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# Korean *Scutellaria baicalensis* water extract inhibits cell cycle G1/S transition by suppressing cyclin D1 expression and matrix-metalloproteinase-2 activity in human lung cancer cells

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ABSTRACT

*Aim of the study: Scutellaria baicalensis* Georgi is a widely used medicinal herb in several Asian countries including Korea. The various medicinal properties attributed to *Scutellaria baicalensis* include anti-bacterial, anti-viral, anti-inflammatory and anti-cancer effects. The present study investigated the cytotoxicity of *Scutellaria baicalensis* water extract (SBWE) on A549 non-small-cell-lung cancer cells and the A549 expression of cyclin D1, cyclin-dependent kinase 4 (CDK4) and matrix metalloproteinase-2 (MMP-2), and the effects of SBWE on cell cycle progression, especially the G1/S phase, and on cell motility.
*Materials and methods:* SBWE cytotoxicity was assessed by a standard colorimetric assay utilizing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and expression of cyclin D1 and CDK4 protein in SBWE-treated A549 cells was assessed by Western blot analysis. Flow cytometry analysis was performed to determine the effect of SBWE on A549 cell cycle progression. A549 cell MMP-2 activity was examined by zymography. Cell motility and migration was assessed by a scratch wound healing assay.
*Results:* SBWE was not cytotoxic. The production of Cyclin D1, CDK4 and MMP-2 activity were significantly decreased in a SBWE dose-dependent manner, with maximum inhibition occurring at SBWE concentrations of 250 μg/ml and 500 μg/ml. SBWE inhibited cell cycle progression in the G1/S phase and significantly inhibited the motility of A549 cells.
*Conclusions:* Cyclin D1 protein may be associated with MMP-2 activity and cell motility. Thus, SBWE promotes a strong protective effect against MMP-2 mediated metastasis and cell proliferation through the down-regulation of cyclin D1. SBWE may be a useful chemotherapeutic agent for lung cancer.


## 1. Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide. Lung adenocarcinoma, which is a subgroup of non-small-cell lung cancer (NSCLC), accounts for approximately 75–85% of all lung cancers (Greenlee et al., 2001; Shieh et al., 2010). NSCLC cells possess aggressive invasion ability and metastatic properties (Shivapurkar et al., 2003; Wang et al., 2009). The most widely used therapies for lung cancer are chemotherapy, surgery and radiation. The disadvantages of these therapies are poor recuperation and prognosis. Greater than 85% of adenocarcinoma patients die within 5 years of diagnosis (Erridge et al., 2007). Although the survival rates for lung cancer diagnosed at earlier stages are higher, there is an urgent need to apply new therapeutic agents and chemotherapy to increase survival rates of adenocarcinoma patients (Luo et al., 2008).

*Scutellaria baicalensis* Georgi is a commonly used medicinal herb in several Asian countries including Korea, Japan and China because of its anti-bacterial, anti-viral, anti-inflammatory and anti-cancer properties (Bensky et al., 1992; Huang, 1993). There is evidence for the role of *Scutellaria baicalensis* in tumor cell lines, particularly cell cycle arrest and inhibition of the progression of tumor angiogenesis (Choi et al., 1999; Liu et al., 2003). *Scutellaria baicalensis* contains flavonoids such as scutellarin, baicalein (5,6,7-trihydroxy-2-phenyl-4*H*-1-benzopyran-4-one), wogonin (5,7-dihydroxy-8-methoxyflavone) and baicalin (5,6-dihydroxy-4-oxygen-2-phenyl-4H-1-benzopyran-7-beta-D-glucopyranose acid) (Ye et al., 2004; Parajuli et al., 2009). Baicalin inhibits human LNCaP prostate cancer and SK-Hep1 hepatoma cell growth (Chen et al., 2001; Chang et al., 2002; Li-Weber, 2009). Baicalein inhibits lipoxygenase and exhibits hypo-tensive and anti-cancer effects. It can also induce cell death in human hepatocellular carcinoma cell lines (Gao et al., 1996; Matsuzaki et al., 1996; So et al., 1997; Inoue and Jackson, 1999). Wogonin inhibits cyclooxygenase-2 and nitric oxide products, prostaglandin E(2) production in macrophages and angiogenesis (Wakabayashi and Yasui, 2000; Chi et al., 2001; Lin et al., 2006).

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The cell cycle is a conserved proliferative signaling cascade pathway (Braun-Dullaeus et al., 1998). In mammals, the cell cycle comprises the G1, S, G2 and M phases. Several cell cycle checkpoints inhibit damaged or incomplete DNA replication. The G1/S transition is a rate-limiting step in the cell cycle and represents the restriction point of the cycle (Kastan and Bartek, 2004). The cell cycle phases are controlled by regulating proteins including cyclin, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) (Nigg, 1995). Cyclin D1 phosphorylates the retinoblastoma protein (pRb), which in turn leads to the release of the E2F family of transcription factors (Motokura and Arnold, 1993; Sherr, 2000; Diehl, 2002; Alao, 2007). These released E2F factors drive the expression of various genes associated with S phase progression. When cyclin D1 is over-expressed, there is an increased risk of tumor progression and metastasis (Motokura and Arnold, 1993; Sherr, 2000; Diehl, 2002; Stacey, 2003; Chung, 2004; Alao, 2007). To our knowledge, this is the first study that demonstrates the inhibition of cyclin D1 expression associated with tumor cell invasion and migration.

The fundamental properties of malignant tumors are that they are invasive and metastatic. Metastasis is a multistep and complex process involving the over-expression of proteolytic enzymes such as matrix metalloproteinases (MMPs). MMP-2 and MMP-9 (also known as type IV collagenases or gelatinases) can degrade most extracellular matrix (ECM) components that form the basal membrane. Excess ECM degradation is one of the identifying factors of tumor invasion and migration. During the metastatic process, tumor cells need to attach to other cells and ECM proteins. Translocation of neoplastic cells across the ECM barrier is necessary due to matrix proteins being degraded by specific proteinases, especially type IV collagen (Woodhouse et al., 1997; McCawley and Matrisian, 2000). MMPs are $Zn^{2+}$-dependent endopeptidases and constitute a major group of enzymes that control cell–matrix composition (Lee et al., 2009). MMPs are frequently associated with the invasive metastatic potential of tumor cells including prostate cancer, lung cancer and malignant mesothelioma (Herbst et al., 2000; Lim and Jablons, 2003; Zhang et al., 2004; Di Carlo et al., 2010).

In Korean folk medicine, *Scutellaria baicalensis* has a long history of anti-cancer and anti-inflammatory prowess. However, the mechanisms relating to cell proliferation and migration have remained unclear. As well, the cytotoxicity of *Scutellaria baicalensis* is unknown. To explore the potential of *Scutellaria baicalensis* water extract (SBWE) as a useful chemotherapeutic agent against lung cancer, the present study examined the effects of SBWE on the expression of cyclin D1, CDK4 and MMP-2 in A549 NSCLC cells. Also, the effects of SBWE on cell cycle progression, especially the G1/S phase, and on cell migration were analyzed.

## 2. Materials and methods

### 2.1. Chemicals

RPMI 1640 was purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS) and antibiotics (streptomycin/penicillin) were obtained from Gibco (BRL Life Technologies, Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Antibodies to cyclin D1 and CDK4, and horseradish peroxidase (HRP)-coupled goat anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Materials and chemicals used for electrophoresis were obtained from BioRad (Hercules, CA, USA). All chemicals used in this experiment were of the purest grade available.

### 2.2. Preparation of SBWE

*Scutellaria baicalensis* cultivated in Korea was obtained from the Animal Bio-Resources Bank (Jinju, Korea). *Scutellaria baicalensis* (30 g) was extracted with 300 mL boiling distilled water for 1 h. The water extract was filtered through filter paper, evaporated *in vacuo*, and lyophilized to give a powdered extract (yield: 26.3%). The powdered extract (representing the SBWE) was dissolved in phosphate buffered saline (PBS, pH 7.4) and then filtered through a 0.45 μm syringe filter and stored at −20 °C until used for various cell culture treatments.

### 2.3. Cell culture and treatment

A549 human lung carcinoma cells obtained from the Korean Cell Line Bank (Seoul, Korea) were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin (P–S) in a 5% $CO_2$ atmosphere at 37 °C. Cells grown to 70–80% confluency were untreated (control) or treated with 10, 50, 100, 250 or 500 μg/ml SBWE for 24 h in complete growth medium.

### 2.4. Viability assay

The cytotoxicity of SBWE on A549 cells was investigated using a standard MTT assay. A549 cells were seeded wells of a 12-well plate and incubated for 24 h at 37 °C. The cells were untreated (control) or treated with 10, 50, 100, 250 or 500 μg/ml SBWE for 24 h at 37 °C prior to the addition of 100 μl of a solution of 5 mg/ml MTT in PBS to each well and incubation for 3 h at 37 °C. After removal of the fluid, 500 μl of dimethyl sulfoxide (DMSO) was added to each well to dissolve the crystalline deposits that had formed. The optical density (OD) of the cells at 540 nm was measured using an enzyme-linked immunosorbent assay plate reader.

### 2.5. DNA cell cycle analysis

Cells grown to 70–80% confluence were untreated or treated with 10, 50, 100, 250 or 500 μg/ml SBWE for 24 h in complete medium. The cells were trypsinized, washed twice with cold PBS and centrifuged. The pellet was fixed in 70% (v/v) ethanol for 24 h at 4 °C. The cells were washed once with PBS and resuspended in cold propidium iodide (PI; 50 μg/ml) containing RNase A (0.1 mg/ml) in PBS (pH 7.4) for 30 min in the dark. Flow cytometry was performed using a FACSCalibur apparatus (Becton Dickinson, San Jose, CA, USA). Forward light scatter characteristics were used to exclude cell debris from the analysis. Approximately 20,000 cells were evaluated for each sample. The DNA histograms were analyzed using ModFitLT software (Verily Software House, Topsham, ME, USA) for cell cycle analysis.

### 2.6. Western blot

A549 cells were cultured in wells of 6-well plates were incubated in PBS as the solvent control or in the presence of 10, 50, 100, 250 or 500 μg/ml SBWE for 24 h at 37 °C. After washing with ice-cold PBS, the cells were lysed in Tris–NaCl buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 1% NP-40] containing a protease inhibitor cocktail. The extracts were centrifuged for 30 min at 13,000 rpm to remove debris and protein concentration was determined using a Bradford assay (Bio-Rad). Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinyldene fluoride (PVDF) membrane (Immunobilon-P, 0.45 mm; Millipore, Billerica, MA, USA) using the TE 77 Semi-Dry Transfer Unit (GE Healthcare Life Sciences, Buckinghamshire, UK).