Study On Detoxification Mechanism of Three Traditional Dai Antidotes

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Research

Keywords: Dai antidote, C. chinense var. simplex, M. tenacissima, A. graminifolia, Detoxification

Posted Date: August 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-751318/v1

Abstract

Background: Dai antidotes are the most distinctive medicine and treatment in traditional Dai medicine. *Clerodendrum chinense* var. *simplex*, *Marsdenia tenacissima (Roxb.) Moon* and *Arundina graminifolia (D. Don) Hochr* are three Dai antidotes widely used for their "detoxifying effects", and their use is rooted in a theoretical system significantly different and much less understood than Western or traditional Chinese medicines. This study aims to provide the mechanistic insights into the detoxifying effects of Dai antidotes, providing a modern scientific basis for understanding and developing Dai medicine as well as encouraging its use in other regions.

Methods: We successively extracted the three Dai antidotes using petroleum ether, ethyl acetate, *n*-butanol, or water, and prepared their decoctions. Their contents of total flavonoids and polyphenols were determined by colorimetric method. Their antioxidant activity were tested by 1,1-diphenyl-2-picrylhydrazyl(DPPH), hydroxyl radical (·OH) and 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radicals scavenging assays. Anti-bacterial activity were evaluated in terms of minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and zones of inhibition (ZOI) against *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. Their anti-inflammatory activity were studied in macrophages stimulated with lipopolysaccharide (LPS) through measuring the production of nitric oxide, tumor necrosis factor-α, interleukin-1β, and interleukin-6.

Results: The content of total flavonoids in three Dai antidotes ranged from 22.41 to 586.39 mg/g, which is higher than the content of total polyphenols (2.76 to 28.66 mg/g). The various extracts were found to scavenge radicals of DPPH, ·OH and ABTS. They scavenged ABTS radicals much more efficiently (IC_{50} 9.54 μg/mL) than other radicals (IC_{50} > 380 μg/mL). They weakly inhibited the growth of *E. coli, P. aeruginosa* and *S. aureus.* Notably, even at low concentration 60 μg/mL, the extracts can significantly down-regulate nitric oxide production, tumor necrosis factor-α, interleukin-1β, and interleukin-6 by macrophages stimulated with lipopolysaccharide.

Conclusion: In conclusion, our results provide the first mechanistic insights into the detoxifying effects of three Dai antidotes, providing a basis for their optimization and for future research to strengthen Dai medicine through modern scientific practices.

Background

As one of the four major ethnomedicines in China [1], Dai medicine dates back more than 2500 years [2] and continues to play an important role in health care in China and many other countries of southeast Asia, especially in the Mekong River valley. Dai antidotes, which include *Bin Hao* (*C. chinense* var. *simplex)*, *Dai Bai Jie* (*M. tenacissima)* and *Zhu Ye Lan* (*A. graminifolia)* (Fig. 1), are widely used among Dai medical practitioners to detoxify body toxins obtained from food or animal bites [3]. Mainly, *Bin Hao* is used to treat cough, sore throat, rheumatic arthralgia and jaundice [4], *Dai Bai Jie*, to treat cough,

swelling and throat pain[5], and *Zhu Ye Lan*, to treat all kinds of poisoning caused by food and medicine, abdominal pain, diarrhea, dizziness and other diseases[6].

There are few studies, using modern biomedical techniques to analyze the clinical benefits of Dai antidotes limiting the use of Dai medicine. In fact, the Dai theoretical system remains completely outside from modern medical science and research. Compared with other types of traditional Chinese medicine, which already have a large number of literature and clinical practice integrated with western medicine, we are unaware the overlap and complementarity between the Dai theoretical system and traditional Chinese medicine or western medicine.

Dai medical theory holds that disease is related to imbalance among the four cosmic elements in the body, and that such imbalance can arise due to the presence of toxins [3]. This imbalance may be attributed to imbalance between free radicals and anti-oxidant defenses [7] or imbalance between pro- and anti-inflammatory factors, such as in excessive inflammation or infection with pathogens. All these imbalances can lead to a disease initiation and progression. Therefore, the present study used standard and well-established laboratory methods to assess the *in vitro* antioxidant, anti-bacterial and anti-inflammatory effects of the three Dai antidotes. In addition, the material basis of three Dai antidotes was explored by determining their contents of total polyphenols and flavonoids and their antioxidant, antibacterial and antiinflammatory potentials, as described in Fig. 1. The results may help explain the clinical efficacy of these traditional medicines, providing a modern scientific basis for understanding and developing Dai medicine for the treatment of various diseases as well as promoting their use in other regions of the world.

Methods

Plant Material, Reagents and Cells

The *C. chinense* var. *simplex*(*Bin Hao*), *M. tenacissima*(*Dai Bai Jie*) and *A. graminifolia*(*Zhu Ye Lan*) were purchased from the Institute of Ethnic Medicine (Xishuangbanna, Yunnan Province) and identified by Mrs. Lin Yanfang, Chief Expert of Dai Medicine. They were washed and dried for two weeks in the shade. Before extraction, the plants were cut into small pieces and crushed using a floor-standing continuous feed grinder (DF-35, Wenling Linda Machinery, Zhejiang, China).

DPPH, ABTS, hydrogen peroxide (H_2O_2, 30% wt), rutin, gallic acid and potassium persulfate were purchased from Aladdin (Shanghai, China); Luria–Bertani (LB) broth, from Hopebio (Qingdao, China); and *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), and *S. aureus* (ATCC 25923), from Huankai Guangzhou Microbial. RAW 264.7 macrophage cells were purchased from the Laboratory Animal Center of Sun Yat-sen University (Guangzhou, China).

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Gibco-Thermo-Fisher Scientific (Grand Island, NY, USA). LPS was purchased from Sigma Chemical (St. Louis,

USA). NO Griess reagent was acquired from Beyotime Institute of Biotechnology (Shanghai, China). IL-1β, IL-6 and TNF-α PicoKine ELISA kits were purchased from Boster Biological Technology (Wuhan, China). Deionized water (18.2 MΩ·cm) was used to prepare all aqueous solutions. All other reagents were purchased as analytical reagent grade and used without further purification.

Extraction of Dai antidotes

Crushed *Bin Hao, Zhu Ye Lan* and *Dai Bai Jie* (100 g) were extracted three times with 95% ethanol at a mass-to-volume ratio of 1:10 for 2 h at 98 ℃. The extracts were filtered, combined, and evaporated under reduced pressure to obtain crude ethanol extract.

The polar extracts were obtained from the corresponding crude extract at room temperature by successive extraction with the same volume of solvents of increasing polarity: petroleum ether, ethyl acetate, n-butanol and distilled water. The solvent was evaporated to dryness under reduced pressure in a rotary evaporator.

In this way, we obtained from *Bin Hao* a petroleum ether extract (PE, 1.1 g), ethyl acetate extract (EE, 1.3 g), *n*-butanol extract (BE, 4.2 g), and water extract (WE, 10.3 g). We obtained from *Zhu Ye Lan* a PE (1.7 g), EE (6.4 g), BE (8.6 g), and WE (9.3 g). We obtained from *Dai Bai Jie* an EE (13.5 g), BE (4.2 g), and WE (7.6 g). All polar extracts were stored at 4 ℃ until use.

Aqueous decoctions of the three Dai antidotes were prepared by mixing 100 g dried *Bin Hao, Zhu Ye Lan* or *Dai Bai Jie* with 1000 mL of distilled water and boiling for 0.5 h. This process was performed three times. The decoctions were filtered with gauze, combined and concentrated to 50 mL, giving crude Dai antidotes 2 g/mL. The extracts were stored at -20 ℃ until use.

Determination of total flavonoids content

The total flavonoids content of the samples was determined based on colorimetric method as described by Mohammad et al[8] with modifications. Briefly, 200 μL of samples, 200 μL of ethanol and 40 μL of 10% $NaNO_2$ was mixed to 48-well plates and allowed to stand for 7 minutes. Then, 40 μL of 5% $Al(NO_3)_3$ solution was added. After 7 minutes, 400 μL of 1mol/L NaOH and 120 μL ethanol was added to the solution. The obtained mixture was shaken for 20 minutes and absorbance was measured at 510 nm. In the same way, the standard solution was prepared with rutin in a series of concentration gradients, and the standard curve was drawn to calculate the flavonoids content (mg RE /g DW).

The linear equation was obtained by taking rutin concentration (x) as the horizontal coordinate and absorbance (y) as the ordinate. The linear equation was: y = 0.00169x-0.01121 and the correlation coefficient R^2 = 0.996. The experimental results showed that the absorbance of rutin was good linear relation in the range of 0.008 ~ 0.04 mg / mL. According to linear equation, the content of total flavonoids in three Dai antidotes was obtained.

Determination of total polyphenols content

The total polyphenols content of Dai antidotes was determined by using Folin–Ciocalteu reagent as described by Mohammad et al[9] with modifications. Briefly, 0.2 mL of samples, 6 mL of ethanol and 0.5 mL of Folin reagent was mixed to the 10 mL volumetric flask. After 5 minutes, 1.5mL of 20% (w/v)Na□CO□ was added,dilute with water to volume and incubated at room temperature for 60 minutes, then absorbance was measured at 765 nm. In the same way, the standard solution was prepared with gallic acid in a series of concentration gradients, and the standard curve was drawn to calculate the polyphenols content (mg GAE /g DW).

The linear equation was obtained by taking gallic acid concentration (x) as the horizontal coordinate and absorbance (y) as the ordinate. The linear equation was: y = 0.0374x-0.02602 and the correlation coefficient R^2 = 0.9995. The experimental results showed that the absorbance of gallic acid is good linear relation in the range of 0.001 ~ 0.006 mg / mL. According to linear equation, the content of total polyphenols in three Dai antidotes was obtained.

In Vitro **Anti-oxidant Activity**

The *in vitro* antioxidant activities of different polar extracts and decoctions of the three Dai antidotes were evaluated based on ability to scavenge DPPH free radicals, ·OH radicals and ABTS radicals. Absorbance was determined on an ultraviolet spectrophotometer (UV-2600, Techcomp, Shanghai, China).

The scavenging effects on DPPH free radical was determined by the method as described by Shimada et al [10]with modifications 3.0 mL of DPPH(0.1mM)was intermingled with 1 mL of each sample (with final concentrations ranging from 0.2 to 1.2mg/mL) and allowed to stand at 37℃ for 30 min. The absorbance was then measured at 517 nm.

The scavenging DPPH free radical effect was calculated according to the following equations:

$$\mathrm{Scavengingeffect}(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\%$$

Where A_0 is the absorbance of the control (water rather than the sample), and A_1 is the absorbance of the samples, A_2 is the absorbance of the sample only(water rather than DPPH).

The scavenging effects on hydroxyl radical(·OH) was determined based on Fenton's reaction as described by Aquino-Martins et al [11] with modifications. 1 mL of samples with different concentrations was mixed with 1mL 9 mM $FeSO_4$ solution, 9 mM salicylic acid ethanol solution, and 8.8 mM H_2O_2 solution, respectively, and incubated at 37℃ for 30min, then absorbance was measured at 510 nm.

The scavenging ·OH effect was calculated according to the following equations:

$$\text{Scavengingeffect}(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\%$$

Where A_0 is the absorbance of the control, A_1 is the absorbance of the samples, A_2 is the absorbance of the sample background.

The protocol of scavenging effects on ABTS free radical was adapted from Roberta Re et al.[12], ABTS reagent (7.0 mM) was mixed with 2.45 mM potassium persulfate in a volume ratio of 1: 1, and allowing the mixture to stand in the dark at room temperature overnight to obtain an ABTS stock solution. Then the ABTS stock solution was diluted with deionized water to obtain ABTS working solution with an absorbance value of 0.70 ± 0.05 at 734 nm. 4.0 mL of ABTS working solution was intermingled with 1 mL of each sample, and incubated at 37℃ in dark for 30 min. The absorbance was then measured at 734 nm. The scavenging ABTS free-radical effect according to the following equations:

$$\text{Scavengingeffect}(\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100\%$$

Where A_0 is the absorbance of the control (ABTS), A_1 is the absorbance of the samples, A_2 is the absorbance of the sample background.

In Vitro **Anti-bacterial Activity**

The antibacterial activity of three Dai antidotes were evaluated by determining the MICs, MBCs and ZOI against *E. coli*, *P. aeruginosa* and *S. aureus*.

The MICs was determined by a microtiter broth dilution method. In brief, 100 μL of bacteria suspension with the dilution of 1: 10 was inoculated in the 96-well plates, the extracts were diluted serially from 50 to 0.196 mg/mL, then 100 μL of the diluted extracts solutions were added subsequently. The inoculated microplates were incubated under microaerobic conditions at 37℃ for 24 h with shaking (100 rpm). The lowest concentration resulting in no visible growth of tested organisms was recognized as MIC.

For determination of MBC, an aliquot (10 μL) of the bacterial suspension and sample (which shown no visible growth) inoculated onto the appropriated agar and incubated at 37℃ for 24 h. The lowest concentration that completely prevented microbial growth in LB Broth agar was recognized as MBC.

To assess ZOI in an agar diffusion model, bacterial lawns (50 μL) were prepared on a nutrient agar plate using the spread plate method. After soaking the sterile double-layer circular filter paper (diameter 6 mm) in each sample solution(with the concentration of 25mg/mL) for 2 h, the filter paper was removed, dried, and gently put it on the corresponding position of the plate. Then, these petri dishes were incubated at 37℃ for 24 h. Then, the ZOI diameter was measured by digital calipers, and was recorded in cm. Each

ats of each material-bacteria combination were assessed.

In Vitro **Anti-Inflammatory Activity**

Cell Culture

The RAW 264.7 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (streptomycin 100 U/mL and penicillin 100 U/mL) in a humidified atmosphere of 5% CO_2 at 37°C.

Determination of NO Production

Herein, we evaluated the ability of the three Dai antidotes to inhibit the NO production by the Griess method. The nitrite assay was carried out according to the manufacturer's instructions. Briefly, Cells were seeded in 96 well plates at a density of 5×10^4 cells/mL and incubated for 24 h. Then, the cells were incubated with respective extracts of three Dai antidotes at different concentrations and exposed to LPS (1 μg/mL) for 24 h. The blank control cells were treated with DMEM only. LPS-induced NO production was determined by using Griess reagent, and the absorbance at 540 nm was measured using a microplate reader (Molecular Devices, Flex Station 3).

Determination of IL-1β,IL-6, TNF-α Production

The generation of IL-1β,IL-6, TNF-α was detected according to the manufacturer's instructions. Briefly, Cells were seeded in 96 well plates at a density of 5×10^4 cells/mL and incubated for 24 h. Then, the cells were incubated with respective extracts of three Dai antidotes and exposed to LPS (1 μg/mL) for 24 h. The blank control cells were treated with DMEM only. LPS-induced IL-1β□IL-6, TNF-α production was determined by using ELISA Kit, and the absorbance at 450 nm was measured using a microplate reader.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics for Windows, Software Version 25.0 (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) and the least significant difference test were employed to analyze the data.

Results

Extracts of *Bin Hao*, *Dai Bai Jie* and *Zhu Ye Lan* were prepared with 95% ethanol and different polarities solvents: petroleum ether, ether ethyl acetate, *n*-butanol and water were used successively to prepare the respective extracts, which were abbreviated as PE, EE, BE, and WE, respectively. Decoctions (DE) was obtained by boiling the Dai antidotes in water under atmospheric pressure. However, we were unable to obtain PE extract from *Dai Bai Jie*, perhaps due to the high polarity of its components.

The contents of total flavonoids and polyphenols

The contents of total flavonoids and polyphenols in three Dai antidotes were determined by colorimetric method. The total flavonoids content of different extracts was expressed as mg of rutin equivalents (RE) ng RE/g DW). While, the total polyphenols content of different

extracts was expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW) of plant material (mg GAE/g DW).

As shown in Fig. 2, the contents of total flavonoids in three Dai antidotes were much higher than that of total polyphenols and *Zhu Ye Lan* was higher than *Bin Hao* and *Dai Bai Jie*. In general, with the increase polarity of solvents, the total polyphenols and flavonoids content decreased and the contents in decoctions was higher than that of water extracts.

As shown in Fig. 2A, the content of total flavonoids in three Dai antidotes ranged from 22.41 to 586.40 mg RE/g DW. In the case of *Zhu Ye Lan*, the content of total flavonoids ranged from 109.52 ± 4.02 to 586.40 ± 23.30 mg RE/g DW, which was the highest. The total flavonoids content of *Bin Hao* and *Dai Bai Jie* were 42.53 ± 0.74 to 469.63 ± 37.27 and 22.41 ± 0.28 to 218.57 ± 2.79 mg RE/g DW, respectively.

Intriguingly, the content of total polyphenols in three Dai antidotes ranged from 2.76 to 28.66 mg GRE/g DW. In the case of *Zhu Ye Lan*, the content of total polyphenols ranged from 5.66 ± 0.06 to 28.66 ± 0.06 mg GAE/g DW, and PE extract from the *Zhu Ye Lan* hold the highest content. The total polyphenols content of *Bin Hao* and *Dai Bai Jie* ranged from 2.76 ± 0.03 to 20.06 ± 0.19 and 2.76 ± 0.03 to 9.32 ± 0.06 mg GAE/g DW, respectively, Fig. 2B. Similar with the results of total flavonoids content, the content of total polyphenols in *Dai Bai Jie* also was the lowest.

Antioxidant activity

The antioxidant activity of extracts were tested in three commonly used DPPH, ·OH, and ABTS radicals scavenging assays and the activity was quantified in terms of the half-maximal inhibitory concentration (IC_{50}).

Ability to scavenge DPPH radicals

All extracts showed dose-dependent DPPH scavenging activity (Fig. 3). In the case of *Bin Hao*, the scavenging activity was approximately 30%, similar across the various polar extracts at 0.2 mg/mL. However, as the concentration increases, extracts with lower polarity showed greater scavenging activity than that higher polarity., At 1.2 mg/mL, the scavenging activities of EE, BE, PE, WE and DE were 89.86%, 88.03%, 79.3%, 71.82% and 64.59%, respectively. While, the DE showed the weakest scavenging at every tested concentration, as shown in the Fig. 3A.

In the case of *Dai Bai Jie*, more polar extracts showed less scavenging activity (Fig. 3B). The DE showed greater scavenging ability than WE, but less than that of EE or BE. At 1.2 mg/mL, the scavenging activities of EE, BE, WE and DE were 63.26%, 56.92%, 45.96% and 35.58%, respectively.

In case of *Zhu Ye Lan*, more polar extracts also showed less scavenging activity (Fig. 3C). The DE showed greater scavenging activity than any of the polar extracts. At 1.2 mg/mL, the scavenging activities of EE, BE, PE, WE and DE were 65.51%, 56.35%, 53.3% and 75.74%, respectively.

Table 1
Quantitation of DPPH radical scavenging activity by various extracts from three Dai antidotes

Dai antidote	IC_{50}, mg/mL				
	PE	EE	BE	WE	DE
Bin Hao	0.53	0.48	0.53	0.67	0.86
Zhu Ye Lan	0.88	0.83	1.01	1.17	0.38
Dai Bai Jie	ND	0.75	1.34	2.55	2.18

Furthermore, the analysis in term of IC_{50} values showed that the scavenging DPPH radicals' ability of *Bin Hao* alcohol extracts was better than that of the other two Dai antidotes (Table 1). At 1.2 mg/mL, these extracts scavenged more than 70% of DPPH radicals, and IC_{50} was lower than 0.7 mg/mL. However, the scavenging DPPH radicals' ability of *Zhu Ye Lan* decoction (IC_{50} 0.38 mg/mL) was better than that of *Bin Hao* (0.86 mg/mL) or *Dai Bai Jie* (2.18 mg/mL).

Ability to scavenge ·OH radicals

Extracts from the three Dai antidotes showed a weak, dose-dependent scavenging activity of ·OH radicals (Fig. 4). In case of *Bin Hao*, polar extracts and decoction showed similar scavenging activity (Fig. 4A). At 1.2 mg/mL, the scavenging activities of PE, EE, BE, WE and DE were 59.65%, 54.50%, 49.43%, 46.56% and 53.37%, respectively.

In the case of *Dai Bai Jie*, as the polarity increased, the scavenging activity of extracts was decreased (Fig. 4B). The scavenging activities of DE and EE were similar. At 1.2 mg/mL, the scavenging activities of EE, BE, WE and DE were 63.13%, 54.60%, 36.37% and 61.38%, respectively. Similarly, in the case of *Zhu Ye Lan*, greater polarity was associated with weaker scavenging (Fig. 4C), and the scavenging activities of DE were also like EE. At 1.2 mg/mL, the scavenging activities of PE, EE, BE, WE and DE were 64.49%, 55.88%, 51.58%, 36.37% and 57.46%, respectively.

Table 2
Quantitation of ·OH scavenging by various extracts from three Dai antidotes

Dai antidote	IC_{50}, mg/mL				
	PE	EE	BE	WE	DE
Bin Hao	1.01	1.15	1.32	1.40	1.18
Zhu Ye Lan	0.92	1.09	1.22	2.27	1.19
Dai Bai Jie	ND	0.98	1.09	2.15	0.71

Comparison of IC_{50} values showed that polar extracts of the three Dai antidotes scavenged ·OH with similar efficacy (Table 2). While for WEs and DEs IC_{50} values ranged from 0.71 mg/mL for *Dai Bai Jie* DE

i Bai Jie.

Ability to scavenge ABTS free radicals

All extracts and decoctions of the three Dai antidotes strongly scavenged ABTS radicals (Fig. 5). Scavenging activity differed substantially between extracts of different polarity from the same Dai antidote, while activity was similar between extracts of similar polarity from different Dai antidotes.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

Table 3
Quantitation of ABTS radical scavenging by various extracts from three Dai antidotes

Dai antidote	IC_{50}, μg/mL				
	PE	EE	BE	WE	DE
Bin Hao	45.95	38.28	9.539	108.4	1523
Zhu Ye Lan	45.57	70.60	17.45	65.46	445.6
Dai Bai Jie	ND	126.9	33.15	143.3	1748

Comparison of IC_{50} values showed that in general, scavenging strength was greater for *Zhu Ye Lan* than for the other two Dai antidotes. All the BEs showed the strongest scavenging ability (IC_{50} 35 μg/mL, Table 3). DEs scavenged more weakly than polar extracts (IC_{50} 400 μg/mL).

Table 3
Quantitation of ABTS radical scavenging by various extracts from three

Dai antidote	IC_{50}, mg/mL				
	PE	EE	BE	WE	DE
Bin Hao	1.01	1.15	1.32	1.40	1.18
Zhu Ye Lan	0.92	1.09	1.22	2.27	1.19
Dai Bai Jie	ND	0.98	1.09	2.15	0.71
Dai antidotes					

Anti-bacterial activity

Anti-bacterial activity of Dai antidote extracts and decoctions was evaluated in terms of minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and zones of inhibition (ZOI) against the Gram-negative bacteria *E. coli* and *P. aeruginosa* as well as the Gram-positive bacterium *S. aureus*.

MICs and MBCs

All polar extracts of *Bin Hao* showed bactericidal effects against the three strains, with activity weakening with greater polarity (Table 4). They were most effective against *E. coli*, followed by *P. aeruginosa* and *S. aureus*. DE showed bactericidal activity only against *E. coli*, while WE showed anti-bacterial activity, but no bactericidal effects.

Table 4
MICs and MBCs (mg/mL) of Bin Hao **extracts against three bacteria**

Bacterium	PE		EE		BE		WE		DE	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	0.391	3.125	0.391	3.125	0.195	3.125	3.125	-	0.781	6.25
P. aeruginosa	3.125	25.0	1.563	12.5	1.563	12.5	12.5	-	3.125	-
S. aureus	1.563	6.25	3.125	6.25	12.5	25.0	6.25	-	1.563	-

The inhibitory effect of *Dai Bai Jie* extracts on *E. coli* was better than that of the other two bacteria (Table 5). All polar extracts except BE showed bactericidal effect against only *E. coli*. BE had the strongest antibacterial effects against all three bacteria, while DE showed no bactericidal effect against any of them.

Table 5
MICs and MBCs (mg/mL) of Dai Bai Jie **extracts against three bacteria**

Bacterium	EE		BE		WE		DE	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	0.391	6.25	0.195	1.563	1.563	12.5	1.563	-
P. aeruginosa	3.125	-	0.781	6.25	6.25	-	3.125	-
S. aureus	25.0	-	12.5	25.0	6.25	-	3.125	-

Zhu Ye Lan extracts also showed stronger effects against *E. coli* than against the other two bacteria (Table 6). All polar extracts showed bactericidal effect against *E. coli*, while PE also showed bactericidal effect against *P. aeruginosa*, and EE and BE also showed bactericidal effect against *S. aureus*. DE did not show bactericidal effect against any of the bacteria.

Table 6
MICs and MBCs (mg/mL) of Zhu Ye Lan **extracts against three bacteria**

Bacterium	PE		EE		BE		WE		DE	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	0.391	3.125	1.563	12.5	0.391	6.25	3.125	25.0	0.391	-
P. aeruginosa	3.125	25.0	6.25	-	3.125	-	12.5	-	1.563	-
S. aureus	1.563	-	3.125	6.25	12.5	25.0	6.25	-	0.781	-

The inhibitory effect of all three Dai antidotes to *E. coli* was better than that of the other two strains. *Bin Hao* and *Zhu Ye Lan* had similarly effective, while *Dai Bai Jie* was less effective. WEs and DEs showed the weakest anti-bacterial activity.

ZOIs

The ZOIs for different Dai antidotes and extracts generally mirrored the trends observed with MICs and MBCs (Fig. 6). Anti-bacterial effect was stronger against *E. coli* than the other two bacteria, and DEs showed negligible anti-bacterial effect.

Anti-inflammatory activity

Inflammation depends on the release of NO and inflammatory cytokines such as TNF-α, IL-1β and IL-6. Therefore, we assessed the ability of the Dai antidote extracts to inhibit release of these pro-inflammatory factors by macrophage cultures stimulated with lipopolysaccharide (LPS).

NO

Bin Hao inhibited NO production in a dose-dependent manner (Fig. 7A), and the inhibition was significant at low extract concentration (p□0.001). At 1.25 mg/mL, NO production was 85.8% lower than in LPS cultures.

In the case of *Dai Bai Jie*, greater polarity was associated with weaker inhibition of NO production for extracts at concentrations of 125 μg/mL or less (Fig. 7B), and EE significantly inhibited the production of NO. At 1.25 mg /mL, BE and WE reduced NO production to the level in control cultures, while EE led to higher NO production than in LPS cultures.

In the case of *Zhu Ye Lan*, greater polarity was again associated with weaker inhibition of NO production for extracts at concentrations of 125 μg/mL or less (Fig. 7C). PE and EE significantly inhibited NO production, although levels remained much higher than in control cultures, while DE inhibited production only weakly. However, at 1.25 mg/mL, NO production was higher in PE and EE cultures than in LPS cultures.

TNF-α

Based on the NO experiments above, extracts at concentrations of 16.125, 32.25, and 62.50 μg/mL were used to assess Dai antidote ability to inhibit secretion of the pro-inflammatory cytokines TNF-α, IL-1 β and IL-6 by LPS-stimulated macrophage cultures.

Bin Hao decreased TNF-α production in a dose-dependent manner (Fig. 8A). Greater polarity was associated with stronger inhibition: at extract concentrations of 62.50 μg/mL, WE and DE suppressed production by nearly 70%, whereas BE caused negligible inhibition.

In the case of *Dai Bai Jie*, an extract concentration of 15.6 μg/mL actually increased TNF-α production, while higher concentration inhibited it (Fig. 8B). EE at 62.50 μg/mL reduced TNF-α level by 62%.

In the case of *Zhu Ye Lan*, extracts at 15.6 μg/mL (except for EE) actually increased TNF-α production, while higher concentrations of polar extracts inhibited it (Fig. 8C). EE at 62.50 μg/mL reduced TNF-α levels by nearly 60%. DE negligibly inhibited TNF-α production.

IL-1β

At 62.5 μg/mL, EE, BE and DE from *Bin Hao* inhibited IL-1β production by nearly 80%, while PE and WE inhibited it by approximately 70% (Fig. 9A). Extracts of *Dai Bai Jie* showed weaker inhibition: DE, BE, EE and WE at 62.5 μg/mL inhibited production by 60–80% (Fig. 9B). In contrast, extracts of *Zhu Ye Lan* at 62.5 μg/mL reduced IL-1β production to nearly undetectable levels (Fig. 9C).

IL-6

All extracts and decoctions of all three Dai antidotes strongly inhibited IL-6 production (Fig. 10). In the case of *Bin Hao*, inhibition increased with polarity, with inhibition ranging from 60–100% at 62.5 μg/mL (Fig. 10A). Similarly, inhibition by extracts from *Dai Bai Jie* or *Zhu Ye Lan* was greater with greater polarity, with inhibition ranging from 80–100% at 62.5 μg/mL (Fig. 10B-C). Notably, *Zhu Ye Lan* DE at 15.6 μg/mL actually increased IL-6 production.

Discussion

Oxidative stress has been associated with various diseases[11], and many drugs exert therapeutic effects by scavenging free radicals. All three Dai antidotes showed dose-dependent ability to scavenge DPPH, ·OH and ABTS radicals. They scavenged ABTS radicals most effectively. These results suggest that anti-oxidation may help explain the clinical benefits of Dai antidotes.

Infections, such as those involving the bacteria *E. coli*, *P. aeruginosa* and *S. aureus*, can cause a range of health problems [13]. Many medicines can inhibit bacterial growth. Here we showed that the three Dai antidotes, at least at higher extract concentrations, showed some anti-bacterial activity against the three [illegible] when extracts were less polar. Most WEs and DEs did not

show bactericidal effects. The relative inefficacy of the Dai antidotes may reflect low intrinsic bactericidal activity, as well as the presence of sugars in the extracts, which may aid bacterial growth. We conclude that the observed detoxifying effects of the Dai antidotes are not due primarily to anti-bacterial effects.

The signaling molecule NO mediates and regulates inflammatory responses [14], while the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 can lead to tissue damage when their levels become excessive or remain chronically high. Using bacterial LPS to stimulate the production of NO and these cytokines [15], we found that all three Dai antidotes inhibited their production. NO production was inhibited most strongly by *Bin Hao*, while IL-1β production was inhibited most strongly by *Zhu Ye Lan*. The three Dai antidotes inhibited IL-6 production to similar extents. These results may help explain the detoxifying effects of Dai antidotes.

As polyphenols and flavonoids have significant antioxidant and anti-inflammatory activities. To explore the material basis of three Dai antidotes, the contents of total polyphenols and flavonoid in three Dai antidotes were determined. The results showed that with the increase of polarity the contents decreased, which was correlated with the antioxidant and anti-inflammatory activities of three Dai antidotes. These results suggested that Dai antidotes may have antioxidant and anti-inflammatory activities by containing more polyphenols and flavonoids.

Conclusions

Our analysis of the commonly used Dai antidotes *Bin Hao*, *Dai Bai Jie* and *Zhu Ye Lan* showed that all three were effective at scavenging radicals of DPPH,·OH and ABTS, as well as inhibiting production of NO, TNF-α, IL-1β and IL-6 by macrophages in response to a bacterial trigger. However, the Dai antidotes showed only weak bactericidal activity against Gram-positive or -negative bacteria. We found that the anti-oxidant, anti-bacterial and anti-inflammatory activities of the Dai antidotes depended on the polarity of the solvent used to extract them. In addition, the contents of total flavonoids and total polyphenols in three Dai antidotes were determined and the contents were correlated with the antioxidant and anti-inflammatory activities. These results may also pave the way for the study of components in Dai antidotes and related in-depth research. Our results begin to provide a modern scientific perspective on the clinical efficacy of Dai medicines, and they provide a guide for future studies to optimize the extraction of active compounds from Dai antidotes and other medicines. Ultimately, these studies should examine the safety, efficacy and mechanisms of action of Dai medicines in preclinical models.

Abbreviations

PE
petroleum ether extract; EE:ethyl acetate extract; BE:*n*-butanol extract; WE:water extract; DE:decoction; ND:not done; DPPH:1,1-diphenyl-2-picrylhydrazyl; ABTS:2,2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid); MICs:minimum inhibitory concentrations; MBCs:minimum bactericidal concentrations; ZOI:zones

of inhibition; NO:nitric oxide; TNF-α:tumor necrosis factor-alpha; IL-1β:interleukin-1 beta; IL-6:interleukin-6; LPS:lipopolysaccharide.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was funded by the Yunnan Provincial Department of Education Science Research Fund Project (2018JS709), Scientific Research Foundation of School of Traditional Dai-Thai Medicine, West Yunnan University of Applied Sciences (2019DYKY003), National Natural Science Foundation of China (81773659; 81973264), Guangdong Basic and Applied Basic Research Foundation, China (2019A1515011954 2020A1515010593).

Author Contributions

X.-F.Z and H.-Y.H designed the study; X.-F.Z performed the experiments, analyzed the data, and wrote the initial draft. J.-K.Z and Imran performed the antibacterial and anti-inflammatory experiments; Y.Z, L.Y and S.-H.Y mainly performed extraction experiments; Q.D, J.W and H.-Y.H interpreted the data and revised the paper. All authors read and agreed to the published version of the manuscript.

Acknowledgments

We thanks to Mrs. Yanfang Lin, Chief Expert of Dai Medicine and Mrs. Xiaohua Li from School of Traditional Dai-Thai Medicine, West Yunnan University of Applied Sciences for identification of plant material.

References

1. Wang L, Suo S, Li J, Hu Y, Li P, Wang Y, Hu H. An investigation Into Traditional Chinese Medicine Hospitals in China: Development Trend and Medical Service Innovation. *International Journal of Health Policy and Management* 2017, 6, (1), 19–25.
2. Li Y, Ma G, Lv Y, Su J, Li G, Chen X, Efficacy of Obcordata A from Aspidopterys obcordata on Kidney Stones by Inhibiting NOX4 Expression. *Molecules* 2019, 24, (10).
3. Zhang C, Chongsuvivatwong V, Keawpradub N, Lin Y. Analysis of prescription database extracted from standard textbooks of traditional Dai medicine. *Journal of Ethnobiology and Ethnomedicine* 2012, 8.
4. LI B, HUANG S, PANG X, XI B, CAO H, ZHANG K. Optimization of Extraction Process of Clerodendrum philippinum Schauer var. simplex Mlodenke Total Flavonoids(CPTF) by Central Composite Design-Response Surface Methodology. Medicinal Plant. 2018;9(03):17–20.
5. Li HT, Kang LP, Guo BL, Zhang ZL, Zhang LX. Original plant identification of Dai nationality herb "Daibaijie". China Journal of Chinese Materia Medica. 2014;39(8):1525–9.
6. QU Rui; LUO Wen; ZHANG Long-wang; HE Rong-bao; Yan-qing Y, Determination of the Contents of Trace Elements in Arundina graminifolia (D.Don) Hochr. *Medicinal Plant* 2011, 2, (06), 57–58 + 61.
7. Oszmianski J, Wojdylo A, Juszczyk P, Nowicka P, Roots and Leaf Extracts of Dipsacus fullonum L. and Their Biological Activities. *Plants-Basel* 2020, 9, (1).
8. Hossain MA, Rahman SMM. Total phenolics, flavonoids and antioxidant activity of tropical fruit pineapple. Food Res Int. 2011;44(3):672–6.
9. Hossain MA, Kalbani MSAA, Farsi SAJA, Weli AM, Al-Riyami Q, Comparative study of total phenolics, flavonoids contents and evaluation of antioxidant and antimicrobial activities of different polarities fruits crude extracts of Datura metel L. *Asian Pacific Journal of Tropical Disease* 2014, 4, (5).
10. Shimada. K, Yahara FK, Nakamura K. a. T., Antioxidative Properties of Xanthan on the Autoxidation of Soybean Oil in Cyclodextrin Emulsion. J Agric Food Chem. 1992;40:945–8.
11. de Q Aquino-Martins, Moura VG, de Melo LF, Pereira Silva LM, Targino de Lima, TR, Queiroz MF, Silva Viana RL, Zucolotto SM, Andrade VS, Oliveira Rocha HA, Scortecci KC, In Vitro Antioxidant, Anti-Biofilm, and Solar Protection Activities of Melocactus zehntneri (Britton & Rose) Pulp Extract. *Antioxidants* 2019, 8, (10).
12. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med. 1999;26(9–10):1231–7.
13. Pendleton JN, Gorman SP, Gilmore BF. Clinical relevance of the ESKAPE pathogens. Expert Review of Anti-Infective Therapy. 2013;11(3):297–308.
14. Yin L, Han H, Zheng X, Wang G, Li Y, Wang W. Flavonoids analysis and antioxidant, antimicrobial, and anti-inflammatory activities of crude and purified extracts from Veronicastrum latifolium. Ind Crops Prod. 2019;137:652–61.
15. Gao X-h, Zhang S-d, Wang L-t, Yu L, Zhao X-l, Ni H-y, Wang Y-q, Wang J-d, Shan C-h, Fu Y-j. Anti-Inflammatory Effects of Neochlorogenic Acid Extract from Mulberry Leaf (Morus alba L.) Against

LPS-Stimulated Inflammatory Response through Mediating the AMPK/Nrf2 Signaling Pathway in A549 Cells. *Molecules* 2020, 25, (6).

Figures

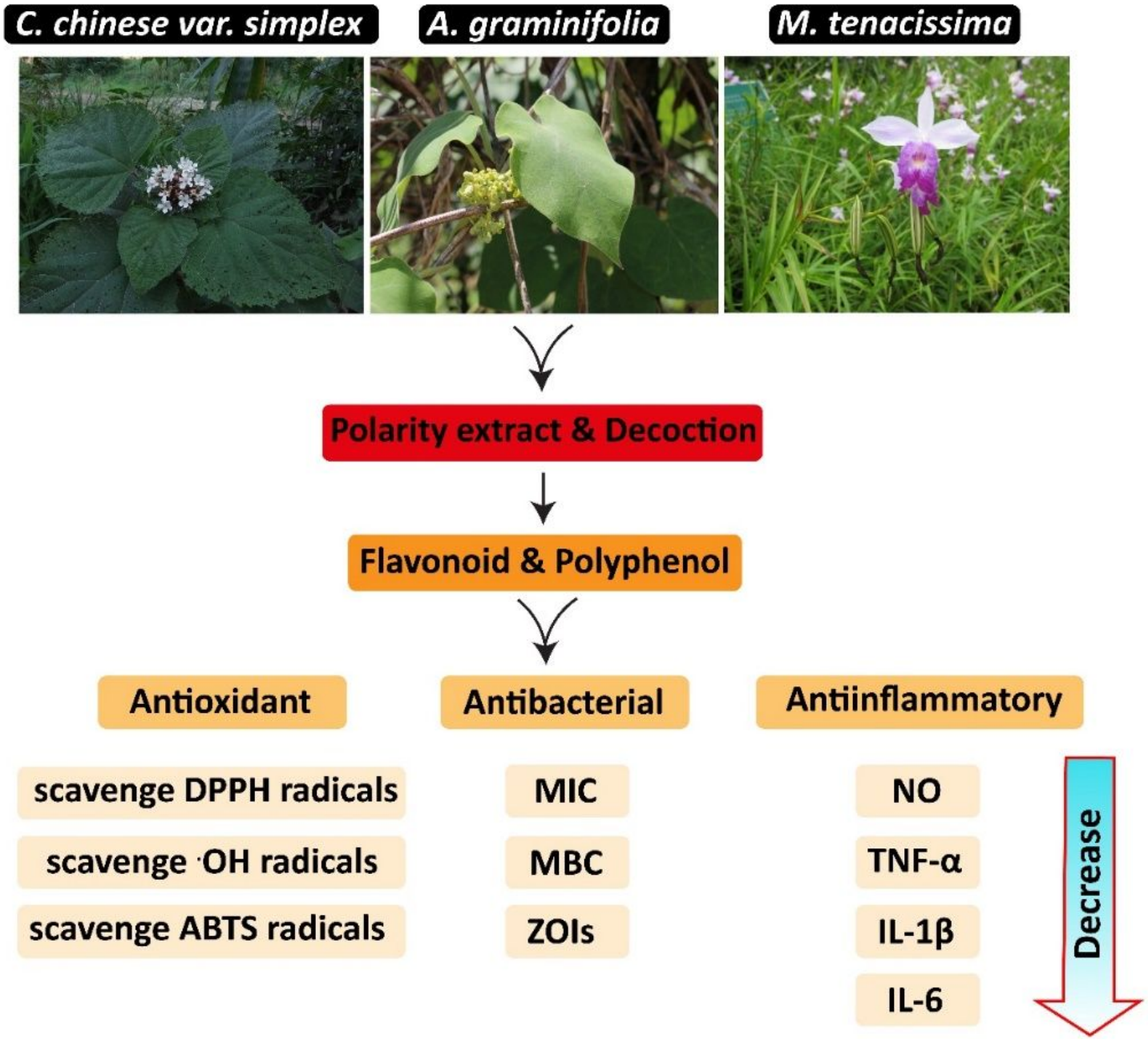

Figure 1

Schematic representation of total polyphenols and flavonoids contents and their antioxidant, antibacterial and antiinflammatory potentials of three Dai antidotes Bin Hao (C. chinense var. simplex), Dai Bai Jie (M. tenacissima) and Zhu Ye Lan (A. graminifolia)

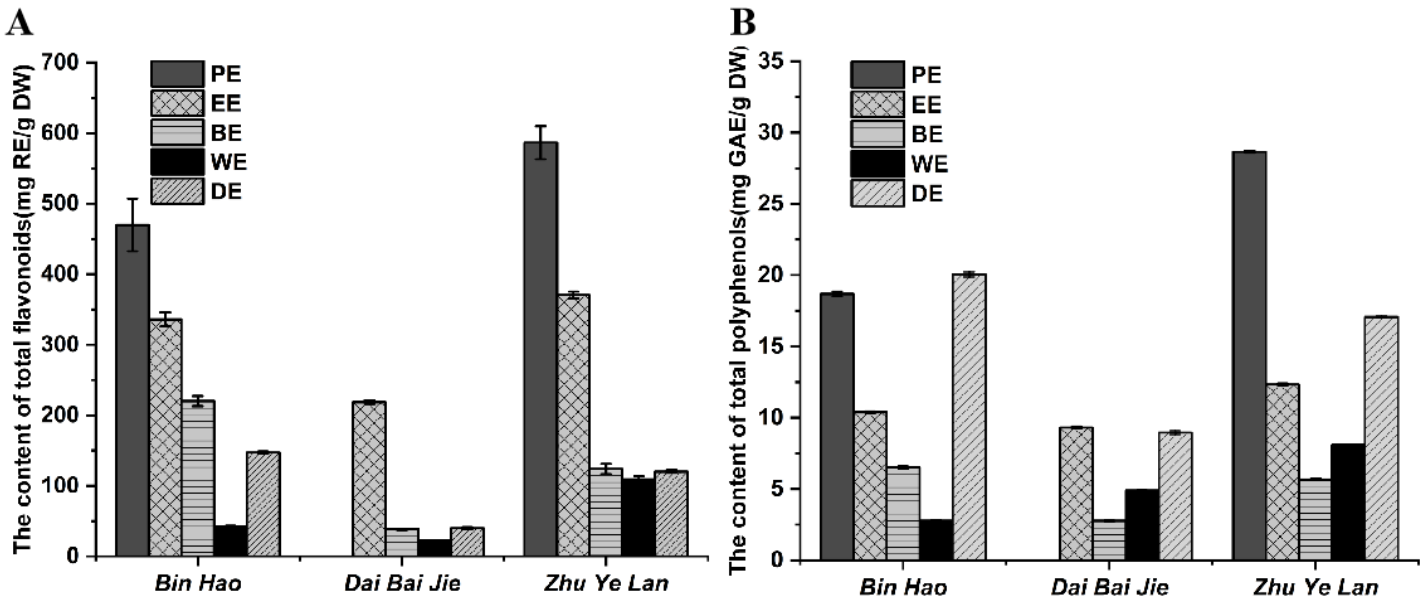

Figure 2

Contents of total flavonoids and polyphenols in three Dai antidotes (A)Total flavonoids content, (B) Total polyphenols content.

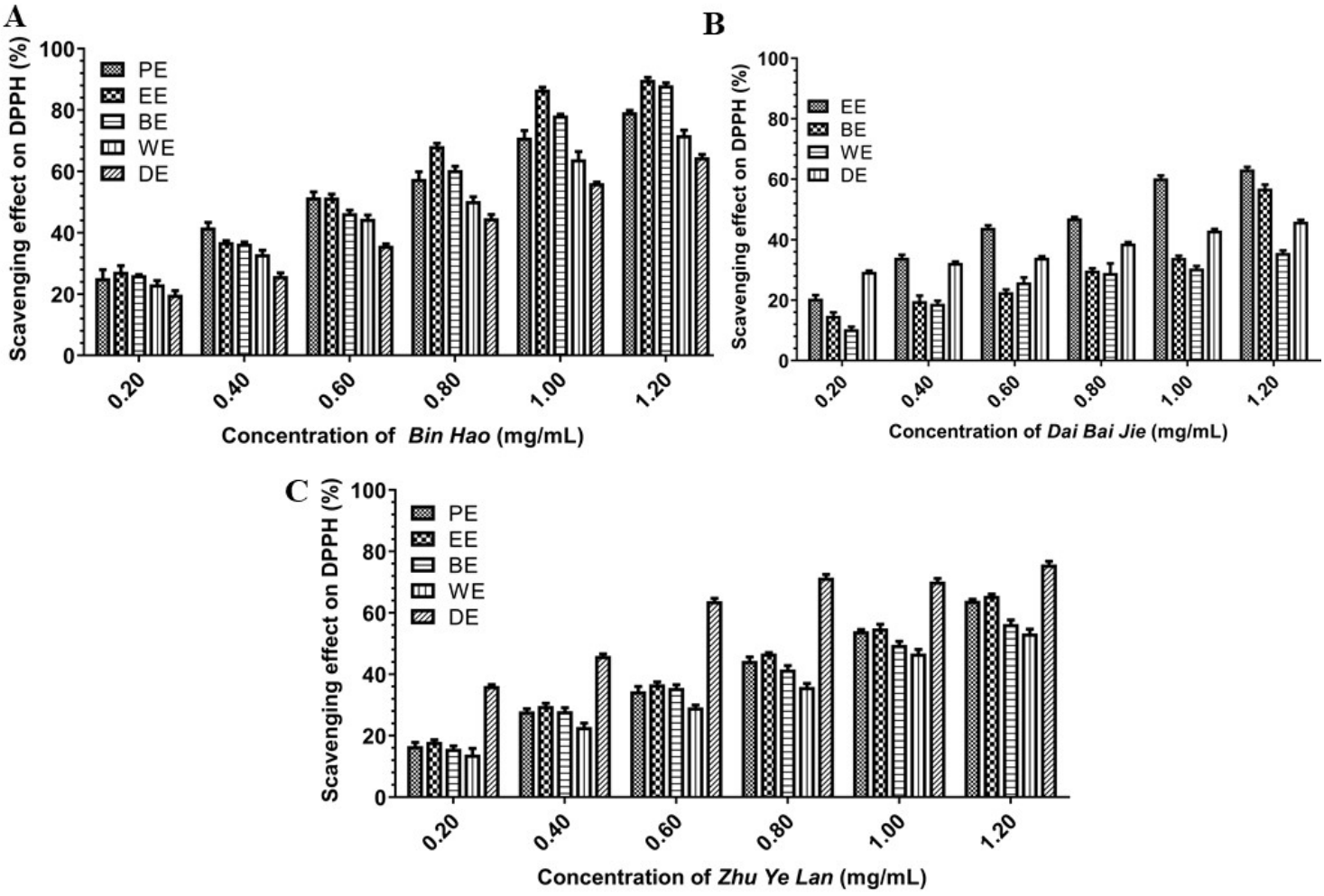

Figure 3

Ability of various extracts of the Dai antidotes (A) Bin Hao, (B) Dai Bai Jie, or (C) Zhu Ye Lan to scavenge DPPH radicals. Various concentrations of extracts (0-1.2 mg/mL) were mixed with 75 μM DPPH for 30 min, then absorbance was measured at 517 nm.

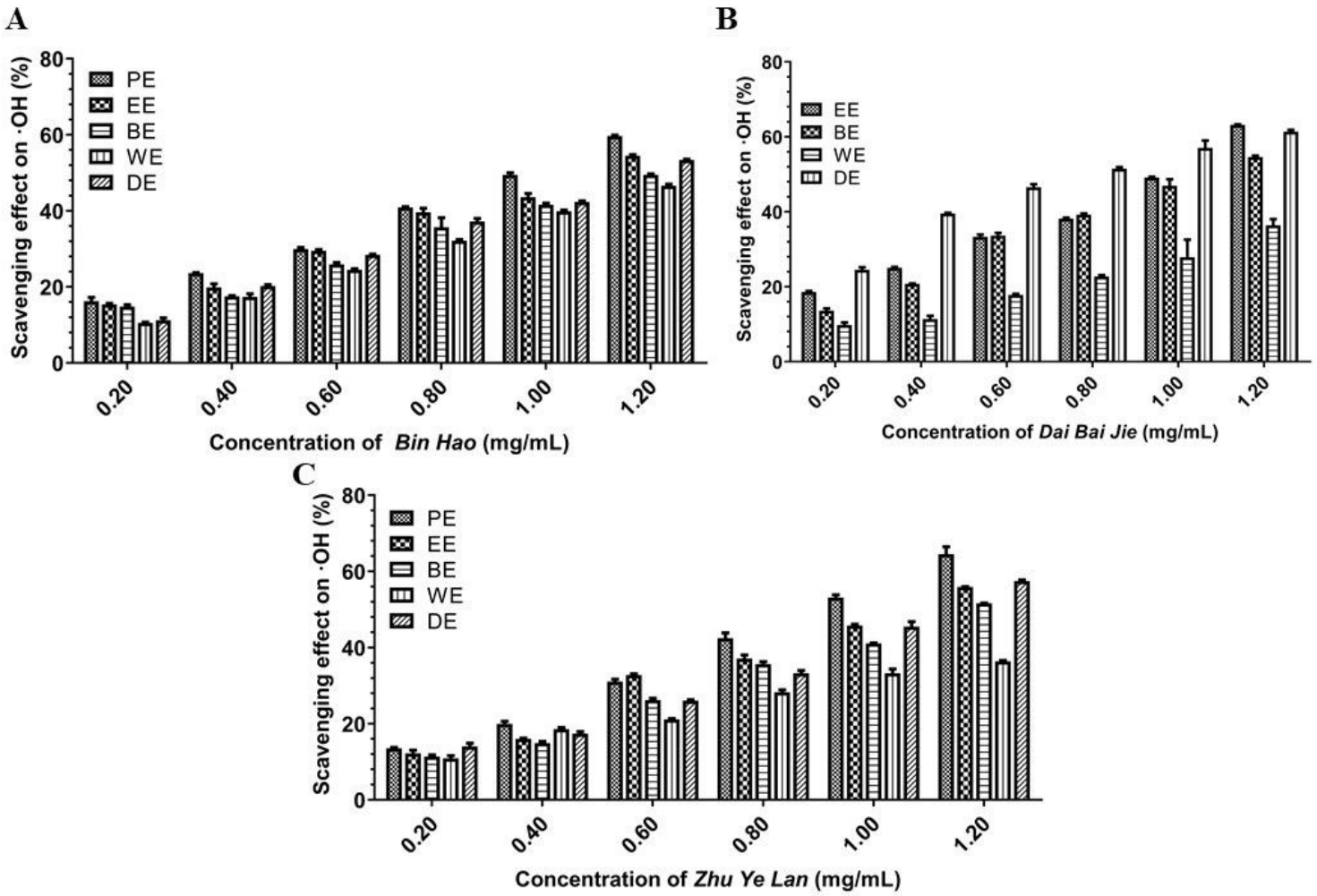

Figure 4

Ability of various extracts of the Dai antidotes (A) Bin Hao, (B) Dai Bai Jie, or (C) Zhu Ye Lan to scavenge ·OH. Various concentrations of extracts (0-1.2 mg/mL) were mixed with solution containing 9 mM FeSO4, 9 mM salicylic acid and 8.8 mM H2O2 for 30 min, then absorbance was measured at 510 nm.

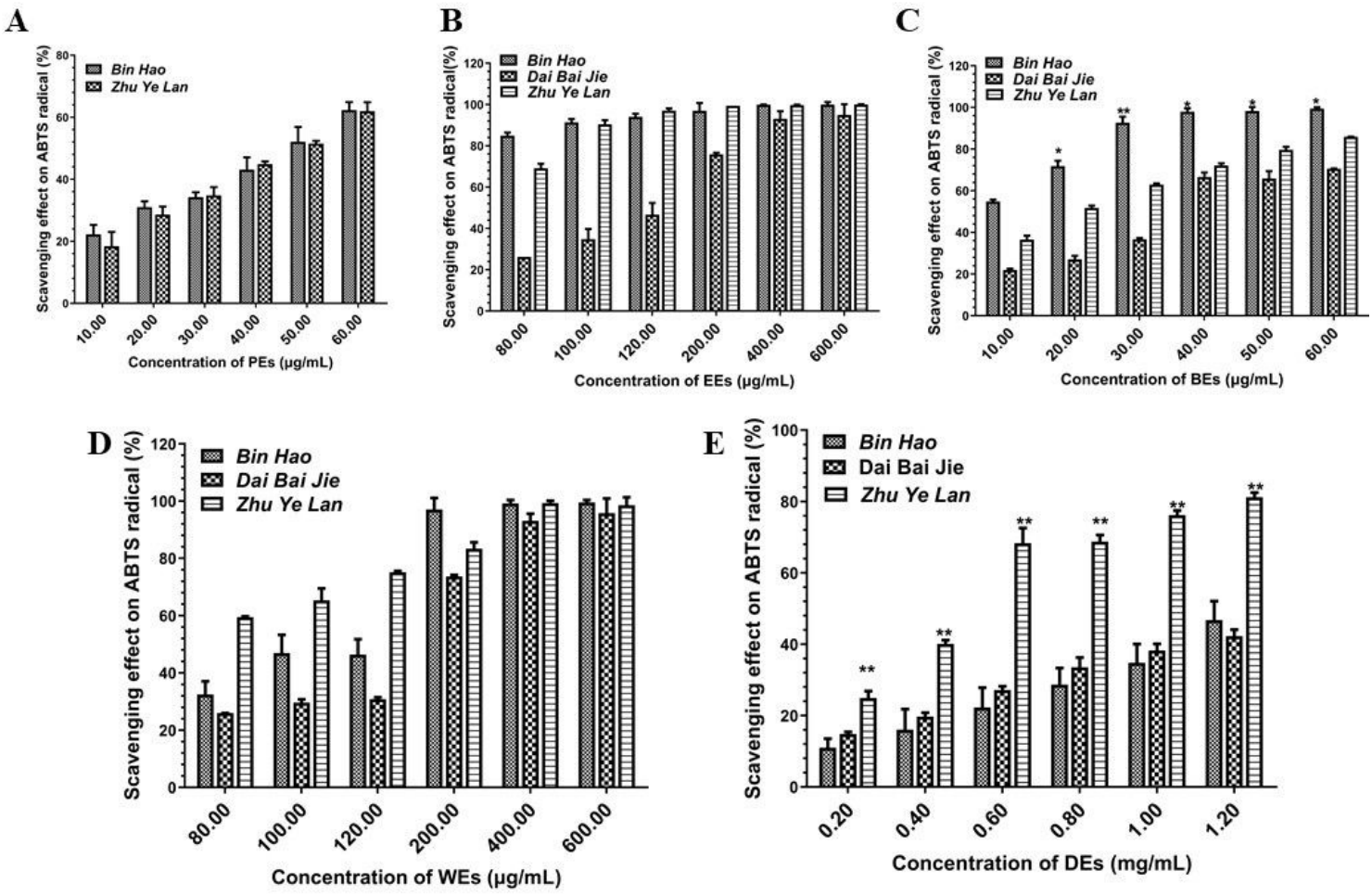

Figure 5

Ability of various extracts of the Dai antidotes(A) PE, petroleum ether extract; (B) EE, ethyl acetate extract; (C) BE, n-butanol extract; (D) WE, water extract; (E) DE, decoction to scavenge ABTS radicals. Various concentrations of extracts were mixed with ABTS and potassium persulfate for 30 min, then absorbance at 734 nm was measured.

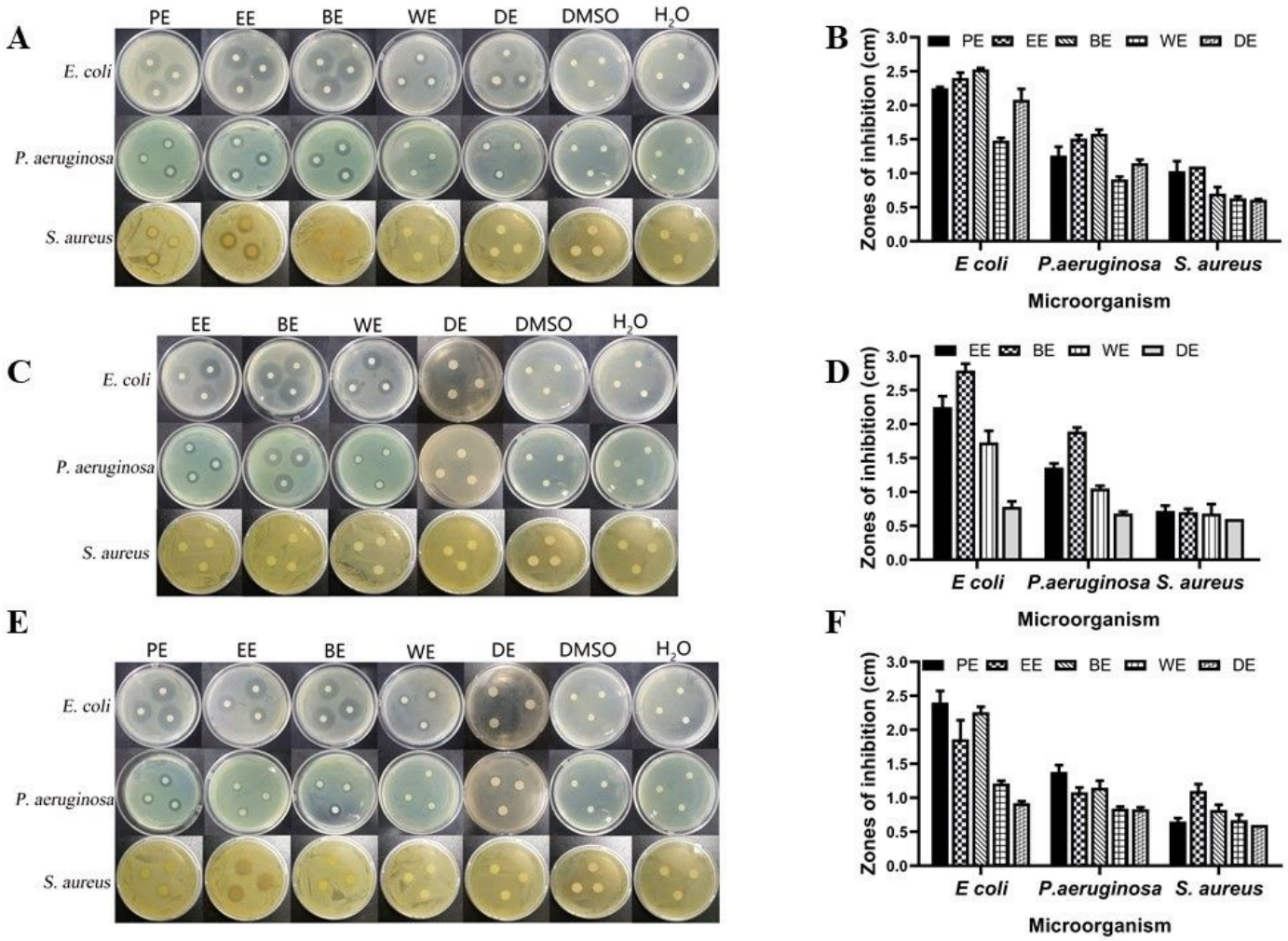

Figure 6

Inhibition zone assay to assess activity of various extracts of the Dai antidotes (A,B) Bin Hao, (C,D) Dai Bai Jie, and (E,F) Zhu Ye Lan. Negative controls were DMSO and H2O. Zones of inhibition were measured using digital calipers.

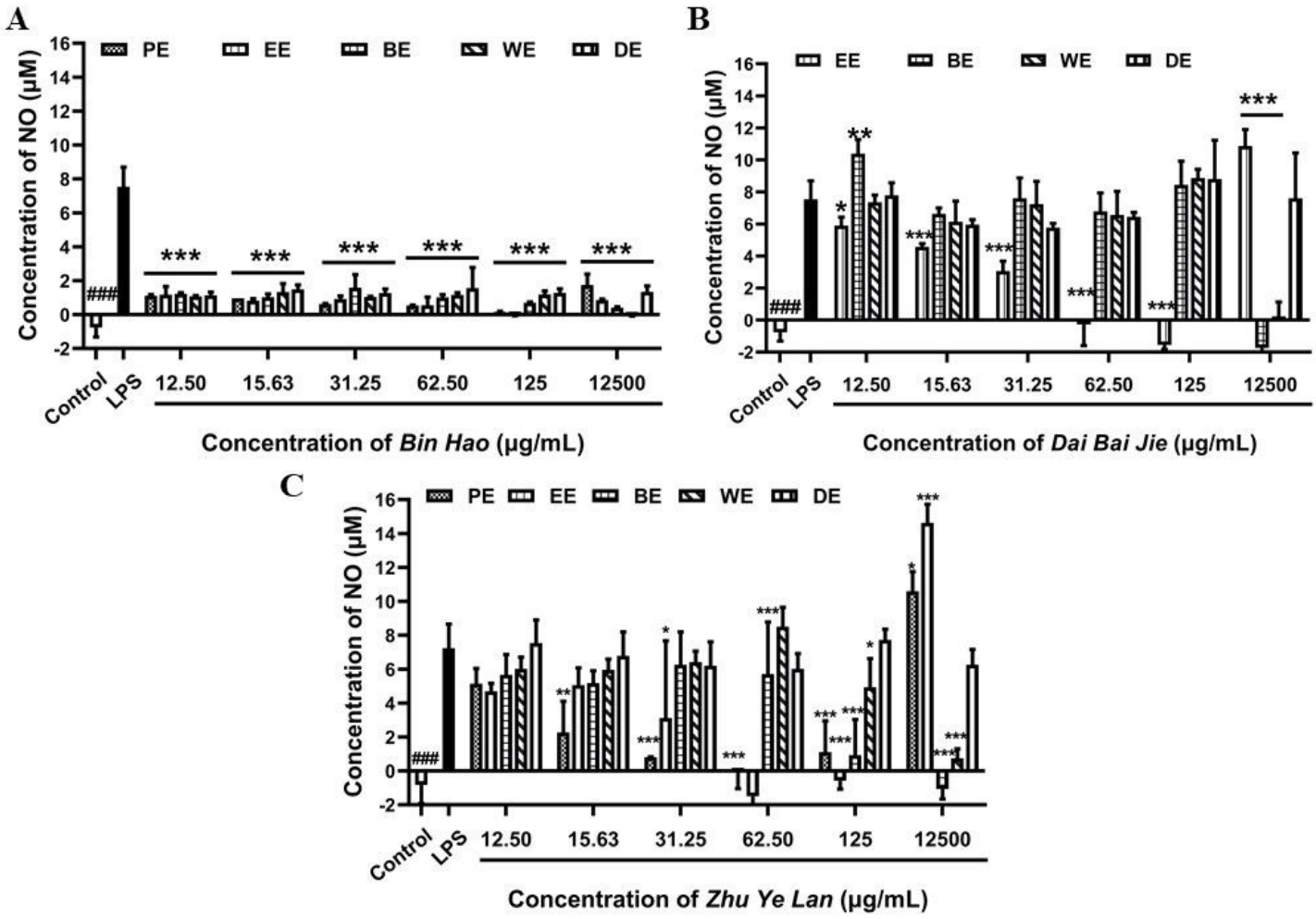

Figure 7

Ability of Dai antidotes to inhibit production of pro-inflammatory NO by LPS-stimulated macrophages in culture. RAW264.7 cells were cultured in 96-well plates, exposed to LPS (1 μg/mL), then treated with various extracts of the Dai antidotes (A) Bin Hao, (B) Dai Bai Jie or (C) Zhu Ye Lan. NO production was assayed in culture medium using Griess reagent. *p<0.05, **p<0.01, ***p<0.001 vs. cultures treated only with LPS.

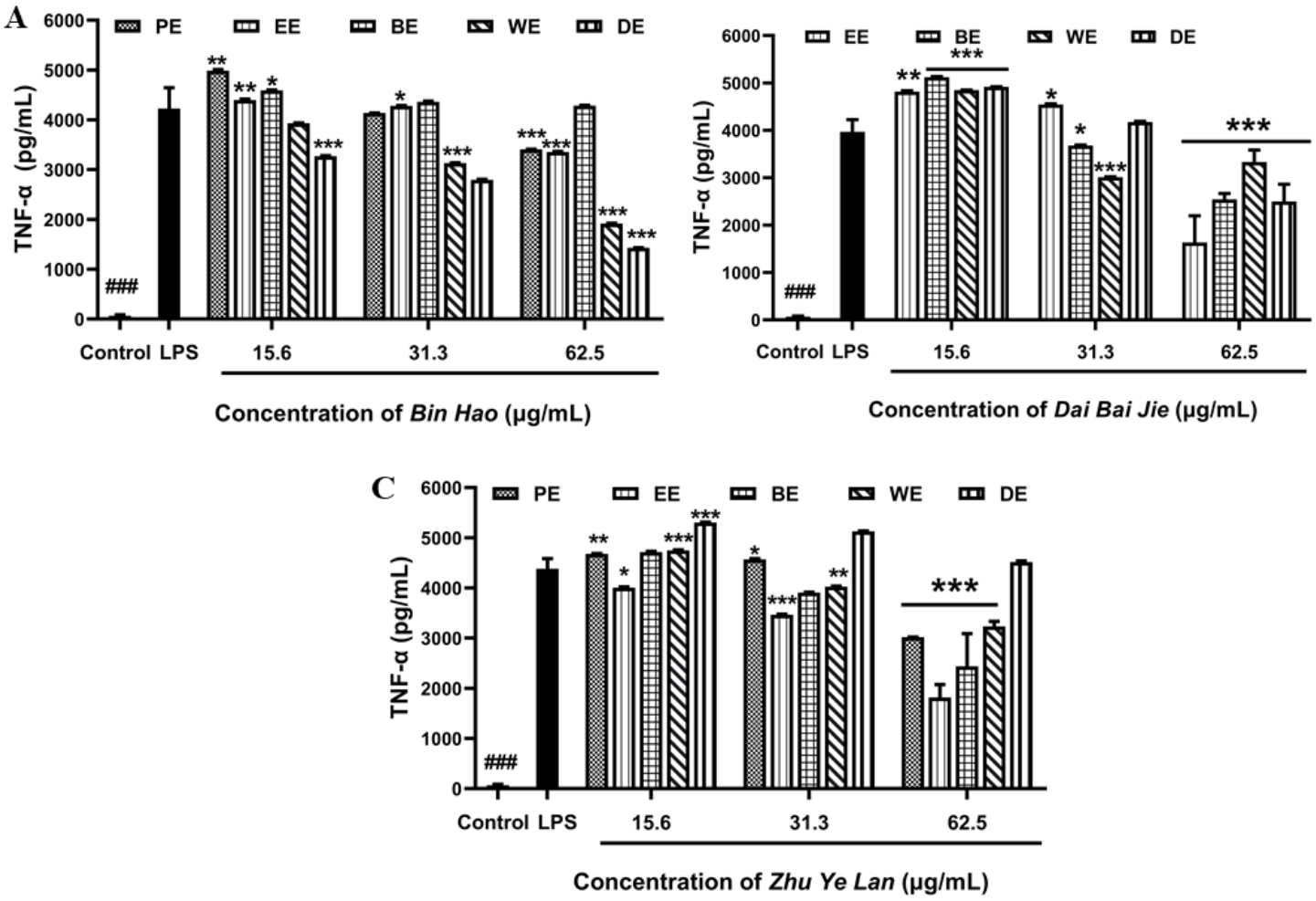

Figure 8

Ability of extracts of the Dai antidotes (A) Bin Hao, (B) Dai Bai Jie and (C) Zhu Ye Lan to inhibit production of pro-inflammatory TNF-α by LPS-stimulated macrophages in culture. RAW264.7 cells were cultured in 96-well plates, exposed to LPS (1 μg/mL), then treated with various extracts of the Dai antidotes. TNF-α in culture medium was assayed by ELISA. *p<0.05, **p<0.01, ***p<0.001 vs. cultures treated only with LPS.

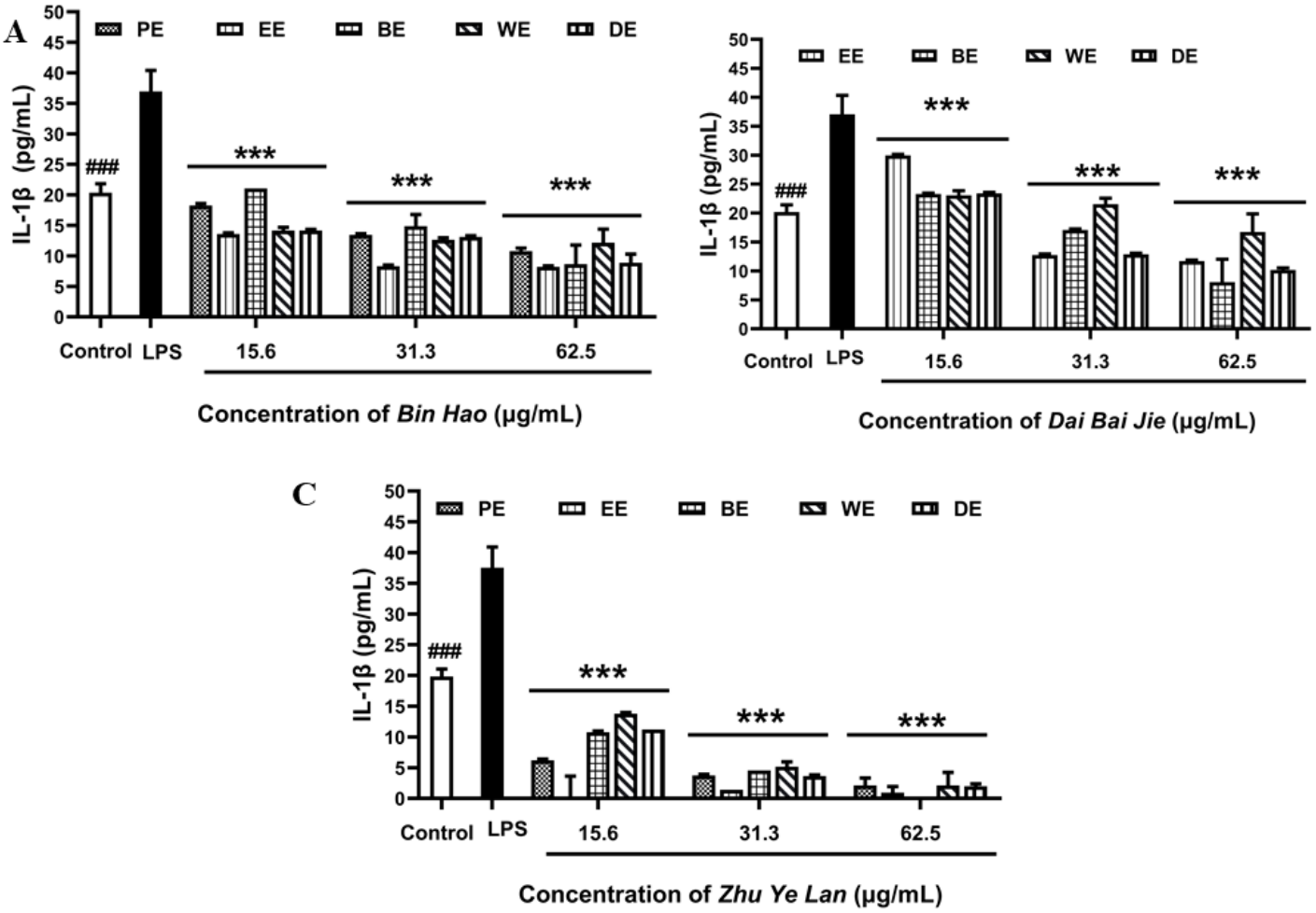

Figure 9

Ability of extracts of the Dai antidotes (A) Bin Hao, (B) Dai Bai Jie and (C) Zhu Ye Lan to inhibit production of pro-inflammatory IL-1β by LPS-stimulated macrophages in culture. RAW264.7 cells were cultured in 96-well plates, exposed to LPS (1 μg/mL), then treated with various extracts of the Dai antidotes. IL-1β in culture medium was assayed by ELISA. *p<0.05, **p<0.01, ***p<0.001 vs. cultures treated only with LPS.

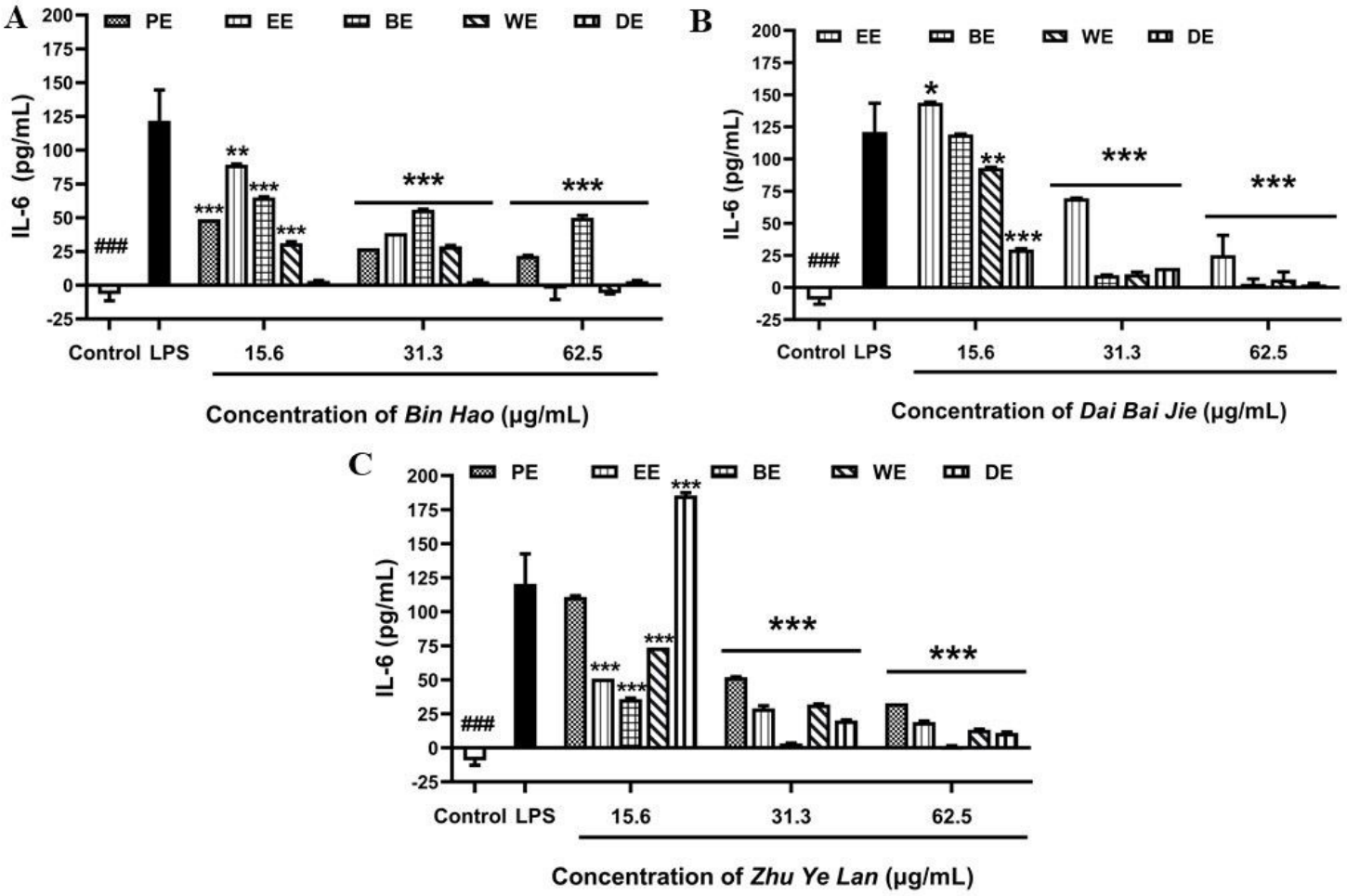

Figure 10

Ability of extracts of the Dai antidotes (A) Bin Hao, (B) Dai Bai Jie and (C) Zhu Ye Lan to inhibit production of pro-inflammatory IL-6 by LPS-stimulated macrophages in culture. RAW264.7 cells were cultured in 96-well plates, exposed to LPS (1 μg/mL), then treated with various extracts of the Dai antidotes. IL-6 in culture medium was assayed by ELISA. *p<0.05, **p<0.01, ***p<0.001 vs. cultures treated only with LPS.