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Spectrum-effect relationship between components and antitumor activity of Lonicerae Japonicae Flos based on orthogonal partial least squares regression

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Abstract

Lonicerae Japonicae Flos is a significant food and traditional Chinese medicine, known as plant antibiotics. It has rich chemical constituents and significant pharmacological effects. The antitumor activity of Lonicerae Japonicae Flos has been clarified, but the study on its spectrum-effect relationship has not been reported. The compounds responsible for its antitumor activity are still unknown. In this study, processed products of Lonicerae Japonicae Flos at different temperatures were taken as experimental materials, and SMMC-7721, A549, and MGC80-3 cells were tested. The orthogonal partial least squares regression method was used to analyze the common compounds in different processed products and the antitumor activity. The results show that processed products have a stronger inhibitory effect on A549 cells and MGC80-3 cells than SMMC-7721 cells. Compounds such as secologanic acid, isochlorogenic acid A, serotonin, and chlorogenic acid play an important role in their antitumor effects.

Keywords: Lonicerae Japonicae Flos; Processed products; Antitumor activity; Spectrum-effect relationship; Orthogonal partial least squares regression

1. Introduction

Lonicerae Japonicae Flos is the dried bud or flower with the initial blooming of *Lonicera japonica* Thunb. It has been used as tea and medicine for more than 1500 years. Lonicerae Japonicae Flos is a significant food and traditional Chinese medicine (TCM), known as plant antibiotics.[1] Pharmacological studies have shown that Lonicerae Japonicae Flos has the effects of antitumor, antioxidation, antibacteria, antivirus, anti-inflammation, antiallergy, heat clearing, and detoxification, and it can protect the liver and gallbladder, reduce lipids and blood glucose, and regulate immunity.[2–4] The pharmacological activity of Lonicerae Japonicae Flos is closely related to its rich chemical composition groups. Phytochemical studies have shown that Lonicerae Japonicae Flos mainly contains essential oils, organic acids, flavonoids, iridoids, saponins, and so on.[5]

In recent years, facing a series of public health emergencies, such as COVID-19, avian influenza, and severe acute respiratory syndrome, TCM has made important contributions to the prevention and treatment of these epidemics, among which Lonicerae Japonicae Flos, as the representative of TCM for heat clearing and detoxification, has played an important role.[6] So far, the importance of TCM is self-evident, and research on Lonicerae Japonicae Flos has been increasing year by year. At present, the spectrum-effect relationship models of antibacterial and antioxidant functions of Lonicerae Japonicae Flos have been successfully established.[7,8] However, these models mainly use chlorogenic acid and luteolin as evaluation indexes, which fail to reflect the holistic view of TCM and the characteristics of multiple components and targets. In addition, the antitumor activity of Lonicerae Japonicae Flos has been clarified, but the study of its spectrum-effect relationship has not been reported.

In this study, a more systematic and complete experiment was designed to establish the antitumor spectrum-effect relationship model of Lonicerae Japonicae Flos. Meridian theory is similar to that of target organs in modern medicine. The clinical practice of Lonicerae Japonicae Flos for thousands of years has indicated that the main target organs of Lonicerae Japonicae Flos are the liver, lung, and stomach.[9–11] Lonicerae Japonicae Flos is also applied flexibly due to different processing methods. The common processing methods include stir frying with wine, stir baking for yellowish, and carbonizing by stir frying.[12] According to the systematic study on the component changes of processed products of Lonicerae Japonicae Flos based on fingerprint, the chromatographic component model of regular differences in processed products at different temperatures was used in this study, and human hepatoma SMMC-7721 cells, human non–small cell lung cancer A549 cells, and human gastric cancer MGC80-3 cells were tested. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to screen the antitumor activity in vitro, and the spectrum-effect relationship model based on orthogonal partial least squares

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(OPLS) regression was established. It can provide a reference for expanding the study on the material basis of the antitumor effect of Lonicerae Japonicae Flos and the pharmacological mechanism of different processed products, so as to explore the potential quality markers and improve the quality control system of Lonicerae Japonicae Flos.

2. Results

2.1 High-performance liquid chromatography fingerprints of different processed products of Lonicerae Japonicae Flos

The high-performance liquid chromatography (HPLC) fingerprints of Lonicerae Japonicae Flos sample (b) and mixed reference substance (a) are shown in Fig. 1. The HPLC fingerprints of C1–C5 samples were analyzed, and the control chromatograms were generated after full-spectrum peak matching. A total of 43 peaks were matched. The results are shown in Fig. 2. In order to eliminate the interference of the solvent peaks, the peaks after 10 minutes were selected for analysis. Cluster analysis of 31 common peaks in HPLC fingerprints of different processed products (C1–C5) of Lonicerae Japonicae Flos shows that the components of Lonicerae Japonicae Flos can be divided into three types as the processing temperature rises (Fig. 3): (1) The content of the first type decreases gradually with the increase in processing temperature. Some of the components degrade at 320°C ± 5°C, and the others decrease sharply at 370°C ± 5°C; (2) With the increase in processing temperature, the content of the second type first increases and then decreases, and most of the components reach the maximum at 170°C ± 5°C and 220°C ± 5°C; (3) The content of the third type in the processed products at different temperatures is higher than that in the unprocessed Lonicerae Japonicae Flos samples, and some of components show a gradually increasing trend with the increase in processing temperature. In addition, the similarity results of different processed Lonicerae Japonicae Flos (C1–C5) and the control atlas are 0.992, 0.998, 0.995, 0.881, and 0.503, indicating that the components of Lonicerae Japonicae Flos change greatly with the increase in processing temperature. When the processing temperature is 320°C ± 5°C, the similarity is less than 0.900, and when the processing temperature reaches 370°C ± 5°C, the similarity is only 0.503.

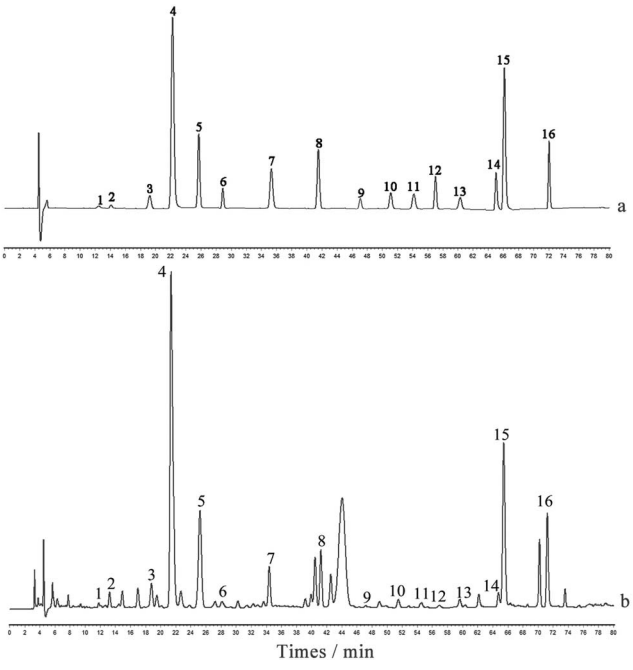

Figure 1. High-performance liquid chromatography chromatograms of mixed standard solution (a) and sample solution (b). 1, cumalic acid; 2, neochlorogenic acid; 3, morroniside; 4, chlorogenic acid; 5, secologaninsaeure; 6, caffeic acid; 7, sweroside; 8, secoxyloganin; 9, ferulic acid; 10, troxerutin; 11, isoquercetin; 12, cynaroside; 13, xylostein; 14, isochlorogenic acid B; 15, isochlorogenic acid A; 16, isochlorogenic acid C.

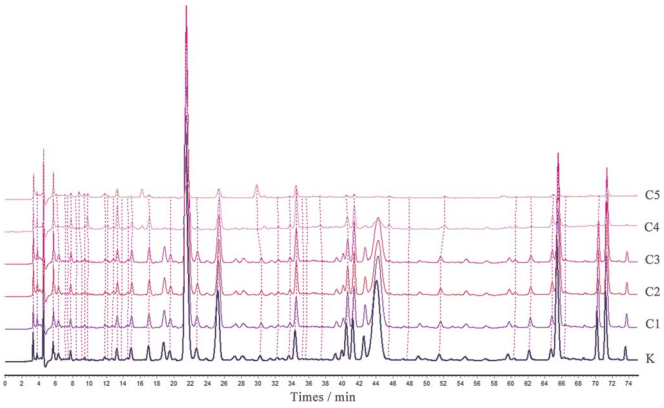

Figure 2. High-performance liquid chromatography fingerprint of Lonicerae Japonicae Flos processed at different temperatures. K, normal drying; C1, processed at 170°C; C2, processed at 220°C; C3, processed at 270°C; C4, processed at 320°C; C5, processed at 370°C.

2.2. Effects of different processed products of Lonicerae Japonicae Flos (C1–C5) on survival rate of SMMC-7721 cells

Fig. 4 shows that with the increase in extracts of different processed products of Lonicerae Japonicae Flos (C1–C4), the survival rate of SMMC-7721 cells decreases, whereas the effect of C5 on the survival rate of SMMC-7721 cells is increased with the increase in drug concentration. According to Fig. 5, with the increase in processing temperature (except for C5), the inhibitory effect of processed Lonicerae Japonicae Flos (C1–C4) extracts gradually weakened. In addition, when the concentration of the drug reaches 1800 μg/mL, the survival rate of SMMC-7721 cells is promoted by different samples, and the promotion is enhanced with the increase in processing temperature.

2.3. Effects of different processed products of Lonicerae Japonicae Flos (C1–C5) on survival rate of A549 cells

Fig. 6 indicates that with the increase in extracts of different processed Lonicerae Japonicae Flos (C1–C4), the survival rate of A549 cells decreases, while the effect of C5 on the survival rate of A549 cells is first inhibited and then promoted with the increase in concentration. According to Fig. 7, with the increase in processing temperature, the inhibitory effect of extracts of different processed products of Lonicerae Japonicae Flos on A549 cells increases gradually. However, when the processing temperature is 270°C ± 5°C, the effect of different concentrations of Lonicerae Japonicae Flos extracts on the survival rate of A549 cells is weak and almost does not reach half of the inhibitory effect.

2.4. Effects of different processed products of Lonicerae Japonicae Flos (C1–C5) on survival rate of MGC80-3 cells

Fig. 8 reveals that with the increase in extracts of different processed products of Lonicerae Japonicae Flos (C1–C5), the survival

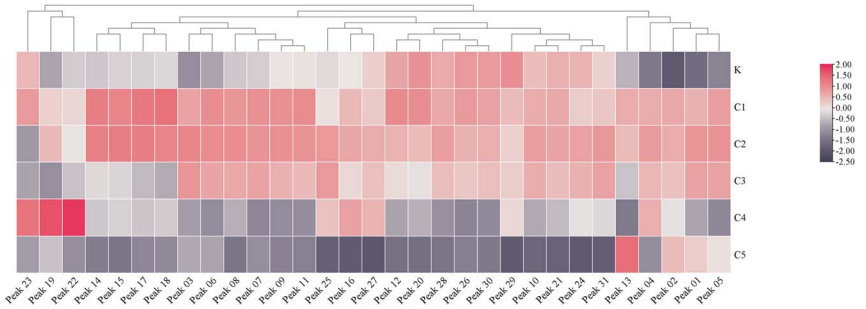

Figure 3. Clustering analysis of common peaks of Lonicerae Japonicae Flos at different temperatures. Peak 1, cumalic acid; Peak 4, neochlorogenic acid; Peak 10, chlorogenic acid; Peak 12, secologaninsaeure; Peak 16, sweroside; Peak 21, secoxyloganin; Peak 24, troxerutin; Peak 27, isochlorogenic acid B; Peak 28, isochlorogenic acid A; Peak 31, isochlorogenic acid C. Other peaks are not identified.

rate of MGC80-3 cells decreases. Compared with the other two cell lines, extracts of different processed products of Lonicerae Japonicae Flos have a stronger inhibitory effect on MGC80-3 cells. According to Fig. 9, with the increase in processing temperature, the inhibitory effect of extracts of different processed products of Lonicerae Japonicae Flos on MGC80-3 cells gradually increases. In addition, when the concentration of the drug reaches 1600 μg/mL, the survival rate of SMMC-7721 cells is promoted by different samples, and the promotion is enhanced with the increase in processing temperature.

2.5. OPLS regression analysis

In order to eliminate the differences between the variable dimensions, the peak areas of 31 common peaks and half maximal inhibitory concentration (IC_{50}) values of different processed products of Lonicerae Japonicae Flos at different concentrations were standardized by Z score. The standardized common peak area of the fingerprint was taken as the independent variable X, and the standardized IC_{50} was taken as the dependent variable Y, which was imported into SIMCA-P 14.1. Partial least squares regression analysis was used to analyze the correlation of the data, and the regression coefficients of each variable X to the dependent variable Y were calculated. The model parameters R^2X and R^2Y represented the interpretation rate of the established model to the X and Y matrices, respectively; Q^2 represented the prediction ability of the model, and variable importance for the projection (VIP) represented the influence intensity and interpretation ability of different components on the classification and discrimination of each group of samples (usually with VIP value >1 as the screening criteria). The regression coefficient represented the contribution of each variable to the antitumor activity. A greater regression coefficient indicated a greater contribution to the antitumor activity. The positive independent variable of the coefficient was positively correlated with the antitumor activity, and the negative value was negatively correlated with the antitumor activity.

The spectrum-effect relationship model of extracts of different processed products of Lonicerae Japonicae Flos and SMMC-7721 was manifested as $R^2X = 0.961$, $R^2Y = 0.998$, and $Q^2 = 0.979$, which indicated that the model was established successfully, and the regression model had strong fitting interpretation ability and model prediction ability. The results of VIP and regression coefficient analysis are shown in Fig. 10 and Fig. 11, in which Peak 20 > Peak 26 > Peak 12 (secologanic acid) > Peak 30 > Peak 21 > Peak 28 (isochlorogenic acid A) > Peak 29 > Peak 22 > Peak 10 (chlorogenic acid) > Peak 27 (isochlorogenic acid B) > Peak 19 > Peak 11 > Peak 16 (sweroside) > 1. The regression coefficients of Peak 22, Peak 27 (isochlorogenic acid B), Peak 19, and Peak 11 are positive, whereas those of Peak 20, Peak 26, Peak 12 (secologanic acid), Peak 30, Peak 21 (secoxyloganin), Peak 28

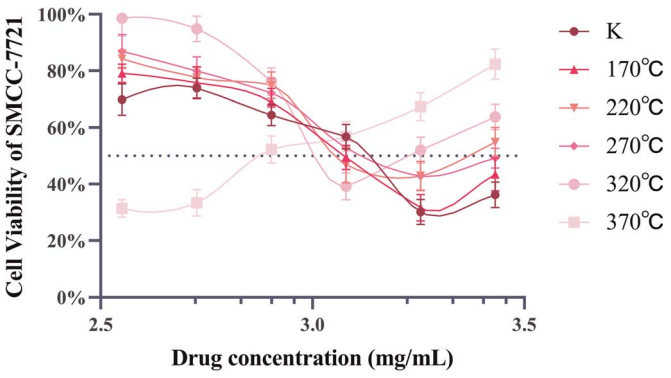

Figure 4. Survival rate of SMCC-7721 cells after administration of different processed products. K, normal drying.

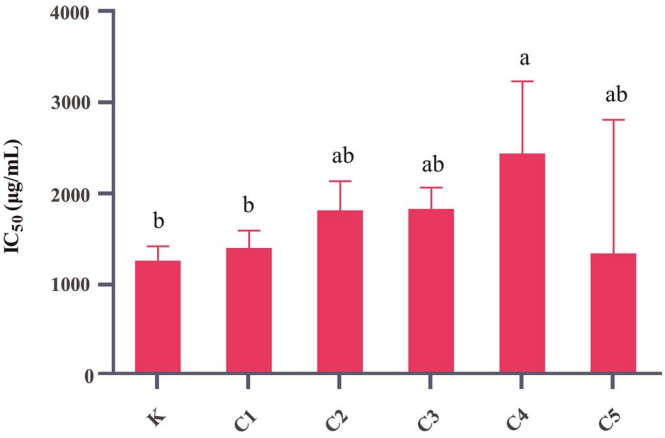

Figure 5. IC_{50} values of SMCC-7721 cells treated with different processed products (n > 3). K, normal drying; C1, processed at 170°C; C2, processed at 220°C; C3, processed at 270°C; C4, processed at 320°C; C5, processed at 370°C. The different lowercase letters indicate significant differences between treatments ($p < 0.05$).

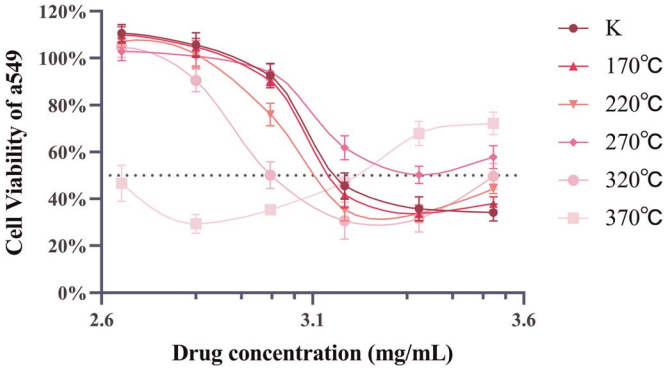

Figure 6. Survival rate of A549 cells after administration of different processed products. K, normal drying.

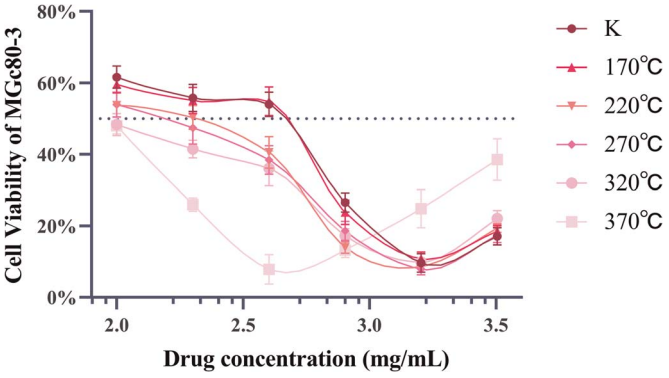

Figure 8. Survival rate of MGC80-3 cells after administration of different processed products. K, normal drying.

(isochlorogenic acid A), Peak 29, and Peak 10 (chlorogenic acid) are negative. The regression equation is as follows:

$$\begin{aligned} Y = & -0.104\,3X_1 - 0.084\,3X_2 - 0.093\,5X_3 \\ & - 0.079\,6X_4 - 0.065\,5X_5 - 0.063\,7X_6 \\ & - 0.099\,3X_7 + 0.052\,9X_8 - 0.046\,3X_9 \\ & + 0.105\,4X_{10} + 0.052\,6X_{11} - 0.043\,3X_{12} \\ & + 0.066\,7X_{13} \end{aligned}$$

In the above formula: X_1, Peak 20; X_2, Peak 26; X_3, Peak 12 (secologanic acid); X_4, Peak 30; X_5, Peak 21; X_6, Peak 28 (isochlorogenic acid A); X_7, Peak 29; X_8, Peak 22; X_9, Peak 10 (chlorogenic acid); X_{10}, Peak 27 (isochlorogenic acid B); X_{11}, Peak 19; X_{12}, Peak 11; X_{13}, Peak 16 (sweroside).

The spectrum-effect relationship model of extracts of different processed products of Lonicerae Japonicae Flos and A549 was manifested as $R^2X = 0.975$; $R^2Y = 0.991$, and $Q^2 = 0.956$, which indicated that the model was established successfully, and the regression model had strong fitting interpretation ability and model prediction ability. The results of VIP and regression coefficient analysis are shown in Fig. 12 and Fig. 13, in which Peak 20 > Peak 12 (secologanic acid) > Peak 30 > Peak 21 (secoxyloganin) > Peak 28 (isochlorogenic acid A) > Peak 26 > Peak 10 (chlorogenic acid) > Peak 29 > Peak 24 (rutin) > Peak 31 (isochlorogenic acid C) > Peak 11 > Peak 9 > Peak 8 > Peak 27 (isochlorogenic acid B) > Peak 15 > Peak 7 > Peak 18 > Peak 17 > Peak 14 > 1. The regression coefficients of all independent variables X are positive. The regression equation is as follows:

$$\begin{aligned} Y = & 0.093\,3X_1 + 0.085\,9X_2 + 0.088\,8X_3 + 0.069\,8X_4 \\ & + 0.074\,9X_5 + 0.091\,3X_6 + 0.064\,8X_7 + 0.080\,6X_8 \\ & + 0.053\,8X_9 + 0.039\,2X_{10} + 0.051\,3X_{11} + 0.045\,4X_{12} \\ & + 0.027\,0X_{13} + 0.030\,2X_{14} + 0.021\,9X_{15} \\ & + 0.035\,5X_{16} + 0.027\,8X_{17} + 0.022\,6X_{18} \\ & + 0.020\,4X_{19} \end{aligned}$$

In the above formula: X_1, Peak 20; X_2, Peak 12 (secologanic

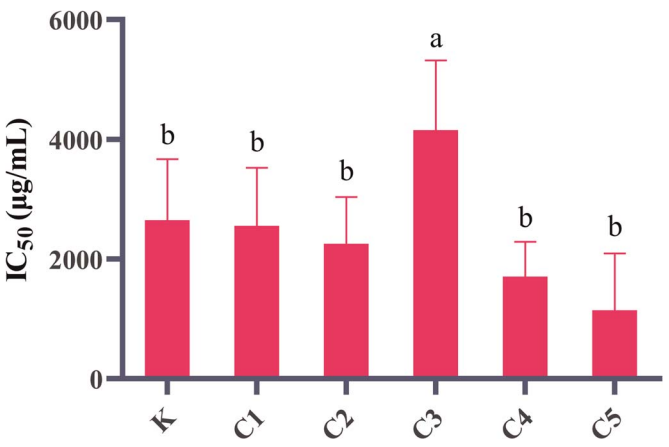

Figure 7. IC_{50} values of A549 cells treated with different processed products (n > 3). K, normal drying; C1, processed at 170°C; C2, processed at 220°C; C3, processed at 270°C; C4, processed at 320°C; C5, processed at 370°C. The different lowercase letters indicate significant differences between treatments ($p < 0.05$).

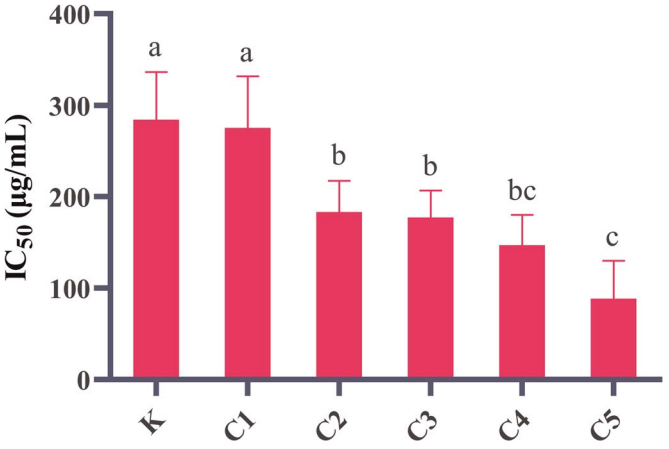

Figure 9. IC_{50} values of MGC80-3 cells treated with different processed products (n > 3). K, normal drying; C1, processed at 170°C; C2, processed at 220°C; C3, processed at 270°C; C4, processed at 320°C; C5, processed at 370°C. The different lowercase letters indicate significant differences between treatments ($p < 0.05$).

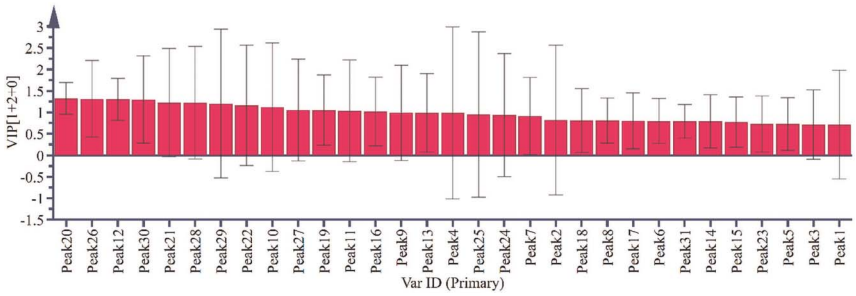

Figure 10. Spectrum-effect relationship VIP values of different processed products against SMMC-7721 cells. VIP, variable importance for the projection.

acid); X_3, Peak 30; X_4, Peak 21 (secoxyloganin); X_5, Peak 28 (isochlorogenic acid A); X_6, Peak 26; X_7, Peak 10 (chlorogenic acid); X_8, Peak 29; X_9 Peak 24 (rutin); X_{10}, Peak 31 (isochlorogenic acid C); X_{11}, Peak 11; X_{12}, Peak 9; X_{13}, Peak 8; X_{14}, Peak 27 (isochlorogenic acid B); X_{15}, Peak 15; X_{16}, Peak 7; X_{17}, Peak 18; X_{18}, Peak 17; X_{19}, Peak 14.

The spectrum-effect relationship model of extracts of different processed products of Lonicerae Japonicae Flos and MGC80-3 was manifested as R^2X = 0.899, R^2Y 0.956, and Q^2 = 0.825, which indicated that the model was established successfully, and the regression model had strong fitting interpretation ability and model prediction ability. The results of VIP and regression coefficient analysis are shown in Fig. 14 and Fig. 15, in which Peak 20 > Peak 12 (secologanic acid) > Peak 26 > Peak 30 > Peak 28 (isochlorogenic acid A) > Peak 29 > Peak 21 (secoxyloganin) > Peak 10 (chlorogenic acid) > Peak 24 (rutin) > Peak 11 > Peak 9 > Peak 31 (isochlorogenic acid C) > 1. The regression coefficient of Peak 31 (isochlorogenic acid C) is negative, and that of other independent variables X is positive. The regression equation is as follows:

$$Y = 0.140\,8X_1 + 0.124\,0X_2 + 0.119\,8X_3 + 0.106\,8X_4 + 0.075\,8X_5 + 0.085\,7X_6 + 0.063\,9X_7 + 0.047\,6X_8 + 0.013\,9X_9 + 0.056\,3X_{10} + 0.042\,5X_{11} - 0.011\,0X_{12}$$

In the above formula: X_1, Peak 20; X_2, Peak 12 (secologanic acid); X_3, Peak 26; X_4, Peak 30; X_5, Peak 28 (isochlorogenic acid A); X_6, Peak 29; X_7, Peak 21 (secoxyloganin); X_8, Peak 10 (chlorogenic acid); X_9 Peak 24 (rutin); X_{10}, Peak 11; X_{11}, Peak 9; X_{12}, Peak 31 (isochlorogenic acid C).

OPLS regression is a multivariate data analysis method integrating multiple dependent variables to regression modeling of multiple independent variables, multiple linear regression analysis, principal component analysis, and canonical correlation analysis. It can eliminate the multiple correlations between independent variables, identify the information and noise in the system, and better reflect the correlation between the components and efficacy of TCM.[13,14] OPLS regression is an effective method to construct the spectrum-effect relationship of TCM. In conclusion (Fig. 16), compared with extracts of unprocessed Lonicerae Japonicae Flos, Lonicerae Japonicae Flos extracts at different temperatures have stronger inhibitory effects on A549 cells and MGC80-3 cells than SMMC-7721 cells. In contrast, the extracts of unprocessed Lonicerae Japonicae Flos have a strong inhibitory effect on MGC80-3 cells. Among them, the common characteristic peaks such as desbrucine acid, isochlorogenic acid A, desoxyloganin, and chlorogenic acid, as well as the unmarked characteristic peaks such as Peak 20, Peak 26, and Peak 30, all play an important role in the antitumor effect of Lonicerae Japonicae Flos extracts.

3. Discussion

Processing is one of the characteristics of TCM, and appropriate processing can reduce toxicity and increase efficiency.[15–17] However, there are few relevant research data on the selection of fire power and optimal fire in the processing of Lonicerae Japonicae Flos.[18] Based on the study of color-composition correlation, previous researchers have discussed the scientific connotation of the survivability of Lonicerae Japonicae Flos in the process of carbonizing by stir frying. The results show that there is a certain correlation between the changes in physical and chemical properties and color characteristics of Lonicerae Japonicae Flos in the process of carbonizing by stir frying.[19] In this study, based on the textual research records and previous studies, Lonicerae Japonicae Flos was

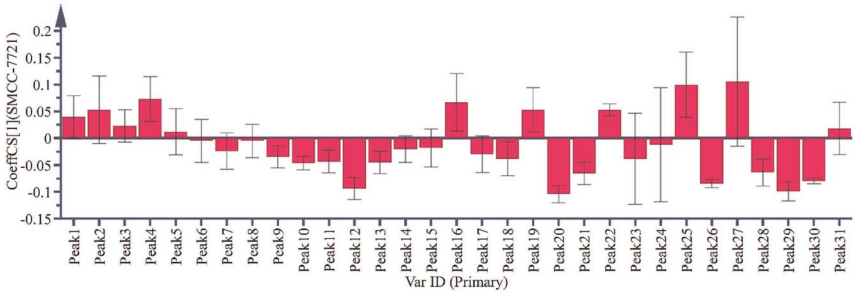

Figure 11. Spectrum-effect relationship regression coefficients of different processed products against SMMC-7721 cells.

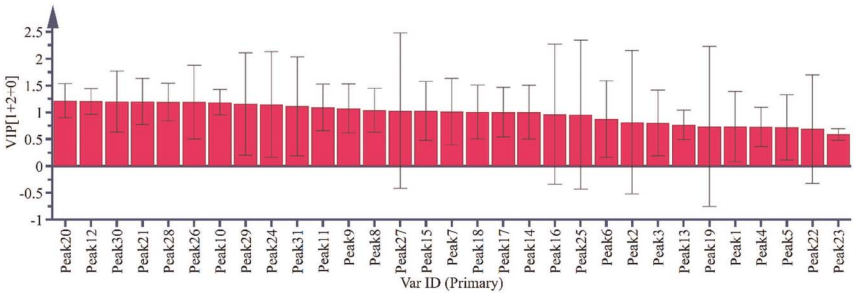

Figure 12. Spectrum-effect relationship VIP values of different processed products against A549 cells. VIP, variable importance for the projection.

processed at a temperature range from 170°C to 370°C, and processed products with different degrees of frying were obtained. The HPLC fingerprints of different processed products of Lonicerae Japonicae Flos were established, with unprocessed Lonicerae Japonicae Flos as control, which provided a reference for the objectification and digitization of processing parameters and the standardization of processed products of Lonicerae Japonicae Flos.

During the experiment, almost all of the curves (C1–C4) showed that the inhibitory effect of extracts of processed products of Lonicerae Japonicae Flos on different types of the cell increased gradually along with the rising concentration and then decreased when a specific content was reached. This phenomenon is often observed, especially in MTT assays in which herbal drugs or compound drugs are administered. The possible reason is that many herbal medicines have a two-way regulation effect, namely, "Yin-Yang transformation" in TCM theory. The inhibitory effect of C5 on SMCC-7721, A549, and MGC80-3 appeared an opposite tendency to the other processed product, and the cell viability increased gradually with increasing content. This result can be explained by differences in composition between samples processed at different concoction temperatures.

Interestingly, with the increase in processing temperature, the changing pattern of the components contained in Lonicerae Japonicae Flos was clustered into three categories. The essential oils, organic acids, flavonoids, iridoids, saponins, and other substances contained in Lonicerae Japonicae Flos had different chemical structures and functional groups, resulting in differences in heat resistance of these substances. As the processing temperature increased, the structure of some compounds was unstable, and the chemical bonds were broken after heating, resulting in a large amount of loss. In other cases, some compounds were converted to other compounds, or new compounds were produced. The first category showed a trend of first increasing and then decreasing, which peaked at 270°C–320°C, corresponding to the change of IC_{50} value of the SMCC-7721 cell group, and it was negatively correlated with it. The second category first gradually increased and then decreased, reaching a peak at 220°C–270°C, corresponding to the change of IC_{50} value of the A549 cell group, and it was positively correlated with it. The third category gradually decreased, corresponding to the change in the IC_{50} value of the MGC80-3 cell group. It was the overall variation in chemical composition that caused C5 and C1–C4 to exhibit marked differences in cytostatic results.

The results of previous *in vivo* experiments can be mutually corroborated with this study. Evidence suggests that a combination of active ingredients in Lonicerae Japonicae Flos, including quercetin, kaempferol, and luteolin, may alleviate the symptoms of liver fibrosis, and its main targets are α-smooth muscle actin, cyclooxygenase 2, formyl-peptide receptor 2, prostaglandin-endoperoxide synthase 1, nuclear receptor coactivator 2, interleukin β (IL-β), tumor necrosis factor α, CXC motif chemokine ligand 14, and transforming growth factor β1.[9] Furthermore, the repair rate of lung index and the improvement rate of interferon γ and IL-6 improve after administration of the Lonicerae Japonicae Flos, indicating that isochlorogenic acid B, isochlorogenic acid C, secoxyloganin, chlorogenic acid, and loganic acid are speculated to be the main effective ingredients in treating respiratory syncytial virus.[10] Moreover, active ingredients in Lonicerae Japonicae Flos reveal a prokinetic effect by enhancing the contractile responses of esophageal smooth muscle cells, increasing tone, strengthening the carbachol-induced or electric field stimulation–induced contractile responses of lower esophageal sphincter muscle strips, and accelerating gastric emptying, and gastrointestinal transit.[11]

The regression equation of the spectrum-effect relationship established by the OPLS method shows that the main components with the antitumor effect of Lonicerae Japonicae Flos are desbrucine acid, isochlorogenic acid A, desoxyloganin, and chlorogenic acid, but some components with important contribution value, such as characteristic Peak 20, Peak 26, and Peak 30,

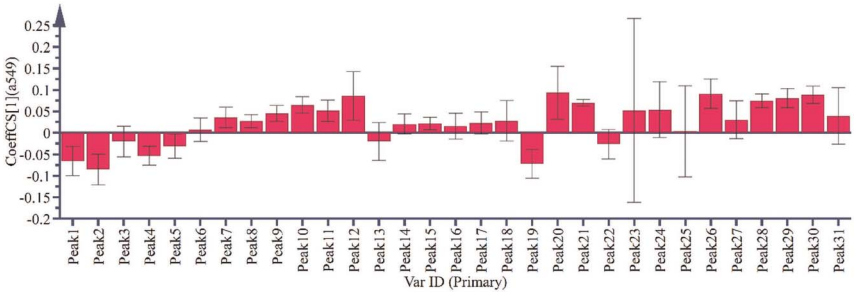

Figure 13. Spectrum-effect relationship regression coefficients of different processed products against A549 cells.

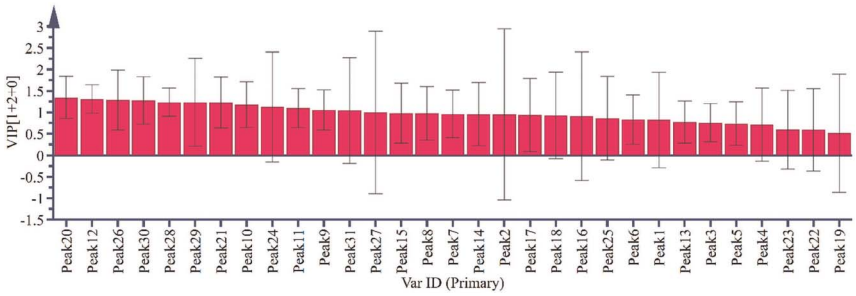

Figure 14. Spectrum-effect relationship VIP values of different processed products against MGC80-3 cells. VIP, variable importance for the projection.

need to be further identified. In addition, based on the traditional dosage form of Lonicerae Japonicae Flos, 50% methanol was selected as the extraction solvent. Under the premise of ensuring multiple peaks and high resolution of fingerprint chromatogram, which was in line with the characteristics of integrity and fuzziness of TCM,[20,21] it could provide ideas and methods for screening pharmacodynamic substances and pharmacological research on different solvent parts of Lonicerae Japonicae Flos.

Furthermore, the search for natural medicines from TCM has become a trend in recent years. The pharmacodynamic effect of TCM is the result of multicomponent and multitarget interaction.[22,23] TCM contains a large number of congeneric compounds, which often act on the same or close targets and produce excellent pharmacological effects. Certainly, for a multicomponent complex system of TCM, due to the diversity of the contained ingredients, there must be multiple targets that can act in vivo. However, there is not necessarily an inevitable connection between multiple components and targets, and multiple components have the potential to act on a single target and multiple targets. The targets of action of a chemical component are likely to be diverse, and this has been confirmed in massive research. Therefore, this study established the spectrum-effect relationship model of Lonicerae Japonicae Flos and antitumor activity, so as to provide a theoretical basis for exploring potential quality markers of Lonicerae Japonicae Flos, applying TCM in cancer prevention and rehabilitation, and developing new anticancer drug formulations related to antitumor active ingredients.

4. Materials and Methods

4.1. Processing method of samples

The samples of Lonicerae Japonicae Flos were collected from the Jinyinhua Cooperative of Jiazhuang Village, Huangde Town, Fengqiu County, Xinxiang City, Henan Province. They were identified as genuine Lonicerae Japonicae Flos by Professor Dong Chengming. The samples were processed at 170°C ± 5°C, 220°C ± 5°C, 270°C ± 5°C, 320°C ± 5°C, and 370°C ± 5°C for 10 minutes in the Processing Laboratory of Henan University of TCM, numbered C1–C5, and unprocessed samples were numbered K (Fig. 17).

4.2. Establishment of HPLC fingerprints of different processed products of Lonicerae Japonicae Flos

Preparation of reference solution: coumaric acid, neochlorogenic acid, morroniside, chlorogenic acid, desbrucine acid, caffeic acid, Dangyao glycoside, desoxyloganin, ferulic acid, rutin, isoquercetin, luteolin, Lonicerae Japonicae Flos glycoside, isochlorogenic acid B, isochlorogenic acid A, and isochlorogenic acid C were precisely weighed and dissolved in 50% methanol to the concentration of 0.590, 0.252, 0.966, 0.480, 0.396, 0.420, 1.172, 0.314, 0.352, 0.954, 0.271, 0.373, 0.284, 0.448, 0.430, and 0.672 mg/mL, respectively. In addition, an appropriate amount of each reference solution was taken and added with 50% methanol to 5 mL as the mixed reference solution.

Chromatographic conditions: Analysis was performed on a Waters e2695-2489 liquid chromatograph system (Waters, USA). A Symmetry C_{18} column (4.6mm × 250 mm, 5 μm) was used. The binary gradient elution system consisted of acetonitrile (A) and 0.2% formic acid aqueous solution (B), and separation was achieved using the following gradient program: 0–10 minutes, 8%–9% A; 10–25 minutes, 9%–11% A; 25–35 minutes, 11%–15% A; 35–50 minutes, 15%–16% A; 50–65 minutes, 16%–20% A; 65–75 minutes, 20%–24% A; 75–80 minutes, 24%–38% A. The injection volume was 10 μL. The flow rate was 0.5 mL/min, and the system was operated at 38°C. The detection wavelength was set at 240 nm.

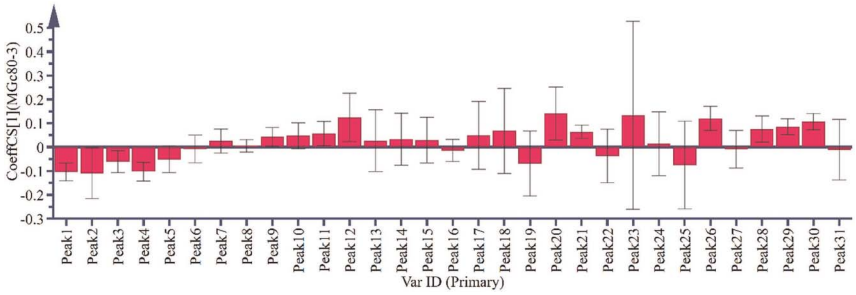

Figure 15. Spectrum-effect relationship regression coefficients of different processed products against MGC80-3 cells.

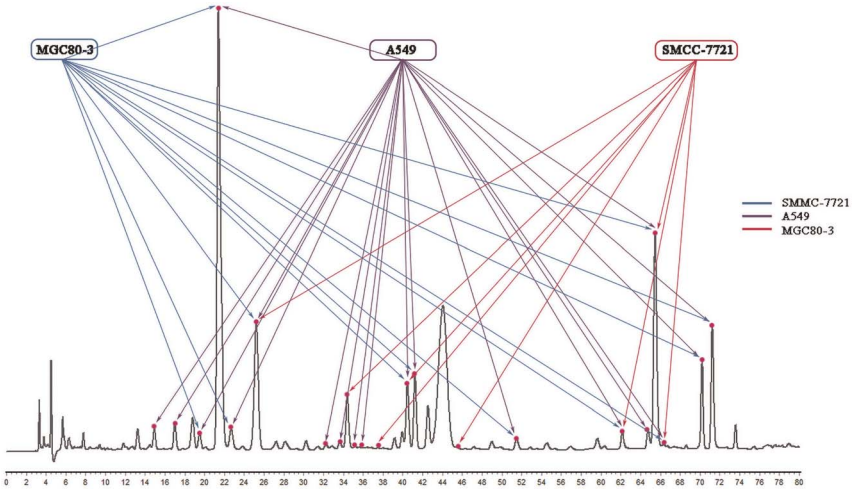

Figure 16. Antitumor spectrum-effect relationship "target-point" of Lonicerae Japonicae Flos.

Preparation and determination of Lonicerae Japonicae Flos samples: 1.0 g of Lonicerae Japonicae Flos samples were weighed and put into a conical flask. According to the ratio of material to liquid of 1:20, 50% methanol was added. The solution was weighted and recorded and received ultrasonic extraction for 30 minutes (power of 250 W and frequency at 35 kHz). After the conical flask was cooled, the weight loss was made up with 50% methanol, and the solution was shaken well and filtered, so as to obtain the sample extract solution, which was then processed through a 0.45 μm filter prior to HPLC analysis according to the above chromatographic conditions.

Establishment of HPLC fingerprints: The results of HPLC fingerprints of different processed products of Lonicerae Japonicae Flos (C1–C5) and unprocessed Lonicerae Japonicae Flos (K) were imported into the Similarity Evaluation System for Chromatographic Fingerprint of TCM (2012.130723). The time window width of 0.5 was set to remove the influence of solvent peak time region, and the fingerprint was generated after full-spectrum peak matching.

4.3. Antitumor activity of Lonicerae Japonicae Flos extracts in vitro

Preparation of extracts of different processed products of Lonicerae Japonicae Flos (C1–C5): 50 mL of the sample extract solution in "1.2" was taken, which was concentrated and evaporated to dryness, and then 20 mL ultrapure water was added to make suspension, respectively. The solution was frozen in an ultralow temperature refrigerator at −80°C for 3 days, placed in the freeze dryer for 1 day, and then frozen dry into powder to get the sample extract. The Lonicerae Japonicae Flos sample extracts were diluted with RPMI-1640 medium to prepare different concentrations of antitumor activity test solution in vitro.

Experimental scheme of antitumor activity in vitro: SMMC-7721, A549, and MGC80-3 cells in the logarithmic growth phase

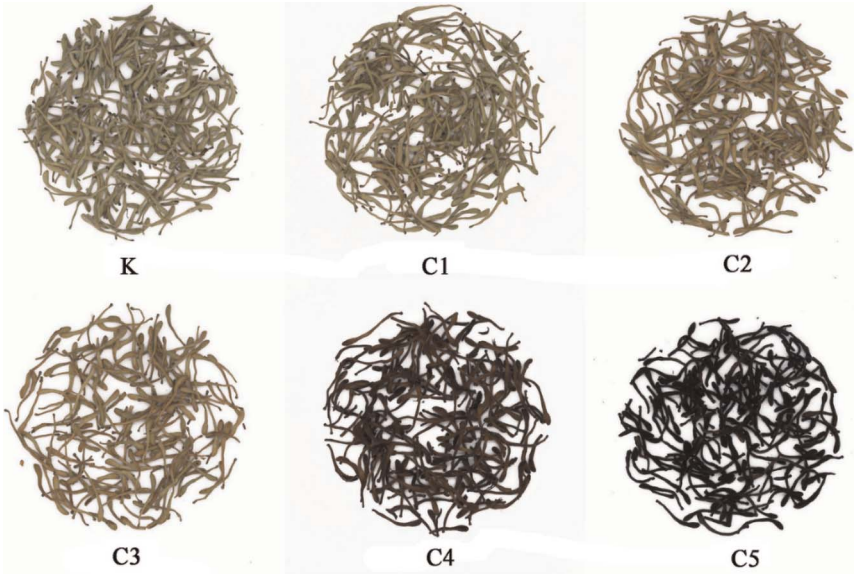

Figure 17. Lonicerae Japonicae Flos processed at different temperatures. K, normal drying; C1, processed at 170°C; C2, processed at 220°C; C3, processed at 270°C; C4, processed at 320°C; C5, processed at 370°C.

Table 1

Concentration gradient of test solution for different cell strains.

Cell strains	Concentration (mg/mL)					
SMMC-7721	0.355	0.533	0.800	1.200	1.800	2.700
A549	0.444	0.666	1.000	1.500	2.250	3.375
MGC80-3	0.100	0.200	0.400	0.800	1.600	3.200

were digested with 0.25% trypsin to prepare cell suspension. The cell density was adjusted to 5×10^4 cells/mL and inoculated in 96-well plates with 100 μL in each well. The cells were cultured in a CO_2 incubator for 24 hours. After that, the original culture medium was removed, and the above test solutions (100 μL in each well) with different Lonicerae Japonicae Flos concentrations were added (Table 1). At the same time, the blank control was added with an equal volume of serum-free medium. After being cultured in a CO_2 incubator for 48 hours, 20 μL of 5 mg/mL MTT solution was added to each well. After mixing, it was cultured in a CO_2 incubator for 4 hours. Then, the supernatant was removed, and 150 μL dimethyl sulfoxide was added and then oscillated on a vortex oscillator for 15 minutes. After the purple crystal in each well was completely dissolved, the absorbance (*A*) in each well was measured by a VARIOSKAN LUX automatic microplate reader (Mettler Toledo, Switzerland) at a wavelength of 490 nm. The process was repeated three times, and the cell survival rate was calculated according to the following formula: cell survival rate = *A* value of experimental group/*A* value of control group × 100%.

4.4. Statistical analysis

The Similarity Evaluation System for Chromatographic Fingerprint of TCM (2012.130723) was used to determine the common peaks and evaluate the similarity of HPLC fingerprint data. The IC_{50} values of each group were calculated and plotted by GraphPad Prism 7.0. The OPLS regression analysis was performed by SIMCA-P 14.1 software.

5. Conclusion

In this study, processed products of Lonicerae Japonicae Flos at different temperatures (170°C ± 5°C, 220°C ± 5°C, 270°C ± 5°C, 320°C ± 5°C, and 370°C ± 5°C) are taken as experimental materials, and MTT assay is used to test the antitumor activity in vitro of Lonicerae Japonicae Flos on SMMC-7721 cells, A549 cells, and MGC80-3 cells. Interestingly, it is found that the key temperature nodes where the chemical composition of the tested materials changes dramatically are consistent with the changing trend of IC_{50} of the three tested cells, respectively. In addition, some related pharmacological studies of previous researchers can corroborate the results of this study. At the same time, the study provides antitumor proof for some potential components in Lonicerae Japonicae Flos.

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Statement of ethics

This article does not contain any studies with human or animal subjects.

Conflict of interest statement

The authors declare that they have no conflicts of interest.

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Data availability statement

All data supporting the findings of this study are available upon request.

Author contributions

Chengming Dong, Linlin Yang, and Tianliang Liu conceived and designed the experiments. Tianliang Liu performed most of the experiments and analyzed the data. Linlin Yang completed the first draft. Daming Qi and Baoyu Ji worked together with Tianliang Liu to accomplish the statistical analysis, and Qiguo Gao provided experimental materials and financial support.

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