Interaction between cytotoxic effects of Xanthomicrol and Noscapine on PC12 cells

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Abstract

In this study, the cytotoxic effects of methanolic leaf extract of Dracocephalum kotschyi Boiss and methanolic seed extract of Peganum harmala Linn. Alone and in combination, was studied against PC12 and B65 cell lines using MTT assay. In addition, the cytotoxicity of harmine, major β indol alkaloid in Peganum harmala was studied against these cells. Furthermore, the cytotoxicity of xanthomicrol, a flavone in methanolic leaf extract of Dracocephalum kotschyi and noscapine, and the interaction between them was also investigated against PC12 cell line. Dracocephalum kotschyi and Peganum harmala and their combination, Spinal-Z, showed cytotoxicity against cell lines tested in a concentration dependent manner. Cytotoxic effects of harmine and xanthomicrol could explain the cytotoxic effect of P. harmala and Dracocephalum kotschyi on these cells. Although, combination of these extracts showed cytotoxicity, but there was a decrease in comparison to each of the extracts alone. P. harmala seeds extract showed a greater preferential cytotoxic effect than D. kotschyi leaves extract on PC12 cells. Such a preferential cytotoxicity was also seen for harmine on the same cell line. Xanthomicrol and noscapine were able to inhibit proliferation of PC12 cells with IC50 of 24.5 µg/mL and 2.8 µg/mL respectively. The cytotoxic effects of xanthomicrol increased in the presence of noscapine. Flow cytometry analysis showed that xanthomicrol induced apoptosis in HL60 cell lines.

Keywords: Dracocephalum kotschyi, Peganum harmala, Spinal-Z, Xanthomicrol, Noscapine, MTT.

1 Introduction

Agents that preferentially inhibit proliferation of neoplastic cells may offer means of developing drugs effective against cancer in human. We have already reported that Peganum harmala L. and Dracocephalum kotschyi Boiss. Alone and in combination with each other (as an Iranian anticancer remedy named Spinal-Z) were able to inhibit proliferation of a number of malignant cells [7]. P. harmala, a member of the
family Zygophyllaceae, is a poisonous plant that is native to Iran [17]. Harmine, the major β-carboline alkaloid in *P. harmala* extract is a DNA topoisomeras type I inhibitor [15], specific cyclin-dependent kinase inhibitor [18], genotoxic [2, 11, 12] and a recombinogenic [3, 4] agent. It has shown cytotoxic activity against a series of tumour cell lines [9]. Also, we have shown that, a flavone called xanthomicrol is a cytotoxic component of *D. kotschyi* methanolic leaf extract, [7]. Leaves extract of *D. kotschyi*, a member of Labiatae family and a native Iranian plant [5] had been previously shown to have immunomodulatory [1, 6] and trypanocidal effects [12, 16]. The cytotoxic effects of xanthomicrol were more selective towards malignant cells than normal cells [9, 14]. In addition, xanthomicrol induced a dose dependent apoptotic cell death using fluorescence staining method [7, 8, 9, 16].

In this study the cytotoxic effects of Spinal-Z were compared with the effects of its components (*D. kotschyi* methanolic leaf extract and *P. harmala* methanolic seeds extract) against B65, PC12 and P19. In addition, the cytotoxicity of harmine (the major β-carboline alkaloid in *P. harmala*) was studied against these cell lines. Furthermore, the cytotoxicity of xanthomicrol and noscapine (an alkaloid derived from opium) and the interaction between their cytotoxic effects were also investigated against PC12 cell line. Finally, flow cytometric analysis was used to study the effects of xanthomicrol, on cell cycle.

2 Materials and Methods

RPMI-1640 medium, FBS (Fetal bovine serum), penicillin, streptomycin, MTT [3-(4,5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], harmine, propidium iodide (PI) and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Noscapine was obtained from TEMAD pharmaceutical company, Tehran, Iran. Cell lines (PC12: Rat adrenal fibroblast pheochromocytoma, B65: Rat nervous tissue neuronal, P19: Mouse embryonal carcinoma and HL60: Human promyelotic leukemia) were obtained from Pasteur institute, Tehran, Iran. Commercial Spinal-Z remedy was a gift from Darou Paksh Pharmaceutical Company and contained the dried methanolic extract of an 80:20 mixture of dried powdered seeds of *P. harmala* and leaves of *D. kotschyi*.

2.1. Preparation of *P. harmala* and *D. kotschyi* methanolic extracts

The leaves of *D. kotschyi* and seeds of *P. harmala* were collected from Esfahan and Tehran provinces respectively, and identified by Dr. G. Amin, the head of herbarium at the school of pharmacy, Tehran University. Voucher specimens of *D. kotschyi* (6537 TEH) and *P. harmala* (6536 TEH) have been deposited in the Herbarium of Department of Pharmacognosy, School of Pharmacy, University of Tehran. 100g of dried pulverized leaves of *D. kotschyi* or seeds of *P. harmala* were reflushed with 400mL methanol for 1h. The suspension was filtered and dried in vacuo and stored at -80°C until needed.

2.2. Isolation and purification of xanthomicrol in *D. kotschyi* leaves extract

Xanthomicrol was isolated from *D. kotschyi* leaf extract as described previously [7]. Briefly, 5g of methanolic *D. kotschyi* extract was dissolved in 15mL Methanol. The solution was applied to a silica coated glass plate (20cmX20cm) as a strip, 2 cm from the plate edge. The plate was developed in a TLC tank using chloroform: methanol: 14M NH₄OH (15:4:1). The band including xanthomicrol with Rf= 0.69 was eluted from silica using 100mL methanol and was dried in vacuo and 100mg of this fraction was dissolved in 10mL mobile phase. To remove particulate matter, the mixture was centrifuged at 2000g for 10 min. The clear supernatant was subjected to semi-preparative HPLC. The chromatographic system comprised of: Two Wellchrom Knaur K-1001 HPLC Pumps, a Linear 200 UVIS UV/Visible detector and a Macherey-Nagel Ecoprep HPLC column (Nucleosil 100-10, 25cm X 16mm). Mobile phase Acetonitrile: 0.05M HCl (75:25) was pumped at 10 mL/min (each pump set at 5mL/min). Detection was at 220nm. 0.4mL sample was loaded into the column during each run. Peak IV was collected, dried in vacuo and its structure was determined using spectral analysis [7].
2.3. MTT assay

MTT assay [13] was used to measure cytotoxicity of Spinal-Z, its component (P. harmala methanolic seeds extract and D. kotschyi methanolic leaves extract), harmine, xanthomicrol and noscapine. Each cell lines was maintained and treated in suspension in RPMI-1640 medium supplemented with 10% FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. Cells were grown under an atmosphere of 5% CO2/95% air at 37°C. These cells were then plated in 96 well plates (5x10^3 cells/well) and incubated under 5% CO2/95% air at 37°C for 24 h. The cells were treated with the test compounds for 72 h. After addition of MTT (10 μl/well, 5 mg/mL in phosphate-buffered saline), the plates were incubated for 4 h under 5% CO2/95% air at 37°C. Oxidized dye was dissolved by addition of 100 μL DMSO per well. Absorbance was measured at 570 nm using a Dynex MRX micro plate reader.

2.4. Fixing and staining of HL60 cells for the flow Cytometry Analysis

2x10^5 HL60 cells, untreated or treated with xanthomicrol or camptothecin for 4 and 24 h, were centrifuged for 5 min at 500 x g for 4°C. Supernatant was aspirated and discarded. Cells were washed twice with ice-cold PBS at 500 x g for 5 min at 4°C and fixed in 70% ethanol. Tubes containing the cell pellet were stored at -20°C for at least 24 h. After this, the cells were centrifuged at 350 x g for 5 minutes and the supernatant was discarded. Cells were washed twice with ice-cold PBS again at 350 x g for 5 minutes. Finally, 1 mL propidium iodide (PI) satin solution (20 μg/mL PI, 0.1% Triton X-100, 2 mM EDTA, 8 μg/mL DNase-free RNAase (sigma) in D-PBS) was added to the samples and the samples were analyzed by flow cytometry (FACScalibur, Becton Dickinson). Results were obtained as the percentage of subdiploid nuclei, which represent the apoptotic cells [4].

3 Result

3.1. Isolation and identification of xanthomicrol

The methanolic extract of D. kotschyi was subjected to TLC. 13 fractions were obtained Fraction 10 was further purified using semi-preparative HPLC which yielded 9 peaks (figure 5). The structure of peak IV, with a retention time of 41 min was determined using spectral analysis:

5-Hydroxy-2-(4-hydroxyphenyl)-6, 7, 8-trimethoxy-4H-chromene-4-one.

Yellow powder, m.p: 227-230°C, IR (KBr) (vmax, cm⁻¹): 1691 (C=O) Ms, m/z (%): 345 (m^+1, 15), 344 (M+, 30), 329 (20), 301 (15), 284 (5), 211 (20). Anal. Calcd for C_{16}H_{16}O_7 (344.8): C, 62.64; H, 4.64; CH3O, 26.97%. Found : C, 62.7; H, 4.71; CH3O, 27.07%. 1HNMR (500 MHz, CDCl3): δ = 3.96, 3.98, and 4.12 (9H, 3 s, 3 OMe), 5.63 (1H, brs, OH), 6.60 (1H, s, CH), 7.00 (2H, d, J_{HH} = 8.4 Hz, CH2), 7.85 (2H, d, J_{HH} = 8.4 Hz, CH2), 12.55 (1H, s, OH). 13 CNMR (125.7 MHz, DMSO-d6): δ = 60.04, 61.96, and 62.40 (3 OMe), 103.12 (C3), 106.82 (C10), 116.65 (CH3C5), 121.50 (C1), 128.97 (CH2C6), 133.15 (C3), 136.191 (C6), 145.69 (C7), 149.021 (C9), 152.99 (C5), 162.05 (C4), 164.8 (C2) and 183.03 (C4). The compound was identified to be xanthomicrol [2, 5].

3.2. In vitro cytotoxicity tests

The cytotoxicity of Spinal-Z, D. kotschyi leaves extract, P. harmala seed extracts and its major β-carboline alkaloid harmine were examined in two rat cell lines (PC12 and B65). Also, the cytotoxicity of D. kotschyi leaves extract was studied against P19 cells. All the compounds showed cytotoxicity in a dose dependent manner. The IC50 values against each cell line are presented in table 1. Also, a cytotoxic index was calculated for each agent as IC50 of test compound against B65 divided by its IC50 against PC12 cell line.

Of all herbal extracts used, P. harmala showed the greatest preferential cytotoxicity toward PC12. IC50 values obtained for harmine, the major β-carboline alkaloid found in P. harmala extract against PC12 and
B65 cell lines were found to be 4.55±0.9 and 13.7±1.2 μg/mL respectively and it showed the greatest selective cytotoxicity of all compounds tested.

Cytotoxic effects of xanthomicrol and noscapine were examined in PC12 cell lines. Both drugs showed cytotoxic effects. IC_{50} values obtained for xanthomicrol and noscapine against PC12 cell line were found to be 24.5 μg/ml and 2.8 μg/mL (table 2). The data obtained in the current study showed that in the presence of noscapine (2.8 μg/mL =IC50 of noscapine against PC12), IC50 of xanthomicrol decreased to 10.59μg/ml (fig. 1).

Figure 1: The cytotoxic of xanthomicrol (■) and xanthomicrol + noscapine (2.8 μg/mL) (□) on PC12 cell line. Noscapine made a decrease statistically in IC50 of xanthomicrol.

Table 1: The cytotoxic effect of Spinal-Z, Dracocephalum kotschyi leaves extract, Peganum harmala seeds extract and harmine against a panel of mouse cell lines.

<table>
<thead>
<tr>
<th>CellLine</th>
<th>Dracocephalum kotschyi</th>
<th>Peganum haramala</th>
<th>Spinal-Z</th>
<th>Harmine</th>
</tr>
</thead>
<tbody>
<tr>
<td>B65</td>
<td>48.5±3.5</td>
<td>63.3±1.6</td>
<td>71.6±4.1</td>
<td>13.7±1.2</td>
</tr>
<tr>
<td>PC12</td>
<td>59.7±2.8</td>
<td>35±3.2</td>
<td>60.4±2.6</td>
<td>4.55±0.9</td>
</tr>
<tr>
<td>P19</td>
<td>69.7±1.6</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
</tr>
</tbody>
</table>

IC_{50} value was calculated by measuring cell proliferation using the MTT assay and is defined as the drug concentration (μg/mL) causing a 50% inhibition of cell growth. Data represent mean values ± SD for 3 independent experiments. NM: Not Measured

Table 2: The cytotoxic effect of xanthomicrol and noscapine against PC12 cell line.

<table>
<thead>
<tr>
<th>Drug</th>
<th>IC50 (μg/mL) against PC12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xanthomicrol</td>
<td>24.5±1.1</td>
</tr>
<tr>
<td>Noscapine</td>
<td>2.8±0.5</td>
</tr>
</tbody>
</table>

IC_{50} value was calculated by measuring cell proliferation using the MTT assay and is defined as the drug concentration (μg/mL) causing a 50% inhibition of cell growth. Data represent mean values ± SD for 3 independent experiments.
3.3. Flow cytometry results

The flow cytometric analysis indicated that the treatment with 0.5µg/mL or 1 µg/mL xanthomicrol induced an accumulation of cells in the sub-G1 phase, which has been considered to be the marker of cell death. As shown in Fig. 2, the untreated cells exposed to the vehicle solvent (DMSO) showed normal cell cycle profile with 4.4% of cells in sub-G1 phase. However after 24-h xanthomicrol (500ng/mL) treatment, 18.7% of cells were in sub-G1 phase, strongly suggesting DNA degradation, an event reminiscent of apoptosis. After treatment with the same concentration of camptothecin for 24h, 36.7% of cells were in sub-G1 phase.

![Flow cytometry results](image.png)

Figure 2: The cytotoxic indices of Spinal-Z, *Dracocephalum kotschyi* leaves extract, *Peganum harmala* seeds extract and harmine.

The cytotoxic index of *dracocephalum kotschyi* was statistically different from *peganum harmala*. (** p<0.01, *** p<0.001)

![Flow cytometric analysis](image.png)

Figure 3: The flow cytometric analysis of HL60 cells treated with medium alone (control) or medium containing 500ng/mL xanthomicrol or camptothecin after 24 h. Accumulation of cells in the sub-G1 phase is considered to be the marker of apoptotic death.
4 Discussion

In this work, the cytotoxicity of Spinal-Z, an Iranian ethnobotanical anti-cancer remedy, and its constituents was studied against PC12, P19 and B65. In vitro cell proliferation inhibition test using MTT viability assay confirmed that Spinal-Z and its constituents (D. kotschyi leaves extract, P. harmala seeds extract and harmine, the major β-carboline alkaloid in P. harmala extract) have cytotoxic activities against cell lines tested. Also, D. kotschyi leaves extract showed cytotoxicity against mouse embryonal carcinoma (P19). The results obtained thus far suggested that: P. harmala seeds extract acted more selectively on the PC12 cancer cells compared to B65 (as normal cells), than Spinal-Z or D. kotschyi leaves extract. The selective cytotoxicity of harmine against PC12 could explain the preferential cytotoxic effects of P. harmala. Additionally, in this study, the cytotoxic effects of xanthomicrol, a flavone isolated and purified from methanolic leaf extract of D. kotschyi was investigated against PC12 cell lines. The results were compared with the cytotoxic effects of noscapine as a positive control. Furthermore, the interaction between cytotoxicity of xanthomicrol and noscapine was also studied. Our previous findings showed that xanthomicrol was the most active component in D. kotschyi leaves extract and had selective and preferential cytotoxicity against a panel of cancer cell lines [2], and like noscapine, could cause apoptosis in some cell types [2, 6]. Noscapine, a phthalideisoquinoline alkaloid derived from opium, has been used as an oral anti-tussive agent and has shown very few toxic effects in animals or humans. Recently, it was discovered that noscapine binds stoichiometrically to tubulin, alters the dynamics of microtubule assembly, and arrests mammalian cells at mitosis causing apoptosis, and exhibiting potent antitumor activity [6, 8, 9]. It is noteworthy that, noscapine showed little or no toxicity to kidney, liver, heart, bone marrow, spleen or small intestine at tumor-suppressive doses. Furthermore, oral noscapine did not inhibit primary immune responses, which are critically dependent upon proliferation of lymphoid cells. Thus, noscapine has the potential to be an effective chemotherapeutic agent for the treatment of some human cancers. In addition, the low toxicity, water solubility, and feasibility for oral administration are very valuable advantages of noscapine over many other microtubule drugs for future clinical use in cancer chemotherapy [6, 10]; Data obtained in this work confirmed the cytotoxic effects of xanthomicrol and noscapine on PC12 cells. Also, it was found that noscapine not only did not compete with xanthomicrol but also increased the anti proliferative effects of xanthomicrol against PC12 cell line. In our previous work using fluoroscence microscopy, it was shown that xanthomicrol induced apoptosis in HL60 cell line (with IC50 880 ng/mL) [2]. In this study, it was shown that, incubation of HL60 cells with xanthomicrol (0.5 and 1µg/mL) for 4h and 24h caused apoptotic cell death. So, further investigation is needed in order to evaluate the significance of this synergistic effect between noscapine and xanthomicrol, in the treatment of cancer in man.

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