Induction of Apoptosis in Human Leukemia Cell (Jurkat) by Neolignans Isolated from Seeds of Licaria puchury-major

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Ethanol extract of the seeds of Licaria puchury-major, a Brazilian herbal medicine, was found to inhibit cell proliferation in human leukemia cell line (Jurkat) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Bioassay-guided fractionation of the active components led to the isolation of one phenylpropanoid (1) and ten neolignans (2—11). The apoptosis-inducing activity of the compounds showing cytotoxicity in Jurkat cells was assessed by flow cytometric analysis. Among the identified neolignans, compounds 3, 4, 6 and 7 which have similar molecular structures, showed apoptotic activity. To elucidate the mechanism of apoptosis induction by neolignans, intracellular caspase-3, -8 and -9 activity in Jurkat cells was evaluated. Compound 4 markedly elevated the activity of caspase-3 and -9.

Key words neolignan; apoptosis; Licaria puchury-major; leukemia; caspase

Apoptosis is an essential physiological process that leads to programmed cell death through the activation of an evolutionarily conserved intracellular pathway distinct from necrosis. Dysregulation of the apoptotic pathway may cause serious diseases, such as neuronal conditions, autoimmune disease, and cancer. Cell death caused by apoptosis is characterized by distinct morphological changes including chromatin condensation, cell shrinkage, and cellular fragmentation into apoptotic-bodies. The process of apoptosis is regulated by the expression of several proteins. Of these, caspase (cysteinyl aspartate-specific protease) plays a pivotal role in the execution of apoptosis. Of particular interest is caspase-3, the most widely studied member of the caspase family and one of the key executioners of anticancer drug-induced apoptosis.

Natural products of plant origin offer a wide variety of bioactive compounds that could meet the demand for base compounds of drugs. Over the past few decades, much attention has been focused on natural products as potential sources of novel anticancer drugs. The therapeutic application of apoptosis has currently been used as a model for developing anticancer drugs, because many chemotherapeutic drugs have been shown to induce apoptosis in cancer cells. Thus, the identification of potential chemotherapeutic agents using mechanism-based study holds great promise for elucidating mechanisms and devising more specific and effective treatments for cancer-related diseases.

The seeds of Licaria puchury-major (Lauraceae), called Puchuri-grosso or Puchurim in Brazil, have been used in folk medicine as a carminative and stomachic. Yet only a few reports on the essential oil constituents of this plant have been published. In our continued search of apoptosis-inducing substances from plants resources, we found that the ethanol extracts of the seeds of Licaria puchury-major exhibit cytotoxic activity against human leukemia cell line (Jurkat). In this study, we performed bioassay-guided fractionation of the ethanol extract and identified one phenylpropanoid (1) and ten neolignans (2—11). Flow cytometric analysis of the isolated compounds showed that neolignans, containing a unique furanocyclohexenone structure with hemiacetal in the molecule, such as ferrearin C (4), have caspase dependent apoptosis-inducing activity. Although many neolignans have also served as lead compounds for the development of new drugs, the present work is the first study evaluating the cytotoxic activity of “ferrearin”-type neolignans against human cancer cells.

MATERIALS AND METHODS

Plant Material The plant material was purchased from Farmacervas Ltd. in São Páuoro, Brazil and identified by Pharmacist Girberto Rubens Biancalana. A voucher specimen (YF0067) was deposited in the herbarium of College of Pharmacy, Nihon University.

Extraction and Isolation Seeds of L. puchury-major (2.8 kg) were crushed and extracted with EtOH (15 l) under ultrasonication. After filtration, the solvent was removed under reduced pressure to give a crude extract (276.8 g, Ext. 1). The EtOH extract dissolved in 90% MeOH (1.0 l) was partitioned into n-hexane (1.0 l × 3) to afford the lipid containing fraction (182.4 g, Fr. 1). The 90% MeOH soluble fraction (93.8 g, Fr. 2) obtained after evaporation was applied to Diaion HP-20 CC (1.5 l), eluted successively with MeOH–H2O (4 : 6, Fr. 3), MeOH–H2O (7 : 3, Fr. 4), MeOH (Fr. 5), and acetone (6 l, Fr. 6). Growth inhibition of the extract (Ext. 1) and each fraction (Fr. 1—Fr. 6) against human leukemia cells (Jurkat) was tested, and the acetone eluted fraction (Fr. 6, 8.3 g) was found to be the most active (82.7% inhibition at 30 μg/ml and 14.8% inhibition at 3 μg/ml). The acetone eluted fraction was chromatographed on a silica gel column using n-hexane : ethyl acetate (50 : 1 to 0 : 100, gradient) to obtain 15 fractions (Fr. 7—Fr. 21). Fr. 8 afforded compound 1 (269 mg) without further purification. Fr. 10 (86 mg) and Fr. 11 (211 mg) on Capcellpack octadecyl silica (ODS) column (80% aq. MeOH) afforded compound 2 (31 mg). A portion of Fr. 11 on Capcellpack ODS...
(Fr. 11-1, 98 mg) was further purified by Capcellpack Ph (60% aq. CH₃CN) to afford compound 3⁵⁹ (49 mg). Fr. 13 (386 mg) on Capcellpack Ph (75% aq. MeOH) afforded compound 4²⁵,²⁶ (72 mg). Fr. 14 (259 mg) on Capcellpack ODS column (75% aq. MeOH) afforded compound 5³⁰,³¹ (63 mg). Fr. 15 (260 mg) and Fr. 16 (443 mg) on Capcellpack ODS column (70% aq. MeOH) afforded compound 6²⁵ (188 mg). A portion of Fr. 15 on Capcellpack ODS (Fr. 15-3, 34 mg) was further purified by Capcellpack ODS (55% aq. CH₃CN) to afford compound 7³⁵ (8 mg). Fr. 17 (673 mg) on Capcellpack ODS column (65% aq. MeOH) afforded compound 8⁴⁰ (66 mg). A portion of Fr. 17 on Capcellpack ODS (Fr. 17-1, 25 mg) was further purified by Capcellpack ODS (50% aq. CH₃CN) to afford compound 9³⁵ (22 mg). Fr. 18 (515 mg) on Capcellpack ODS column (65% aq. MeOH) and Capcellpack Ph (45% aq. CH₃CN) afforded compound 10³²,³⁶ (49 mg). Fr. 20 (667 mg) on Capcellpack ODS column (60% aq. MeOH) afforded compound 11³⁵ (25 mg). The structures of isolated compounds were elucidated by extensive analyses of EI-MS, ¹H-, and ¹³C-NMR spectra and identified by spectral data and specific rotations in the literature.

Bromide (MTT) Assay Jurkat cells (1 x 10⁶/well) were spread onto a 96-well culture plate with phenol red-free RPMI 1640 medium with 10% fetal bovine serum (FBS) and maintained for 24 h. Then neolignan (final concentration 10⁻⁵—10⁻⁴M) and vehicle was applied for 48 h. After addition of 0.5% MTT solution as a 1/10 volume of medium in medium containing 5% CO², the well, incubation was continued for a further 4 h at 37 °C within 24 h as shown by flow cytometric analysis.

Flow Cytometry Apoptosis was detected using Viability™ MTT assay kit #3 (Molecular Probes). Cells (1 x 10⁶ cells/dish) were exposed to neolignan (10⁻⁵—10⁻⁴ M) or vehicle for 4 h. To prepare the cell sample for flow cytometry, cells were washed with annexin-binding buffer and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) for 15 min. The cell samples were analyzed by a FC500 flow cytometer (Beckman Coulter) using the FL1 and FL4 range for annexin V FITC and PI, respectively.

Immunoblotting Cells were collected and lysed with lysis buffer (20 mM Tris–HCl pH 8.0, 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonylfluoride (PMSF), protease inhibitor cocktail I (1:200; Sigma), phosphatase inhibitor cocktail II (1:100; Sigma), and 1 mM dithiothreitol). Protein concentration was determined using the Bradford method. Cell lysates containing 20 μg total protein were loaded onto 12.5% or 15/25% sodium dodecyl sulfate (SDS)-polyacrylamide gels with Tris/glycine running buffer and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Bioscience). Each membrane was blocked with blocking buffer (5% skim milk, 137 mM NaCl and 20 mM Tris–HCl, pH 7.6) for 1 h at room temperature and incubated with the primary antibody (anti-caspase-3 1:3000 (cell signaling), anti-caspase-8 1:500 (cell signaling), anti-caspase-9 1:1000 (cell signaling), diluted in 5% bovine serum albumin) at 4 °C overnight. After washing with Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated with a secondary antibody conjugated with horseradish peroxidase (1:10000, (Sigma), diluted in 5% skim milk) at room temperature for 1 h. The signal was detected using an enhanced chemiluminescence Western blotting detection system (Amersham Bioscience). The density of the band was analyzed by NIH image-J software.

RESULTS AND DISCUSSION

The EtOH extract (E1) of the seeds of L. puchury-major showed the growth inhibitory activity against human leukemia cells (Jurkat) (53.3% inhibition at 30 μg/ml). The extract dissolved in 90% MeOH was partitioned into n-hexane to afford lipid containing fraction (Fr. 1) (6.7% inhibition at 30 μg/ml). After removal of the lipid containing fraction, the 90% MeOH soluble fraction (Fr. 2) (65.2% inhibition at 30 μg/ml) was applied to Diaion HP-20 CC (1.5 l), eluted successively with 40% MeOH (Fr. 3), 70% MeOH (Fr. 4), MeOH (Fr. 5) and acetone (Fr. 6). Growth inhibition of each fraction (Fr. 1—Fr. 6) against Jurkat cells was tested, and the acetone eluted fraction (Fr. 6) was found to be the most active (82.7% inhibition at 30 μg/ml). To clarify whether Fr. 6 induced cell death involving apoptosis, early apoptosis was examined by flow cytometry after annexin V and PI double staining. As shown in Fig. 1, the acetone eluted fraction (Fr. 6) induced early apoptosis at 30 μg/ml within 24 h against Jurkat cells.

Further purification of Fr. 6 by silica gel column chromatography followed by HPLC purification gave phenylpropanoid, apiole (1), and ten neolignans: (7S,8S)-Δ²⁻⁴,⁵⁻dimethyl-3,4,4'-methyleneoxy-7,0,3',8,4',1',O,7'-neolignan (2), ferrearin B (3), ferrearin C (4), licarin A (5), rel(7S,8S)-1'2'S)-2'-hydroxy-3,4-dimethoxy-3'-oxy-4',8'-8.1',7-O,2'-neolignan (6), ferrearin G (7), oxaguanin (8), rel(7S,8S)-1'2'R,5'-8'-5'-methoxy-3,4-methyleneoxy-4'-oxy-Δ²',8'-8.1',7-O,2'-neolignan (9), armenin B (10), 3'-methoxyburchellin (11) (Fig. 2). The cytotoxic activity of isolated compounds 1—11 against Jurkat was tested by MTT assay (Table 1). Interestingly, compounds 3, 4, 6 and 7 having furanocyclohexenone structure with hemiacetal in the molecule showed cytotoxic activity at 10 μM. As shown in Fig. 3, all of these four neolignans induced early apoptosis at 10 μM within 24 h as shown by flow cytometric analysis. Compound 2 also induced apoptosis at 100 μM within 48 h (data not shown). Moreover, Jurkat cells treated with apoptosis-inducing neolignans (10 μM) for 48 h were observed by Hoechst 33342 staining. As shown in Fig. 4, the cells treated with neolignans exhibited condensed nuclei within 24 h, compared with the control cells.

Two major pathways of caspase activation have been revealed at present. One pathway is the death receptor-mediated apoptosis pathway where tumor necrosis factor family activates upstream caspase-8.³⁷ Activation of caspase-8 can in turn directly activate caspase-3. The alternative is the mitochondrial-mediated apoptosis pathway where cytochrome c is released from mitochondria and activates procaspase-9.³⁸ Caspase-9 forms an active holoenzyme that processes and activates downstream caspase-3. To examine the mechanisms of apoptosis in Jurkat cells treated with neolignans, caspase-3, -8, -9 and -12 activity was evaluated. Ferrearin C (4) clearly...
showed the activity of both caspase-3 and -9 at 10 μM (Fig. 5). Although it was less clear, the other three “ferrearin”-type neolignans: ferrearin G (7) having an additional methoxy group on the benzene ring of ferrearin C (4); ferrearin B (3), a diastereomer of ferrearin C (4); and compound 6 containing dimethoxy group in place of methylenedioxy group in ferrearin C (4), showed activation of caspase-3 and -9 at 10 μM (data not shown). These results suggest that these “ferrearin”-type neolignans exert cytotoxicity through caspase-dependent pathways. It should be noted that the isolated apoptosis-inducing “ferrearin”-type neolignans (3, 4, 6, 7) have three characteristics common in their molecules: 1) furanocyclohexenone structure with α,β-unsaturated carbonyl group, 2) an angular allyl group, and 3) bicyclo-hemiacetal structure. Further studies of structure–activity relationship including stereochemistry for “ferrearin”-type neolignans will be required to investigate the mechanisms.

In the present study, we showed that the EtOH extract of the L. puchury-major seed exhibited cytotoxic activity against human leukemia cells (Jurkat). Bioassay-guided fractionation of the extract gave a phenylpropanoid and ten neolignans. Using flow cytometry, we revealed that among the isolated compounds, four neolignans (3, 4, 6, 7) induce apoptotic cell death. We further determined that apoptosis induced by compound 4 at 10 μM is mediated by procaspase-9 processing. The apoptosis-inducing activity of these “fer-
Fig. 3. Effect of Compounds 3, 4, 6 and 7 (10 μM) on the Induction of Apoptosis in Jurkat Cells

Fig. 4. Cell Shrinkage and Fragmentation Revealed by Compounds 3, 4, 6 and 7 (10 μM) with Hoechst 33342 in Jurkat Cells (Top: Phase-Contrast Image; Bottom: Fluorescent Image)

Table 1. Cytotoxicity of Isolated Compounds against Jurkat Cells

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Cell survival (%)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0.3 (μM)</td>
</tr>
<tr>
<td>1 (Apiol)</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>98.7±0.4</td>
</tr>
<tr>
<td>3 (Ferrearin B)</td>
<td>ND</td>
</tr>
<tr>
<td>4 (Ferrearin C)</td>
<td>94.5±1.6*</td>
</tr>
<tr>
<td>5 (Licarin A)</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>ND</td>
</tr>
<tr>
<td>7 (Ferrearin G)</td>
<td>ND</td>
</tr>
<tr>
<td>8 (Oxaguianin)</td>
<td>ND</td>
</tr>
<tr>
<td>9 (Armenin B)</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
</tr>
<tr>
<td>11 (3'-Methoxyburchellin)</td>
<td>ND</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>94.5±0.1**</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values represent means±S.E.M. *p<0.05, **p<0.01 vs. control, compared by one-way ANOVA followed by Bonferroni’s post hoc test.  
<sup>b</sup> ND, not done.
neolignans has not been reported previously. These results suggested the “ferrearin”-type neolignan possessing a unique structure in the molecule may become a candidate as a lead compound in the treatment of patients with leukemia.

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