Full Length Research Paper

Citotoxic activity of isoflavan-cinnamylphenols from *Dalbergia congestiflora* on HeLa cells

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A systematic molecular fragmentation pathway (SMFP) was designed as an identifier to detect isoflavan-cinnamylphenols in methanol extract of *Dalbergia congestiflora*. Comprehensive mass spectral fragmentation patterns for extract of *D. congestiflora* were established using high-performance liquid chromatography (HPLC)/mass spectrometry (MS) equipment. Seven (1 to 7) tautomeric forms of isoflavan-cinnamylphenols (IFCPs) were identified by electron ionization mass spectroscopy (ESI-MS). These compounds were cytotoxic to the HeLa cervical cancer cell line. Compounds 1, 2 and 3 showed significant activity against HeLa cancer cell line *in vitro* with IC₅₀ values in the range 1.70 to 3.34 μg/ml. Compounds 4 to 7 showed moderate cytotoxicity with IC₅₀ values ranging from 2.23 to 8.67 μg/ml.

Key words: *Dalbergia congestiflora*, isoflavan-cinnamylphenols, tautomeric form, mass spectrometry.

INTRODUCTION

*Dalbergia* species (Leguminosae) are known for their deeply pigmented heartwood of varying colours, which are valued for use in wooden crafts, decoration and traditional medicine (Flynn and Holder, 2001). The genus has a wide distribution, native to the tropical regions of Central and South America, Africa, Madagascar and Southern Asia, but has become threatened due to overexploitation of its valuable timber (Buzatti et al., 2012). In Mexico, the wood of *Dalbergia congestiflora* is also used to manufacture musical instruments such as guitars and their purple pigment is used for hand-made crafts. Ethnobotanical reports mentioned, among other uses, the bark as a treatment to snakebites, and as a diuretic, febrifuge and anti-diarrhea remedy. Other cultures have been using *Dalbergia* spp. in their traditional medicine. In China, for example, medicinal remedies derived from this genus of plants are named Jianxiang for the treatment of various diseases, including cardiovascular diseases (Li et al., 2012). The heartwood of *Dalbergia candenatensis*, which is a deep red colour, has been used in folkloric medicine as an antimicrobial (Rasamiravaka et al., 2013) and an anti-inflammatory (Lee et al., 2013), and exhibits cytotoxic activity against HT-29 (colon cancer), KB (human oral cancer), MCF-7 (breast adenocarcinoma) and HeLa (human cervical cancer) cell lines (Songsiang, 2011).

Chemical investigations of *D. congestiflora* have led to the isolation of obtusaquinone and vestitol (Bekker et al., 2002) and neocandenatone (Barragan-Huerta et al., 2001). Isoflavan-cinnamylphenols (IFCPs) are a kind of isoflavones that exhibit oestrogenic, antiangiogenic, antioxidant and anticancer activities (Dixon, 1999; Dixon and Ferreira, 2002). Because of these properties, they have recently become popular as dietary supplements.

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Dalbergia spp. have exhibited cytotoxicity against various cancer cell lines in vitro (Song et al., 2009, 2011), therefore this study was intended to examine the cytotoxic activity of the isoflavancinnamylophenols isolated from D. congestiflora, in an assay with HeLa cells. By considering the general use of hyphenated techniques such as HPLC-MS in the analysis of plant extracts, a systematic molecular fragmentation pathway (SMFP) for seven candelatone and retusapurpurin A tautomeric forms under electron impact is reported for the first time. This SMFP was used as an identifier to detect isoflavancinnamylophenols in a methanol extract obtained from the dried bark of D. congestiflora.

MATERIALS AND METHODS

General experimental procedures

The HPLC/MS equipment was an HPLC 1100 series by Agilent Technologies, (Waldborn, Germany) equipped with an autoinjector, quaternary HPLC pump, column heater, UV detector and HP Chem Station for data collection and handling. The HPLC was interfaced to a mass-selective detector equipped with an API-ES detector directly into the MS detector. The spectra were scanned through a range of m/z 100 to 600.

Plant

Heartwood of D. congestiflora (Leguminosae) was purchased from a medicinal plants store in Mexico City. The plant was identified in the herbarium of the National School of Professional Studies, Ixtaca, UNAM, and a voucher specimen was deposited with the number 7653.

Extraction and isolation

A section of heartwood (350 g) of D. congestiflora was powdered; the obtained powder was extracted with methanol at room temperature with overnight agitation. Extracts were evaporated under reduced pressure conditions and the resulting crude methanol extract was passed through a 45 cm x 4 column of Sephadex LH-20 that was pre-equilibrated with water (yield was 15.2%). The column was successively eluted with a gradient of chloroform-methanol with increasing amounts of methanol. The final elution was performed with methanol and increasing amounts of water at a flow rate of 100 ml/h. Each fraction was collected and monitored by two-dimensional thin-layer chromatography. Extracts were concentrated in a rotary evaporator to produce nine pooled fractions (F1 to F9). Further HPLC/MS analysis was performed to obtain seven compounds: 1 (30 mg), 2 (37 mg), 3 (42 mg), 4 (23 mg), 5 (12 mg), 6 (9 mg) and 7 (18 mg).

**Compound 1**


**Compound 2**


**Compound 3**


**Compound 4**


**Compound 5**


**Compound 6**


**Compound 7**


Cytotoxic assay

The human cervical cancer cell line, HeLa, was grown in RPMI 1640 medium (Gibco Invitrogen: Carlsbad, CA, USA) containing 10% foetal bovine serum (FBS, HyClone: Logan, Utah, USA), 100 units/ml penicillin, and 100 µg/ml streptomycin sulphate. Cultures were incubated at 37°C in 5% CO2/air. HeLa cells (1.5 x 10^5) were seeded in 96-well tissue culture plates. After 24 h, 100 µl of dimethyl sulfoxide (DMSO) solution containing the test compounds was added to final concentrations of 0.01 to 10 µmol/ml. DMSO (100 µl) was added into control wells. The cells were treated with various concentrations of the test compounds for 96 h, after which cell growth was evaluated by an 3-[4,5-dimethylthiazol–2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay procedure (Tengchaisri et al., 1998). Then 200 µl of 0.2% MTT was added to every well with RPMI 1640 medium, and the plate was further incubated at
37°C in 5% CO₂/air for an additional 4 h. The plate was centrifuged to precipitate cells and formazan. An aliquot of 150 µl of the supernatant was removed from every well, and 200 µl DMSO was added to dissolve the formazan crystals. The plate was mixed on a microshaker for 10 min and measured on a microplate reader at 570 nm. The tautomeric forms, 1 to 7, were tested at five different concentrations, and each compound was tested in triplicate. The results were plotted and a dose-response curve was obtained for each compound, and the IC₅₀ value was calculated as the concentration of the tested compound that resulted in 50% reduction of optical density compared with the negative control. Etoposide (Sigma, St. Louis, USA; purity >98%) was used as the reference drug.

RESULTS AND DISCUSSION

The methanol extract of the bark of *D. congestiflora* was extensively chromatographed over Sephadex LH-20 columns, followed by HPLC-MS, to yield seven (1 to 7) tautomeric forms of isoflavon-cinnamylphenols. The interpretation of the MS of IFCPs previously isolated from *Dalbergia retusa* (Czako and Marton, 2001), *Dalbergia parviflora* (Czako and Marton, 2001), *D. congestiflora* (Barragan-Huerta et al., 2004), *Dalbergia nitidula* (Bekker et al., 2002) and *Dalbergia horrida* (Narayanan et al., 2007) revealed a systematic molecular fragmentation pathway analogous to that observed for candentone and retusapurpurin A. Similarly, the SMFP permits structures of fragment ions for IFCPs under electronic ionization (EI) to be proposed. The molecular formula indicates that compounds 1 to 7 (Figures 1 and 2) have the same substitution pattern, which only differ from each other in the positions of the methoxyl and hydroxyl groups. Figures 3 and 4 summarize the most characteristic fragments observed for the [M+H]+ ions of the seven types of isoflavon-cinnamylphenols, and as an example, the fragmentation pathways proposed for candentone and retusapurpurin A are shown in Figures 3 and 4, respectively. Its fragmentation behaviour could be extended to the other isoflavon-cinnamylphenols. The variety of minor fragment ions could be explained by the fact that there are many possible cleavage sites in these compounds and that different fragments can be lost simultaneously. In the positive mass, some common features, such as the protonated molecular ion fragment of the isoflavon-cinnamylphenols 1 to 6 [M+H]+ at m/z 523 (100%) was always the dominant peak and therefore it was chosen to monitor these components, whereas in compound 7, the most abundant peak was at m/z 537 [C₃₃H₃₅O₇]. Ion 242 [C₁₃H₁₄O₃] was observed as a common fragment in derivatives from candentone, while it was absent in the case of retusapurpurin A tautomeric forms. The positive mass spectra of all compounds display a quasi-molecular ion [M+1] at m/z 524, which
observed in retusapurpurin A. The molecular formula of the MS of isoflavan-cinnamylphenols revealed a systematic molecular fragmentation pathway analogous to that observed for candenateone (1) previously isolated from D. candidatensis (Hamburger et al., 1988) where its molecular formula was deduced from the pseudo-molecular ion [M + H]+ peak at m/z 523 in the MS spectra. Upon electron impact, the molecular ions of the candenateone derivative, dissociate through a limited number of assumed pathways, and as a rule, the origin of diene reaction (RDR) in the molecular ions and in the the origin of flavonoids (Saitoh et al., 1976). Cleavage of the pyran ring at the F-ring gave a peak at m/z 349.

The MS data established that retusapurpurin A is a quinonemethide represented by the structural formula 4 (Figure 2) previously isolated from D. candidatensis (Czako and Marton, 2001). A retro-diene reaction was not observed in retusapurpurin A. The molecular formula of retusapurpurin A (C_{32}H_{27}O_7) was deduced from the pseudo-molecular ion [M+H]^+ peak at m/z 523. The MS spectrum indicated four tautomeric structures of 4. The positive ion of 4 exhibited abundant [M + H]^+ ions, and fragmentation occurs predominantly by cleavage of interflavonoid bonds to produce sequence ions. The fragmentation characteristics for 4 are shown in Figure 4. M/z 359 ions are produced by quinonemethide (QM) fission, and m/z 149 by heterocyclic ring (HRF) fission (Karchesy et al., 1986).

The cytotoxic activity of the aforementioned seven isoflavonoids (1 to 7) against HeLa cells was evaluated by the MTT method. The results showed that the seven compounds were cytotoxic against HeLa cells in vitro. In particular, compounds 1, 2 and 3 were highly cytotoxic, with IC_{50} values ranging from 1.70 to 3.34 µg/ml (Table 1). Despite their structural differences in the F-, A- and C-rings, compounds 4 to 7 showed moderate cytotoxicity with IC_{50} values ranging from 2.23 to 8.67 µg/ml (Table 1). Therefore, further anti-tumour activity of IFCPs should be performed to elucidate the structure/activity relationship and the possible action mechanisms that may explain their cytotoxic activity.

In conclusion, in the present study, HPLC/MS analysis was found to be a suitable method for the characterization of isoflavon-cinnamylphenols from D. congestiflora. Compounds 1, 2 and 3 showed better cytotoxic activity than compounds 4 to 7 against HeLa cells.
Figure 3. Proposed fragmentation of tautomeric from candenate.
Figure 4. Proposed fragmentation of tautomeric from retusapurpurin A
Table 1. Cytotoxicities of compounds 1 to 7.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cytotoxicity (IC&lt;sub&gt;50&lt;/sub&gt; µg/ml) HeLa cell line</th>
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<tbody>
<tr>
<td>1</td>
<td>1.70 ± 0.07</td>
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<tr>
<td>2</td>
<td>3.34 ± 0.98</td>
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<tr>
<td>3</td>
<td>2.25 ± 0.21</td>
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<tr>
<td>4</td>
<td>16.72 ± 4.32</td>
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<tr>
<td>5</td>
<td>18.67 ± 3.19</td>
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<tr>
<td>6</td>
<td>14.43 ± 2.67</td>
</tr>
<tr>
<td>7</td>
<td>12.23 ± 4.02</td>
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<tr>
<td>Etoposide</td>
<td>0.50 ± 0.01</td>
</tr>
</tbody>
</table>

Etoposide was a positive control.

REFERENCES

Computational information technologies for environmental sciences (CITES) (2009).