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A novel “target constituent knock-out” strategy coupled with TLC, UPLC–ELSD and microcalorimetry for preliminary screening of antibacterial constituents in *Calculus bovis*

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ABSTRACT

A novel “target constituent knock-out” strategy was proposed and applied for preliminary screening of antibacterial constituents in *Calculus bovis* (*C. bovis*). This strategy was accomplished through the following steps: (1) the single constituents (A–F) in *C. bovis* samples were knocked out on the Silica Gel thin-layer plates by thin-layer chromatography (TLC); (2) these knocked-out constituents were identified by ultra performance liquid chromatography–evaporative light scattering detection (UPLC–ELSD); (3) the antibacterial activities of these knocked-out constituents and *C. bovis* samples on *Staphylococcus aureus* (*S. aureus*) were evaluated by microcalorimetry combined with principal component analysis (PCA); (4) the activities of these knocked-out constituents and the total extract of *C. bovis*, also the interaction properties between these single constituents and the total extract were elucidated. The results showed that the sum of inhibitory ratio (*I*) of constituents A–F (202.0%) was 5-fold of the *I* of *C. bovis* sample (38.01%), showing that these knocked-out constituents had strong antagonistic effects on each other in *C. bovis* sample and the antagonistic extent was 81.18%. And we found that the key antibacterial composition of *C. bovis* was not a single component, also not the high content component (cholic acid, CA), but constituent F, which was the combinatorial composition of deoxycholic acid (DCA) and hyodeoxycholic acid (HDCA). Constituent F revealed over 33-fold high activity of the sum of DCA and HDCA activity in solo-use, showing strong synergistic effect between DCA and HDCA. In addition, constituents A–E had significant antagonistic effects on constituent F. Our study indicates that this proposed “target constituent knock-out” strategy is a useful approach for screening active constituents and elucidating the multi-component interactions in *C. bovis*, further providing some reference for understanding the pharmacodynamic actions, controlling the quality of Chinese materia medicas (CMMs) and discovering new drugs.

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1. Introduction

Calculus bovis (Niuhuang in Chinese), dried pigment gallstones of cattle, is a valuable Chinese materia medica (CMM) with multiple pharmacodynamic actions, including sedation, relieving fever, diminishing inflammation and immunoregularity, etc. [1–3]. It was firstly listed in *Shennong Bencao Jing* (the Divine Farmer's Herbal Classic, 22-250$_{AD}$, the earliest monograph on medical materials in China). Because of the good clinical therapy of *C. bovis*, it has been extensively used in China for over 2000 years, and many big pharmaceutical companies are using it as an excellent pool for discovering natural bioactive compounds or new drugs. However, *C. bovis* is a complicated system of complex chemical constituents and the therapeutic effects of it are expressed by multi-components with complicated interactions [4], so it is difficult to explain which active constituents are responsible for the holistic efficacy of this CMM. In addition, the natural resources of *C. bovis* are rare and the obtainment of it from cattle is inhumane; hence, there are great needs to develop artificial products as substitutes to reduce the utilization of natural resources. Though the researchers have explored many artificial products, they were

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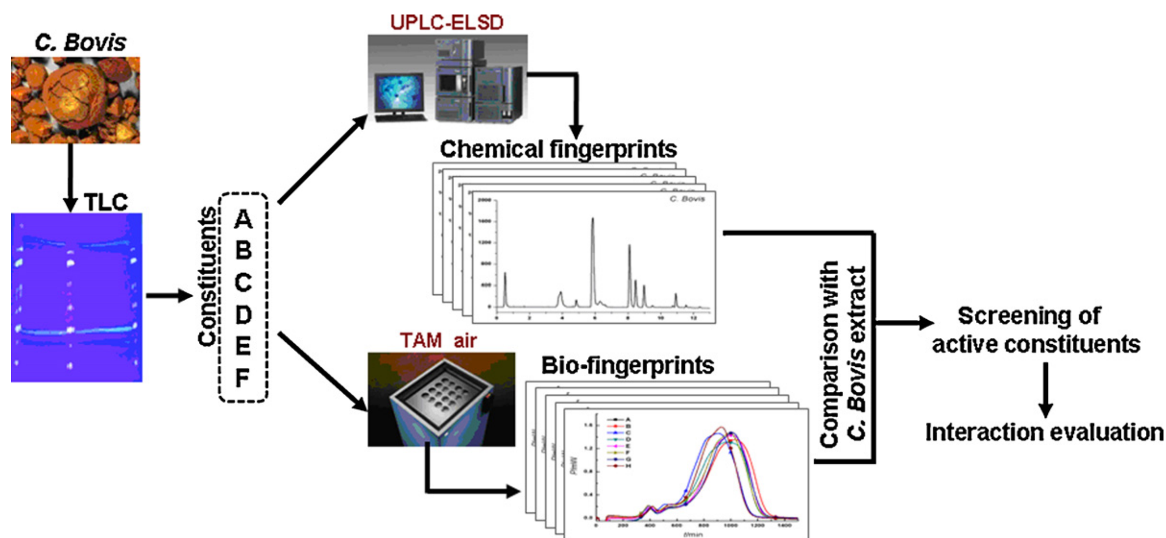

Fig. 1. Flowchart of screening for antibacterial constituents in *C. bovis* in this study.

designed and prepared by simple mixing of cholic acid, deoxycholic acid, hyodeoxycholic acid and other substances to simulate the composition of natural *C. bovis*. The matching ratio of these substances, also the quantity–activity relationships of these constituents in artificial products are disordered and not clear. Since last century, large numbers of 'single constituents' in *C. bovis* have been isolated and the chemical structures, contents and pharmacodynamic actions of them have been reported [5,6]. But the success stories unfortunately have been few and far between, and the data about authentication, efficacy and safety of this CMM are far from sufficient to meet the criteria needed to support its use worldwide.

This predicament is possibly caused by the inappropriate strategies of the existing screening approaches for pharmacodynamic constituents in CMMs. The principal interests driving such existing approaches focus on the high activity of single constituent, rather than the holistic therapeutic effect of the CMMs. Briefly, there are three problems of the existing approaches needing to resolve. Firstly, the key pharmacodynamic constituents (KPCs) of the CMMs are still not clear although many chemical structures have been identified. The KPCs may include the main constituents (of high contents in CMMs and possibly active), the active ones (of high activity in solo-use), and the synergistic ones (maybe inactive but of synergistic effect to other active ones). The synergistic constituents, which are very important to elucidate the molecular basis of the holistic therapeutic potential of traditional medicines, are usually ignored by researchers using the existing approaches. Secondly, the interaction properties of the multiple components in CMMs have not been emphasized and remain uncertain, which include two sides, synergistic and/or antagonistic effects. Both are non-observable in the existing approaches. Thirdly, for quality assessment of CMMs, the marker constituents assigned on the basis of the existing screening approaches are usually not responsible for the whole effect of the CMMs indeed. For example, cholic acid, the main component in *C. bovis*, is determined as index to assess the quality of this CMM stipulated in the Chinese Pharmacopoeia. But this abundant component might not be the most important chemical structure responsible for the whole effect of *C. bovis*, at least based on our findings.

In order to overcome these disadvantages of the existing screening strategies, we initially proposed the "target constituent knock-out" strategy [7], which is taken reference to the molecular biology method and mode of "gene knock-out" or "gene deletion" [8–10] but completely different. The major principles and aims of this novel screening strategy can be summarized to answer three *W*s below.

W-1, who is (are) responsible for the whole effect of the CMM? This could be concluded through comparing the target constituent to the total extract of the CMM. The responsible constituents are the KPCs aforementioned. Usually, the KPCs are the mixture of multiple components rather than a single one.

W-2, who is (are) good or bad? This could be deduced through comparing the target constituent to the minus extract (knocking out the target constituent from the total extract). For a "good" or synergistic constituent, its absence from the minus extract would lead to the decrease of additional activity away from the total extract over the loss of activity itself. For a "bad" or antagonistic constituent, the minus extract would reveal additional enhancement over the loss of activity itself.

W-3, what composition of KPCs is optimal? Since the possible existing interactions, the ratios of the KPCs in the CMM are important and the optimized combinatorial compounds might acquire even higher activity compared to the total extract of the CMM in original proportion.

In our previous study, we have found that artificial *C. bovis* had significant antibacterial activity on many microbes [11,12]. But, the corresponding antibacterial constituents of it, also the interactions of these antibacterial constituents and *C. bovis* were not definite. So, in this study, the novel "target constituent knock-out" strategy was introduced for screening the antibacterial constituents in *C. bovis*. The overall flowchart was shown in Fig. 1. Firstly, *C. bovis* sample was extracted by proper solvent and the single constituents in it were separated and knocked out on the Silica Gel thin-layer plates by thin-layer chromatography (TLC). Secondly, these knocked-out constituents were analyzed and identified by ultra performance liquid chromatography–evaporative light scattering detection (UPLC–ELSD). Thirdly, the antibacterial activities of these knocked-out constituents and *C. bovis* sample on *Staphylococcus aureus* (*S. aureus*) were evaluated by microcalorimetry combined with principal component analysis (PCA). Finally, the antibacterial constituents or components in *C. bovis* were clearly found, further, their interaction properties (synergistic and/or antagonistic effect) were elucidated. This study provided a novel and useful strategy for screening the KPCs of *C. bovis* or other CMMs, further provided some help for quality assessment of them to meet the criteria needed for their use worldwide.

2. Materials and methods

2.1. Materials

Artificial *C. bovis*, produced in Shanghai city, were purchased from Tongren Tang Pharmaceutical Co. Ltd. (lot 20080311). Cholic acid (CA), deoxycholic acid (DCA), hyodeoxycholic acid (HDCA), ursodeoxycholic acid (UDCA) and taurocholate sodium (TCANa) were purchased from National Institute for the Control of Pharmaceutical and Biological Products of China, Beijing, China. Their purities were all over 98% by UPLC–ELSD analysis. Acetonitrile of chromatographic grade were purchased from Fisher Chemicals (Pittsburg, PA, USA), other reagents of analytical grade from Beijing Chemical Factory (Beijing, China). Double-distilled water was used in all experiments. Glass backed 0.4 mm Silica Gel 60 preparative thin-layer chromatography plates were purchased from Yantai Chemical Industry Research Institute, Shandong province.

S. aureus (CCTCC AB910393) was provided by China Center for Type Culture Collection, Wuhan University, Wuhan. Briefly, the broth culture medium was consisted of 10 g peptone, 6 g beef extract and 5 g NaCl dissolving in 1000 mL deionized double-distilled water and was adjusted pH to 7.0–7.2. The culture medium was sterilized by autoclaving at 0.1 Megapascal (MPa) and 121 °C for 30 min. Initially, *S. aureus* were grown in 25 mL broth culture medium and incubated in the shaker for 8 h at 37 °C, respectively. The rotation speed of incubator shaker was 110 rpm. The flask was enveloped with a cotton plug, so there was enough oxygen to be used by *S. aureus*. Luria–Bertani (LB) culture medium was consisted of 10 g peptone, 6 g yeast extract and 5 g NaCl dissolving in 1000 mL deionized double-distilled water and was adjusted pH to 7.0–7.2, and was also sterilized by autoclaving at 0.1 MPa and 121 °C for 30 min. Before the experiment, *S. aureus* and the broth and LB culture medium were stored in a refrigerator at 4 °C.

2.2. Apparatus

UPLC analysis was performed using a Waters Acquity system equipped with a binary solvent delivery pump, a column oven and an auto sampler, connected to the Waters Empower 2 software. An Alltech 2000ES ELSD (Deerfield, IL, USA) detector was connected to this LC system through an 800 mm × 0.1 mm PEEK tube.

A 3114/3236 thermal activity monitor (TAM) air isothermal calorimeter (Thermometric AB, Sweden) was employed to record the thermal output, which was used as an indicator of metabolic activity of *S. aureus* in the presence of samples. This microcalorimeter has eight calorimetric channels which can keep the temperature within ±0.02 °C. All channels are mounted together to form a single heat-sink block housed in a temperature controlled air thermostat. Each calorimetric channel is constructed in twin configuration with one side for the sample and the other side for a static reference. The twin configuration of sample and reference within a channel allows the heat flow from the active sample to be compared directly with the heat flow from the inert reference. The power difference is a quantitative expression of the overall rate of thermal production in the sample. Details of the performance and construction of the instrument have been documented before [13].

2.3. Preparation of mixed standard and sample solutions

The mixed standard solution containing TCANa, CA, HDCA, DCA and UDCA was prepared by adding an accurately weighed amount of each standard substance into a volumetric flask and dissolved with methanol (MeOH), and then filtered through 0.22 μm Millipore membrane to yield the mixed standard solution.

About 2 g dried powder of raw *C. bovis* medical material was ultrasonicated in 50 mL ethanol for 1 h and the procedure was repeated for triplicates. The supernatant fluid for each time was filtered, merged and concentrated to 40 mL to yield the sample solution, which was filtered through 0.22 μm Millipore membrane for UPLC–ELSD analysis.

2.4. TLC separation of constituents in C. bovis

For routine compositional analysis of CMMs, thin layer chromatography (TLC) is often the good method of choice. As a simple, quick and inexpensive procedure, TLC method has been developed and successfully applied to the study of CMMs or implemented into pharmacopoeias all over the world [14–16].

To separate and obtain the single constituents in *C. bovis*, preparative TLC plates were employed. 1.0 mL *C. bovis* sample solution was deposited as coarse line onto the preparative TLC plate (20 cm × 20 cm), which was then developed in a presaturated solvent tank with toluene–acetic acid–water (6:5:0.4, v/v/v) as developing reagents until the solvent front reached the top of plate. The developed TLC plate was removed from the solvent, and allowed to air-dry. Some bands were observed under 365 nm by coloring on the two edges and middle of TLC plate using sulphuric acid–ethanol (3:7, v/v) as chromogenic agent and activating at 105 °C for 3 min. The single band was marked with a pencil, then, was removed and collected. All the above procedures were repeated for triplicate. Then, the merged silica chips of each band were extracted with methanol and the suspension was centrifuged. The supernatants were filtered through 0.22 μm Millipore membrane and concentrated to 2 mL to get the solutions of single target constituents for the following analysis.

2.5. UPLC–ELSD analysis

The chromatographic separation and detection of samples was performed on a Waters Acquity UPLC HSS T3 column (2.1 × 50 mm, 1.8 μm) at a column temperature of 35 °C and liquid flow-rate of 0.6 mL/min using acetonitrile (solvent A) and 0.2% aqueous formic acid (solvent B) as mobile phase with a linear gradient: 0–9 min (25–45%, A), 9–13 min (45–60%, A). The drift tube temperature for the ELSD was set at 100 °C with the gas flow-rate of 1.9 L/min.

The solutions of mixed standard, *C. bovis* sample and the knocked-out constituents were all injected into the UPLC system for UPLC–ELSD analysis.

2.6. Microcalorimetric measurement

The thermogenic curves of *S. aureus* metabolism were recorded using the isothermal calorimeter with ampoule method [11]. The microcalorimeter was brought to equilibrium temperature overnight in advance. 10 mL LB culture medium containing 100 μL *S. aureus* suspension was put into the 20 mL glass ampoule, then 80 μL sample solution was added into this ampoule. All the ampoules were sealed, shaken up and put into the microcalorimeter. The heat-flow power (HFP)–time signals were recorded at interval of 1 min. The temperature of the microcalorimeter was controlled at 37 °C. All apparatus were cleaned and sterilized by autoclaving before use.

2.7. Principal component analysis (PCA)

From the HFP–time curves of *S. aureus* growth, many quantitative parameters can be obtained to represent the activities of samples on the bacteria. But, these parameters have no same changing trends, which provide some difficulty for the activity evaluation. In order to reduce the parameters and quickly evaluate the antibacterial activity, the main parameters should be obtained. PCA extracts data, removes redundant information, highlights hidden

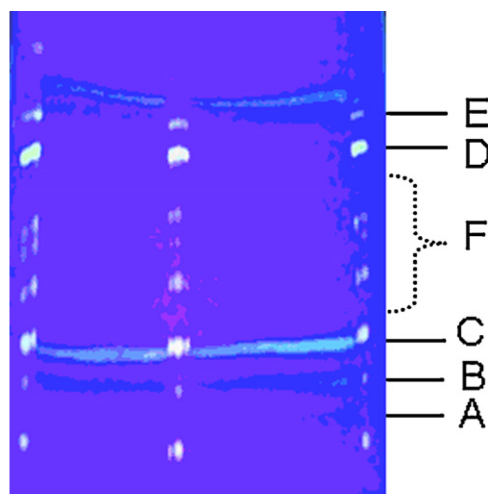

Fig. 2. TLC separation of constituents in *C. bovis*. These constituents were separated on glass backed 0.4 mm Silica Gel 60 preparative TLC plates using the solvent system of toluene–acetic acid–water (6:5:0.4, v/v/v) and visualized under 365 nm. Bands A–F represented the different single constituents A–F in *C. bovis*.

features, and visualizes the main relationships that exist between observations among a large number of variables in terms of a smaller number of underlying factors (PCs) without losing much information [17]. Here, PCA was performed on the mean-centered data with no scaling of many quantitative parameters taken from the HFP–time curves to find out the main parameters using software of SIMCA-P 11.5 (Umetrics AB, Umea, Sweden). These main parameter(s) is/are this/these furthest away from the main cluster of variables.

3. Results

3.1. TLC separation

The developing solvent system is very important for TLC separation of any complicated constituents. Satisfactory TLC separation should be obtained from the optimized developing solvent system [14]. Firstly, several solvent systems and their different ratios (v/v), such as ethyl acetate–acetic acid–methanol, ethyl acetate–acetic acid–methanol–water, toluene–ethyl acetate–acetic acid–water, toluene–acetone–acetic acid–water and toluene–acetic acid–water, were assessed to select the most suitable solvent system for the simultaneous resolution of the single constituent in *C. bovis*. Finally, toluene–acetic acid–water (6:5:0.4, v/v/v) was chosen as the simplest and quickest solvent system. These constituents were well resolved without tail formation in any band.

The bands in Fig. 2, which presented the different single constituents A–F, got good separation under the optimized condition. Among these knocked-out constituents, constituent F was the residuum of *C. bovis* by excluding constituents A–E. Next, the six knocked-out constituents were treated to prepare the samples for UPLC analysis and antibacterial evaluation.

3.2. UPLC–ELSD detection

The major active components of *C. bovis* are bile acids (BAs) and their derivatives, including CA, DCA, HDCA, UDCA, TCANa and others [18–20], which are often used as the chemical markers for quality control of this medicinal material. Several analytical methods have been reported for the determination of BAs based on liquid chromatography (LC) with UV detector or LC coupled to other detectors [21–23]. But, due to the absence of a chromophore in these BAs, evaporative light scattering detector (ELSD), as a universal detector with high sensitivity of detection and low cost, is in great need. Also, for the objective of reducing analysis time and maintaining good efficiency, the quick chromatographic separation using ultra-performance liquid chromatography (UPLC) is necessary. The combination of UPLC and ELSD could make up some disadvantages and inconveniences in the above-mentioned analytical methods for the determination of these BAs. And the UPLC–ELSD method has been established and validated and successfully applied for the determination of the BAs in *C. bovis* and its medicinal preparations [19,24].

In this study, the knocked-out constituents in *C. bovis* samples were separated, detected and identified using UPLC–ELSD method by comparing their retention times and ELSD responses with those presented in the chromatogram of the mixture standards solution. The satisfactory UPLC–ELSD chromatograms of mixture standards, *C. bovis* sample and six knocked-out constituents were shown in Fig. 3. In the analysis time of 13.0 min, all the components got good separation. From this figure, it could be seen that constituent A was identified as compound TCANa, constituents B and C as unknown compounds, constituent D as compound CA, constituent E as compound UDCA and constituent F as the mixture of compounds HDCA and DCA. The antibacterial activities of these knocked-out constituents and *C. bovis* sample were evaluated in the following part.

3.3. Antibacterial activities based on microcalorimetric determination

3.3.1. Selection of isothermal microcalorimetry (IMC)

Some traditional techniques have been applied for the antibacterial evaluation of drugs or screening of novel antibacterial agents. But, almost all of these techniques still have some disadvantages [12,25–28]. An alternative to these techniques that is universal, real-time, simple, non-invasive and non-destructive is IMC. Firstly, this technique can continuously monitor the heat flow generated or consumed by a sample while the sample is kept at constant temperature [29]. Secondly, IMC provides a universal tool for real-time determination of the heat production in bacterial growth process, thermo-dynamic/kinetic information can be obtained from the heat-flow power (HFP)–time curves of bacterial growth. This information could be used to synthetically present the antibacterial activities of drugs on microbes, and has become a reliable confirmation of MIC determination [30–32]. Thirdly, IMC is highly sensitive and about 2×10^4 bacteria/mL are usually enough for IMC. Also, IMC is a simple method and studies of bacterial growth processes frequently can be carried out in sealed ampoules and the HFP–time curves can be detected and recorded continuously and automatically.

In view of the potential benefits of IMC, the microcalorimetric technique was used, in this study, to evaluate the antibacterial activities of these knocked-out constituents and *C. bovis* sample on *S. aureus* growth, further to find the interaction properties of these knocked-out constituents and *C. bovis*.

3.3.2. Method validation and choosing the volume of MeOH

Based on our previous study [33], we have found that the stability of this TAM air isothermal microcalorimeter was good and the reproducibility of IMC was satisfied under identical experimental conditions.

Because of the poor water solubility of the main constituents of *C. bovis*, we got the solutions of *C. bovis* and these knocked-out constituents dissolved in MeOH. But, MeOH itself has some influence on *S. aureus*. The repeated preliminary experiments showed that the influence of 80 μL MeOH on *S. aureus* could be neglected.

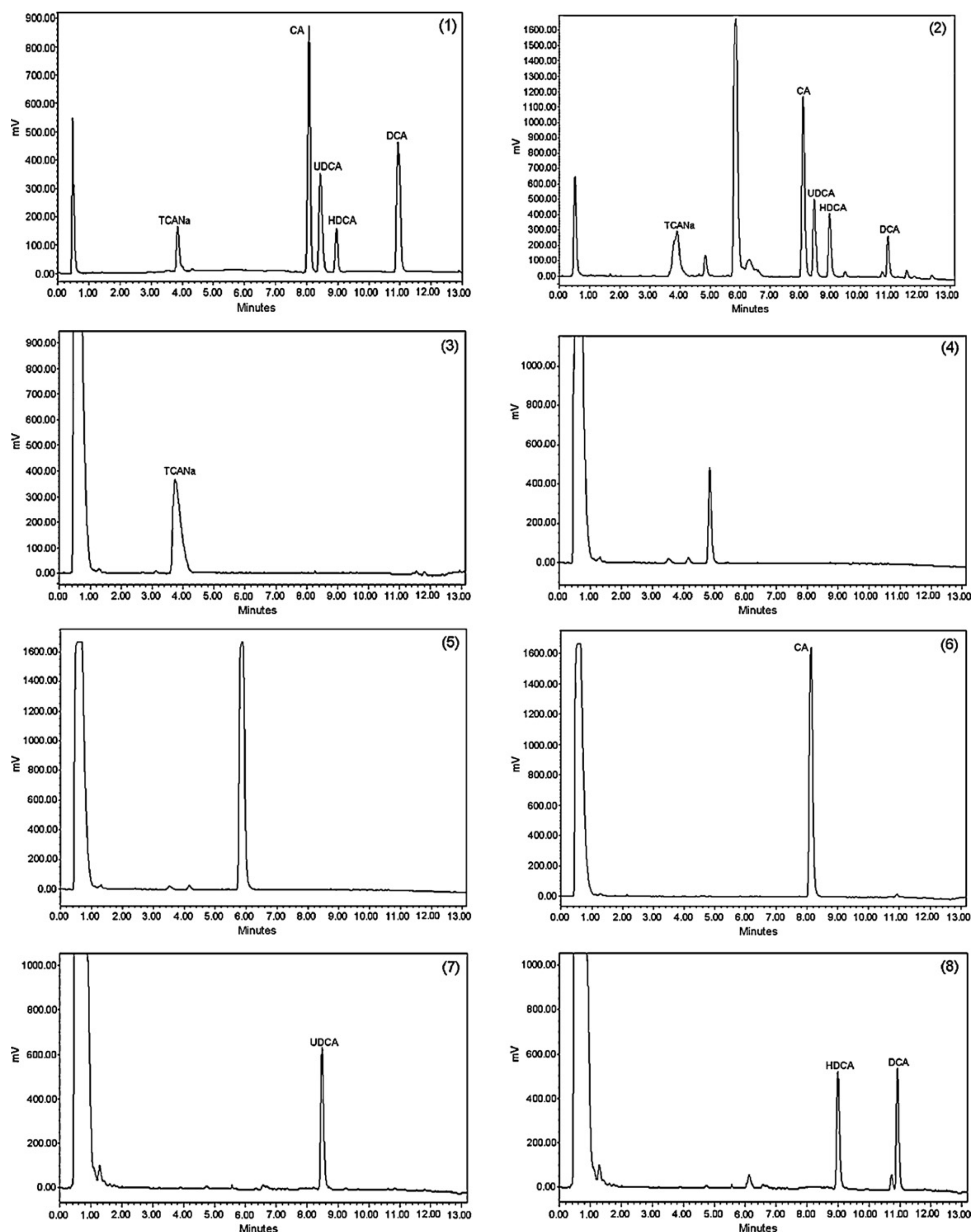

Fig. 3. Typical UPLC–ELSD chromatograms of (1) mixed standards, (2) *C. bovis* sample, (3) constituent A, (4) constituent B, (5) constituent C, (6) constituent D, (7) constituent E and (8) constituent F. The peaks were identified and marked in this figure. Column: Waters Acquity UPLC HSS T3 column (2.1 × 50 mm, 1.8 μm), temperature of 35 °C, mobile phase of acetonitrile and 0.2% aqueous formic acid in gradient mode, liquid flow-rate of 0.6 mL/min; detector: ELSD, drift tube temperature of 100 °C, nebulizing gas flow-rate 1.9 L/min.

So, the maximum added volume of sample solutions dissolved with MeOH into the ampoule was controlled within 80 μL.

3.3.3. Thermogenic HFP–time curves of S. aureus growth at 37 °C

The thermogenic HFP–time curve of *S. aureus* growth at 37 °C in the absence of any substance was shown in Fig. 4. It is the metabolic profiles of *S. aureus* culturing in LB culture medium supplemented without any substance monitored by the microcalorimeter at 37 °C, and could be divided into the first exponential growth phase, a transition phase, the second exponential growth phase and a decline phase.

Correspondingly, the HFP–time curves of *S. aureus* growth in the presence of *C. bovis* sample and the knocked-out constituents (A–F) were recorded and shown in Fig. 5. It could be observed that the four phases of the curves still existed when the sample and different constituents were added into the internal system

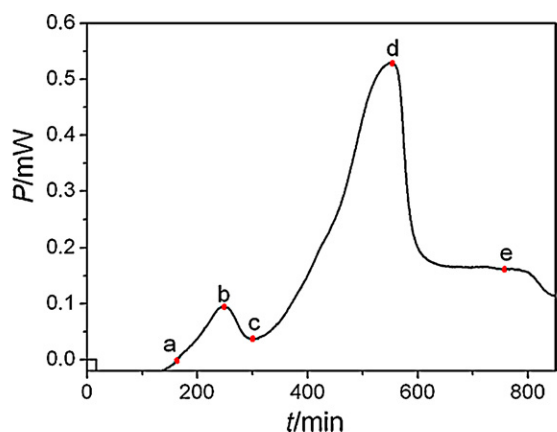

Fig. 4. HFP–time curve for *S. aureus* growth at 37 °C in the absence of any substance. It is the typical metabolic profiles of *S. aureus* culturing in LB culture medium supplemented without any substance monitored by the microcalorimeter at 37 °C, respectively, and both could be divided into the first exponential growth phase (a and b), a transition phase (b and c), the second exponential growth phase (c and d) and a decline phase (d and e).

of *S. aureus* growth in the glass ampoules. But, the height of the highest peaks changed, illustrating that *C. bovis* and different constituents had different activities on the growth of the bacteria.

3.3.4. Activities of C. bovis and the knocked-out constituents on S. aureus growth

From the HFP–time curves of *S. aureus* in Fig. 5, one could find the influences of *C. bovis* sample and the knocked-out constituents on the bacterial growth. Further, the activities of these samples on *S. aureus* could be evaluated from the changes of some quantitative thermokinetic parameters obtained from the HFP–time curves.

The two exponential growth phases of *S. aureus* both obeyed the following equation [34]:

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

where P_0 and P_t were the heat-flow power at time 0 and t (min), respectively. Using this equation, the growth rate constants k_1, k_2 of the first and second exponential phase for *S. aureus* were calculated and shown in Table 1. Then, other thermokinetic parameters for *S. aureus* including the maximum heat-flow power P_1, P_2 and the appearance time t_1, t_2 of the first and second highest peak, the total heat output Q_t (which could be obtained by integrating the peak area under the curve) were obtained from the HFP–time curves and also shown in Table 1.

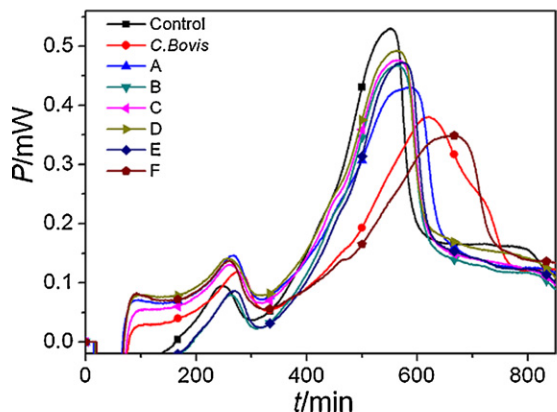

Fig. 5. HFP–time curves for *S. aureus* growth at 37 °C in the presence of *C. bovis* sample and the knocked-out constituents A–F.

From Fig. 5, one could intuitively find the changes of the heights of the two peaks with the addition of different samples, and these changes could be quantitatively reflected from the values of thermokinetic parameters P_1 and P_2, also k_1, k_2, t_1, t_2 and Q_t in Table 1. Generally, the degression of the peak would result in the decrease of P_1, P_2, k_1, k_2 and Q_t and the increase of t_1, t_2 [34]. But, the changing trends of these parameters were complex and disorder. It was difficult to find the internal change regularity. So, PCA provided some help.

3.3.5. PCA

The seven quantitative parameters (k_1, k_2, P_1, P_2, t_1, t_2 and Q_t in Table 1) were analyzed by PCA. With the first two principal components explaining 91.37% of the variability in the original seven parameters, the loading plot in Fig. 6 showed the distribution of these parameters. This plot indicated that parameters k_2 and P_2, which were furthest away from the cluster of other five parameters, might be the two main parameters playing more important role in evaluating and comparing the antibacterial effects of these samples.

Returning to the two main parameters k_2 and P_2 in Table 1, it could be found that the sequence of k_2 and P_2 values for the samples was: (control) > constituent D > constituent C > constituent E > constituent B > constituent A > *C. bovis* > constituent F. The values of k_2 and P_2 for these samples were all lower than those of the control, illustrating that these samples all had antibacterial activities on *S. aureus*. The potency of antibacterial activities for these samples was constituent F > *C. bovis* > constituent A > constituent B > constituent E > constituent C > constituent D.

3.4. Screening of antibacterial constituents

According to the results mentioned above, it could be concluded that:

1. Constituent F had the strongest antibacterial activities on *S. aureus* among these knocked-out constituents, even stronger than *C. bovis* (without knocked-out constituents). Fig. 3(8) displayed that constituent F was the mixture of compounds HDCA and DCA, showing that the main antibacterial composition of *C. bovis* might be certain constituents other than single component, which provided some helpful inspirations for the screening of pharmacodynamic substances of CMMs.
2. The antibacterial activities of the single constituents A–E were all poorer than that of *C. bovis*, while constituent A (TCANa) had the strongest antibacterial activity among these five constituents, which also might be the main antibacterial component of *C. bovis*.
3. Constituent B (unknown compound) and constituent E (UDCA) also had strong antibacterial activities, but whether they were the main antibacterial components of *C. bovis* needed further study.
4. Constituent C with poor antibacterial activity might not be the antibacterial component of *C. bovis*. Although constituent D (CA) was the main component with high content in *C. bovis* [3,5,19,24], it had the poorest antibacterial activity among these knocked-out constituents and might not be the antibacterial component of *C. bovis*. So, based on this finding, if CA was the index for quality control of *C. bovis* needed more studies and validations.

Although constituents F and A might be the main antibacterial constituents of *C. bovis*, their antibacterial activities might be influenced by other antibacterial constituents or constituents with poor antibacterial activities [35,36]. The interaction properties (synergistic and/or antagonistic effect) of them would be explained in the following part.

Table 1
Quantitative thermo-kinetic parameters for *S. aureus* growth at 37 °C in the presence of *C. bovis* sample and the knocked-out constituents.

Constituents	k_1 (min^{-1})	R^a	t_1 (min)	P_1 (mW)	k_2 (min^{-1})	R^a	t_2 (min)	P_2 (mW)	Q_t (J)	I (%)
Control	0.02542	0.9963	250.0	0.0953	0.01055	0.9981	552.0	0.5307	7.44	0
C. bovis	0.01197	0.9993	276.0	0.1188	0.00654	0.9976	620.7	0.3810	6.90	38.01
A	0.00940	0.9974	268.3	0.1466	0.00732	0.9993	584.3	0.4309	7.07	30.62
B	0.02604	0.9970	260.7	0.0802	0.00808	0.9996	562.3	0.4680	6.03	23.41
C	0.00987	0.9968	260.0	0.1314	0.00941	0.9996	562.0	0.4765	7.20	10.81
D	0.01071	0.9967	257.3	0.1420	0.00964	0.9996	562.3	0.4934	7.11	8.63
E	0.02505	0.9973	270.0	0.0871	0.00882	0.9996	571.7	0.4728	6.15	16.40
F	0.00958	0.9961	260.7	0.1377	0.00609	0.9975	662.0	0.3496	6.87	42.27

k_1, k_2, the growth rate constants of the first and second exponential phase; P_1, P_2, the maximum heat-flow power of the first and second highest peak; t_1, t_2, the appearance time of the two highest peaks; Q_t, the total heat output; I, inhibitory ratio.
[a] Correlation coefficient.

3.5. Interaction properties of these antibacterial constituents

The activity extents of these knocked-out constituents and total extract of *C. bovis* on *S. aureus* growth could also be expressed as inhibitory ratio (I, %) which was calculated based on k_2 in Table 1:

$$I = \frac{k_{(2,0)} - k_{(2,m)}}{k_{(2,0)}} \times 100\%$$

where $k_{(2,0)}$ was the growth rate constant of *S. aureus* without sample (the control), $k_{(2,m)}$ was the growth rate constant of *S. aureus* inhibited at different samples (m represented *C. bovis* sample or these knocked-out constituents). The I values of *C. bovis* sample and these knocked-out constituents were also summarized in Table 1.

From the data of I shown in Table 1, it could be concluded that:

1. The I values could be divided into three segments: 30–43% for *C. bovis* sample and constituents F, A, 16–24% for constituents B and E, 8–11% for constituents C and D, which further illustrated the strongest antibacterial activities of constituents F and A, the strong antibacterial activities of constituents B and E, and the poorest antibacterial activities of constituents C and D.
2. Although *C. bovis* sample was consisted of constituents A–F, the total I value of constituents A–F (202.0%) was 5-fold of the I value of *C. bovis* sample (38.01%), showing that these knocked-out constituents had strong antagonistic effects each other in *C. bovis* sample and the antagonistic extent was 81.18%.
3. Constituents C and D with low I values might have small or no influence on the antibacterial activities of the other constituents in *C. bovis* sample, but the total I value of constituents C and D was large, showing that constituent C might have synergistic effect with constituent D.
4. Constituent F was the residue of *C. bovis* sample by knocking out constituents A–E, but the I value of constituents F was larger than that of *C. bovis* sample, showing that constituents A–E could antagonize the antibacterial activity of constituent F.

According to our previous study [19] and Fig. 3(8), DCA and HDCA were isolated from constituent F and the contents of them in constituent F were calculated. Then, the antibacterial activities of the isolated components DCA and HDCA on *S. aureus* were investigated and compared with those of synthetic compounds DCA and HDCA, which could be evaluated from half-inhibitory concentration, IC_{50}. IC_{50} could be calculated based on I using the microcalorimetric method mentioned above. I was re-calculated as:

$$I = \frac{k_{(2,0)} - k_{(2,c)}}{k_{(2,0)}} \times 100\%$$

where $k_{(2,0)}$ was the growth rate constant of *S. aureus* without compound (the control), $k_{(2,c)}$ was the growth rate constant of *S. aureus* inhibited at compound concentration c. When I was 50%, the corresponding concentration was called IC_{50}. From the data in Table 2 (the HFP–time curves of different concentrations of isolated and synthetic DCA, HDCA on *S. aureus* were not shown), it could be found that the antibacterial activities of isolated and synthetic DCA and HDCA were almost same, showing the reliability of the design and experiment in this study.

The total I value (1.28%) of isolated DCA and HDCA on *S. aureus* at concentrations of 3.3 μg/mL and 3.7 μg/mL (the contents of them in constituent F) was 1/33 of I value (42.27%) of constituent F on *S. aureus* (Table 1), showing that DCA had strong synergistic effect with HDCA in constituent F.

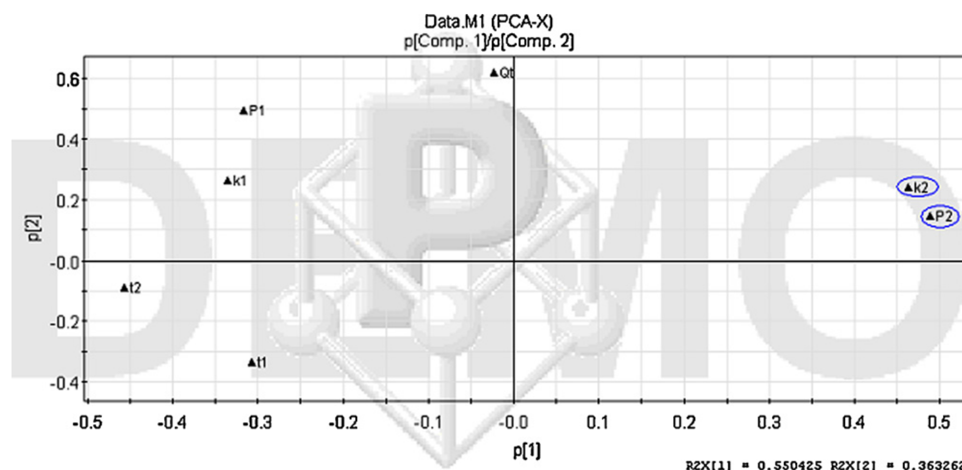

Fig. 6. Loading plot generated from PCA on the seven quantitative parameters k_1, t_1, P_1, k_2, t_2, P_2 and Q_t. This plot was obtained using software of SIMCA-P 11.5 (Umetrics AB, Umea, Sweden). The main two parameters were marked with a circle.

Table 2
The values of I and IC_{50} of DCA and HDCA from constituent F on *S. aureus*.

c[a] (μg/mL)	Compounds[c]	k_2 (min^{-1})	R[b]	I (%)	IC_{50} (μg/mL)	Compounds[d]	k_2 (min^{-1})	R[a]	I (%)	IC_{50} (μg/mL)
0	Control	0.00817	0.9981	0		Control	0.00835	0.9990	0	
3.3	DCA	0.00808	0.9961	1.16		DCA	–	–	–	
20.0		0.00654	0.9978	19.95	57.73		0.00638	0.9989	23.59	55.69
40.0		0.00532	0.9983	34.88			0.00517	0.9991	38.08	
50.0		0.00499	0.9972	38.92			0.00473	0.9992	43.35	
60.0		0.00382	0.9979	53.24			0.00366	0.9988	56.17	
70.0		0.00293	0.9965	64.14			0.00281	0.9995	66.35	
80.0		0.00178	0.9977	78.21			0.00163	0.9990	80.48	
3.7	HDCA	0.00816	0.9957	0.12		HDCA	–	–	–	
20.0		0.00703	0.9979	13.95	67.81		0.00698	0.9992	16.41	65.43
40.0		0.00611	0.9988	25.21			0.00602	0.9992	27.90	
50.0		0.00533	0.9983	34.76			0.00521	0.9990	37.60	
60.0		0.00465	0.9976	43.08			0.00455	0.9989	45.51	
70.0		0.00391	0.9981	52.14			0.00373	0.9990	55.33	
80.0		0.00304	0.9989	62.79			0.00291	0.9993	65.15	

c, concentration; IC_{50}, half-inhibitory concentration.
[a] The final concentration of compounds in the ampoule containing LB culture medium and *S. aureus*.
[b] Correlation coefficient.
[c] DCA and HDCA were isolated from *C. bovis*.
[d] DCA and HDCA were from synthetic compounds.

5. The interaction properties (synergistic/antagonistic effect) of these antibacterial constituents with each other in *C. bovis* might be the mechanism to explain the basis of the therapeutic efficacy of it, which needed further study in our next work.

4. Discussions

The “target constituent knock-out” strategy in this study provided a useful idea for screening active constituents and evaluating the interaction properties of them in *C. bovis*, which was selected as a model drug. First of all, we found that the key antibacterial composition(s) of *C. bovis* was not a single component, and also not the high content component (CA), but was the combinatorial constituent of DCA and HDCA. These findings provided us some useful inspirations that the potential synergistic or antagonistic effect might be the mechanism often claimed to represent the molecular basis of the “holistic” therapeutic efficacy of CMMs [37–39].

Secondly, it was found that the combination of DCA and HDCA (constituent F) revealed over 33-fold high activity of the sum of their activities in solo-use. This finding would inspire an appealing idea to design the artificial product to substitute natural resource of *C. bovis*, based on the proper composition of the KPCs, which would possess competitive therapeutic effect and complete assurance of quality control comparing to natural resources. In addition, we found that DCA and HDCA had weak antibacterial activities, respectively, but their combination revealed strong antibacterial activity. But, such two compounds might be judged as non-active components and put away out the experiment through the existing activity-oriented screening approaches. This gave a powerful illustration for the valuable feature of the “target constituent knock-out” strategy to avoid false-negative results.

Thirdly, the isolated constituents from *C. bovis* revealed high activities even surpassed the activity of total extract, which suggested that the different constituents might interfere with each other’s activity, and highlighted the existence of antagonistic effects of these constituents. Since the isolated constituents were both active and antagonistic, the judgment of whether the target constituent was the KPC needed integrated consideration. This feature of the “target constituent knock-out” strategy was important to facilitate avoiding false-positive results. For instance, CA has some activity to inhibit *S. aureus* growth and might be categorized as active constituent, but it could not be considered as KPC for its relative weak activity and strong antagonistic effect to other constituents [40–42]. It also suggested that the high content of some components would not be beneficial to the “holistic” effect of *C. bovis*, and their contents in *C. bovis* should be controlled within a proper range, especially under the upper limitation. To achieve the purpose, future work in our lab would introduce the “target constituent knock-in” strategy to study the quantity–activity relationships of the KPCs in *C. bovis*. The distinct feature of the “target constituent knock-in” strategy was reflected in the design of quantity–activity relationship study that the quantity of the target constituent was added into the minus extract rather than blank matrix. This assured involvement of the multiple-component interactions in the CMMs for the desired quantity–activity relationship in a holistic view. Usually, the quantity–activity relationship based on minus extract was not a monotone curve, and the range might be delimited for the aim of quality control. Standing in vivid contrast against the current quality standards set on the basis of the statistical sampling distribution, the quality criteria deduced from the “target constituent knock-in” experiment would be closely related to the real activity of the CMMs. Finally, the combination of target constituent “knock-out” and “knock-in” strategy could more precisely and quickly recognize the key pharmacodynamic constituents, facilitate developing new drugs with definite pharmacodynamic constituents, and provide effectiveness-related quality control standard for *C. bovis* or other CMMs.

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References

[1] P.G. Xiao, Modern Chinese Materia Medica, vol. 4, Chemical Industry Press, Beijing, 2001.
[2] Y.W. Li, X.Y. Zhu, H.W. Yeung, J. Ethnopharmacol. 47 (1995) 27.
[3] China Pharmacopoeia Committee, Pharmacopoeia of the People’s Republic of China 1st Div (2005 Edition), China Chemical Industry Press, Beijing, 2005, pp. 4–5, 47–48.
[4] T.H. Xue, R. Roy, Science 300 (2003) 740.
[5] F. Wei, M. Zhong, J.T. Ni, R.C. Lin, Chin. J. Pharm. Anal. 29 (2009) 21.
[6] J.L. Zhang, H. Wang, H.F. Pi, H.L. Ruan, P. Zhang, J.Z. Wu, Steroids 74 (2009) 424.
[7] X.H. Xiao, D. Yan, H.L. Yuan, J.B. Wang, C. Jin, Chin. Tradit. Herb. Drugs 40 (2009) 1345.

[8] H.L. Yuan, M. Yang, X.Y. Li, R.H. You, Y. Liu, J. Zhu, H. Xie, X.H. Xiao, Chem. Pharm. Bull. 54 (2006) 1592.
[9] S.A. Bura, A. Burokas, E. Martín-García, R. Maldonado, Eur. Neuropsychopharmacol. 20 (2010) 369.
[10] A. Minokadeh, L. Funkelstein, T. Toneff, S.R. Hwang, M. Beinfeld, T. Reinheckel, C. Peters, J. Zadina, V. Hook, Mol. Cell. Neurosci. 43 (2010) 98.
[11] W.J. Kong, C. Jin, X.H. Xiao, Y.L. Zhao, Z.L. Li, P. Zhang, W. Liu, X.F. Li, J. Hazard. Mater. 179 (2010) 742.
[12] W.J. Kong, J.B. Wang, X.Y. Xing, C. Jin, X.H. Xiao, Y.L. Zhao, P. Zhang, Q.C. Zang, Z.L. Li, J. Hazard. Mater. 185 (2011) 346.
[13] W.J. Kong, Y.L. Zhao, L.M. Shan, X.H. Xiao, W.Y. Guo, Chin. J. Biotechnol. 24 (2008) 646.
[14] M.L. Latorre-Moratalla, S. Bover-Cid, T. Veciana-Nogués, M.C. Vidal-Carou, J. Chromatogr. A 1216 (2009) 4128.
[15] P. Płocharz, A. Klimek-Turek, T.H. Dzido, J. Chromatogr. A 1217 (2010) 4868.
[16] G.D. Xiao, G.W. Li, L. Chen, Z.J. Zhang, J.J. Yin, T. Wu, Z.H. Cheng, X.H. Wei, Z.T. Wang, J. Chromatogr. A 1217 (2010) 5470.
[17] K.V. Mardia, J.T. Kent, J.M. Bibby, Multivariate Analysis, Academic, New York, 1979.
[18] F. Feng, Y.J. Ma, M. Chen, Z.X. Zhang, D.K. An, Acta Pharm. Sin. 35 (2000) 216.
[19] W.J. Kong, C. Jin, W. Liu, X.H. Xiao, Y.L. Zhao, Z.L. Li, P. Zhang, X.F. Li, Food Chem. 120 (2010) 1193.
[20] W. Liu, C. Jin, W.J. Kong, H.T. Wang, Q.F. Gong, X.H. Xiao, Chin. J. Anal. Chem. 38 (2010) 522.
[21] K.Y. Ni, J. Wang, J. Yu, S.Z. Tu, Acta Pharm. Sin. 29 (1994) 629.
[22] Z. Hu, L.C. He, J. Zhang, G.A. Luo, J. Chromatogr. B 837 (2006) 11.
[23] X. Zhou, T. Lü, Z.G. Chen, J. Zhang, Chin. J. Anal. Lab. 26 (2007) 5.
[24] W.J. Kong, C. Jin, X.H. Xiao, Y.L. Zhao, W. Liu, Z.L. Li, P. Zhang, J. Sep. Sci. 33 (2010) 1518.
[25] A. Coates, Y.M. Hu, R. Bax, C. Page, Nat. Rev. Drug Discov. 1 (2002) 895.
[26] M.J. Pallen, B.W. Wren, Nature 449 (2007) 835.
[27] D.J. Payne, Science 321 (2008) 1644.
[28] O. Braissant, D. Wirz, B. Göpfert, A.U. Daniels, FEMS Microbiol. Lett. 303 (2010) 1.
[29] I. Wadsö, R.N. Goldberg, Pure Appl. Chem. 73 (2001) 1625.
[30] W.J. Kong, Y.L. Zhao, L.M. Shan, X.H. Xiao, W.Y. Guo, Acta Chim. Sin. 66 (2008) 1111.
[31] F.M. Aarestrup, N.E. Jensen, Vet. Microbiol. 64 (1999) 299.
[32] W.J. Kong, Y.L. Zhao, X.H. Xiao, Z.L. Li, C. Jin, H.B. Li, J. Appl. Microbiol. 107 (2009) 1072.
[33] W.J. Kong, J.B. Wang, C. Jin, Y.L. Zhao, C.M. Dai, X.H. Xiao, Z.L. Li, Appl. Microbiol. Biotechnol. 83 (2009) 1183.
[34] C.L. Xie, H.K. Tang, Z.H. Song, S.S. Qu, Thermochim. Acta 123 (1988) 33.
[35] J. Qiu, Nature 448 (2007) 126.
[36] J. Qiu, Nature 446 (2007) 590.
[37] F.R. Stermitz, J. Tawara-Matsuda, P. Lorenz, P. Mueller, L. Zenewicz, K. Lewis, J. Nat. Prod. 63 (2000) 1146.
[38] F.R. Stermitz, P. Lorenz, J.N. Tawara, L.A. Zenewicz, K. Lewis, PNAS 97 (2000) 1433.
[39] Y. Yanagawa, Y. Yamamoto, Y. Hara, T. Shimamura, Curr. Microbiol. 47 (2003) 244.
[40] C. Thomas, R. Pellicciari, M. Pruzanski, J. Auwerx, K. Schoonjans, Nature 7 (2008) 678.
[41] C.G. Liu, T.H. Zhang, R.G. Ding, J. Zhao, M. Huang, Pharm. J. Chin. PLA 24 (2008) 49.
[42] T.H. Zhang, J. Lin, C.G. Liu, R.G. Ding, J.X. Ruan, X.H. Sun, C.Q. Huang, X.C. Zhang, Int. J. Pharmacol. 4 (2008) 407.