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## Development of personalized health monitoring using ultra-weak photon emission based on systems medicine concepts

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## Chapter 5

### **Effects of growth altitude on chemical constituents and delayed luminescence properties in medicinal rhubarb**

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## Abstract

To improve the quality control of herbal drugs, there has been a major shift from evaluating individual chemicals to evaluating multiple-constituent chemicals, given the multi-pharmacology nature of herbal drugs. Therefore, rapid, systematic assays are needed in order to assess the quality of medicinal herbs using a comprehensive, integrated approach. Light-induced delayed luminescence (DL) is used to measure decaying long-term ultra-weak photon emissions following excitation with light. DL is considered to be a sensitive indicator for characterizing the properties of biological systems and herbal medicines with various therapeutic properties. The aim of this study was to examine the feasibility of using DL as a novel quality-assessment tool using rhubarb material as a model system, and to establish the correlation between DL parameters and the chemical constituents of rhubarb. Raw roots and rhizomes were collected from rhubarb (*Rheum palmatum* L.) at various elevations in western China. HPLC analysis was used to identify fourteen bioactive constituents. Five DL parameters were calculated from the DL decay curves of the rhubarb samples. Statistical tools, including principal component analysis, were used to classify the rhubarb samples using data obtained using two different assays. Finally, Spearman's correlation coefficient was calculated to quantify the correlation between the bioactive compounds and corresponding DL parameters. We found that both the chemical analysis and DL measurements reflect variations in the quality of rhubarb due to environment factor. The DL parameters were correlated significantly with the bioactive chemical constituents. Our results indicate that DL is a promising tool for evaluating multiple constituents and for assessing the therapeutic properties of herbal medicines. Thus, DL may be used as part of a comprehensive system for assessing the quality and/or therapeutic properties of herbal medicines.

**Key words:** rhubarb, delayed luminescence, quality control, active constituents, environmental factor.

## 1. Introduction

Environmental factors related to growth conditions are closely associated with the quality and therefore therapeutic properties of medicinal plants.<sup>1,2</sup> Since ancient times in China, medicinal plants have been produced and/or collected in specific geographic regions with unique ecological conditions; these so-called “indigenous medicinal materials” are used to represent the optimal quality and therapeutic properties of Chinese herbal drugs based on clinical practices and experiences.<sup>1</sup> It is generally accepted that secondary metabolites are important pharmacologically active constituents in medicinal plants.<sup>3</sup> Plants produce secondary metabolites as a means to adapt to their growth conditions, for example to provide protection from environmental stressors.<sup>2</sup> In addition, changes in environmental factors can be reflected in the accumulation of secondary metabolites.<sup>4</sup> Therefore, focus should be shifted towards controlling environmental conditions in order to manage quality and standardize herbal drugs.<sup>5</sup>

Rhubarb (scientific name: *Rhei Rhizoma*) is an herbal drug that has been widely used for thousands of years throughout China.<sup>6,7</sup> The dried roots and rhizomes of *Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., and *Rheum officinale* Bail. are officially included in the Chinese Pharmacopoeia<sup>8</sup> and European community monograph.<sup>9</sup> According to the principles of traditional Chinese medicine, rhubarb is a typical herbal drug with many therapeutic properties, including catharsis, heat-clearing effects, detoxification, and removal of blood stasis.<sup>8</sup> In recent decades, chemical and pharmacological studies have shown that the pharmacological activities of secondary metabolites in rhubarb—including anthraquinone derivatives and polyphenol constituents—correspond with the traditional therapeutic functions associated with rhubarb (Fig 1).<sup>10–14</sup> For example, rhubarb’s detoxification property is reflected largely by its antibacterial activity, which is related primarily to free anthraquinones such as rhein, emodin, and aloe-emodin.<sup>10</sup> Rhubarb’s cathartic property is due to the presence of both anthraquinone glycosides and

sennosides.<sup>10,11,13</sup> Rhubarb's blood stasis-relieving properties have been attributed to (+)-catechin.<sup>14</sup> In addition, gallic acid has been proposed to have antiplatelet activity,<sup>15</sup> which may also explain rhubarb's blood stasis-relieving properties. Moreover, additional pharmacological effects have been attributed to compounds isolated from rhubarb, including anti-cancer activity,<sup>16</sup> anti-inflammatory activity,<sup>17</sup> antioxidant activity,<sup>18</sup> liver-protective properties,<sup>19</sup> and improved renal dysfunction.<sup>20</sup>

Given these important therapeutic properties, rhubarb has become one of most commonly used herbal medicines, and the demand for rhubarb has grown both in China and in global markets.<sup>11</sup> However, rhubarb is produced primarily in a limited region in western China at an altitude of 1000-5000 m,<sup>21</sup> and overharvesting has caused a significant decline in rhubarb crops; in addition, overharvesting also causes considerable damage to the growing environment, causing variation in the quality of rhubarb grown.<sup>11</sup> Therefore, developing an effective tool for measuring quality control in rhubarb is very important. Given the high number of bioactive constituents in rhubarb and their high number of therapeutic properties, comprehensive, systematic methods for analyzing rhubarb quality are needed.<sup>11</sup>

Delayed luminescence (DL) is long-term decaying, weak photon emission from various materials following exposure to light with a wavelength of 400–800 nm.<sup>22–25</sup> DL has been used as a tool for directly and rapidly assessing biological systems and has been found to provide a sensitive indicator of food quality.<sup>24–26</sup> Recent studies examined the DL signatures of dry powders prepared from Chinese herbal medicines.<sup>27,28</sup> These studies demonstrate that distinct DL properties can be measured between the same herbal medicines prepared under different conditions, including the age of the herb, environmental factors, and the processing method.<sup>27</sup> These differences in DL properties reflect variations in both the bioactive constituents contained in the herb and the therapeutic properties of herbal drugs.<sup>29–32</sup>

Importantly, DL can be used to predict the herb's energetically therapeutic properties

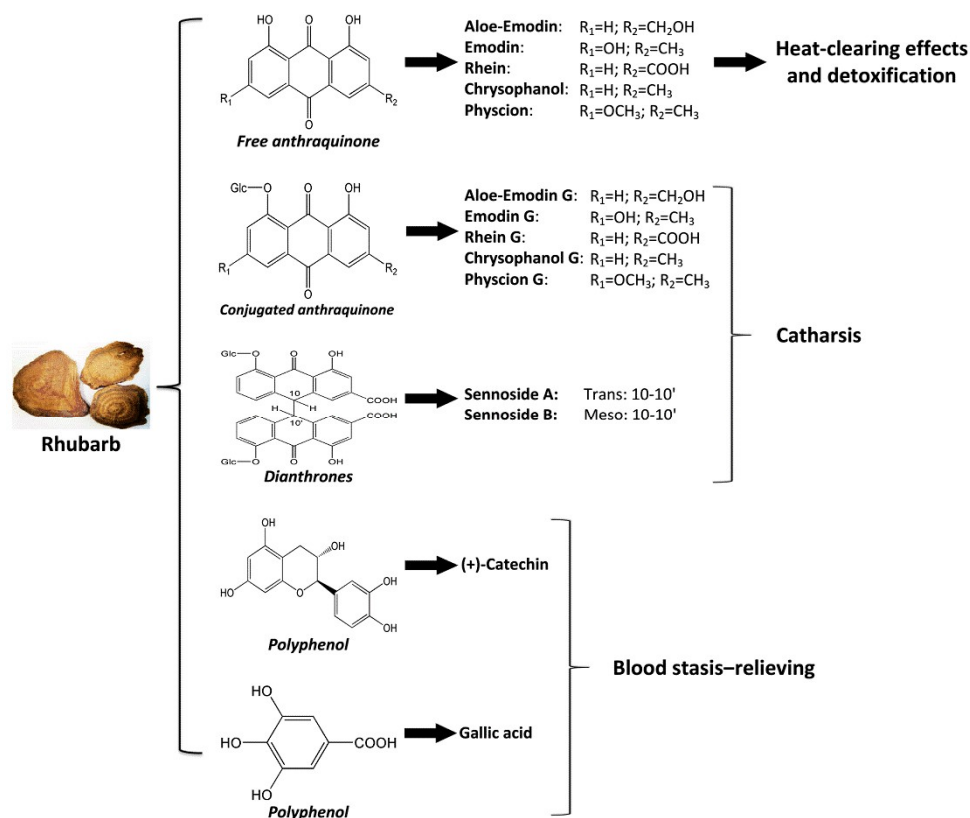


Fig. 1 Schematic diagram of the chemical constituents in rhubarb and their role in traditional Chinese medicine-based concepts. Fourteen chemical constituents in rhubarb correspond to various therapeutic properties in the principle of traditional Chinese medicine. The terms in italics under the chemical structure indicate the structural characteristics of the chemical constituents. “Glc” in the chemical structure indicates a glycoside. “G” in the names of chemical constituents indicate glycosides. Conjugated anthraquinone represents anthraquinone glycosides with O-glycosides, where the aglycone moiety is an 8-dihydroxyanthraquinone derivative. Additional structures of conjugated anthraquinones not shown here are published elsewhere.<sup>11</sup>

(i.e., “Cold” or “Heat”) based on the principles of traditional Chinese medicine.<sup>28</sup> Thus, DL may contribute to our understanding of why herbs with different chemical constituents can have the same therapeutic properties and—conversely—why herbs with different therapeutic properties can contain similar chemical constituents.<sup>28</sup> Importantly, DL may provide an integrated, comprehensive picture of the bioactive constituents, thereby reflecting the herbal medicine’s therapeutic properties.

Here, we performed both chemical analyses and DL measurements in order to evaluate the effect of the growth environment on the constituents contained in rhubarb (*Rheum palmatum* L.) The aim of this study was to determine whether DL parameters can be used to create a signature of rhubarb quality, and to determine whether these parameters correspond to key chemical constituents. Our results show a clear correlation between DL and the chemical constituents, reflecting variations in the quality of rhubarb, thus providing new insights into measuring quality control in herbal medicines.

## 2. Materials and Methods

### 2.1 Rhubarb materials and chemicals

Rhubarb (*Rheum palmatum* L.) samples were collected from twelve locations in western China (Table 1) during the summer months (from July through August) to minimize the effect of seasonal changes on the concentrations of chemical constituents.<sup>33</sup> All rhubarb samples were verified by Prof. Chunsheng Liu (Beijing University of Chinese Medicine) and deposited at the Beijing Institute of Chinese Medicine.

Seven reference compounds (aloe-emodin, rhein, emodin, chrysophanol, physcion, gallic acid, and (+)-catechin) were supplied by the National Institutes for Food and Drug Control (Beijing, China). Two additional reference compounds (sennoside A and sennoside B) were purchased from Nature Standard Biotechnology Co., Ltd. (Shanghai, China). The purity of all reference compounds was >98%.

### 2.2 High-performance liquid chromatography (HPLC)

#### 2.2.1 Specimen preparation for determination of bioactive constituents

Extraction of total anthraquinones: Total anthraquinones contain five constituents (aloe-emodin, rhein, emodin, chrysophanol, and physcion). Powdered rhubarb samples (250 mg each) were extracted with 25 ml of 15% sulfuric acid (v/v) and

Table 1. Summary of the 12 sampling sites in China, including the number of batches collected at each site

| Sample ID | Location          | Batch | Longitude      | Latitude      | Altitude (m) |
|-----------|-------------------|-------|----------------|---------------|--------------|
| SXCJW     | Caojiawan, Shanxi | 3     | E106°38'45.02" | N35°51'14.43" | 1441         |
| GSLX      | Lixian, Gansu     | 3     | E104°52'52.12" | N33°57'16.91" | 2136         |
| GSTC      | Tanchang, Gansu   | 5     | E104°43'50.33" | N33°57'17.50" | 2331         |
| NXLD      | Longde, Ningxia   | 3     | E106°07'01.69" | N35°33'28.71" | 2358         |
| GSMX      | Minxian, Gansu    | 5     | E104°06'54.69" | N34°23'01.86" | 2530         |
| SCLH      | Luhuo, Sichuan    | 5     | E100°39'56.91" | N31°23'54.51" | 3166         |
| GSDB      | Deibu, Gansu      | 3     | E103°39'40.62" | N34°05'41.26" | 3227         |
| SCLD      | Luding, Sichuan   | 5     | E102°02'14.81" | N29°51'50.07" | 3590         |
| SCDB      | Danba, Sichuan    | 4     | E101°32'55.86" | N30°51'35.83" | 3636         |
| SCKD      | Kangding, Sichuan | 3     | E101°45'40.80" | N30°13'36.41" | 4077         |
| SCDF      | Daofu, Sichuan    | 4     | E101°22'01.14" | N30°41'16.71" | 4094         |
| SCLT      | Litang, Sichuan   | 4     | E100°15'09.17" | N30°13'35.91" | 4265         |

methanol at a ratio of 2:3 (v:v) using a model KQ5200D ultrasonication device (200 W, 40 HZ; Kunshan Ultrasonic Instruments Co., Ltd., Kunshan City, China) for one hour. The resulting solution was extracted four times with 10 ml chloroform, and the combined extract was then dried by evaporation. The residue was dissolved in methanol and transferred to a 25-ml volumetric flask. The solution was then filtered through a 0.22- $\mu$ m membrane and analyzed by HPLC.

Extraction of free anthraquinones:<sup>34</sup> The five aforementioned constituents were also extracted as free anthraquinones. Powdered rhubarb samples (250 mg each) were extracted in 60% methanol (v/v) using ultrasonication for one hour. The extracted solution was prepared by the method of weight relief, in which we compensated for any weight lost during the extraction procedure. The weight lost during the extraction procedure was replaced with 60% methanol (v/v). The solution was then filtered through a 0.22- $\mu$ m membrane and analyzed by HPLC. This extraction method was also used to isolate gallic acid, (+)-catechin, sennoside A, and sennoside B using the same powdered rhubarb.<sup>34</sup>

## 2.2.2 HPLC analysis

HPLC analysis was performed using an Agilent 1100 system (Agilent Technologies, Palo Alto, CA) equipped with a micro-vacuum degasser, quaternary pump, auto-sampler, thermostatted column compartment, and diode array detector, which was connected to an Agilent ChemStation. Chromatographic analysis was conducted using an Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, particle size: 5 µm; Agilent Technologies) maintained at 40°C. The detection wavelength was 280 nm. The mobile phase consisted of 0.05% (v/v) aqueous phosphoric acid (A) and acetonitrile (B) with a gradient program of 3%–11% (B) at 0–10 min, 11%–15% (B) at 10–30 min, 15%–17% (B) at 30–45 min, 17%–22% (B) at 45–60 min, 22%–36% (B) at 60–75 min, 36%–60% (B) at 75–90 min, 60% (B) at 90–105 min, and 60%–3% (B) at 105–110 min. The injected volume was 10 µl, and the standard solution containing nine bioactive reference compounds were prepared in methanol. The method used to analyze the aforementioned chemical constituents is well-established and validated,<sup>34</sup> showing reasonable reproducibility and repeatability for each chemical constituent.

## 2.3 Delayed luminescence measurements

### 2.3.1 Sample preparation

Rhubarb samples were prepared by crushing using a model QE-100 grinder (Yili Company, Zhejiang Province, China), and 150-µm particles were selected using a standard sieve (Tongrentang Company, Beijing, China). Thereafter, the samples were stored in a dark box containing 3-5-mm silica gel (Boom BV, Meppel, the Netherlands) at room temperature for ≥16 hours before DL measurements were performed.<sup>27</sup>

### 2.3.2 DL measurement

DL was measured using a previously established protocol for herbal drugs.<sup>27</sup> The device for measuring DL (Meluna Research, the Netherlands) included a dark sample chamber with a vertically positioned photomultiplier tube (PMT) (model

9558QB; Electron Tubes Enterprises Ltd., Ruislip, UK). The sample chamber was kept at 22°C. The cathode end of the PMT has a diameter of 51 mm and is sensitive at 160-870 nm. The PMT was cooled to -25°C in order to reduce the dark count rate to 10 counts per second. The photon emission signal was amplified using a model 9301 fast preamplifier (ORTEC, Oak Ridge, TN). A personal computer containing a model 6602 counting card (National Instruments, Austin, TX) was used for data acquisition. Each batch of rhubarb was used to prepare five 1-g samples. Each 1-g sample was placed in a Petri dish and illuminated for 10 seconds using a model 284-2812 white halogen excitation source (Philips, Germany). The DL of each sample was measured three consecutive times. The total number obtained from all fifteen measurements in each batch was used to analyze the DL parameters of that particular rhubarb batch. DL kinetics were obtained by recording the number of counts in consecutive 0.05-second periods for a total of 30 seconds, resulting in a total of 600 data points.

## 2.4 Data processing and statistical analysis

### 2.4.1 Statistics of fourteen bioactive compounds

The semi-quantitative contents of the five anthraquinone glycosides—aloe-emodin glycosides, rhein glycosides, emodin glycosides, chrysophanol glycosides, and physcion glycosides—were calculated using the difference between the total anthraquinone and free anthraquinone contents measured using HPLC.<sup>10</sup> The calculated five anthraquinone glycosides and nine constituents identified using HPLC were investigated using statistical tools. Hence, the contents of fourteen compounds in each rhubarb sample were analyzed. Principal component analysis (PCA) was used to discriminate among the fourteen compounds in rhubarb using the tools provided in the MetaboAnalyst software package (<http://www.metaboanalyst.ca>).<sup>35</sup> Thereafter, the rhubarb samples were stratified into two groups based on their growing altitude (i.e., above or below 3000 m). Partial least square discriminant analysis (PLS-DA) was used to further investigate the



differences in the chemical compositions of rhubarb (<http://www.metaboanalyst.ca>). The PLS-DA model was validated using cross-validation in order to avoid overfitting of the model.<sup>36</sup> Variable Importance in the Projection (VIP) scores based on the PLS-DA analysis were used to indicate the compounds that significantly contributed to group separation.<sup>37</sup> Subsequently, a two-tailed, unpaired Student's *t*-test was performed (SPSS version 23.0; IBM, Armonk, NY) to compare the two altitude groups using the contents of each constituent; differences were considered significant at  $p < 0.05$ .

#### 2.4.2 Statistics of five DL parameters

The photon counts measured during the first 30 seconds of each decay curve were used to calculate the parameters of the following double-exponential decay function:<sup>27</sup>

$$y = y_0 + A_1 e^{-\frac{x}{t_1}} + A_2 e^{-\frac{x}{t_2}}$$

where  $t_1$  and  $t_2$  are time constants for the exponential decays,  $A_1$  and  $A_2$  are the amplitudes of the exponential decay components, and  $y_0$  is the final value of photon emissions in the DL decay curve. Curve-fitting was performed using ExpDecay 2 in Origin version 9.0 (OriginLab Corporation, Northampton, MA). The parameters of the fifteen batch measurements were averaged and used to represent the DL properties of each rhubarb batch. PCA was used to indicate the level of discrimination between DL parameters using tools provided in the MetaboAnalyst software package (<http://www.metaboanalyst.ca>). A two-tailed, unpaired Student's *t*-test was used (SPSS version 23.0) to compare the DL parameters between the two altitude groups; differences were considered significant at  $p < 0.05$ .

#### 2.4.3 Correlation between compounds and DL parameters

Spearman's rank correlation ( $\rho$ ) was used to quantify the correlation between the bioactive constituents and DL parameters (SPSS version 23.0). Moderately strong,

significant correlations were defined as Spearman's  $|\rho| > 0.35$  and  $p < 0.01$ , respectively.<sup>38,39</sup> Thereafter, Cytoscape version 3.2.1 ([www.cytoscape.org](http://www.cytoscape.org)) was used to draw a network view, which was used to visualize these correlations.<sup>40</sup>

### 3. Results

To evaluate the effects of growth environment on the quality of rhubarb, chemical analyses and DL measurements were conducted using 47 batches of rhubarb collected at various altitudes. The concentrations of 14 chemical constituents were quantified in each batch of rhubarb using HPLC. Unsupervised PCA was applied to the chemical data in order to visualize the variations between the different batches of rhubarb. Fig 2A displays the PCA results in the form of a score plot. The results show that the variance in chemical components of rhubarb due to the first two principal components (PC1 and PC2) accounted for 34% and 18.2%, respectively, of the total variance. The results also show two clusters based on the growth altitude, with a threshold of 3000 meters. Given the significant clusters obtained using PCA, a supervised clustering approach (PLS-DA; Fig 2B) was performed to optimally separate and identify the constituents that contributed significantly to the separation. PLS-DA evaluation with cross-validation revealed predictive accuracy of 0.90 and goodness-of-fit ( $R^2$ ) of 0.80. Six chemical constituents with a VIP score  $> 1$  were found to contribute to the classification; three of these constituents (gallic acid, emodin, and rhein) had a VIP score  $> 1.5$  (S1 Table). The results obtained from the PLS-DA analysis revealed a similar classification effect as the results obtained from the PCA analysis. This is likely because the most variation in response to altitude were well represented by the first two principal components of the PCA. In addition, the PCA and PLS-DA analyses contained the same misclassified batches of rhubarb. Specifically, three batches (GSLX1, NXLD2, and NXLD3) in the  $< 3000$ -m group were misclassified into the  $> 3000$ -m group, and one batch (SCLD5) in the  $> 3000$ -m group was misclassified into the  $< 3000$ -m group. Next, the estimated differences in chemical data between the two altitude groups were analyzed using a two-tailed,

unpaired Student's *t*-test. The analysis revealed that the contents of nine constituents in the >3000-m group were significantly higher than in the <3000-m group ( $p < 0.05$ ); these results are shown in Fig 3.



Fig 2. PCA and PLS-DA scores obtained from the chemical data. (A) PCA and (B) PLS-DA score plots of the chemical data obtained from all rhubarb batches, showing two general clusters separated by growth altitude (3000m). The individual samples were marked with a “Δ” (<3000 m) or a “+” (>3000 m) symbol, together with the sample ID number.

Next, to calculate the parameters of the rhubarb DL curves, a double-exponential decay function was used to fit the observed decay curves; an example of such a fit is illustrated in Fig 4. To display the differences in DL parameters using an unsupervised approach, PCA was used to obtain a focused view of the variance in the five DL parameters. Fig 5 shows a score plot of PCA using DL parameters with PC1 and PC2 values of 60.1% 32.9%, respectively. The reasonable group clusters in the PCA plot also indicates the differences in DL parameters between the two groups, with a threshold altitude of 3000 m. However, the misclassified rhubarb batches based on the DL parameters differed from the misclassified batches based on the PCA analysis using chemical data. Specifically, DL parameters of three rhubarb batches from location SXCJW were misclassified into the >3000-m group, whereas

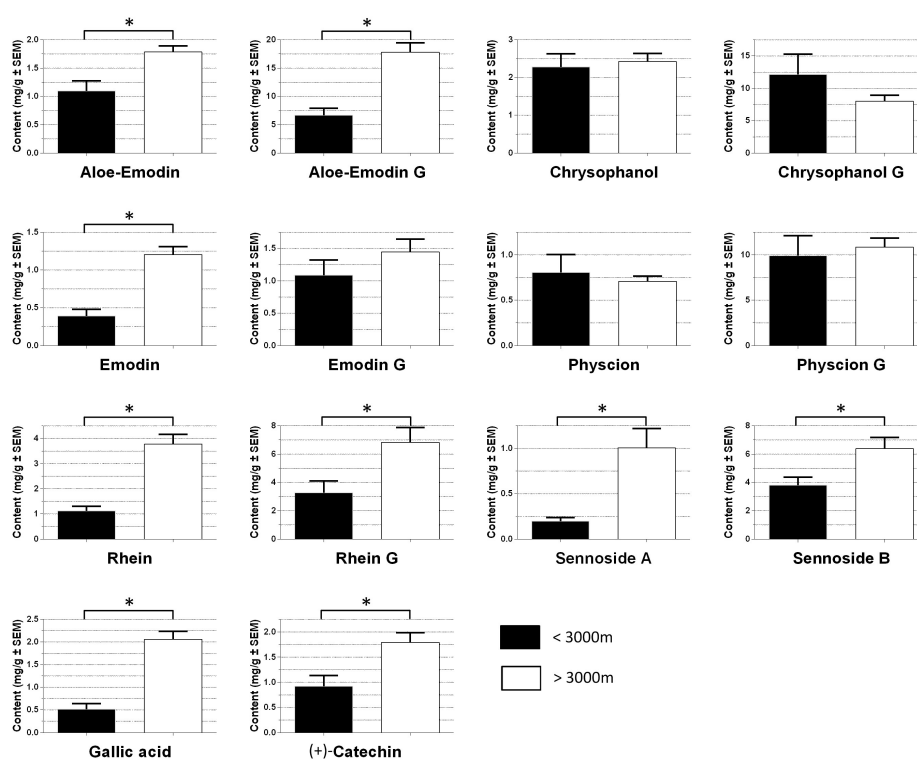


Fig. 3 Histograms comparing the contents of chemical constituents between rhubarb samples in two altitude groups. \*,  $p < 0.05$  (two-tailed, unpaired Student's *t*-test). "G" in the names of chemical constituents indicate glycosides.

the other three batches (SCLH1, SCKD1, and GSDB3) were misclassified into the <3000-m group. To visualize the differences in DL kinetics caused by altitude, 20 consecutive data points were accumulated and are expressed as photon emissions per second.<sup>27</sup> Fig 6 illustrates the different decay curves between the two altitude groups following excitation. To analyze further the difference between DL parameters, a two-tailed, unpaired Student's *t*-test was used to compare the five DL parameters between the two altitude groups. The analysis revealed that four parameters differed significantly between the two groups; only one parameter ( $t_2$ ) did not differ significantly ( $p=0.42$ ). On average, the DL parameters in the <3000-m group were larger than in the >3000-m group (Fig 7, S2 Table).

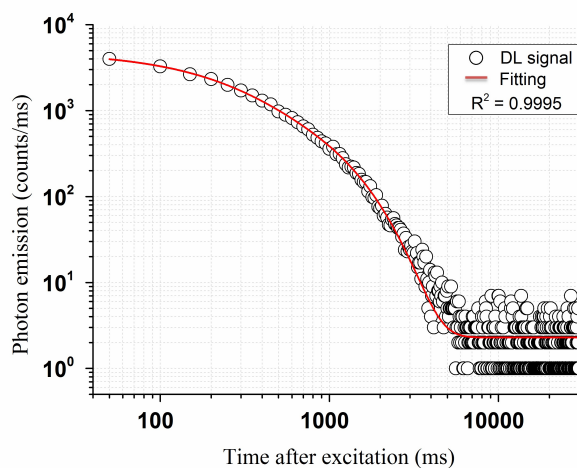


Fig. 4 Fitting effect of the DL decay curve of a rhubarb sample (ID: GSTC5). The sample was excited with light, after which photon emissions were counted. The red line shows a double-exponential fit of the data. Note that the data are plotted on a log-log scale.

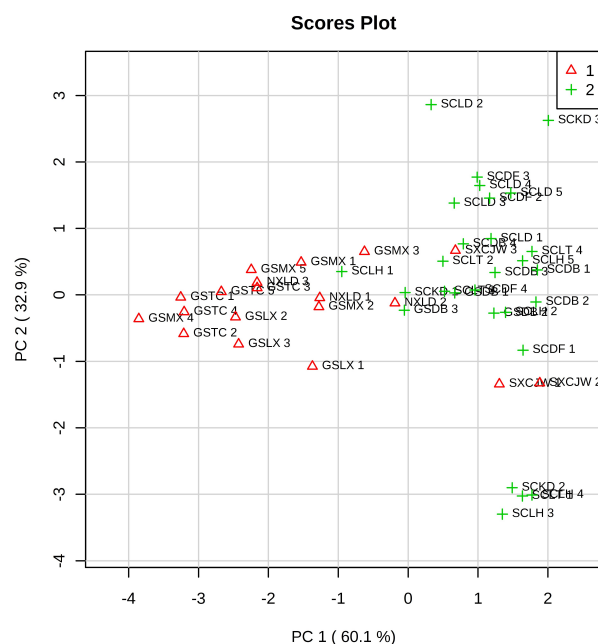


Fig. 5 PCA scores of the DL parameters measured for the individual rhubarb batches. The PCA score plot of DL parameters shows two general clusters. The individual samples are marked with a “Δ” (<3000 m) or a “+” (>3000 m) symbol, together with the sample ID number.

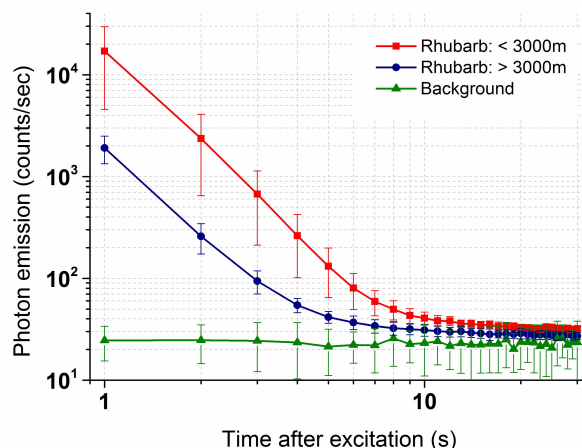


Fig. 6 DL decay curves obtained from rhubarb samples collected at an altitude <3000 m and >3000 m. The sample was excited with light, after which photon emissions were counted. Background emission were stable throughout the experiments. Note that these data are plotted on a log-log scale.

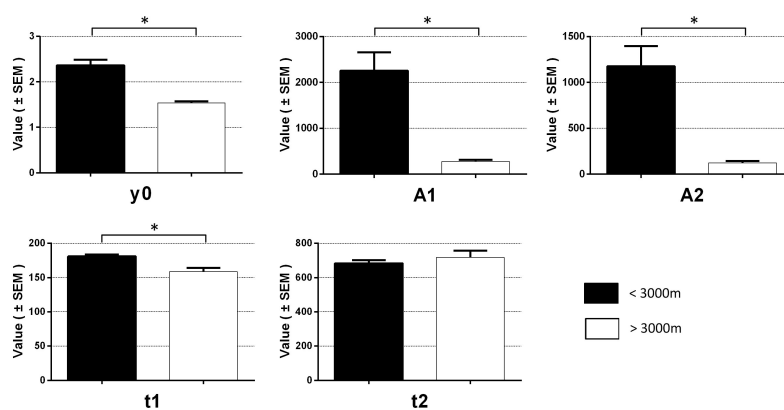


Fig. 7 Histograms comparing the DL parameters obtained between the two different altitude groups. \*,  $p < 0.05$  (two-tailed, unpaired Student's t-test).

Both the chemical analysis and the DL measurements successfully identified differences in rhubarb based on altitude. Next, we determined the correlations between the chemical constituents and DL parameters for all rhubarb batches using the Spearman's correlation method. We found moderately strong and significant correlations between DL parameters and constituents (Table 2). These correlations

are depicted visually in Fig 8. Interestingly, the compounds that differed significantly due to altitude generally had a negative correlation with the significantly different DL parameters; in particular, gallic acid, emodin, and rhein—the three compounds with the highest VIP scores—had stronger correlations with the DL parameters related to photon counts (i.e.,  $y_0$ , A1, and A2). On the other hand, the DL parameter that was not significantly different (t2, which represents the decay time of the DL curve) had a relatively stronger positive correlation with sennoside A.

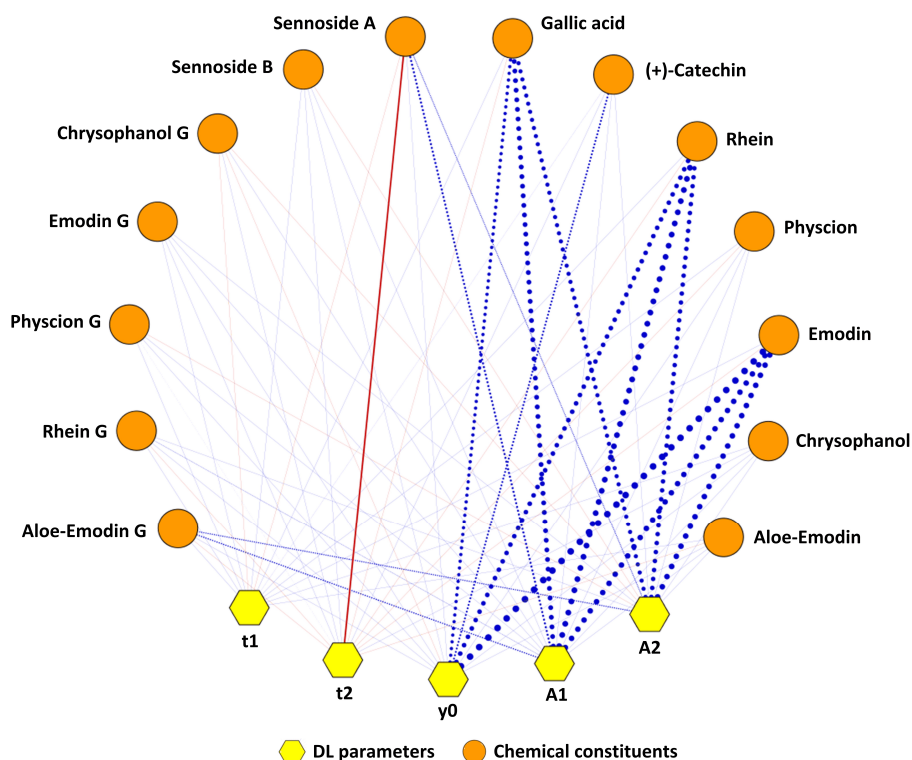


Fig. 8 Correlation network between the 14 chemical constituents and 5 DL parameters measured in the rhubarb samples. Visualization of data concentrated on the correlations between chemical constituents in relation to DL parameters. The negative correlations are indicated with blue lines, and positive correlations are indicated with red lines; thicker lines indicate a higher correlation. The length of each line has no meaning. “G” in the names of chemical constituents indicate glycosides.

Table 2. Moderately strong and significant correlations between DL parameters and chemical constituents

| Chemical constituent | DL Parameter             |                          |                          |                         |
|----------------------|--------------------------|--------------------------|--------------------------|-------------------------|
|                      | y0                       | A1                       | A2                       | t2                      |
| Rhein                | $\rho=-0.57$<br>$p<0.01$ | $\rho=-0.63$<br>$p<0.01$ | $\rho=-0.66$<br>$p<0.01$ |                         |
| Emodin               | $\rho=-0.67$<br>$p<0.01$ | $\rho=-0.61$<br>$p<0.01$ | $\rho=-0.62$<br>$p<0.01$ |                         |
| Gallic acid          | $\rho=-0.51$<br>$p<0.01$ | $\rho=-0.58$<br>$p<0.01$ | $\rho=-0.55$<br>$p<0.01$ |                         |
| (+)-Catechin         | $\rho=-0.43$<br>$p<0.01$ |                          |                          |                         |
| Aloe-Emodin G        |                          | $\rho=-0.41$<br>$p<0.01$ | $\rho=-0.40$<br>$p<0.01$ |                         |
| Sennoside A          |                          | $\rho=-0.44$<br>$p<0.01$ | $\rho=-0.39$<br>$p<0.01$ | $\rho=0.41$<br>$p<0.01$ |

#### 4. Discussion

Our preliminary analysis indicates that altitude is an important environmental factor, significantly affecting both the chemical composition of rhubarb and its DL parameters. Specifically, the concentrations of nine chemical constituents in rhubarb were significantly higher in rhubarb samples collected at an altitude higher than 3000 m. The increased contents of two anthraquinone derivatives (aloe-emodin glycoside and rhein glycoside) in rhubarb grown at high altitude are consistent with results reported by Wang et al.<sup>30</sup> It has been reported that altitude is able to affect the contents of anthraquinone glycosides in all three medicinal rhubarb species (*Rheum palmatum* L., *Rheum tanguticum* Maxim. ex Balf., and *Rheum officinale* Bail.) included in the Chinese Pharmacopoeia<sup>30</sup>, therefore we may hypothesis that altitude is able to influence the other chemical constituents in these three rhubarb species. Since different species of rhubarb may have variations in their chemical constituents.<sup>41</sup> Therefore, in our present study only *Rheum palmatum* L. is used. Our analysis of rhubarb DL curves revealed that four of the five DL parameters decreased in value at the higher altitude. Moreover, the misclassified batches of rhubarb based



on the PCA analysis differed between the chemical data and DL data. This indicates that DL may be correlated with unknown mechanisms that may not be characterized completely by chemical analysis. In addition, our data suggest that chemical constituents in rhubarb are correlated with DL parameters. The most significant negative correlations were found between DL intensity parameters and gallic acid, rhein, and emodin, the three constituents with the highest contributions in the PLS-DA analysis (S1 Table, Fig 8). One significant positive correlation was also identified; this correlation was between the DL parameter t2 and the compound sennoside A. Significant correlations between chemical constituents and DL properties indicate that specific DL parameters are correlated with the contents of bioactive constituents and that these parameters may be correlated with specific therapeutic properties of rhubarb.

In our study, we found that the contents of anthraquinone derivatives and polyphenols in rhubarb are affected by altitude. Variations among such chemical constituents have also been reported in other plants that are affected by altitude and altitude-related environmental factors. Altitude is believed to be an important environmental factor, as altitude is related both comprehensively and closely to several environmental features, including the intensity of solar radiation and fluctuations in ambient temperature.<sup>42,43</sup> In turn, these environmental factors cause the accumulation of phenolic compounds in plants that grow at the timberline.<sup>44,45</sup> As altitude increases, temperature gradually decreases, whereas light intensity increases.<sup>4</sup> This increasing light intensity increases the contents of free anthraquinones and sennosides in *Rhamnus purshiana* and *Cassia angustifolia* Vahl., respectively.<sup>46,47</sup> Additionally, an increase in the concentration of anthraquinone glycosides with increasing altitude (and the resulting decrease in temperature) has also been demonstrated in *Rumex dentatus* L. and *Lavandula officinalis* L..<sup>43</sup> Moreover, many other pharmaceutically active secondary metabolites in medicinal plants—including quercitrin, total flavonoid, total hypericin, terpenes, and

antioxidant compositions—are positively correlated with altitude.<sup>4,43,48,49</sup> Importantly, variations in the concentration and/or ratio of specific chemical constituents can directly affect the therapeutic effects of herbal drugs;<sup>29,50</sup> thus, growing conditions and environmental factors are likely also closely correlated with the pharmacological activity of herbal drugs. For example, ethanol extracts of rhubarb (*Rheum tanguticum* Maxim. ex Balf.) grown at 4500 m are more potent at inhibiting the proliferation of adenocarcinoma cells than extracts of rhubarb grown at 3200 m.<sup>51</sup> Therefore, the idea of using indigenous medicinal materials is deeply rooted in traditional Chinese medicine. In ancient China, based on clinical observations, doctors believed that using only indigenous medicinal materials could achieve optimal medical results.<sup>1</sup> Although the notion of producing regions of indigenous medicinal materials (for example, growing specific medicinal in mountain areas, valleys, or in a specific province) was relatively simple in ancient times,<sup>1</sup> even today this notion indicates that growing conditions and environmental factors play an important role in the quality and therapeutic properties of medicinal herbs. Therefore, because environment factors can be closely correlated with the overall quality of the herbal drug, comprehensive quality control requires measuring both the chemical constituents and the chemical bioactivities under specific growth conditions.<sup>52</sup>

DL is believed to reflect the overall properties of a biological system,<sup>22,53</sup> and differences in the physiological status of plants in response to environmental stressors have been detected by measuring DL in fresh leaves and twigs.<sup>54,55</sup> Interestingly, altitude-related variations in dried herbal materials can also be measured using DL. The underlying environmental factor could be the different growth rates of rhubarb depending on the growth altitude. Variation in plant growth rates are closely related to ambient temperature.<sup>56,57</sup> Since average temperatures differ at different altitudes<sup>4</sup>, the growth rates of rhubarb will vary in the 1000 – 5000 meter range. As a consequence, this results in varying concentration of plant

metabolites but possibly also in polysaccharide / protein content i.e. carbon-to-nitrogen ratio.<sup>58,59</sup> Hence, the concentration of rhubarb chemical composition is related to altitude and at the same time it partially correlated to DL parameters suggesting DL might be a suitable tool for quality control. However, whether variations in DL parameters could be correlated to specific chemical constituents requires further research and validation.

The molecular absorption of the illuminating energy defines the dynamics of delayed luminescence, whereas changes in emission may be due to conformational changes in macromolecules in the cell, including proteins and nucleic acids.<sup>60</sup> These conformational changes can influence the interactions between molecules, thereby affecting the radiant (resonance) transfer of energy from one excited molecule to another.<sup>60</sup> On the other hand, secondary metabolites in herbal plants often interfere with protein conformation via highly reactive functional groups such as aldehydes and phenolic hydroxyls.<sup>61,62</sup> For example, polyphenols contain several hydroxyl groups and phenolic OH groups, which can form hydrogen bonds with amino acid residues in proteins.<sup>61,62</sup> Similarly, most anthraquinones contain phenolic OH groups and can also affect the conformation of proteins.<sup>62</sup> Accordingly, we conclude that amino acid residues in rhubarb contain more hydrogen bonds with increasing contents of anthraquinone derivatives and polyphenols, and the resulting changes in conformation and molecular interactions cause a change in DL dynamics. It may partially explain the correlation between DL parameters and the chemical constituents.

In our study, the relative high correlations have been found between DL properties and chemical constituents such as gallic acid, rhein, and emodin. This results suggest that DL parameters may be suitable for estimating both the quality and therapeutic properties of rhubarb, however, further validation is required. Rhein and emodin are two free anthraquinones that are commonly used to evaluate the quality of rhubarb in the Chinese Pharmacopoeia.<sup>8</sup> The significant correlations between DL parameters

and both rhein and emodin indicate that DL may serve as a tool for assessing the quality control of rhubarb. In addition, both rhein and emodin have heat-clearing and detoxifying properties, which are rooted in traditional Chinese medicine-based therapies,<sup>10</sup> as well as more general pharmacological properties, including anti-cancer and antioxidant activities.<sup>63–73</sup> In addition, gallic acid may have similar properties, inhibiting both human prostate carcinoma cells and human glioma cells, as well as scavenging free radicals.<sup>74–76</sup> Therefore, measuring DL parameters may reveal one or more specific therapeutic properties that are related to specific chemical constituents in rhubarb. If DL parameters can be comprehensively linked to both the chemical constituents and bioactivities of other Chinese herbs, these parameters may serve to explain why different Chinese medicinal herbs can have different bioactive constituents but can synergistically affect similar therapeutic targets and diseases.<sup>77</sup> Depending on the identified correlations between DL and both the chemical constituents and therapeutic properties of Chinese herbal medicines, DL may provide new opportunities to understand Chinese medicinal herb-based concepts based on biochemistry, thereby improving our understanding of healthcare.

## 5. Conclusions

Environmental stressors—particularly altitude—can have significant effects on the quality of many plants, including rhubarb. Here, we found that both chemical analyses and DL measurements revealed that the quality and composition of rhubarb are affected by altitude. Thus, DL parameters may represent a novel tool for assessing the quality of rhubarb. The identified potential correlations between DL parameters and chemical constituents suggest that DL may also be correlated to the bioactive properties of rhubarb. To date, approximately 200 constituents have been isolated from rhubarb species, including stilbenes, tannins, and anthocyanins.<sup>11,78</sup> Therefore, this proof-of-concept study should be repeated using a wider range of rhubarb samples and species, and the comprehensive network between secondary metabolites and DL parameters should be established. Additional studies for

validation of this methods are also required. Because of the multi-pharmacology nature of herbal drugs, there is currently a shift toward analyzing multiple constituents in herbal drugs.<sup>79</sup> Therefore, DL provides a rapid, direct, systematic tool for analyzing overall property, which may provide a novel means to measure quality control in medicinal herbs.

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## Supplementary Table

S1 Table. The concentrations of each bioactive constituent measured in the rhubarb samples collected below 3000 m and above 3000 m

| Chemical constituent | Altitude (m) |              | <i>P</i> -value <sup>a</sup> | VIP score <sup>b</sup> |
|----------------------|--------------|--------------|------------------------------|------------------------|
|                      | <3000        | >3000        |                              |                        |
| Aloe-Emodin          | 1.10 ± 0.18  | 1.78 ± 0.11  | <b>&lt;0.05</b>              | <b>1.09</b>            |
| Rhein                | 1.11 ± 0.19  | 3.78 ± 0.39  | <b>&lt;0.05</b>              | <b>1.55</b>            |
| Emodin               | 0.39 ± 0.09  | 1.21 ± 0.10  | <b>&lt;0.05</b>              | <b>1.58</b>            |
| Chrysophanol         | 2.28 ± 0.34  | 2.43 ± 0.21  | >0.05                        | 0.36                   |
| Physcion             | 0.80 ± 0.20  | 0.71 ± 0.06  | >0.05                        | 0.49                   |
| Aloe-Emodin G        | 6.67 ± 1.25  | 17.80 ± 1.66 | <b>&lt;0.05</b>              | <b>1.24</b>            |
| Rhein G              | 3.28 ± 0.83  | 6.83 ± 1.05  | <b>&lt;0.05</b>              | 0.57                   |
| Emodin G             | 1.09 ± 0.24  | 1.45 ± 0.20  | >0.05                        | 0.33                   |
| Chrysophanol G       | 12.16 ± 3.10 | 8.00 ± 0.91  | >0.05                        | 0.17                   |
| Physcion G           | 9.90 ± 2.21  | 10.86 ± 1.00 | >0.05                        | 0.49                   |
| Sennoside A          | 0.20 ± 0.04  | 1.01 ± 0.21  | <b>&lt;0.05</b>              | 0.99                   |
| Sennoside B          | 3.80 ± 0.58  | 6.38 ± 0.80  | <b>&lt;0.05</b>              | 0.43                   |
| Gallic acid          | 0.52 ± 0.12  | 2.06 ± 0.18  | <b>&lt;0.05</b>              | <b>1.7</b>             |
| (+)-Catechin         | 0.92 ± 0.21  | 1.79 ± 0.19  | <b>&lt;0.05</b>              | <b>1.12</b>            |

Values are presented in mg/g (mean ± SEM). <sup>a</sup> Two-tailed, unpaired Student's *t*-test. Significant *p*-values are shown in bold. <sup>b</sup> VIP score obtained by PLS-DA. A VIP score >1 (shown in bold) indicates a significant contribution to the classification. "G" in the names of chemical constituents indicate glycosides.

S2 Table. Values obtained for the five DL parameters measured in the rhubarb samples collected below 3000 m and above 3000 m

| DL parameter | Altitude (m)     |                | <i>P</i> -value <sup>a</sup> |
|--------------|------------------|----------------|------------------------------|
|              | <3000            | >3000          |                              |
| y0           | 2.36 ± 0.13      | 1.54 ± 0.03    | <b>&lt;0.05</b>              |
| A1           | 2254.64 ± 405.30 | 279.69 ± 34.81 | <b>&lt;0.05</b>              |
| t1           | 180.95 ± 2.86    | 158.88 ± 5.20  | <b>&lt;0.05</b>              |
| A2           | 1176.64 ± 218.37 | 124.95 ± 17.92 | <b>&lt;0.05</b>              |
| t2           | 683.70 ± 18.39   | 718.49 ± 38.84 | >0.05                        |

Values are presented as the mean ± SEM. <sup>a</sup> Two-tailed, unpaired Student's *t*-test. Significant *p*-values are shown in bold