Evaluation of the Antibacterial Effects of Flavonoid Combination from the Leaves of *Dracontomelon dao* by Microcalorimetry and the Quadratic Rotary Combination Design

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Skin infectious disease is a common public health problem due to the emergence of drug-resistant bacteria caused by the antibiotic misuse. *Dracontomelon dao* (Blanco) Merr. et Rolfe, a traditional Chinese medicine, has been used for the treatment of various skin infectious diseases over 1000 of years. Previous reports have demonstrated that the leaves of *D. dao* present favorable antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Bacillus subtilis*. The flavonoids are the main components of the ethyl acetate extract of *D. dao* leaf. However, the correlation between flavonoids and antibacterial activities is yet to be determined. In this study, the combined antibacterial activities of these flavonoids were investigated. Three samples with the different concentrations of flavonoids (S1–S3) were obtained. By microcalorimetric measurements, the results showed that the IC₅₀ value of S2 was lower than those of S1 and S3. The contents of main flavonoids (including Luteolin, L-Epicatechin, Cianidanol, and Quercetin) in S1–S3 were various, confirmed by the method of the Ultra High Performance Liquid Chromatography (UPLC). Based on the method of quadratic general rotary unitized design, the antibacterial effect of single flavonoid, and the potential synergistic effects between Luteolin and Quercetin, Luteolin and Cianidanol were calculated, which were also proved by microcalorimetric analysis. The antibacterial activities of main flavonoids were Luteolin > Cianidanol > Quercetin > L-Epicatechin. Meanwhile, the synergistic effects of Luteolin and Cianidanol ($P_{L+C} = 1.425$), Quercetin and Luteolin ($P_{L+Q} = 1.129$) on anti-microbial activity were validated. Finally, we found that the contents of Luteolin, L-Epicatechin, Cianidanol, Quercetin were 1061.00–1061.00, 189.14–262.86, 15,990.33–16,973.62, 6799.67–7662.64 ng·ml⁻¹ respectively, with the antibacterial rate over 60.00%. In conclusion, this study could provide reference for quality evaluation and pharmacodynamics research of *D. dao*.

Keywords: *Dracontomelon dao* (Blanco) Merr. et Rolfe, antibacterial activity, *E. coli*, microcalorimetry, quaternary quadratic general rotary unitized design
INTRODUCTION

Traditional Chinese medicine has a long history in the treatment of skin infections. The ancient Chinese people treated skin and soft tissue infections (SSTIs) with Kushen (Sophora flavescens Ait.) and Baixianpi (Dictamnus dasycarpos Turcz.) 2000 years ago. Dracontomelon dao (Blanco) Merr. et Rolfe belongs to the Anacardiaceae family, a traditional Chinese medicinal material with regional feature, and has been widely used to treat various infectious diseases, such as decubitus and skin ulcers. Previous studies have showed that the ethanol extracts of the leaves of D. dao demonstrate anti-Staphylococcus aureus and anti-Bacillus subtilis activities (Khan and Omoloso, 2002). The subsequent studies from our research team have also indicated that the different extracts from the leaves of D. dao by means of systematic solvent extraction (including petroleum ether, chloroform, ethyl acetate, n-butanol, and water) show anti-Escherichia coli (the greatest IC₅₀ value is 83.93 µg·ml⁻¹) and anti-Staphylococcus aureus (the greatest IC₀ value is 98.5 µg·ml⁻¹) activities, especially the ethyl acetate fraction, the main components of which were flavonoids, including Cianidanol, L-Epicatechin, Quercetin, and Luteolin (which were flavonoids, including Cianidanol, L-Epicatechin, Quercetin, and Luteolin (which were flavonoids, including Cianidanol, L-Epicatechin, Quercetin, and Luteolin)). The basic principle of microcalorimetry is that the growth or/and metabolism of organism consistent with the change of heat is real-time online recorded and further evaluated. Under a set of growth conditions, unique power–time (P–t) curves of growth were established (Kong et al., 2009b; Br assisted by systematic solvent extraction, including petroleum ether, chloroform, ethyl acetate, n-butanol, and water, and after the combined extract filtered and analyze the interaction of flavonoids from leaf extracts, specifically to provide reference for the future research.

MATERIALS AND METHODS

Samples, Chemicals, and Reagents

The leaves of D. dao (Blanco) Merr. et Rolfe were purchased from the Chinese herbal medicine market in Guangdong province of China and were authenticated by Professor Xiaohe Xiao (Department of Integrative Medical Center, 302 Military Hospital of China, 100, the 4th Ring Road, Beijing 100039, China.). The leaves were dried in shade and stored at room temperature. Leaves of D. dao were crushed into powder, and decoked with eight times ultra-pure water by refluxing for 1.5 h with the procedure repeated again. After the combined extract filtered and evaporated, the water decoction was further extracted by ethyl acetate. Then ethyl acetate fraction was eluted by 70% alcohol within column chromatography of polyamide and three samples (S1–S3) were collected in succession by removing the solvent.

Escherichia coli (ATCC 25922) was provided by the American Type Culture Collection, Manassas, Virginia, USA. E. coli was inoculated in Luria-Bertani (LB) culture medium prepared by dissolving 10 g peptone, 5 g yeast extract, and 5 g NaCl in 1,000 ml ultra-pure water (pH 7.0–7.2) and sterilized by autoclaving at 0.1 MPa and 121°C for 30 min and then stored in a refrigerator at 4°C (Zhao et al., 2011). Before microcalorimetric measurements, E. coli was added to a 50 ml sterilized flask containing 30 ml LB culture medium and then incubated in incubator shaker for 2.5 h at 37°C (Liu T. et al., 2013) with the rotation speed at 75 rpm.

Polyamide for chromatography (60–100 mesh) was obtained from Mosu (the batch number: 20160223, Shanghai Mosu Science Equipment Co., Ltd., Shanghai, China). Ninety five percent ethanol was from Lircon (the batch number: 151111A, ShanDong LIRCON Medical Technology Co., Ltd., Shandong, China). Pure water was from Wahaha (the batch number: 20160185200TW, Hangzhou Wahaha Group Co., Ltd., Zhejiang, China). Chromatographic grade methanol was from Sigma Chemicals (the batch number: WXBC2019V, Sigma Scientific Co., L.L.C, USA).

The information of reference substance: Quercetin (prepared by laboratory, the degree of purity ≥98%), L-Epicatechin (the batch number: B-020-140923, the degree of purity ≥98%), Cianidanol (the batch number: E-011-140728, the degree of purity ≥98%), Luteolin (the batch number: M-007-150730, the degree of purity ≥98%). All reference substances but Quercetin were purchased from Chengdu Herbpurify bio-technology Co. Ltd., Chengdu, China.

Instruments

A microcalorimeter of type TAM 3114/3236 Bio-activity monitor (TA, Sweden) was used to determine the metabolic power–time (P–t) curves of E. coli growth. The TAM air microcalorimeter is an eight-channel heat conduction calorimeter for flow measurements under isothermal conditions. This microcalorimeter was thermostated at 37°C with a temperature error of ±0.02°C. The baseline stability was lower than 40 µW over 24 h (Kong et al., 2015). For more details of the performance and construction of the instrument, see the instruction and the report by Xie et al. (1988).
UPLC were performed using a Waters Acquity UPLC™ system (Waters, Milford, MA, USA), including binary solvent delivery pump, auto sampler manager, column compartment, and photo diode array detector, connected to Waters Empower 2 software.

**Microcalorimetric Measurements**

**Sample Preparation**

50.0 mg powder of flavonoids from *D. dao* was dissolved in 10 mL of LB culture medium. After filtration through millipore filter (pore size: 0.45 µm), sample solution was conducted at the concentration of 5.0 mg/mL for microcalorimetric measurements.

**Experimental Procedure**

This experiment was performed using the ampoule method and the microcalorimeter was thermostated at 37°C. One 20 mL sterilized glass ampoule was filled with 10 mL LB culture medium, while others were filled with 0.5 mL LB culture medium containing *E. coli* at a cell density of 1 × 10⁶ colony forming units (CFU)/ml (Kong et al., 2011) and different weight of flavonoids were added into each ampoule with a final volume of 10 mL. Eventually, each ampoule was sealed up and put into the eight-channel calorimeter block. When the temperature of ampoules reached 37°C, the P-t curves were recorded at an interval of 1 s until the recorder returned to the baseline. All data were collected continuously using the dedicated software package.

**UPLC Analysis**

**Preparation of Reference Standard Solution**

The standard solutions were prepared by accurately weighing. 20.0 mg of Cianidanol, L-Epicatechin, Quercetin, or Luteolin was added respectively into 10 mL of methanol to prepare the solutions with a concentration of 2 mg/ml, and then filtered through millipore filter with the pore size of 0.22 µm.

**UPLC Conditions**

The chromatographic separation was performed using a Waters Acquity BEH C₁₈ column (50 × 2.1 mm, 1.7 µm) with Column temperature of 30°C. The mobile phase composed of solvent A (Chromatographic grade methanol) and solvent B [water solution of 0.15% (V/V) formic acid] was performed with a linear gradient: 0–3.8 min (5%, A), 3.8–4 min (5–30%, A), 4–14.8 min (30%, A), 14.8–15 min (30–50%, A), 15–18.8 min (50%, A), 18.8–19 min (50–95%, A), 19–20 min (95%, A) at a rate of 0.2 mL/min. The detection wavelength was set at 280, 350, 360 nm and the sample injection volume was designated as 2.0 µl, based on the Chinese Pharmacopoeia 2015 edition.

**Statistical Analysis: Principal Component Analysis (PCA)**

PCA is a famous technique used for reducing the dimensionality of the data, which can visualize the information of the dataset in a few principal components retaining the maximum possible variation within that set (Yi L. Z. et al., 2007; Yi Z. B. et al., 2007; Chen J. et al., 2008; Chen Y. et al., 2008). Hence, the metabolic P-t curves were analyzed to obtain the key thermodynamic parameters which reduced the dimensionality by PCA statistics (Kong et al., 2009a; Liu S. X. et al., 2013). The SPSS statistics software (SPSS for Windows 17.0, SPSS) was used during this process.

**Quaternary Quadratic General Rotary Unitized Design**

There are many restrictions on the orthogonal test, for instance, the multiple test numbers should be considered and the contribution of factors to the index is always uncertain and uncontrollable. Although the uniform design test could save test duration, the accuracy of results cannot be guaranteed. The statistical model of quaternary quadratic general rotary unitized design could not only solve the problems above, but also minimize the shortcomings that the variance of the prediction value of quadratic regression heavily depends on the position of the experimental point in factor space and the interference in error. The quaternary quadratic general rotary unitized design is effective and useful to get a better combination because of its high precision and less test times.

**RESULTS**

**Microcalorimetry**

**Normal Power–Time Curves of *E. coli***

When the suspensions of *E. coli* are introduced into the ampoules with LB culture medium, the heat-output signals are recorded to form the normal metabolic P-t curves of *E. coli* (Figure 1). The heat of bacteria represents its metabolic strength. The curve in Figure 1 shows the typical growth characteristics of *E. coli* and could be divided into five phases: the lag phase (from point A to point B in Figure 1), the first exponential growth phase (from B to C), the transition phase (from C to D), the second exponential growth phase (from D to E), and the decline phase (from E to F).
**P-t Curve and Quantitative Thermo-Kinetic Parameters for E. coli Growth with Flavonoids**

When the suspensions of *E. coli* are introduced into the ampoules with different concentrations of flavonoids, there exists corresponding changes in the *P-t* curve. As could be seen in Figure 2, Table 1, the *P-t* curves of low concentration flavonoids on *E. coli* had slight changes compared with control group. The *P-t* curves and quantitative thermo-kinetic parameters had obvious changes in high concentration, such as the peak of the second exponential growth phase reducing, the time of peak postponing, the slope of curves diminishing and the area of curves lowering. Besides, curves and parameters changed with the concentration increasing. All these demonstrated that the growth of *E. coli* was influenced by flavonoids in different degrees.

**Dose—Response Relationship among Thermo-Kinetic Parameters**

The influence of flavonoids on the growth of *E. coli* could be quantitatively reflected from the changes of eight thermo-kinetic parameters in Figure 2. The *P-t* curve of *E. coli* growth could be fitted by two exponential functions. They both obey the following equation.

\[
P_t = P_0 \times e^{kt}
\]

\[
\ln P_t = \ln P_0 + kt
\]

In this equation, \(P_0\) and \(P_t\) are the power at time 0 and \(t\). Using this equation, the quantitative thermo-kinetic parameters such as the power of the first and second peak (\(P_1\) and \(P_2\)) represent the strength of *E. Coli* metabolism.

The growth rate constants (\(k_1\) and \(k_2\)) of the first and second exponential phase for *E. coli* growth at 37°C represent the rate of *E. coli* metabolism which is always a thermo-kinetic parameter. The heat outputs in stage 1 and stage 2 (\(Q_1\) and \(Q_2\)) are obtained from the *P-t* curve of *E. coli* growth affected by different concentrations of flavonoids. The growth rate constant \(k_1\) and \(k_2\) and the correlation coefficient \(R\) (not listed, \(R > 0.9\)) are also calculated.
Eight thermo-kinetic parameters (P₁, P₂, t₁, t₂, Q₁, Q₂, k₁, and k₂) which profile the metabolism of *E. coli* were obtained from the curves (Figure 2). Obviously, these parameters changed in line with the concentration of flavonoids. It is necessary to find out the main parameter(s) playing crucial role in evaluating the anti-bacterial effects, but the key information reflected from these changes is hard to figure out. Hopefully, PCA could solve these problems well.

The eight quantitative parameters (P₁, P₂, t₁, t₂, Q₁, Q₂, k₁, and k₂) were analyzed by PCA with the results indicating that the two principal components (Z₁ and Z₂) contained 90.284, 85.777, and 93.387% information of the original parameters in each sample. A component correlation matrix of the principal components (Table 9) was obtained by PCA analysis. The component correlation of each factor is equivalent to the coefficient of the factor (normalized) in the principal component equation. The equation of main component was formed according to this matrix. Each quantitative parameter is normalized in this equation.

\[
Z_{S1} = -0.913B_{t1} - 0.082B_{Q1} + 0.932B_{P1} + 0.880B_{k1} - 0.924B_{t2} - 0.364B_{Q2} + 0.961B_{P2} + 0.942B_{k2}
\]

\[
Z_{S2} = -0.337B_{t1} + 0.906B_{Q1} + 0.266B_{P1} + 0.404B_{k1} + 0.222B_{t2} + 0.806B_{Q2} - 0.110B_{P2} - 0.248B_{k2}
\]

\[
Z_{S3} = -0.804B_{t1} + 0.123B_{Q1} + 0.907B_{P1} + 0.956B_{k1} - 0.572B_{t2} + 0.220B_{Q2} + 0.930B_{P2} + 0.949B_{k2}
\]

\[
Z_{S2} = -0.540B_{t1} + 0.773B_{Q1} + 0.276B_{P1} + 0.000B_{k1} + 0.783B_{t2} + 0.776B_{Q2} - 0.283B_{P2} - 0.253B_{k2}
\]

\[
Z_{S3} = -0.931B_{t1} - 0.784B_{Q1} + 0.610B_{P1} + 0.851B_{k1} - 0.927B_{t2} - 0.584B_{Q2} + 0.971B_{P2} + 0.933B_{k2}
\]

\[
Z_{S3} = -0.235B_{t1} + 0.451B_{Q1} + 0.786B_{P1} + 0.504B_{k1} - 0.052B_{t2} + 0.794B_{Q2} - 0.047B_{P2} - 0.334B_{k2}
\]

\[x = \frac{x_i - \bar{x}}{s_i}\]

The equation indicated that P₃, k₁, and k₂ might be the main parameters playing more important role in evaluating the anti-bacterial effects of *D. dao* leaf extracts.
Inhibition Ratio $I$ of Samples S1–S3 on *E. coli*

According to the result of PCA, $P$, $k_1$, $k_2$, and $Q_2$ might be the main parameters. By further comparison of $k_1$ and $k_2$ from the equation, we found that $k_2$ contributed more than $k_1$ to PCA. So, the growth inhibition ratio $I$ (%) could be calculated on the basis of $k_2$ and can be defined as:

$$I = \left( \frac{k_0 - k_c}{k_0} \right) \times 100$$

Where $k_0$ refers to the growth rate constant of the control, $k_c$ refers to the growth rate constant in the second exponential growth phase at an inhibitor concentration of $c$.

The Probit regression with SPSS statistical analysis software was conducted to calculate the half-inhibitory concentration ($IC_{50}$) after antibacterial rate counted. $IC_{50}$, representing the sensitivity of bacteria to flavonoids was one of the most important indexes in evaluating the anti-bacterial activity of flavonoids. Table 2 showed the 95% confidence limit of $IC_{50}$ for S1–S3.

**UPLC Analysis**

The UPLC method in this research was newly established with main reference to Chinese Pharmacopoeia 2015 edition and the guidelines of the State Food and Drug Administration of China. We carried out the methodology validation of the UPLC method by Quercetin. External standard calibration lines were generated by injection of standard solutions at five concentrations in triplicate, plotting the peak area ($y$) obtained from the UPLC analysis against the concentration ($x$) of the standard material displaying a linear regression with correlation coefficient ($r$) calculated out.

The results of methodology validation showed that accuracy was obtained by 6 detection resulting in relative standard deviation (RSD) value $<$0.99%. Repeatability was determined by analyzing six independent prepared solutions which resulted in RSD value $<$1.32%, suggesting that this method had high repeatability. Stability was measured by repeating the analysis of the same sample solution at 0, 3, 6, 12, 24, and 48 h at room temperature with RSD value $<$0.96%.

As shown in Table 3, the calibration curves displayed strong linearity over the concentration range of 1–100 $\mu$g.mL$^{-1}$ ($r = 0.9987$) for Luteolin, 20–100 $\mu$g.mL$^{-1}$ ($r = 0.9999$) for L-Epicatechin, 100–2000 $\mu$g.mL$^{-1}$ ($r = 0.9996$) for Cianidanol, 20–800 $\mu$g.mL$^{-1}$ ($r = 0.9999$) for Quercetin.

The content of Luteolin, L-Epicatechin, Cianidanol, and Quercetin in S1–S3 were determined by external standard method. The linear equations of each standard substance were established through the peak area of five concentration gradient (Table 4).

**Results of Quadratic General Rotary Unitized Design Analysis**

### Determination of Code Level

Based on the $IC_{50}$ of sample, S2 exhibited favorable anti-bacterial effect. The content of Luteolin, L-Epicatechin, Cianidanol,
and Quercetin was defined as 0-code, and Luteolin ($X_1$), L-Epicatechin ($X_2$), Cianidanol ($X_3$), and Quercetin ($X_4$) were chosen as the four factor. The general quaternary quadratic designs of rotary combination statistical model (1/2 implement) was introduced to investigate the combined effect of four flavonoids with the use of microcalorimeter figuring out the antibacterial rate ($Y$-value) on different code level. The content of four flavonoids on different code level was illustrated on Table 5 with Table 6 and Figure 4 displaying experimental design scheme and the analysis result in Table 7.

### Results of Statistical Analysis

The regression equations of antibacterial rate were obtained from statistical model of quaternary quadratic general rotary unitized design. That was

\[ Y = 37.99545 + 5.86714X_1 + 2.12554X_2 + 4.02043X_3 + 0.21225X_4 - 0.61375X_1X_2 - 0.17375X_1X_3 - 1.08375X_1X_4 + 2.32473X_2^2 + 0.10265X_2^2 + 0.10265X_3^2 - 0.21225X_4^2 \]

and the result of variance analysis with this model showed that $F_1 = 2.85323$ (testing of lack of fit for regression equation of antibacterial rate) was < $F_{0.05}(5, 3) = 9.01$, which suggested that the X factor had little influence on experimental result. Moreover, the test result for significance was that $F_2 = 6.98973 > F_{0.01}(11, 8) = 5.74$, demonstrating that the regression equation had significant difference. The mathematic model with good fitting degree could reflect the experimental
In single-factor analysis procedure, the $P$-values of $X_1$ (Luteolin) and $X_3$ (Cianidanol) were <0.01. Surprisingly, the $P$-values of (L-Epicatechin) and $X_4$ (Quercetin) were >0.05. Moreover, the simplified four quaternary regression equation was calculated out without no-significance items of $\alpha = 0.10$. That was

$$Y = 37.99545 + 5.86714X_1 + 4.02043X_3 + 2.59711X_4 + 2.32473X_1^2$$

The four flavonoids had independent anti-bacterial effect with Luteolin and Cianidanol showing the main and better anti-bacterial effect from the analysis of variance table. In addition, the $P$-value of $X_1$ (Luteolin) was less than $X_3$ (Cianidanol) and the concentration range of $X_1$ was 296–1061 ng·ml$^{-1}$ far less than that of $X_3$ (4482–17,506 ng·ml$^{-1}$).

The diagram of single factor effect for S1–S3 was rationally calculated from the rotational combination design model. The single factor under different code level was predicted to make out the antibacterial rate ($Y$-value) with others factors as 0-code in this method.

The effect of different factors on bacteria could be observed via the range of each factor (the difference of $Y$-value for the highest code between the lowest one) and change tend single factor in the analysis of single factor effect. The result showed that $X_1$ (Luteolin) mainly contributed to the antibacterial rate ($Y$-value) with $X_3$ (Cianidanol) and $X_4$ (Quercetin) contributing only marginally. Unexpectedly, $X_2$ (L-Epicatechin) had little influence on antibacterial rate. Based on the range result, the sequence of antimicrobial activity was $X_1$ (Luteolin) > $X_3$ (Cianidanol) > $X_4$ (Quercetin) > $X_2$ (L-Epicatechin) consistent with the $P$-value of analysis of variance model, further validating the different antibacterial effect among the four individual compounds (Figure 5).

Analysis of the effect of two-factor interaction that defined other factor as 0-code for the four flavonoids was obtained from the statistical model of quaternary quadratic general rotary design.

### TABLE 6 | Quaternary quadratic general rotary unitized design.

| No. | $X_1$ | $X_2$ | $X_3$ | $X_4$ | $Y$ (%) |
|-----|-------|-------|-------|-------|---------|
| 1   | 1     | 1     | 1     | 1     | 49.69   |
| 2   | 1     | 1     | -1    | -1    | 41.51   |
| 3   | 1     | -1    | 1     | -1    | 47.80   |
| 4   | 1     | -1    | -1    | 1     | 44.03   |
| 5   | -1    | 1     | 1     | -1    | 35.85   |
| 6   | -1    | 1     | -1    | 1     | 35.72   |
| 7   | -1    | -1    | 1     | 1     | 40.25   |
| 8   | -1    | -1    | -1    | 1     | 27.04   |
| 9   | -1.6818 | 0     | 0     | 0     | 34.59   |
| 10  | 1.6818 | 0     | 0     | 0     | 55.97   |
| 11  | 0     | -1.6818 | 0   | 0     | 31.45   |
| 12  | 0     | 1.6818 | 0     | 0     | 48.54   |
| 13  | 0     | 0     | -1.6818 | 0   | 30.19   |
| 14  | 0     | 0     | 1.6818 | 0     | 47.80   |
| 15  | 0     | 0     | 0     | -1.6818 | 33.96  |
| 16  | 0     | 0     | 0     | 1.6818 | 44.65   |
| 17  | 0     | 0     | 0     | 0     | 37.11   |
| 18  | 0     | 0     | 0     | 0     | 40.88   |
| 19  | 0     | 0     | 0     | 0     | 35.22   |
| 20  | 0     | 0     | 0     | 0     | 37.11   |

$X_1$, Luteolin; $X_2$, L-Epicatechin; $X_3$, Cianidanol; $X_4$, Quercetin; $Y$, antibacterial rate.

### TABLE 7 | The analysis of variance table for quadratic general rotary unitized design.

| Variable | $$SS$$ | $f$ | $$MS$$ | $r$ | $F$-value | $p$-value |
|----------|--------|-----|--------|-----|-----------|-----------|
| $X_1$    | 293.8219 | 1   | 293.8219 | 0.9103 | 24.1644 | 0.0012 |
| $X_2$    | 38.5630  | 1   | 38.5630  | 0.6230 | 3.1715  | 0.1128 |
| $X_3$    | 137.9669 | 1   | 137.9669 | 11.3468 | 10.098  | 0.0023 |
| $X_4$    | 57.5721  | 1   | 57.5721  | 0.6974 | 4.7348  | 0.0612 |
| $X_1^2$  | 48.0861  | 1   | 48.0861  | 0.6646 | 3.9547  | 0.0519 |
| $X_2^2$  | 0.9383   | 1   | 0.9383   | 0.0392 | 0.0777  | 0.9322 |
| $X_3^2$  | 0.9383   | 1   | 0.9383   | 0.0392 | 0.0777  | 0.9322 |
| $X_4^2$  | 0.4008   | 1   | 0.4008   | 0.0809 | 0.0330  | 0.8640 |
| $X_1X_2$ | 1.8834   | 1   | 1.8834   | -0.1733 | 0.1549  | 0.7042 |
| $X_1X_3$ | 0.1509   | 1   | 0.1509   | -0.0498 | 0.0124  | 0.9140 |
| $X_1X_4$ | 5.8726   | 1   | 5.8726   | -0.2968 | 0.4830  | 0.5068 |

Linear correlation between $X_2$ and $X_3$
Linear correlation between $X_2$ and $X_3$
Linear correlation between $X_2$ and $X_3$
Regression $$R^2 = 6.95, 973$$ 0.0021
Residual term 97.2741 8 12.1593
Lack of fit 80.3727 5 16.0745 0.0907
Error 16.9014 3 5.6338

Total 1032.1639 19

$X_1$, Luteolin; $X_2$, L-Epicatechin; $X_3$, Cianidanol; $X_4$, Quercetin.
unitized design. The changes of different factors on Y-value as well as the relationship between two factors could be found out via Y-value of two factors under different levels of code by mathematical model. The results showed that there were two couple of factors obvious and similar, such as the pair of Quercetin and Luteolin between Cianidanol and Luteolin (Figure 6).

The diagram of two-factor interaction effect for $X_1$ (Luteolin) and $X_3$ (Cianidanol) was rationally calculated from the rotational combination design model. When $X_1$ and $X_3$ factor were in high code level, the synergistic activity was obvious between $X_1$ and $X_3$ with the antibacterial rate (Y-value) in the top level, which suggested that there was a positive effect between high concentration of $X_1$ (Luteolin) and $X_3$ (Cianidanol) and antibacterial rate (Y-value). With the decline of code of Luteolin and Cianidanol, Y-value (antibacterial rate) decreased rapidly with a smaller degree. When the $X_1$ code number was $-1$ to $-1.6820$, the Y-value decreased at first and increased later. When the $X_1$ code number was $-1.3410$ and the $X_3$ code number was $-1.6818$, the Y-value was 27.5466, which is the lowest point in this analysis model. Therefore, we predicted that there existed positive and synergistic effect between Luteolin and Cianidanol. When Luteolin and Cianidanol was in a higher concentration, there existed obvious synergistic effect between them. But when Luteolin and Cianidanol in low concentration, no marked synergistic effect was observed, even presenting slight antagonistic effects similar with the relationship of $X_1$ (Luteolin) and $X_4$ (Quercetin).

Obviously, the analysis showed that there existed both single factor effect and interaction among two factors. So it was difficult to find out the best antibacterial concentration and combinations from the results of the single factor effect and interaction analysis, and quaternary quadratic regression mathematical model did not have the maximum value of bacterial inhibition rate. The analysis method of frequency domain was performed to analyze and confirm this regression model and the combined optimum concentration (Table 8).

Table 8 showed the frequency distribution of 325 cases with antibacterial rate exceeding 39.87%. when antibacterial rate was over 39.87%, most cases could be obtained, verifying the mathematical regression model. Code value of factors was that $X_1$: 0.633 – 0.864, $X_2$: $-0.135$ – $0.135$, $X_3$: 0.374 – 0.627, $X_4$: 0.200 – 0.460 under the condition that the antibacterial rate $>39.87$ within 95% confidence limits. In other words, Luteolin was 822.32 – 874.99 ng·ml$^{-1}$, L-Epicatechin was 215.74 – 236.26 ng·ml$^{-1}$, Cianidanol was 12,597.62 – 13,547.13 ng·ml$^{-1}$, and Quercetin was 5674.6 – 6138.18 ng·ml$^{-1}$.

To minimize the error for this experiment, the combined range was defined as Luteolin 850 ng·ml$^{-1}$ (Code = 0.754), L-Epicatechin 220 ng·ml$^{-1}$ (Code = $-0.079$), Cianidanol 13,000 ng·ml$^{-1}$ (Code = 0.481), and Quercetin 6,000 ng·ml$^{-1}$ (Code = $-0.079$).

![FIGURE 5 | The analysis of single factor effect diagram.](image)

![FIGURE 6 | The analysis of two factors interaction effect diagram. (A) Luteolin and Cianidanol. (B) Luteolin and Quercetin.](image)
Inhibition rate of L-Epicatechin was still finally obtained the combination that Luteolin was 1,061.00 – further verified the rationality of regression model. The exact IC value for the combination of Luteolin and Cianidanol was 15,990.33–16,973.62 ng·ml\(^{-1}\) with Y-value (inhibition rate) >60.00 within 95% confidence limits.

**Anti-Microbial Activity of Single Flavonoid and Combination of Two Flavonoids**

**Anti-Microbial Activity of Single Flavonoid**

To verify the result that antibacterial activity of the four major components from the leaves of *D. dao* is Luteolin > Cianidanol > Quercetin > L-Epicatechin based on the quadratic general rotary unitized design model, we determined the anti-bacterial activity of the four flavonoid independently using the microcalorimetry method (Figure 7) with the same parameter as above. The Probit regression with SPSS statistical analysis software was conducted to calculate the half-inhibitory concentration (IC\(_{50}\)) after antibacterial rate counted. IC\(_{50}\) representing the sensitivity of bacteria to flavonoids was one of the most important indexes in evaluating the anti-bacterial activity of flavonoids. Table 9 showed the 95% confidence limit of IC\(_{50}\) for each flavonoid. The result showed that Luteolin (15.642 \(\mu\)g·ml\(^{-1}\)), Cianidanol (17.584 \(\mu\)g·ml\(^{-1}\)), and Quercetin (19.002 \(\mu\)g·ml\(^{-1}\)) had lower IC\(_{50}\) values, except for L-Epicatechin. When the concentration of L-Epicatechin was increased to 32 \(\mu\)g/ml, the inhibition rate of L-Epicatechin was still <50%. In the final calculation, only Probit regression can be used to predict the IC\(_{50}\) of L-Epicatechin (44.247 \(\mu\)g·ml\(^{-1}\)). The exact IC\(_{50}\) of L-Epicatechin remains to be further analyzed yet, but that the antibacterial activity of L-Epicatechin is much weaker than that of Luteolin, and Cianidanol and Quercetin can be confirmed. By comparing the IC\(_{50}\) values, we can confirm that antibacterial activity of the four major components from the leaves of *D. dao* (Luteolin > Cianidanol > Quercetin > L-Epicatechin) based on the quadratic general rotary unitized design model is correct.

**Anti-Microbial Activity of the Combination of Two Flavonoids**

In the quadratic general rotary unitized design model, we analyzed two factors interaction effect: Luteolin and Cianidanol, Quercetin, and Luteolin had synergistic effects on antibacterial activity at high doses. In order to verify this result, we independently measured the antimicrobial activity of the two combinations. We referred to the proportion of flavonoids the anti-bacterial rate > 39.87 within 95% confidence limits from the quadratic general rotary unitized design model to determine the ratio of Luteolin and Cianidanol was 1:15, and the ratio of Luteolin and Quercetin was 1:7. microcalorimetry determination of half inhibition rate was also conducted to determine the anti-bacterial activity of flavonoids with the same anti-microbial activity indicators as above (Figure 8). The Probit regression with SPSS statistical analysis software was conducted to calculate the half-inhibitory concentration (IC\(_{50}\)) after antibacterial rate counted. IC\(_{50}\) representing the sensitivity of bacteria to flavonoids was one of the most important indexes in evaluating the anti-bacterial activity of flavonoids. Table 9 showed the 95% confidence limit of IC\(_{50}\) for Luteolin and Cianidanol, Quercetin and Luteolin. The result showed that the IC\(_{50}\) values of the combination of Luteolin and Cianidanol was 11.566 \(\mu\)g/ml (722.9 ng·ml\(^{-1}\) for Luteolin, 10,843.1 ng·ml\(^{-1}\) for Cianidanol), and that of the combination of Luteolin and Quercetin was 14.721 \(\mu\)g·ml\(^{-1}\) (1840.1 ng·ml\(^{-1}\) for Luteolin, 12,880.9 ng·ml\(^{-1}\) for Cianidanol) within 95% confidence limits. The ratio of their anti-microbial activity P (in IC\(_{50}\)) through the concentration ratio weighted average method based on the IC\(_{50}\) values of the four flavonoids alone and two combinations of two flavonoids was calculated. The results displayed that the IC\(_{50}\) value for the combination of Luteolin and Cianidanol was increased to 1.425-fold than the theoretical antibacterial activity of the same concentration of the compound alone and the IC\(_{50}\) value for the combination of Luteolin and Quercetin was 1.129-fold as much as the theoretical antibacterial activity of

### Table 8 | Distribution frequency and interval of factor.

| Code | \(X_1\) | \(X_2\) | \(X_3\) | \(X_4\) |
|------|--------|--------|--------|--------|
| \(-1.6818\) | 25 | 0.0769 | 65 | 0.2000 | 35 | 0.1077 | 45 | 0.1385 |
| \(-1.0000\) | 25 | 0.0769 | 65 | 0.2000 | 40 | 0.1231 | 45 | 0.1385 |
| 0.0000 | 50 | 0.1538 | 65 | 0.2000 | 60 | 0.1846 | 65 | 0.2000 |
| 1.0000 | 100 | 0.3077 | 65 | 0.2000 | 85 | 0.2615 | 85 | 0.2615 |
| 1.6818 | 125 | 0.3846 | 65 | 0.2000 | 105 | 0.3231 | 85 | 0.2615 |

Weight mean: 0.7480, 0.0000, 0.5010, 0.3300
Standard Error: 0.0590, 0.0690, 0.0650, 0.0660
95% confidence limits: 0.633–0.864, −0.135–0.135, 0.374–0.627, 0.200–0.480

\(c/\text{ng·ml}^{-1}\): 822.32–874.99, 215.74–236.26, 12,597.62–13,547.13, 5674.60–6136.18

\(X_1\), Luteolin; \(X_2\), L-Epicatechin; \(X_3\), Cianidanol; \(X_4\), Quercetin.
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**FIGURE 7 |** The $P$-$t$ curves of *E. coli* intervened by single flavonoid. (A) Luteolin, (B) L-Epicatechin, (C) Cianidanol, (D) Quercetin.

the same concentration of the compound alone. The synergistic effect of Luteolin and Cianidanol ($P_{L+C} = 1.425$), Quercetin and Luteolin ($P_{L+Q} = 1.129$) on anti-microbial activity could be confirmed, since the $P$-values of which were $> 1$.

$$
P_{L+C} = \frac{IC_{50(L)}^{1} + IC_{50(C)}^{15}}{IC_{50(L+C)}}$$

$$
P_{L+Q} = \frac{IC_{50(L)}^{1} + IC_{50(Q)}^{7}}{IC_{50(L+Q)}}$$

Cianidanol ($C$), Luteolin ($L$), Quercetin ($Q$).

**DISCUSSION**

*Escherichia coli*, a Gram negative bacteria, is common and important to screen flavonoids with anti-bacterial activity as a model organism (Sondi and Salopek-Sondi, 2004). In the study, we investigated the anti-*E. coli* effect of three samples from the leaves of *D dao* (Blanco) Merr. et Rolfe by microcalorimetry and UPLC, and demonstrated that the anti-bacterial activity of the three samples (S1–S3) was various after *E. coli* was treated with three samples independently via microcalorimetry. According to the $P$-$t$ curves of *E. coli* in **Figure 2** and thermal-kinetic parameters in Table 1, it could be seen that the anti-bacterial

| Parameters | Component | $Z_{S1-1}$ | $Z_{S1-2}$ | $Z_{S2-1}$ | $Z_{S2-2}$ | $Z_{S3-1}$ | $Z_{S3-2}$ |
|------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| $t_1$      | -0.913    | -0.337    | -0.804    | -0.540    | -0.931    | -0.235    |
| $Q_1$      | -0.082    | 0.906     | 0.123     | 0.773     | -0.784    | 0.451     |
| $P_1$      | 0.932     | 0.266     | 0.907     | 0.276     | 0.610     | 0.786     |
| $k_1$      | 0.880     | 0.404     | 0.966     | 0.000     | 0.851     | 0.504     |
| $t_2$      | -0.924    | 0.222     | -0.572    | 0.783     | -0.927    | -0.052    |
| $Q_2$      | -0.364    | 0.806     | 0.220     | 0.776     | -0.584    | 0.794     |
| $P_2$      | 0.961     | -0.110    | 0.930     | -0.283    | 0.971     | -0.047    |
| $k_2$      | 0.942     | -0.248    | 0.949     | -0.253    | 0.933     | -0.334    |
activities of different simples varied in a dose-dependent manner with the IC₅₀ value sequence S1 > S3 > S2.

The previous study has demonstrated that flavonoids are abundant in the leaves of *D. dao*, and their anti-bacterial activities have been reported by cumulative evidences. Ethanol extract from *Mentha longifolia* containing the components of Luteolin, Apigenin, Quercetin, and Kaempferol was the most active fraction against the tested bacteria (Akroum et al., 2009). The combination of Luteolin and Amoxicillin displayed synergistic activity against *E. coli* (Eumkeb et al., 2012). Furthermore, the novel Luteolin derivatives showed favorable antibacterial activity *in vitro* against *B. subtilis, S. aureus, P. fluorescens*, and *E. coli* (Lv et al., 2009).

In terms of IC₅₀ values, the IC₅₀ value of S2 was lower than those of S1 and S3, because the content and ratio of Luteolin, L-Epicatechin, Cianidanol, and Quercetin in S1-S3 were quite different. To be specific, the content of Luteolin in S2 was 22.5- and 10.8-fold than that of S1 and S3, respectively. The content of Quercetin in S2 was 20.1- and 1.8-fold than that of S1 and S3, respectively. However, the content of flavonoids in extracts did not show a linear relationship with the IC₅₀ value, indicating that there existed unknown compounds with significant antibacterial activity from the leaves of *D. dao*.

The statistical model of quadratic general rotary unitized design was conducted to analyze the compounds interaction based on the content of four flavonoids in S2. We observed that the *p*-value of Luteolin (*p* = 0.0012) and Cianidanol (*p* = 0.0098) were <0.01, which indicated that Luteolin and Cianidanol were very important factors (Table 7). Further research is needed to explore their antibacterial mechanism. Of note, Quercetin (*p* = 0.0612) and L-Epicatechin (*p* = 0.1128) were not the major factors of the antibacterial activity in the extract of the leaves of *D. dao*. The ultimate result showed that the antibacterial activity of four flavonoids was Luteolin > Cianidanol > Quercetin > L-Epicatechin from the result of single factor analysis. And the IC₅₀ values of each flavonoid evaluated by microcalorimetry confirmed the results above (the IC₅₀ value of Luteolin was 15.642 µg·ml⁻¹; the IC₅₀ value of Cianidanol was 17.584 µg·ml⁻¹; the IC₅₀ value of Quercetin was 19.002 µg·ml⁻¹; the IC₅₀ value of L-Epicatechin was 44.247 µg·ml⁻¹; Table 10).

The correlation analysis of two factors could be obtained from this model, indicating that the synergistic effects and concentrations of flavonoids were in a dose-dependent manner in a certain concentration range. In order to verify this deduction, we determined the IC₅₀ value of the combination of Luteolin and Cianidanol (1:15), Luteolin and Quercetin (1:7) by microcalorimetry. The results showed that the combination of Luteolin and Cianidanol (*P* = 1.425), Luteolin and Quercetin (*P* = 1.129) had synergetic effects, and the former couple showed stronger synergistic effect. Flavonoids had many different types of chemical structure. Luteolin and Quercetin had similar structural framework, but Quercetin had one more hydroxyl than Luteolin, thus we speculated that the antibacterial action sites of these two compounds may well be semblable. The chemical structures of Cianidanol and Luteolin are various, Luteolin has a unique carbon-carbon double bond and carbonyl compared to Cianidanol in a conjugate relationship. Compared with Luteolin, Cianidanol with a unique hydroxyl has a distinct synergistic effect on its anti-bacterial activity. The results could lay the foundation for exploring the synergistic mechanism of Luteolin and Cianidanol.

There may well exist other uncontrollable factors in UPLC with much larger peak area of substances not included as marker,
thus their antimicrobial activities were not displayed under the application of quaternary quadratic general rotary unitized design.

The results of two-factor interaction effect analysis showed that Luteolin and Cianidanol had synergistic effect at high concentration. Interestingly, they were antagonistic in low concentration. Herein, a conclusion that the four flavonoids have anti-bacterial activity could be drawn. Together, this work could contribute to the quality evaluation and exploring the interaction among Luteolin, Quercetin, Cianidanol, and L-Epicatechin.

**AUTHOR CONTRIBUTIONS**

YL did the writing of paper and polyamide column chromatography, Ultra High Performance Liquid Chromatography (UPLC) as well as micro-calorimetry and quaternary quadratic general rotary unitized design; YL, HX, YZ, and XX did the quaternary quadratic general rotary unitized design. MW, JW, SL, KL, LW, RW, and PZ did the Ultra High Performance Liquid Chromatography (UPLC) as well as micro-calorimetry. HX, YZ, and XX supervised the project. All the authors read and approved the final manuscript.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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