RESEARCH ARTICLE

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Spectrum-effect relationship between UPLC fingerprints and anti-lung cancer effect of *Panax ginseng*

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Abstract

Objectives: Lung cancer has the highest mortality rate among the various types of cancer. *Panax ginseng* (C. A. Mey). is a popular anti-cancer herbal supplement. The quality control of ginseng is crucial to ensure its clinical efficacy. This study aimed to establish new quality control methods for ginseng and to identify its main active components responsible for lung cancer treatment.

Methods: Ultra-high-performance liquid chromatography (UPLC) was used to establish fingerprints of 18 batches of ginseng. CCK-8 test was performed to evaluate the inhibitory activity of ginseng on Lewis lung cancer (LLC) cells. The spectrum-effect relationship analysis of ginseng was assessed by canonical correlation analysis (CCA) and bioactivity validation.

Key findings: Six common peaks were identified and the variation coefficients were determined. The 18 batches of ginseng inhibited the proliferation of LLC cells to different degrees, showing different half maximal inhibitory concentration (IC_{50}) values. Spectrum-effect relationship analysis showed that ginsenoside Ro is the main anti-proliferative constituent of LLC cell.

Conclusions: Spectrum-effect relationship is suitable for quality control of ginseng used for lung cancer. It is also effective in discovering the active ingredients related to the clinical efficacy of traditional Chinese medicine.

KEYWORDS
ginsenoside Ro, Lewis lung cancer cells, *Panax ginseng*, spectrum-effect relationships, traditional Chinese medicine

1 | INTRODUCTION

Lung cancer is the most common type of malignancy worldwide, with nearly two million new cases diagnosed each year.[1] Surgery, chemotherapy and targeted therapy are currently the main treatment methods. However, toxic effects and drug resistance have been a bottleneck. Traditional Chinese medicine (TCM), as a complementary therapy, has survival and quality of life benefits for cancer patients,

and has attracted great research interest.[2,3] The detection of one or several index components has been used as the main method in the quality of TCM.[4] However, the multiple chemical components and targets of TCM pose a challenge in establishing quality control methods especially in targeting ingredients associated with clinical efficacy.[5,6] The fingerprint method provides a comprehensive characterisation of the TCM components and has been approved for assessing the quality of herbal medicines by the World Health Organisation (WHO).[7]

Moreover, ultra-high-performance liquid chromatography (UPLC), a highly efficient and accurate technique, can be used to obtain chemical fingerprints, evaluate quality, and identify the authenticity of TCM.[8] The spectrum-effect relationship is a new method that can integrate the chemical fingerprints with pharmacological effects, to ensure the quality and find functional constituents in TCM.[9]

The dry root and rhizome of *Panax ginseng*, have been widely used in TCM and have multiple pharmacological activities, including: antioxidant, anti-inflammatory, anti-ageing, and immunity boost properties.[10,11] Extensive preclinical and clinical evidence shows that ginseng has beneficial effects against lung cancer;[12–15] the main active ingredient is ginsenoside Rg3.[15–18] However, its quantity is too low to be determined by general detection methods. Therefore, it is important to explore other compounds that are active against lung cancer.

Inhibition of tumour cell proliferation is regarded as one of the main mechanisms of antitumour agents.[19,20] In this study, UPLC was performed to generate fingerprints of ginseng samples from various sources. The half maximal inhibitory concentration (IC_{50}) of ginseng was determined on Lewis lung cancer (LLC) cells to evaluate its anti-lung cancer effect. The spectrum-effect relationship of ginseng was constructed using correlation analysis statistical method by correlating UPLC data with anti-lung cancer activity. This work provides a new perspective on the active components and clinical efficacy oriented quality control of TCM in the treatment of lung cancer.

2 | MATERIAL AND METHODS

2.1 | Materials and reagents

Eighteen batches of ginseng were collected from China Medico Technology Co. Ltd (Tianjin, China) and various Chinese herbal medicine markets in several provinces of China, such as Sichuan, Guangxi, Heilongjiang, Jilin, Hunan and Shenyang. The species were identified by Professor Xiao-he Xiao, a taxonomist at the China Military Institute of Chinese Medicine. Acetonitrile and phosphoric acid (HPLC grade) were obtained from Fisher Scientific Co. (Fair Lawn, NJ, USA) and Beijing Chemical Works (Beijing, China), respectively. Ultrapure distilled water was prepared using a Millipore water purification system (Millipore, Bedford, MA, USA). The ginsenosides Rg1, Re, Rb1, Rc, Ro, and Rd were acquired from Chengdu Chroma-Biotechnology Co. Ltd (Chengdu, China). Foetal bovine serum (FBS), DMEM (Dulbecco's modified Eagle's medium) high-sugar medium, penicillin, and streptomycin were purchased from Gibco (Carlsbad, CA, USA). CCK-8 kit was purchased from Dojindo Laboratories (Kumamoto, Japan).

2.2 | Plant sample preparation and reference standard solution

Powdered sample (5 g) of ginseng was added to 100 mL of 70% (*v/v*) ethyl alcohol and extraction done by ultrasonic wave for an hour at 25°C. The extracted liquid was filtered and concentrated using a

Highlights

- Chemical fingerprints revealed variations in the contents of six main peaks contained in 18 batches of *Panax ginseng*.
- Ginsenosides Ro might be the active constituents of anti-proliferative effect of *Panax ginseng* on Lewis lung cancer cells.
- Using cells viability assay and chromatographic fingerprint to establish the spectrum-effect relationship method to improve the quality control method of *Panax ginseng* in the treatment of lung cancer.

rotary evaporator. The lyophilised powder of the extract was dissolved before use.

Accurately weighed amounts of ginsenosides Rg1, Re, Rb1, Rc, Ro and Rd were dissolved in methanol to final concentrations of 78.9 μg/mL (Rg1), 86.8 μg/mL (Re), 95.7 μg/mL (Rb1), 94.1 μg/mL (Rc), 87.2 μg/mL (Ro) and 190.6 μg/mL (Rd).

2.3 | UPLC conditions

The dry powder was dissolved in methyl alcohol to a suitable concentration. The prepared samples were injected into a Waters ACQUITYUPLC system (Waters, Milford, MA, USA) with a photodiode array (PDA) detector. The chromatographic separation was performed with a Waters ACQUITYUPLCBEHC18 column (2.1 mm × 100 mm, 1.7 μm) (Waters), operated at 30°C. The mobile phases were water (solvent A) and acetonitrile (solvent B) with gradient elution as follows: 0–10.0 min, 10–19% B; 10.0–16.0 min, 19–21% B; 16.0–21.0 min, 21–32% B; 21.0–26.0 min, 32–41%; 26.0–32.0 min, 41–45%; 32.0–36.0 min, 45–50%. The flow rate was kept constant at 0.25 mL/min and UV measurements were obtained at 203 nm.

2.4 | Cell culture

LLC cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM high-sugar medium supplemented with 10% FBS, with 1% penicillin–streptomycin added. They were incubated at 37°C under a humidified atmosphere containing 5% carbon dioxide (CO_2).

2.5 | Cells viability assay

All 18 batches of lyophilised powder of the extract were dissolved in 1 mL DMEM high-sugar medium and diluted with medium to the

appropriate concentrations. The anti-proliferative effect of these batches was evaluated *in vitro* on LLC cells, using the CCK-8 test. LLC cells were incubated in 5% CO_2 at 37°C for 24 h, and then seeded in a 96-well plate at a density of 1×10^4 cells per well in DMEM high-sugar medium for another 24 h. The supernatant was discarded and the cells were treated with ginseng samples over a wide range of concentrations to establish a 48-h growth curve. Afterwards, 10 μL of CCK-8 reagent (100 μL/mL medium) was added to each well and the cells were incubated for 1 h. Absorbance (*A*) was measured at 450 nm using Synergy HTX Multi-Mode Reader (BioTek Instruments, Winooski, VT, USA). The proliferative inhibition rate was measured using the formula: Inhibition rate (%) = $[(A_{control} - A_{sample})/(A_{control} - A_{blank})] \times 100\%$. The IC_{50} was calculated by non-linear regression analysis using Graph-Pad Prism software (San Diego, CA, USA).

2.6 | Hierarchical clustering analysis

Hierarchical clustering analysis (HCA) is a multivariate analysis method that classifies specimens according to their closeness in nature.[4,21] The analysis maximises the similarity between the comparable data elements and the differences between different data elements.[22] In this study, HCA of the 18 batches of ginseng was performed using online Metabo Analyst 3.0 (http://www.metaboanalyst.ca/) to estimate the correlation to the UPLC fingerprints.

2.7 | Canonical correlation analysis

Canonical correlation analysis (CCA) is a multivariate statistical model used to extract factors with the greatest impact on the outcome variables and to maximise the relationship between the two sets of variables.[23] Taking the Pearson correlation coefficient as an index, the common peaks in the fingerprints of different ginseng samples were regarded as one set of variables, and the reciprocal of IC_{50} values as the other set. SPSS statistics software (SPSS for Windows 17.0, SPSS Inc., Chicago, IL, USA) was used to analyse the correlation between peaks and the reciprocal of IC_{50} values.

3 | RESULTS

3.1 | UPLC fingerprint analysis

The fingerprint of a mixture of reference substances is shown in Figure 1(A). The test sample showed satisfactory segregation between its peaks (Figure 1B). Under the optimal conditions, the UPLC fingerprints of the 18 ginseng samples from different areas were generated (Figure 1C). Six common peaks were observed at 1 to 30 min interval by comparing their retention time and UV spectrum with those of standard compounds. Six common peaks (a, b, c, d, e, and f) were identified as ginsenosides Rg1, Re, Rb1, Rc, Ro, and Rd, respectively. Some differences were noted in the data collected (Table 1) and the

percentage relative standard deviation (RSD%) of common peaks in the range 30.53–66.17%, which indicates that there was a difference in the chemical constituents of ginseng obtained from different production areas.

3.2 | Inhibitory effect of ginseng on LLC cells

Cell viability was tested by CCK-8 assay. All 18 batches of ginseng inhibited LLC cells in a dose-dependent manner (Figure 2A). The curve fitting method was used to evaluate the inhibitory activity and to estimate the IC_{50} value. The samples showed different biological activities on LLC cells, with IC_{50} values ranging from 0.06 to 13.20 mg/mL. The sample, S6, had the highest inhibition rate as it had the lowest IC_{50}. In contrast, S7 showed the weakest inhibitory effect with a difference of more than 200-fold (Figure 2B) The biological activity varied greatly amongst batches, indicating that the sample source has a great influence on the quality of ginseng.

3.3 | Hierarchical clustering analysis

HCA was used to distinguish ginseng from different sources by generating different clusters, according to the similarity of fingerprints. The results were visualised using a heat map. Samples were divided into three main clusters based on their inherent differences in their chemical composition (Figure 3). Cluster 1 consisted of S1 to S4 and S7; cluster 2 of S6, S11, S15 and S16; and cluster 3 of S5, S8–10, S12–14 and S17–18. A heat map with annotation and labels can map individual values to colours.[24] The map reveals that there are significant differences in the distribution of the main components among different samples; ginseng from different production regions has different chemical composition. HCA may be useful in preliminarily distinguishing the chemical composition of samples.

3.4 | Correlation analysis of the spectrum-effect

Pearson correlation analysis was performed between the reciprocal of IC_{50} values and peak area values for six common peaks in UPLC data, to find the major active components involved in LLC cell inhibition. The reciprocal of IC_{50} is positively correlated with the inhibitory activity. We analysed the peaks with positive correlation coefficients using SPSS statistics software. The correlation coefficients of ginsenosides Rg1, Re, Rb1, Rc and Ro, were positive, which shows that they correlate with the anti-proliferative activity of ginseng (Figure 4A). Online Metabo Analyst 3.0 (http://www.metaboanalyst.ca/) was used to correlate the reciprocal of IC_{50} values and the area of common peak. Thermographs with annotation labels mapped individual values to colours. The results in Figure 4(B) were consistent with those in Figure 4(A). Pearson correlation analysis showed that ginsenosides Ro, Rg1, and Rb1 were significantly correlated. Ginsenoside Ro had the highest correlation (*R* = 0.584) to inhibition, followed by Rg1 (*R* = 0.496), Rb1
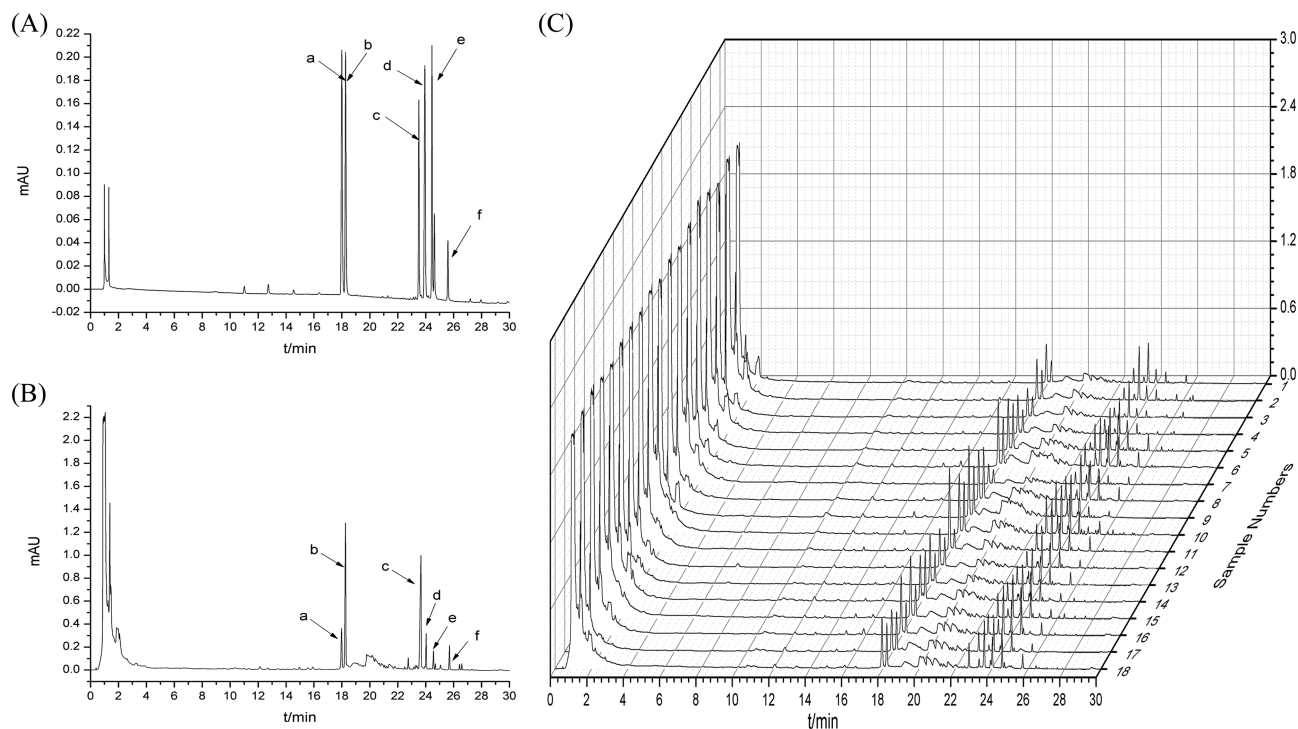
FIGURE 1 UPLC fingerprints of reference standards (A), test samples (B) and all ginseng samples (C) six peaks were represented as follows: (a) ginsenoside Rg1; (b) ginsenoside Re; (c) ginsenoside Rb1; (d) ginsenoside Rc; (e) ginsenoside Ro; (f) ginsenoside Rd

TABLE 1 Peak area of six common peaks identified by ultra-high-performance liquid chromatography (UPLC)

Sample	Peak area of each compound					
	a	b	c	d	e	f
t_R (min)	18.39	18.63	23.48	24.07	24.41	25.42
S1	1563	980	1112	605	328	464
S2	1667	1302	2053	595	331	587
S3	1362	985	972	377	265	325
S4	1497	1116	1093	505	293	339
S5	2644	2228	1850	2023	893	445
S6	3553	2351	2366	1620	1311	248
S7	951	660	887	745	263	468
S8	2487	2395	2003	1381	818	326
S9	3208	2890	2059	1263	663	298
S10	2819	2497	2165	1598	709	303
S11	3758	1941	2231	1302	688	220
S12	1895	1323	1192	911	557	421
S13	2243	1706	1518	1039	701	357
S14	2106	1979	1445	1066	669	327
S15	2928	1431	1567	1116	843	283
S16	2796	1367	1791	1078	1137	434
S17	1891	1483	1095	890	598	498
S18	2531	2193	1955	1128	938	470
RSD%	33.41	36.37	30.53	41.87	45.93	66.17

RSD = $\sigma/\mu \times 100$; RSD, relative standard deviation; σ, standard deviation; μ, average value of peak area. (a) Ginsenoside Rg1, (b) ginsenoside Re, (c) ginsenoside Rb1, (d) ginsenoside Rc, (e) ginsenoside Ro, (f) ginsenoside Rd.
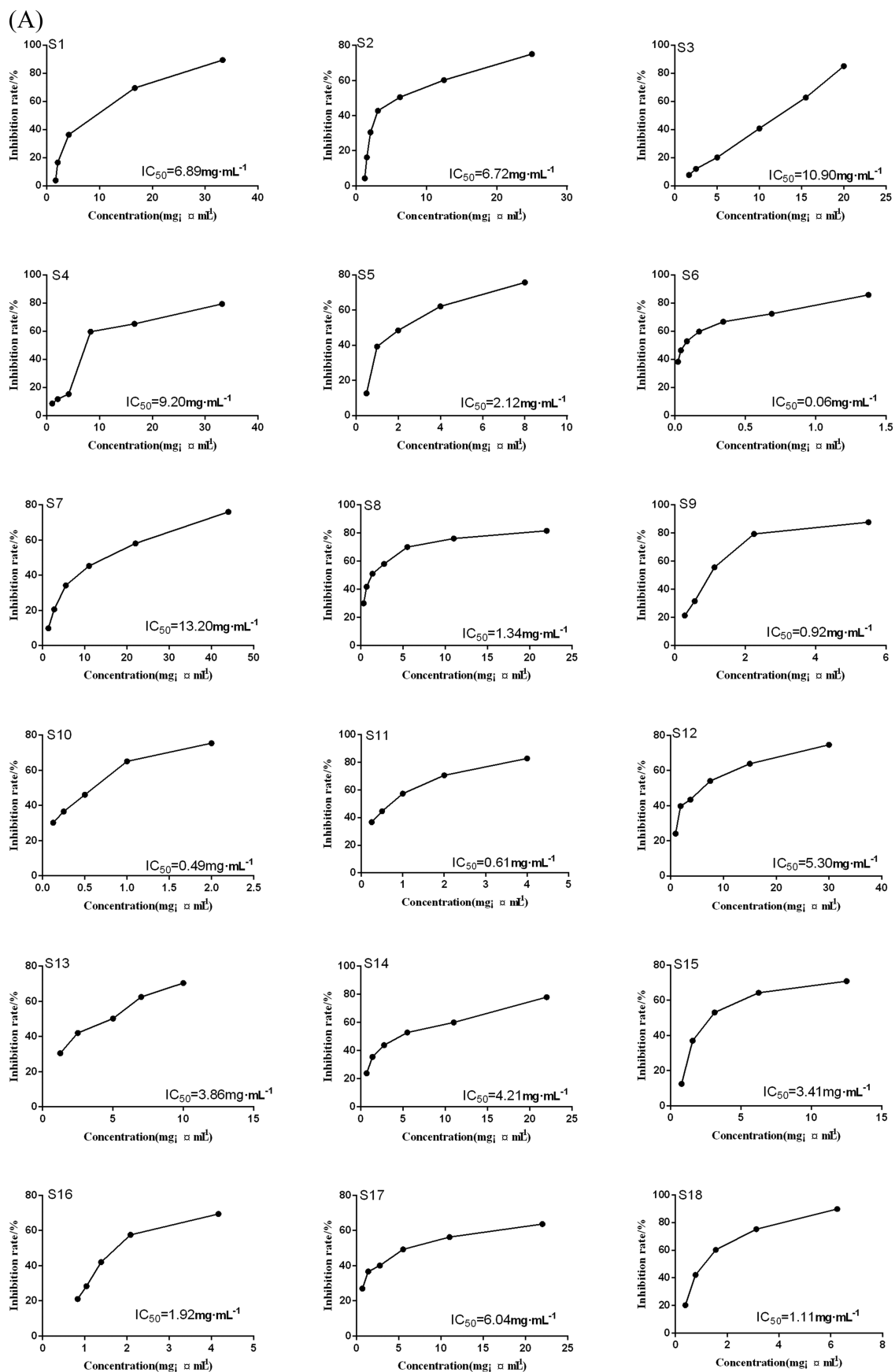
(A)

FIGURE 2 Inhibitory effect of ginseng on LLC cells. (A) Effects of 18 batches of ginseng samples on the viability of LLC cells. (B) IC_{50} values of ginseng samples on LLC cells
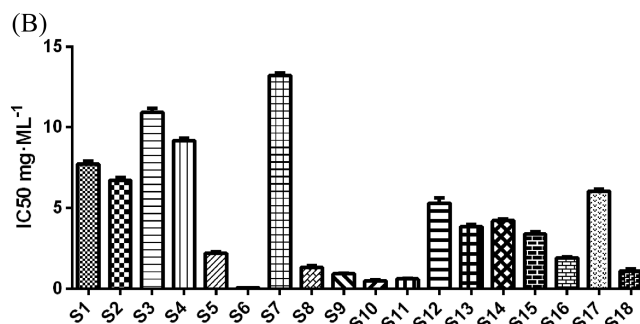
(B)

FIGURE 2 (Continued)

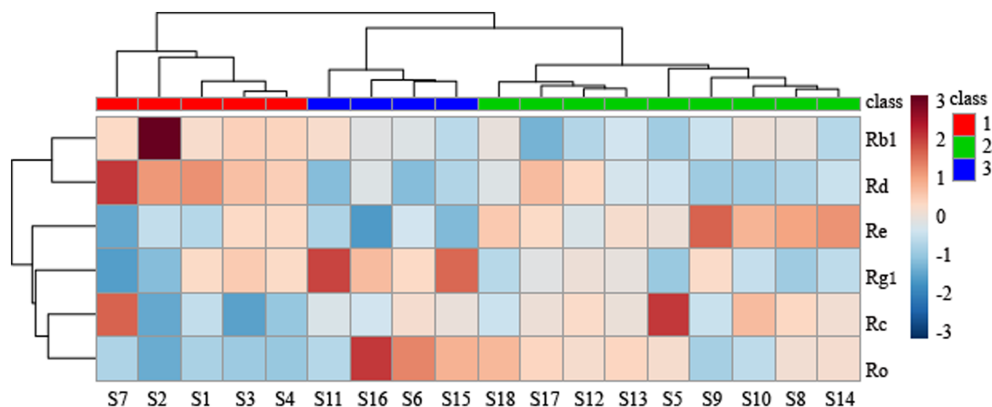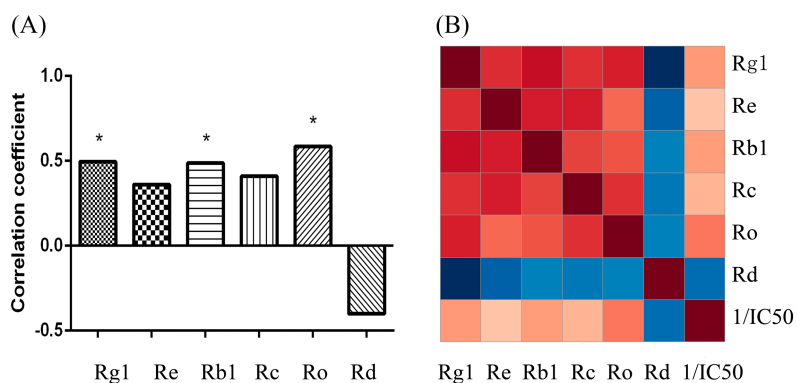**FIGURE 3** Heat map of 18 batches of ginseng and six chemical compounds (right of the map) [Colour figure can be viewed at wileyonlinelibrary.com]

(A)

(B)

FIGURE 4 Correlation analysis. (A) Pearson correlation coefficient between the IC_{50} value and peak area of six common peaks. (B) Thermograph of the correlation analysis of all factors [Colour figure can be viewed at wileyonlinelibrary.com]

(R = 0.487); indicating that the biological activity increased with the increasing content of ginsenoside Ro. Therefore, the earlier results suggest that ginsenoside Ro is the major active constituent that inhibits LLC cells in ginseng.

3.5 | Experimental validation

The CCK-8 assays were performed to determine their inhibition of ginsenosides Rg1, Rb1, and Ro on LLC cells to verify their anti-lung cancer activity. Four concentrations of each compound were used. The three compounds were found to possess dose-dependent anti-proliferation activity against LLC cells. Ginsenoside Ro had the highest inhibitory effect on LLC cells, which was consistent with the results of spectrum-effect correlation analysis (Figure 5).

4 | DISCUSSION

Quality control of TCM mainly utilises chemical analysis to determine index components. Some of these constituents may not be associated with clinical efficacy; therefore, chemical analysis alone is not adequate for clinical quality control of TCM. For example, ginseng is often used as an adjuvant treatment for lung cancer, but its main active ingredients related to anti-lung cancer activity is not clear. It is important to identify the quality of ginseng in the market because it is expensive and the quality varies amongst different sources. A quality control method for the evaluation and standardisation of ginseng components is therefore necessary.

Chromatographic fingerprint can reveal the chemical composition of various test samples and has been accepted as a quality control method for TCM by the WHO.[25] According to the People's Republic
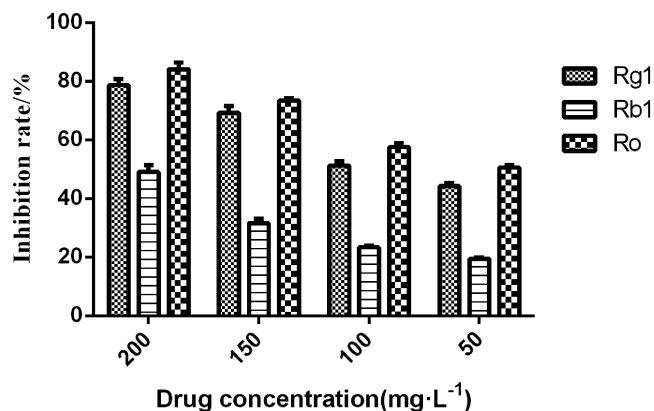
FIGURE 5 Validation of inhibitory effect of ginsenosides Rg1, Rb1, and Ro on LLC cells

of China Pharmacopoeia, the determination of ginsenosides Rg1, Re, and Rb1 is used as the quality control standard of ginseng. In this study, fingerprints of 18 batches of ginseng were established using UPLC and six common peaks were obtained. The peaks had relatively high contents of Rg1, Re and Rb1, and these varied significantly among different samples. The effects of these variations on lung cancer cells need to be verified.

Biological detection methods for anti-lung cancer activity were constructed to supplement the lack of chemical analysis of ginseng. The results showed that the 18 batches of ginseng had different inhibitory effects against LLC cells, and the variation in the anti-proliferative activity of S6 and S7 was by > 200 times among the batches. This method accurately reflected the difference in anti-lung cancer activity among ginseng samples, and could be used to evaluate the efficacy. Moreover, this method may provide a paradigm for the quality control of other TCM modalities used in lung cancer.

Spectrum-effect relationship analysis can combine the chromatographic fingerprint with pharmacological efficacy through multiple statistical analysis methods. This helps to assess the internal quality and find the major active constituents involved in clinical efficacy.[4,5,26] Spectrum-effect relationship has been successfully applied to analyse a variety of bioactive constituents in TCM. Li *et al*. found that *trans*-2,3,5,4'-tetrahydroxy-stilbene-2-O-β-D-glucoside and catechin are the active constituents of *Polygonum multiflorum* in inhibiting platelet aggregation.[5] Wang *et al*. found sinomenine, magnoflorine, menisperine and stepharanine as the major anti-inflammatory compounds in Sinomenii Caulis[27] and Wu *et al*. found that praeruptorin A, schisanddrin, arctiin and pseudo-ephedrine were the quality control markers for the Suhuang anti-tussive capsule in anti-inflammation.[25] In this study, we found ginsenoside Ro to be the main active ingredient against LLC cells. Our previous research showed that Si Jun Zi Tang (SJZ) can inhibit tumour growth in LLC-bearing mice.[28] Ginsenosides Ro and Rg1 were the two major active components of SJZ in inhibiting PC9 proliferation by spectrum-effect relationship analysis.[29] This effect may be attributed to ginseng, the main herb in SJZ. Ginsenoside Ro is also one of the bioactive components of Shenmai injection that

is often used as an adjuvant treatment for various cancers.[30] Ginsenoside Ro can suppress the HT29 cell metastasis[31] and markedly inhibits tumour growth in B16F10-transplanted mice.[32] Oleanolic acid (OA), an aglycone of ginsenoside Ro, has been shown to inhibit the proliferation and induce apoptosis of cancer cells.[33] Some studies suggested that ginsenoside Ro may be a novel autophagy inhibitor, with the potential to be used as an antitumour drug.[34] Thus, ginsenosides Ro could be used as a new quality control marker for ginseng treatment in lung cancer.

In conclusion, the chemical composition content and biological activity of ginseng varies greatly across regions. The bioactive components against lung cancer were revealed using the spectrum-effect relationship, which is a reliable method for quality control of ginseng. However, ginsenoside Ro is the only quality control indicator of the efficacy of ginseng against lung cancer, which is a limiting factor. Many other antitumour constituents such as ginsenoside Rg3, were not detected using a PDA detector due to low content;[35] there may be other components that promote the activity of Ro (the crude sample). In conclusion, modern analytical technology such as UPLC–mass spectrometry should be used in the future research to analyse the active constituents or metabolites of ginseng with activity against lung cancer and to identify their interaction.

CONFLICT OF INTEREST

The authors declared that they have no conflicts of interest to this work.

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