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研究报告

微量热法研究板蓝根中四种有机酸对微生物生长代谢的影响

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摘　要: 微量热法研究传统中药板蓝根中四种有机酸对大肠杆菌、金黄色葡萄球菌和痢疾杆菌生长代谢的影响。得到加药与不加药时大肠杆菌、金黄色葡萄球菌和痢疾杆菌生长代谢的“效-时”曲线, 以生长速率常数(k_1, k_2)、最大产热功率(P_m)和最大达峰时间(t_m)等热力学参数来评价四种有机酸对微生物生长代谢抑制的强度和程度。四种有机酸抗微生物活性作用的顺序为: 丁香酸>邻氨基苯甲酸>水杨酸>苯甲酸, 其中苯甲酸对金黄色葡萄球菌和痢疾杆菌的生长代谢具有促进作用。本研究对板蓝根的进一步研究提供了基础和依据。

关键词: 板蓝根, 微量热法, 有机酸, 微生物, 抗微生物活性

Microcalorimetric Studies of the Action on Four Organic Acids in *Radix isatidis* on the Growth of Microorganisms

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Abstract: The actions of four organic acids in *Radix isatidis,* a traditional Chinese medicinal (TCM) herb, on *Escherichia coli*, *Staphylococcus aureus* and *Shigella dysenteriae* growth were investigated by microcalorimetry. The four organic acids were syringic acid, 2-amino-benzoic acid, salicylic acid and benzoic acid. The power-time curves of *Escherichia coli*, *Staphylococcus aureus* and *Shigella dysenteriae* growth with and without organic acids were acquired, meanwhile the extent and duration of inhibitory effects on the metabolism were evaluated by growth rate constants (k_1, k_2), maximum heat-output[0] power (P_m) and peak time (t_m). The inhibitory activity varied with different drugs. The sequences of anti-microbial activity of the four organic acids on *Escherichia coli*, *Staphylococcus aureus* and *Shigella dysenteriae* were all: syringic acid > 2-amino-benzoic acid > salicylic acid > benzoic acid. And benzoic acid promoted the growth of *Staphylococcus aureus* and *Shigella dysenteriae*. This study provides a basis for the further study on *Radix Isatidis*.

Keywords: *Radix isatidis*, microcalorimetry, organic acids, microorganism; anti-microbial activity

Radix isatidis (Banlangen in Chinese), the root of *Isatidis indigotica* Fort, is a traditional Chinese

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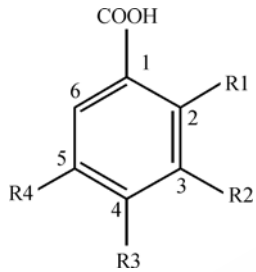
medicinal (TCM) herb, and is officially listed in the Chinese Pharmacopoeia[1]. It has heat clearing, detoxicating, cooling the blood and anti-inflammatory activity[2]. The chemical constituents of *Radix Isatidis* with various pharmacologic actions are very complicate. The four organic acids (OAs): syringic acid, 2-amino-benzoic acid, salicylic acid and benzoic acid have been segregated and obtained from *Radix Isatidis*[3–5], and their structures were given in Fig.1. The four OAs had strong anti-endotoxic effects[6].

In order to investigate the anti-microbial effect, in this study, the four OAs in *Radix Isatidis* were tested against *Escherichia coli* (*E. coli*), *Staphylococcus aureus*(*S. aureus*) and *Shigella dysenteriae* (*S. dysenteriae*) growth.

E. coli, *S. aureus* and *S. dysenteriae* are perhaps the human pathogenic microorganisms that have been studied extensively. It is a good choice for studying the effects of four OAs in *Radix Isatidis* on their growth in vitro. This may help us to understand the general effects that these OAs may have on other microorganisms[7].



	R1	R2	R3	R4
Benzoic acid	H	H	H	H
Salicylic acid	OH	H	H	H
2-amino-benzoic acid	NH_2	H	H	H
Syringic acid	H	OCH_3	OH	OCH_3

Fig. 1 Chemical structures of investigated OAs from *Radix isatidis*

Microcalorimetry provides a general analytical tool for the characterization of the microbial growth process. It has been used extensively to investigate drug and the microbial cell interaction and has furnished much useful information[7–9].One of the most prominent features of the microbial growth process is the production of heat. If the heat is monitored by microcalorimeter, much useful information, both qualitative and quantitative, may be obtained. Each type of microbial has a unique power-time trace, as recorded by the microcalorimeter, under a defined set of growth conditions.

Any substance that can modify the metabolic growth processes involved in cell will change the power-time curve obtained from the microcalorimeter. From the power-time curves, not only thermodynamic but also kinetic information can be obtained.

Microcalorimetry has been previously used to investigate the interaction between drugs and microbial cells[10]. This technique has also been used to investigate the inhibitory effects of selenomorpholine compounds on *E. coli*[11], *S. aureus*[12] and *S. dysenteriae*[13]. In this paper, the power-time curves produced by *E. coli, S. aureus* and *S. dysenteriae* under the action of four OAs at the same concentration (200 μg/mL) were obtained with a TAM Air Bioactivity Monitor. From these power-time curves, the growth rate constant k and the generation time t_G of microorganism growth were calculated.

1 Materials and methods

1.1 Instrument

A TAM Air Bioactivity Monitor (Thermometric AB, Sweden, Fig.2) was used to determine the metabolism of *E. coli, S. aureus* and *S. dysenteriae*. It is an eight-channel heat conduction calorimeter for heat flow measurements in the milliwatt range under isothermal conditions, was held together in a single removable block. The system is very sensitive, the detection limit is 2 μW and baseline stability (over a period of 24 h) is 6 μW. All calorimetric channels were of twin type, consisting of a sample and a reference vessel. Each vessel was connected to the surrounding heat sink by a Peltier module, and when heat was produced or consumed due to any process, the temperature of the sample vessel was to change. The surrounding temperature was constant and thus a temperature gradient across the Peltier module was developed. This would generate a measurable voltage and the voltage was proportional to the heat flow across the Peltier module and to the rate of the processes taking place in the sample vessel. Such voltage signal was recorded continuously and in real-time through an eight-channel data logger. The software supplied to the TAM air was used to monitor and record the heat flow over the Peltier module. For details of the performance and structure of the instrument, see reference [14].

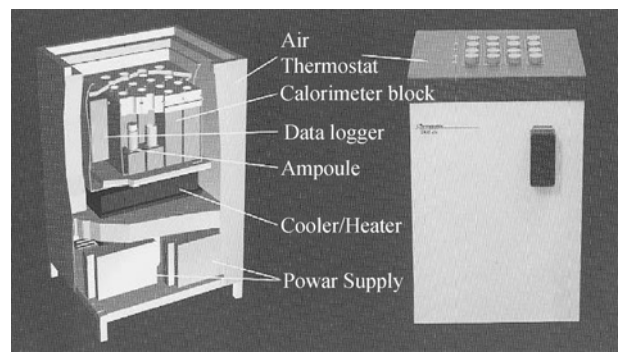


Fig. 2 TAM Air Isothermal Calorimeter
(Thermometric AB, Sweden)

1.2 Materials

Lactose Broth (LB) medium, consisting of 5 g NaCl, 10 g tryptone, 5 g yeast extract per liter, pH 7.0, was sterilized by autoclaving for 30 min at 121°C.

E. coli (*Escherichia coli* CMCCB 44103), *S. aureus* (*Staphylococcus aureus* AB 910393) and *S. dysenteriae* (*Shigella dysenteriae* AB 210562) were provided by the

National Institute for the Control of Pharmaceutical and Biological Products. The three strains were grown in a peptone culture medium, which contained 10 g peptone, 6 g beef extract and 5 g NaCl. Medium pH was adjusted to 7.0~7.2 with 1mol/L NaOH before autoclaving, these suspensions were used as the inocula for the microbiological assay.

Radix Isatidis was the dried root of *Isatis indigotica* Fort, which was accredited by Xiaohe Xiao, one of the authors, institute of Chinese Materia Medica in 302 Hospital of PLA, Beijing 100039, China. The four OAs were extracted from *Radix Isatidis* with the purity > 98%[3] and their structures were given in Fig. 1.

1.3 Batch experiments of bacteriostatic activity by microcalorimetry

The inocula were homogeneously distributed into 50 mL of LB medium by gentle shaking. Aliquots of 5 mL of the suspensions were added into 20 mL sterilized ampoules containing test samples and sealed tightly. The ampoules were placed in the calorimeter and signals obtained during growth were detected. The experiments were run at 37°C for bacteria and the thermogenic curves were recorded until the recorder returned to the baseline. Since the bacterial metabolic process was monitored under the isothermal and isochoric conditions, the nutrient and oxygen consumed by cells was surely limited. All data were collected continuously using the dedicated software package.

2 Results

2.1 Growth rate constants (k_1, k_2) of *E. coli*

Fig. 3 showed the thermogenic curves of *E. coli*, *S. aureus* and *S. dysenteriae* growth at 37°C without drugs and with the four OAs at the same concentration (200 μg/mL), respectively. The heat-production growth curves of *E. coli*, *S. aureus* and *S. dysenteriae* could be divided into four phases, *i.e*. lag phase, first exponential phase, second exponential phase and decline phase. The exponential model of metabolism of *E. coli*, *S. aureus* and *S. dysenteriae* could be used in the two growth processes[15]:

$$P_t = P_0 \exp(kt) \quad \text{or} \quad \ln P_t = \ln P_0 + kt \qquad (1)$$

where P_0 is the heat-output power at time 0, and P_t at time t. The thermogenic curve formula of the exponential phase of growth was Eq. (1). The calorimetric power (P), which reflects the multiplication of the cells, can be used as a parameter to characterize the growth of the cells. Since $P = dQ/dt$, the area under the curve records the heat output Q released during the experimental period. The growth rate constants (k_1, k_2) were obtained by fitting ln P_t and t to a linear equation. The second exponential phase could be regarded as a part of stationary phase because the growth rate constant of phase II (k_2) was much less than that of phase I (k_1).

The different inhibitory actions of four OAs could be clearly demonstrated by the plots shown in Fig. 4 and Fig. 5. These two figures respectively showed the *k*-*c* plots and P_m-*c* plots of *E. coli*, *S. aureus* and *S. dysenteriae* in the presence of four OAs at the same concentration (200 μg/mL).

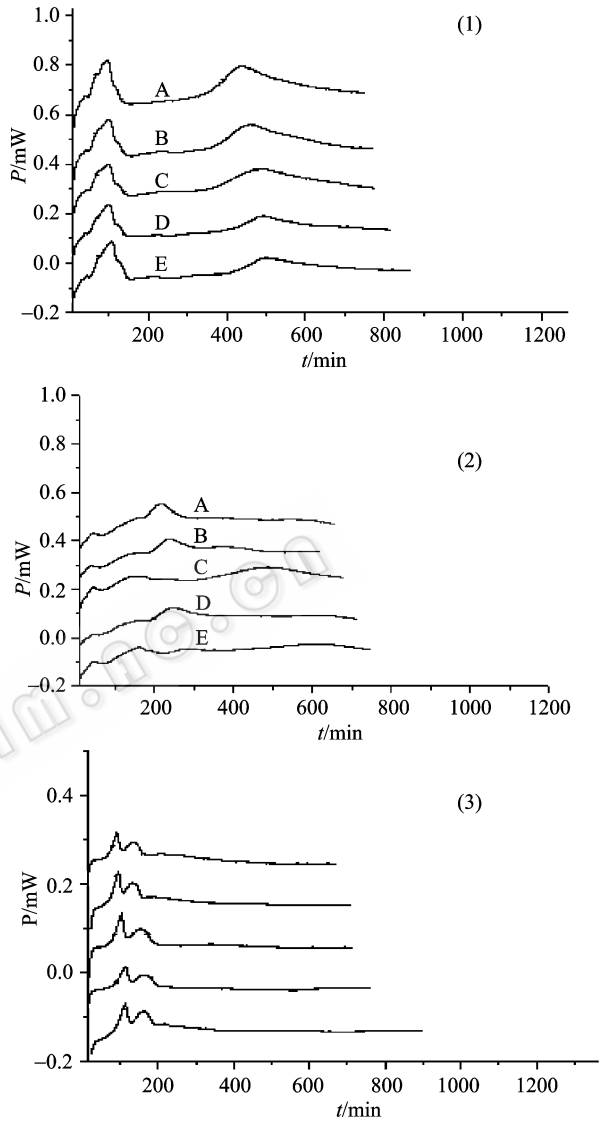


Fig. 3 Power-time curves of bacterium in the presence of four OAs with same concentration (200 μg/mL)
(1) *E. coli*; (2) *S. aureus*; (3) *S. dysenteriae*

2.2 Inhibitory ratio *I* and the half inhibitory concentration IC_{50}

Inhibitory ratio I was defined as:

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

where k_0 was growth rate constant of the control, k_c was rate constant in the exponential phase of bacterial growth inhibited by inhibitor concentration (c). IC_{50} is the inhibitor concentration causing a 50% decrease of the growth rate constant. The IC_{50} of four OAs on *E. coli* growth were 56 μg/mL for syringic acid, 65 μg/mL for 2-amino-benzoic acid, 86 μg/mL for salicylic acid and 224 μg/mL for benzoic acid; on *S. aureus* growth were 25 μg/mL for syringic acid, 45 μg/mL for 2-amino-benzoic acid, 68 μg/mL for salicylic acid; on *S. dysenteriae* growth

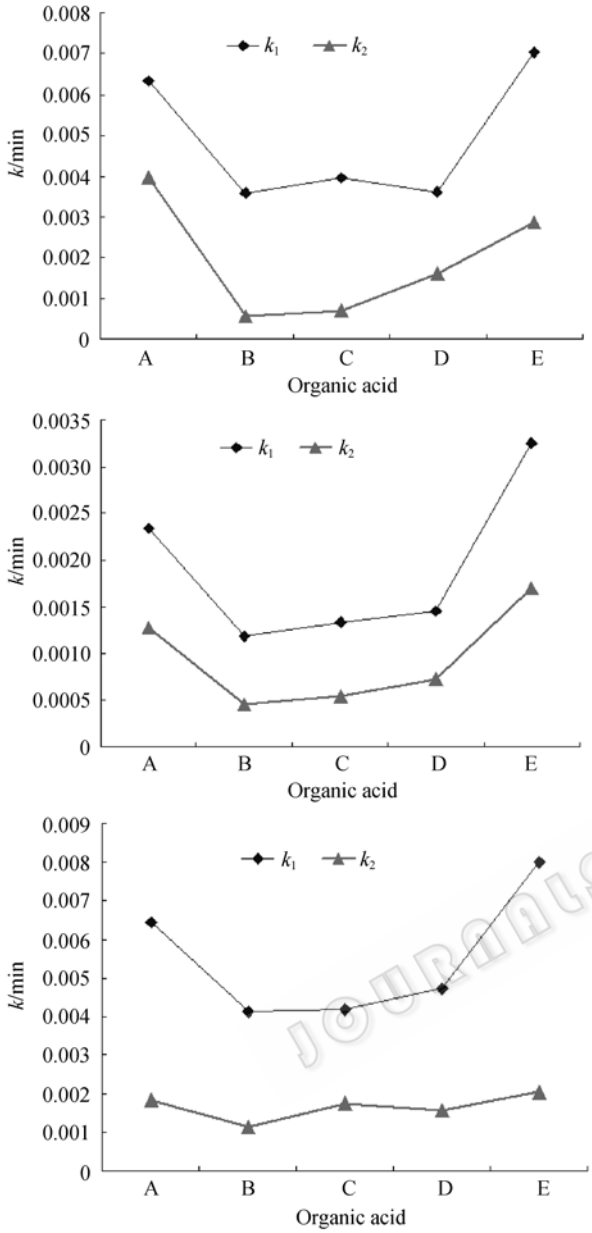


Fig. 4 The *k*-*c* plots of bacterium in the presence of four OAs with same concentration (200 μg/mL)
(1) *E. coli*; (2) *S. aureus*; (3) *S. dysenteriae*
A:,blank; B: syringic acid; C: 2-amino-benzoic acid; D: salicylic acid; E: benzoic acid

were 45 μg/mL for syringic acid, 60 μg/mL for 2-amino-benzoic acid, 95 μg/mL for salicylic acid, respectively, and benzoic acid had no IC_{50} on *S. aureus* and *S. dysenteriae* growth. Thus, the sequence of anti-microbial activity of the four OAs was: syringic acid > 2-amino-benzoic acid > salicylic acid > benzoic acid; and benzoic acid promoted *S. aureus* and *S. dysenteriae* growth.

2.3 The maximum heat-output power(P_m) and peak time (t_p) of thermogenic curve

E. coli, *S. aureus* and *S. dysenteriae* produced more energy in the second exponential growth phase than in other phases, so P_m was chosen as the maximum power output in that phase and t_p as the peak time of the thermogenic curves. The P_m-*t* curves, *k*-*c* and P_m-*c* plots of *E. coli*, *S. aureus* and *S. dysenteriae* growth showed the values of P_m decreased, k_1, k_2 and t_p increased with different drugs.

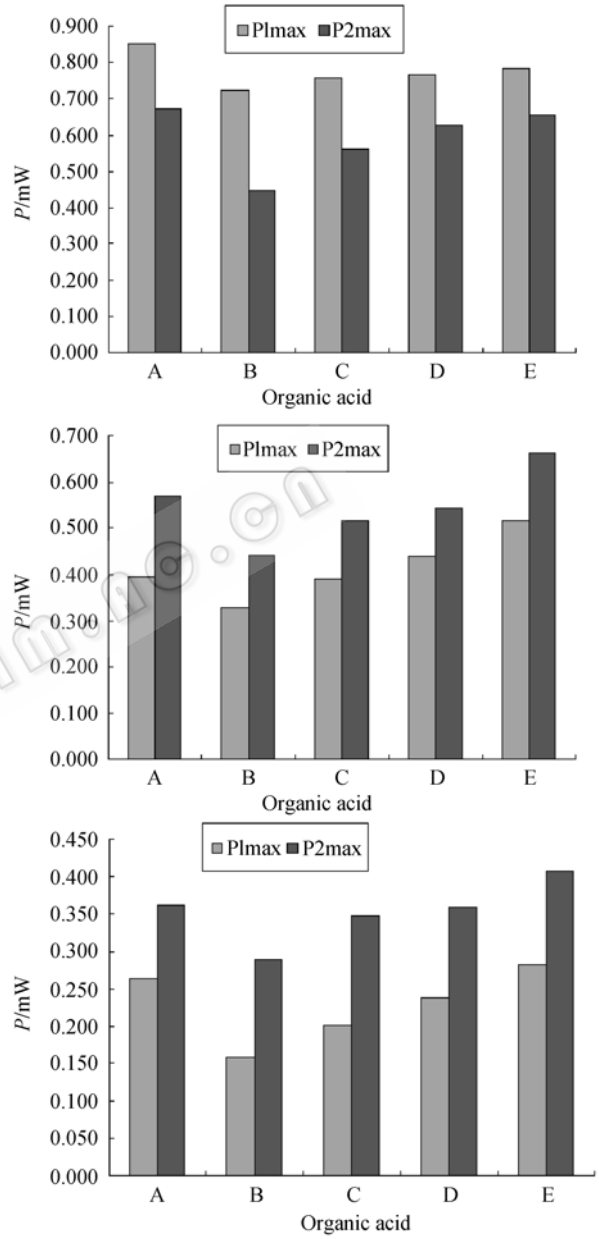


Fig. 5 The *P*-*c* plots of bacterium in the presence of four OAs with same concentration (200 μg/mL)
(1) *E. coli*; (2) *S. aureus*; (3) *S. dysenteriae*
A: blank; B: syringic acid; C: 2-amino-benzoic acid; D: salicylic acid; E: benzoic acid

3 Discussion

The thermogenic curves of *E. coli*, *S. aureus* and *S. dysenteriae* growth affected by various OAs from *Radix isatidis* indicated that all tested drugs hadinhibitory effects and benzoic acid had promotion effects on the tested bacteria. The lag phase of bacteria growth

prolonged with tested OAs. This results, probably were due to excess drugs inhibited the growth of microorganisms or killed the bacteria, indicating that the four OAs all have the capacity to inhibit the growth of *E. coli S. aureus* and *S. dysenteriae* and the inhibitory extent varied with different drugs.

Compared to the blank, adding the four OAs led to a decrease of P_m at log growth phase and the lag phase became longer, indicating that the OAs had inhibitory effects on the growth of *E. coli*. *S. aureus* and *S. dysenteriae*. But, in the presence of benzoic acid, the power-time curves of *S. aureus* and *S. dysenteriae* growth became high and the values of P_m, k_1, k_2 increased, indicating that the microbial growth was promoted. The sequences of anti-microbial activity of the four OAs on *E. coli, S. aureus* and *S. dysenteriae* were all: syringic acid > 2-amino-benzoic acid> salicylic acid>benzoic acid, but benzoic acid promoted the growth of *S. aureus* and *S. dysenteriae.* Syringic acid and 2-amino-benzoic acid showed stronger inhibitory effects on the three bacteria than the other two OAs, especially benzoic acid. Syringic acid, 2-amino-benzoic acid and salicylic acid suppressed the growth of *E. coli*, *S. aureus* and *S. dysenteriae* more than benzoic acid. These results also confirmed that the functional groups on phenyl ring improved the anti-microbial activity. It was obvious that not only the oxatyl on phenyl ring inhibited the growth of the microorganisms, but the functional groups at C2, C3, C4 and C5 of phenyl ring improved anti-microbial activity strongly.

Syringic acid with the functional groups methoxyl at C3 and C5 had strongest anti-microbial activity among the four OAs and the amino at C2 had stronger anti-microbial activity than the hydroxyl.

Effect of OAs on *E. coli* growth is not so effective as that on *S. aureus* and *S. dysenteriae*, which might be attributed to their different cell walls. *S. aureus* and *S. dysenteriae* are typical Gram-positive bacteria, the cell walls of which are fully composed of peptide polyglycogen. And *E. coli* is a typical Gram-negative bacterium, the cell wall of which is made up of a thin layer of peptide polyglycogen and an outer lipopolysaccharide layer. The outer LPS layer of *E. coli* cell wall is a potential barrier against foreign molecules with high molecular weight.

Our research provides a new thermochemical method-microcalorimetry-for the investigation of promoter structure and function. The microcalori-metric detection system is very sensitive, the detection limit is 2 μW and baseline stability (over a period of 24 h) is 6 μW. Thus the little change of the expression level of reporter gene can be monitored efficiently by the change of thermokinetic parameters, which can reflect the little difference of activity among various promoters. Microcalorimetry is much sensitive and easily performed. We think that it is also promising to be applied in other biological research fields, such as studying the regulation of gene transcription and several different and important goals might be reached in studying the energy flow in natural environments.

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