In vitro Antitumor Mechanisms of Various Scutellaria Extracts and Constituent Flavonoids

Abstract

Scutellaria is a traditional herbal remedy with potential anti-cancer activity. The purpose of this study was to evaluate anticancer mechanisms of thirteen Scutellaria species and analyze their leaf, stem and root extracts for levels of common biologically active flavonoids: apigenin, baicalein, baicalin, chrysin, scutellarein, and wogonin. Malignant glioma, breast carcinoma and prostate cancer cells were used to determine tumor-specific effects of Scutellaria on cell proliferation, apoptosis and cell cycle progression, via the MTT assay and flow cytometry-based apoptosis and cell cycle analysis. The extracts and individual flavonoids inhibited the proliferation of malignant glioma and breast carcinoma cells without affecting primary or non-malignant cells. The flavonoids exhibited different mechanisms of antitumor activity as well as positive interactions. The antitumor mechanisms involved induction of apoptosis and cell cycle arrest at G1/G2. Of the extracts tested, leaf extracts of S. angulosa, S. integrifolia, S. ocmulgee and S. scandens were found to have strong anticancer activity. This study provides basis for further mechanistic and translational studies into adjuvant therapy of malignant tumors using Scutellaria leaf tissues.

Introduction

Plants of the genus Scutellaria constitute one of the most common components of Eastern and traditional American medicines against various human ailments, including cancer. Extracts and isolated active components from Scutellaria have antiinflammatory [1], [2], antioxidative [1], [2], [3], anxiolytic [4], [5], and antiviral [2] activity. Among its various species, Scutellaria baicalensis is the most widely studied [6], [7], [8], [9], [10], followed by S. barbata [11], [12], [13]. A recent study illustrated the antiproliferative effect of S. baicalensis root extract in drug-resistant gliomas when used alone and in conjunction with the alkylating drug, carmustine (BCNU) [7]. Scutellaria extract comprises one of the eight constituents of the herbal mixture PC-SPES, which is widely used by prostate cancer patients as a supplementary treatment [14]. A phase I clinical trial has recently been concluded using BZL101, an aqueous extract from S. barbata, in patients with advanced breast cancer [15]. Studies on Scutellaria have most commonly utilized the roots. Although aerial parts are also known to be substantially rich in phytochemicals of medicinal value, antitumor activities of leaf and stem extracts have not been explored. Flavonoids constitute a major phytochemical component of Scutellaria extracts. The two most common, baicalein and baicalin, have shown antiproliferative and proapoptotic activities against various cancer cells [16], [17], [18], [19]. Baicalein has also shown a favorable effect in cisplatin-induced cell death of human glioma cells [19]. There are few reports on the anticancer activity of apigenin [20], [21], [22] and wogonin [13], [23], whereas the antitumor properties of other Scutellaria flavonoids, including chrysin and scutellarein, have rarely been studied. Paucity of knowledge on the phytochemical composition and mechanisms of antitumor activity of Scutellaria triggered our study, to further explore the therapeutic benefits of Scutellaria. We report a comprehensive analysis of the anticancer activity of leaf, stem and root extracts ob-
tained from thirteen different *Scutellaria* species using various
human cell cultures, including gliomas, breast carcinoma, pro-
tate carcinoma, normal primary human astrocyte, and non-ma-
lignant human mammary epithelial cell lines. The phytoche-
nical composition of the extracts, with respect to the presence
of six known flavonoids (apigenin, baicalein, baicalin, chrysin, scu-
tellarein and wogonin), and their antineoplastic activity, either
individually or in combination, are also reported.

**Materials and Methods**

**Cell lines and flavonoids**

Human malignant glioma cell lines U87-MG and U251; malig-
nant breast carcinoma MDA-MB-231, non-malignant human
mammary epithelial cell (HMEC) and human prostate cancer
cell line PC-3 were purchased from the American Type Culture
Collection and were cultured in DMEM/F12 supplemented with
10% fetal bovine serum (FBS). Normal human astrocytes (NHA)
cultures were purchased from Lonza Inc. and maintained in their
patented medium. Apigenin (purity 95%), baicalein (purity 98%),
baicalin (purity 98%), chrysin (purity 96%) and genistein (purity
98%) were purchased from Sigma Chemical Co.; scutellarein
(purity 96%) was from Indofine Chemical Co.; and wogonin (pu-
rity 98%) was purchased from Wako Chemicals. The flavonoids
were dissolved in dimethyl sulfoxide to make a 20 mM stock sol-
dition and diluted in DMEM/F12 before use.

**Collection and cultivation of *Scutellaria* plants**

Potted plants of *S. montana* and *S. costaricana* were obtained
from Atlanta Botanical Garden (ABG) and were identified by
Ron Galliardo (ABG). *S. ocmulgee*, *S. ovata* and *S. integrifolia*
were identified and received from Tom Patrick, Botanist, Georgia
Natural Heritage Program. Seeds of *S. baicalensis*, *S. barbata* and
*S. lateriflora* were purchased from Horizon Herbs, LLC. David
Creech, Stephen F. Austin State University, identified and provid-
ed cuttings of *S. suffrutescens*. *S. racemosa* were collected at the
FVSU campus. *S. alpina* was identified and provided by Dr. Ikhlas
A Khan, University of Mississippi, *S. angulosa* and *S. scandens*
were procured as tissue cultured plantlets from Nepal Biotech
Nursery, Kathmandu, Nepal. They were identified by Dr. Gopal
S. Rawat (plant taxonomist) at the Wildlife Institute of India,
Dehradun. All these specimens are preserved as herbaria and
photographed at FVSU (contact N. Joshee). Herbarium sheets
are arranged in alphabetical order as shown in Table 1.

**HPLC analysis of flavonoids**

Dried extracts (10 mg) were dissolved in 450 µL of 0.1% acetic
acid in methanol and 50 µL of a solution of 6-hydroxyflavone (in-
ternal standard, 600 µg/mL 0.1% acetic acid in methanol). Sam-
ple were analyzed by HPLC (Hewlett-Packard 1050) using an In-
ertsil ODS-2 5 µ, 250 mm x 4.6 mm, column (MetaChem Technol-
ologies, Inc.), and monitored for their flavonoid content at
λ = 270 nm. The mobile phase was 0.005% phosphoric acid (A)
and acetonitrile (B), eluted as follows: 30% B for 5 min, ramped
to 45% B from 5 min to 25 min, ramped to 100% B from 25 min
to 30 min and held for 3 min. Solvent flow rate was 1 mL/min; vol-
ume of injection was 10 µL. Apigenin, baicalein, baicalin, chrysin,
scutellarein and wogonin were quantitated from a calibration
curve of commercial samples of the flavonoids.

**Cell proliferation assay**

Cells were seeded to 96-well flat-bottom plates (2 x 10^4 cells/
well), and cultured in the presence of *Scutellaria* extracts or fla-
ovoids. After incubation at 37 °C for 4 days, cell viability was
evaluated with the MTT assay, as described elsewhere [24].
The absorbance was measured at 570 nm in an Infinite-200 mi-
croplate reader (Tecan Systems Inc.). Cell viability was ex-
pressed as a percent of control (cells cultured with medium
alone).

**Apoptosis assay**

U87-MG cells were plated (5 x 10^5 cells/mL/well) in 12-well
plates in the presence of *Scutellaria* extracts or flavonoids at
indicated doses. After 72 or 96 h, cells were washed, stained with
FITC-conjugated Annexin-V and propidium iodide (PI) (BD Phar-
mingen) and then analyzed in a flow cytometer (Becton-Dick-
inson FACScan), as previously described [25]. All cells that
bound Annexin-V were considered apoptotic. Cells were also
distributed into early apoptotic (bound with Annexin-V only)
and late apoptotic/necrotic (bound with both Annexin-V and
PI) cohorts.

| Table 1 | Inhibition of malignant glioma cell proliferation by *Scutellaria* extracts (500 µg/mL). *
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<tr>
<td></td>
<td>Leaf</td>
<td>Stem</td>
</tr>
<tr>
<td><em>S. alpina</em></td>
<td>91 ± 31</td>
<td>82 ± 16</td>
</tr>
<tr>
<td><em>S. angulosa</em></td>
<td>56 ± 13</td>
<td>65 ± 20</td>
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<tr>
<td><em>S. baicalensis</em></td>
<td>90 ± 28</td>
<td>62 ± 16</td>
</tr>
<tr>
<td><em>S. barbata</em></td>
<td>62 ± 19</td>
<td>73 ± 28</td>
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<tr>
<td><em>S. costaricana</em></td>
<td>86 ± 34</td>
<td>110 ± 39</td>
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<tr>
<td><em>S. integrifolia</em></td>
<td>50 ± 7</td>
<td>61 ± 16</td>
</tr>
<tr>
<td><em>S. lateriflora</em></td>
<td>49 ± 13</td>
<td>84 ± 17</td>
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<tr>
<td><em>S. montana</em></td>
<td>53 ± 14</td>
<td>74 ± 26</td>
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<tr>
<td><em>S. ocmulgee</em></td>
<td>44 ± 13</td>
<td>58 ± 9</td>
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<tr>
<td><em>S. ovata</em></td>
<td>65 ± 24</td>
<td>70 ± 18</td>
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<tr>
<td><em>S. racemosa</em></td>
<td>72 ± 34</td>
<td>81 ± 10</td>
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<tr>
<td><em>S. scandens</em></td>
<td>44 ± 13</td>
<td>75 ± 16</td>
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<tr>
<td><em>S. suffrutescens</em></td>
<td>78 ± 13</td>
<td>69 ± 12</td>
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* Results are expressed as percent proliferation relative to the control, which was taken as 100% proliferation. Proliferation less than 60% is considered significant, and indicated in bold numbers. Data are means ± s. d. of three ex-
periments.
Cell cycle analysis
U87-MG cells were plated \((5 \times 10^5 \text{ cells/mL/well})\) in 12-well plates in medium without serum for 12 h in order to synchronize at the quiescent or G0 phase of cell cycle. After 12 h, serum-free medium was replaced by medium containing 10% FBS. \(S\) cutellaria extracts and flavonoids were added at indicated doses. After 72 h, cells were fixed and permeabilized with cold absolute ethanol for one hour. Following treatment with RNase (100 \(\mu\)L at 100 mg/mL) for 15 minutes, cells were stained with PI (50 \(\mu\)g/mL). DNA content was analyzed in a flow cytometer (FACScan; Becton-Dickinson). Data analysis was performed using the Cell-Quest and WinMDI software packages.

Statistical analysis
Wilcoxon's log-rank test was performed to determine statistical differences between various experimental and control groups using the SPSS package (SPSS Inc.) [26]. A 'p' value less than 0.05 was considered significant.

Results and Discussion

In the initial evaluation of the anticancer activity of the extracts at 500 \(\mu\)g/mL concentration using U87-MG, nine extracts showed inhibitory activity, measured as <60% cell proliferation compared to the control (100% proliferation). These extracts were: \(S\) angulosa leaf (SanL), \(S\) integrifolia leaf (SinL), \(S\) lateriflora leaf (SlaL), \(S\) lateriflora root (SlaR), \(S\) montana leaf (SmoL), \(S\) ocmulgee leaf (SocL), \(S\) ocmulgee stem (SocS), \(S\) scandens leaf (SscL), and \(S\) suffrutescens root (SsuR) (Table 1). At 250 \(\mu\)g/mL concentration, only four leaf extracts (SanL, SinL, SocL, and SscL) showed significant anti-proliferative activity. The dose for 50% inhibition (IC\(_{50}\)) of proliferation for these four extracts was approximately 500 \(\mu\)g/mL (Table 1, Fig. 1).

HPLC analysis of the levels of apigenin, baicalein, baicalin, chrysins, scutellarein, and wogonin in leaf, stem, and root extracts showed various combinations of only three or four of the flavonoids in most extracts, except for SsuS and SsuR, which contained all six flavonoids (Table 2 and Supporting Information Table S2). The maximum concentration of a flavonoid detected in any extract was that of baicalein in SsuL (48.07 ± 4.12 \(\mu\)g/mg extract) followed by \(S\) baikalensis root (SbaR: 47.46 ± 0.10 \(\mu\)g/mg extract). The flavonoid concentrations were comparable to those reported in earlier studies [27], [28]. Of the four extracts that showed significant anti-proliferative activity, all but SocL had detectable levels of baicalin and chrysins, combined with other flavonoids. On the other hand, SocL extract, which showed some of the best anti-proliferative activity, contained only wogonin. These results suggest that: 1) wogonin could potentially have very high anticancer activity among the flavonoids examined; or wogonin, even at low concentration, could have positive interaction with other phytochemicals; 2) while there is positive interaction among some flavonoids, some may antagonize each other; 3) the six flavonoids examined could not account for the antiproliferative activity of the extracts, strongly suggesting the participation of other flavonoids and/or non-flavonoid active phytochemicals.

All the flavonoids dose-dependently inhibited proliferation of U87-MG cells (Figs. 2A and 2B), among which apigenin and

<table>
<thead>
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<th>Table 2</th>
<th>Flavonoid composition of selected (S)cutellaria extracts. *</th>
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<tbody>
<tr>
<td></td>
<td>Apigenin</td>
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<tr>
<td>(S) angulosa</td>
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<tr>
<td>Leaf</td>
<td>0.99 ± 0.45</td>
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<td>Stem</td>
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<td>Root</td>
<td>0.21 ± 0.03</td>
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<td>(S) integrifolia</td>
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<tr>
<td>Leaf</td>
<td>2.64 ± 0.22</td>
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<tr>
<td>Stem</td>
<td>0.64 ± 0.01</td>
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<tr>
<td>Root</td>
<td>1.47 ± 0.02</td>
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<tr>
<td>(S) ocmulgee</td>
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<tr>
<td>Leaf</td>
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<tr>
<td>Stem</td>
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<tr>
<td>Root</td>
<td></td>
</tr>
<tr>
<td>(S) scandens</td>
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</tr>
<tr>
<td>Leaf</td>
<td>2.34 ± 0.20</td>
</tr>
<tr>
<td>Stem</td>
<td>0.46 ± 0.02</td>
</tr>
<tr>
<td>Root</td>
<td>0.50 ± 0.02</td>
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* Values are \(\mu\)g/mg extract. Data are means ± s.d. of two experiments.
wogonin were the most potent with an IC$_{50}$ of approximately 100 nM, while the IC$_{50}$ for other flavonoids varied from 125 to 250 nM (Fig. 2). This is in agreement with another study, where apigenin and wogonin showed better anti-proliferative activity than baicalein in human leukemia HL-60 cells [29]. It was apparent from the dose-response curves (Fig. 2) that the lowest concentration of a single flavonoid required to inhibit tumor cell proliferation to the 70% level of control value was 15.6 nM apigenin (Fig. 2A) followed by 40 nM chrysin (Fig. 2B), which are equivalent to approximately 4.2 pg/mL of apigenin and 10.2 µg/mL of chrysin. This concentration was more than ten times higher than that detected in the active extracts required to achieve similar inhibition of proliferation (inhibition to 70% of control value was achieved with 125 µg/mL SinL extract (Fig. 1), which contained only 0.26% apigenin (2.64 µg/mg, w/w) and 0.05% chrysin (0.54 µg/mg, w/w) (Table 2)). These data indicate potential additive/synergistic interactions among the flavonoids. A combination of the four flavonoids detected in SinL, namely, apigenin, baicalin, chrysin, and scutellarein, each 5 µM, inhibited the proliferation of glioma cells by almost 50% (Fig. 2C). However, individually, at 5 µM, none showed any anti-proliferative activity. This result strongly suggested positive interaction among these flavonoids. The nature of the interaction, whether additive or synergistic, needs to be elucidated.

In order to examine whether the antiproliferative activity is tumor-specific, two human malignant tumor cell lines (U87-MG glioma and MDA-MB-231 breast carcinoma) and the corresponding non-malignant cells (NHA, normal human astrocytes primary culture, and HMEC, non-malignant human mammary epithelial cell line) were cultured in the presence of selected Scutellaria extracts and isolated flavonoids for 96 h. Baicalein as well as SanL, SinL, and SscL extracts significantly inhibited proliferation of the malignant glioma (U87-MG) cells (Fig. 3A). Similarly, all four Scutellaria extracts and six flavonoids significantly inhibited proliferation of breast cancer (MDA-MB-231) cells (Fig. 3C). On the other hand, proliferation of non-malignant cells, NHA and HMEC, was not affected by any of the extracts or flavonoids tested (Figs. 3B and 3D). The antiproliferative activity of the extracts and individual flavonoids was comparable to that of genistein (Fig. 4), a soy isoflavone known to have antiproliferative activity against breast cancers.
The antiproliferative activity of selected Scutellaria extracts and flavonoids against another malignant glioma (U-251) and a prostate carcinoma (PC3) cell lines were also examined. Except for scutellarein, all flavonoids significantly inhibited the proliferation of both U-251 and PC3 cells at 100 μM concentration (Figs. 3E and 3F). Among the extracts, SanL and SocL, at 500 μg/mL, significantly inhibited the proliferation of U-251 cells (Fig. 3E). Extracts of SanL, SinL and SocL significantly inhibited the proliferation of PC3 cells at 500 μg/mL, while inhibition by SscL was not significant (Fig. 3F).

These results indicate that Scutellaria extracts or flavonoids target molecular mechanisms specific to the malignant phenotype. In this context, we have observed inhibition of Akt/PKB and TGF-β activity in malignant gliomas following treatment with Scutellaria (Parajuli et al., unpublished data). Further studies on specific anti-tumor molecular mechanisms of Scutellaria are in progress.

Scutellaria extracts and isolated flavonoids have demonstrated anticancer activity via inhibition of proliferation and induction of apoptosis in various tumor cells [6], [7], [9], [18]. In our study, of the nine Scutellaria extracts that showed significant antiproliferative activity, four extracts (SanL, SinL, SocL, and SscL) induced significantly high apoptosis in U87-MG glioma cells (Fig. 4A). Incidentally, these four extracts were also those with relatively better antiproliferative activity compared to other extracts examined.
Among the six flavonoids examined, all but scutellarein displayed significantly higher apoptotic activity compared to the control (Fig. 4B). Induction of apoptosis was significantly enhanced upon increasing the dose of Scutellaria extract or flavonoids, which was further enhanced by prolonging the treatment from 72 h to 96 hours (Figs. 4C - 4G).

Baicalein and baicalin displayed markedly higher induction of apoptosis compared to apigenin and wogonin (Fig. 4), although apigenin and wogonin demonstrated better anti-proliferative activity than baicalein and baicalin (Fig. 1). This indicates a differential mechanism of anticancer activity among flavonoids, which in fact could also contribute to their positive interaction.

The differential antitumor mechanism of flavonoids was also reflected on their effect on cell cycle progression in gliomas. U87-MG cells, initially serum-starved for 12 h and then cultured in serum-containing medium for 72 h, had low number of apoptotic cells (4%) and an even distribution of cells in G1 (33%) and G2 (24%) phases (Fig. 5A). There was a significant enhancement in the number of cells in the G2 phase following treatment with apigenin (Fig. 5B), baicalin (Fig. 5C) and wogonin (Fig. 5G). A significant increase in the percentage of apoptotic cells (22%) was observed following treatment with baicalein (Fig. 5C). On the other hand, there was only marginal enhancement of cells in the G2 phase after treatment with chrysin (Fig. 5E) and scutellarein (Fig. 5F).

Our results are in agreement with some published studies, which have also shown enhancement of cells at G2/M phase after treatment with apigenin, baicalein, baicalin and wogonin [20], [23]. Another study has shown baicalein to induce dual G1/G2 arrest in rat heart endothelial cells [16].

Various Scutellaria extracts also differed in their effect on cell cycle progression. A significant enhancement of cells in both G1 and G2 phases was observed after treatment with SanL extract (Fig. 5H), while there was a significant enhancement in apoptotic cells following treatment with SinL extract (Fig. 5I). The number of cells in G1 phase was significantly enhanced following treatment with SocL (45%; Fig. 5J) as well as SscL (47%; Fig. 5K) compared to controls. Interest-
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**References**


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**References**

23 Chang WH, Chen CH, Lu FJ. Different effects of baicalein, baicalin and wogonin on mitochondrial function, glutathione content and cell cycle progression in human hepatoma cell lines. Planta Med 2002; 68: 128 – 32
27 Bergeron C, Gafner S, Clausen E, Carrier DJ. Comparison of the chemical composition of extracts from Scutellaria lateriflora using accelerated solvent extraction and supercritical fluid extraction versus standard hot water or 70% ethanol extraction. J Agric Food Chem 2005; 53: 3076 – 80