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Differential Protein Expression of the Inhibitory Effects of a Standardized Extract from *Scutellariae radix* in Liver Cancer Cell Lines Using Liquid Chromatography and Tandem Mass Spectrometry

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The purpose of this study is to verify the inhibitory effect of a chemically standardized extract from *Scutellariae radix* in liver cancer cell lines (HepG2). The botanical extract was prepared using pressurized liquid extraction (PLE). A method using proteolytic digest with single dimensional and two-dimensional liquid chromatography with tandem mass spectrometry was used to characterize differential protein expression in mammalian cells in response to the botanical extract. The whole cell lysates were digested with trypsin, and the peptides were separated by one-dimensional (reversed phase) or by two-dimensional (cation exchange and reversed phase) solid-phase extraction (SPE) cleanup and separated by liquid chromatography with UV detection and mass spectrometry. In the presence of the botanical extracts, drug-induced apoptosis was not observed, and a number of proteins that played an important role in the metabolic pathways in HepG2 cell line had been affected. The data, as presented, suggest that the inhibitory effects of the standardized extracts from *Scutellariae radix* resulted from expression of heat shock protein and other proteins related to energy metabolism. The proposed platform had the potential to provide significant information about the particular proteome such as human hepatoma HepG2. At the molecular level, it was possible to study the proteins and how their levels and modifications change in response to the effects of the botanical extract.

KEYWORDS: ***Scutellariae radix*; differential protein expression; liquid chromatography; tandem mass spectrometry; inhibitory effects; liver cancer cell lines**

INTRODUCTION

Scutellariae radix or *Scutellaria baicalensis* is a medicinal plant found in the Chinese Pharmacopoeia (*1*) and Japanese Pharmacopoeia (*2*). The active components consist of major flavonoids such as baicalein, baicalin, wogonin, and wogonosides. For botanical drugs and herbal preparations, there is a need to approach scientific proof and clinical validation with chemical standardization, biological assays, animal models, and clinical trials. Hence, the evaluation of the biological activities of botanical extracts is an important parameter.

The oxidative modification of proteins plays a major role in a number of human diseases including Alzheimer's disease. Flavones in *Scutellariae radix* or *Scutellaria baicalensis* can attenuate apoptosis and protein oxidation in neuronal cell lines (*3*). The flavonoid baicalin isolated from dried roots of *Scutellaria baicalensis* was found to have a direct antitumor effect on human prostate cancer cells (*4*). Other findings suggested that baicalin, baicalein, and wogonin from *Scutellaria baicalensis* might be effective candidates for inducing apoptosis or inhibiting proliferation in various human hepatoma (*5*, *6*). The effects of baicalin on the gene expression of surfactant protein A (SP-A) in lung adenocarcinoma cell lines H441 had been reported (*7*). The inhibitory effects of cancer cell proliferation and antitumor effects of the total extract from *Scutellariae radix* were well studied (*8*−*10*). In an in vivo experiment, significant inhibition of tumor growth had been observed (*8*−*10*). However, studies using chemically standardized botanical extracts were limited.

To study the proteins and how their levels and modifications change in response to the external factors, proteomics approach using two-dimensional gel electrophoresis and mass spectrometry (MALDI/MS and ESI/MS) in classifying the biochemical basis of the anti-cancer activity of the new olomoucine-derived synthetic cyclin-dependent kinase inhibitor, bohemine, was reported (*11*). The protein pattern changes of Burkitt lymphoma cell lines (DG 75) and modification of membrane-associated proteins following treatment with 5′-azycytidine was monitored using two-dimensional gel electrophoresis and mass spectrometry (*12*, *13*). A pancreatic adenocarcinoma cell line (Paca44) was treated with trichostatin-A, a potent inhibitor of histone

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deacetylases, to evaluate the effect of this drug on protein expression with two-dimensional gel electrophoresis and mass spectrometry (*14*).

The isotope-coded affinity tag (ICAT) technology enables the concurrent identification and comparative quantitative analysis of proteins present in cell and other biological samples. The ICAT technology with multidimensional liquid chromatography was used to identify and determine the ratio of proteins in microsomal fractions of naïve and in-vitro differentiated human myeloid leukemia (HL-60) cells. For most proteins identified in the microsomal fraction in the HL-60 cells, the abundance in the control and 12-phorbol 13-myristate acetate (PMA) treated cells did not change significantly. Unchanged abundance was particularly prevalent for ribosomal proteins, cytoskeletal proteins, metabolic enzymes, the majority of cell surface receptors, and channel proteins. In contrast, some of the membrane associated signal transduction proteins and enzymes showed significant changes in their d0:d8 ratio, pointing toward a regulatory mechanism that is directly or indirectly due to PMA treatment (*15*).

Nonporous (NPS) reversed-phase HPLC had been used to rapidly separate proteins from whole cell lysates of human breast cell lines. It was demonstrated that the expressed protein profiles change during neoplastic progression and that many oncoproteins were readily detected. It was shown that the response of premalignant cancer cells to estradiol could be rapidly screened by this method (*16*). A multidimensional chromatographic 2-D liquid-phase separation method had been developed for differential display of proteins from cell lysates and applied to a comparison of protein expression between Peninsularinone-treated and untreated HCT-116 human colon adenocarcinoma cells. The method involved fractionation according to pI using chromatofocusing with analytical columns in the first dimension followed by separation of proteins in each pI fraction using nonporous reversed-phase HPLC (*17*).

The aim of the current work was to study the inhibitory effects of chemically standardized *Scutellariae radix* extracts in liver cancer cell lines. A method using proteolytic digest with single dimensional and two-dimensional liquid chromatography with tandem mass spectrometry was used to characterize differential protein expression in mammalian cells such as HepG2 in response to the botanical extract. The whole cell lysates were digested with trypsin, and the peptides were separated by one-dimensional (reversed phase) or by two-dimensional (cation exchange and reversed phase) liquid chromatography with ultraviolet detection and tandem mass spectrometry.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Dulbecco's modified Eagles medium (DMEM), penicillin, streptomycin, and trypsin-EDTA were bought from Hyclone (Logan, UT). Fetal bovine serum (FBS) was obtained from Biological Industries (Israel). Dimethyl sulfoxide (DMSO), methanol, and acetonitrile of HPLC grade were purchased from APS (NSW, Australia). Pure water was obtained from Millipore Alpha-Q water system (Bedford, MA). Sequencing grade modified trypsin was purchased from Promega (Madison, WI). Formic acid and ammonium acetate were purchased from Merck (Darmstadt, Germany).

Cell Cultures. All cell lines were obtained from ATCC. Human hepatoma HepG2 was maintained with Eagle's Minimum Essential Medium with Earle's BSS and 2 mM L-glutamine (EMEM) that is modified to contain 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, and 1.5 g/L sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL stretomycin, incubated at 37 °C and 5% CO_2 atmosphere.

Preparation of Plant Extracts. To prepare a homogeneous sample, the roots of *Scutellariae radix* were ground using an IKA MF10 microfine grinder (Staufen, Germany) with sieve insert of hole size 0.5 mm. The botanical extract was prepared using pressurized liquid extraction (PLE) as reported earlier (*18*). Briefly, methanol was used as the solvent for pressurized liquid extraction of 0.5 g of plant material. The extraction cell was prefilled with methanol to check for possible leakage before setting the temperature of the oven to 120 °C. Extraction with methanol was carried out for a period of 20 min, and 20−25 mL of methanol was collected into a 25 mL volumetric flask. Between runs, the system was washed with methanol for 5 min. The content of baicalein present in *Scutellariae radix* was determined by reversed-phase HPLC as in an earlier report (*18*). Two milliliters of the solution was transferred into a test tube. Excess methanol was evaporated under a stream of nitrogen and dried in a vacuum oven at 50 °C for 12 h before use.

For in-vitro studies, the standardized extract obtained was dissolved into 200 μL of DMSO and 800 μL of media to form solution A. The solution was prepared before in-vitro study. Two hundred microliters of solution A was mixed with 5 mL of media to form solution B for time-dependent and other interaction studies. The final concentration of DMSO was kept at less than 0.8% to avoid inhibitory effects of the cells.

Cell Growth Analysis. Cell growth inhibition assays were performed by plating 1×10^5 cells on 25 cm^2 tissue culture flask, in EMEM supplemented with 10% FBS for HepG2. Cells were treated with extracts from *Scutellaria baicalensis* or control with DMSO for 24, 48, and 72 h, respectively. For dose-dependent studies, 50, 100, and 200 μL of solution A with 5 mL of media was used and cells were treated with extract or control for 48 h. The number of cells was determined with a hemocytometer. Cell viability was measured by tryphan blue dye exclusion.

DNA Fragmentation Analysis. Cells were plated on 75 mm tissue culture plates treated with *Scutellaria baicalensis* extract or control with DMSO for 48 h. Briefly, 1×10^7 cells were lysed in lysis buffer (10 mM Tris-Cl, 0.1 M EDTA, 20 μg/mL pancreatic RNAse A, 0.5% SDS, with added 100 μg/mL Proteinase K) at 55 °C for 3 h, and then lysate was centrifuged for 2×1000 rpm for 3 min. The supernatant containing fragmented DNA was collected and was extracted with phenol/chloroform (50:50) and chloroform. The aqueous layer was then precipitated with 100% alcohol and 5 M sodium chloride at −20 °C overnight. Centrifugation was done at 12 000 rpm for 30 min to pellet down the DNA, which was semi-dried and dissolved in 10 μL of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) buffer, then with electrophoresis in a 1.5% agarose gel contained ethidium bromide (1 μg/mL) in TE buffer. After electrophoresis, the gel was imaged using the UV light.

Flow Cytometry Analysis. A total of 1×10^6 cells were plated on a 75 cm^2 tissue culture flask and incubated for 24 h at 37 °C. Cells were treated with extracts from *Scutellaria baicalensis* or control with DMSO for another 48 h. For treatment with *Scutellaria baicalensis*, 400 μL of solution A with 10 mL of media was used for dose-dependent studies. Cells were then washed, pelleted, and fixed with cold 70% ethanol for at least 30 min. Before analysis, the 70% ethanol was removed by spinning at 2000 rpm and washed with phosphate saline buffer (PBS). The resulting solution was incubated with 100 μg/mL of Rnase A and 50 μg/mL of propidium iodide at room temperature for 30 min. Samples were immediately analyzed by flow cytometry (Becton Dickinson, San Jose, CA). Cell cycle distribution was determined using Modfit software (Verify Software House, Topsham, ME).

Preparation and Digestion of Total Cell Lysates. Cells (1×10^5) were plated on a 25 cm^2 tissue culture flask treated with *Scutellaria baicalensis* extract or control with DMSO for 48 h. Hep G2 cell extract was prepared from cultured cells using a commercial kit, M-Per (Pierce, Rockford, IL). To harvest the cells, the growth medium was aspirated and the cells were washed with PBS. The proteins were extracted from the cells with 1.5−2 mL of M-Per reagent with the aid of a cell scrapper. The extracts were sonicated for 15 min and centrifuged at 13 000 rpm for 20 min. The supernatant was removed from the cell debris, and the cell lysates were used immediately. Protein concentration was assayed using Bradford assay reagent (Pierce).

The proteins were reduced with DTT (3 μL of 1000 mM in water). The mixture was incubated at 37 °C for 30 min. To alkylate the protein, idoacetamide (7 μL of 1000 mM in water) was added and the mixture

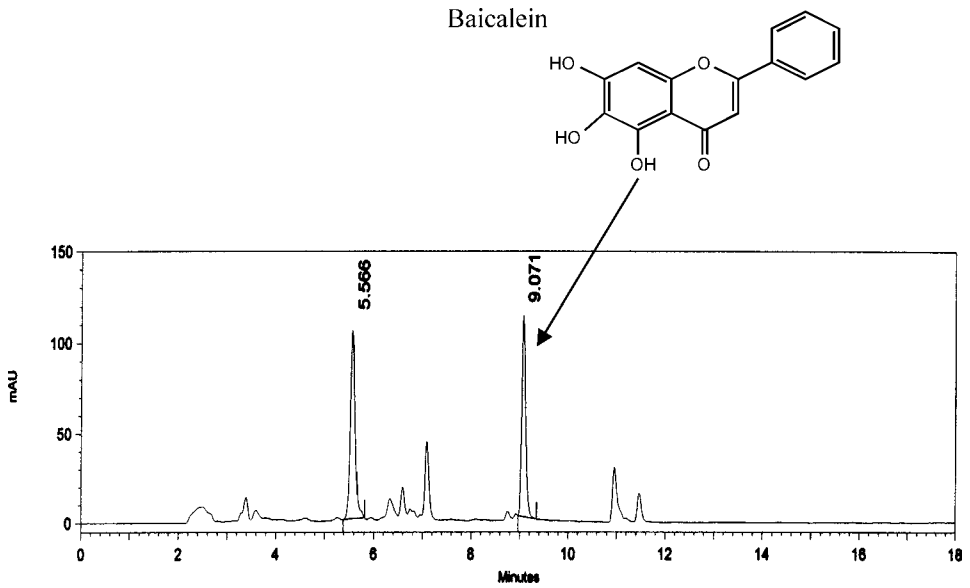

Figure 1. Chromatogram obtained for baicalein in *Scutellariae radix* by PLE. Mobile phase of (**A**) 25 mM NaH_2PO_4 at pH 2.5 and (**B**) acetonitrile. Initial condition was set at 30% of B, gradient up to 100% B in 15 min before returning to initial condition for 10 min. Detection was at 254 nm. Oven temperature was at 40 °C, and flow rate was 1.0 mL/min.

was incubated at room temperature for an additional 30 min in the dark. An additional 13 μL of DTT (1000 mM in water) was added to react with the excess idoacetamide. The reduced and alkylated proteins were digested with sequencing grade trypsin (1:50) for 18 h. For single dimensional liquid chromatography platform, the enzymatically digested samples were subjected to solid-phase extraction, 500 mg Strata (Phenomenex, Torrence, CA) C-18 SPE column conditioned with 10 mL of methanol, water, and water with 0.1% acetic acid before loading the enzymatically digested samples. The loaded SPE columns were washed with 5 mL of water with 0.1% acetic acid before eluting with 1.0 mL of acetonitrile. Excess solvent was evaporated under a stream of nitrogen, and the sample was reconstituted in 100 μL of water with 0.1% acetic acid.

For the two-dimensional liquid chromatography platform, 5 mL of 5 mM of ammonium acetate was added to the whole cell lysates. The 500 mg solid-phase extraction cartridges, Strata (Phenomenex, USA) SCX column was conditioned with 10 mL of water, 5 mL of 5 mM ammonium acetate, 5 mL of 500 mM ammonium acetate, followed by 20 mL of 5 mM ammonium acetate before loading the whole cell lysates. The whole cell lysates with 5 mM of ammonium acetate added were allowed to pass through the SCX column and the first fraction was collected. The peptides on the SCX column were eluted with 5 mL of 10, 25, 50, 100, 250, and 500 mM of ammonium acetate, respectively. For all seven fractions obtained, the samples were subjected to C-18 SPE column cleanup. Excess solvent was evaporated under a stream of nitrogen, and the sample was reconstituted in 200 μL of water with 0.1% acetic acid as stated above before analysis by LCMS.

Reversed-Phase HPLC/MS or HPLC/MSMS Analysis for Protein Expression. For LC/MS, a Shimadzu LC 10 series (Kyoto, Japan) equipped with a binary gradient pump, autosampler with sample cooler, column oven, and diode array detector was coupled with a Shimadzu LCMS-2010A quadruple mass spectrometer. The gradient elution used a mobile phase consisting of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The initial condition was set at 5% of B, gradient up to 40% in 90 min, up to 90% in the next 5 min, and returning to initial condition for 15 min. Detection was at 214 nm. Oven temperature was set at 40 °C, and flow rate was set at 100 μL/min. For all experiments, 100 μL of sample extract was injected. The column used for the separation was a reversed-phase C18 Jupiter, 150 $\times$ 1.0 mm, 5 μ, 300 Å (Phenomenex). The ESI-MS was acquired in the positive ion mode. The probe voltage was set at 4.5 kV; curve desolvation line temperature was at 250 °C. The scanning mass range was from 400 to 1600.

For LC/MSMS experiments, an Agilent 1100 series (Waldbronn, Germany) equipped with a quaternary gradient pump, autosampler with sample cooler, column oven, and diode array detector was coupled with a LC/MSD Trap VL ion trap mass spectrometer. The gradient elution used a mobile phase consisting of (A) water with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The initial condition was set at 5% of B, gradient up to 40% in 70 min, up to 90% in the next 5 min, and returning to initial condition for 15 min. Detection was at 214 nm. Oven temperature was set at 40 °C, and flow rate was set at 200 μL/min. For all experiments, 30 μL of sample was injected. The column used for the separation was a reversed-phase C18 Jupiter, 150 $\times$ 2.0 mm, 5 μ, 300 Å (Phenomenex). The ESI-MS was acquired in the positive ion mode. The scanning mass range was from 400 to 1500. The heated capillary temperature was maintained at 350 °C, and the drying gas and nebulizer nitrogen gas flow rates were 10 L/min and 50 psi, respectively. Data were acquired using automated MSMS. The target was set at 30 000; maximum accumulation time, 300 ms; the number of average scans, five; and SmartSelect was used.

Database Searching Procedure of MS/MS Data for Protein Identification. Mass data collected during a LC/MSMS run were submitted to the search software Mascot (http://www.matrixscience-.com/). Preliminary protein identifications were obtained by comparing experimental data to NCBInr database and Swiss-prot. The taxonomy was set to Homo sapien (human), one missed cleavage was allowed, and carbamidomethyl was selected for fixed modifications. Searches were done with a tolerance on mass measurement of 1.0 Da in MS mode. A sequence tag of several continuous amino acids (5−20 residue) and the peptide mass were generally sufficient to identify the protein of a peptide. The raw data were inspected manually for confirmation prior to acceptance.

RESULTS AND DISCUSSION

Preparation of Botanical Extracts. In the study of the biological activity and toxicity of botanical extracts, the method of extraction is an important step. It had been observed that extracts with varying active components or marker compounds are obtained when using different methods of extraction (*18*−*21*). This will in turn affect the bioactivity and toxicity of the botanical extracts obtained. Hence, the method of extraction used should be validated to ensure that it has the required precision and extraction efficiencies.

Pressurized liquid extraction (PLE) was used to extract the components present in *Scutellariae radix*. The technique used

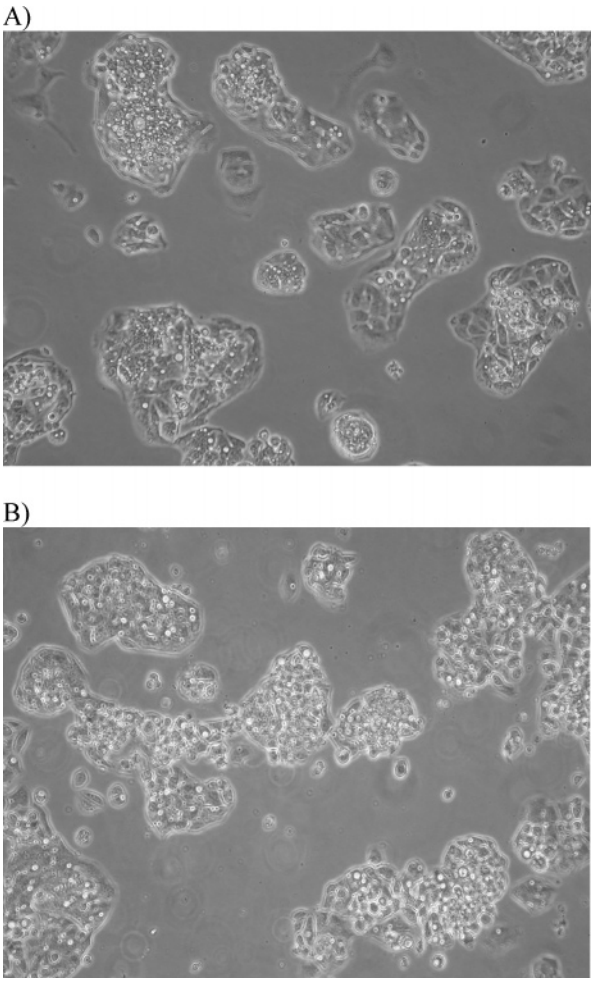

Figure 2. Morphology of HepG2 (10× magnification) (**A**) control with DMSO and (**B**) treated with extract from *Scutellariae radix* (solution B) after 48 h.

organic solvent such as methanol at elevated temperature and pressure, which drastically improved the speed of the extraction process. The method for solid sample preparation was found to have good method precision and comparable or higher extraction efficiencies with reference to Soxhlet extraction and sonication for medicinal plants such as *Scutellariae radix*, *radix glycyrrhizae*/liquorice, and others (*18*−*21*). The amount of extract obtained from the medicinal plant was found to be 112.5 ± 12.6 mg ($n = 4$), and the baicalein was determined by HPLC to be 14.26 ± 0.48 mg/g ($n = 5$). A chromatographic fingerprint was shown in **Figure 1**.

Standardized Extracts from *Scutellariae radix* Inhibit Cell Growth. The effects of the extract from *Scutellariae radix* on HepG2 cell lines are shown in **Figure 2**. It was observed that there was no significant morphological change between the control and treated cell. However, more floating cells were observed in the treated HepG2 cell line. Cells were treated with DMSO and botanical extracts, and the growth rate was evaluated after 1, 2, 3, and 4 days. As shown in **Figure 3**, the growth rate of HepG2 cells line treated with the botanical extract was significantly lower as compared to control cells treated with vehicle alone. The results were repeated on a different day, and a growth rate similar to that in **Figure 3** was observed. Extract from *Scutellariae radix* was observed to display a dose-dependent growth of the HepG2 cell (data not shown).

Using a DNA fragmentation assay, we observed that extract from *Scutellariae radix* did not cause the DNA genomic

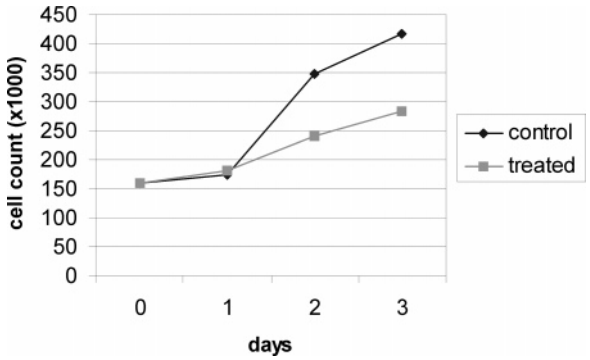

Figure 3. Inhibition of HepG2 cell proliferation by extract from *Scutellariae radix*. Cell growth inhibition assay was performed by plating 2×10^4 cell in a 25 cm^2 flask, in DMEM with 10% FBS. Cells were treated with extract from *Scutellariae radix* (solution B) or DMSO every 48 h for 4 days, and the number of cell was determined at day 0, 1, 2, 3, and 4 by counting with a hemocytometer. The viability of cells was measured by trypan blue dye exclusion.

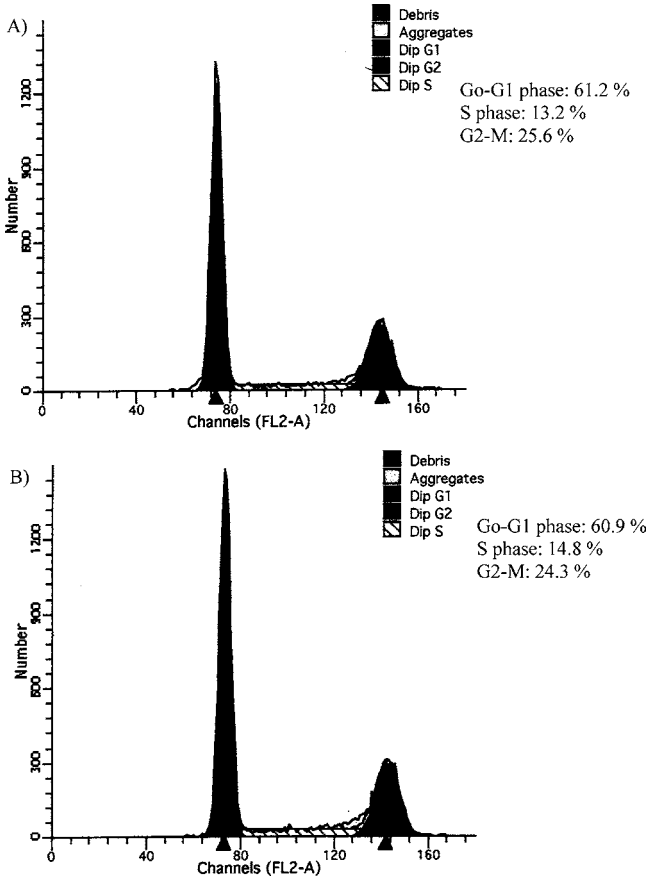

Figure 4. Cell cycle analysis by flow cytometry. The HepG2 cells lines were (**A**) treated with DMSO for control and (**B**) treated with extract from *Scutellariae radix* (solution B) for 48 h. The histogram showed that there was no significant change in the G_0–G_1, S, and G_2–M phase in the control and treated sample.

extracted and separated by gel electrophoresis to have significant DNA fragmentation (data not shown). The effects of the botanical extract on the cell cycles were analyzed using flow cytometry. From **Figure 4**, it was found that significant changes in the G_0-G_1, S, and G_2-M phase were not observed in both the control and the treated sample.

Our data on the inhibitory effects of standardized *Scutellariae radix* extract on HepG2 cell line were consistent with other

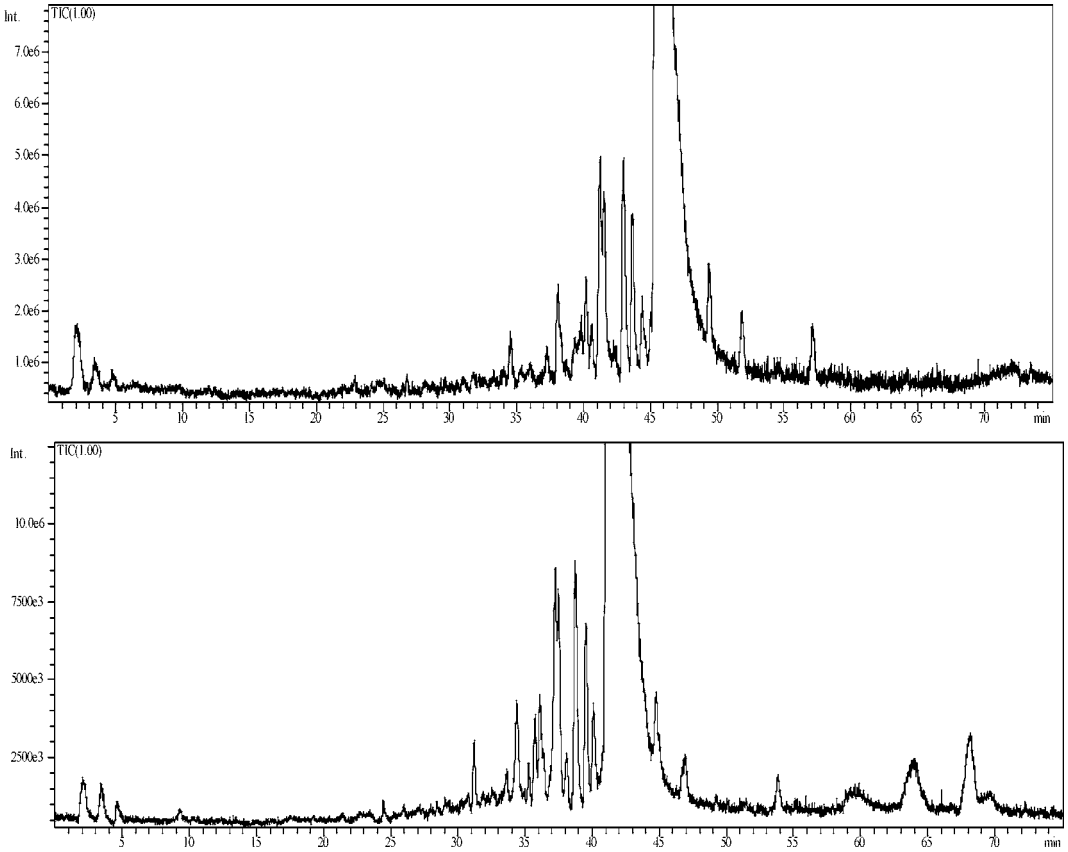

Figure 5. Chromatograms (TIC) of (**A**) fraction 2 of whole cell lysate from HepG2, flask 1, and (**B**) fraction 2 of whole cell lysate from HepG2, flask 2, with two-dimensional HPLC/MS.

works where it was reported to inhibit the growth of head and neck squamous cell carcinoma such as SCC-25 and KB (*10*) and bladder cancer cells lines such as KU-1 and EJ-1 (*8*). Cell cycle analysis by flow cytometry with HepG2 cell lines showed that a significant increase in the sub G_1 phase was not observed between the control and treated sample. A significant increase in the sub G_1 phase is indicative of induction of apoptosis as observed in baicalein, baicalin, and others in HepG2 cell lines (*5*, *6*). Our data suggested that no significant induction of apoptosis was observed at the concentration of *Scutellariae radix* used in the current work. It was reported that inhibition of cancer cell growth for SCC-25 and KB cell lines is because of its ability to cause G_0-G_1 phase arrest using flow cytometry rather than apoptosis. *Scutellariae radix* was reported to inhibit cyclooxygenase (COX)-2 expression. With flow cytometry, terminal deoxynucleotidyl tranferase-mediated nick end labeling (TUNEL) assay, and DNA ladder assay, no significant induction of apoptosis was observed at all concentrations of *Scutellariae radix* extract on the cancer cells such as SCC-25 and KB studied (*10*). However, extract from *Scutellariae radix* was reported to have differences in biological effects as compared to individual componets such as baicalin, baicalein, and others present. This suggested the synergistic effect among components present in the plant (*4*−*6*, *10*).

Proteomic Analysis of HepG2 by Single and Two-Dimensional HPLC. The method using proteolytic digest with one-dimensional and two-dimensional liquid chromatography with tandem mass spectrometry had been applied to the profiling of differential protein expression of liver from mouse in the control group and groups treated with botanical extracts. The proposed method was reported to be able to identify changes at the molecular level and have a high level of reproducibility (*22*). Quantitative profiling of tryptic digest of proteins in complex mixtures without isotope labeling using liquid chromatography and mass spectrometry had been reported. Expected and calculated protein ratios differ no more than 16% (*23*). Identification of biological markers in human urine with whole mixture proteolysis followed by two-dimensional liquid chromatography and tandem mass spectrometry without the use of stable isotope was reported (*24*). Global protein identification and quantification technology in epidermal cell lines grown in the presence or absence of epidermal growth factor using two-dimensional liquid chromatography nanospray mass spectrometry without the use of stable isotope was reported (*25*).

The proteins present in HepG2 cell line had been identified using 2-D gel electrophoresis and mass spectrometry (*26*) and multidimensional liquid chromatography mass spectrometry (*27*). A representative selection of proteins and protein families such as (A) Chaperones, Chaperoins, and folding catalyst, (B) heterogeneous nuclear ribonucleoproteins, (C) 14-3-3 proteins, (D) Rab family, and (E) proteasome were identified through various shotgun proteomic methods (*27*). It was observed that different compounds such as indomethacine, cisplatin, tetracycline, and paracetamol would cause different changes in the expression of proteins present in HepG2 cell line (*26*).

The chromatograms in **Figure 5A**,**B** showed the fraction 2 of the tryptic digest of HepG2 cell line from two different cell lysates with two-dimensional HPLCMS. The data in **Figure 6** obtained were consistent with other reports (*22*, *23*) and showed that good reproducibility was obtained for fraction 2 and other fractions. With one-dimensional HPLC, it was possible to identify any changes in the expression of the respective peptides between the control and the treated cell. Using prefractionation on the SCX cartridges on a vacuum manifold, it was possible

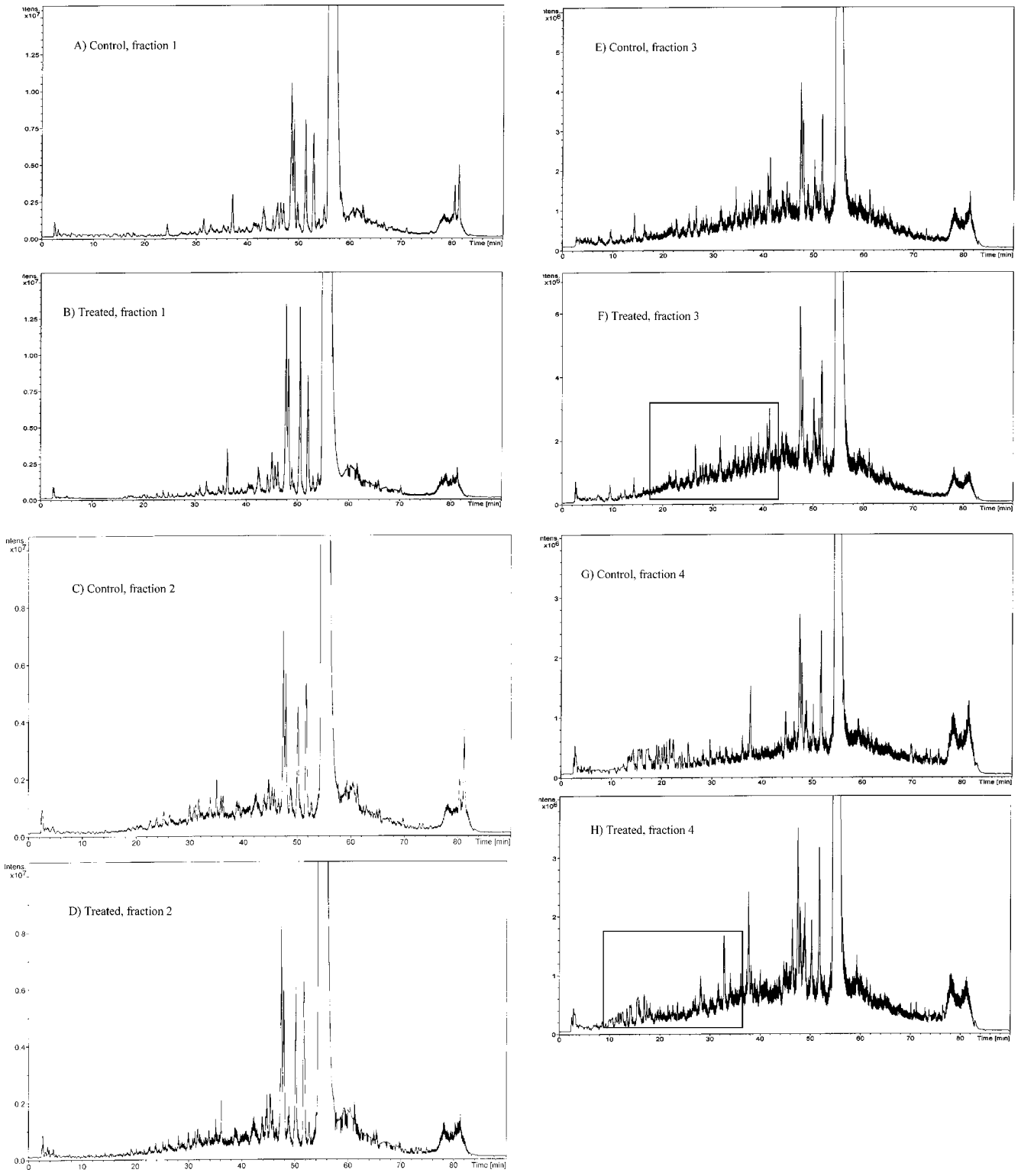

Figure 6. Chromatograms (TIC) from LCMSMS of (**A**) fraction 1 of HepG2 cell line, control with DMSO, (**B**) fraction 1 of HepG2 cell line, from treated group, (**C**) fraction 2 of HepG2 cell line, control with DMSO, (**D**) fraction 2 of HepG2 cell line, from treated group, (**E**) fraction 3 of HepG2 cell line, control with DMSO, (**F**) fraction 3 of HepG2 cell line, from treated group, (**G**) fraction 4 of HepG2 cell line, control with DMSO, and (**H**) fraction 4 of HepG2 cell line, from treated group from two-dimensional HPLC/MSMS.

to identify the expression changes for any minor components as shown in **Figure 6**. With LCMSMS and the assistance of the software by superimposing the chromatograms from the control sample and treated group, it was possible to identify any changes in the peptides present. It was observed that a profile similar to that in **Figure 6** from different fractions was observed for whole cell lysates prepared on different days. As seen in **Figure 6A**,**B**, a number of peptides remained unchanged in the control and treated group for fraction 1. It was proposed that they were likely to be house-keeping proteins as observed in our earlier work (*22*) and other reports (*15*−*17*). The presence of peptides from house-keeping proteins assisted in the identification of peptides from proteins where their expression had been modified. Similarly, a number of other peptides whose

Table 1. Identification of Proteins in the HepG2 Cell Line Found To Be Significantly Different (More than 2 Times) in the Control and Treated Group

MSDB accession	mass	description	
CAD13240	5624	dJ1092K5.2 (novel protein similar to cytochrome *c* oxidase subunit Vic (COX6C))	up-regulated
S09036	71 209	DnaK-type molecular chaperone HSPA6	up-regulated
CAD54446	45 778	vaccinia-related kinase 2	up-regulated
Q9HB23	71 795	Lysyl-tRNA synthetase	up-regulated
S57342	107 198	endopeptidase La homologue (EC 3.4.21.-) precursor, mitochondrial (version 1)	down-regulated

expressions were modified were observed in the chromatograms obtained from fractions 2, 3, and 4 in **Figure 6C−H**, respectively.

A list of proteins where the expression was modified more than 2-fold identified using tandem mass spectrometry after treatment is given in **Table 1**. The criteria was selected as it was observed that the proposed method did not induce variation higher than 2-fold (peak reduction or increased by two times). The current approach of identification of proteins was similar to other works (*27*−*29*). Proteins such as myosin light chain alkali and others where the expression was unchanged in the treated and control HepG2 cell line were identified using the current methodology. Some of the proteins identified using the current approach were consistent with that obtained by 2-D gel electrophoresis and mass spectrometry for HepG2 cell line (*26*). An apparent weakness of the current approach was that it would be rather difficult to identify expression changes of less than 2-fold due the variation of signals produced by the MS. Using conventional MSMS processing software such as Mascot, the mass and amino acid type for the modifications must be known before the search is performed. It will be difficult or is not possible to identify peptides bearing adducts that may be present of unknown mass or at unanticipated amino acid targets. Hence, there were a number of peptides with expression changes where it was not identified. For DnaK-type molecular chaperone HSPA6, the tandem mass spectrum derived by collision-induced dissociation of the precursor ion, *m*/*z* 796.6, was shown in **Figure 7**.

The inhibitory effects of the standardized extract from *Scutellariae radix* resulted in a change in the expression of a number of proteins present in the HepG2 cell line, all of which are highly expressed in the liver, and most of them played an important role in metabolic pathways. This included the up-regulation of a novel protein similar to cytochrome *c* oxidase subunit Vic (COX6C). Baicalin was reported to induce differential expression of cytochrome *c* oxidase in human lung adenocarcinoma H441 cell. The induction of cytochrome *c* oxidase might increase ATP level in cell, elevating cAMP that up-regulates surfactant synthesis and secretion (*31*). Proteins that had their molecular function related to ATP that were down-regulated included Lon protease homologue, mitochondrial [precursor]. For serine/threonine protein kinase VRK2 and Lysyl-tRNA synthetase, both were up-regulated. For Lon protease homologue, mitochondrial [precursor], its molecular function included ATP-dependent peptidase activity and biological process included proteolysis and peptidolysis. Vaccinia-related kinase 2 (Type IV membrane protein) was reported to be similar to the Ser/Thr protein kinase family (VRK subfamily). Its catalytic activity included ATP + a protein = ADP + a phosphoprotein. As for Lysyl-tRNA synthetase, its molecular function involved aspartyl-tRNA synthetase activity. It catalyzed the reaction, ATP + L-aspartate + tRNA(Asp) = AMP + diphosphate + L-aspartyl-tRNA(Asp), interacting selectively with ATP, adenosine 5′-triphosphate, and a universally important coenzyme and enzyme regulator (http://us.expasy.org/sprot). The data obtained were consistent with other reports in the profiling of protein patterns following treatment of Burkitt lymphoma cell line (DG 75) with 5′azycytidine where a number of proteins such as transaldolase, malate dehydrogenase, and others regulating energy metabolism were up-regulated (*12*). The expression

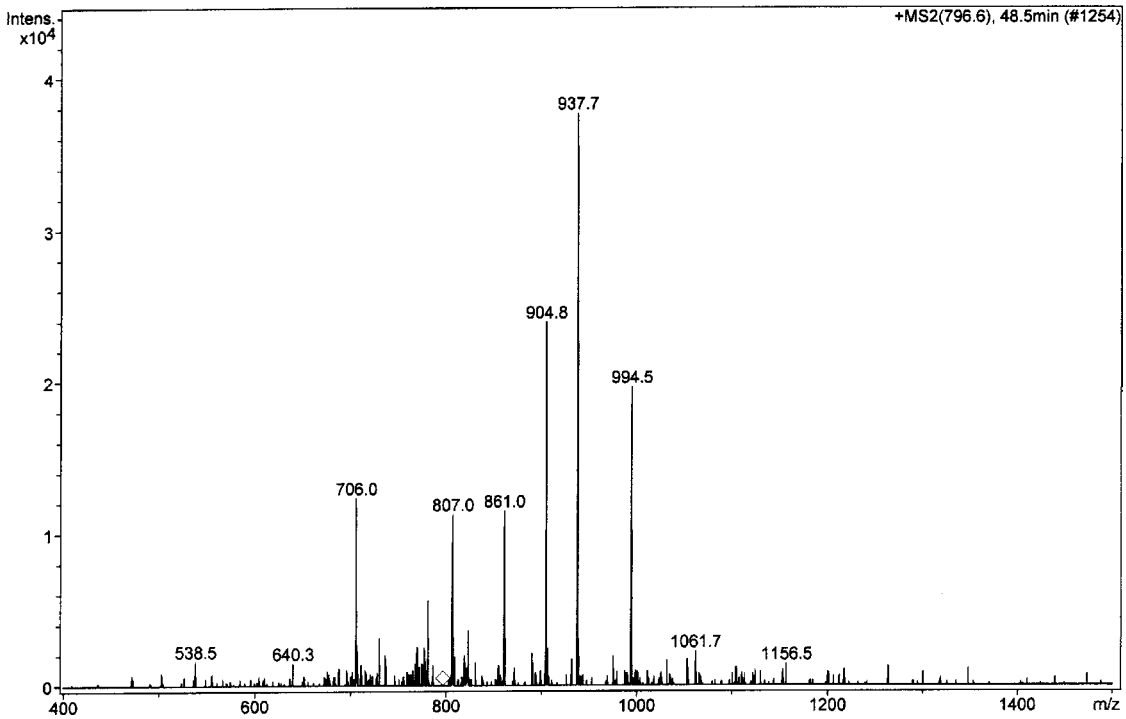

Figure 7. Tandem mass spectrum derived by collision-induced dissociation of the precursor ion, *m*/*z* 796.6. The sequence identification of the peptide and database search showed that this peptide was derived from DnaK-type molecular chaperone HSPA6.

change in proteins such as trisephosphate isomerase that played an important role in metabolic pathways was identified from the CEM T-lymphoblastic leukemia cell line after treatment with the new olomoucuine-derived synthetic cyclin-dependent kinase inhibitor, bohemine (*11*). Protein involved in metabolic pathways was identified for pancreatic ductal carcinoma cell line treated with trichostatin-A (*14*). However, for human myeloid leukemia (HL-60) cells treated with 12-phorbol 13-myristate acetate (PMA), it was observed that unchanged abundance was particular prevalent for metabolic enzymes (*15*).

All living cells respond to stress by triggering rapid changes in protein profile characterized by induction of heat shock proteins. Cellular damage or stress results in two fundamental cellular responses. With apoptosis, it results in a precisely programmed form of cell death and stress response, or expression of the heat shock protein (HSP), that protected cell and mediates an accelerated recovery following stress or damage. It was observed that induced heat shock protein 70 confers cytoprotection by suppressing c-jun N-terminal kinase and inhibiting apoptotic cell death (*31*). The down-regulation of 60 kDa heat shock protein, mitochondrial precursor was reported in the proteomic profiling of pancreatic ductal carcinoma cell lines treated with trichostatin-A (*14*). For HCT-116 human colon adenocarcinoma cells treated with drugs, heat shock protein 71-kDa protein was observed to be down-regulated (*16*). Farnesyl transferase inhibitors such as manumycin inhibit ovarian cancer cell growth with up-regulation of heat shock protein 70 (HSP70) and reported that HSP70 played an important role in protecting cells under stress (*32*). The up-regulation of DnaK-type molecular chaperone HSPA6 with the treatment of the botanical extracts may be the result of oxidative stress and indicated the possibility of its protective mechanism against drug-induced apoptosis as observed in farnesyl transferase inhibitors such as manumycin (*32*).

To conclude, identification of differential protein expression was possible with the method using proteolytic digest with single dimensional and two-dimensional liquid chromatography with tandem mass spectrometry. In the presence of the botanical extracts, drug-induced apoptosis was not observed, and a number of proteins that played an important role in the metabolic pathways in HepG2 cell line had been affected. The data in the current work proposed that the inhibitory effects of a standardized extract from *Scutellariae radix* involved the expression change of heat shock protein 70 and other proteins related to energy metabolism. In investigating biological events, the platform had the potential to provide significant information about the particular proteome. This included the proteins and how their levels and modifications change in response to the effects of external factors. The current methodology will provide a basis for further investigation into metabolic pathways of the inhibitory or antitumor effects of botanical extracts.

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