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پروپوزال نویسی
Investigation of Cytotoxic Activity in Four Stachys Species from Iran

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Abstract

The aerial parts of Stachys laxa Boiss. and Buhse. from Siah-bishe in Mazandaran province, Stachys trinervis Aitch. and Hemsl. from Karaj in Alborz province, Stachys subaphylla Rech. F. and Stachys turcomanica Trautv. from Golestan province have been collected in May 2008. Total extracts were obtained through MeOH/H₂O (80/20) and then partitioned between CHCl₃, EtOAc and MeOH. These fractions and total extracts have been investigated for *in vitro* cytotoxic activity against the colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2), breast ductal carcinoma (T47D) and Swiss mouse embryo fibroblast (NIH 3T3) cell lines using MTT assay (3-(4,5-di methyl thiazol-2-yl)-2,5-di phenyltetrazolium bromide). At each cell line, doses of 3.125, 6.25, 12.5, 25, 100, 200, 400 and 800 µg/mL in 1% (v/v) DMSO of all samples were tested. Ethyl acetate and chloroform fractions of *Stachys laxa* against proliferation of T47D and HT-29 cell lines and chloroform fraction of *Stachys subaphylla* and *Stachys subaphylla* ethyl acetate fraction toward T47D cell line exhibited highest cytotoxic activity (IC₅₀ < 50 µg/mL). Ethyl acetate and chloroform fractions of *Stachys turcomanica* against HT-29 cell line, except methanol fraction of *Stachys subaphylla*, the other extracts on T47D cell line, represented moderate cytotoxic activity (IC₅₀ < 70 µg/mL). All fractions of *S. trinervis* demonstrated no effective cytotoxic activity. IC₅₀ values confirmed that the growth and proliferation of HT-29 and T47D cells were most affected by chloroform and ethyl acetate fractions of *Stachys laxa* and *Stachys turcomanica* due to their nonpolar compounds.

Keywords: Cytotoxic activity; *Stachys laxa* Boiss. and Buhse.; *Stachys turcomanica* Trautv.; *Stachys subaphylla* Rech. F.; *Stachys trinervis* Aitch. and Hemsl.; MTT assay.

Introduction

The genus *Stachys* belongs to the plant family of Lamiaceae. The most species of this genus has been previously analyzed in numerous studies concerning their chemical composition, pharmacological properties and therapeutic uses. This family is well represented in the flora of Iran, at least with 200-300 species in the world (1) and 34 species in Iran (2). Phytochemical investigation of some *Stachys* species has demonstrated phenolic acids, tannins (3, 4), flavonoids (5) and phenyl ethanoid glycosides (6, 7). There are some reports about pharmacological activities of this genus including anticancer (8,
were collected in May 2008. The plants have been identified and deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.

**Extraction**

Freshly collected aerial parts of four species of *Stachys* were cleaned and shade dried. These parts were coarse powdered in a hand mill and stored at room temperature. Two hundred grams of powdered plants were extracted through percolation method with 80% aq. MeOH three times at room temperature. The extract was evaporated using rotary evaporator and consequently partitioned between CHCl$_3$, EtOAc and MeOH. Each fraction evaporated with rotary evaporator and has been stored at refrigerator for the investigation of cytotoxic activity.

**Cytotoxicity assay**

The colon carcinoma (HT-29), colorectal adenocarcinoma (Caco-2) and ductal carcinoma (T47D) cell lines were mentioned as exponentially growing cultures in RPMI 1640 cell culture medium (PAA, Germany), supplemented with 10% fetal bovine serum (FBS: Gibco, USA), for HT-29 cells and 15% FBS for Caco-2 and T47D cells. The Swiss mouse embryo fibroblast (NIH 3T3) cell line was kept in Dulbecco’s modified Eagle’s medium (DMEM; PAA, Germany) supplemented with 10% FBS. 100 IU/mL penicillin and 100 µg/mL streptomycin (Roche, Germany) were added to the media. All the cell lines were cultured at 37°C in air /carbon dioxide (95:5) atmosphere.

Cytotoxic activity was measured using modified MTT assay (31). 1×10$^4$ cells/well were plated in 96-well plates (Nunc, Denmark) and incubated for 24 h before the addition of drugs. After 96 h of incubation in Caco$_2$ cells and 48 h of incubation in HT-29, NIH/3T3 and T47D cells, the Swiss mouse embryo fibroblast (NIH 3T3) cell line was kept in Dulbecco’s modified Eagle’s medium (DMEM; PAA, Germany) supplemented with 10% FBS. 100 IU/mL penicillin and 100 µg/mL streptomycin (Roche, Germany) were added to the media. All the cell lines were cultured at 37°C in air /carbon dioxide (95:5) atmosphere.

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**Experimental**

**Plant material**

The aerial parts of *S. laxa* Boiss. and Buhse., from Siah-bishe in Mazandaran province, *S. trinervis* Aitch. and Hemsl from Karaj in Alborz province, and *S. subaphylla* Rech. F. and *S. turcomanica* Trautv. from Golestan province were collected in May 2008. The plants have been identified and deposited at the Herbarium of Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran.
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The cell viability in MTT assay was calculated as the percentage of control value. Methotrexate was used as the positive control. Cytotoxicity was expressed as the concentration of extract inhibiting cell growth with 50% (IC\textsubscript{50} ± SD). All tests and analysis were run in triplicate.

Statistical analysis

IC\textsubscript{50} (the median growth inhibitory concentration) values were calculated from the IC\textsubscript{50} of dose-response curve in the sigma plot 11 software. Data representative of three independent experiments with similar results were presented as mean ± SD.

Result

The effects of these plant extracts on the proliferative response of the HT-29, Caco-2 and T47D cell lines have been analyzed by treating the cells with different concentrations of the extracts and significant decrease in cell lines proliferation were observed. IC\textsubscript{50} ± SD are reported in Table 1. The chloroform and ethyl acetate fractions of \textit{S. laxa} Boiss. showed high cytotoxicity on T47D, HT-29 (IC\textsubscript{50} < 50 µg/mL).

Statistical analysis

IC\textsubscript{50} (the median growth inhibitory concentration) values were calculated from the IC\textsubscript{50} of dose-response curve in the sigma plot 11 software. Data representative of three independent experiments with similar results were presented as mean ± SD.

Table 1. Cytotoxic activity of total extract and fractions of four species of \textit{Stachys}.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell Lines* (MTT assay)</th>
<th>HT-29</th>
<th>Caco-2</th>
<th>T47D</th>
<th>NIH/3T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Stachys laxa}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total extract</td>
<td>421.97 ± 8.71</td>
<td>&gt; 1000</td>
<td>239.78 ± 16.92</td>
<td>508.77 ± 45.56</td>
<td></td>
</tr>
<tr>
<td>Methanol fr.</td>
<td>265.83 ± 46.52</td>
<td>&gt; 1000</td>
<td>254.1 ± 7.45</td>
<td>405.7 ± 74.18</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate fr.</td>
<td>134.004 ± 1.764</td>
<td>116.53 ± 18.23</td>
<td>18.079 ± 2.248</td>
<td>31.452 ± 1.554</td>
<td></td>
</tr>
<tr>
<td>Chloroform fr.</td>
<td>27.007 ± 2.096</td>
<td>101.65 ± 12.4</td>
<td>21.106 ± 2.491</td>
<td>41.294 ± 8.391</td>
<td></td>
</tr>
<tr>
<td>\textit{Stachys subaphylla}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total extract</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>60.15 ± 3.72</td>
<td>771.26 ± 164.67</td>
<td></td>
</tr>
<tr>
<td>Methanol fr.</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>771.26 ± 197.38</td>
<td>&gt; 1000</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate fr.</td>
<td>-</td>
<td>116.52 ± 2.78</td>
<td>51.05 ± 8.89</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Chloroform fr.</td>
<td>234.86 ± 11.28</td>
<td>183.85 ± 8.87</td>
<td>43.411 ± 9.99</td>
<td>74.27 ± 2.34</td>
<td></td>
</tr>
<tr>
<td>\textit{Stachys trinervis}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total extract</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>358.1 ± 14.14</td>
<td>&gt; 1000</td>
<td></td>
</tr>
<tr>
<td>Methanol fr.</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>630.96 ± 29.99</td>
<td>649.23 ± 17.91</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate fr.</td>
<td>241.66 ± 14.71</td>
<td>338.22 ± 1.02</td>
<td>128.35 ± 6.65</td>
<td>110.05 ± 5.56</td>
<td></td>
</tr>
<tr>
<td>Chloroform fr.</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td>383 ± 4.01</td>
<td>674.84 ± 67.37</td>
<td></td>
</tr>
<tr>
<td>\textit{Stachys turcomanica}</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Total extract</td>
<td>219.58 ± 14.21</td>
<td>&gt; 1000</td>
<td>103.67 ± 12.43</td>
<td>308.7 ± 1.34</td>
<td></td>
</tr>
<tr>
<td>Methanol fr.</td>
<td>693.57 ± 56.91</td>
<td>&gt; 1000</td>
<td>708.60 ± 25.8</td>
<td>802.58 ± 26.84</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate fr.</td>
<td>66.10 ± 5.43</td>
<td>87.08 ± 8.9</td>
<td>30.14 ± 1.78</td>
<td>50.45 ± 3.45</td>
<td></td>
</tr>
<tr>
<td>Chloroform fr.</td>
<td>66.84 ± 7.92</td>
<td>187.89 ± 11.72</td>
<td>51.38 ± 9.49</td>
<td>58.22 ± 4.06</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>0.23 ± 0.02</td>
<td>0.32 ± 0.04</td>
<td>0.16 ± 0.09</td>
<td>0.24 ± 0.013</td>
<td></td>
</tr>
</tbody>
</table>

*Results are expressed as IC\textsubscript{50} values (µg/mL), Key to cell Lines employed: HT-29 and Caco-2 (colon Adenocarcinoma), T47D (breast carcinoma), NIH 3T3 (Swiss embryo fibroblast).

Discussion

Among all the samples, nonpolar (chloroform and ethyl acetate fractions) fractions of \textit{S. laxa} exhibited greatest cytotoxicities on T47D and HT-29 cell lines compared with polar fraction and total extract. According to the data, the
cytotoxic activity of chloroform and ethyl acetate fractions on HT-29 and T47D cell lines were much stronger than that of Caco-2. It indicated that chloroform and ethyl acetate fractions of S. laxa had potential cytotoxic selectivity on T47D cell line. There was a report about the antioxidation and total phenol content of some Stachys spp. The research implied that total phenol content and FRAP value of methanolic extract are in this order: S. laxa > S. turcomanica > S. subaphylla > S. trinervis (33). Except S. subaphylla total extract against T47D cell line, the other methanolic extracts have indicated the same order of cytotoxic activity on T47D and HT-29. Higher cytotoxic activity of nonpolar fraction of S. laxa and S. turcomanica may be due to the high content of germacrene D in their essential oil, same as the Stachys cretica ssp. (34, 35), but main components of S. subaphylla and S. trinervis essential oils were identified as monoterpenic hydrocarbons. In comparison with another fraction, methanolic and total fractions of all samples demonstrated slightly cytotoxic effect on cell line tested. The real IC_{50} values of fractions of four species Stachys may be considerably lower than the positive control (Methotrexate) since its pharmacological active compounds are not pure and further researches are needed for defining potential component as cytotoxic natural medicines.

References

(22) Skaltsa HD, Bermejo P, Lazari DM, Silvan AM, Skaltsounis AL, Sanz A and Abad MJ. Inhibition of prostaglandin E, and leukotriene C, in mouse peritoneal macrophages and thromboxan B, production in human


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