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Antibacterial effect of different extracts from *Wikstroemia indica* on *Escherichia coli* based on microcalorimetry coupled with agar dilution method

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Abstract The root of *Wikstroemia indica* has been widely used in China as folk medicine for the treatment for arthritis, whooping cough, cancer, and bacillosis. However, the constituents which have antibacterial activity were not clarified yet. In this study, the antibacterial effect of five extracts from *W. indica* on *Escherichia coli* was evaluated by microcalorimetry coupled with agar dilution method. The ethanol extract of *W. indica* was isolated with organic solvents of different polarities including petroleum (P.E.) extract, chloroform ($CHCl_3$) extract, ethyl acetate (EtOAc) extract, n-butylalcohol (nBuOH) extract, and residue extract. The metabolic profiles of *E. coli* growth at 37 °C were measured by microcalorimetry. According to the principal component analysis, k_1, k_2, and P_1 were obtained from heat flow power–time (HFP–time) curve. The agar dilution method was performed to verify the results of thermodynamics. The results of microcalorimetric experiment indicated that EtOAc fraction demonstrated the strongest antibacterial activity with half-inhibitory concentration of 92.4 μg mL^{-1}. Meanwhile, similar results were gained from the common method of agar diffusion, which suggested that EtOAc extract could be further developed as antibacterial bioactive fraction of *W. indica*. Altogether, microcalorimetry is a useful technique to provide sufficient quantitative information and evaluate the antimicrobial effect with its sensitive.

Keywords *Wikstroemia indica* · Anti-*E. coli* · Bioactive fraction · Microcalorimetry · Agar dilution method

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Introduction

Wikstroemia indica (L) C.A.Mey. (*W. indica*) has been applied as a folk medicine for many years in China. According to the recent studies, the effects of *W. indica* mainly focused on anticancer [1], anti-inflammatory [2], antiviral [3], and antimalarial [4]. Several studies also showed that ethanol extraction and active components of *W. indica* had an inhibitory effect on bacteria [5]. So the evaluation of pharmacodynamics action including antimicrobial effects of this folk medicine should be studied deeply and widely by useful and sensitive methods.

As a nondestructive and noninvasive tool, microcalorimetry evaluates aerobic and anoxic microbial growth process in a real-time manner, with reproducibility and long-term baseline stability [6, 7]. To increase sensitivity and accuracy, most microcalorimetries generally use "twin instrument" [8]. Among the wide range of microcalorimetry applications, it has been used to study the antimicrobial activities of many drugs and heavy metal on microorganism (including bacteria, cellular organelles, and animal cells) [9–12], and can supply an

automatic and continuous metabolic curve to indicate the growth process with ideal living environment of the biosystem. According to the power–time curves, the effects of drugs on microbe growth can provide abundant quantitative and qualitative information to evaluate the antimicrobial activities of drug and screen for novel antibacterial agents. Therefore, microcalorimetry may be regarded as one of the most sensitive tools in the study of bacterial growth.

Due to the bacteria-producing heat, monitoring power changes plays an important role in evaluating antimicrobial activity and has been attracting increasing attention [13, 14]. Monitoring the metabolic activity of microbe to evaluate the antibacterial mode of *W. indica* had never been explored. Therefore, in this study, the term isothermal microcalorimetry has been performed to evaluate the antibacterial activities of five extract portions of *W. indica* on *E. coli*. The heat flow power–time (HFP–time) curves reflect the dynamic changes in the growth process of *E. coli* quantitatively under the action of agents. Then, principal component analysis (PCA) is carried out on the quantitative parameters obtained from the metabolic profile of *E. coli* to distinguish five extracts according to their antimicrobial effects. Half-inhibitory concentration of *E. coli* metabolism of each sample is calculated. Simultaneously, the common method of agar dilution is used to verify the results of thermodynamics. Figure 1 is the experimental flowchart of this study. Above all, this study aims to explore the different antimicrobial effects among five fraction extracts from *W. indica*.

Materials and methods

Samples and reagents

W. indica was taken from Hunan Province, and the crude materials were authenticated by Prof. Xiao-he Xiao (China Military Institute of Chinese Materia Medica, 302 Military Hospital of China). The air-dried root of *W. indica* (400 g) was powdered and extracted with 75 % EtOH two times under heat reflux. The filtrates were mixed together and concentrated as extractum which was further extracted by different organic solvents, and five kinds of extraction liquids are low-temperature-dried, grind to powder, and stored at 4 °C for microcalorimetric measurement and agar doubling dilution. The quantity of each extraction is, respectively, P.E. (1.02 g), EtOAC (10.11 g), $CHCl_3$ (1.13 g), nBuOH (6.68 g), and water layer residue (10.85 g).

Strain *E. coli* [CMCC (B) 44102] was offered by the National Institutes for Food and Drug Control, Beijing 100051, China. Luria–Bertani (LB) culture medium was prepared by 5 g NaCl and 5 g yeast extract, and 10 g peptone was dissolved in 1000 mL distilled water. And medium pH was adjusted to 7.0–7.2, and the culture medium was sterilized in high-pressure steam at 121 °C for 30 min and stored in a refrigerator at 4 °C. Mueller-Hinton (MH) culture medium contained 17.5 g acid-hydrolyzed casein, 1.5 g soluble starch, 6 g beef extract, 13 g agar in 1000 mL deionized water. The culture medium was sterilized in high-pressure steam at 121 °C for 30 min before it was used.

The extraction solvents such as petroleum ether (P.E.; the batch number: 20140402), chloroform ($CHCl_3$; 20140930), ethyl acetate (EtOAc; 20140622), and n-butyl alcohol (n-BuOH; 20140121) were provided by Beijing Chemical Factory (Beijing, China). All chemicals used were of analytical grade. Ultrapure water was obtained from a Milli-Q Plus system (Bedford, MA, USA), fed by pure water from a Millipore Elix system.

Microcalorimetric measurements

Sample solution preparation

About 20 mg powder of water layer residue of *W. indica* was dissolved with 20 mL LB culture medium and then lautered through 0.22-μm Millipore film to remove the bacteria in the liquid. And the sample solution was yielded at the concentration of 1 mg mL^{-1} for microcalorimetric measurements. All the operations had been done in aseptic conditions. P.E. extract, EtOAC extract, $CHCl_3$ extract, and nBuOH extract were prepared with the same procedure.

Microcalorimetric measurement

The microcalorimetric experiments were performed on the thermal activity monitor (TAM) air isothermal calorimeter (Thermometric AB, Sweden),which was equipped with eight twin calorimetric channels, of which one side was used for the sample and the other for a static reference. The generated signal was recorded in situ by a computer. The microcalorimetric measurement was taken using the ampoule method and brought to equilibrium temperature overnight in advance. All 20-mL glass ampoules were cleaned and sterilized in high-pressure steam (0.1 MPa) at 121 °C prior to use. Table 1 shows that 3 mL LB culture medium containing *E. coli* at the cell density of 1×10^6 colony-forming units (CFU)/mL was inoculated in a sterilized 20-mL glass ampoule, and then different volumes (0, 500, 1000, 1500, 2000, 2500, 3000, 3500 μL) of sample solution were put into each ampoule at a final volume of 10 mL [15]. Ampoules were sealed with wax, shaken-up gently, and placed in

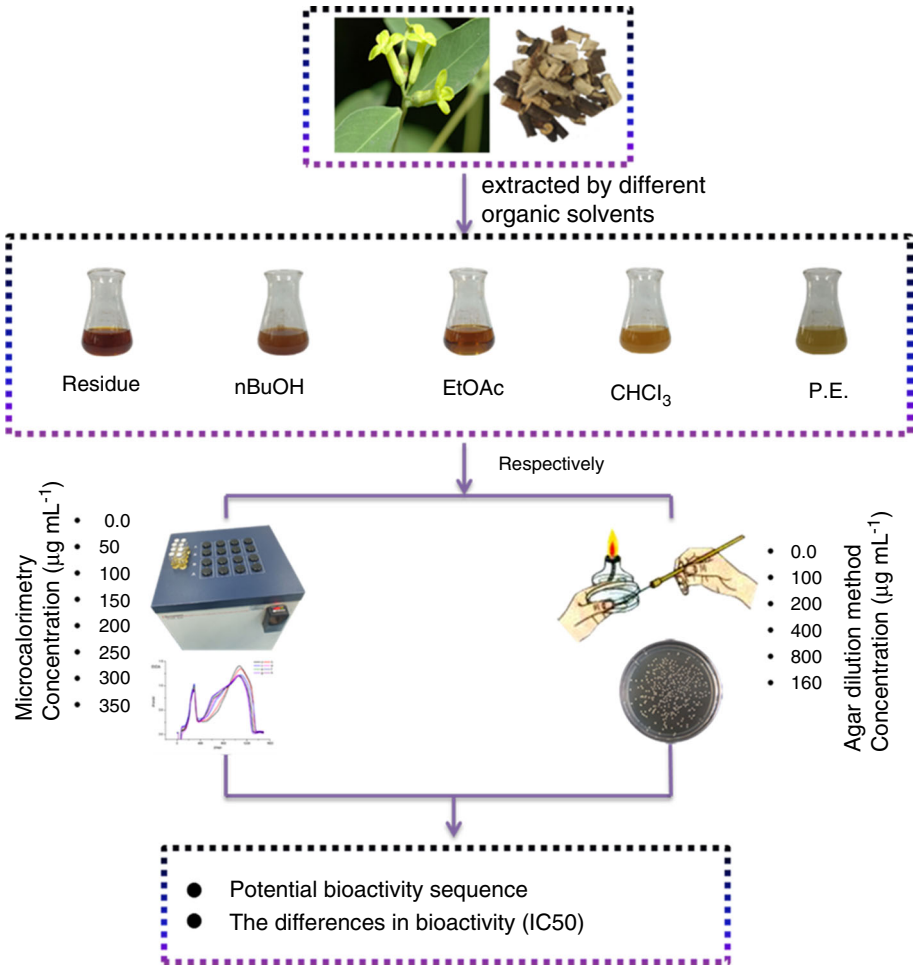

Fig. 1 Experimental flowchart

Table 1 Reagent addition to ampoule of each channel

Channel no.	Bacteria suspension/ mL	LB culture/ μL	Sample solution/ μL
1	3	7000	0
2	3	6500	500
3	3	6000	1000
4	3	5500	1500
5	3	5000	2000
6	3	4500	2500
7	3	4000	3000
8	3	3500	3500

measuring channels. All the operations had been done in aseptic conditions.

Principal component analysis

Principal component analysis (PCA) is a multivariate and unsupervised pattern recognition method used for analyzing, classifying, and reducing the dimensionality of numerical datasets in a multivariate problem, and allows the representation of the original dataset with a set of new orthogonal variables called principal components (PCs) generated as linear combinations of the original variables [16]. In this paper, PCA was performed to reduce the computation burden and further carried out to search for the main parameters which were obtained by analyzing the HFP–time curves of *E. coli* growth affected by roots of *W. indica* extracts using SPSS statistics software (SPSS for Windows 18.0, SPSS).

Antibacterial effects of different extracts from *W. indica* were evaluated by agar doubling dilution method.

With sterile technique, extracts from *W. indica* at different concentrations were poured into aseptic plate. The MH agar medium was taken to the plate containing different kinds of extracts with various concentrations and mixed together for solidification; 18 h later, solvent with bacteria was adjusted to the concentration of 10^5 CFU/mL; same volume of bacteria liquid was inoculated on agar plate containing different extracts at various concentrations. The plates were incubated overnight at 35 °C for 18–24 h. The agar plate containing extracts without bacterial growth was considered as

minimum inhibitory concentration (MIC). Each experiment was tested in triplicate.

Results

Metabolic HFP–time curve of *E. coli* without drugs

Figure 2 shows the metabolic HFP–time curves of *E. coli* in medium as control group absence of any substance are measured by microcalorimeter. There are two curves in Fig. 2: the HFP-*t* curve (the lower one) and the corresponding ln*P*-*t* curve (the upper one) of *E. coli* at 37 °C. The ln*P*-*t* curve indicated the changing character of the metabolic heat power and the HFP–time curve indicated the metabolism profile of *E. coli* which is the typical growth curve for *E. coli* and is usually divided into six growth phase: the lag phase (I), the first exponential phase (II), the transition phase (III), the second exponential phase (IV), the stationary phase (V), and the decline phase (VI). From the standard curve, P_1, P_2, t_1, t_2, k_1, and k_2, six key thermokinetic parameters were obtained and would be further analyzed. [14, 17].

Since the *E. coli* was cultured in the ampoule and replication progress was monitored in isothermal and isochoric conditions, the nutrient and oxygen consumed by bacteria were in limited supply. When the bacteria are inoculated into fresh medium, the bacteria exist a lag phase to adapt to new condition (I). Regrettably, it cannot be exactly displayed for the thermal balance in the ampoule method. Because of sufficient nutrient and oxygen, the bacteria enter into the first exponential growth phase (II), which represents the aerobic proliferation metabolism of *E. coli* [17]. With multiplying rapidly and running out of the oxygen, the bacteria will adjust themselves to adapt to the anaerobic condition and go into a transition phase (II).

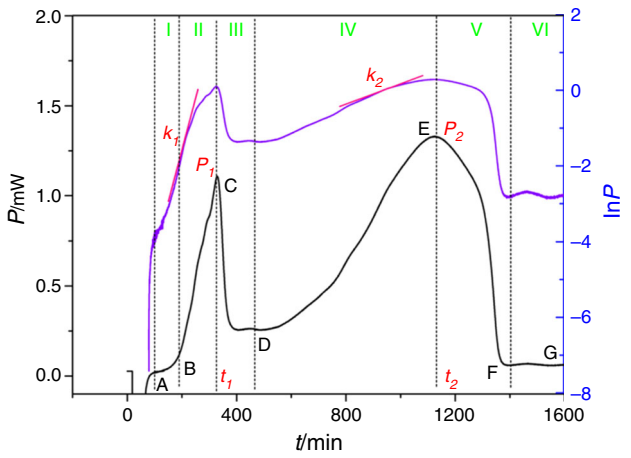

Fig. 2 Metabolic HFP–time curves of *E. coli* in the absence of any substance

While plenty of nutrients remain in this growth system, the bacteria will adapt another way to accommodate the growth environment and begin the second exponential growth phase (IV) [18]. In this phase, all the bacteria sufficiently utilize the nutrients to grow actively and multiply swiftly. When the limited nutrients are exhausted, these bacteria will even produce many toxic materials and go into the decline phase (V). When no further bacterial metabolic activity is measurable, power curves get to the baseline and the following dormant phase appears (VI). Therefore, the experiment is finished.

Quantitative thermokinetic parameters for *E. coli* growth

From the metabolic power–time profiles of *E. coli* growth (Fig. 3), seven quantitative thermokinetic parameters were obtained and are listed in Table 2. And the seven important thermokinetic parameters could reflect the influences of five fractions on the growth of *E. coli*. P_1 and P_2 (mW) are heat flow powers of first and second highest peaks, and t_1 and t_2 (min) are the corresponding appearance time. And k_1 and k_2 present the metabolism rate constant of the first and the second exponential growth phase for *E. coli* growth. Then, total heat output (Q) was obtained from the *P*–*t* curve of *E. coli* growth.

During the exponential phase, the *P*-*t* curve of *E. coli* growth could obey the following equation [19]:

$$P_t = P_0 \exp(kt) \quad (1)$$

$$\ln P_t = \ln P_0 + kt \quad (2)$$

where P_0 and P_t were the power at initial time and any time, respectively. Thus, using the data lnP_t and t taken from the curves to fit a linear equation, k_1 and k_2 represent the metabolism rate constant or growth rate constant of the first and the second exponential phase for *E. coli* growth under the action of five fractions at different concentrations. We could see that in comparison with control, the growth of *E. coli* was decreased with the increasing concentration of samples including EtOAc portion, nBuOH portion, and $CHCl_3$ portion, indicating that those portions may have antibacterial effect.

PCA

In order to further show the tendency and internal change rule of different polarities on the effect, correspondence analysis the thermokinetic parameters k_1, k_2, t_1, t_2, P_1, P_2, Q in Table 2 were set as X_1, X_2, X_3, X_4, X_5, X_6, X_7 in correspondence analysis. The result of the PCA showed that the first three principal components (Z_1, Z_2, and Z_3) contained 90.44 % of the information of the original seven

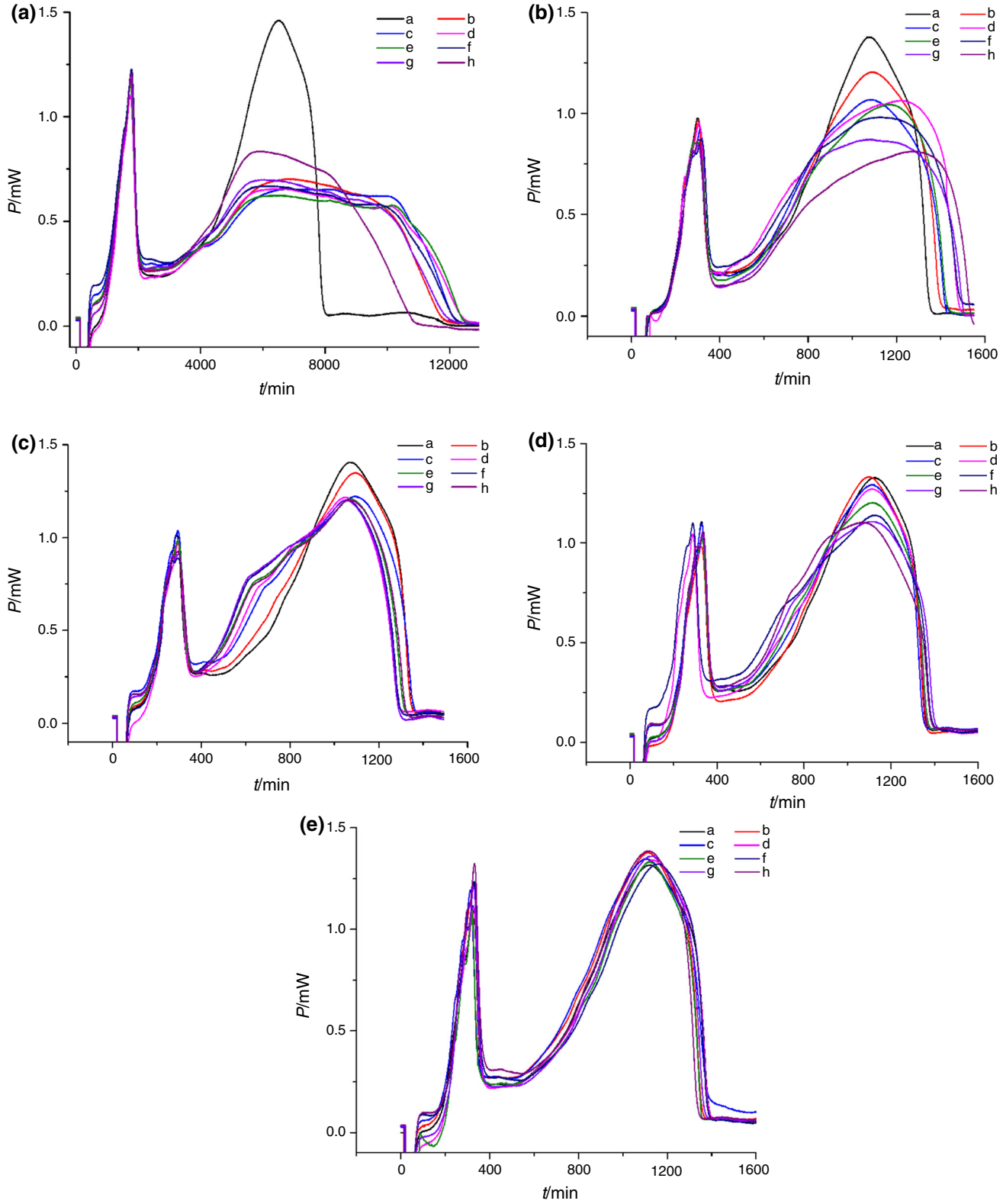

Fig. 3 HFP–time curves of *E. coli*, in the presence of different concentrations of A P.E. fraction, B $CHCl_3$ fraction, C EtOAc fraction, D nBuOH fraction, E residue fraction

Table 2 Thermokinetic parameters obtained from the HFP–time curves of the growth of *E. coli* in the presence of five extracts of *Wikstroemia indica*

Extracts	C/μg mL^{-1}	k_1/min^{-1}	k_2/min^{-1}	t_1/min	t_2/min	P_1/mW	P_2/mW	Q/J	I/%	IC$_{50}$/μg mL^{-1}
P.E. fraction	0	0.0133	0.00245	295.5	1081.33	1.2132	1.4611	51.13	0	–
	50	0.01317	0.00204	292	1111.83	1.1505	0.7011	55.73	16.7	
	100	0.01152	0.00197	290.67	1124.67	1.1714	0.6567	57.87	19.6	
	150	0.01233	0.00181	290.17	1124.83	1.1009	0.6558	55.58	26.1	
	200	0.01162	0.00173	291	1119.83	1.1533	0.6234	58.05	29.4	
	250	0.01006	0.00195	296.33	1058.33	1.2269	0.6662	56.24	20.4	
	300	0.01188	0.00219	293.5	991	1.1819	0.6979	56.14	10.6	
	350	0.01348	0.00232	296.67	982.17	1.2049	0.8354	54.74	5.3	
$CHCI_3$ fraction	0	0.02152	0.00339	299.17	1074.83	0.9783	1.4066	50.32	0	109.1
	50	0.02327	0.00248	299.17	1084	0.9648	1.3499	50.76	26.8	
	100	0.02618	0.00158	310.17	1082.33	0.9241	1.223	48.75	53.4	
	150	0.02052	0.00127	304.33	1210.17	0.9528	1.2182	55.14	62.5	
	200	0.01565	0.00125	296.33	1162.17	0.8594	1.2091	47.87	63.1	
	250	0.01966	0.00113	315.5	1134.17	0.8727	1.2039	52.33	66.7	
	300	0.01573	0.00092	302.17	1066.5	0.8434	1.2034	46.27	72.9	
	350	0.01852	0.00081	300.67	1289.17	0.8724	1.2026	43.87	76.1	
EtOAc fraction	0	0.02397	0.00319	289.17	1068	1.0223	1.4066	48.57	0	92.4
	50	0.02619	0.00228	291.5	1091.67	0.9759	1.3499	48.15	28.5	
	100	0.02856	0.00119	293.67	1091.83	1.0387	1.223	43.25	62.7	
	150	0.02109	0.00113	295.17	1046.5	0.9772	1.2182	51.93	64.6	
	200	0.02261	0.00108	293.17	1067.83	0.9849	1.2091	47.27	66.1	
	250	0.0265	0.000983	293.17	1049.83	0.8894	1.2039	48.93	69.2	
	300	0.02184	0.00096	296.67	1048.33	0.9117	1.2034	44.16	69.9	
	350	0.03153	0.000869	294.17	1061.5	0.9263	1.2026	42.86	72.3	
nBuOH fraction	0	0.01982	0.00327	329	1124.83	1.1079	1.3298	50.7	0	218.8
	50	0.02257	0.00298	326	1102	0.9792	1.333	51.88	8.9	
	100	0.02439	0.00295	329	1111.33	1.0979	1.2935	46.56	9.8	
	150	0.01862	0.00203	288.67	1110.67	1.0455	1.2732	51.24	37.9	
	200	0.02467	0.00171	329.33	1111	1.0247	1.2024	50.47	47.7	
	250	0.02829	0.00137	288.83	1122.33	1.1006	1.1401	41.99	58.1	
	300	0.02689	0.0012	336	1117	1.044	1.1084	52.97	63.3	
	350	0.03005	0.00101	335.5	1066.67	1.0596	1.1055	45.48	69.1	
Residue fraction	0	0.0192	0.00315	324.83	1125.83	1.1268	1.3289	52.91	–	–
	50	0.02625	0.00321	313.17	1120.83	1.1166	1.3597	51.99	–	
	100	0.02569	0.00328	313.17	1119.5	1.1457	1.2979	50.83	–	
	150	0.0241	0.00308	326.83	1137.5	1.1979	1.4293	58.03	–	
	200	0.0363	0.00436	317.83	1123	1.0706	1.1867	38.58	–	
	250	0.02587	0.0036	329.17	1151.67	1.1617	1.2454	45.57	–	
	300	0.02405	0.00335	326.83	1133.67	1.2477	1.3992	53.29	–	
	350	0.02523	0.00371	331.83	1114.5	1.2446	1.3071	46.27	–	

indexes. The equation of three principal components showed the distribution of these seven indexes. The equations were:

$$Z1 = 0.1X_1 + 0.943X_2 - 0.913X_3 + 0.637X_4 + 0.715X_5 + 0.940X_6 + 0.261X_7$$

$$Z2 = 0.858X_1 - 0.185X_2 - 0.004X_3 + 0.623X_4 + 0.166X_5 - 0.146X_6 - 0.851X_7$$

$$Z3 = -0.417X_1 - 0.167X_2 + 0.296X_3 + 0.273X_4 + 0.628X_5 - 0.196X_6 - 0.261X_7$$

These relations indicate that parameters k_2, k_1, and P_1 might be the main parameters, which play a more important role in evaluating the antimicrobial effects of *W. indica* extracts.

Inhibition ratio I and the half-inhibitory concentration IC_{50} on *E. coli*

According to the results of PCA, parameters, k_2, k_1, and P_1 might be the focal parameters. By further comparison of k_2, k_1, and P_1 in Table 3, we could see that parameter k_2 contributed more than k_1 and P_1, indicating k_2 played a focal role. Then, based on the main parameter k_2, another important parameter, inhibitory ratio (I), was calculated and could be defined as:

$$I = [(k_0 - k_c/k_c)] \times 100\ \% \quad (3)$$

where k_0 is the growth rate constant of the control, k_c is the growth rate constant of the second exponential growth phase at inhibitor concentration C. Inhibition ratio I on *E. coli* at different concentrations of different polarities of *W. indica* was calculated from Eq. (3). When the inhibitory ratio I is 50 %, the corresponding concentration of inhibitor is expressed as IC_{50}, another necessary parameter to evaluate the activity of antimicrobial agent. Because the inhibitory rate of P.E. fraction did not reach 50 % and residue fraction had no anti-*E. coli* activity, we only calculated the IC_{50} of $CHCl_3$, EtOAc, and nBuOH fractions. The sequence of IC_{50} of the three extracts was EtOAc fraction < $CHCl_3$ fraction < nBuOH fraction. EtOAc fraction had the smallest value of IC_{50} (92.40 μg mL^{-1}), while nBuOH fraction had the biggest value of IC_{50} (218.8 μg mL^{-1}) among the three extracts of *W. indica* (Table 2), which indicated that EtOAc fraction might have the strongest anti-*E. coli* activity among them.

Table 3 Inhibitory effects of different extracts from *W. indica* on *E. coli*

Extracts	MIC/mg mL^{-1}		
	Range	MIC_{50}	MIC_{90}
P.E.	4–256	128	>256
$CHCl_3$	2–256	64	>256
EtOAc	2–256	32	256
nBuOH	2–256	64	>256
residue	–	–	–

The result of agar dilution method

The MIC ranges and the 50 % (MIC_{50}s) and 90 % (MIC90 s) MICs for different extracts of *W. indica* are shown in Table 3. Except for residue part, the sizes of colonies decreased sharply with the concentration of samples on agar plates increasing. And residue extracts had no antibacterial activities. *E. coli* was susceptible to EtOAc extract (MIC_{50}, 32 mg mL^{-1}; MIC_{90}, 256 mg mL^{-1}), $CHCl_3$ extract (MIC_{50}, 64 mg mL^{-1}), nBuOH extract (MIC_{50}, 64 mg mL^{-1}), and P.E. extract (MIC_{50}, 128 mg mL^{-1}). MIC_{50} was used for tentatively evaluating the antibacterial activity of the five extracts from *W. indica* on *E. coli*. The lower the MIC_{50}, the stronger the inhibitory effect on the *E. coli* among the five portions in this study. And a potential antimicrobial activity sequence, EtOAc extract > $CHCl_3$ extract/nBuOH extract > P.E. extract was obtained.

Discussion

W. indica is distributed in the southeast of China. It has long been used as a traditional crude drug for the treatment for pneumonia, rheumatism, and antibacterial in China. The antibacterial activity of *W. indica* has been reported previously, but the constituents that have antibacterial activity have not been clarified yet. [20] In this study, the ethanol extracts of *W. indica* were fractionated, and various fractions were examined for their activity against *E. coli based on* microcalorimetry coupled with agar dilution method.

In this study, microcalorimetry was performed due to its high sensitivity and accuracy especially when the concentration of sample was low. From the results of the study, in the power–time curves of *E. coli*, we could see that in comparison with control, the k_2 of the second exponential phase of *E. coli* was decreased with the increasing concentration of samples including EtOAc portion, nBuOH portion, and $CHCl_3$ portion, indicating that *E. coli* decreased the growth speed. Simultaneously, sufficient quantitative parameters were obtained to estimate the antibacterial effects of different extractions of *W. indica.* Based on PCA, k_1, k_2, P_1 were considered as the main parameters, which played a more important role in evaluating the antibacterial effect of *W. indica* extraction. Further analyzed, parameters k_2 contributed more than k_2 and P_1, indicating k_2 played a focal role. So, a potential

antimicrobial activity sequence, EtOAc extract > $CHCl_3$ extract > nBuOH extract > P.E. extract, of the tested samples was obtained by microcalorimetry. At the same time, in Table 3, similar results were obtained from common pharmacological experiments.

As a useful tool, microcalorimetry is proven to be a higher efficient and valuable tool in several fields of microbiology [8] compared with conventional techniques. IMC microbiology, using the methods that we describe, entails real-time, continuous measurement of microbial metabolic heat produced in sealed ampoules containing growth medium, bacteria, and antibiotic. And it can distinguish bacterial species based on the heat flow power–time curves. One noteworthy environment application is efficient assessment of bacterial activities directly without adding radiolabelled, fluorescent, or chromogenic substrates [6]. For medical application, microcalorimetry is a rapid detection way of bacterial infection or contamination, which is of vital importance in quickly implementing the correct treatment [21].

In summary, our study illustrates that EtOAc extract from *W. indica* demonstrated stronger antibacterial effects on *E. coli* growth than each other parts. The antibacterial effects of *W. indica* were not limited in one active portion, showing that *W. indica* exerted antibacterial effects by different active components working coordinately in different ways. The *W. indica* and the actual action mechanism of active compounds on microorganisms need further investigation. This study also illustrated that it was possible for microcalorimetry coupled with agar dilution method to evaluate the antimicrobial effect, which further provided the basis to develop a new method to assess the antibacterial effect of other Chinese material medicas.

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