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Tracking antibacterial components of *Pteris multifida* Poir and its antibacterial mechanism

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***Pteris multifida* is an effective Chinese herb for treating dysentery caused by *Escherichia coli*. It might be a good veterinary antibiotic alternative. In this study, we optimized the extraction condition based on the antibacterial activity of crude extracts from *P. multifida*, tracked its antibacterial components, and investigated their antibacterial activity, as well as its antibacterial mechanism. The results revealed that the crude extracts from *P. multifida* displayed the highest antibacterial activity against *Escherichia coli* when *P. multifida* was extracted with 70% ethanol whose ratio to *P. multifida* powder was 30:1 (V/W), followed by reflux extraction three times at 50°C (0.5 h each time). The major antibacterial components might be alkaloids, flavonoids, organic acid, anthraquinones, and cardiac glycosides which were enriched in *n*-butyl alcohol extracts. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *n*-butyl alcohol extracts against *Escherichia coli* were 12.5 and 50 mg/ml, and the antibacterial mechanism might be the damage of bacteria cell membrane, leading to electrolyte exosmosis and in turn bacteria cell death.**

Key words: *Pteris multifida*, antibacterial activity, *Escherichia coli*, antibacterial mechanism.

INTRODUCTION

In the past seven decades, antibiotics have been essential in the fight against infectious diseases in human and animal medicine. However, due to excessive and inappropriate use, bacteria have become more and more resistant to antibiotics, and we are now gradually facing a time when antibiotics are no longer effective (Hansen et al., 2015; Wang et al., 2021), which means that routine infections might be fatal. That is why infectious diseases remain a threat to public health worldwide. In such a situation, new antimicrobial agents with novel mechanisms of action are urgently needed. Since new antibiotics are hard to find, natural products, especially plants, which are always regarded as an important source of drug discovery, might be a good alternative to antibiotics for they have been proved to be effective in infectious diseases (Ngezahayo et al., 2017; Xu et al., 2021).

Pteris multifida also known as spider brake, or Feng-wei-cao in China, belongs to the genus *Pteris* of the Pteridaceae family. It is a perennial fern mainly distributed in southern regions of China, South Korea, and Japan (Kim et al., 2017) and has been traditionally used in China

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Table 1. Factors and levels for orthogonal tests.

Levels	Factors				
	A	B	C	D	E
	Ethanol concentration (%)	Extraction temperature (°C)	Ratio of extraction solvent to *Pteris multifida* powder (g: ml)	Extraction times	Duration of extraction (h)
1	50	50	10	1	0.5
2	60	60	20	2	1
3	70	70	30	3	2

Source: Authors (2022).

in China as an antimicrobial agent for treatment of cholecystitis, tonsillitis, parotitis, hepatitis, rheumatism, eczema, bacterial dysentery, hematemesis, enteritis, haematuria, diarrhea and asthma (Lu et al., 1999; Harinantenaina et al., 2008; Hao-bin et al., 2009; Shen and Qiu, 2021). Although several constituents such as diterpenoids, sesquiterpenoids, flavonoids, coumarins, lignans, and sterolshave been found in *P. multifida* previously (Lu et al., 1999; Chen et al., 2007; Harinantenaina et al., 2008; Kim et al., 2017), numerous components must remain unknown due to the complex chemical composition of plants. In this study, antibacterial activity of crude extracts against *Escherichia coli* was taken as screening index to optimize the extraction condition of *P. multifida*, and phytochemical analysis was conducted to track the active constituents of the extract which were potentially responsible for the antimicrobial activity, followed by the investigation of the antibacterial mechanism of the extracts, which had good antibacterial effect.

MATERIALS AND METHODS

Plant material

The whole plant of *P. multifida* was collected in Nanchang city of Jiangxi province, PR China, in October 2012 and identified at the College of Forestry, Jiangxi Agricultural University.

Chemicals and bacterial strains

All chemicals were of analytical grade and purchased from Sinopharm Chemical Reagent CO., LTD (ShangHai, China). Mueller Hinton broth and agar were obtained from AOBOX Biotechnology CO., LTD. The following microorganisms were used as test bacteria: *Escherichia coli* ATCC 25922 was purchased from Guangdong Culture Collection Center (GuangZhou, China). Clinical isolated strains including *E. coli* K99, *Salmonella* and *Staphylococcus aureus* (MRSA) were obtained from the Chinese Academy of Agricultural Sciences (CAAS) located in Beijing.

Preparation of extracts

The collected plant were washed and dried at 60°C in a constant temperature electric oven before being grounded into fine powder. The crude extracts were prepared as follows: A total of 5 g powder of *P. multifida* was soaked in 150 ml 70% ethanol for 30 min, followed by reflux extraction for three times at 50°C (0.5 h each time). The supernatants were combined and filtered. The filtrates were evaporated to dryness at 60°C and the crude extracts were stored at 4°C and kept away from light until use (Nan et al., 2017).

Orthogonal experiments design for optimization of extraction conditions

The factors influencing antimicrobial activity of *P. multifida* extracts were examined using the orthogonal test method (Yu et al., 2007). A L_{18} (3^5) orthogonal test was designed using statistical analyses which were conducted by SPSS Statistical Software Package, version 17.0 (SPSS, Chicago, IL, USA). The ethanol concentration, extraction temperature, ratio of extraction solvent to *P. multifida* powder, extraction times and duration of extraction were taken as five inspecting factors and each factor was set at three levels (Tables 1 to 2). The size of the inhibition zone diameter was used as a test index. The range method and analysis of variance were employed for analyzing the results. The influence order of levels and factors could be estimated by the K and R values. KJm represented the sum of corresponding experiments of m levels of a column J factor and was used to evaluate the quality of the J factor where: kJm was the KJm average (kJm = KJm/3). RJ represented the range of the column J factor that responded to fluctuations at each level, that is, RJ = kJmax – kJmin, where kJmax and kJmin represents maximum value and minimum value, respectively. The highest average k value of the inhibition zone diameter represents the optimal level, and the greater R value represents the greater influence of the level on the test index. In the analysis of variance, the impact of the factor was considered statistically significant at $p < 0.05$ (Wang et al., 2019).

Determination of in vitro antibacterial activity of the extracts

The Oxford cup method was employed to investigate the antibacterial activity *of P. multifida* extracts against test microorganism strain (Nan et al., 2017). Briefly, 0.1 ml of diluted inoculum (10^6 CFU/ml) from *Escherichia coli* suspension was added on the surface of MacConkey Agar, and then spread it with a sterile cotton swab. After that, sterilized Oxford cups (Φ 5 mm) were placed on the agar medium and filled with 0.2 ml of *P. multifida* extracts. After standing for 30 min, the plates were incubated at 37°C for 18-24 h. The antibacterial activity was assessed by measuring the diameter zone. Three replicates of each sample were determined and the data were calculated as means ± standard deviation (SD).

Tracking the polarity of antibacterial active components of *P. multifida*

The crude extract of *P. multifida* (10 g) was dissolved in 50 ml distilled water, followed by successive extraction with four solvents of varying polarity including petroleum ether, trichloromethane, ethyl acetate and *n*-butyl alcohol for three times, the organic layers were combined and evaporated to paste at 60℃ under vacuum, while the aqueous layer of each extraction was further extracted by the next solvent (Qiu, 2013). The antibacterial activity of organic fractions and the remaining aqueous fraction were screened using the Oxford cup method described previously. The screening of chemical constituents was carried out for the faction showing the highest antibacterial activity.

Phytochemical screening of *n*-butyl alcohol extracts

The crude n-butyl alcohol extract of *P. multifida* was phytochemically screened according to the methodology described by Wagner and Bladt (1996) and Wang et al., (2019). The presences of glycosides, terpenoids, alkaloids, flavonoids, phytosterols, tannins, phenolics, anthraquinones, organic acid, coumarin, inner ester, cardiac glycosides, polypeptide, etc., were analyzed.

Determination of antibacterial activity of *n*-butyl alcohol extracts

Antibacterial activities of the *n*-butyl alcohol extract from *P. multifida* against *E. coli* ATCC 25922, *E. coli* K99, *Salmonella* and *S. aureus* (MRSA) were measured according to CLSI (2013) at a final inoculum of 5 × 104 colony forming units (CFU)/ml. The extracts were dissolved in 10% dimethyl sulfoxide (DMSO) and then were 2-fold diluted in a microdilution plate (96 wells) using Mueller Hinton Broth (MHB). Then 100 µl of inoculums prepared in MHB were added to each well. Afterwards, the plates were covered and incubated at 37°C for 18-24 h. Three replicates of each extract concentration were investigated against each bacterial strain. MIC was defined as the lowest concentration resulting in the inhibition of bacterial growth which was determined by visual inspection.

The DMSO was evaluated and did not impact the antibacterial activity of the extracts, so it was taken as the negative control. MBC was determined by subculturing 100 µl of the culture from the wells in which concentrations of n-butyl alcohol extracts were above the MIC on new plates of Mac Conkey Agar. These preparations were then incubated at 37°C for 18-24 h. MBC was defined as the lowest concentration of the extracts associated with no bacterial culture (Djeussi et al., 2013). The experiment was performed in triplicate.

Antibacterial mechanism of *n*-butyl alcohol extracts

In order to investigate the bactericidal mechanism of *n*-butyl alcohol extracts from *P. multifida,* a time–kill curve and a electrical conductivity curve were performed, taking *E. coli* ATCC 25922 as an example.

Time-kill curve assay

The *n*-butyl alcohol extracts from *P. multifida* were added to the tube containing suspension of *E. coli* ATCC 25922 at concentration of approximately 10^5 CFU/ml to obtain final concentrations of 0 × MIC, 0.5 × MIC, 1 × MIC, and 2 × MIC. The tubes were then incubated at 37°C. At 0, 2, 4, 8, 12, and 24 h, 100 µl of the sample were taken from each tube and diluted following a tenfold serial dilution in normal saline (0.9% NaCl). Then, 100 µl from each of the dilution were cultured on Mac Conkey agar plates and incubated at 37°C for 18-24 h. Colony count of the bacterial culture was carried out after incubation. The number of colonies between 30 and 300 was used to calculate bacterial concentration at that hour. A time-mortality curve was built by plotting log 10 of bacterial concentration (CFU/ml) *versus* time (hour).

Electrical conductivity curve assay

The *n*-butyl alcohol extracts from *P. multifida* were added to the tube containing suspension of *E. coli* ATCC 25922 at concentration of approximately 10^5 CFU/ml to obtain final concentrations of 0 × MIC, 0.5 × MIC, 1 × MIC, and 2 × MIC. The tubes were then incubated at 37°C. At 0, 2, 4, 8, 12, and 24 h, 2 ml of the sample were taken from each tube and centrifuged for 5 min at 5000 rpm. Thereafter, the supernatant was diluted 20 times before determination of its electrical conductivity. MHB was taken as control. A time- electrical conductivity curve was built by plotting electrical conductivity *versus* time (hour).

RESULTS

Optimization of extraction conditions

Range analysis results of the orthogonal tests showed that the best level combination of the 5 test factors was A3B1C3D3E1, namely, the crude extracts of *P. multifida* showed the strongest antibacterial activity against *E. coli* ATCC 25922 using the following extraction condition: the ethanol concentration, extraction temperature, ratio of extraction solvent to *P. multifida* powder, extraction times and duration of extraction of were 70%, 50°C, 30:1 (V/W), three times, and 0.5 h, respectively. When ranked by the R values for each factor, their influence on antibacterial activity of the crude extracts decreased in the order: duration of extraction > extraction times > extraction temperature > ratio of extraction solvent to *P. multifida* powder > ethanol concentration (Table 2).

Tracking the polarity of antibacterial components of *P. multifida*

The polarity of antibacterial components of *P. multifida* was traced by liquid-liquid extraction. The results shown in Table 3 suggested that the extracts of different polarity showed different degree of antibacterial activity. The ethyl acetate and *n*-butyl alcohol extracts showed significantly stronger antibacterial activity than the other extracts ($P<0.05$).

Phytochemical screening of *n*-butyl alcohol extracts

The preliminary phytochemical screening for various constituents showed that *n*-butyl alcohol extracts were rich in a wide variety of secondary metabolites such as alkaloids, flavonoids, organic acid, anthraquinones, and cardiac glycosides, while phytosterols, terpenoid, coumarin, and inner ester were not detected in the

Table 2. L_{18} (^{35}) orthogonal test.

Experiment	Factors					Results
	Ethanol concentration (%)	Extraction temperature (°C)	Ratio of extraction solvent to *Pteris multifida* powder (g: ml)	Extraction times	Duration of extraction (h)	
1	1	1	1	1	1	15.71
2	1	2	2	2	2	14.00
3	1	3	3	3	3	15.69
4	2	1	1	2	2	17.13
5	2	2	2	3	3	16.00
6	2	3	3	1	1	16.00
7	3	1	2	1	3	15.12
8	3	2	3	2	1	15.95
9	3	3	1	3	2	14.70
10	1	1	3	3	2	16.68
11	1	2	1	1	3	15.00
12	1	3	2	2	1	17.00
13	2	1	2	3	1	16.02
14	2	2	3	1	2	15.42
15	2	3	1	2	3	15.03
16	3	1	3	2	3	17.32
17	3	2	1	3	1	18.89
18	3	3	2	1	2	13.72
K1	15.680	16.330	16.077	15.162	16.595	-
K2	15.933	15.877	15.310	16.072	15.275	-
K3	15.950	15.357	16.177	16.330	15.693	-
R	0.270	0.973	0.867	1.168	1.320	-

Source: Authors (2022).

Table 3. Antibacterial activity of different polar fractions on Escherichia coli and the extraction rate.

Extracts of different polarity	Inhibition zone(mm)	Extraction amount(g)	Extraction rate(%)
Petroleum ether extracts	15.06±0.02^a	1.0901	11.38
Trichloromethane extracts	14.00±0.00^a	0.7064	7.37
Ethyl acetate extracts	19.71±0.01^b	0.1336	1.39
N-butyl alcohol extracts	19.10±0.02^b	0.2798	2.92
Aqueous extracts	13.35±0.03^a	7.3732	76.94

Different letters represent that significant difference was found between groups (P < 0. 05).
Source: Authors (2022).

extracts (Table 4).

Determination of antibacterial activity of *n*-butyl alcohol extracts

MIC and MBC of *n-butyl alcohol* extracts from *P. multifida* against *E. coli* ATCC 25922, *E. coli* K99, *Salmonella* and *S. aureus* (MRSA) are shown in Table 5. It can be seen that the MIC of *n-butyl alcohol* extracts from *P. multifida* against *E. coli* ATCC 25922, *Salmonella and S. aureus* (MRSA) was 12.5 mg/ml, which was half of that against *E. coli* K99 (25 mg/ml). And its MBC against *S. aureus* (MRSA) (25 mg/ml) was half of those against the other bacteria (50 mg/ml).

Antibacterial mechanism of *n*-butyl alcohol extracts

Time-kill curve assay

The time-kill curve of *n*-butyl alcohol extract from *P.*

Table 4. Phytochemical screening of n-butyl alcohol extracts of *P. multifida*.

S/N	Major phytoconstituents detected	Score
1	Tannins, phenolics	±
2	Organic acid	+
3	Alkaloids	+
4	Flavanoids	+
5	Carbohydrate	±
6	Phytosterols, terpenoid	-
7	Anthraquinones	+
8	Coumarin, inner ester	-
9	Cardiac glycosides	+
10	Polypeptide, glycosides	±

+, presence; -, absence; ±, presence in traces.
Source: Authors (2022)

Table 5. MIC and MBC of n-butyl alcohol extracts from P. multifida against four bacteria.

	E. coli ATCC 25922	*E. coli* K99	*Salmonella*	*S. aureus* (MRSA)
MIC(mg/ml)	12.5	25	12.5	12.5
MBC(mg/ml)	50	50	50	25

MIC, Minimal inhibitory concentration; MBC, Minimal bactericidal concentration.
Source: Authors (2022).

multifida against *E. coli* ATCC 25922 shown in Figure 1 revealed that the log rates significantly decreased. The *n*-butyl alcohol extract significantly inhibited the bacterium at the concentration of 1 × MIC after 4 h incubation. The *n*-butyl alcohol extract completely eradicated the bacterium at the concentration of 2 × MIC after 4 h incubation. The *n*-butyl alcohol extract at the concentration of 0.5 × MIC had no antibacterial effect against *E. coli* ATCC 25922.

Electrical conductivity curve assay

It can be seen from the Figure 1 that with the extension of time, the conductivity of *E. coli* treated with 0.5 MIC *n*-butanol and untreated samples increased slowly and steadily; the conductivity of *E. coli* treated with 1 MIC *n*-butanol increased continuously until 8 h, and then developed steadily. Therefore, under 1 MIC, the *n*-butanol part can destroy the cell membrane of *E. coli* and make a lot of electrolytes in the cell seep out, and the *n*-butanol part at 0.5 MIC has no obvious effect on the cell membrane of *E. coli*. As shown in Figure 2, the electrical conductivity of *E. coli* ATCC 25922 treated with *n*-butyl alcohol extract at the concentration of 0.5 × MIC increased slowly and steadily as that in the control group; while the conductivity of *E. coli* ATCC 25922 treated with *n*-butyl alcohol extract at the concentration of 1 × MIC showed an obvious increase before 8 h incubation.

DISCUSSION

Recently, the large-scale application of veterinary antibiotics leads to drug resistance and drug residue in animal-derived food and the environment, in turn causing public health problems, and ecological and environmental problems. Developing alternatives to antibiotics in both animal agriculture and human medicine is of great significance. It has been reported that *P. multifida* was effective in treatment of dysentery caused by *S. aureus* and *E. coli* (Hu et al., 2008; Yin et al., 2018; Wei et al., 2020). In this paper, we aimed to preliminarily study a veterinary antibiotic alternative by tracking antibacterial components from *P. multifida*. Based on the antibacterial activity of crude extracts against *E. coli*, further study on chemical composition, antibacterial activity against different bacteria and antibacterial mechanism of the extracts showing good antibacterial effect were further studied.

In most studies on extraction of *P. multifida*, the amount of total flavonoids extracted was traditionally taken as screening index for antibacterial constituent tracking. As well known, however, there are many other antibacterial components in herb extracts such as terpenoids, organic acids, alkaloids, anthraquinones, and cardiac glycosides.

Therefore, we might miss some constituents showing strong antibacterial activity if only flavonoids were evaluated. Since diarrhea caused by *E. coli* is an important disease in animal husbandry, we used the

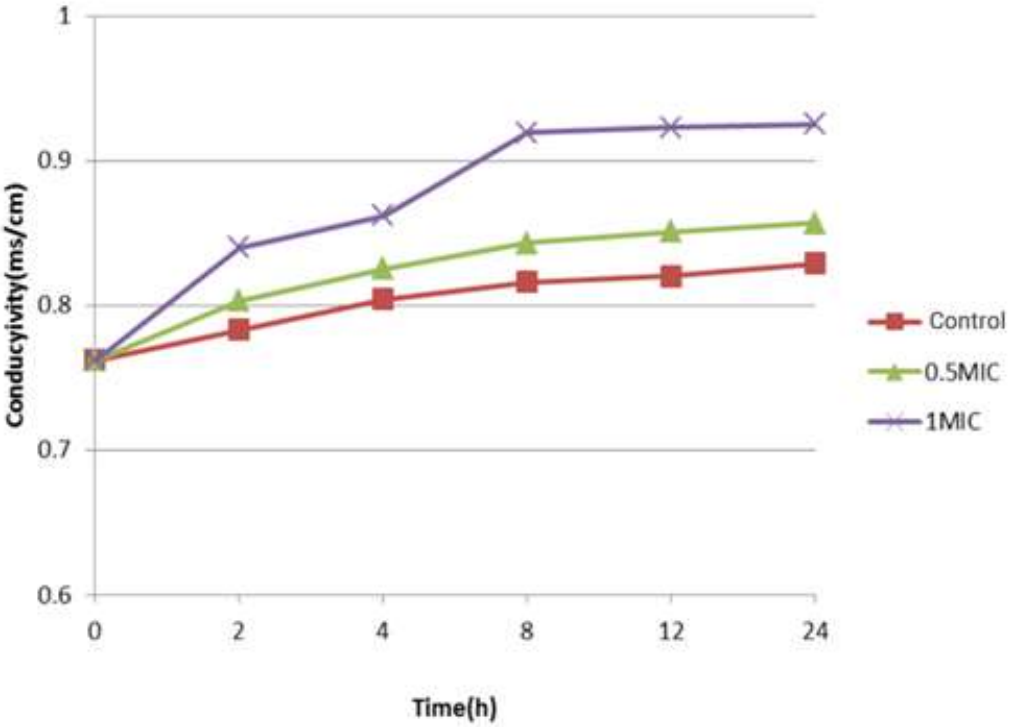

Figure 1. Time-kill curves of n-butyl alcohol extracts against Escherichia coli ATCC 25922.
Source: Authors (2022).

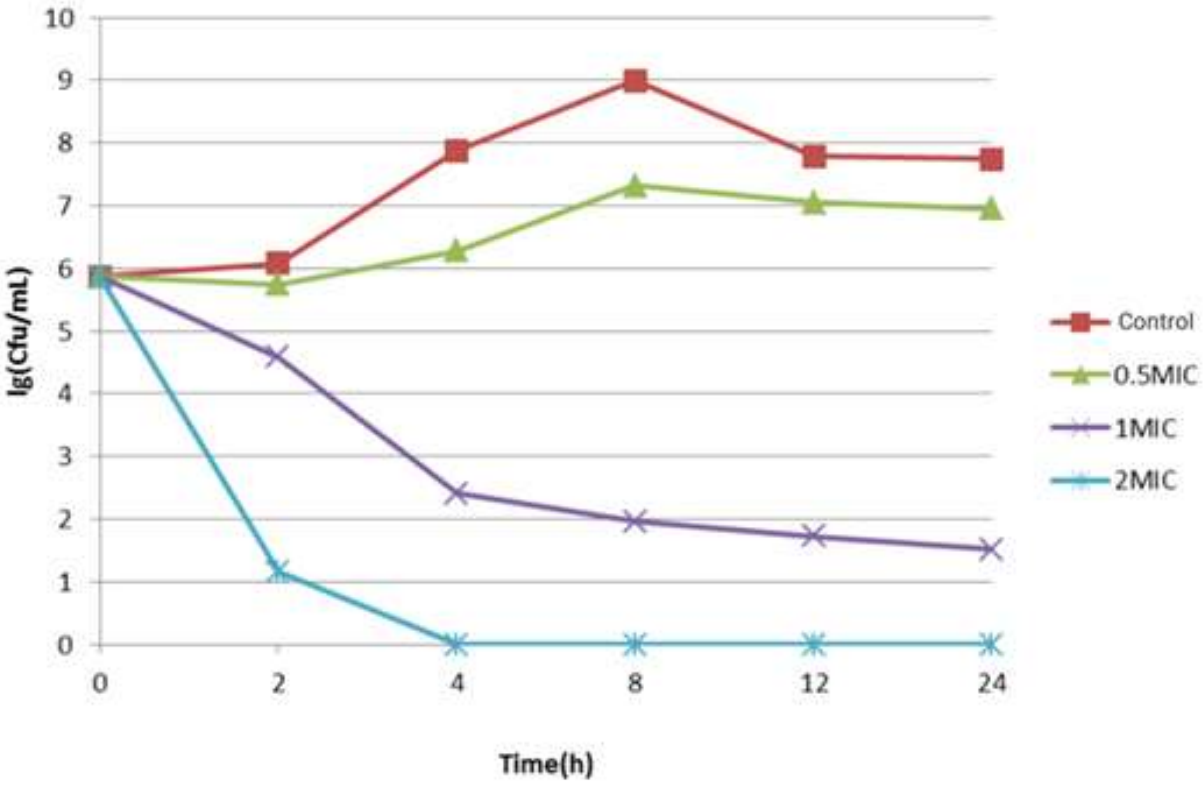

Figure 2. Conductivity curves of n-butyl alcohol extracts against Escherichia coli ATCC 25922.
Source: Authors (2022).

optimize the extraction of components with strong antibacterial activity.

The extracts from ethyl acetate and *n*-butyl alcohol showed stronger antibacterial activity than the other extracts, suggesting that antibacterial components are of medium polarity. Since *n*-butyl alcohol extracts had higher extraction efficiency, they were selected for further investigation. Various constituents such as alkaloids, flavonoids, organic acid, anthraquinones, and cardiac glycosides were found in n-butyl alcohol extracts, laying a foundation for the later separation of antibacterial components. Hu et al. (2008) reported that MIC and MBC of methanol extracts of *P. multifida* and essential oils of *P. multifida* extracted with diethyl ether against *E. coli* ATCC 25922 were both > 72 mg/ml, which were higher than those of *n*-butyl alcohol extracts in this study (MIC 12.5 mg/ml, MBC 50 mg/ml). This indicated that component with stronger antibacterial activity against *E. coli* causing diarrhea might be investigated from *n*-butyl alcohol extracts.

Cell membrane is the most important protective barrier in living cells. It plays a significant role in maintaining the stability of cell environment. The permeability of cell membrane is a representative indicator of physiological function of cells. Conductivity is a parameter to measure ion strength inside and outside cells. It is related to the number of ions and the concentration of electrolytes inside and outside cells. The conductivity of bacterial suspension can reflect the electrolyte penetration in bacterial cells (Zhou et al., 2001). The results of conductivity curve indicated that *n*-butyl alcohol extract at the concentration of 1 × MIC could destroy bacteria cell membrane, leading to electrolyte exosmosis and in turn bacteria cell death, while *n*-butyl alcohol extract at the concentration of 0.5 × MIC had no impact on bacteria cell membrane. This explained why 0.5 × MIC could not inhibit the growth of bacteria while 1 × MIC and 2 × MIC showed significant antibacterial activity.

Conclusion

When *P. multifida* was extracted with 70% ethanol whose ratio to *P. multifida* powder was 30:1 (V/W), followed by reflux extraction for three times at 50°C (0.5 h each time), the crude extracts showed the highest antibacterial activity against *E. coli* ATCC 25922. Taking antibacterial activity as index for extraction would be helpful to find out the antibacterial components. It was also discovered that antibacterial components were enriched in ethyl acetate and *n*-butyl alcohol extracts, which were of medium polarity. Further study showed that *n*-butyl alcohol extracts were rich in various constituents such as alkaloids, flavonoids, organic acid, anthraquinones, and cardiac glycosides. The *n*-butyl alcohol extracts displayed strong antibacterial effect against *E. coli* ATCC 25922 when its concentrations were above 1× MIC. The antibacterial mechanism of *n*-butyl alcohol extracts might be the damage of bacteria cell membrane, leading to electrolyte exosmosis and in turn bacteria cell death.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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