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Application of microcalorimetry and principal component analysis

Antibacterial evaluation of *Benzoinum* and *Styrax* on *Staphylococcus aureus* growth

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Abstract A useful microcalorimetric technique based on the bacterial heat production was applied to evaluate the antibacterial effects of *Benzoinum* and *Styrax* on the growth of *Staphylococcus aureus* (*S. aureus*). The thermogenic power-time curves of *S. aureus* growth in the presence of the two drugs were determined by a thermal activity monitor (TAM) air isothermal microcalorimeter, ampoule mode, at 310 K. Some quantitative metabolic parameters, such as growth rate constant k, the heat-flow power P, the appearance time for the heat power t, and the heat production Q were obtained from these curves. By analyzing these curves and some quantitative parameters using principal component analysis (PCA), the antibacterial effects of *Benzoinum* and *Styrax* on *S. aureus* growth were accurately evaluated from the change of the two main parameters, the heat-flow power for the second peaks P_{2nd} and total heat production Q_t: the antibacterial effects of the two drugs at concentrations of 0–125 mg mL^{-1} were both enhanced with increasing the concentration, and *Benzoinum* with IC_{50} of 132.2 mg mL^{-1} had stronger antibacterial effect than *Styrax* with IC_{50} of 179.8 mg mL^{-1}. This study provides some useful references for the application of *Benzoinum* and *Styra* as potential antibacterial agents. Microcalorimetry is a powerful analytical tool for the characterization of the microbial growth progress and the evaluation of the drugs' efficiency.

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Introduction

Due to the complementary therapeutic effects to western medicines and the capability to deal with many essential problems that have not yet been solved by conventional medicinal practices, traditional Chinese medicine (TCM) has attracted more and more attention in recent years. *Benzoinum* (Anxixiang in Chinese) and *Styrax* (Suhexiang in Chinese) are among the most commonly used herbal drugs in TCM and listed in the Chinese Pharmacopoeia [1]. *Benzoinum* is the dried balsamic resin obtained from *Styrax tonkinensis* (Pierre) Craib ex Hart (Styracaceae) trees, which are mainly produced in Asia [2, 3]. *Styrax* is also the dried resin of the species *Liquidambar orientalis* Mill. (Hamamelidaceae) trees, which are grown in Central America, Mexico, and the Mediterranean region including West and South Anatolia [4]. They are the pathological products, which flow out from the stem when deep gashes are made in the bark. The fluid resins harden upon exposure to air and are harvested 3–5 months later by scrapping the trunk. After dried, the finished products of resins are obtained. *Benzoinum* and *Styrax* are both the dried resins and have some similar efficacies, including unconsciousness-waking, mind-improving, cold-eliminating, and acesodyne actions etc. [1]. However, they have different pharmacological actions and applications, such as, *Benzoinum* is extensively used for healing wounds, erythema, and cough [2, 3]. *Styrax* has the reported anti-matrix metalloproteinase-1 [5], anti-complementary [6], and antioxidant [7] activities, etc. Also, it is not clear whether they have different antimicrobial effects and the research on the

treatment of microorganism with them as potential antimicrobial agents is scarce. It is believed that the antimicrobial effects of them should be accurately evaluated by suitable methods to warrant their accurate use in therapeutics.

Biological investigation is very important for assessing the effects of other substances on microbe. In any living systems including microbe, the various metabolic events occurring within the cells are heat-producing reactions. By monitoring the heat production of the growing cells with a sufficiently sensitive microcalorimeter, the thermogenic growth curve could be obtained to reflect the effect of any substance on the cells [8]. It has been widely applied to determine the effects of drugs, metal ion, and various materials on biological subjects including microorganisms, cultured tissue cells, and tissue pieces because of its high sensitivity, accuracy, and full automation [9–12]. And, the growing and metabolic progress of bacteria can be reflected from the thermogenic curves, which was determined by the microcalorimetric method [13]. Based on these thermogenic curves, some qualitative and quantitative thermodynamic/kinetic information about the characterization of the microbial growth under a certain set of growth conditions can be observed [14]. This information, which cannot be obtained by other existing methods [15–17], is helpful to understand the thermodynamical rules of bacteria during the metabolic process and the interaction between drugs and the bacterial cells [18–20].

In this study, the power–time curves of *Staphylococcus aureus* (*S. aureus*) affected by different concentrations of *Benzoinum* and *Styrax* were measured by a thermal activity monitor (TAM) using microcalorimetry. By analyzing some quantitative parameters from these curves using principal component analysis (PCA), the antibacterial effects of *Benzoinum* and *Styrax* were well evaluated and differentiated. Our studies show that the microcalorimetric method is a powerful tool to study the effects of drugs on microbial growth.

Experimental

Microorganism and materials

Staphylococcus aureus (*S. aureus* CCTCC AB910393) was provided by China Center for Type Culture Collection, Wuhan University, Wuhan, P. R. China. It was cultured in Luria–Bertani (LB) culture medium (pH 7.0–7.2), which was composed of 5 g L^{-1} yeast extract, 10 g L^{-1} tryptone, and 5 g L^{-1} NaCl. This culture medium was sterilized by autoclaving at 0.1 MPa and 394 K for 30 min before the experiment.

Benzoinum was produced in Sumatra, Indonesia. *Styrax* was produced in Montreal, Vietnam. They were both purchased from the Hehuachi market for Chinese crude drug, Chengdu, P. R. China. Ethanol (EtOH) was used as a solvent for preparing the original solution of the two drugs and the original concentrations of *Benzoinum* and *Styrax* were both 250 mg mL^{-1}. All other chemicals used were of analytical grade and available locally.

Microcalorimetric studies

The experiments were performed at 310 K using a 3114/3236 TAM Air Isothermal Calorimeter (Thermometric AB, Sweden) with ampoule method [18]. This calorimeter is an eight-channel isothermal heat conduction microcalorimeter operating in the milliwatt range. The thermal stability of the apparatus is ±273.02 K. The performances and the details of this instrument have been described previously [21]. *S. aureus* were inoculated in 100 mL LB medium with the density of 1×10^6 colony forming units (CFU) mL^{-1}. The cell suspension of 10 mL was added into each sterilized 20-mL glass ampoule. Then, the solution of different concentrations of *Benzoinum* and *Styrax* was introduced into this suspension. Eventually, each ampoule containing the cell suspension of *S. aureus* and one of the two drugs was sealed up and put into the eight-channel calorimeter block. After about 30 min (the temperature of ampoules reached 310 K), the metabolic power–time curves of *S. aureus* growth in the presence of the drugs were recorded until the recorder returned to the baseline. All data were collected continuously using the dedicated software package.

Principal component analysis

Many quantitative thermokinetic parameters could be obtained from the power–time curves of *S. aureus* growth to represent the effect of the drugs on the bacteria. However, the accurate conclusions were difficult to get from these too many parameters because of the overlapping of information and different change trends of these parameters [22]. To reduce the parameters and accurately evaluate the antibacterial effect of the drugs, the main parameter(s) should be obtained. Therefore, PCA, as a sophisticated technique, was introduced for reducing the dimensions of multivariate problems. It can transform the correlated data set to a smaller set of variables, the principal components (PCs), which are uncorrelated and contain nearly all of the original information. An eigenvector is established (PC1) that accounts for the maximum amount of variance in the data. Next, a vector orthogonal to the first is chosen (PC2) that describes the maximum amount of variance remaining in the data. Only the principal components that describe 80–90% of the total variance are typically selected to

describe the data [22–26]. Here, PCA was performed using the software Statistica 6.0 (StatSoft, Tulsa, OK) on unit variance scaled data of these quantitative thermokinetic parameters. From the score plots, the concentration distribution can be found and the antibacterial effect the two drugs can be differentiated, and from the loading plot, the main parameter(s), which is/are this/these furthest away from the main cluster of variables, can be obtained to present the antibacterial effect of the drugs.

Results

Power–time curves of *S. aureus* growth

The thermogenic power–time curve of *S. aureus* growth in LB culture medium at 310 K without drug was shown in Fig. 1 and can be divided six phases, that is, a lag phase (I), the first exponential growth phase (II), a transition phase (III), the second exponential growth phase (IV), a stationary phase (V), and a decline phase (VI).

Correspondingly, the thermogenic power–time curves of *S. aureus* growth in the presence of different concentrations of *Benzoinum* and *Styrax* were determined and depicted in Fig. 2. As could be seen from the profiles of these curves, the growth of *S. aureus* was influenced by the two drugs.

Quantitative thermokinetic parameters for *S. aureus* growth

As shown in Figs. 1 and 2, the bacterial growth was exponential during the two exponential growth phases. If P_0 and P_t are the heat-flow powers at time = 0 and time = t, respectively, then $P_t = P_0 \exp(kt)$ or $\ln P_t = \ln P_0 + kt$. The growth rate constant (k_1 and k_2) for the first and second exponential growth phase can be obtained from the rise part of the first and second peak by plotting a linear curve between $\ln P_t$ and t. Other quantitative thermokinetic parameters, such as the heat-flow powers for the first and second peaks P_{1st}, P_{2nd}, the appearance times for the first and second peaks t_{1st}, t_{2nd}, the heat production Q_1, Q_2 for the first and second exponential phase, and total heat production Q_t for all the metabolic progress were obtained from the power–time curves of *S. aureus* growth in the presence of different concentrations of *Benzoinum* and *Styrax* and shown in Table 1.

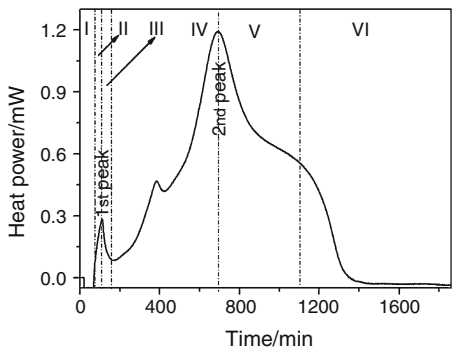

Fig. 1 The power–time curve of *S. aureus* growth at 310 K without drug. It is a typical metabolic profile of *S. aureus* culturing in LB culture medium supplemented without any drug monitored by the TAM Air microcalorimeter, and can be divided six phases, that is, a lag phase (I), the first exponential growth phase (II), a transition phase (III), the second exponential growth phase (IV), a stationary phase (V), and a decline phase (VI). There are two highest peaks, the first peak and the second peak in this curve

Relationships between quantitative parameters and concentration c of Benzoinum

It could be seen the values of nine quantitative parameters for *Benzoinum* and *Styrax* in Table 1 that k_2, P_{2nd}, and Q_2 decreased with the increase of the concentration of the two drugs, while, k_1, P_{1st}, t_{1st}, t_{2nd}, and Q_1 and Q_t fluctuated irregularly (increasing or decreasing). This phenomenon made it difficult to objectively evaluate the effect of *Benzoinum* and *Styrax* on *Benzoinum*. Therefore, PCA provides some help.

PCA

PCA models the significant variation in a data set and aims to reduce the dimensionality yet retain a maximum amount of the variance in the data set. Thus, only the important characteristics of the original data are retained. Therefore, PCA was performed on nine quantitative parameters in Table 1. The two-dimensional plane accounted for 88.37% of the original nine parameters. The scores plot (Fig. 3a) shows the distribution of concentration of *Benzoinum* and *Styrax*. It could be seen from Fig. 3a that different concentrations of the two drugs were classified into two groups, *Benzoinum* of different concentrations were distributed in the left area and *Styrax* in the right area. The antibacterial effects of *Benzoinum* and *Styrax* could be well differentiated based on PCA. The loadings plot (Fig. 3b) indicated that parameters P_{2nd} and Q_t might be the main two parameters, which would play more important role in evaluating the effects of *Benzoinum* and *Styrax*. Then, returning to the values of P_{2nd} and Q_t in Table 1, one could clearly and quickly find that both of the antibacterial effects of *Benzoinum* and *Styrax* were enhanced with increasing the concentration of them, and *Benzoinum* had stronger antibacterial effect than *Styrax*.

Then, to describe the extent of the inhibition of *Benzoinum* and *Styrax* on *S. aureus* growth, we defined the inhibitory ratio (I/%) as: $I = [(Q_{t(0)} - Q_{t(c)})/Q_{t(0)}] \times 100\%$, where $Q_{t(0)}$ and $Q_{t(c)}$ represent the total heat production in the whole metabolic progress of the bacteria

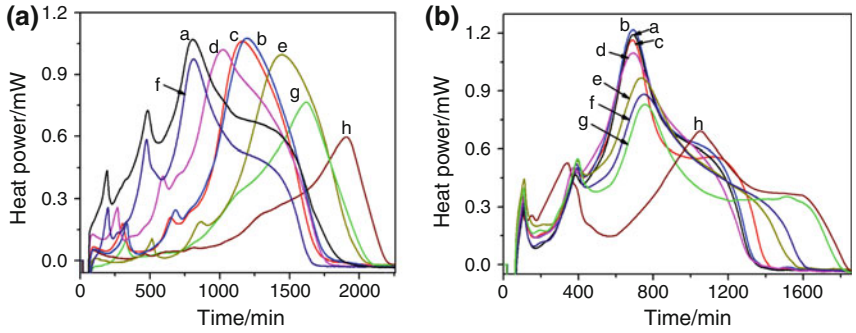

Fig. 2 The power–time curves of *S. aureus* growth at 310 K in the presence of **a** *Benzoinum* and **b** *Styrax*. The concentrations of the two drugs are both (*a*) 0 mg mL^{-1}, (*b*) 2.0 mg mL^{-1}, (*c*) 3.9 mg mL^{-1}, (*d*) 7.8 mg mL^{-1}, (*e*) 15.6 mg mL^{-1}, (*f*) 31.3 mg mL^{-1}, (*g*) 62.5 mg mL^{-1}, and (*h*) 125.0 mg mL^{-1}, respectively

Table 1 Quantitative thermokinetic parameters for *S. aureus* growth at 310 K in the presence of *Benzoinum* and *Styrax*

Drugs	c/mg mL^{-1}	k_1/min^{-1}	P_{1st}/mW	t_{1st}/min	k_2/min^{-1}	P_{2nd}/mW	t_{2nd}/min	Q_1/J	Q_2/J	Q_t/J	I/%
Benzoinum	0	0.01909	0.4984	189.7	0.00439	1.0699	804.0	2.07	20.96	55.25	0
	2.0	0.01548	0.1805	332.3	0.00471	1.0766	1199.0	0.80	18.67	47.16	14.64
	3.9	0.01399	0.1809	304.3	0.00431	1.0615	1163.3	0.86	16.76	43.63	21.03
	7.8	0.01254	0.2574	266.0	0.00409	1.0216	1023.3	1.40	19.36	41.16	25.50
	15.6	0.01942	0.1063	515.0	0.00395	0.9963	1438.0	0.32	18.37	38.87	29.65
	31.3	0.02857	0.2578	198.3	0.00347	0.9751	812.7	0.62	15.76	37.36	32.38
	62.5	0.02669	0.1006	327.3	0.00193	0.7635	1640.0	0.13	13.98	34.92	36.80
	125.0	0.01300	0.0331	463.0	0.00122	0.6354	2018.7	0.39	12.03	28.45	48.51
Styrax	0	0.02152	0.2891	111.7	0.00511	1.1938	692.0	0.48	16.15	49.32	0
	2.0	0.01308	0.3407	110.7	0.00529	1.2192	691.7	0.74	17.65	42.18	14.48
	3.9	0.02171	0.3365	108.0	0.00508	1.1657	688.7	0.61	16.94	41.30	16.26
	7.8	0.02294	0.3541	111.0	0.00469	1.0977	693.3	0.69	17.44	40.60	17.68
	15.6	0.01676	0.4446	110.7	0.00428	0.9682	730.3	0.57	16.84	38.15	22.65
	31.3	0.02838	0.3165	109.7	0.00396	0.8831	748.7	0.53	15.45	37.54	23.88
	62.5	0.02359	0.3956	108.3	0.00303	0.8297	752.7	0.72	15.18	35.49	28.04
	125.0	0.02247	0.3699	100.7	0.00218	0.687	1054.3	0.51	11.26	30.21	38.75

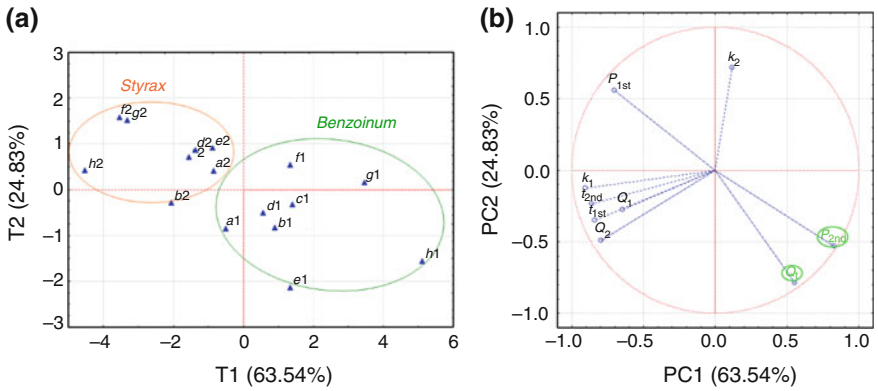

Fig. 3 **a** Scores plot shows the distribution of concentration of *Benzoinum* and *Styrax*. In the plot, we see two largest components (PC1, *horizontal*, and PC2, *vertical*), and the scores $t1$ and $t2$ are new variables aiming at describing as much of the original variation as possible without losing information. *Letters* $a1$–$h1$ represent the values of concentrations (0–125.0 mg mL^{-1}) of *Benzoinum* and *Styrax*. **b** Loadings plot indicates the contribution of the original variables (parameters) for the first two principal components PC1 and PC2. The main two parameters were marked with a circle

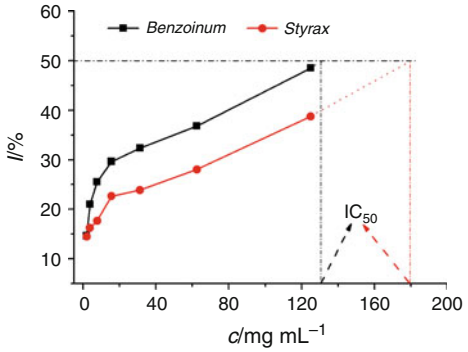

Fig. 4 Relationships of *I* and *c* for *Benzoinum* and *Styrax*

under the condition of control and final concentration *c* of the two drugs. And we can also obtain the *I*–*c* curves, from which the value of IC_{50} (the half-inhibitory concentration) of the drugs can be obtained. The higher the IC_{50} is, the stronger the antibacterial effect is. From Fig. 4, the values of IC_{50} of 132.2 mg mL^{-1} for *Benzoinum* and 179.8 mg mL^{-1} for *Styrax* were obtained, respectively, further showing the stronger antibacterial effect of *Benzoinum* on *S. aureus* growth than *Styrax*.

Discussions

Due to the non-water-solubility of *Benzoinum* and *Styra*, we got its solution dissolved in EtOH. First, the influence of this organic solvent on *S. aureus* growth was investigated to eliminate the negative interference on the experiments. By repeat experiments, the volume of EtOH was controlled within 30 μL (0.6% *V*/*V*) during all the experiments.

The microcalorimetric method is successfully applied to analyze the antibacterial effect of different concentrations of *Benzoinum* and *Styra* on the growth of *S. aureus*. Some useful information, such as the power–time curves and some quantitative thermokinetic parameters, were obtained to depict the metabolic progress of *S. aureus* and to evaluate the antibacterial effect of *Benzoinum* and *Styra* on the bacteria. By analyzing this qualitative and quantitative information coupled with component analysis (PCA), the antibacterial effects of *Benzoinum* and *Styra* were well and quickly evaluated: the antibacterial effects of *Benzoinum* and *Styrax* were both enhanced with the increase of the concentration of them, and *Benzoinum* with IC_{50} of 132.2 mg mL^{-1} had stronger antibacterial effect than *Styrax* with IC_{50} of 179.8 mg mL^{-1}, respectively. All the results provide some useful references for the application of *Benzoinum* and *Styra* as potential antibacterial agents. However, a validation study with more additional clinical strains from different body sites is needed before introduction into the clinical routine.

In conclusion, the microcalorimetric method, which is used to evaluate the antimicrobial effect of more TCMs on more microorganisms, is possible and promising. We believe that microcalorimetry is a useful analytical tool for the characterization of the microbial growth progress and the evaluation of the drugs' efficiency.

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