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Microcalorimetry studies of the antimicrobial actions of *Aconitum* alkaloids[*]

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Abstract: The metabolic activity of organisms can be measured by recording the heat output using microcalorimetry. In this paper, the total alkaloids in the traditional Chinese medicine *Radix Aconiti Lateralis* were extracted and applied to *Escherichia coli* and *Staphylococcus aureus.* The effect of alkaloids on bacteria growth was studied by microcalorimetry. The power-time curves were plotted with a thermal activity monitor (TAM) air isothermal microcalorimeter and parameters such as growth rate constant (μ), peak-time (T_m), inhibitory ratio (I), and enhancement ratio (E) were calculated. The relationships between the concentration of *Aconitum* alkaloids and μ of *E. coli* or *S. aureus* were discussed. The results showed that *Aconitum* alkaloids had little effect on *E. coli* and had a potentially inhibitory effect on the growth of *S. aureus.*

Key words: *Aconitum* alkaloids, *Escherichia coli*, *Staphylococcus aureus*, Microcalorimetry
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1 Introduction

Radix Aconiti Lateralis (RAL) (also known as monkshood or aconite), which is the daughter root of *Aconitum carmichaeli* Debx. (Ranunculaceae), is one of the most useful herbal medicines. It has been reported that RAL Preparata have some pharmacological activities for the treatment of heart failure, congestion, colds, neuralgia, polyarthralgia, rheumatism, and gout (Wadsö, 2002; Zhao *et al.*, 2006; Chen *et al.*, 2008). Up until now, the most investigated pharmaceutical ingredients in RAL Preparata were a series of alkaloids, which are also found to be the major toxic ingredients in the medicine. Inappropriate use of RAL Preparata could result in unwarranted toxicity (Ameri,

1998; Singhuber *et al.*, 2009; Wang *et al.*, 2009). However, there are few studies on the effect of alkaloids on bacteria. Therefore, the aim of this study is to investigate the effect of alkaloids extracted from RAL Preparata on bacteria.

In recent years, microcalorimetric techniques have been widely applied in biochemistry, biophysics and other fields (Wadsö, 1995; 1997; 2001). These techniques have also increasingly been used to study the interactions between drugs and microorganisms. Microcalorimetry is a highly sensitive, quantitative, and versatile tool for assessing the activity of microorganisms. Further advantages of this method are that it is simple and non-invasive *vis-a-vis* the sample. As a result, the power-time curves of bacteria can be continuously plotted in real time (Critter *et al.*, 2001; Yan *et al.*, 2007). It can provide abundant thermodynamic and kinetic information, such as the growth rate constant (μ), inhibitory ratio (I), generation time (T), minimal inhibitory concentration (MIC), peak-height (H_m), peak-time (T_m), and total heat (Q_{tot}).

Microcalorimetry therefore appears to be a suitable technique for studying microbial activity (Xi *et al.*, 2002; Wu *et al.*, 2005).

In this paper, we investigate the antimicrobial activity of alkaloids extracted from RAL Preparata on *Escherichia coli* and *Staphylococcus aureus* growth using microcalorimetry. *Aconitum* alkaloids were obtained using the water extraction method and determined with aconitine reference substance by ultraviolet (UV). The metabolic power-time curves for *E. coli* and *S. aureus* growth in different concentrations of extractions were obtained and compared with bacterial growth curves without the addition of samples. The relationships between the thermokinetic parameters of *E. coli* growth and sample concentrations were discussed, and the correlations between the antimicrobial effects were determined.

2 Materials and methods

2.1 Instrumentation

A thermal activity monitor (TAM) air device (Thermometric AB, Sweden), an 8-channel heat-conduction calorimeter for heat-flow measurements in isothermal conditions, was used to assess the metabolism of *E. coli* and *S. aureus*. The device was equipped with eight twin calorimetric channels with one side for the sample and the other for a static reference. Measurements were made in sealed 24-ml glass ampoules between 5 °C and 90 °C. The error in the temperature measurements was ±0.02 °C. The detection limit was 2 μW and the baseline stability was 2×10^6 μW over a period of 24 h (Kong *et al.*, 2008). The heat output was recorded continuously using a PLW32 recorder in real time.

2.2 Materials and methods

The bacteria *E. coli* (CMCC(B)44102) and *S. aureus* (CMCC(B)26003) were provided by the Shandong Institute for Drug Control, China. They were routinely cultured in a Luria-Bertani (LB) culture medium, which contained 10 g of peptone, 5 g of yeast extract, and 5 g of NaCl per liter (pH 7.0–7.2). The LB culture medium was sterilized by autoclaving at 121 °C for 20 min. The *Aconitum* alkaloids were provided by Jiangyou Zhongba Ltd. (Sichuan, China).

The purified *Aconitum* alkaloid test samples were prepared according to the following procedure. The powdered commercial RAL Preparata (10.0 g) was extracted at boiling conditions with 180 and 90 ml of water. The combined filtrates were concentrated to approximately 120 ml by rotary vacuum evaporation. The concentrated filtrate was then fractionated overnight with 60% ethanol at 4 °C. The supernatant was concentrated and extracted with chloroform five times to obtain the *Aconitum* alkaloids, which were then dried under vacuum. The average yield of *Aconitum* alkaloids was determined by UV spectroscopy at 490 nm. According to the standard curve established by the aconitine reference substance ($A=0.0181c+0.1353$, $r=0.9989$, where A is the absorbance and c is the concentration of aconitine reference substance), the content of *Aconitum* alkaloids in RAL Preparata was 81.2%.

The microcalorimetric measurements were made with the ampoule method. The LB culture medium containing the bacteria was placed in 20-ml glass ampoules. Then, different concentrations of *Aconitum* alkaloids were mixed into each ampoule to a final volume of 10 ml. One ampoule without *Aconitum* alkaloids was used as the blank control. The ampoules were then sealed and placed in the microcalorimeter. The temperature was maintained at 37 °C. The power-time signals were recorded every minute until the recordings returned to the baseline measurement (Yang *et al.*, 2008).

3 Results and discussion

3.1 Power-time curves

The power-time curves of the growth of *E. coli* and *S. aureus* without *Aconitum* alkaloids are shown in Figs. 1 and 2. The shapes of the two curves are different. The *E. coli* growth curves can be divided into four stages: the first growth, first decrease in metabolic activity, second growth, and second decrease in metabolic activity stages. The output power was stronger than that of *S. aureus* in the first 30 min. The power-time curves of the *S. aureus* growth process were comparatively simple with only an increased growth stage and a decreased metabolic stage. In the exponential phase (the first rapid growth stage), the bacteria were in a good environment with sufficient nutrients and limited metabolic products. The shapes
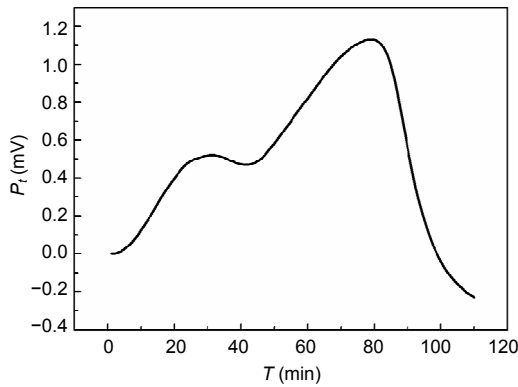
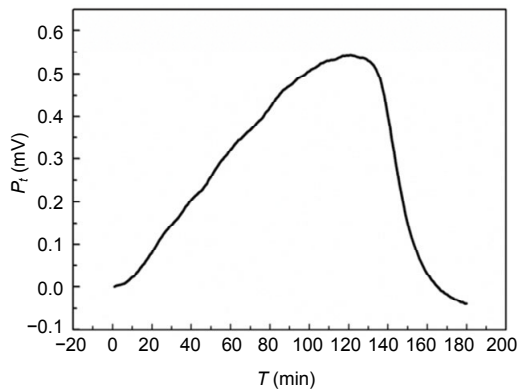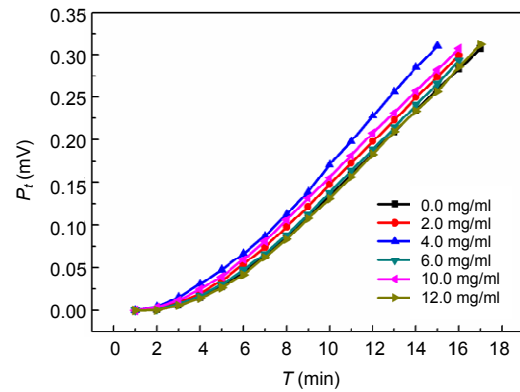**Fig. 1 Power-time curve of *E. coli* without *Aconitum* alkaloids**

Fig. 3 Exponential phase of *E. coli* with different concentrations of *Aconitum* alkaloids

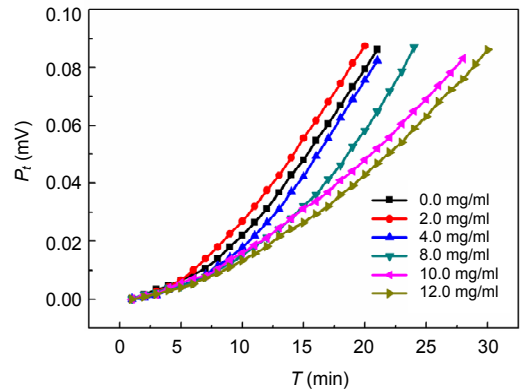**Fig. 2 Power-time curve of *S. aureus* without *Aconitum* alkaloids**

Fig. 4 Exponential phase of *S. aureus* with different concentrations of *Aconitum* alkaloids

of the power-time curves made it possible to study the effect of drugs on the bacteria. The exponential growth phases of *E. coli* and *S. aureus* with the addition of different concentrations of *Aconitum* alkaloids are shown in Figs. 3 and 4, respectively. In the exponential growth phase, *S. aureus* had a distinct downward trend with increases in the *Aconitum* alkaloid concentrations, and a high concentration of samples needed more time to reach the same heat output. However, a concentration-dependent delay was not clearly observed in the exponential growth phases of *E. coli* with the *Aconitum* alkaloid samples.

3.2 Thermokinetics

In the exponential growth phase, the power-time curves for *E. coli* and *S. aureus* obey the following law:

$$dN_t/dt = \mu N_t - \beta N_t^2, \tag{1}$$

where N_t is the bacterial number at time t, μ is the

growth rate constant, and β is the bacterial static-rate constant. The integral of Eq. (1) is

$$N_t = K/(1+\alpha e^{-\mu t}), \tag{2}$$

where K is the maximum density and α is integral constant. If the power produced by every bacterium is P, then $PN_t = KP/(1+\alpha e^{-\mu t})$, making $P_t = PN_t$ and $P_m = PK$ (P_t is the power output at time t and P_m is the maximum power output). This leads to the following relationship:

$$P_t = P_m/(1+\alpha e^{-\mu t}). \tag{3}$$

Eq. (3) is the logistic equation. If the values of P_t and t can be obtained from the bacterial growth curves, the rate constant μ can then be calculated.

The inhibition ratio, I, is an excellent index of the inhibition of different *Glycyrrhiza* extracts on the metabolism of *E. coli*. The inhibition ration can be defined as:

$$I=(\mu_0-\mu_c)/\mu_0\times100\%. \qquad (4)$$

The enhancement ratio, *E*, is an excellent index of the inhibition of *Aconitum* alkaloids on *E. coli* and *S. aureus*, and can be defined as:

$$E=(\mu_c-\mu_0)/\mu_0\times100\%, \qquad (5)$$

where μ_0 and μ_c are the growth rate constants of the bacteria without and with *Aconitum* alkaloids, respectively.

The corresponding values of μ, T_m, P_m, *I*, *E* and the calculated results are shown in Table 1.

3.3 Relationships between growth rate constant (μ) and concentration (*c*)

The relationships between μ and *c* are demonstrated in Figs. 5 and 6. The rate constant, μ, of *E. coli* changed slightly with an increase in the concentration of *Aconitum* alkaloids. It could be inferred that *Aconitum* alkaloids had little influence on *E. coli* growth. Fig. 6 shows that μ for *S. aureus* growth increased with a low concentration of *Aconitum* alkaloids and decreased as the concentration was increased. This reveals that *Aconitum* alkaloids promoted *S. aureus* growth at low concentrations and inhibited growth at higher concentrations. When the concentration of *Aconitum* alkaloids was between 0 and 12 mg/ml, the relationship between μ and *c* was

$$\mu=0.0002c^3-0.0050c^2+0.0173c+0.2452$$
$$(r=0.9990). \qquad (6)$$

According to Eqs. (4) and (5), the inhibitory (*I*) and enhancement (*E*) ratios were calculated, as shown in Table 1. From the data, we concluded that *Aconitum* alkaloids had a minimal effect on *E. coli* growth and a potent antibacterial activity against *S. aureus*.
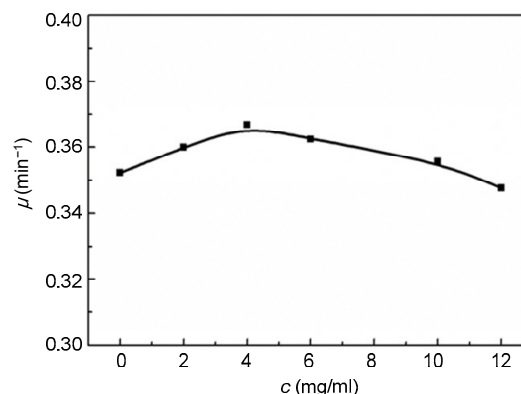

Fig. 5 Relationship between growth rate constant (μ) and concentration (*c*) of *Aconitum* alkaloids for *E. coli*
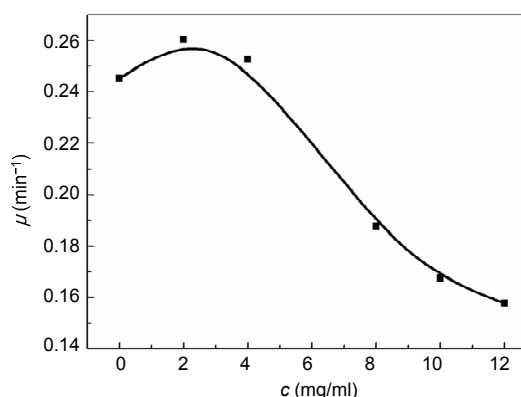

Fig. 6 Relationship between growth rate constant (μ) and concentration (*c*) of *Aconitum* alkaloids for *S. aureus*

Table 1 Parameters of *E. coli* and *S. aureus* growth at different concentrations of *Aconitum* alkaloids

Bacteria	*c* (mg/ml)	μ (min^{-1})	*r*	T_m (min)	P_m (mW)	*I* (%)	*E* (%)
E. coli	0	0.3522	0.9975	77	1.1346		
	2.0	0.3599	0.9973	78	1.1187		2.19
	4.0	0.3667	0.9980	76	1.1507		4.12
	6.0	0.3623	0.9976	75	1.1032		2.87
	10.0	0.3557	0.9972	77	1.1195		0.99
	12.0	0.3474	0.9973	78	1.1032	1.36	
S. aureus	0	0.2455	0.9990	122	0.5414		
	2.0	0.2604	0.9979	129	0.5045	5.50	6.07
	4.0	0.2526	0.9991	145	0.5467	16.29	2.89
	8.0	0.1876	0.9988	154	0.5488	22.71	
	10.0	0.1675	0.9973	158	0.5411	29.80	
	12.0	0.1577	0.9978	163	0.5321	34.68	

c: concentration; μ: growth rate constant; *r*: correlation coefficient; T_m: peak-time, P_m: maximum heat output; *I*: inhibitory ratio; *E*: enhancement ratio

3.4 Relationships between peak-time (T_m) and concentration (*c*)

As seen in Table 1, *Aconitum* alkaloids had no effect on the first T_m of *E. coli* growth, while an increasing trend was observed in the values of T_m for *S. aureus* growth as *c* was increased. This indicates that *Aconitum* alkaloids had a concentration-dependent inhibitory effect on *S. aureus*.

4 Conclusions

In this research, the microcalorimetric method was used to study the effect of different concentrations of *Aconitum* alkaloids on the growth of *E. coli* and *S. aureus*. By recording the heat output, the metabolic activity of the bacteria was evaluated, and the effect of extracts was investigated. By the kinetic and thermodynamic information from the microcalorimetric method, a series of kinetic parameters, such as the rate constant μ, peak-time T_m, maximum power output P_m, inhibitory ratio I, and enhancement ratio E, were obtained. We also discussed the relationship between the growth rate constant (μ) and the concentration of *Aconitum* alkaloids (*c*). The rate constant of *E. coli* growth showed little change with increase in the concentration of *Aconitum* alkaloids. However, the growth rate constant of *S. aureus* increased and then decreased as the concentration of *Aconitum* alkaloids was increased. The increased concentration of *Aconitum* alkaloids changed the T_m of *E. coli* slightly, but the T_m of *S. aureus* was prolonged compared with the control. This indicated that the *Aconitum* alkaloid treatment slowed the growth and metabolism of *S. aureus*. We could draw the conclusion that *Aconitum* alkaloids had no effect on the growth of *E. coli* but a potential inhibitory effect on *S. aureus*.

This work has shown that microcalorimetry is a useful technique, which can be applied to the study of microbial growth. It also offered useful information for research and the application of the traditional herbal medicine RAL and its active ingredients.

Compliance with ethics guidelines

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中文概要

题　目：附子生物碱抗菌活性的微量热研究

目　的：研究附子生物碱对大肠杆菌和金黄色葡萄球菌生长代谢作用的影响，并对其抗菌活性进行评价。

创新点：首次应用微量热的方法研究了大肠杆菌和金黄色葡萄球菌在附子生物碱中的生长代谢作用，并从热力学角度对附子生物碱的抗菌活性进行了评价。

方　法：微量量热仪绘制了 37 °C 时大肠杆菌和金黄色葡萄球菌在不同浓度的附子生物碱作用下的热谱曲线，根据热动力学模型，分别得到了生长速率常数 μ、抑制率 I、最大产热功率 P_m 以及最大产热功率所对应的时间 T_m 等热动力学参数，从热动力学的角度探讨了附子生物碱对大肠杆菌和金黄色葡萄球菌代谢作用的影响。

结　论：实验结果显示，附子生物碱对大肠杆菌的生长代谢基本没有影响。但是对于金黄色葡萄球菌，随着附子生物碱浓度的增大，其生长速率常数呈现先升高后降低的趋势，同时达峰时间也被延长，说明附子生物碱对金黄色葡萄球菌存在潜在的生长抑制作用。

关键词：附子生物碱；大肠杆菌；金黄色葡萄球菌；微热量热法