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Inhibitory Effects of a Chemically Standardized Extract from *Scutellaria barbata* in Human Colon Cancer Cell Lines, LoVo

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Scutellaria barbata (SB) is a medicinal plant that contains flavonone compounds such as scutellarein, scutellarin, carthamidin, isocarthamidin, and wogonin. A functional proteomic approach was used to study the inhibitory effects of a chemically standardized extract from SB in human colon adrencarcinoma, LoVo. In this work, a stable isotope was not used in the proposed method developed. The whole cell lysates from the control and treated cells were digested with trypsin, and the peptides were separated by two-dimensional (cation-exchange and reversed-phase) liquid chromatography and tandem mass spectrometry. The differentially expressed proteins identified using the current approach supported the data obtained from cell-cycle analysis with flow cytometry. With flow-cytometry analysis, a significant increase in the sub G_1 phase was observed with a higher dose of extract from SB. Our results suggest that the chemically standardized extract from SB can induce cell death in the human colon cancer cell line. Our current work showed that the proposed platform provided a rapid approach to study the molecular mechanism because of the inhibitory effects of different doses of the botanical extracts on LoVo cell lines. This included a network of proteins involved in metabolism, regulation of the cell cycle, and transcription-factor activity.

KEYWORDS: Proteomic analysis; cell death; human colon cancer cell lines (LoVo); two-dimensional liquid chromatography tandem mass spectrometry; *Scutellaria barbata*

INTRODUCTION

Scutellaria barbata (SB) is a medicinal plant found in the Chinese Pharmacopoeia (*1*) and others (*2*, *3*). The herb contains flavonone compounds scutellarein, scutellarin, carthamidin, isocarthamidin, and wogonin. A diterpene substance neoandrographolide and a cytotoxic constituent *E*-1-(4′-hydroxyphenyl)-but-1-en-3-one were also isolated from the herb (*2*). The antibacterial activity against methicillin-resistant *Staphylococcus aureus* from a crude extract prepared from SB was reported (*4*). The antimicrobial activity of the essential oil of SB was evaluated against 17 microorganisms. The Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, were more sensitive to the oil than Gram-negative bacteria and yeast (*5*). An aqueous extract of SB was reported to induce apoptosis of ovarian cancer cell lines such as SKOV-3 and CAOV3. Transfection of the most sensitive ovarian cancer cell lines (A2780) with Bcl-2 protein resulted in noticeable protection against apoptosis (*6*). The anticancer activity and mechanism of SB extract in human lung cancer cell lines, A549, were investigated (*7*). The methylene chloride fraction from SB can induce apoptosis in human leukemia cells, U937, via the mitochondria signaling pathway (*8*). It was also suggested that that SB extract reduces tumor volume in smooth muscle cells by inducing a concomitant increase in the rate of apoptosis (*9*).

For botanicals and herbal preparations, there is a need to approach scientific proof and clinical validation with chemical assay, biological assays, animal models, and clinical trials. Quality assurance of botanicals with the chemical assay using well-validated analytical methods is the prerequisite of a credible biological assay and clinical trials (*10*, *11*). However, works using standardized extracts are still limited. Currently, works with some botanical extracts have progressed to phase II and III clinical trials sponsored by the National Center for Complementary and Alternative Medicine (NCCAM) (http://nccam.nih.gov).

To study the signaling pathway leading to cell growth, a functional proteomics approach using two-dimensional gel electrophoresis was used to delineate the pathways and mediators involved in butyrate action in colon cancer cells, HT-29, at 24 h post-treatment. MALDI−TOF mass spectrometry was used to identify butyrate-regulated spots (*12*). A method with two-dimensional liquid-phase separation was developed and applied to a comparison of protein expression between peninsularinone-treated and untreated human colon adenocarcinoma cells, HCT-116 (*13*). In search of the molecular targets of flavone and quercetin action in human colon cancer cells, HT-29, proteome analysis using two-dimensional gel electrophoresis with MALDI−TOF mass spectrometry was used to study the differential protein expression (*14*, *15*).

Quantitative profiling of tryptic digest of proteins in complex mixtures without isotope labeling using liquid chromatography

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and mass spectrometry had been reported. Expected and calculated protein ratios differ no more than 16% (*16*). An approach using proteolytic digest with two-dimensional liquid chromatography nanospray mass spectrometry without the use of a stable isotope was applied for the study of differential protein expression in epidermal cell lines grown in the presence or absence of epidermal growth factor (*17*). In our laboratory, a method using proteolytic digest with single-dimensional and two-dimensional liquid chromatography with tandem mass spectrometry without the use of a stable isotope was used to characterize differential protein expression in liver cancer cell lines (HepG2) in response to the standardized extract from *Scutellariae radix* (*18*). At the same time, the method was applied for the profiling of differential protein expression of liver from a mouse in the control group and groups treated with standardized extract from *S. radix* (*19*). The proposed method was reported to be able to identify changes at the molecular level and have a high level of reproducibility (*18*, *19*).

The aim of the current study was to use a functional proteomic approach to study the inhibitory effects of a chemically standardized extract from SB in human colon adrencarcinoma, LoVo. A stable isotope would not be used in the proposed method developed. The whole cell lysates from the control and treated cells were digested with trypsin, and the peptides were separated by two-dimensional (cation-exchange and reversed-phase) liquid chromatography and tandem mass spectrometry. The molecular mechanism because of the effects of different doses of the botanical extracts will be investigated. The differentially expressed proteins identified using the current platform will be used to correlate with the cell viability assay and cell-cycle analysis with flow cytometry.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Ham's F12K medium, penicillin, streptomycin, and trypsin-EDTA were bought from Hyclone (Logan, UT). Fetal bovine serum (FBS) was obtained from Biological Industries (Israel). Methanol and acetonitrile of HPLC grade were purchased from APS (New South Wales, 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 was purchased from Merck (Darmstadt, Germany). Reference standard scutellarein was purchased from Chromadex (Santa Ana, CA).

Cell Cultures. All cell lines were obtained from ATCC. Human colon adrencarcinoma, LoVo (ATCC number CCL-229) was maintained with Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL stretomycin, incubated at 37 °C, and 5% CO_2 atmosphere.

Preparation of Plant Extracts. To prepare a homogeneous sample, the leafs of SB were ground using an IKA MF10 microfine grinder (Staufen, Germany) with a sieve insert of hole size 0.5 mm. A total of 1.0 g of sample was weighed in the thimble. Methanol was selected as the solvent with Soxhlet extraction. The co-extract gave a green color with the extraction solvent. The green color turned continuously lighter through the course of the extraction. Hence, after extraction with 120−150 mL of methanol for 5−6 h, the extraction solvent was essentially colorless. The excess solvent was evaporated with the rotary evaporator, and the extract was transferred into a 50 mL volumetric flask.

The content of scutellarein present in SB was determined by reversed-phase HPLC with external standard calibration. For all experiments, a Shimadzu LC 10 series (Shimadzu, Kyoto, Japan) equipped with a binary gradient pump, autosampler, column oven, and diode array detector was used. The gradient elution using a mobile phase consisted of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The initial condition was set at 20% of B, with a gradient up to 100% in 15 min and returning to the initial condition for 12 min. Detection was at 285 nm. Oven temperature was set at 40 °C, and a flow rate was set at 1.0 mL/min. For all experiments, 20 μL of standards and sample extract were injected. The column used for the separation was a reversed-phase C18 Luna, 150 × 4.6 mm, 5 μm (Phenomenex, Torrance, CA). For scutellarein, the working solutions were prepared in the range of 0−60 mg/L in methanol. Linearity of scutellarein was established between 0 and 60 mg/L with a correlation coefficient $r^2 \geq 0.99$.

A total of 1.5 mL of the solution was transferred into a test tube. Excess methanol was evaporated under a stream of nitrogen and dried in an oven at 50 °C for 1−2 h before use. The amount of extract obtained from the medicinal plant was found to be 3.72 ± 0.30 mg ($n = 16$). For *in vitro* studies, the standardized extract obtained was dissolved into 750 μL of medium to form solution A. The solution was prepared before *in vitro* study. A total of 100, 300, and 500 μL of solution A were mixed with 6 mL of medium to form dose B (82.7 mg/L), C (248.1 mg/L) and D (413.3 mg/L) for time-dependent and other interaction studies.

Cell-Growth Analysis. Cell-growth inhibition assays were performed by plating 1×10^5 cells on a 25 cm^2 tissue culture flask, in Ham's F12K medium with 10% FBS for LoVo. Cells were treated with extracts from SB or the control with medium for 24, 48, and 72 h, respectively. For dose-dependent studies, 100, 300, and 500 μL of solution A with 6 mL of medium was used and cells were treated with the extract or control for 48 h. The number of cells was determined with a hemocytometer. Cell viability was measured by tryphan blue dye exclusion.

Flow-Cytometry Analysis. A total of 1×10^6 cells were plated on a 75 cm^2 tissue culture flask and incubated for 48 h at 37 °C. Cells were treated with extracts from SB or the control with medium for another 48 h. For treatment with SB, 300, 900, and 1500 μL of solution A with 15 mL of media were 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-buffered saline (PBS). The resulting solution was incubated with 100 μg/mL 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 SB extract (dose B and D) or the control with medium for 48 h. The LoVo 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 1.0−1.5 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. The protein concentration was assayed using a Bradford assay reagent (Pierce, Rockford, IL).

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 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 the single-dimensional liquid chromatography platform, the enzymatically digested samples were subjected to solid-phase extraction, 500 mg of Strata (Phenomenex, Torrance, 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 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 ammonium acetate with 5% acetonitrile was added to the whole cell lysates. The 500 mg of solid-phase extraction cartridges, Strata

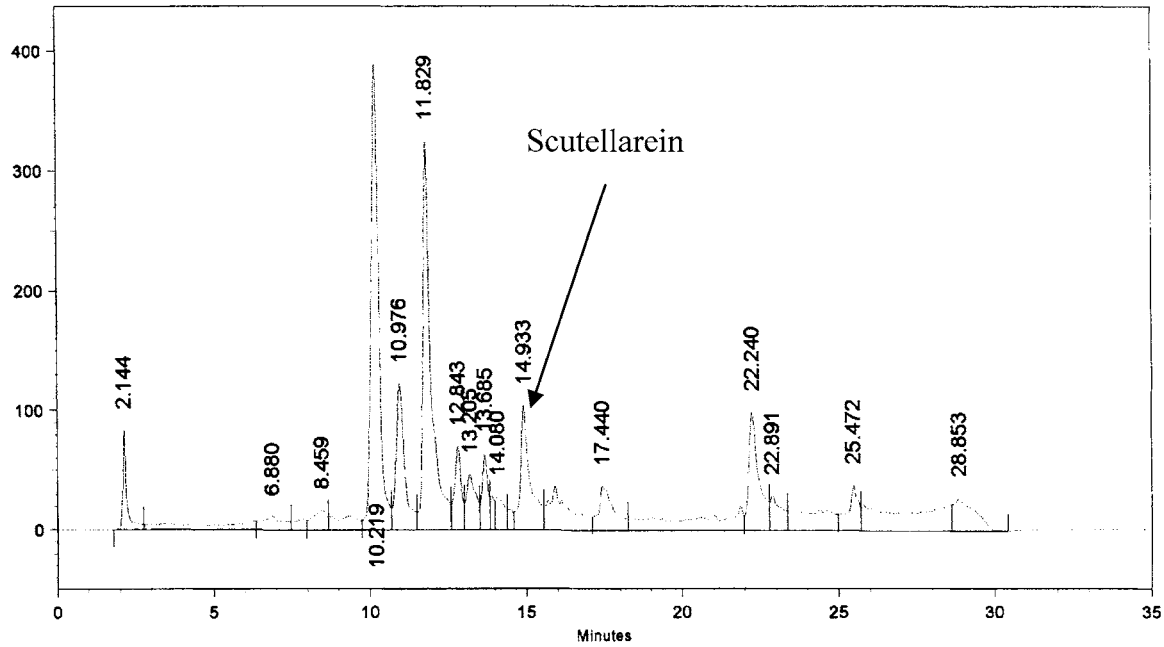

Figure 1. Chromatogram obtained for scutellarein in SB by Soxhlet extraction. The amount of scutellarein in the extract was determined by external standard calibration. The gradient elution using a mobile phase consisting of (A) 0.1% formic acid in water and (B) 0.1% formic acid in methanol. The initial condition was set at 20% of B, with a gradient up to 100% in 15 min and returning to the initial condition for 12 min. Detection was at 285 nm. Oven temperature was set at 40 °C, and flow rate was set at 1.0 mL/min.

(Phenomenex, Torrance, CA) SCX column, was conditioned with 10 mL of 5 mM ammonium acetate before loading the whole cell lysates. The whole cell lysates with 5 mM ammonium acetate with 5% acetonitrile 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 ammonium acetate with 5% acetonitrile, 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 100 μL of water with 0.1% acetic acid with 5% acetonitrile as stated above before analysis by LC/MSMS.

Reversed-Phase HPLC/MSMS Analysis for Protein Expression. 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 using a mobile phase consisted 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, with a gradient up to 40% in 70 min, up to 90% in the next 5 min, and returning to the initial condition for 15 min. Detection was at 214 nm. Oven temperature was set at 40 °C, and the 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 × 2.0 mm, 5 μm, 300 Å (Phenomenex, Torrance, CA). 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, with a maximum accumulation time of 300 ms, number of average scans of 5, and SmartSelectTM.

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 the NCBInr database and Swiss-prot. The taxonomy was set to *Homo sapien* (human); 1 missed cleavage was allowed; and carbamidomethyl was selected for fixed modifications. Searches were done with a tolerance on the 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 tissue specificity of the proteins identified was examined using Swissprot. The raw data were inspected manually for confirmation prior to acceptance.

RESULTS AND DISCUSSION

In the study of the biological activities of botanical extracts, the method of extraction is an important step. It had been observed that extracts with varying active components or marker compounds were obtained when using different methods of extraction (*20*−*23*). This will in turn affect the biological activities and toxicity of the botanical extracts obtained. Hence, the method of extraction used should be sufficiently validated to ensure that it has the required precision and extraction efficiencies. The current method of the chemical assay was based on suggestions in guidance documents issued by regulatory authorities (*8*, *9*). The combination of a chromatographic fingerprint and the assay of a specific compound in the botanical extracts ensured the quality of the extract used. For the current work, the content of scutellarein in SB was determined by HPLC. The amount of scutellarein determined by HPLC was found to be 2.26 ± 0.17 mg/g (n = 3). A representative chromatographic fingerprint of the extract from SB was shown in **Figure 1**.

Standardized Extract from *S. barbata* Inhibits Cell Growth. Flavonoids such as flavone, quercetin, baicalin, and baicalein had been reported to induce apoptosis in colon and liver cancer cell lines (*14*, *15*, *24*, *25*). From our earlier work and other paper, significant induction of apoptosis in cancer cells lines was not observed with extracts from *S. radix*, which contained bioactive components such as baicalin and baicalein (*18*, *25*). Hence, for flavonoids present in *S. radix*, differences in biological effects on cell cycles from the extract from *S. radix* compared with individual components such as balcalein and baicalin suggested the synergistic effects among components present in the botanical extract (*26*).

For flavonoids present in the extract from SB, the inhibitory effects on LoVo cell lines are shown in **Figure 2**. Cells were

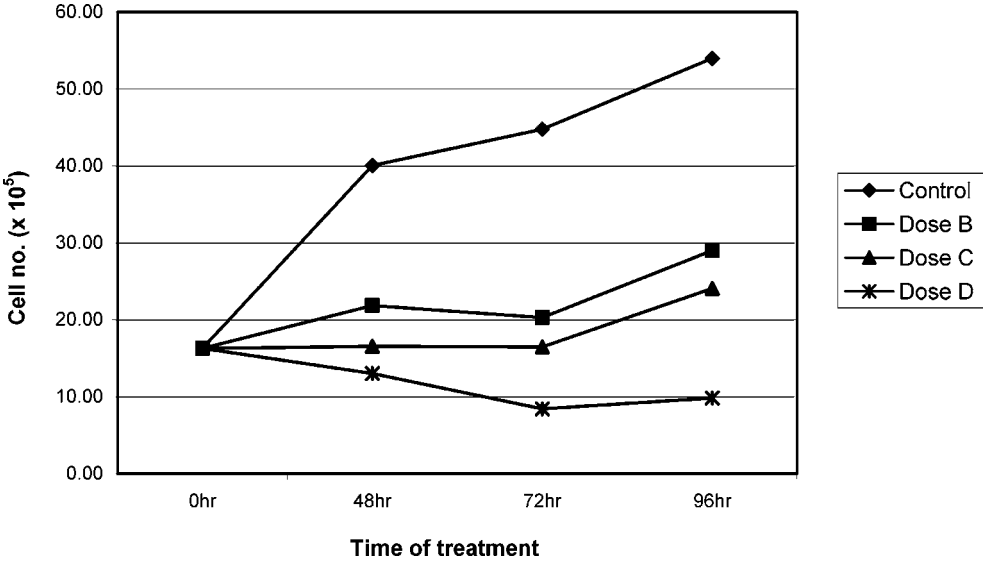

Figure 2. Inhibition of LoVo cell proliferation by the extract from different doses of SB (Dose B, C, and D). The cell-growth inhibition assay was performed by plating 2×10^4 cells in a 25 cm^2 flask, in Ham's F12K medium with 10% FBS. Cells were treated with an extract from SB (dose B, 82.7 mg/L; C, 248.1 mg/L; and D, 413.3 mg/L) or media every 48 h, and the number of cell was determined at day 0, 48, 72, and 96 by counting with a hemocytometer. The viability of cells was measured by trypan blue dye exclusion.

treated with medium and different doses of botanical extracts, and the growth rate was evaluated after 1, 2, 3, and 4 days. As shown in **Figure 2**, the growth rate of LoVo cell lines treated with different doses of the botanical extract was significantly lower compared to control cells treated with medium alone. Our data suggested that there was not much of a difference in the data points obtained after treatment with 82.7 and 248.1 mg/L of botanical extract. The results were repeated on a different day, and a similar growth rate as in **Figure 2** was observed. The extract from SB was observed to display a dose-dependent growth of the LoVo cells (data not shown).

The effects of the botanical extract on the cell cycles were analyzed using flow cytometry. From **Figure 3**, with an increase in the sub-G_1 phase, no extensive changes in the G_0-G_1, S, and G_2-M phases were observed in both the control and low-dose group (dose B, 82.7 mg/L). However, a more significant change in the sub-G_1 phase, concurrent with a decrease in the G_0-G_1 phase and an increase in the S phase, was observed between the control and high-dose group (dose D, 413.3 mg/L). The sub-G_1 method relies on the fact that, after DNA fragmentation, there are small fragments that are able to be eluted following washing in PBS. A significant increase in the sub-G_1 phase is indicative of induction of cell death. Our current data in cell-cycle analysis (sub-G_1 and G_0-G_1) with flow cytometry was similar to propolin-C-induced apoptosis in human melanoma cells (*27*). The effects of botanical extract on LoVo cells were consistent with other work, where an aqueous extract of SB was reported to induce apoptosis of ovarian cancer cell lines such as SKOV-3, CAOV3, and human leiomyomal smooth muscle cell in a dose-dependent manner (*6*, *9*). The data obtained in our current work suggested that extracts from SB were observed to give a different biological effects compared to *S. radix*.

Proteomic Analysis of Differential-Expressed Proteins in the LoVo Cell Line by Two-Dimensional HPLC/MSMS. Proteomic analysis of the human colon carcinoma cell lines and human colon cancer patients had been reported (*28*, *29*). The global profiling of the cell-surface proteome of colon cancer cells such as LoVo uncovers an abundance of proteins with chaperone functions (*30*). Alkaline proteins present in human colon crypt were analyzed by two-dimensional gel electrophoresis, and protein spots were identified by mass spectrometry (*31*).

LoVo cell lines were treated with a high dose (dose D at 413.3 mg/L) and low dose (dose B at 82.7 mg/L) of extract from SB. With LC/MSMS 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. The chromatograms in parts **A**−**D** of **Figure 4** showed the fraction 1 and 3 of the tryptic digest of the LoVo cell line from two different cell lysates with two-dimensional HPLC/MSMS from the LoVo cell lines treated with different doses of botanical extract. As seen in parts **A**−**D** of **Figure 4**, a number of peptides remained unchanged in the control and treated group for fraction 3 and other fractions. It was proposed that they were likely to be house-keeping proteins as observed in our earlier works (*18*, *19*). The presence of peptides from house-keeping proteins assisted in the identification of peptides from proteins where their expression had been modified. A list of proteins where the expression was modified more than 2-fold identified using tandem mass spectrometry after treatment with different doses of extracts from SB was tabulated in **Tables 1** and **2**, respectively. The criteria was selected because it was observed that the proposed method did not induce variation higher than 2-fold (peak reduction or increased by 2 times). The current approach of identification of proteins was based on our earlier works (*18*, *19*) and other papers (*16*, *32*−*35*). The potential for false positive identifications from large databases through tandem mass spectra data has been discussed (*34*). Hence, for the searches obtained from Mascot, websites such as Swiss-prot (http://us.expasy.org/sprot) and NCBInr database (http://www.ncbi.nlm.nih.gov) were used to examine domains and motifs present in identified proteins.

An apparent weakness of the current approach was that it would be rather difficult to identify expression changes of less than 2-fold because of the variation of signals produced by MS. Using conventional MSMS processing software such as Mascot,

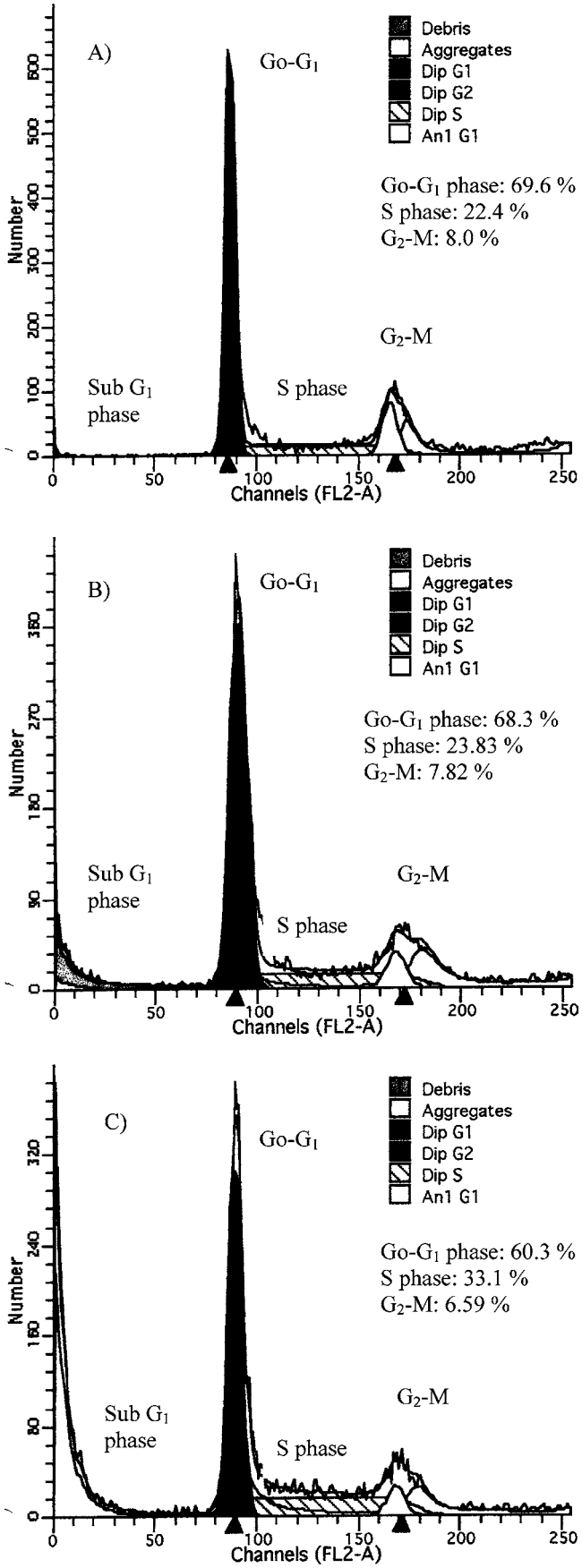

Figure 3. Cell-cycle analysis by flow cytometry. The LoVo cells lines were (A) treated with medium for a control, (B) treated with an extract from SB (dose B, 82.7 mg/L), and (C) treated with an extract from SB (dose D, 413.3 mg/L) for 48 h. An increase in sub-G_1 was observed in C for LoVo cell lines treated with dose D (413.3 mg/L) of the botanical extract.

the mass and amino acid type for the modifications must be known before the search is performed. It will be difficult or impossible to identify peptides bearing adducts that may be present of an unknown mass or at unanticipated amino acid targets. Hence, there were a number of peptides with expression changes where it was not identified.

With a higher dose of SB (dose D at 413.3 mg/L), the protein expressions that were affected included those involved in metabolism, regulation of the cell cycle, and transcription-factor activity. The differential protein expression from proteomic analysis supported the data obtained from cell-cycle analysis using flow cytometry. A cell-cycle transition is often driven by the cyclin-dependent kinases (CDKs). CDKs can be controlled by dissociation of the active cyclin−CDKs complex and subsequent proteolysis of the cyclins. A herbal extract such as Huanglian (*Coptidis rhizome*) inhibits cell growth by the suppression of the expression of cyclin B1 and inhibiting CDC2 kinase activity in human gastric cancer cell lines. The mRNA of cyclin B1 was not changed after treatment with the herbal extract. At the same time, there was no change in the protein expression of cyclins A and E (*35*). For piceatannol, it inhibits cell growth in colon cancer cell lines with downregulation of cyclin D1, cyclin B1, and CDK4. At the same time, CDK2, CDK6, and CDC2 were expressed at steady state in cells treated with piceatannol (*37*).

In our current work, protein kinase (EC 2.7.1.37) CDC2-related PITALRE was downregulated. It is a member of the CDK pair (CDK9/cyclin T) complex, also called positive transcription elongation factor B (P-TEFB). It is proposed to facilitate the transition from abortive to production elongation by phosphorylating the CTD (carboxy-terminal domain) of the large subunit of RNA polymerase II (RNAP II). It is involved in biological processes such as cell proliferation and regulation of the cell cycle. Its catalytic activity involved the reaction: ATP + protein = ADP + phosphoprotein (*36*). At the same time, the expression of CDK9 in colon cancer cell lines such as HCT116 had been examined using Western blots (*39*). Another signaling protein whose expression was downregulated was protein-tyrosine kinase (EC 2.7.1.112) abl. It is involved in biological processes such as regulation of the cell cycle and catalysis of the reaction: ATP + protein tyrosine = ADP + protein tyrosine phosphate. It is also proposed to be involved in protein-tyrosine kinase activity, DNA damage response, and signal transduction resulting in induction of apoptosis (*38*).

Proteasomes play central roles in proteolysis of ubiquitinated cellular proteins and are responsible for cleaving many regulatory proteins such as cyclins and oncogenic products. Thus, they play an important role in transcriptional regulation, the cell cycle, and apoptosis. Expression of proteasome subunits was changed in lymphoma-derived cell lines following treatment with a demethylating drug, 5′-azacytidine (*40*). The downregulation of human 26S proteasome subunits was observed in butyrate-treated human colon cancer cell lines (*12*). It was proposed that the downregulation of 26S protease regulatory subunit S10B in the LoVo cell line may be a mechanism by which the extract from a higher dose of SB affected the cell cycle and apoptosis. At the same time, it is proposed to be involved in the catalysis of the reaction: ATP + H_2O = ADP + phosphate (*38*).

Other than signaling proteins, DNA-binding protein (fragment) and transcription-factor mammalian MafA that were proposed to be involved in transcription-factor activity and regulation of DNA-dependent transcription were downregulated (*38*). Two proteins, α-1,4-glactosyltransferase and similar to CG14740 gene product (EC 4.1.3.7) (citrate synthase) involved

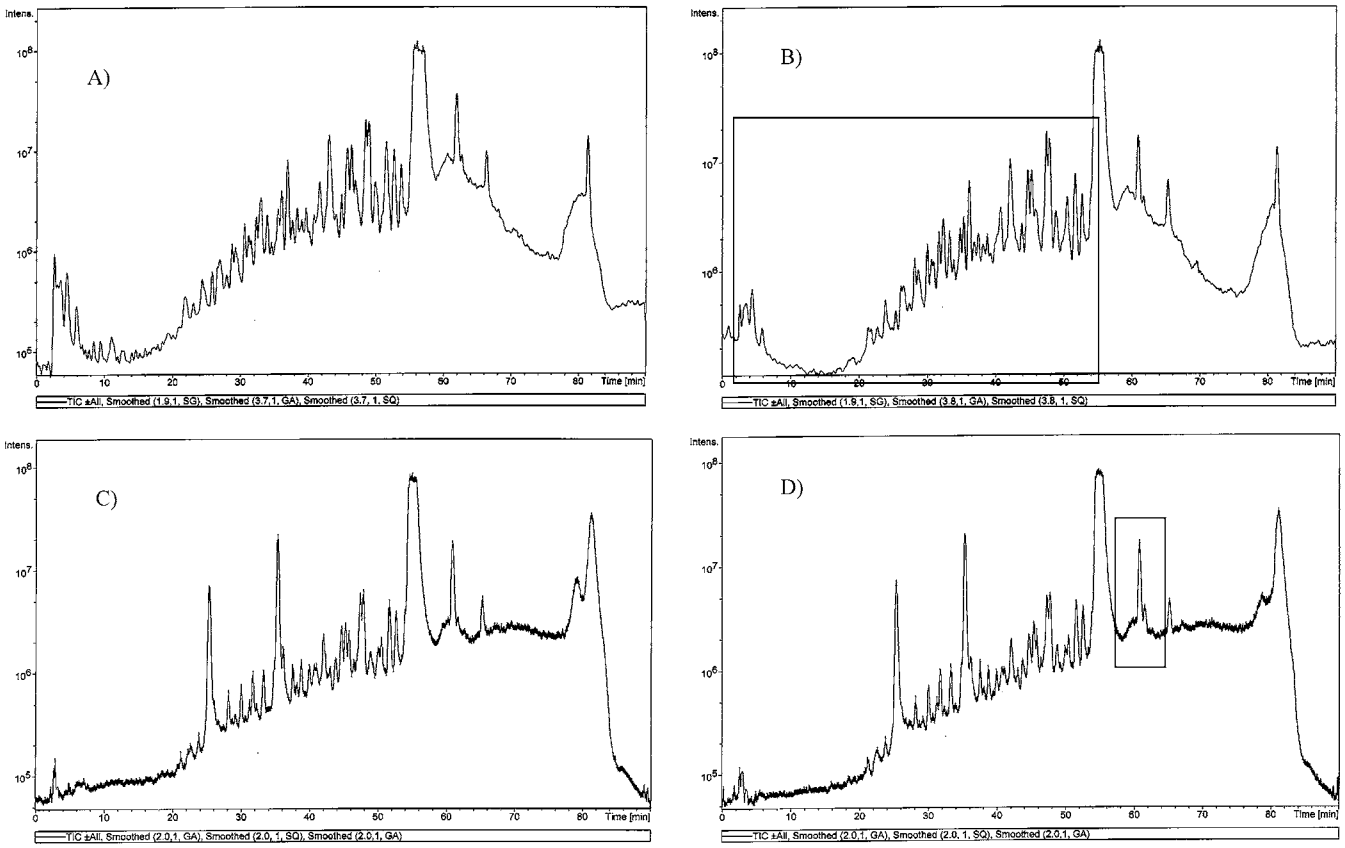

Figure 4. Chromatograms (TIC) from LCMSMS of (A) fraction 1 of the LoVo cell line, control with medium, (B) fraction 1 of the LoVo cell line, from a high dose (dose D at 413.3 mg/L) of extract from SB, (C) fraction 3 of the LoVo cell line, control with medium, and (D) fraction 3 of the LoVo cell line, from a low dose (dose B at 82.7 mg/L) of extract from SB, from two-dimensional HPLC/MSMS. The box regions are where differential expressed peptides (up- or downregulated more than 2 times) were observed.

Table 1. Identification of Proteins in the LoVo Cell Line Found To Be Significantly Different (More Than 2 Times) in the Control and SB-Treated Group (Dose D at 413.3 mg/L)

accession	mass	description	
Q7Z2I1	40 782	α-1,4-glactosyltransferase	upregulated
Q9BWN8	29 552	similar to CG14740 gene product (EC 4.1.3.7) (citrate synthase)	upregulated
A55262	43 149	protein kinase (EC 2.7.1.37) CDC2-related PITALRE (cell division protein kinase 9)	downregulated
TVHUA	123 681	protein-tyrosine kinase (EC 2.7.1.112) abl (proto-oncogene tyrosine-protein kinase ABL1)	downregulated
Q9UDY3	25 859	DNA-binding protein (fragment)	downregulated
Q8NHW3	37 107	transcription-factor mammalian MafA	downregulated
S71316	44 418	proteasome endopeptidase complex (EC 3.4.25.1) p42 chain (26S protease regulatory subunit S10B)	downregulated
AAA52503	37 419	HUMG6PDX NID: (GMP reductase 1)	downregulated
RGHUT2	40 720	GTP-binding regulatory protein Gt α-2 chain-human	downregulated
S10114	8347	hypothetical protein (Bcr-abl mRNA 5′ [fragment])	downregulated

Table 2. Identification of Proteins in the LoVo Cell Line Found To Be Significantly Different (More Than 2 Times) in the Control and SB-Treated Group (Dose B at 82.7 mg/L)

accession	mass	description	
G02371	29 432	U1-snRNP-binding protein homologue (transcription-factor mammalian MafA)	downregulated
JC1169	19 060	DNA-damage-inducible protein GADD153	downregulated
S10114	8347	hypothetical protein (Bcr-abl mRNA 5′ [fragment])	downregulated
A55262	43 149	protein kinase (EC 2.7.1.37) CDC2-related PITALRE (cell division protein kinase 9)	downregulated
TVHUA	123 681	protein-tyrosine kinase (EC 2.7.1.112) abl (proto-oncogene tyrosine-protein kinase ABL1)	downregulated

in transferase activity, were upregulated. For the protein similar to CG14740 gene product (EC 4.1.3.7) (citrate synthase), it was also proposed to be involved in the tricarboxylic acid cycle. It is a nearly universal metabolic pathway in which the acetyl group of acetyl coenzyme A is effectively oxidized to two CO_2 and four pairs of electrons are transferred to coenzymes. The acetyl group combines with oxaloacetate to form citrate, which undergoes successive transformations to isocitrate, 2-oxoglutarate, succinyl-CoA, succinate, fumarate, malate, and oxaloacetate again, thus completing the cycle. The protein, GMP reductase 1, was proposed to be involved in GMP reductase activity and catalyze the irreversible NADPH-dependent deamination of GMP to IMP. It functions in the conversion of nucleobase, nucleoside, and nucleotide derivatives of G to A nucleotides and in maintaining the intracellular balance of A and G nucleotides (*38*). Another protein that was proposed to be involved in cell signaling and the metabolism pathway was the GTP-binding regulatory protein Gt α-2 chain human. It belongs to the G proteins that are a family of guanine nucleotide-

binding proteins that relay signals from cell-surface receptors to intracellular effectors (*41*).

A number of proteins involved in metabolism pathways were affected in LoVo cell lines treated with a high dose of the botanical extract. Our observed data were consistent with other papers. For flavonoids such as flavone and quercetin that have been identified as potent apoptosis inducers in human colon cancer cell lines, it was observed that a number of proteins involved in intermediate metabolism were downregulated (*14*, *15*). In our earlier work, the inhibitory effect of a chemically standardized extract from *S. radix* in liver cancer cell lines (HepG2) was investigated, drug-induced apoptosis was not observed, and a number of proteins that played an important role in the metabolic pathways in HepG2 cell lines had been affected (*18*). In the profiling of protein patterns following the treatment of the Burkitt lymphoma cell line (DG 75) with 5′-azycytidine, a number of proteins such as transaldolase, malate dehydrogenase, and others regulating energy metabolism were upregulated (*43*). Expression change in proteins such as trise-phosphate isomerase that played an important role in metabolic pathways were identified from the CEM T-lymphoblastic leukemia cell line after treatment with the new olomoucuine-derived synthetic CDK inhibitor, bohemine (*44*). The protein involved in metabolic pathways was identified for the pancreatic ductal carcinoma cell line treated with trichostatin-A (*45*).

The effects of various concentrations of chemical substances such as propolin C, piceatannol, geraniol, β-ionone, and huanglain (a herbal extract) on the expressions of different cyclins, CDKs, and other proteins in various cell lines had been investigated. In comparison with a higher dose of the various chemical substances, a lower dose of each in various treated cell lines was observed to have different biological activities (*27*, *36*, *37*, *42*). From flow-cytometry analysis, a lower dose of extract from SB (dose B at 82.7 mg/L) did not induce extensive changes as a higher dose of the botanical extract. For LoVo cells treated with a lower dose of SB, the number of differentially expressed proteins identified (**Table 2**) was less compared to a higher dose of SB. In comparison with a higher dose of botanical extract, it was proposed that a lower dose of SB may have affected the protein expression in the LoVo cell line in a slightly different way. The data obtained from proteomic analysis was consistent with that obtained from cell-cycle analysis with flow cytometry. Using the current platform, it was observed that the number of peptides that were differentially expressed was much less than in LoVo cells treated with a high dose of SB. Other than protein kinase (EC 2.7.1.37) CDC2-related PITALRE and protein-tyrosine kinase (EC 2.7.1.112) abl, proteins such as DNA-damage-inducible protein GADD153 involved in the regulation of DNA-dependent transcription factor were downregulated. The identification of protein kinase (EC 2.7.1.37) CDC2-related PITALRE and protein-tyrosine kinase (EC 2.7.1.112) abl in the LoVo cells treated with a low dose of SB once again demonstrated the reproducibility of the proposed platform.

The combination of proteomic analysis with a chemically standardized botanical extract using chromatographic fingerprint and an assay of specific compounds allows us to study the signaling pathway for the inhibitory effects of SB in human colon cancer cell lines. Our results suggest that the chemically standardized extract from SB can induce cell death in the colon cancer cell line. The data obtained from cell-cycle analysis with flow cytometry give us some idea of the proteins that may be differentially expressed in LoVo cell lines treated with different doses of the botanical extract. In our earlier work, we did not identify any differentially expressed proteins that were associated with signal transduction, the cell cycle, and cell death when significant changes in G_0-G_1, S, and G_2-M phases were not observed in cell-cycle analysis with flow cytometry (*18*). From the number of fractions collected, it was observed that the current platform would not be able to identify as many proteins as in other papers (*17*, *32*−*35*). However, the proposed platform was able to identify differentially expressed proteins in LoVo and HepG2 cell lines treated with botanical extracts (*18*) and in mouse liver (*19*). Without the use of stable isotope labeling, the proposed platform provided a rapid approach to study the molecular mechanism because of the inhibitory effects of different doses of the botanical extracts on LoVo cell lines. This included a network of proteins involved in metabolism, regulation of the cell cycle, and transcription-factor activity.

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