Latin American Journal of Pharmacy
(formerly *Acta Farmacéutica Bonaerense)*
Lat. Am. J. Pharm. **32** (6): (2013)

Regular Article

Received: May 16, 2013
Accepted: May 23, 2013

Influence of the Endophytic Fungus *Phomopsis* sp. in the Production of Secondary Metabolites in *Erythrina crista-galli*

Flavia REDKO [1], María L. CLAVIN [1], Gustavo C. GIBERTI [2], Timm ANKE [3] & Virginia S. MARTINO [1]*

[1] *Cátedra de Farmacognosia, IQUIMEFA (UBA CONICET), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires Junín 956, 2° P, C1113AAD Buenos Aires, Argentina.*
[2] *Museo de Farmacobotánica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 2° P, C1113AAD Buenos Aires, Argentina.*
[3] *Institut für Biotechnologie und Wirkstoff-Forschung IBWF, Paul Ehrlich Str. 23, Kaiserslautern, Germany.*

***SUMMARY*. The aim of this investigation was to determine if the production of daidzein, genistein and coumestrol, compounds reported as antimicrobial principles in this species, is affected by the presence of the endophyte *Phomopsis* sp. in the young twigs of *Erythrina crista-galli*. HPLC profiles of the acetone extracts from cultivated plants and plants over infected with *Phomopsis* sp. were obtained. Daidzein, coumestrol, genistein, biochanin A, and formononetin were identified in all the acetone extracts. Coumestrol and daidzein content (0.04 and 0.05 %) was higher in over infected plants than in control plants (0.01 and 0.02%). A major peak was observed in the HPLC/DAD profile of the acetone extract from over infected plants. This was partially identified as a 3-glucosyl-rhamnosyl isorhamnetin derivative.**

INTRODUCTION

Endophytes are microorganisms that reside in the tissues of living plants in a variety of relationships ranging from symbiotic to pathogenic. These microorganisms seem to occupy virtually every living and non-living site in the world but only a small percentage of the endophyte biodiversity has been explored and there is little understanding of the endophytic biology and its relation with their plant hosts. The endophytes produce bioactive substances that are involved in a host-endophytic relationship and may make contributions to their host plant by providing protection to it by virtue of the antimicrobial compounds that it produces. Host specificity is a known phenomenon in endophytic-higher plant relationships and such specificity implies that complex interactions are occurring between the host and its associate microorganisms. Significance and implications of endophytes are their ecological relevance and the potential of yielding metabolites with diverse structure and biological function [1].

From a natural products perspective, the interactions between plants and endophytes may lead to the production of certain metabolites of interesting biological properties. Enhancement of secondary products accumulation in plants is of great importance in the medicinal plants cultivation industry [2]. The discovery of a taxol producing endophyte fungus Taxomyces andreanae in yew *(Taxus brevifolia),* points out the economic importance of the discovery, since the production of a given bioactive product by fermentation is much easier and economic than its obtaining from the plant material. It is conceivable that medicinal plants have microbes that mimic the chemistry of their respective host plants and make the same bioactive natural products [3]. Thus investigation in this emerging area seems promising and has a great potential in agriculture and medicine.

The genus *Erythrina* is a rich source of isoflavonoid compounds. These are a large and distinctive class of flavonoids, almost entirely restricted in the plant kingdom to the family Papilionatae. These metabolites are usually produced in the presence of a fungal infection and are thus called phytoalexins [4-6].

Erythrina crista-galli L. (Papilionatae) is a

KEY WORDS: Erythrina crista-galli, Phomopsis sp., Isoflavonoids, Isorhamnetin glycoside.

* Author to whom correspondence should be addressed. *E-mail:* vmartino@ffyb.uba.ar

tree that grows in South America and is used in folk medicine for wound healing, as astringent, narcotic and analgesic [7]. Alkaloids, pterocarpans, cinnamoylphenols and triterpenoids have been reported as the major compounds in bark and leaflets [8-11]. Erycristin, sandwisencin and erythrabyssin II, pterocarpans from the EtOH extract of its bark, have shown antimicrobial activity against *Mycobacterium smegmatis* and *Staphylococcus aureus* [12]. Besides, antinociceptive and anti-inflammatory activities [13] as well as crown gall tumour inhibition and antifungal activity [14,15] have been reported for this species.

The isolation of *Phomopsis* sp., an endophytic fungus, 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* anti-inflammatory activities and eight new compounds have been identified from this same endophyte [16,17]. Daidzein, genistein and coumestrol have been isolated, by bioassay guided fractionation from the acetone extract of of *Erythrina crista-galli* young twigs infected with *Phomopsis* sp. [18].

The aim of this investigation was to determine if the presence of *Phomopsis* sp. in the young twigs of *E. crista-galli* affects the production of daidzein, genistein and coumestrol, isoflavonoids reported as antimicrobial principles in this species.

MATERIALS AND METHODS

General Experimental Procedures

HPLC was performed on a Waters equipment with photodiode-array detector (Waters 2996), Pump (Waters Delta 600), Waters 600 controller. ^{1}HNMR, ^{13} CNMR spectra were run using a Bruker Avance 500 NMR equipment and UV analysis using a Jasco UV 630 spectrophotometer. Coumestrol, daidzein, genistein, formononetin, and biochanin A were purchased from Sigma-Aldrich Inc., St Louis, USA.

Plant material

Young twigs (CS) and seeds samples from *Erythrina crista-galli* were collected at the surroundings of Ciudad Universitaria, Buenos Aires, Argentina on November 2003. A voucher specimen is deposited at the Herbarium of Museo de Farmacobotánica. Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Series 2012 N°11.

Seed treatment

Ten seeds were submitted to successive washes with EtOH 70% during 1 min, 5% ClONa 3 min and EtOH 70% for 30 s, in order to sterilize the seed surface. Seeds were finally washed with sterile distilled water.

Cultivation of Erythrina crista galli

Sterilized and non-sterilized *E. crista-galli* seeds were separately wrapped in sterile gauze, submerged in sterile water and kept for 24 h under a constant air flow. Seeds (3-4) were placed in 10 flower pots (25 x 15 cm). Plants were grown in separate green houses for 6 months and watered regularly. Plants obtained from non-sterilized and sterilized seeds were labeled CC and CE respectively.

Preparation of Phomopsis suspension

Phomopsis strain was isolated from CS samples and grown as described by Weber *et al.* [16]. Spores were scrapped from the plates with a loop and suspended in a 0.9% NaCl solution.

Contamination of CE plants with Phomopsis sp.

A group of plants from CE group, consisting of 6 individuals in the 12th month of growth, was sprayed with a suspension of *Phomopsis* spores twice every fifteen days. This group of plants was named CI.

Determination of the presence of Phomopsis sp.

Young twig samples were collected from the cultivated plants at 12th (CC and CE) and 18th month of growth (CC and CI). Isolation of the endophytic fungi from CC, CE, and CI young twigs was performed as previously described [16].

Extraction of the plant material

Samples of young twigs from each set of plants CC and CI were harvested 6 months after the starting of the spraying treatment. Young twigs from both control CC and over infected samples CI (5 g) were dried and ground and were separately extracted with acetone (3 x 50 mL) at room temperature for 24 h each. CC acetone extract (CCA) and CI acetone extract (CIA) were taken to dryness under pressure in a rotatory evaporator. Also, 5 g of powdered young twigs from CS samples were extracted using this same procedure and the CS acetone extract was labeled CSA.

HPLC analysis

The acetone extracts CSA, CCA and CIA were re-suspended in MeOH at 1 mg/mL concentration. Then they were filtered through syringe filters non-sterile RC-membrane (Minisart RC 15, Sartorius). The samples were submitted to HPLC analysis employing a C_{18} reverse phase column (Luna 5 µ, 250 x 4.60 mm, Phenomenex) operated at 1 mL/min. Elution was accomplished with a gradient of H_2O/MeOH 50:50 (v/v) up to 100% MeOH in 30 min.

Eluted compounds were monitored by UV detection at 210, 280, and 344 nm. The injection volume was 20 µL. Coumestrol, daidzein, genistein, formononetin and biochanin A were used as standards. Each sample was injected by triplicate. The concentration of coumestrol and daidzein were calculated by the external standard calibration method. Peak areas were calculated using the Empower software. Standard deviation was calculated and was < 0.02%.

Isolation and identification of Compound 1

This compound was isolated from CIA extract by analytical HPLC/DAD using a C_{18} column eluted with 95% H_2O during 3 min to 40% ACN in 40 min during 10 min, back to initial conditions in 5 min. Detection was made at λ 260 and 340 nm. Sample injection 100 µL of CIA (10 mg/mL). Flow rate: 1 mL/min. Fractions were collected manually as peaks came out of the column according to their UV spectrum. Three fractions were obtained CIA-1, CIA-2, and CIA-3. The major fraction CIA-2 was re-chromatographed on a C18 column eluted with 100% H_2O during 3 min; 40% ACN in 10 min up to 98% ACN in 30 min for 10 min, back to initial conditions in 5 min. Detection was made at λ 260 and 340 nm. Flow rate: 1 mL/min. Sample injection: 100 µL of CIA-2 (5 mg/mL). Peaks were collected manually according to their UV spectrum. A yellow precipitate was obtained. Its purity was checked using three different chromatographic systems by HPLC/DAD.

RESULTS AND DISCUSSION

HPLC fingerprint is a highly recommended analysis for metabolic profiling of secondary metabolites of fungi-infected plants to determine relevant changes in the metabolite pattern in comparison to non-infected plants [2]. *Erythrina crista-galli* cultivated plants, obtained under different experimental conditions, were investigated for their coumestrol and daidzein content by means of HPLC-DAD analysis.

Two groups of cultivated plants were obtained: a control group (CC) and a group of plants obtained from sterilized seeds (CE), in order to demonstrate if the induction or accumulation of daidzein and coumestrol in *E. crista-galli* is influenced by the presence of *Phomopsis* sp. in the young twigs of the plant. Samples of these groups were taken at the 12th and 18th month of plant growth and the presence of *Phomopsis* sp in their young twigs were investigated. The endophytic fungus was detected in both groups, CC and CE, demonstrating that though seeds had been sterilized on their external surface, the vertical transmission of the endophytic fungi could not be avoided since this may be present in the inner tissues of the seeds. As no cultivated plants free from *Phomopsis* sp. could be obtained a group of CE plants were sprayed with a fungal suspension and samples of the young twigs were taken 6 months later.

HPLC profile of the acetone extracts CCA and CIA were compared with an acetone extract obtained from young twigs from wild plants (CSA) and daidzein and coumestrol contents were calculated. The presence of biochanin A and formononetin were also investigated in these extracts. Results are shown in Fig. 1-3. The HPLC profile at 260 nm was quite similar for CSA (Fig. 1), CCA (Fig. 2) and CIA (Fig. 3). Daidzein, genistein, coumestrol, formononetin and biochanin A were identified in all of the three samples.

Quantitative evaluation of coumestrol and daidzein in CCA (0.02 % and 0.01%, respectively) and CIA (0.04 and 0.05 %, respectively) indicated that CIA plants present a higher content of daidzein and coumestrol than control plants, thus indicating that the presence of the endophyte increases the production of these

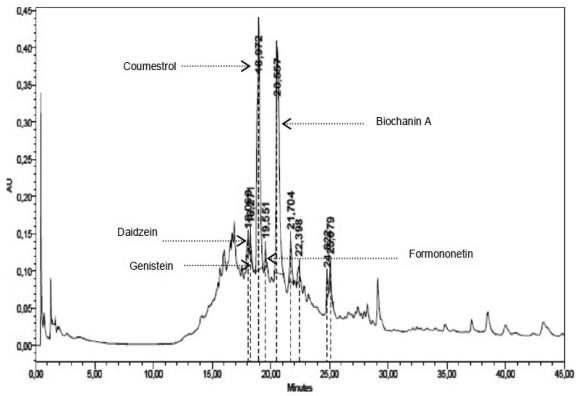

Figure 1. Chromatographic profile of the acetone extract (CCA) from cultivated *Erythrina crista-galli* plants.

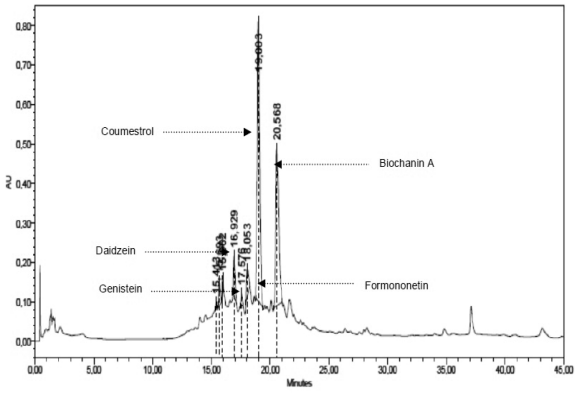

Figure 2. Chromatographic profile of the acetone extract (CSA) of *Erythrina crista-galli* young twigs wild sample.

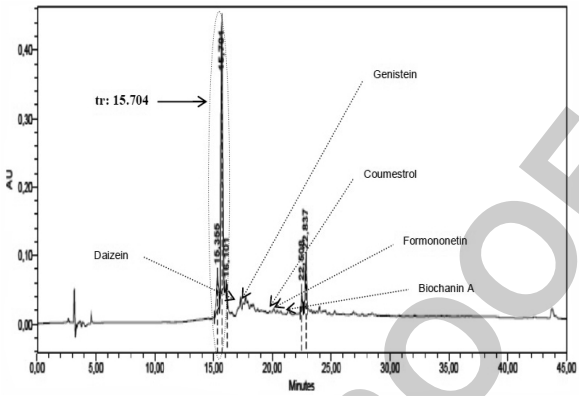

Figure 3. Chromatographic profile of the acetone extract (CIA) of *Erythrina crista-galli* cultivated plants over infected with *Phomopsis* sp.

Samples	Coumestrol (% p/p)	Daidzein (% p/p)	Genistein (% p/p)
CCA	0.02	0.01	*n.q.*
CIA	0.04	0.05	*n.q.*
CSA	0.12	0.02	*n.q.*
Rt (min)	18.0	18.9	18.2

Table 1. Isoflavone content of wild and cultivated *E. crista-galli* young twigs samples as determined by HPLC. *n.q.:* not quantified.

isoflavonoids in the over infected plants. Besides, CSA extract showed a higher content of coumestrol and daidzein (0.12 and 0.02 %, respectively) than cultivated control plants. This may be probably due to infections produced by other fungal organisms, besides *Phomopsis* sp., in the natural environment. Genistein was present in traces and could not be quantified in any of the extracts, under these experimental conditions (Table 1).

A major peak was observed in the HPLC/DAD profile of CIA extract. Characterization of this compound was based on chromatographic (TLC, HPLC) and spectroscopic analysis (UV, ^{1}H-NMR, ^{13}C-NMR spectra and bidimensional experiments).

Compound 1. UV (λ máx., nm): MeOH: 257, 267 (sh), 296 (sh), 361 nm; UV (λ máx., nm): MeOH + NaOMe: 274, 321 (sh), 416 nm; UV (λ máx., nm): MeOH + AlCl3: 274, 316 (sh), 362, 429 nm; UV (λ máx., nm): MeOH + $AlCl_3$ / HCl: 268, 306 (sh), 362 nm; UV (λ máx., nm): MeOH + NaOAc : 267, 400 nm; UV (λ máx., nm): MeOH + NaOAc / H_3BO_3: 271, 414 nm. ^{1}H-NMR (500 MHz) (MeOD): 1.33 ppm (3 H); 3.63 ppm (3 H); 4.54 ppm (1 H); 5.14 ppm (1 H, d, *J* = 7.5 Hz); 6.24 ppm (1 H, d, *J*= 2Hz); 6.43 ppm (1 H, d, *J*= 2 Hz); 6.90 ppm (1 H, d, *J*= 8.5 Hz); 7.64 ppm (1 H, d, *J* = 8.5 Hz); 7.69 ppm (1 H, d, *J* = 2Hz). ^{13}C-NMR (500 MHz) (MeOD): 16.47 (–CH_3, rhamnose), 59.42 (–OCH_3), 93.45 (C-8), 98.55 (C-6), 101.02 (C_1-rhamnose), 103.28 (C-10), 104.22 (C_1-glucose), 114.65 (C-5'), 116.26 (C-2'), 121.72 (C-6'), 122.13 (C-1'), 133.00 (C-2), 144.46 (C-4'), 148.42 (C-3'), 157.16 (C-1), 157.49 (C-4), 161.61 (C-7), 164.68 (C-9), 178.03 (C-3). HMBC spectrum revealed significant correlations of: H-6 with C-7; H-7 with C-6; H-8 with C-10; H-5' with C-1', 3', 4' and 6'; H-6' with C-3'; anomeric glucose proton with C-3. HSQC spectrum showed correlations of H-6 with C-6; H-8 with C-8; H-2'with C-2'; H-5' with C-5'; H-6' with C-6'; glucose protons with its anomeric carbon; rhamnose protons with its anomeric carbon.

This compound showed an Rf corresponding to a glycoside in AcH 15%. UV spectrum of the compound in MeOH indicated its structure as corresponding to a flavonol derivative with hydroxyl functions at C5, C7, C3' and C4'. Two metacoupled protons at δ 6.24 and 6.43 correlated with the ^{13}C-NMR signals at δ 98.55 and 93.45 in the HSQC spectrum and were characteristic of a 5,7-disubstituted A ring. Signals at δ 7.69, 7.64, and 6.90, corresponding to B ring protons, were assigned to protons at 6', 2', 5', respectively. A signal at δ 3.63 in the ^{1}H-NMR spectrum, which integrated for 3 protons, corresponded to a methoxyl group which was assigned to the 3' position of B ring. Two signals corresponding at least to two anomeric protons were present in the ^{13}C-NMR spectrum, one at 104.22 and another at 101.02 ppm correlated with the signals at 5.14 (*J* = 7.5 Hz) and 4.54 (*J* = 1Hz) in the ^{1}H-NMR spectrum. These signals

could indicate a β-glucosyl and a α-rhamnosyl linkage, since a methyl signal at δ 1.33 in the ^{1}H-NMR spectrum, typical of a rhamnosyl moiety, was also observed. A crossed correlation between signal at 133 ppm and signal at δ 5.14 in the HSQC spectrum indicated the glucose moiety was linked at C3 of the flavonoid nucleus. Other signals in the ^{1}H-NMR and ^{13}C-NMR spectra could not be fully assigned. MS peak in the MS spectrum (70 ev) corresponded to the aglycone isorhamnetin.

Based on spectroscopic evidence, the aglycone was identified as isorhamnetin and the sugars as glucose and rhamnose. Thus the compound was partially characterized as a 3-*O*-glucosyl-rhamnosyl glycoside, with at least two or three sugar moieties. Identification of the compound could not be completed since not enough amount of it was isolated. Further studies need be undertaken (FABS-MS) in order to determinate the number of sugars attached to the C-3 position of the flavonoid and the type of sugar linkages.

Flavonoids are a group of secondary metabolites that are generally involved in plant defense mechanisms. Pathogenic microorganisms interact with these compounds in response to the fungitoxic effect of flavonoids and transform them into less toxic metabolites [19]. Glycosylation is one of these detoxification mechanisms adopted by fungi. So the presence of the isorhamnetin glycoside in the young twigs of *E. crista-galli* may be explained as a consequence of the over infection with *Phomopsis* sp. in this species.

CONCLUSIONS

The results obtained in this study indicate that coumestrol and daidzein are constitutive metabolites in *Erythrina crista-galli* young twigs and the presence of the endophyte, *Phomopsis* sp., increases their production in the plant. Glycosylation of flavonol compounds may also occur in response to an over infection with *Phomopsis* sp.

REFERENCES

1. Gunatilaka, A.A.L. (2006) *J. Nat. Prod.* **69**: 509-26.
2. Yuan, Z.-L. C.-C. Dai and L.-Q. Chen (2007) *Afr. J. Biotechnol.* **6**: 1266-71.
3. Strobel, G. & D. Bryn (2003) *Microbiol. Mol. Biol. Rev.* **67**: 491-502.
4. Van Etten, H., E. Temporini & C. Wasmann (2001) *Physiol. Mol. Plant. P.* **59**: 83-93.
5. Veitch, N. (2007) *Nat. Prod. Rep.* **24**: 417-64.
6. Mazid, M., T. Khan & F. Mohammad (2011) *Biol. Med.* **3**: 232-49.
7. Toursarkissian, M. (1980) *Plantas Medicinales de la Argentina,* Ed. Hemisferio Sur S. A., Buenos Aires, p.70.
8. Ingham, J. & K. Markham (1980) *Phytochemistry* **19**: 1203-7.
9. Iinuma, M., Y. Okawa & T. Tanaka (1994) *Phytochemistry* **37**: 1153-5.
10. Tanaka, H., T. Tanaka & H. Etgh (1997) *Phytochemistry* **45**: 835-8.
11. Imamura, H., Y. Hibino, H. Ito & H. Ohashi (1981) *Res. Bull. Fac. Agric. Gifu Univ.* **45**: 77-9.
12. Mitscher, L.A., S.R. Gollapudi, D.C. Gerlach, S.D. Drake, E.Véliz & J. A. Ward (1988) *Phytochemistry* **27**: 381-5.
13. Miño, J., S. Gorzalczany, V. Moscatelli, G. Ferraro, C. Acevedo & O. Hnatyszyn (2002) *Acta Farm. Bonaerense* **21**: 93-8.
14. Mongelli, E., S. Pampuro, J. Coussio, H. Salomon & G. Ciccia (2000) *J. Ethnopharmacol.* **71**: 145-51.
15. Portillo, A., R. Vila, B. Freixa, T. Adzet & S. Cañigueral (2000) *J. Ethnopharmacol.* **76**: 93-8.
16. Weber, D., O. Sterner, T. Anke, S. Gorzalczany, V. Martino & C. Acevedo (2004) *J. Antibiot.* **57**: 559-63.
17. Weber, D., S. Gorzalczany, V. Martino, C. Acevedo, O. Sterner & T. Anke (2005) *Z. Naturforsch.* **60** C: 467-77.
18. Redko, F., M. Clavin, D. Weber, F. Ranea, T. Anke & V. Martino (2007) *Z. Naturforsch.* **62 C**: 164-8.
19. Siana, M., Z. D., T. Makovec & H. Lenasi (2011) *J. Basic Microbiol.* **51**: 433-41.