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Study of Antimicrobial Effects of Different Types of Glycyrrhiza Extracts by Microcalorimetry

Ting Wei,[†,a] Guimei Lin,[†,a,*] Lian Liu,[‡] and Zhongxi Zhao[†,*]

[†]*School of Pharmaceutical Sciences and Center for Pharmaceutical Research & DDS, Shandong University, Jinan, Shandong 250012, China. [*]E-mail: uszxzhao@gmail.com (Z. Zhao); guimeilin@sdu.edu.cn (G. Lin)*
[‡]*Beijing Health Vocational College, 94 Southern Cross Street, Xuanwu District, Beijing 100053, China*
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Recently studies indicate that the microcalorimetry is a suitable measurement for metabolic activities of organisms by recording the rate of heat outputs. In this work, we investigated the growth thermogenic curves of *Escherichia coli* (*E. coli*) affected by three kinds of Glycyrrhiza extracts by microcalorimetry. The power-time and exponential phase power-time curves of the *E. coli* growth at various concentrations of extracts were generated. The kinetic parameters such as the growth rate constants (*C*), maximum power outputs (*Pm*), peak times (*Tm*), and inhibition ratios were calculated and the relationships between *Pm* or *Tm* and *C* were established. Also, the clear correlations among the antimicrobial effects, *Pm* and *C* were obtained. The results revealed the Glycyrrhiza extracts had inhibitory activities towards *E. coli* while the Glycyrrhiza polysaccharides showed the most potent effects.

Key Words : Microcalorimetry, Glycyrrhiza aqueous extract, Glycyrrhiza polysaccharides, Glycyrrhiza flavonoids, *E. coli*

Introduction

Licorice (Glycyrrhiza) species are perennial herbs and belong to the family of leguminous. The roots and rhizomes of licorice species have long been used for the treatment of various diseases worldwide, which has also been one of the most widely used herbal medicines found in traditional formulas since antiquity. Glycyrrhiza has been used mainly for the treatment of hepatitis C, dry cough and pulmonary diseases. The Glycyrrhiza extracts have been shown to have some pharmacological properties including anti-inflammatory, antiulcer, antiviral, antimicrobial, antioxidative, anticarcinogenesis, immunomodulatory, hepatoprotective and cardioprotective effects to a certain extent.[1-5]

The term "Isothermal microcalorimetry" usually implies measurements on a calorimeter in the microwatt range in thermochemistry and thermodynamic study, this technique is especially suitable for the survey of heat outputs of slightly exothermic or endothermic processes. As a non-specific, non-invasive, non-destructive, quantitative, inexpensive and versatile technique for life sciences applications, it has been confirmed to be a valuable tool in the research of metabolism of microorganisms, cells and whole organisms.[6-9]

Microcalorimetry permits the continuous monitoring of a metabolic process in situ without disturbing the biological system. The heat evolved or adsorbed is proportional to the rate of the biological processes.[10,11] It is an online, kinetic and precise method to measure the bioactivity of compounds, which could be a useful tool in drug discovery.

Microcalorimetry has recently been applied in the clinical analysis, pharmacology, ecology, biotechnology and agriculture.[12-16] In this work, we investigated the antimicrobial activities of Glycyrrhiza aqueous extract (G) and Glycyrrhiza polysaccharides (GP) and Glycyrrhiza flavonoids (GF) purified from Glycyrrhiza on the *E. coli* growth. Glycyrrhiza aqueous extract (G) was obtained by water extraction method while the samples of purified Glycyrrhiza polysaccharides (GP) and Glycyrrhiza flavonoids (GF) were directly purified from the Glycyrrhiza powdered roots. The metabolic power–time curves of the *E. coli* growth under different concentrations of three extract samples were attained. The relationships between thermokinetic parameters of the *E. coli* growth and sample concentrations were discussed and the correlations among the antimicrobial effects were obtained. The results from this study indicate that the microcalorimetric method could potentially be used in the microbiological evaluation of the complicated traditional Chinese medicines (TCM).

Experimental

Instrument. A 3114/3236 TAM air microcalorimeter (Thermometric AB, Sweden) was used to determine the thermal effect of the three extracts from Glycyrrhiza on *E. coli* metabolism. The TAM air microcalorimeter is an 8-channel heat conduction calorimeter for heat flow measurements in the milliwatt range under isothermal conditions. It was held together in a single removable block. This block was placed in an air thermostat, which kept the temperature within 0.02 °C. Measurements were carried out in the sealed 20-mL glass ampoules. The reactions could be carried out in the temperature range of 5 to 90 °C. The software supplied

[a]These authors contributed equally to this work.

to TAM air (PLW32) was used to monitor and record the heat flow output over the Peltier module. The detection limit was 2 μW and baseline drift was less than 20 μW over a period of 24 h,[17] respectively.

Materials. The licorice species (Glycyrrhiza uralensis) was provided by Hangjinqiliangwai LTD (Ordos, Inner Mongolia, China). It was a light brown powder and easily soluble in water. The Glycyrrhiza aqueous extract (G) was isolated from Glycyrrhiza uralensis with the following preparation procedure: the powdered roots of the commercial Glycyrrhiza uralensis (3 g) were extracted for 1 h at 100 °C twice with the water volumes of 30 and 15 mL, respectively. The final volume of the filtrate was removed using a rotary vacuum evaporator to give the concentrated extract, which was then dried under vacuum and frozen until use. The test sample of the Glycyrrhiza aqueous extract consisted of glycyrrhetinic acid, polysaccharides, flavonoids, and other miscellaneous compounds.

The purified Glycyrrhiza polysaccharide (GP) test sample was prepared according to the following procedure: the powdered roots of the commercial Glycyrrhiza uralensis (10.0 g) were subsequently extracted under boiling conditions with 150, 150, 300, 150 mL of petroleum ether, 80% ethanol and water (twice), respectively. The combined filtrates were concentrated by evaporation to about 100 mL. The concentrated filtrate was fractionated with 80% ethanol at 4 °C overnight. The precipitate was washed with ethanol, acetone and diethyl ether twice to give the crude polysaccharides and dried under vacuum. The average yield of Glycyrrhiza polysaccharides was determined by UV spectroscopy at 490 nm and the yield of the purified Glycyrrhiza polysaccharides was determined to 2.04%. As the name implies, the test sample of purified Glycyrrhiza polysaccharides was a mixture of different polysaccharides.

The purified Glycyrrhiza flavonoid (GF) test sample was prepared according to the following procedure: the powdered roots of the commercial Glycyrrhiza uralensis (10.0 g) were subsequently extracted under boiling conditions with 300 and 150 mL of 80% ethanol and 100% ethanol, respectively. The combined filtrates were concentrated by evaporation to the paste. The concentrated paste was suspended in the hot water and extracted with petroleum ether and ethyl acetate each three times. The flavonoid fraction was obtained after the solvent removal and dried under vacuum. The average yield of Glycyrrhiza flavonoids from the purification process was determined by UV spectroscopy at 509 nm and the yield was found to be 1.90%. It was found that the test sample of the purified Glycyrrhiza flavonoid (GF) contained various flavonoids.

Bacteria *E. coli* [CMCC (B) 44102] were used as the test organism, which was obtained from Shandong Institute for Drug Control. *E. coli* CMCC (B) 44102 was routinely cultivated in a Luria-Bertani (LB) culture medium consisting of 5 g NaCl, 5 g yeast extract, 10 g peptone per liter. The medium pH was adjusted to 7.0-7.2 and. The LB culture medium was sterilized by autoclaving at 121 °C for 20 min before the experiment.

Methods. The microcalorimetric measurement was performed using the ampoule method, thermostated at 37 °C. The test samples were prepared by adding the different concentrations of various active ingredients isolated from the Glycyrrhiza uralensis into the *E. coli*-containing LB culture media in 10 mL placed in the 20-mL glass ampoules. One ampoule containing 10 mL sterile LB medium was used as a blank control. All the ampoules were sealed up and put into the 8-channel calorimeter block. The power-time signals were recorded at an interval of 1 min until the recorder returned to the baseline.[18]

Results and Discussion

Power-Time Curves of *E. coli* Growth with Different Glycyrrhiza Extracts.

Power-Time Curves of *E. coli* Growth with Different Concentrations of the Glycyrrhiza Aqueous Extract: Figures 1 and 2 show the power–time curves and exponential phase of the *E. coli* growth in the LB medium containing various concentrations of the Glycyrrhiza aqueous extract (G). The effects of different concentrations of G on the *E. coli* growth can be clearly seen. The *E. coli* growth curves can be divided into four stages: the first growth, first decreased metabolic activity, second growth, and second decreased metabolic activity stages. The *E. coli* growth increased very fast while the power produced decreased rapidly at the decreased stage (Figure 1). The power–time curves were found to be delayed gradually and the maximum metabolic power increased with the increase of the G concentration. At the first exponential phase, the growth of *E. coli* showed a distinct downward trend with the increased G concentrations (Figure 2).

Power-Time Curves of *E. coli* Growth with Different Concentrations of Glycyrrhiza Polysaccharides: The power–time curves and and exponential phase of different concentrations of Glycyrrhiza polysaccharides (GP) on the *E. coli* growth are plotted in Figures 3 and 4. The *E. coli* growth curves with the addition of the GP could also be

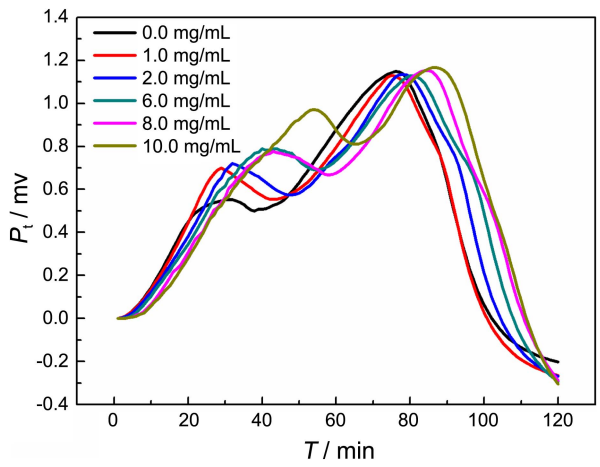

Figure 1. Power-time curve of *E. coli* with different concentrations of G.

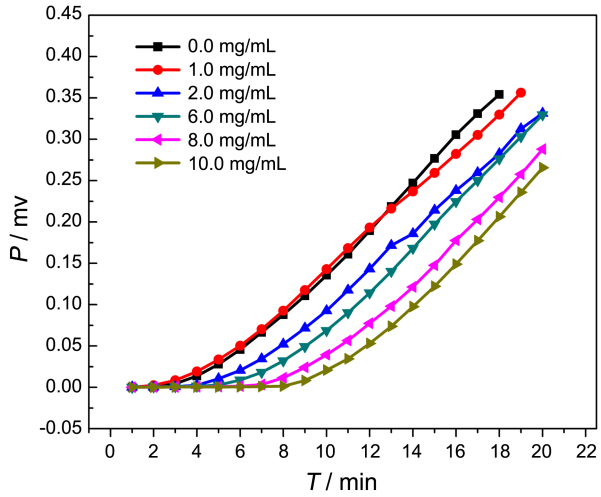

Figure 2. Exponential phase of *E. coli* with different concentrations of G.

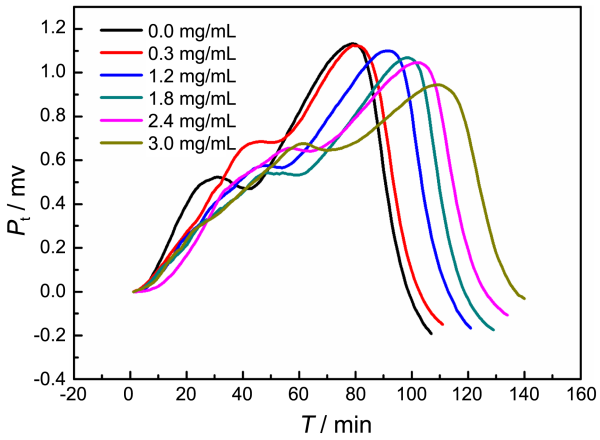

Figure 3. Power-time curve of *E. coli* with different concentrations of GP.

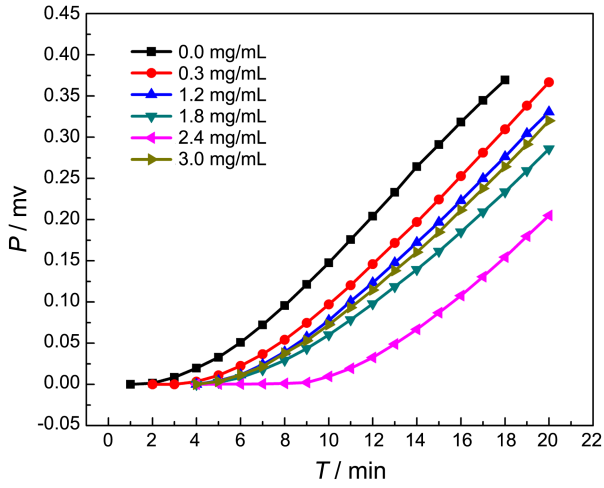

Figure 4. Exponential phase of *E. coli* with different concentrations of GP.

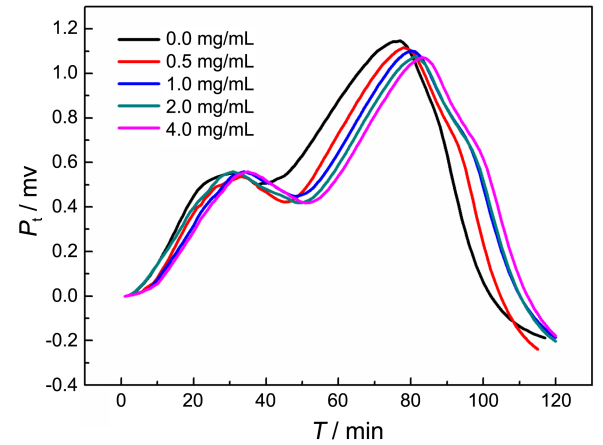

Figure 5. Power-time curve of *E. coli* with different concentrations of GF.

divided into 4-5 stages depending upon the concentration range of the Glycyrrhiza polysaccharides: the first growth that might be broke into two growth stages with different slops, first decreased stage, second growth, and decreased stages (Figure 3). The first decayed stage seems to be gentle as shown in the concentration range of 0.3-2.4 mg mL^{-1}. Comparing to the effects of the Glycyrrhiza aqueous extract (G) on the *E. coli* growth, the power–time curves of the *E. coli* growth show the similar delay patterns as the increase of the GF concentrations, however, the metabolic power increase rates became sequentially slower. As is shown in Figure 4, the first exponential phase of the *E. coli* growth showed a downward trend with the increased GP concentrations.

Power-Time Curves of *E. coli* Growth with Different Concentrations of Glycyrrhiza Flavonoids: The power–time curves from the various concentrations of GF were showed in Figures 5 and 6. The diauxie of *E. coli* growth curves with the addition of the GF can clearly be depending upon the concentration range (Figure 5). Comparing to the effects of the G and GP on the *E. coli* growth, the differences of the metabolic power increase rates among concentrations of GP became relatively smaller, the concentration-dependent delay could not clearly observed. As shown in Figure 6,

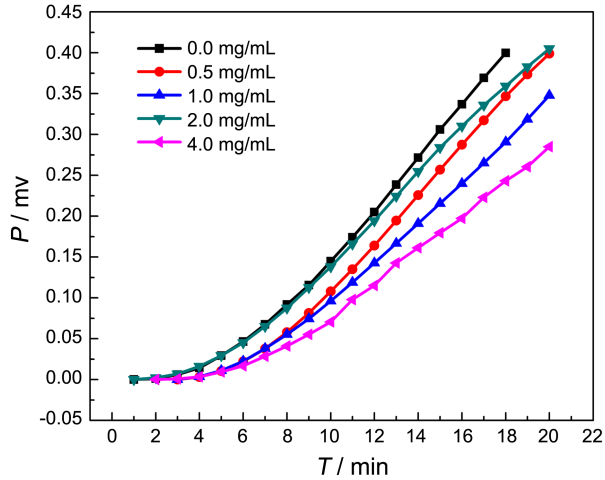

Figure 6. Exponential phase of *E. coli* with different concentrations of GF.

the first exponential phase of the *E. coli* growth also showed a downward trend with the concentration increase of the GP, while the trend was inconspicuous in contrast to the exponential growth phase of GF.

Thermokinetics and Inhibition of *E. coli* Growth by Different Glycyrrhiza Extracts. In the growth phase, a theoretical model used is in accordance with the following law:[4]

$$dNt/dt = \mu Nt - \beta Nt^2 \quad (1)$$

where Nt is the bacterial number at time t, μ is the growth rate constant, and β is the bacterial static rate constant. The integration of Eq. (1) is given by

$$Nt = K/(1 + \alpha e - \mu t) \quad (2)$$

Where K is the maximum density, α is integral constant. If the power produced by every bacterium is P, then $P Nt = K P/(1 + \alpha e - \mu t)$, making $Pt = P Nt$, $Pm = P K$, where Pt is the power output at time t, Pm is the maximum power output, then

$$Pt = Pm/(1 + \alpha e - \mu t) \quad (3)$$

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

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

$$I = [(\mu 0 - \mu c)/\mu 0] \times 100\% \quad (4)$$

where $\mu 0$ and μc are the growth rate constants of *E. coli* without and with the Glycyrrhiza extract, respectively.

Based on the kinetic equations listed above, the thermokinetic parameters of μ, Tm, Pm and I were calculated from the effects of the Glycyrrhiza extracts on *E. coli* growth curves, the data of which are shown in Table 1. Two new terms r and Tm are introduced here: r is the correlation coefficient and Tm is the peak time, which is the time of reaching the maximum power output.[19] As can be seen from Table 1, the values was greater than 0.99 for the correlation coefficients indicated that the thermokinetic models used in this work were good fit. The time of reaching the maximum power outputs (Tm) of the *E. coli* growth for three Glycyrrhza extracts became longer with the increase of the extract concentrations.

The inhibition ratios listed in Table 1 indicate that the antimicrobial effects of G, GP, and GF on the *E. coli* growth showed various trends in relationship to the extract concentrations. The inhibition effects of G on the *E. coli* growth were found to decrease with the increase of the extract concentrations, which might be related to different compositions in the Glycyrrhiza extracts. The *E. coli* growth inhibition by GP showed a clear concentration dependency, indicating that the Glycyrrhiza polysaccharides had a relatively potent antibacterial activity against *E. coli*. The antimicrobial effects of GF on the *E. coli* growth showed a similar trend as GP but in a rather less dramatic manner, indicating less potent inhibitory activities to the *E. coli* growth. Other themokinetic parameters such as the growth rate constants and the maximum power outputs will be discussed in more details in following subsections.

Relationship between *E. coli* Growth Rates and Glycyrrhiza Extract Concentrations. The *E. coli* growth rates at various concentrations of three Glycyrrhiza extracts are presented in Figure 7. The *E. coli* growth rates showed significant different trends among different Glycyrrhiza extracts. The *E. coli* growth dramatically declined with the increase of concentrations of GP, indicating that GP had a relatively potent antibacterial activity against *E. coli*. The effects of GF on the *E. coli* growth showed a similar trend as the GP, but in a rather less conspicuous manner, indicating less potent inhibitory activities to the *E. coli* growth. However, the growth rate constant (μ) of *E. coli* with the addition of G initially decreased and then increased with the increased G concentrations. The reason for biphasic inhibition effects of

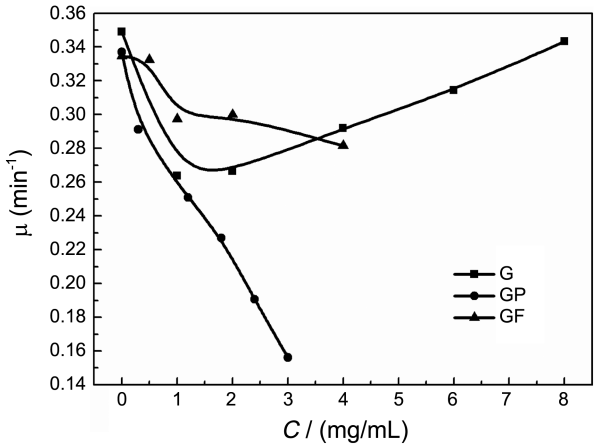

Figure 7. Relationship between μ and c for *E. coli* with G, GP and GF.

Table 1. Parameters of *E. coli* growth at different concentrations of active compositions

Drug	c/(mg/mL)	μ/min^{-1}	r	T_m/min	P_m/mw	I/%
G	0	0.3490	0.9972	76	1.1476	—
	1.0	0.2638	0.9985	76	1.1232	22.18
	2.0	0.2665	0.9964	77	1.1252	21.39
	4.0	0.2919	0.9977	80	1.1276	17.67
	6.0	0.3145	0.9972	84	1.1547	9.19
	8.0	0.3433	0.9973	89	1.1604	–
GP	0	0.3370	0.9964	80	1.1313	–
	0.3	0.2913	0.9934	81	1.1226	13.56
	1.2	0.2509	0.9971	89	1.0938	25.55
	1.8	0.2270	0.9971	98	1.0678	32.64
	2.4	0.1906	0.9974	100	1.0410	43.44
	3.0	0.1562	0.9983	111	0.9403	53.65
GF	0	0.3347	0.9975	77	1.1475	–
	0.5	0.3325	0.9976	78	1.1162	1.51
	1.0	0.2973	0.9969	80	1.1015	8.87
	2.0	0.3000	0.9978	81	1.0778	12.04
	4.0	0.2815	0.9979	83	1.0721	17.86

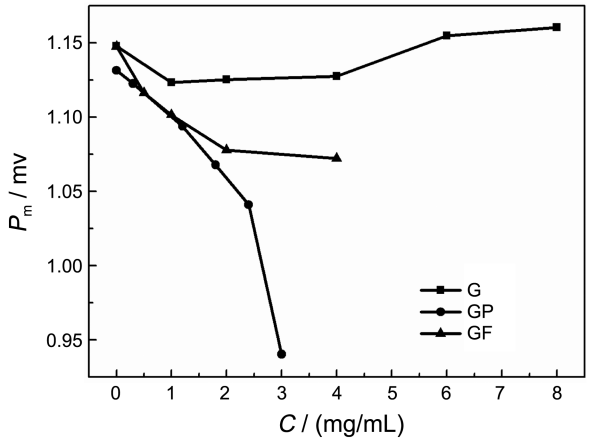

Figure 8. Relationship between P_m and c for *E. coli* with G, GP and GF.

the *E. coli* growth by G requires further investigations. One possible explanation may be related to its rather diversified compositions such as triterpene saponins, flavonoids, isoflavonoids, chalcones, polysaccharides, the net inhibition effects of which could show the concentration dependency.[3]

All the effects of different Glycyrrhiza extracts on the *E. coli* growth were graphically shown in Figure 7 are consistent with the quantitative inhibition data listed in Table 1.

Relationship between the Maximum Power Outputs (Pm) and Glycyrrhiza Extract Concentrations. The relationship between the maximum power outputs (*Pm*) of the *E. coli* growth and Glycyrrhiza extract concentrations are shown in Figure 8. The effects of different Glycyrrhiza extracts at various concentrations on the *E. coli* growth rates (Figure 7) and maximum power outputs (Figure 8) showed the similar trends. The maximum heat outputs (*Pm*) for the *E. coli* growth effected by G had little changes when the concentration was increased. The Glycyrrhiza polysaccharides (GP) inhibited the *E. coli* growth dramatically as increased GP concentrations, revealing significant antimicrobial activities on *E. coli*. The maximum power outputs of the *E. coli* growth with the addition of GF showed a different trend of concentration dependency, which decreased greatly at the lower concentration and then slowly at the high concentration range. Once again, the different inhibition trends of the *E. coli* growth by G, GP and GF may be attributed to various components of the different extracts.

Conclusion

In this work, microcalorimetry was used to study the inhibitory effects of three samples involved in Glycyrrhiza on the growth of *E. coli*. The power–time curves of the *E. coli* growth at different concentrations were plotted. Diauxie of the *E. coli* curve in relationship to the concentration of each extracts were observed. A series of thermokinetic parameters such as the *E. coli* growth rate constant (μ), the peak-time (*Tm*), the maximum powder outputs (*Pm*), and inhibition ratios (*I*) were obtained. The trends of antimicrobial effects of extracts evaluated were also examined by plotting the curves of the maximum powder outputs or the *E. coli* growth rate constants with the extracts concentrations. There were not clear concentration-dependent inhibitory effects of the Glycyrrhiza aqueous extract on the *E. coli* growth while the Glycyrrhiza polysaccharides and flavonoids showed some downward dependencies between the power outputs of *E. coli* growth rates and the concentrations. This work indicated that microcalorimetry could be a useful technique for studying the microbial growth and estimating the antimicrobial kinetics of drugs in live organisms.

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