Antimicrobial Isoflavonoids from *Erythrina crista galli* Infected with *Phomopsis* sp.

Flavia Redko\(^a\), María L. Clavina\(^a\), Daniela Weber\(^b\), Fernando Ranea\(^c\), Timm Anke\(^b\), and Virginia Martinoa,\(^*\)

\(^a\) Instituto de Química y Metabolismo del Fármaco (IQUIMEFA) (UBA-CONICET), Càtedra de Farmacognosia, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina. Fax: 54 (11)-45083642.

E-mail: vmartino@ffyb.uba.ar

\(^b\) Institut für Biotechnologie und Wirkstoff-Forschung (IBWF), Erwin-Schrödinger-Straße 56, D-67663, Kaiserslautern, Germany

\(^c\) Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina

\(^*\) Author for correspondence and reprint requests

Z. Naturforsch. 62c, 164–168 (2007); received October 4/November 7, 2006

The isoflavonoids coumestrol, genistein and daidzein have been isolated and identified by bioassay-guided fractionation from the acetone extract of *Erythrina crista galli* young twigs infected with *Phomopsis* sp. These compounds showed antimicrobial activity against *Bacillus brevis* (MIC values 16.3, 64.8 and 137.8 \(\mu\)m, respectively). This is the first time that coumestrol, besides lutein and \(n\)-nonacosane, are reported in this species.

**Key words:** *Erythrina crista galli*, *Phomopsis* sp., Isoflavonoids

**Introduction**

The isolation of *Phomopsis* sp., an endophytic fungi, from different collections of young and old twigs of *Erythrina crista galli* has already been reported. Phomol, a compound with antibacterial, antifungal and *in vivo* antiinflammatory activities, has been isolated and identified from the fermentation media of this fungus. Furthermore, eight new compounds have been identified from the same endophyte (Weber et al., 2004, 2005).

As part of an ongoing project in the search for bioactive metabolites from Argentine medicinal plants and their endophytic fungi, the isolation of compounds from young twigs of *E. crista galli*, infected with *Phomopsis* sp., is now described.

*Erythrina crista galli* L. (Leguminosae) is a tree that grows in South America and is used in folk medicine for wound healing, as astringent, narcotic and analgesic (Toursarkissian, 1980). Alkaloids, pterocarpans, cinnamoylphenols and triterpenoids have been reported as the major compounds in bark and leaflets (Ingham and Markham, 1980; Inuma et al., 1994; Tanaka et al., 1997). Erycrisin, sandwisencin and erythrabyssin II, pterocarpans from the EtOH extract of its bark, have shown antimicrobial activity against *Mycobacterium smegmatis* and *Staphylococcus aureus* (Mitscher et al., 1988). Besides, antinociceptive and antiinflammatory activities (Miño et al., 2002) as well as crown gall tumour inhibition and antifungal activity (Mongelli et al., 2000; Portillo et al., 2001) have been reported for this species.

The discovery of a taxol-producing endophytic fungus from the yew (Stierle and Strobel, 1995) brought the attention about the ecological and economic importance of this discovery, since the production of a determinate metabolite from a fungus is a much more interesting source of a drug than the plant material (Strobel et al., 2005). Many medicinal plants have been investigated in recent years for endophytic fungi and attention has been paid to their possible influence on the biological properties of the plants they live in. These findings have encouraged us to investigate the presence of active metabolites in Argentine medicinal plants infected with these microorganisms.

**Experimental**

**General procedures**

Thin layer chromatography (TLC) was performed on Silicagel 60F\(_{254}\) plates (Merck), column chromatography (CC) on Sephadex LH20 (Amersham Biosciences) and Kieselgel MN 60 (0.063–
0.2 mm/70–230 mesh, ATSM). Culture media were: Difco Bacto Nutrient Broth dehydrated and Britania Nutrient Broth dehydrated (Buenos Aires, Argentina).

Preparative HPLC was performed using a Waters equipment with photodiode array detector (Waters 2996), pump (Waters Delta 600), Waters 600 controller and in-line degasser; HPLC-MS was done using an Agilent 1100 equipment with a binary pump, photodiode array detector, mass spectrometer detector, autosampler and column thermostat. GC analysis was performed on a Varian Star 3400 CX and GC-MS on a Hewlett Packard 5890 Series II MSD 5971a instrument.

1H NMR, MS and UV spectra were recorded using a Bruker AM 500, a Shimadzu QP 5000, and a Shimadzu 2101 PC spectrophotometer, respectively.

Plant material

Young twigs of E. crista galli were collected between December 2002 and March 2003 in Buenos Aires surroundings, identified by Ing. G. Giberti and voucher specimens are kept at the Herbarium of the Museo de Farmacobotànica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina.

Extraction and chromatography

1.240 g of the powdered dry material were extracted at room temperature for 24 h (three times) with acetone and MeOH successively. Yields of the acetone and MeOH extracts were 12.5 and 4.4 g w/w, respectively. The acetone extract was submitted to CC on Silicagel eluted with cyclohexane, EtOAc, acetone, MeOH and their mixtures. From this fractionation fractions FI–FVIII were obtained. TLC analysis of the fractions was performed on Silicagel plates with cyclohexane/EtOAc (1:1 v/v). FI afforded a white precipitate which was submitted to GC-MS analysis. FIV was submitted to preparative HPLC using a SPC18 column (250 mm × 10 mm, Nucleosil100–7, Macherey-Nagel) with a gradient of H2O/MeOH (70:30 v/v) up to 100% MeOH in 20 min and UV detection at 210, 280 and 330 nm; flow rate was 4 ml/min. Three fractions were obtained (F6–F8), one of which (F8) presented antimicrobial activity. From this fraction three compounds, named 1, 2, and 3, were isolated.

Antimicrobial assay

Tested microorganisms

Bacillus subtilis ATCC 6633; Bacillus brevis ATCC 9999; Enterobacter dissolvens LMG 2683; Paecilomyces varioti ETH 114646; Micrococcus luteus ATCC 381; Nematospora corioli ATCC10647; Penicillium notatum IBWF collection were used in the screening. Bioassay-guided fractionation and bioautography were carried out using Bacillus subtilis, Bacillus brevis and Sarcina lutea.

Acetone and MeOH extracts, FI–FVIII, FIV subfractions and isolated compounds 1, 2, 3 dissolved in MeOH were assayed in the disc diffusion test (Kupka et al., 1979) at 100 μg/6 mm disc. Petri dishes were incubated at 37 °C in 2% agar in culture medium with the microorganisms. Positive control: penicillin 2.5 μg/6 mm disc. A vehicle control was also performed. Inhibition zone diameter was measured after 24 h.

Bioautography

Chromatography of FIV(35–39) was performed on Silicagel plates developed with cyclohexane/EtOAc (3:7). Chromatograms were dried and placed on Petri dishes containing 2% agar in the culture medium and incubated at 37 °C for 24 h.

Determination of minimum inhibitory concentration (MIC)

Bacillus brevis was cultured in nutrient medium. The optical density (OD) of the bacteria was adjusted to the standard of McFarland No. 0.5 with fresh medium to achieve a concentration of approx. 1 × 10⁸ CFU/ml. A final concentration of bacteria of approx. 5 × 10⁵ CFU/ml was obtained by diluting 200 times with fresh medium. Suspension of bacteria and serial two-fold dilution of the test compounds in fresh medium (280 to 0.5 μg/ml) were dispensed at 0.1 ml/well in 96-well microtiter plates. Plates were incubated during 15 h at 35 °C. Minimum inhibitory concentration (MIC) was determined in triplicate and is defined as the concentration of the test compound that completely inhibits cell growth.

HPLC-MS and GC-MS analysis

FIV(6–8) was analyzed in a column thermostat at 40 °C (LiChroCART 125–2, 4 μm Supersphere 100 RP-18, Merck) with a gradient of H2O/ace-
Antimicrobial Isoflavonoids from Erythrina crista galli

tone (50:50 v/v) up to 100% acetone in 15 min. The flow rate was 0.5 ml/min and the sample volume 20.0 μl. UV detection was at 450 nm and MS detection was with the following conditions: oven temperature, 350 °C (isothermic); drying gas, 6 ml/min; injector temperature, 400 °C; detector temperature, 250 °C; fragmentor, 140 V (G1946D).

GC was performed using: a split/splitless injector; fused silica capillary column 5% phenyl 95% methylpolysiloxane (DB-5 J&W Scientific, Folsom, CA, USA) (60 m × 0.25 mm id, film thickness 0.25 μm); oven temperature, 230 °C (isothermic); N2 flow, 0.8 ml/min; injector temperature, 240 °C; split, 1:90; FID detector temperature, 270 °C. GC-MS analysis was performed using the same column as for the analytical procedure but with the following conditions: oven temperature, 230 °C (isothermic); He flow, 1 ml/min; injector temperature, 250 °C; split, 1:60; detector temperature, 250 °C.

Results and Discussion

Chemical defense agents against pathogenic microorganisms in the Leguminosae include alkaloids, coumarins and mainly isoflavonoid derivatives, such as coumestans and pterocarpans, some of them acting as phytoalexins as a consequence of microorganisms’ attack.

In spite of the numerous compounds isolated from E. crista galli bark, seeds and leaves, nothing about the chemical composition and biological activities of twigs has already been reported nor about the presence of endophytic fungi in this species. In this investigation, E. crista galli acetone and MeOH extracts from young twigs infected with Phomopsis sp. were screened for antimicrobial activity against different microorganisms using the disc diffusion assay. Results are shown in Table I. Bioassay-guided fractionation of the acetone extract was carried out on Bacillus subtilis and B. brevis in order to isolate the antimicrobial compounds. Bioautography of the most active fraction, FIV(35–39), on B. brevis evidenced three active bands with Rf values between 0.3–0.4, corresponding to compounds 1, 2 and 3, isolated by successive CC and preparative HPLC from the acetone extract. These were identified as coumestrol (1), genistein (2) and daidzein (3), respectively (Fig. 1) by comparison of their spectral data (UV, MS and 1H NMR) with literature references (Kinjo et al., 1987) and with authentic samples.

Daidzein and genistein, biosynthetic precursors of coumestans and pterocarpans, have been reported in some Erythrina species (Yenesew et al., 2003; Yu et al., 2000; Nkengfack et al., 2000, 2001) including E. crista galli bark (Imamura et al., 1981).

Besides acting as phytoalexins, daidzein and genistein have been reported having in vitro antibac-

Table I. Screening of antimicrobial activity on Erythrina crista galli.

<table>
<thead>
<tr>
<th>Extract/fraction</th>
<th>Microorganism (inhibition zone in mm)</th>
<th>Bacillus brevis</th>
<th>Bacillus subtilis</th>
<th>Sarcina lutea</th>
<th>Penicillium notatum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td></td>
<td>12</td>
<td>14</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FIII</td>
<td></td>
<td>7</td>
<td>11</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FIV</td>
<td></td>
<td>7</td>
<td>11</td>
<td>11</td>
<td>–</td>
</tr>
<tr>
<td>FV</td>
<td></td>
<td>7</td>
<td>–</td>
<td>–</td>
<td>7</td>
</tr>
<tr>
<td>FIV(35–39)</td>
<td></td>
<td>15</td>
<td>13</td>
<td>10</td>
<td>–</td>
</tr>
<tr>
<td>FIV[(35–39) B]</td>
<td></td>
<td>12</td>
<td>10</td>
<td>11</td>
<td>–</td>
</tr>
</tbody>
</table>

Table II. MIC of the compounds isolated from Erythrina crista galli.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MIC [μg/ml]</th>
<th>MIC [μm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coumestrol (1)</td>
<td>4.4</td>
<td>16.33</td>
</tr>
<tr>
<td>Genistein (2)</td>
<td>17.5</td>
<td>64.81</td>
</tr>
<tr>
<td>Daidzein (3)</td>
<td>35.0</td>
<td>137.81</td>
</tr>
<tr>
<td>Penicilllin</td>
<td>&lt; 0.5</td>
<td>&lt; 3.07</td>
</tr>
</tbody>
</table>

Fig. 1. Antimicrobial compounds from Erythrina crista galli: coumestrol (1), genistein (2), and daidzein (3).
terial activity by Verdrengh et al. (2004) and Ulanowska et al. (2006). These last authors pointed out that genistein, which exhibited a more pronounced effect than daidzein, is a bacteriostatic agent inhibiting DNA, RNA and protein synthesis. Coumestrol is active against S. aureus, B. megaterium and E. coli, and its activity is increased in the presence of multidrug pump inhibitors (Tegos et al., 2002); it inhibits membrane-associated transport processes in E. coli (Weinstein and Albersheim, 1983).

Phytochemical analysis of F1, F2 and F3 showed the presence of lutein which was identified by HPLC/MS. n-Nonacosane was isolated from F1 and identified by GC analysis through its Kovats retention index and analysis of its MS spectrum (Mc Lafferty and Stauffer, 2000).

In conclusion, three compounds antimicrobial against B. brevis have been isolated by bioassay-guided fractionation from the acetone extract of Erythrina crista galli, infected with Phomopsis sp.: coumestrol, genistein and daidzein. Besides, lutein and n-nonacosane have been isolated and identified from the same extract. This is the first time that coumestrol and these compounds are reported in this species.

The close relation between endophytes and its plant hosts involves evolutionary processes that are able to influence physiological mechanisms of plants (Araujo et al., 2001). Based on this evidence, work is in progress in order to evaluate if coumestrol, genistein and daidzein are constitutive compounds in E. crista galli or if their production is induced by the presence of Phomopsis sp. and if they influence the biological activities of this medicinal plant.

Acknowledgements

We gratefully acknowledge M. T. Argerich for processing the plant material, A. Mejfert and Pharmacist C. van Baren for performing the HPLC-MS and GC-MS analyses, respectively. This investigation was supported by VW foundation, grant I/78249, and is part of the collaborative project ALPA BIO 3 between BMBF (Germany) and SETCIP (Argentina).


