Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 723–728

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

# Secondary metabolite mapping identifies *Scutellaria* inhibitors of human lung cancer cells

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ARTICLE INFO

*Article history:*
Received 11 February 2010
Received in revised form 1 April 2010
Accepted 3 April 2010
Available online 20 April 2010

*Keywords:*
*Scutellaria*
Metabolomics
Human lung cancer cells
Baicalein
Baicalin
Wogonin

ABSTRACT

*Scutellaria baicalensis* root is widely used in China as an adjuvant to orthodox chemotherapy of lung cancer. However, functional biomarkers of this plant for anti-lung cancer activity have not yet been reported. We therefore determined the growth inhibition activity by MTT assay of eight solvent extracts of *S. baicalensis* in the human lung cancer cell line SK-MES-1. This activity was then mapped onto the secondary metabolite profile of crude extracts by principal components analysis (PCA) of proton NMR and HPLC-UV data. NMR- and HPLC-PCA maps revealed highest inhibitory activity for the non-aqueous extracts. The first two components of both maps discriminated extract activity mainly based on the differential content of three compounds, which were then tested individually. The $IC_{50}$ values for baicalin ($IC_{50}$: $64 \pm 5$ μM), baicalein ($IC_{50}$: $80 \pm 6$ μM) and wogonin ($IC_{50}$: $39 \pm 10$ μM) were comparable to that of the antineoplastic cisplatin ($IC_{50}$: $79 \pm 16$ μM). A partial least squares regression (PLS)-NMR model highly correlated with the corresponding PLS-HPLC model for prediction of inhibition. Secondary metabolite mapping of lung cancer growth inhibitors in crude extracts may be an important first step to qualify Chinese herbal prescriptions required for meaningful clinical trials of such integrated therapies.



## 1. Introduction

In China, a hot water infusion of *Scutellaria baicalensis* Georgi root (Chinese skullcap) is used as an oral adjuvant in conventional chemotherapy of lung cancer [1]. However, the clinical evidence-base for this traditional herbal use, either alone or as part of a Chinese patent medicine Huang Qin, is sparse [1]. Clearly a confounding variable in evaluating such integrated therapy is the herbal quality, a point rarely addressed in clinical reports. In contrast to Chinese practitioners using oral infusions as an adjuvant to lung cancer chemotherapy, UK medical herbalists use *S. baicalensis* tincture to treat inflammation [2]. There is a similar lack of scientific evidence for this clinical practice although some of us recently reported wide variability in quality amongst commercial products of identical claimed formulation [3]. An ongoing problem concerning herbal products remains the validation of agreed functional biomarkers within the complex natural product and their clinical formulations. These are defined here as chemical constituents shown to have *in vitro* activity relevant to therapeutic use. Commonly, functional biomarkers for a plant product are agreed by government expert panels on the basis of published chemical analysis of major constituents [1,4,5]. However, this is often confounded by species differences in chemical constituents and conflicting results from published *in vitro* pharmacological assays for formulations that are not relevant to clinical use.

Concerning *S. baicalensis* root, the flavonoids, baicalein, baicalin and wogonin (Fig. 1), have been reported as functional biomarkers of anti-cancer activity against a wide range of *in vitro* cancer cell lines except lung cancer. Selective induction of apoptosis and cell cycle arrest in cancer cells compared to normal cells are but two of a myriad of mechanisms that constitute rational anti-cancer drug targets [6]. Baicalein is an inhibitor of one important regulator of human cancer development, platelet-type 12-lipoxygenase (12-LOX) and has already been shown to initiate apoptosis in prostate cancer cell lines DU-145 and PC-3 in a dose-dependent manner. Mechanistic evidence included decreased phosphorylation of Akt, loss of survivin and subsequent activation of caspase-3 and caspase-7 in both lines, decreased Bcl-2 and Bcl-$X_L$ expression only in DU-145, and a shift in Bcl-2/Bax levels favouring apoptosis in PC-3 cells. Baicalein (25 μM) has been shown to induce expression of cyclin D1 and D3 proteins causing $G_0/G_1$ stage arrest in DU-145 and PC-3 cells [7]. Wogonin has also been reported to induce $G_1$ stage arrest for human U-937 leukaemia cells via stimulating the expression of phosphorylated protein kinase C (PKC)γ, upregulating p21 protein and decreasing the cyclin D-CDK 4 complex and p-Rb [8]. However, *in vitro* activity of either *Scutellaria* extracts or the biomarkers in animal or human lung cancer cell lines has not yet been reported. Therefore we employed a multivariate data

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doi:10.1016/j.jpba.2010.04.019

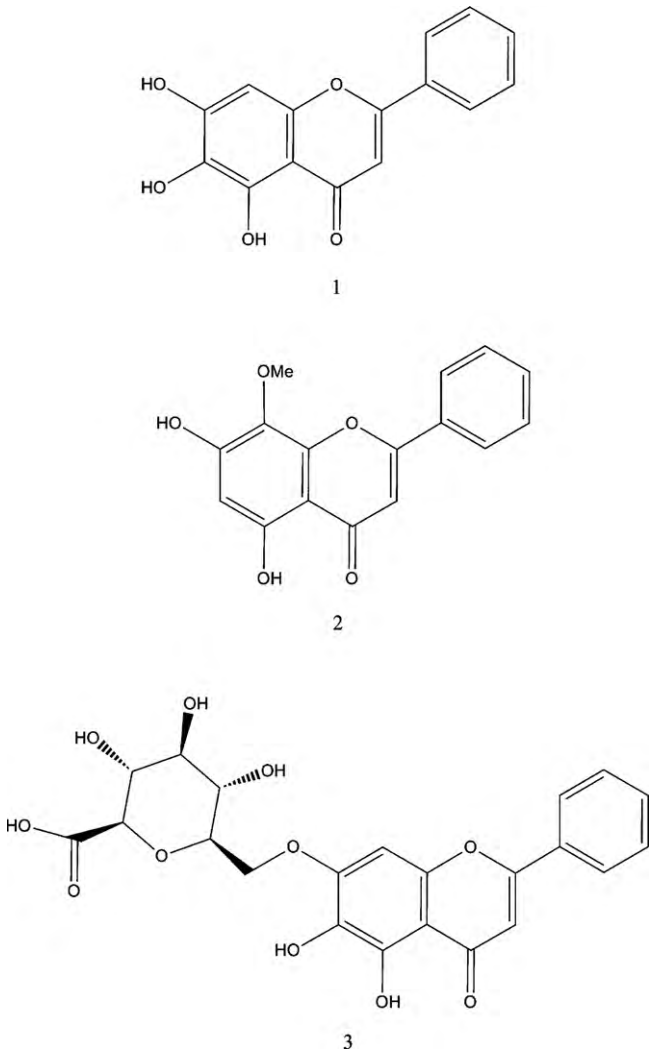


**Fig. 1.** Baicalein (1), wogonin (2) and baicalin (3).

analysis (MVA)-guided method to identify the functional biomarkers of *S. baicalensis* crude extract for the anti-lung cancer activities determined *in vitro*.

The MVA of plant secondary metabolites by metabolic profiling, or mapping, has been exhaustively reviewed elsewhere [9,10]. Nevertheless, the use of MVA to relate pharmacological activity to chemical constituents in crude extracts is a recent development [11,12]. In this study, HPLC and NMR secondary metabolite maps were constructed to correlate the chemistry with the growth inhibitory activity of crude extracts on human lung cancer cell line SK-MES-1 so revealing the functional biomarkers of *S. baicalensis* root.

## 2. Materials and methods

### 2.1. Preparation of extract and standards

*S. baicalensis* was purchased as dried root preparation from Tong Ren Tang Chinese herbal medicine store, 124 Shaftesbury Avenue, London. The original plant was collected in Shandong province, China (August, 2006) and its identity kindly confirmed as *S. baicalensis* Georgi by Ms Christine Leon of the Royal Botanical Gardens, Kew, UK. A range of EtOH extracts was originally prepared in our laboratory to encompass the commercial formulations of 25, 45 and 70% EtOH that are used in UK clinical herbal medicine. Portions of dried root (10 g) were each minced with 100 ml of different solvents: 25, 45, 70 or 100% EtOH and extracted by shaking for 24 h at room temperature. The solvents were completely removed at 35 °C under reduced pressure and the residue was freeze-dried overnight. Early experiments indicated the 100% EtOH and MeOH extracts had the highest growth inhibitory activity and thus a sequence of differing polarity solvent extractions was undertaken at a larger scale. Dried root (200 g) was thus extracted twice with 500 ml methanol and agitated at room temperature for 24 h. The extracts were filtered, solvent was completely removed at 35 °C under reduced pressure and the residue was lyophilised overnight. The dry residues were then redissolved in 100 ml 10% methanol and sequentially extracted twice with 200 ml of dichloromethane, ethyl acetate and butanol. The remaining water phase was retained. Baicalin (99% purity) and baicalein (99% purity) were purchased from Aldrich Chemical Co. (Dorset, UK). Wogonin (98% purity) was purchased from ChromaDex (Irvine, CA, USA). The standards were dissolved in either 100% MeOH or a 0.01% dimethylsulfoxide (DMSO, Sigma–Aldrich Ltd., Gillingham, Dorset, UK) in Hanks' solution. Antiproliferative effects were not observed at this concentration of DMSO in vehicle solution.

### 2.2. Lung cell culture

The human lung squamous cancer cell line SK-MES-1 was purchased from ECACC (Salisbury, Wiltshire, UK). Cells were grown in DMEM medium (Biowhittaker, Verviers, Belgium) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin and 100 μg/ml streptomycin in a 5% carbon dioxide humidified incubator at 37 °C. Experiments were performed when cells were approximately 80% confluent.

### 2.3. Growth inhibition as measured by MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma–Aldrich Ltd., Gillingham, Dorset, UK) assay was performed as described below. Briefly, 200 μl cell suspensions were seeded at a concentration of $1.5 \times 10^4$ cells/ml in a 96-well plate. After overnight incubation, serial doubling dilutions of *S. baicalensis* extract (250–7.8 μg/ml), baicalin, baicalein and wogonin (100–3.1 μg/ml) were added in 10 μl aliquots. The negative control was 10 μl of 0.01% DMSO in Hanks' solution. Each concentration was tested four times in the same plate. Plates were run at least in triplicate. After 72 h incubation, 20 μl MTT solution (5 mg/ml, Sigma–Aldrich Ltd., Gillingham, Dorset, UK) was added and incubated at 37 °C for 4 h. The optical density (OD) was measured at 590 nm using a Multiskan Spectrophotometer. The growth inhibition was determined using: Growth inhibition percentage = ((control O.D. − sample O.D.)/control O.D) × 100%. Statistical comparison between treatments was carried out using analysis of variance (ANOVA).

### 2.4. HPLC quantitation of baicalin, baicalein and wogonin

Details of the HPLC system used for quantification of samples and the calibration curves are reported elsewhere [3]. Dry residues of *S. baicalensis* extracts were dissolved in methanol at 5 mg/ml. The internal standard used was 4-hydroxybenzoic acid (500 μg/ml, Sigma–Aldrich Ltd., Gillingham, Dorset, UK). The sample injection volume was 10 μl: three injections were performed for each sample and standard. Baicalin, baicalein and wogonin calibration curves ($n = 6$) were determined over the range (1000–50 μg/ml) in methanol solution. The linearity of the calibration curves was determined ($r^2 > 0.998$). The limit of detection (LOD) calculated based on a signal-to-noise ratio (S/N: 3.3) was 2.20, 3.32 and 4.34 ng/ml for baicalin, baicalein and wogonin, respectively. The limit of quanti-

tation (LOQ) was then calculated based on the signal-to-noise ratio (S/N: 10) using the lowest concentration in the calibration and the highest noise observed when injecting a blank. The LOQ was 6.68, 10.06 and 13.16 ng/ml for baicalin, baicalein and wogonin, respectively. The intraday precision was calculated by comparing the ratio of the area of standard/area of internal standard obtained for three injections of standards within the day. For the interday precision, the ratios of the area of standard/area of internal standard in the calibration curve for 3 days were summed and compared [3].

### 2.5. NMR spectroscopy

Dried sample (10 mg) was dissolved in 1 ml $CD_3OD$ (99.8%) with 0.05% (v/v) tetramethylsilane (TMS, Cambridge Isotope Laboratories Inc., Hook, Hampshire, UK). One dimensional $^1H$ NMR spectra were measured at 300 K on a Bruker DRX 400 MHz Spectrometer (Bruker, Coventry, UK) equipped with a 5 mm triple resonance inverse detection (TXI) probe. Spectra were the result of the summation of 16 free induction decays (FIDs), with data collected into 65k data points and a sweep width of 20 ppm. Acquisition parameters were 0.126 Hz/point, pulse width was 17.857 kHz (90°) and relaxation delay = 1.0 s. Prior to Fourier transformation, an exponential line broadening equivalent to 0.3 Hz was applied to the FIDs. The signal intensities for all samples were referenced against TMS set as the reference at 0.00 ppm.

### 2.6. Multivariate data analysis (MVA)

Principal components analysis (PCA) was performed using Pirouette 4.0 chemometrics modelling software (Infometrix, USA). Partial least squares (PLS) analysis was performed using SIMCA-P 12.0 MVA software (Umetrics, Sweden) and multiple linear regression models were constructed to predict values for $IC_{50}$.

HPLC chromatograms at 270 nm comprised 2100 discrete regions by data acquisition every second from 0.00 to 35.00 min. Alignment for HPLC chromatograms used LineUp software 3.0 (Infometrix, USA). The resulting data were formatted as an ASCII-text file and exported into Microsoft® Excel 2007 using Totalchrom Convert 6.31 (PerkinElmer, USA). The region concerning the internal standard which had been added to all samples between 3.06 and 4.35 min was eliminated. The remaining 2050 integral regions were retained for further analysis. NMR spectra were reduced to 4250 discrete chemical shift regions by digitisation to produce a series of sequentially integrated regions, $\delta$ 0.002 wide, between $\delta$ 0.500 and 9.000, using MestReC 1.1 (Mestrenova, Spain). The resulting data were exported into Pirouette 4.0 and selected regions removed around the residual solvents including water ($\delta$ 4.566–5.078), methanol ($\delta$ 3.244–3.338), and also the lipid region ($\delta$ 1.192–1.454). The remaining 3813 integral regions were retained for further analysis.

Normalization of NMR spectra was carried out using Pirouette 4.0 (Infometrix, USA). The processing method for PCA and PLS was mean-centring without scaling and rotation. Three principal components were chosen for PLS analysis. The dataset was split into two groups of training (7 out of 8 samples) and test (1 sample). The process of generating a PLS model on randomly chosen samples followed by validation was repeated in order to ensure all extracts were excluded once. Models were constructed to predict $IC_{50}$ values on human lung cancer cell line SK-MES-1 *in vitro*.

## 3. Results and discussion

### 3.1. Identification of Scutellaria inhibitors from crude extract

The flavonoid constituents of *S. baicalensis* have been widely reported to inhibit the growth of a broad spectrum of human cancer cells by numerous and competing degenerative pathways [6–8,13–15]. Here, HPLC- and NMR-PCA secondary metabolite maps were constructed to correlate the growth inhibitory activity of crude extracts on human lung cancer cell line SK-MES-1 so revealing the functional biomarkers of *S. baicalensis* root.

### 3.2. HPLC-PCA map

Similar chemical profiles were observed in most of the samples (data not shown). This precluded visual and manual analysis to correlate certain compounds with the growth inhibitory effects. PCA was thus employed to model both HPLC and NMR data and to aid determination of discriminative contributors. As PCA is an unsupervised method, the scores plot constructed from the HPLC data of crude extracts is presented in Fig. 2. Each point represents one HPLC dataset for a given extract and points with the same letter indicate replicates. The position of each point in 3D space is a statistical function of the chemical profile of each sample at 270 nm. Thus samples with similar composition form clusters, while distinct samples are separated in this scores plot. On supervision, as illustrated by the ellipses superimposed in Fig. 2, all 24 samples can be discriminated into three groups. This is consistent with the experimental $IC_{50}$ value of each sample's inhibitory effects on human lung cancer cell lines *in vitro*. Crude extracts with $IC_{50}$ values (>124 μg/ml) are considered as poor inhibitors: 25 and 45% aqueous EtOH (a and b), and water (h). Those of 70% aqueous EtOH (c), EtAc (f) and butanol (g) show intermediate inhibition (58 < $IC_{50}$ < 124 μg/ml). The more potent inhibition ($IC_{50}$ < 58 μg/ml) is located in the crude extracts from absolute EtOH (d) and dichloromethane (e). This map demonstrates good correlation between composition and the inhibition. Furthermore, as the variance is mainly due to the 1st principal component (PC1, 67%) and the 2nd principal component (PC2, 17%), it is possible to determine the contributing variables (HPLC peaks) using the PCA loading plot (data not shown). Peaks with retention times of 12.6 (baicalin), 16.6 (baicalein) and 21.9 min (wogonin) contribute most to PC1 and PC2, as confirmed by spiking samples with commercial standards. This indicates that the variance of growth inhibitory effect of *S. baicalensis* extracts on lung cancer cell line SK-MES-1 depends on the concentration of these compounds. Moreover, according to the location of correlated peaks in the loading plots, baicalin contributed most to the activity of 70% EtOH (c) and butanol (g), while baicalein and wogonin, in particular, had the highest contribution to the EtOH (d) and dichloromethane (e) extracts.

### 3.3. NMR-PCA map

To cross-validate the results obtained from the HPLC-PCA model, PCA was then performed with 400 MHz $^1H$ NMR data (Fig. 3). The unsupervised scores plot showed a different mapping compared with the HPLC-PCA map yet the crude extracts may be similarly supervised into three groups on the basis of measured $IC_{50}$ values (as shown by ellipses). The NMR loadings plot (data not shown) demonstrated that this spectrum was dominated by signals for lipid compounds centred at $\delta$ 1.28. These lipid signals observable in the NMR spectrum are non-detectable by HPLC-UV and are not related to inhibition activity so this spectral region was removed from further NMR-PLS models. The resulting contributions plot (data not shown) revealed the NMR signals responsible for class variance. The most significant contributing resonances also correlate to the three compounds baicalein, wogonin and baicalin: as identifed by the diagnostic $^1H$ singlet at $\delta$ 6.75 for baicalein H-3, the diagnostic H-1 anomeric hydrogen doublet at $\delta$ 5.20 for the sugar conjugate baicalin and the diagnostic 3H H-8 methoxyl singlet for wogonin at $\delta$ 3.92, respectively. The highest contribution to the variation in activity was determined by baicalein as

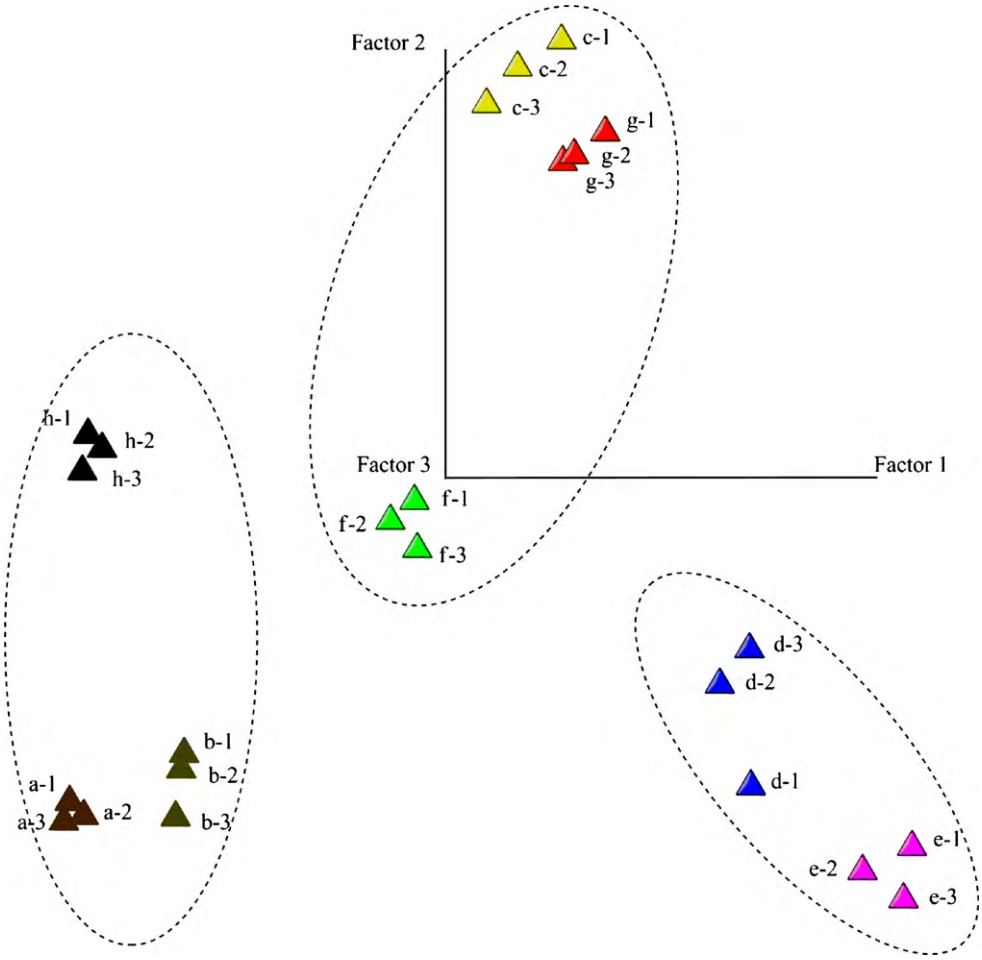


**Fig. 2.** PCA scores plot for *S. baicalensis* crude extracts. Each point on the plot represents one HPLC dataset of an extract and points with the same letter indicate replicates: a: 25% aqueous ethanol; b: 45% aqueous ethanol; c: 70% aqueous ethanol; d: ethanol extract; e: dichloromethane; f: ethyl acetate; g: butanol; h: water.

the signals for H-3 ($\delta$ 6.75) and H-8 ($\delta$ 6.65) in the spectrum of baicalein are of higher intensity than those of H-3 ($\delta$ 7.04) and H-8 ($\delta$ 6.82) in that of baicalin. This NMR-PCA contributions plot therefore cross-validated the HPLC-PCA contributions plot in that baicalein is determined as the most significant inhibitor from the crude extracts.

### 3.4. PLS prediction of inhibition by S. baicalensis crude extracts and constituents

Whilst the non-parametric HPLC-PCA and NMR-PCA models successfully map the high, medium and low bioactivity groups, these PCs do not necessarily align with the best predictive components for class separation. Accordingly, partial least squares regression (PLS) was employed as a type of supervised classification and regression model. This provided a correlation between complex multivariate chemical datasets and the experimental $IC_{50}$. In the corresponding NMR-PLS regression model based on the experimental chemistry data, the $IC_{50}$ values of the crude extracts are imported as the *y*-matrix. Three components were used for all models, with >71% of samples predicted with >99% confidence. Variance ($R^2$) values ranged from 0.992 to 0.997 and cross-validated variance ($Q^2$) values from 0.906 to 0.946. The value of $Q^2$ is considered a measure of the predictive ability of the model and $Q^2 > 0.5$ is generally considered to be good [16]. The model construction process was repeated in order to exclude every extract from the training set once. The overall predictions for each extract were summarised in Table 1. The correlation coefficient between predicted versus actual values is 0.754. This value compares favourably with other predictive models [12,17]. This clearly demonstrated the potential of using orthogonal chemical datasets to predict the pharmacological activity of herbal preparations based on the chemical composition and experimental data. Moreover, if the predicted $IC_{50}$ values were used to classify the extracts again into the high, intermediate and poor activity groups, then all extracts except dichloromethane (e) would be placed in the same group as that of the experimental data model. However, it was only re-mapped from the active into the intermediate activity group. The corresponding HPLC-PLS model, using three PCs, predicted >84% of samples with >99% confidence with values for $R^2$ 0.89–0.99 and $Q^2$ 0.82–0.99. The correlation coefficient of predicted values versus actual values for this model was determined as 0.79, similar to that of the NMR-PLS model. This suggested that by using either NMR spectroscopic or HPLC data in modelling $IC_{50}$ values, it has been possible to reasonably predict the growth inhibitory effects of plant extracts which would normally require larger scale lab-based assays and to specify extracts for further investigation.

### 3.5. Validation of Scutellaria inhibitors of human lung cancer cells

The concentration of baicalin, baicalein and wogonin in the eight extracts were determined by standard calibration curve and summarized in Table 1. In general, increasing activity of extracts correlates with an increasing concentration of baicalin, baicalein and wogonin. To further validate this prediction, the individual components were tested for inhibition on SK-MES-1 cells *in vitro*.

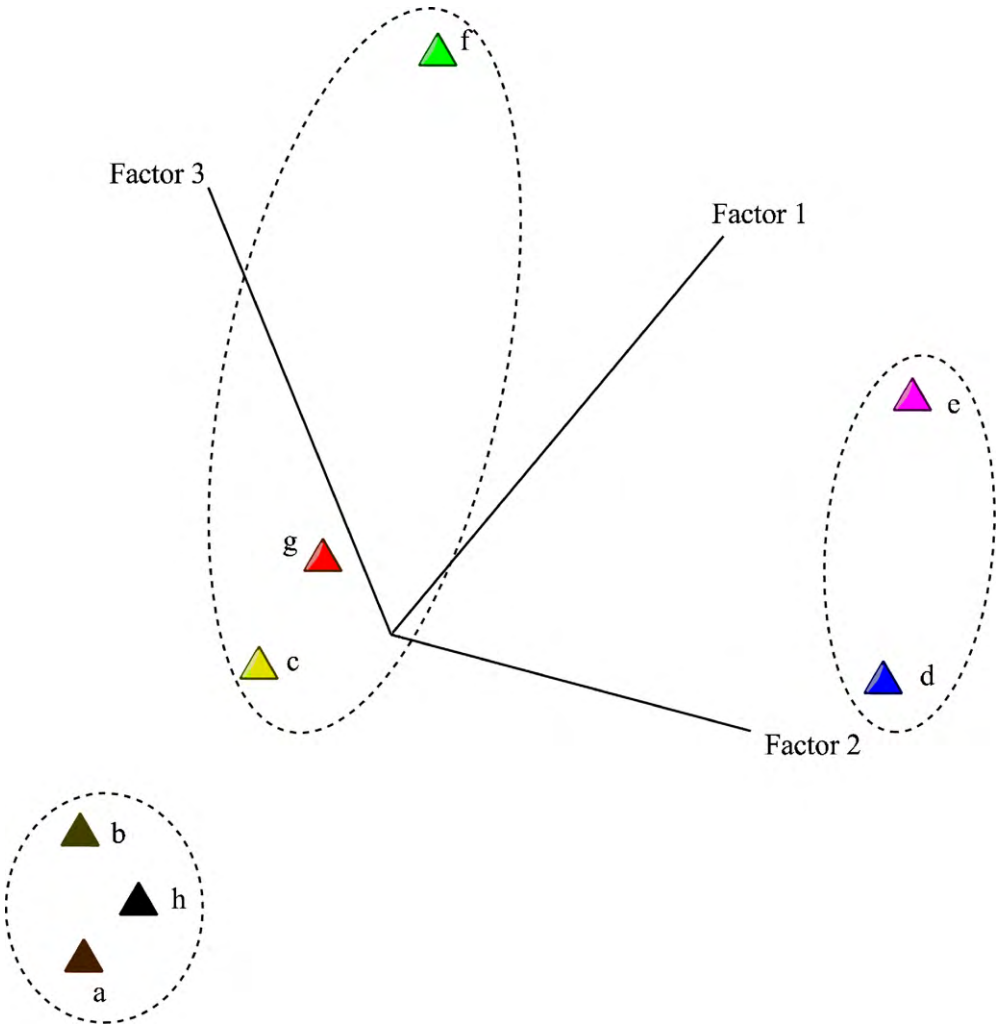


**Fig. 3.** PCA scores plot for *S. baicalensis* crude extracts. Each point on the plot represents one NMR dataset of an extract: a: 25% aqueous ethanol; b: 45% aqueous ethanol; c: 70% aqueous ethanol; d: ethanol extract; e: dichloromethane; f: ethyl acetate; g: butanol; h: water.

**Table 1**
Experimental and predicted $IC_{50}$ values of *S. baicalensis* crude extracts on SK-MES-1 lung cancer cells and concentration of baicalin, baicalein and wogonin in each extract: a: 25% aqueous ethanol; b: 45% aqueous ethanol; c: 70% aqueous ethanol; d: ethanol extract; e: dichloromethane; f: ethyl acetate; g: butanol; h: water. Results represent the mean ± S.D. of three independent experiments.

| Extract | $IC_{50}$ measured value (μg/ml) | $IC_{50}$ predicted by NMR model (μg/ml) | $IC_{50}$ predicted by HPLC model (μg/ml) | Baicalin (μg/ml) | Baicalein (μg/ml) | Wogonin (μg/ml) |
|---|---|---|---|---|---|---|
| a: 25% Aqueous ethanol | 249 ± 5 | 165 | 199 ± 3 | nd | 2 ± 0 | 1 ± 0 |
| b: 45% Aqueous ethanol | 175 ± 15 | 200 | 161 ± 7 | 1 ± 0 | 103 ± 2 | 16 ± 0 |
| c: 70% Aqueous ethanol | 82 ± 14 | 124 | 100 ± 2 | 269 ± 16 | 362 ± 20 | 85 ± 5 |
| d: Ethanol | 57 ± 6 | 34 | 96 ± 5 | 109 ± 8 | 451 ± 23 | 169 ± 8 |
| e: Dichloromethane | 42 ± 13 | 88 | 44 ± 4 | 44 ± 1 | 530 ± 4 | 238 |
| f: Ethyl acetate | 83 ± 19 | 60 | 94 ± 4 | 99 ± 7 | 234 ± 9 | 78 ± 2 |
| g: Butanol | 107 ± 16 | 102 | 45 ± 6 | 228 ± 15 | 333 ± 3 | 90 ± 1 |
| h: Water | 124 ± 11 | 166 | 191 ± 12 | 222 ± 4 | 43 ± 1 | 9 ± 0 |

All three inhibited cancer cell growth in a dose-dependent manner (data not shown). The $IC_{50}$ values for baicalin, baicalein and wogonin were found to be 28.6 ± 2.3 μg/ml (64 ± 5 μM), 21.5 ± 1.5 μg/ml (80 ± 6 μM), and 11.0 ± 2.7 μg/ml (39 ± 10 μM). These are comparable to that of the antineoplastic cisplatin ($IC_{50}$: 79 ± 16 μM). These values are similar to *in vitro* growth inhibition values reported for other cancer cell types. All three functional biomarkers decreased the viability of HOS human osteogenic sarcoma cells at 200 μM [18]. Bonham et al. reported similar $IC_{50}$ values on human prostate carcinoma cells (LNCap) of 13 and 42 μM and on PC-3 cells of 25 and 50 μM, for baicalein and wogonin, respectively [13]. Our data indicated similar growth inhibition activity on the human cancer cell line SK-MES-1 that we have related to the actual concentration of three functional biomarkers in crude extracts.

## 4. Conclusion

*S. baicalensis* constituents baicalein and wogonin are inhibitors of human lung squamous cancer cell line growth with activity comparable to that of cisplatin ($IC_{50}$: 79 ± 16 μM). Secondary metabolite mapping quickly identified functional compounds from crude plant extract. This approach was an important first step

to qualify Chinese herbal prescriptions required for meaningful clinical trials of such integrated therapies. Moreover, this work demonstrated the potential of NMR/HPLC-PLS analysis as a tool for the prediction of growth inhibitory activities of plant extracts.

## Acknowledgements

JG and OC acknowledge PhD scholarship and sabbatical funding, respectively, from the School of Health and Bioscience, University of East London. JG received kind financial assistance from the Sir John Stapely Education Fund (GlaxoWellcome, UK). HZ is grateful for Overseas Research Students Awards Scheme (ORSAS) by Secretary of State for Education and Science and King's Annual Fund Studentship (KAF) from King's College London.

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