNA of the cells pretreated with PM_{2.5}. Detection of mRNA expression with Applied Biosystems ViiATM 7 Real-Time PCR system. cDNA synthesis was performed using a PrimeScript™ RT Reagent Kit according to the manufacturer’s instructions. After reverse transcription, the cDNA was amplified using SYBR-Green Premix (Takara, Otsu, Japan). Level of TLR2, TLR4, MyD88, IRAK1, TRAF6, TAK1, IKKβ, and NF-κB mRNA expressions were examined, and GAPDH was used as an internal control.

Data were analyzed by the $2^{-\Delta\Delta CT}$ method. Sequences of primers used for qRT-PCR are list in Table 1.

2.8 Western blot

After overnight culture in a 6-well plate (2×10^6 cells/well, 3 ml medium/plate), the cells were pre-treated with PM_{2.5} for 48 h and drugs for an additional 24 h, cells were harvested and lysed in lysis buffer for 15 min on ice. After incubation, lysates were centrifuged and supernatant was collected. The protein concentration was measured by the bicinchoninic acid (BCA) method. 20 μg protein sample was separated on 10% SDS-PAGE and transferred to PVDF membranes. At room temperature, the membrane was sealed with 5% skimmed



milk powder dissolved in triple buffered saline containing .1% Tween-20 (TBST) for 1 h. Then incubate the membrane with the primary antibody that recognized TLR2, TLR4, MyD88, IRAK1, TRAF6, TAK1, IKKβ, NF-κB, and GAPDH in a shaking incubator at 4°C overnight, wash it with TBST three times, and incubate it with the secondary antibody conjugated with peroxidase diluted in the closed solution at room temperature for 1 h. After washing, proteins of interest were detected using ECL detection reagent.

2.9 Statistical analysis

Statistical analyses were performed using the SPSS 24.0 software. The data were expressed as the Mean ± SD. Multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett’s posttest. Statistical significance was accepted at *p* < .05 or *p* < .01.

3 Results

3.1 Effect of PM_{2.5} on RAW264.7 cells

After administration of PM_{2.5} at different concentrations, the survival rate of RAW264.7 cells decreased, but there was no statistical difference compared with the control group, indicating that PM_{2.5} within the measurement range could not inhibit the growth of RAW264.7 cells (Figure 2). It was found by flow cytometry that compared with the control group, PM_{2.5} of all concentrations did not induce apoptosis (Figure 3). Fluorescence staining results showed that the number of dead cells (red fluorescence) did not increase significantly after the action of PM_{2.5} of various concentrations. Hochest staining results showed that the nuclear morphology of each PM_{2.5} group did not change significantly, and no apoptotic cells were observed (Figure 4).

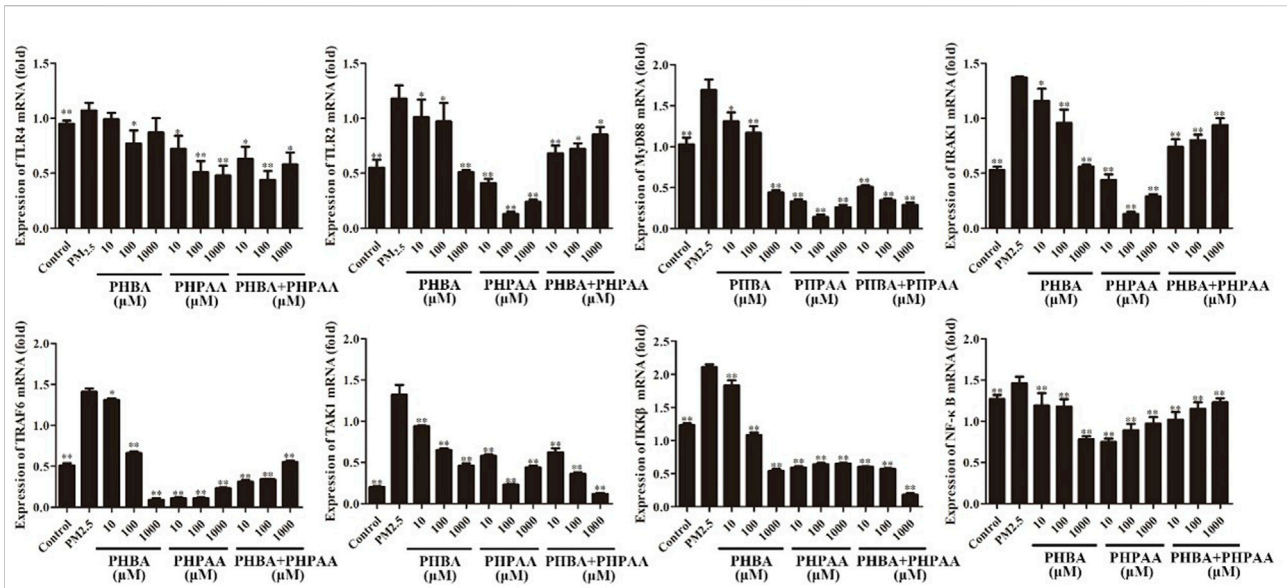


FIGURE 8
Gene expression of TLRs related pathways. Values are means \pm SD from three separate experiments. Compared with PM_{2.5} stimulation group, * $p < .05$, ** $p < .01$.

TABLE 2 Effects of PHBA on gene expression in TLRs related pathways.

Name	Control	PM _{2.5}	PHBA(μ M)		
			10	100	1,000
TLR2	.95 \pm .03**	1.07 \pm .07	.99 \pm .06	.77 \pm .12*	.87 \pm .13
TLR4	.55 \pm .073**	1.18 \pm .12	1.01 \pm .16*	.97 \pm .17*	.51 \pm .02**
MyD88	1.03 \pm .08**	1.69 \pm .13	1.31 \pm .11*	1.17 \pm .08**	.44 \pm .03**
IRAK1	.53 \pm .03**	1.37 \pm .01	1.16 \pm .11*	.96 \pm .12**	.56 \pm .02**
TRAF6	.51 \pm .03**	1.41 \pm .04	1.34 \pm .02*	.66 \pm .02**	.09 \pm .02**
TAK1	.20 \pm .01**	1.32 \pm .12	.94 \pm .01**	.65 \pm .02**	.46 \pm .03**
IKK β	1.23 \pm .03**	2.11 \pm .04	1.83 \pm .08*	1.08 \pm .04**	.54 \pm .03**
NF- κ B	1.27 \pm .05**	1.46 \pm .08	1.19 \pm .15**	1.18 \pm .09**	.78 \pm .04**

Note: Compared with PM_{2.5} stimulation group, * $p < .05$, ** $p < .01$.

3.2 Effect of PM_{2.5} on inflammatory cytokines produced by RAW264.7 cells

The inflammatory factors produced by RAW264.7 cells stimulated by PM_{2.5} were detected by ELISA, and the results showed that 80 μ g/ml and 100 μ g/ml PM_{2.5} produced significantly higher IL-1 β , IL-6, and TNF- α than that of the control group ($p < .01$). Compared with the control group, there was no difference of IL-1 β concentration produced by RAW264.7 cells that stimulated by 10, 20 or 40 μ g/ml PM_{2.5} ($p > .05$) (Figure 5). Through microscopic

observation, high concentration of PM_{2.5} will cause unclear observation field of vision, so 80 μ g/ml of PM_{2.5} was applied in subsequent experiments.

3.3 Inhibitory effect of PHBA and PHPAA on inflammatory factors produced by PM_{2.5}

Compared with the control group, PM_{2.5} significantly increased the production of inflammatory factors ($p < .01$).

TABLE 3 Effects of PHPAA on gene expression in TLRs related pathways.

Name	Control	PM _{2.5}	PHPAA (μM)		
			10	100	1,000
TLR2	.95 ± .03**	1.07 ± .07	.72 ± .12*	.51 ± .1**	.48 ± .09**
TLR4	.55 ± .073**	1.18 ± .12	.41 ± .04**	.13 ± .02**	.24 ± .02**
MyD88	1.03 ± .08**	1.69 ± .13	.33 ± .03**	.14 ± .03**	.26 ± .03**
IRAK1	.53 ± .03**	1.37 ± .01	.44 ± .05**	.13 ± .02**	.29 ± .02**
TRAF6	.51 ± .03**	1.41 ± .04	.11 ± .01**	.11 ± .01**	.23 ± .01**
TAK1	.20 ± .01**	1.32 ± .12	.58 ± .02**	.23 ± .01**	.44 ± .02**
IKKβ	1.23 ± .03**	2.11 ± .04	.59 ± .02**	.64 ± .02**	.65 ± .01**
NF-κB	1.27 ± .05**	1.46 ± .08	.75 ± .04**	.89 ± .08**	.97 ± .08**

Note: Compared with PM_{2.5} stimulation group, **p* < .05, ***p* < .01.

TABLE 4 Effects of PHBA combined with PHPAA on gene expression in TLRs related pathways.

Name	Control	PM _{2.5}	PHBA + PHPAA (μM)		
			10	100	1,000
TLR2	.95 ± .03**	1.07 ± .07	.63 ± .11*	.44 ± .08**	.58 ± .11*
TLR4	.55 ± .07**	1.18 ± .12	.68 ± .07**	.72 ± .05*	.85 ± .07*
MyD88	1.03 ± .08**	1.69 ± .13	.51 ± .02**	.35 ± .02**	.29 ± .03**
IRAK1	.53 ± .03**	1.37 ± .01	.74 ± .07**	.8 ± .05**	.94 ± .06**
TRAF6	.51 ± .03**	1.41 ± .04	.31 ± .02**	.34 ± .01**	.55 ± .02**
TAK1	.20 ± .01**	1.32 ± .12	.62 ± .05**	.36 ± .02**	.12 ± .01**
IKKβ	1.23 ± .03**	2.11 ± .04	.60 ± .01**	.57 ± .01**	.18 ± .02**
NF-κB	1.27 ± .05**	1.46 ± .08	1.02 ± .09**	1.15 ± .08**	1.23 ± .05**

Note: Compared with PM_{2.5} stimulation group, **p* < .05, ***p* < .01.

After different concentrations of PHBA or PHPAA (10, 100, 1,000 μM) treatment, the content of IL-1 β, IL-6, and TNF- α significantly decreased (*p* < .01). After combined treatment of PHBA and PHPAA (the molar ratio of PHBA and PHPAA was 1:1), the content of IL-1 β, IL-6, and TNF- α significantly decreased (*p* < .01) (Figure 6).

3.4 Effect of PHBA and PHPAA on TLRs related signal pathway protein expression

Compared with the control group, the protein expression of TLRs (TLR2 and TLR4) and related receptors MyD88, IRAK1, TRAF6, TAK1, IKKβ, and NF-κB increased significantly after PM_{2.5} stimulation (*p* < .01) (Figure 7). After treating RAW264.7 cells stimulated by PM_{2.5} with different

concentrations of PHBA, the expressions of TLR2, TLR4, MyD88, IRAK1, TRAF6, TAK1, IKKβ, and NF-κB decreased, and the changes showed a dose-effect relationship. However, when treated with a high concentration of PHBA (1,000 μM), the protein expression of TLR2 increased (Figure 7). Treating RAW264.7 cells stimulated by PM_{2.5} with different concentrations of PHPAA (10, 100, 1,000 μM) could make the protein expression of TLR2, TLR4, MyD88, IRAK1, TRAF6, TAK1, IKKβ, and NF-κB significantly decreased (*p* < .01). The protein expressions of TLR4, MyD88, IRAK1, TRAF6, TAK1, IKKβ, and NF-κB were increased when PHPAA was treated with high concentration (1,000 μM) (Figure 7). The combination of PHBA and PHPAA (1:1) also significantly decreased the expression of TLRs pathway proteins (*p* < .01), but increased the expression of TLR2, TLR4, IRAK1 and NF-κB proteins at high concentrations (1000 μM) (Figure 7).

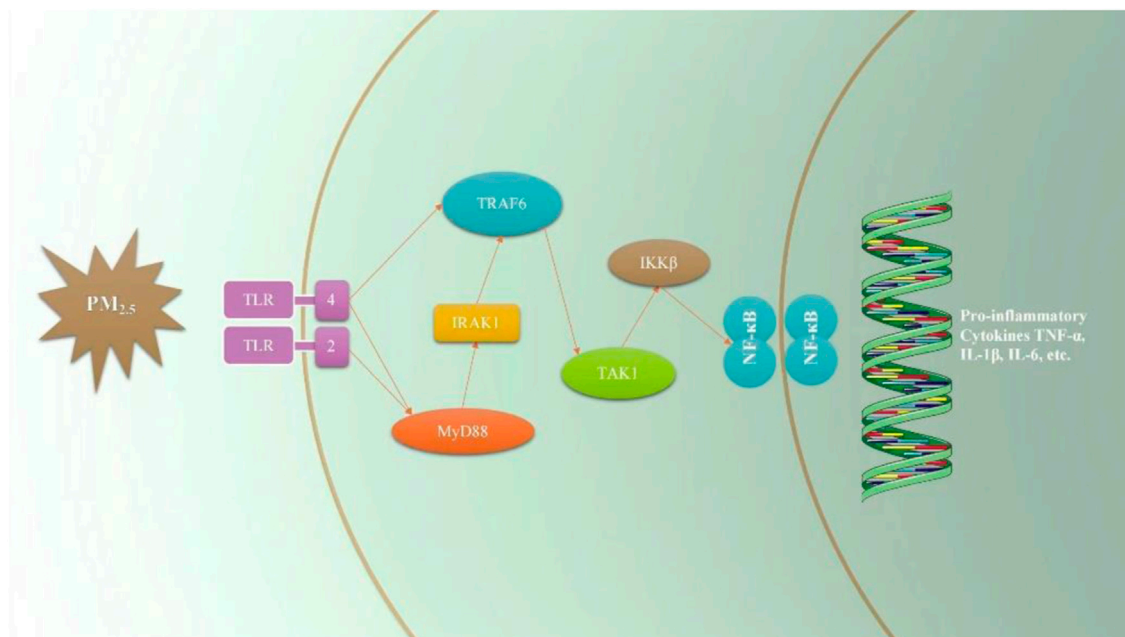


FIGURE 9
Sketch of the effect of $PM_{2.5}$ on TLRs signal pathway.

3.5 Effects of PHBA and PHPAA on TLRs signal pathway gene expression

The gene expressions of TLRs and its downstream related pathway were determined by RT-qPCR. $PM_{2.5}$ can significantly up-regulate the gene expression of TLR2, TLR4, MyD88, IRAK1, TRAF6, TAK1, IKK β and NF- κ B ($p < .01$) (Figure 8, Table2). After treating RAW264.7 cells stimulated by $PM_{2.5}$ with drugs for 24 h, the TLRs and its downstream pathway related genes were detected. The results showed that PHBA could significantly down regulate the TLRs related pathway genes in a dose effect relationship ($p < .01$) (Figure 8; Table2). After the cells stimulated by $PM_{2.5}$ were treated with PHPAA, the gene expression of TLRs pathway was significantly decreased ($p < .01$). When PHPAA concentration is high (1,000 μ M), the expression of related genes TLR4, MyD88, IRAK1, TRAF6, TAK1, IKK β , and NF- κ B increased (Figure 8; Table3). When the mixture of PHBA and PHPAA (1:1) was used, the gene expression of TLRs pathway was significantly decreased ($p < .01$). When the mixture is at high concentration (1,000 μ M), the expression of TLR2, TLR4, IRAK1 and NF- κ B significantly increased (Figure 8; Table4).

4 Discussion

$PM_{2.5}$ has a great impact on the central nervous system, blood system, metabolic immune system, digestive system, skin

and other human bodies, and may lead to a variety of diseases. $PM_{2.5}$ can destroy the integrity of the blood brain barrier (BBB) and make it easy for peripheral systemic inflammation to pass through the BBB to reach the central nervous system (Shou et al., 2019). The study found that the concentration of $PM_{2.5}$ had a certain toxic effect on children's bone marrow stromal cells, which affected the hematopoietic microenvironment of bone marrow. $PM_{2.5}$ may activate PINK1/Parkin pathway signal and induce mitochondrial autophagy by increasing ROS, and further activate HSCs (hepatic stellate cells) to cause liver fibrosis (Qiu et al., 2019). Cholesterol and squalene are key substances that affect the skin barrier function. $PM_{2.5}$ can cause barrier disorder by increasing cholesterol synthesis, leading to the temporary accumulation of epidermal cholesterol and the reduction of squalene (Liao et al., 2019). It can be seen that $PM_{2.5}$ is harmful to human health. It is very important to find effective drugs to treat $PM_{2.5}$ as soon as possible.

It has been reported that $PM_{2.5}$ can activate the inflammatory axis of vascular endothelial cell COX-2/PGES/PGE2, and promote cell apoptosis and inflammatory response (Yin et al., 2017). $PM_{2.5}$ may induce apoptosis by increasing lipid accumulation, ROS level and activating mitochondrial pathway of macrophages. However, the results of MTT, flow cytometry and Calcein AM/PI staining showed that $PM_{2.5}$ used in our study had no cytotoxicity to RAW264.7 and had no effect on apoptosis. The experimental results obtained in this study are different from those in related papers, which may be related to

the physical and chemical properties of PM_{2.5} collected, processing and preservation methods of PM_{2.5}.

It is reported that PM_{2.5} can cause airway inflammation and lung injury in mice, and can also produce cellular inflammation and secrete inflammatory factors from RAW264.7 cells (He et al., 2017; Ogino et al., 2018). In this study, PM_{2.5} was used to stimulate RAW264.7 cells, and the related inflammatory factor IL-1 β , IL-6, and TNF- α secreted by cells significantly increased. However, when PHBA, PHPAA, the mixture of PHBA and PHPAA (1:1) were given in advance, the detection results of inflammatory factors secreted by cells were significantly reduced. It suggests that PHBA and PHPAA can repair the damage caused by PM_{2.5} through inhibiting inflammatory factors.

In the induction of inflammatory response, TLRs play a role mainly through MyD88 and TRIF mediated pathways (Deguine and Barton, 2014). When sensing external stimuli, TLRs allow MyD88 to dock with MAL (MyD88 adapter-like). MAL is a bridging linker, often involved in TLR4 signal pathway, while TLR2 signal pathway is less involved, and interacts with MyD88 through TIR. In addition to the TIR domain, MyD88 also contains a dead domain, which can help it interact with IRAK4 (Janssens and Beyaert, 2003; Lim and Staudt, 2013). The interaction between these domains produces a large polymer, and the nitration of this polymer leads to the activation and dimerization of TRAF6 (Lin et al., 2010). TRAF6 mediates the ubiquitination of TAK1. The lack of TAK1 reduces the inflammatory signal of TLRs, but this phenomenon is not observed when TAB protein is lacking. TAK1 signal then activate NF- κ B and MAPK, respectively. NF- κ B is a molecular center of inflammation signal, and activated by phosphorylated IKK α and IKK β (Kawai and Akira, 2007). TRF signal is a separate branch of TLR signal, which can only be continued through TLR3 and TLR4, where TRIF interacts with TRAF3 and TRAF6 (Akira et al., 2006). In this study, the protein and gene of TLR pathway related factors were determined. It was found that PM_{2.5} can increase the expression of TLR2/4 \rightarrow MyD88 \rightarrow IRAK1 (TRAF6) \rightarrow TAK1 \rightarrow IKK β \rightarrow NF- κ B protein and gene in TLR pathway and eventually lead to inflammation (Figure 9). When PHBA, PHPAA and the mixture of PHBA and PHPAA (1:1) were added to PM_{2.5}-stimulated RAW264.7 cells, the above three factors could reduce the expression of TLRs related pathway proteins and genes. These results indicate that PHBA and PHPAA can reduce inflammation caused by PM_{2.5} by regulating TLRs and their related pathway signal transduction factors.

In this study, we measured the protein and gene of TLR pathway related factors and found that PM_{2.5} can increase the protein and gene expression of TLR pathway and eventually lead to inflammation. PHBA, PHPAA and the mixture of PHBA and PHPAA (1:1) can reduce the expression of TLRs related pathway proteins and genes of RAW264.7 cells stimulated by PM_{2.5}, indicating that PHBA and PHPAA can reduce the inflammation produced by PM_{2.5} by regulating TLRs and its related pathway signal transduction factors.

5 Conclusion

PM_{2.5} can produce inflammatory reaction and secrete inflammatory cytokines. The molecular mechanism of inflammation produced by PM_{2.5} is related to TLRs and its related pathways, and TLRs pathway can become a potential new target for treating inflammation produced by PM_{2.5}. PHBA, PHPAA and their combination can reduce the inflammatory reaction produced by PM_{2.5}. The mechanism of action is related to the inhibition of TLRs and its related pathways, indicating that TLRs signal pathway may be a potential pathway for PHBA and PHPAA to treat inflammation induced by PM_{2.5}.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

Study design: B-LX and X-BW; data collection: B-LX, Y-YW, L-LJ, and ZL, D-RL, HZ, and S-LL; Analysis and interpretation: B-LX, Y-YW, and L-LJ; Statistical analysis: Y-YW; Drafting manuscript: B-LX; Revision manuscript: X-BW.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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