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Calorimetric study of the effect of protoberberine alkaloids in *Coptis chinensis* Franch on *Staphylococcus aureus* growth

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ABSTRACT

The effects of five protoberberine alkaloids (PBAs) from rhizoma of *Coptis chinensis* Franch on *Staphylococcus aureus* growth were investigated by calorimetry. The power-time curves of *S. aureus* with and without PBA were measured in closed glass ampoules in a TAM Air isothermal calorimeter. And, the extent and duration of inhibitory effects on the metabolism were evaluated by growth rate constant (k), half inhibitory ratio (IC_{50}), maximum heat-output (P_{max}) and peak time (t_p). The obtained calorimetric data showed that the inhibitory action varied for different protoberberine alkaloid. The results also revealed that the sequence of antimicrobial activity of five PBAs was: berberine>coptisine>palmatine>epiberberine>jatrorrhizine. One explanation could be substitutions at several positions in the core structure of berberine possess different antimicrobial activity. In conclusion, it can be proposed that this technique should be as a useful analytical method for determining the bioactivity of PBAs.

1. Introduction

Traditional Chinese medicine (TCM) has a long history with rhizoma dating back thousands of years for the medicinal practice in China. *Coptis chinensis* Franch (Huanglian in Chinese), as an important traditional Chinese medicinal herb, has been officially listed in the Chinese Pharmacopoeia [1]. This herbal medicine possesses broad-spectrum antibacterial and antiprotozoal effects, as well as clearing away heat and depriving dampness effects, and is used for treatment of dysentery, hypertension, inflammation, diabetes, cancer, and liver disease [2,3]. The main active constituents of the herb are protoberberine alkaloids (PBAs), including berberine, coptisine, palmatine, epiberberine and jatrorrhizine. PBAs representing a structural class of organic cations, are also active components in numerous plant-derived antimicrobial drugs from the genera *Berberis*, *Mahonia* and *Coptis*. The alkaloidal content, especially berberine, is generally claimed to be responsible for their beneficial effects and many studies have been conducted so far [4-6]. However, some of the PBAs have been used to treat bacterial infectious diseases of the digestive and respiratory tract and gynecological inflammation [7]. Only a few studies regarding the pharmacology of other PBAs such as, coptisine and palmatine, have been carried out [8,9]. So, it would be very interesting to know how microbial activities change under the action of PBAs.

Staphylococcus aureus is one of the human pathogenic microorganisms that has been studied most extensively. A previous study also showed that berberine displayed a significant antibacterial activity against *S. aureus* [10]. Consequently, it is a good choice for studying the effects of PBAs on their growth in vitro. This may help us to understand the general effects that PBAs may have on other microorganisms.

Calorimetry can be used to measure the heat associated with chemical or biochemical reactions, and is applied in different fields of science with high precision and sensitivity [11,12]. In recent years, it has been proven to be a useful tool for investigating the interaction between drugs and a wide range of organisms [13,14]. Furthermore, calorimetric manifestations of microbial activity can be correlated with data obtained using common microbiological methods, such as microbial counting and microbial biomass measurements [15]. In addition, one of the advantage of the calorimetric method is related to the fact that it is simple and the measurement does not affect the sample. The signal is continuously recorded and enables one to continuously follow processes in a sample, something which is not possible for most other methods [16]. Thus, calorimetry may serve as a speedy supplementary method

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to contribute to a better understanding of the biological effect of PBAs.

With this in mind, calorimetry was applied to determine the antimicrobial activity of five PBAs in this paper. By using a TAM Air isothermal calorimeter, the power-time curves produced by *S. aureus* cell suspensions under the action of five PBAs in different concentrations were obtained. Quantitative data reflected the dynamic changes of the growth process of *S. aureus* with and without PBAs, which could help to elucidate the effects of PBAs on the biological processes.

2. Experimental

2.1. Instrument

TAM Air isothermal calorimeter, manufactured by Thermometric AB company of Sweden, was used to obtain the metabolic growth curves of *S. aureus* cells. This instrument is an eight-channel isothermal batch calorimeter. All calorimetric channels are of twin configuration with one side for the sample and the other side for a static reference vessel. Each vessel is connected to the surrounding heat sink by a Peltier module, and when heat is produced or consumed due to any process, the temperature of the sample vessel changes. The temperature of the surrounding is constant and thus a temperature gradient across the Peltier module is developed. This will generate a measurable voltage and the voltage is proportional to the heat flow across the Peltier module and to the rate of the processes taking place in the sample vessel. The voltage signal is recorded continuously through an eight-channel data logger connected to a computer. Baselines are taken before or after each measurement and the calorimeters are calibrated electrically. The PicoLog software (Pico Technology Ltd.) supplied to TAM Air was used to monitor and record the heat flow over the Peltier module.

2.2. Materials

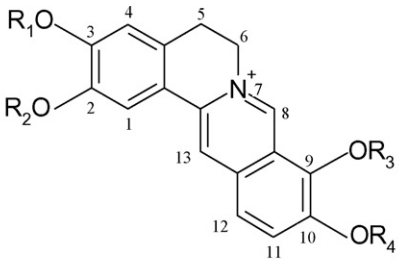
S. aureus (CCTCC AB910393) was provided by the Chinese Center for Type Culture Collections, National Institute for the Control of Pharmaceutical and Biological Products, Beijing, PR China. *S. aureus* was grown in a peptone culture medium, which contained 10 g peptone, 5 g beef extract and 5 g NaCl per 1000 mL. Medium pH was adjusted to 7.0–7.2 with 1 mol L^{-1} NaOH and 1 mol L^{-1} HCl before autoclaving. Berberine, coptisine, palmatine, epiberberine and jatrorrhizine were supplied by National Institute for the Control of Pharmaceutical and Biological Products. The five PBAs were extracted from *C. chinensis* Franch and their structures are shown in Fig. 1.

2.3. Preparation of samples

At the beginning of the experiments, *S. aureus* was inoculated into the peptone medium, with 2×10^6 cells per mL. Cells were suspended in the peptone culture medium, and the prepared PBAs solutions with different concentrations were added to the cell suspension. The solutions of PBAs were prepared in sterilized distilled water, and were prepared freshly every time.

2.4. Experimental procedure

The calorimeter was thermostated at 37 °C, and the measurements were made with the ampoule method. In all experiments, each sterilized 20 mL glass ampoule containing the cell suspension of *S. aureus* and one of the PBAs was sealed up and put into



	R_1	R_2	R_3	R_4
berberine	- CH_2 -		CH_3	CH_3
coptisine	- CH_2 -		- CH_2 -	
palmatine	CH_3	CH_3	CH_3	CH_3
jatrorrhizine	H	CH_3	CH_3	CH_3
epiberberine	CH_3	CH_3	- CH_2 -	

Fig. 1. Chemical structures of investigated PBAs in *C. chinensis* Franch.

eight-channel calorimeter block. The power-time curves of *S. aureus* growth were then recorded continuously.

3. Results

3.1. The power-time curves of S. aureus

The power-time curves of *S. aureus* with and without PBAs at 37 °C are shown in Fig. 2. It can be seen that the growth curve of *S. aureus* could be divided into four phases, *i.e.*, lag phase (the period between the start of the experiment and the ascending phase of the power-time curves), first exponential phase (log phase), second exponential phase and decline phase.

The shape of the power-time curves of *S. aureus* under the action of five PBAs was similar. However, with the increasing concentrations of PBAs, the lag phase became longer. The time of the lag phase suggested that the retarding time of *S. aureus* was longer with the increasing concentrations of PBAs. The results indicated that the five PBAs inhibited the growth of *S. aureus*. Berberine, coptisine and palmatine suppressed the growth of *S. aureus* more than epiberberine and jatrorrhizine. Moreover, Fig. 2 also shows berberine and coptisine completely inhibit growth of *S. aureus* at 150 and 180 μg mL^{-1}, respectively.

3.2. Growth rate constants of S. aureus

The growth curves of *S. aureus* could be fitted by two exponential functions. Each of these obeyed the following equation [17]:

$$\ln P_t = \ln P_0 + kt \quad (1)$$

where t is the time, P_0 represents the heat-output power at the beginning of baseline, P_t is the power at time t, and k is the growth rate constant. The growth rate constants (k_1, k_2) were obtained by fitting ln P_t and t to a linear equation. The value of k_2 for the second exponential phase was much less than k_1 in first exponential phase. Fig. 3 shows experimental results evaluated with Eq. (1) for different PBAs samples. By comparing the plots, we can find that the value of k_1 of *S. aureus* with berberine declines drastically with the increase of the concentration of berberine. On the contrary, k_1 of *S. aureus* with jatrorrhizine declines gently. These results could indicate that inhibitory effect varied for different PBAs. The

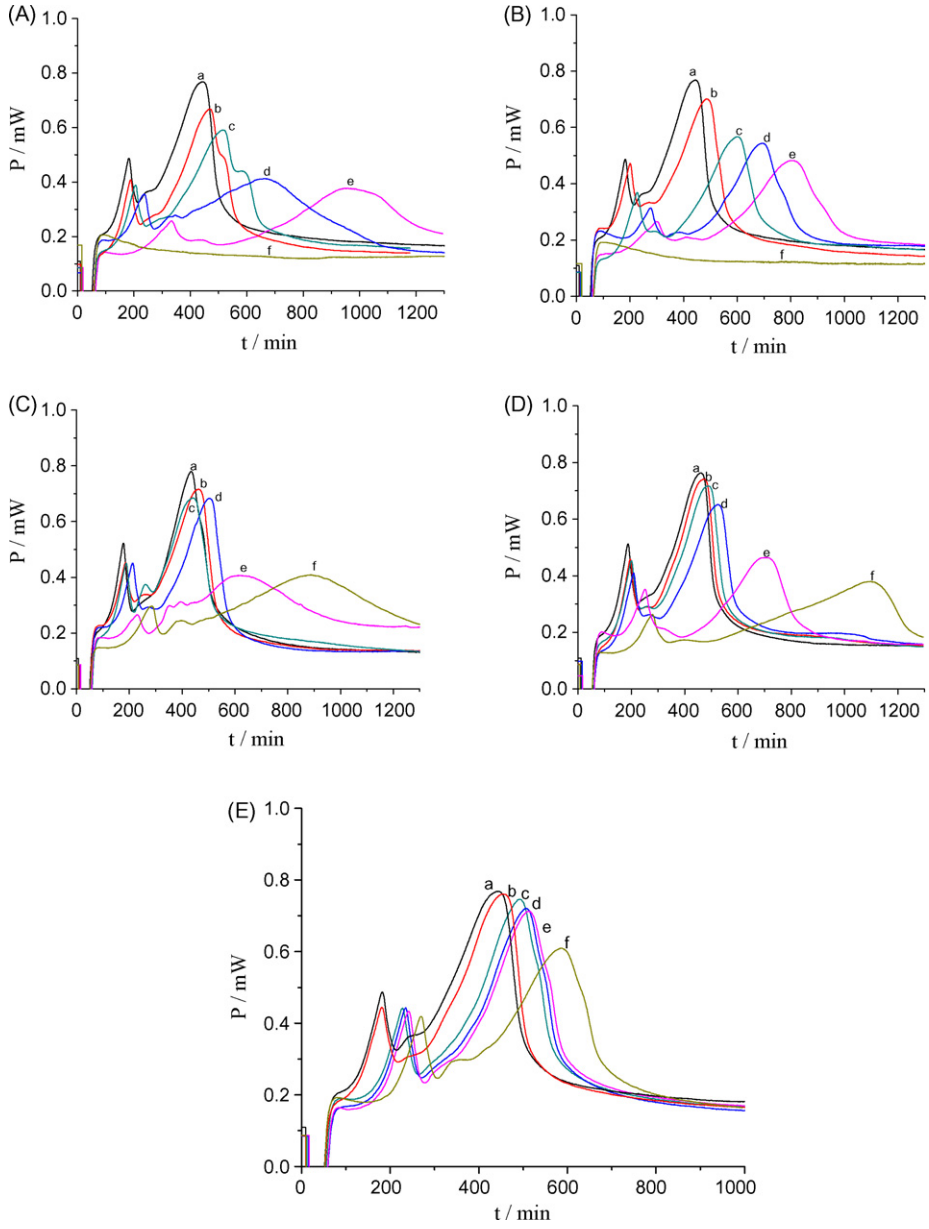


Fig. 2. The power-time curves of *S. aureus* growth in the presence of PBAs at different concentrations: (A) berberine ((a) control, (b) 10 μg mL^{-1}, (c) 20 μg mL^{-1}, (d) 40 μg mL^{-1}, (e) 60 μg mL^{-1}, (f) 150 μg mL^{-1}), (B) coptisine ((a) control, (b) 10 μg mL^{-1}, (c) 25 μg mL^{-1}, (d) 50 μg mL^{-1}, (e) 80 μg mL^{-1}, (f) 180 μg mL^{-1}), (C) palmatine ((a) control, (b) 20 μg mL^{-1}, (c) 30 μg mL^{-1}, (d) 50 μg mL^{-1}, (e) 90 μg mL^{-1}, (f) 130 μg mL^{-1}), (D) epiberberine ((a) control, (b) 50 μg mL^{-1}, (c) 80 μg mL^{-1}, (d) 120 μg mL^{-1}, (e) 180 μg mL^{-1}, (f) 250 μg mL^{-1}), (E) jatrorrhizine ((a) control, (b) 50 μg mL^{-1}, (c) 100 μg mL^{-1}, (d) 150 μg mL^{-1}, (e) 200 μg mL^{-1}, (f) 250 μg mL^{-1}).

different inhibitory actions of five PBAs are clearly demonstrated in Fig. 3.

3.3. Peak time (t_p) and the maximum heat-output (P_{max}) of the power-time curve

The power-time curves of *S. aureus* showed that *S. aureus* produced more heat in the second exponential growth phase than in the first phase, so P_{max} in that phase was chosen as the maximum heat-output and t_p as the peak time in power-time curve. Fig. 2 shows that P_{max} of *S. aureus* in the absence of PBAs is greater than that of *S. aureus* with PBAs. Meanwhile, the quantity of P_{max} decreases and t_p is prolonged with increasing concentrations of five PBAs. Thus, the maximum heat-output and the peak time can reveal the intensity and the time necessary for development of antimicrobial activities of a specific PBA.

3.4. Inhibitory ratio I and the half-inhibitory concentration IC_{50}

All PBAs inhibit *S. aureus* growth to some degree, and the growth rate constants decrease. So, the inhibitory ratio *I* can be defined as:

$$I = \frac{[k_0 - k_c]}{k_0} \times 100\% \quad (2)$$

where k_0 is the growth rate constant of the control, and k_c is the rate constant in the first exponential phase for *S. aureus* growth inhibited by an inhibitor with a concentration *c*. When the inhibitory ratio (*I*) is 50%, the corresponding half-inhibitory concentration of the inhibitor can be represented as IC_{50}. IC_{50} is the inhibiting concentration causing a 50% decrease of the *S. aureus* growth rate constant. From the data in Fig. 3, we obtained that IC_{50} was 57 μg mL^{-1} for berberine, 67 μg mL^{-1} for coptisine, 119 μg mL^{-1} for palmatine,

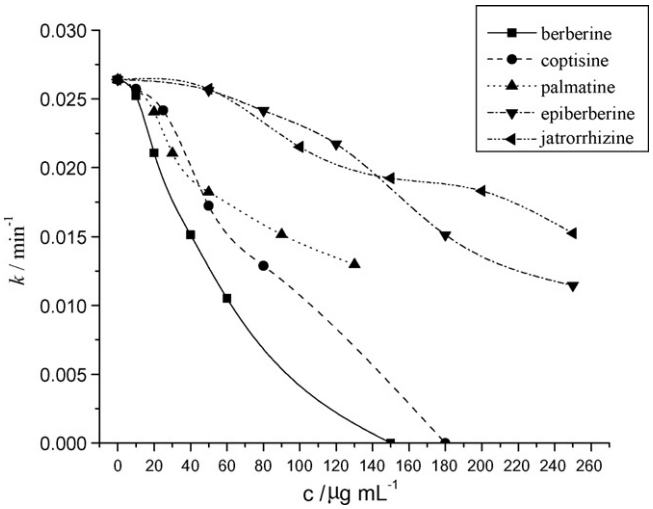


Fig. 3. Plot of k_1 for the growth *S. aureus vs*. concentration (c) for PBAs.

214 μg mL^{-1} for epiberberine and 272 μg mL^{-1} for jatrorrhizine. Thus, the sequence of antimicrobial activity of these five PBAs was berberine>coptisine>palmatine>epiberberine>jatrorrhizine. Compared with the previous studies, these results about the ranges of IC_{50} for the five compounds were basically close to the ranges of minimum inhibitory concentrations (MICs) tested by the often-used standard microbiological methods [10,18].

4. Discussion

Coptis chinensis Franch was documented as antimicrobial in Chinese herbs [2]. PBAs as its main constituents were generally claimed to be responsible for the beneficial effects of this herb. In this work, the power-time curves of *S. aureus* with and without PBAs acquired by calorimetry showed the typical pattern of *S. aureus* growth. Other study of growth of *S. aureus* [19] showed a pattern of growth similar to that reported here. Besides, the power-time curves of *S. aureus* growth affected by various PBAs indicated that all investigated drugs had inhibitory effects on *S. aureus* to different extents. The result that the time of the lag phase of bacteria growth was prolonged and the maximum heat-output (P_{max}) decreased with the increasing concentrations of PBAs indicated that the bacterial culture took longer time to produce a sufficient number of cells for a detectable signal.

The power-time curves and parameters of *S. aureus* under the action of five PBAs demonstrated that the inhibitory extent varied with different PBAs. This could be verified from Fig. 3, which showed berberine and coptisine gave a stronger inhibitory effect on *S. aureus* than the other three PBAs.

Previous papers showed that the action of the drugs on different cells depended on the structure of the drugs [18,20]. This work showed differences in antimicrobial activity after substitutions at several positions in the core structure of berberine, suggesting that different residues of the core structure may affect the role of individual molecules in the antimicrobial activity of *C. chinensis* Franch. Thus, antibacterial activity and structure-activity relationships of PBAs could be evaluated. All PBAs, which belong to protoberberine class of isoquinoline alkaloids, possess different substituted groups at C-2, C-3, C-9 and C-10 of phenyl ring (see Fig. 1). In general, compounds bearing a methylenedioxy functional group at C-2 and C-3 on phenyl ring showed higher activity than those which have methoxy groups at the same positions. For compounds bearing the same functional group at C-2 and C-3 on phenyl ring, a methoxy at C-9 and C-10 on phenyl ring displayed more activity on bacteria than methylenedioxy at C-9 and C-10. Replacement of a methoxy group at C-3 on phenyl ring by a hydroxy group led to a reduction in the activity. As a consequence, a methylenedioxy function at C-2 and C-3 is required for enhanced antibacterial activity of PBAs.

5. Conclusions

Evaluation of the relative antimicrobial efficacy of drugs can be achieved by traditional microbiological techniques that are often complex and time consuming. This work indicates that calorimetry offers an alternative means for the study of the kinetics of the antibacterial action of active components of TCM and for estimation for the relative bioactivity of different drugs. The obtained results indicate that calorimetry as a non-invasive physicochemical method can be a valuable supplement in the study of the effect of PBAs. Calorimetry is an established procedure that offers quantitative measurements and has the distinct advantage over traditional antimicrobial test methodologies in that calorimetric measurement is continuous, real-time measurement, so that the dynamic response of a microorganism to an antimicrobial agents is observed in situ. Moreover, the calorimetric method requires only an observable difference between the power production in the treated and control incubations. Unlike many other procedures, transparent solution is not required. Colored or turbid solutions, even suspensions can be put into the calorimeter [21]. That approximates more closely the vivo state than many other techniques do.

The presentations showed the potential of the applicability of calorimetry to determine the influence of drugs on different cells. Thus, calorimetry would be utilized to screening effectual components pharmacologically extracted from TCM herbs or other medicinal plants.

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