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

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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 μM, 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; Iinuma *et al*., 1994; Tanaka *et al*., 1997). Erycristin, 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.

^{1}H 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 Farmacobotánica, 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 F_I–F_{VIII} were obtained. TLC analysis of the fractions was performed on Silicagel plates with cyclohexane/EtOAc (1:1 v/v). F_I afforded a white precipitate which was submitted to GC-MS analysis. F_{IV} was submitted to CC on Sephadex LH20 eluted with CH_2Cl_2 and MeOH: 80 fractions were obtained. $F_{IV(6-8)}$ was analyzed by HPLC-MS. $F_{IV(35-39)}$ was submitted to preparative HPLC using a SPC18 column (250 mm × 10 mm, Nucleosil100–7, Macherey-Nagel) with a gradient of H_2O/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 (F_A–F_C), one of which (F_B) 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 variotti* ETH 114646; *Micrococcus luteus* ATCC 381; *Nematospora coryli* 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, F_I–F_{VIII}, F_{IV} 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 $F_{IV(35-39)}$ 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 N° 0.5 with fresh medium to achieve a concentration of approx. 1×10^8 CFU/ml. A final concentration of bacteria of approx. 5×10^5 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

$F_{IV(6-8)}$ was analyzed in a column thermostat at 40 °C (LiChroCART 125–2, 4 μm Supersphere 100 RP-18, Merck) with a gradient of H_2O/ace-

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); N_2 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, $F_{IV(35-39)}$, 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 ^{1}H NMR) with literature references (Kinjo *et al.*, 1987) and with authentic samples. Compounds **1**, **2** and **3** inhibited the growth of *B. brevis* and its MIC values were calculated (Table II) being coumestrol the most active compound.

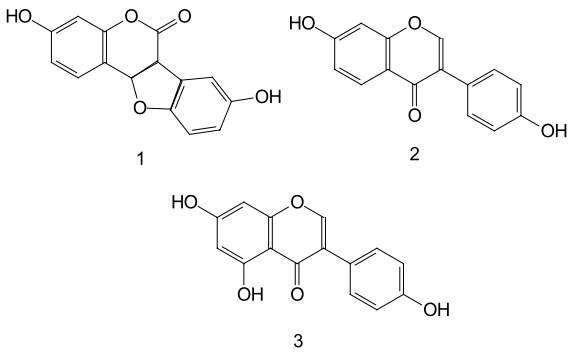

Fig. 1. Antimicrobial compounds from *Erythrina crista galli*: coumestrol (**1**), genistein (**2**), and daidzein (**3**).

Daidzein and genistein, biosynthethic 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.*

Extract/ fraction	Microorganism (inhibition zone in mm)			
	Bacillus brevis	*Bacillus subtilis*	*Sarcina lutea*	*Penicillium notatum*
Acetone	12	14	–	–
Methanol	–	–	–	–
F_{III}	7	11	–	–
F_{IV}	7	11	11	–
F_V	7	–	–	7
$F_{IV\,(35-39)}$	15	13	10	–
$F_{IV\,[(35-39)\,B]}$	12	10	11	–

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

Compound	MIC [μg/ml]	MIC [μM]
Coumestrol (**1**)	4.4	16.33
Genistein (**2**)	17.5	64.81
Daidzein (**3**)	35.0	137.81
Penicillin	< 0.5	< 3.07

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 $F_{IV(6-8)}$ showed the presence of lutein which was identified by HPLC/MS. *n*-Nonacosane was isolated from F_I 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.

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Araujo W., Maccheroni Jr. W., Aguilar-Vildoso C., Barroso P., Saridakis H., and Azevedo J. (2001), Variability and interactions between endophytic bacteria and fungi isolated from leaf tissues of citrus rootstocks. Can. J. Microbiol. **47**, 229–236.

Iinuma M., Okawa Y., and Tanaka T. (1994), Three new cinnamylphenols in heartwood of *Erythrina crista galli.* Phytochemistry **37**, 1153–1155.

Imamura H., Hibino Y., Ito H., and Ohashi H. (1981), Isoflavonoids of *Erythrina crista galli* (Leguminosae). Res. Bull. Fac. Agric. Gifu Univ. **45**, 77–79.

Ingham J. and Markham K. (1980), Identification of the *Erythrina* phytoalexin cristacarpin and a note on the chirality of other 6a-hydroxypterocarpans. Phytochemistry **19**, 1203–1207.

Kinjo J., Furusawa J., Baba J., Takeshita T., Yamasaki M., and Nohara T. (1987), Studies on the constituents of *Pueraria lobata*. III. Isoflavonoids and related compounds in the roots and the voluble stems. Chem. Pharm. Bull. **35**, 4846–4850.

Kupka J., Anke T., Oberwinkler F., Schramm G., and Steglich W. (1979), Antibiotics from basidiomycetes VII. Crinipellin, a new antibiotic from the basidiomycetous fungus *Crinipellis stipitaria* (Fr.) Pat. J. Antibiot. (Tokyo) **32**, 130–135.

Mc Lafferty F. W. and Stauffer D. B. (2000), The Wiley/NBS Registry of Mass Spectral Data, 7th ed. J. Wiley & Sons, Inc., New York.

Miño J., Gorzalczany S., Moscatelli V., Ferraro G., Acevedo C., and Hnatyszyn O. (2002), Actividad antinociceptiva y antiinflamatoria de *Erythrina crista galli* L. ("Ceibo"). Acta Farm. Bonaerense **21**, 93–98.

Mitscher L. A., Gollapudi S. R., Gerlach D. C., Drake S. D., Véliz E., and Ward J. A. (1988), Erycristin, a new antimicrobial pterocarpan from *Erythrina cristagalli.* Phytochemistry **27**, 381–385.

Mongelli E., Pampuro S., Coussio J., Salomon H., and Ciccia G. (2000), Cytotoxic and DNA interaction activities of extracts from medicinal plants used in Argentina. J. Ethnopharmacol. **71**, 145–151.

Nkengfack A. E., Waffo A. K., Azebaze G. A., Fomum Z. T., Meyer M., Bodo B., and van Heerden F. R. (2000), Indicanine A, a new 3-phenylcoumarin from root bark of *Erythrina indica*. J. Nat. Prod. **63**, 855–856.

Nkengfack A. E., Azebaze A. G., Waffo A. K., Fomun Z. T., Meyer M., and van Heerden F. R. (2001), Cytotoxic isoflavones from *Erythrina indica*. Phytochemistry **58**, 1113–1120.

Portillo A., Vila R., Freixa B., Adzet T., and Cañigueral S. (2001), Antifungal activity of Paraguayan plants used in traditional medicine. J. Ethnopharmacol. **76**, 93–98.

Stierle A. and Strobel G. (1995), The search for a taxol-producing microorganism among the endophytic fungi of the pacific yew, *Taxus brevifolia.* J. Nat. Prod. **58**, 1315–1324.

Strobel G., Daisy B., and Castillo U. (2005), Novel natural products from rainforest endophytes. In: Natural Products: Drug Discovery and Therapeutic Medicine

(Zhang L. and Demain A., eds.). Humana Press, Totowa, NJ, pp. 329–351.

Tanaka H., Tanaka T., and Etgh H. (1997), Three pterocarpans from *Erythrina crista galli.* Phytochemistry **45**, 835–838.

Tegos G., Stermitz F., Lomovskaya O., and Lewis K. (2002), Multidrug pump inhibitors uncover remarkable activity of plant antimicrobials. Antimicrob. Agents Chemother. **46**, 3133–3141.

Toursarkissian M. (1980), Plantas Medicinales de la Argentina. Ed. Hemisferio Sur S. A., Buenos Aires, p. 70.

Ulanowska K., Tkaczyc A., Konopa G., and Wegrzyn G. (2006), Differential antibacterial activity of genistein arising from global inhibition of DNA, RNA and protein synthesis in some bacterial strains. Arch. Microbiol. **184**, 271–278.

Verdrengh M., Vincent Collins L., Bergin, P., and Tarkowski A. (2004), Phytoestrogen genistein as an antistaphylococcal agent. Microbes Infect. **6**, 86–92.

Weber D., Sterner O., Anke T., Gorzalczany S., Martino V., and Acevedo C. (2004), Phomol, a new antiinflammatory metabolite from an endophyte of the medicinal plant *Erythrina crista galli.* J. Antibiot. **57**, 559–563.

Weber D., Gorzalczany S., Martino V., Acevedo C., Sterner O., and Anke T. (2005), Metabolites from endophytes of the medicinal plant *Erythrina crista galli.* Z. Naturforsch. **60c**, 467–477.

Weinstein L. and Albersheim P. (1983), XXXIII. The mechanism of the antibacterial action of glycinol, a pterocarpan phytoalexin synthesized by soybeans. Plant. Physiol. **72**, 557–563.

Yenesew A., Irungu B., Derese S., Midiwo J. O., Heydenreich M., and Peter M. G. (2003), Two prenylated flavonoids from the stem bark of *Erythrina burtii*. Phytochemistry **63**, 445–448.

Yu D. L., Yang X. D., Guo J., Xu L. Z., and Yang S. L. (2000), Studies on chemical constituents of *Erythrina arborescens* Roxb. Zhongguo Zhong Yao Za Zhi. **25**, 353–355.