Original article

Cytotoxic activity of the chloroform extract and four diterpenes isolated from *Salvia ballotiflora*

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**A R T I C L E   I N F O**

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**A B S T R A C T**

New compounds with chemotherapeutic activity are sought after, and plants are an important source of these compounds. Four diterpenes, 19-deoxyicetexone, 7,20-dihydroanastomosine, icetexone and 19-deoxyisoicetexone, were isolated from the hexane-washed chloroform extract of *Salvia ballotiflora*. The cytotoxic activity of the hexane-washed chloroform extract and its four diterpenes were tested using the MTT assay against three tumor cell lines: HeLa (cervical cancer), A549 (lung cancer) and MCF7 (breast cancer), and two murine cell line: J774A.1 (epithelial cancer) and CT26 (colon cancer), and their IC\textsubscript{50} values were determined. 19-Deoxyisoicetexone had the greatest effect on HeLa cells with IC\textsubscript{50} of 3.2 μg/ml (9.36 μM), whereas hexane-washed chloroform extract had the best cytotoxic effect on A549 cells with an IC\textsubscript{50} of 2.29 μg/ml. These effects of 19-deoxyisoicetexone and hexane-washed chloroform extract were with similar activity compared to cisplatin (IC\textsubscript{50} = 1.06 μg/ml in HeLa cells, and 4.6 μg/ml (15.21 μM) in A549 cells).

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**Introduction**

Cancer is a collection of several diseases that occur when cells of the body divide without stopping and spread to other tissues. Cancer is one of the primary causes of death worldwide. The World Health Organization estimated that 84 million people died of cancer between 2005–2015 (Danhier et al., 2010). Chemotherapy is an important option in the treatment of cancer. However, the drugs used during this treatment have negative side effects, such as fatigue, changes in taste, diarrhea, and hair loss, among other. For this reason, new chemotherapeutic compounds are sought. Plants have a long history of use in health care (Etkin, 1981). However, the effectiveness of many of these plants has not been evaluated, and the active metabolites have not been characterized. Between 1940 and 2002, most drugs used against cancer were naturally-derived products, while 8% were considered natural product mimics (Newman et al., 2000; Butler, 2004).

*Salvia ballotiflora* Benth., Lamiaceae, commonly known as “mejorana,” is an aromatic shrub. The leaves contain serrated margins and have hairs on the top and bottom. The flowers are blush-purple in color. *S. ballotiflora* is used in Mexican traditional medicine to relieve postpartum symptoms (Biblioteca Digital de la Medicina Tradicional Mexicana). Two diterpene quinones, icetexone (ICT) and conacytone were isolated from *S. ballotiflora* and their structures were elucidated by single X-ray diffraction techniques (Watson et al., 1976). Subsequently, three icetexane diterpenoids, 19-deoxyicetexone (DEOX), 19-deoxyisoicetexone (DIC) and 7,20-dihydroanastomosine (DAM) were isolated from the aerial parts of this plant and their structures were elucidated by spectroscopy techniques (Esquivel et al., 1997). Domínguez and co-workers isolated romulogarzone, icetexone (ICT) and conacytone (Domínguez et al., 1976; Taira et al., 1976). In 2013, the antidiarheal properties of DEOX were reported (Pérez-Gutiérrez et al., 2013).

The main goal of this research was to determine the cytotoxic activities of hexane-washed chloroform extract (ESC), and four diterpenoids isolated from *S. ballotiflora*, as the first step to find new compound that could be used in the treatment of cancer. They were tested against the human cancer cell lines HeLa (cervical...
cancer), MCF7 (breast cancer), and A549 (lung cancer) as well as two murine cell lines: CT26 and J774A.1.

Materials and methods

Reagents

Fetal bovine serum (FBS), Dulbecco Modified Eagle Medium (DMEM), antibiotic, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and dimethylsulfoxide (DMSO) were purchased from Sigma.

Plant material

*Salvia ballotiflora* Blenth., Lamiaceae, was collected in July 2014 in Las Comadres, Municipality of Guadalcazar, San Luis Potosí State, Mexico. The plant identification was confirmed by the taxonomist José García Pérez. A voucher specimen was deposited in the Isidro Palacios Herbarium of the Universidad Autónoma of San Luis Potosí (SLPM43014). The aerial parts of the plant were dried in the shade at room temperature.

Preparation of the extract

Two hundred grams of the dried, ground aerial parts of *S. ballotiflora* were extracted with chloroform by heating at its boiling point for 4 h. Then, the supernatants were filtered and evaporated to dryness under reduced pressure, after which the solid was washed with hexane. The yield was 5.07%. The extract (8 g) was separated by column chromatography using silica gel (Macherey Nagel 70–230 mesh) with hexane as the mobile phase and increasing the polarity with ethyl acetate, and fractions of 100 ml were collected.

Structural analysis

Structural identification was performed by NMR spectroscopy. The $^1$H and $^{13}$C NMR spectra were recorded on Agilent DD2-600 ($^1$H: 599.5 MHz, $^{13}$C: 150.8 MHz) NMR spectrometer at 25°C using CDC$_3$ as solvent and TMS with reference.

Cell lines and culture conditions

J774A.1 and CT26 cell lines were obtained from ATCC and HeLa, MCF7, A549 were obtained from Instituto Nacional del Cáncer of México. The cells were maintained in DMEM supplemented with 10% FBS, penicillin 100 IU/ml, and streptomycin 100 μg/ml. All the cells were cultured at 37°C in an atmosphere of 5% CO$_2$.

Cell cytotoxicity assay

Cells were seeded in DMEM in 96-well microplates at a density of $5 \times 10^3$ cell per well. After 24 h incubation, the cells were treated with concentrations of ESC from 1 to 200 μg/ml, with each compound at concentrations from 1 to 200 μM, and with cisplatin (DDP) at concentrations of 0.1 to 50 μM as a positive control. The cells without treatment were used as negative control. Each compound was dissolved in saline solution. After 48 h of treatment, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) at 5 mg/ml in PBS was added. The plates were then incubated for 3 h at 37°C. Then, the medium was removed, and the formazan crystals were dissolved in DMSO. The optical density (OD) was determined at 540 nm with an ELISA plate reader (Bio-Rad). Six replicates for each group were used to determine viability using the following equation, and the concentration leading to 50% inhibition of the viability (IC$_{50}$) was calculated by linear regression analysis.

\[
\% \text{Viability} = \frac{\text{OD treated cells}}{\text{OD control cells}} \times 100
\]

Statistical analysis

All experimental values are expressed as the mean ± SEM of at least two independent experiments. Statistically significant differences from the vehicle group were identified by Student’s *t*-test or ANOVA with post hoc Tukey test for paired data. The level of *p* < 0.05 was used to determine statistical significance. All calculations were performed using the Graph Pad Prism V.3 software system (GraphPad Software, San Diego, CA, USA).

Results and discussion

ESC was separated by column chromatography, orange crystals were obtained from the fraction with hexane/ethyl acetate (90:10, v/v) and were identified as DEOX (1): yield 0.0043%; m.p. 203–205°C. From the fraction eluting with hexane/ethyl acetate (85:15, v/v), a crystal yellow was obtained. The crystals were identified as DAM (2): yield 0.002%; m.p. 210–214°C. Yellow solid was obtained from the hexane/ethyl acetate (75:25, v/v) fraction. This compound was identified as DIC (3): yield 0.003%; m.p. 208–210°C. The $^1$H, and $^{13}$C NMR chemical shifts were corroborated with previously report (Esquivel et al., 1997). Orange crystals were obtained from the hexane/ethyl acetate (80:20, v/v) fraction, which were identified as ICT (4): yield 0.0009%; m.p. 210–214°C. The $^1$H, and $^{13}$C NMR chemical shifts were corroborated with previously report (Dominguez et al., 1976).

![Structural analysis](image)

White solid was obtained from the hexane/ethyl acetate (50:50, v/v) fraction. The solid was identified as a mixture of ursolic and oleanolic acids: yield 0.015%; m.p. 220–221°C. The $^1$H and $^{13}$C NMR chemical shifts of these triterpenoids were compared with the spectra of the reference samples.

The cytotoxic effects of ESC and the four compounds isolated from *S. ballotiflora* were evaluated against three human cancer cell lines, HeLa, MCF7 and A549, and two murine cell lines J774A.1 and CT26 at different concentrations (Fig. 1) to determine the IC$_{50}$ values (Table 1). ESC and DIC exhibited the highest cytotoxic effect on A549, CT26, HeLa, MCF7 and J774A.1 cells. The IC$_{50}$ values with ESC were 2.29, 6.76, 23.79, 6.57 and 29.91 μg/ml respectively. In the five cell lines, DIC exhibited IC$_{50}$ values of 5.11, 6.17, 3.2, 14.87 and 8.81 μg/ml respectively (Table 1), and these results show that the extract had the best activity on cell lines A549 and MCF7 and the cytotoxic effect on A549 cells was higher than DDP. Methanol extracts from other *Salvia* species, including *S. menthaefolia*, *S. sclarea*, *S. dominica*, *S. spinose*, and *S. palentina* showed IC$_{50}$ values ranging from 89.6 to 405.9 μg/ml in
cancer cell lines (Fiore et al., 2006). Ethanol and aqueous extracts from S. ringens showed IC_{50} values against cancer cell lines ranging from 179 to higher than 500 µg/ml (Alimić et al., 2015). Our results indicate that S. ballotiflora exerts cytotoxic effects on human cancer cell lines with higher potency compared to other Salvia species.

The IC_{50} values of the other three diterpenes showed lower cytotoxic effects compared to DIC or ESC. In summary, there is no significant difference of cytotoxic activity of ESC (A549) and DIC (HeLa) compared to the positive control CDDP. Therefore, we can suggest that ESC and DIC hold promise in the treatment of cancer. Different terpenes isolated from S. pachyphylla, native from Mexico, such as carnosol, 16-hydroxycarnosol, and 20-deoxycarnosol showed IC_{50} values ranging from 1.18 to 3.09 µg/ml in different cancer cell lines (Guerrero et al., 2006). The triterpenoids urmiensolide B and urmiensic acid, isolated from S. urmiensis, exerted cytotoxic effects against cancer cell lines (IC_{50} = 1.1–6.7 µg/ml) (Farimani et al., 2015). DIC showed similar IC_{50} values compared to other terpenes obtained from other Salvia species.

The cytotoxic effect of ursolic and oleanolic acids has been reported (Kassi et al., 2007; Yan et al., 2010). Also, it was found that oleanolic acid prevented colon carcinogenesis in male F344 rats (Jankikiram et al., 2008). Thus, the best cytotoxic activity of ESC in A549 and MCF-7 cells might be due to the effect of the mixture of these two triterpenic acids and the four diterpenes. However, more studies can be carried on in order to know be interactions between these compounds.

**Fig. 1.** Cytotoxicity activity of (A) DEOX, (B) DIC, (C) DAM, (D) ICT, (E) ESC, and CDDP as a positive control, in HeLa, A549, CT26, MCF7 and J774A.1 cell lines.

**Table 1**

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>IC_{50} (µg/ml)</th>
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<tbody>
<tr>
<td></td>
<td>CDDP</td>
</tr>
<tr>
<td>A549</td>
<td>4.6 ± 2.6</td>
</tr>
<tr>
<td>CT26</td>
<td>2.8 ± 0.8</td>
</tr>
<tr>
<td>HeLa</td>
<td>1.06 ± 3.8</td>
</tr>
<tr>
<td>MCF7</td>
<td>2.14 ± 2.7</td>
</tr>
<tr>
<td>J774A.1</td>
<td>2.45 ± 2.3</td>
</tr>
</tbody>
</table>

The results represent the mean ± standard error (SEM) of each of the compounds (six independent experiments). Significant difference *p < 0.05, **p < 0.01 and ***p < 0.001, respectively versus control group. “ns” not significant difference from control group p > 0.05.
Conclusions

The results here presented also suggest that the ESC and terpenes isolated from *Salvia ballotiflora* might be a good alternative for the search of new agents from natural origin to treat cancer.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Author contribution

NC-X, AJA-C, MAZ-S, and ES-M carried out the experimental studies. SP-G conceived the study, participated in its design and coordination, wrote the manuscript, and helped draft the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

References


