Research Article

Received: 6 June 2017 Revised: 3 August 2017 Accepted article published: 11 August 2017 Published online in Wiley Online Library: 27 September 2017

(wileyonlinelibrary.com) DOI 10.1002/ps.4698

Bioassay-guided isolation of potent aphicidal *Erythrina* alkaloids against *Aphis gossypii* from the seed of *Erythrina crista-galli* L.

Delong Wang, Na Xie, Shandong Yi, Chuanyuan Liu, Hui Jiang, Zhiqing Ma, Juntao Feng, He Yan* and Xing Zhang

Abstract

BACKGROUND: The cotton aphid (*Aphis gossypii* Glover) is one of the most invasive pests of cotton. Many botanical phytochemicals have a long history as a source of insecticides, and as templates for new insecticides. This study was undertaken to isolate aphicidal compounds from the seeds of *Erythrina crista-galli* L. using the bioassay-guided isolation method.

RESULTS: Three novel and 11 known *Erythrina* alkaloids were isolated. Erysodine (9), erysovine (10), erysotrine (8) and erythraline (11) showed moderate to excellent aphicidal activity with LD_{50} values of 7.48, 6.68, 5.13 and 4.67 ng aphid^{-1}, respectively. The Potter spray tower bioassay gave corresponding LC_{50} values of 186.81, 165.35, 163.74 and 112.78 μg ml^{-1}. A unique substructure, which presents an sp^3 methylene at C-8, a non-oxygenated site at N-9 and a conjugated dienes group ($\Delta^{1,2}$ and $\Delta^{6,7}$), plays a crucial role in the aphicidal activity. Application of erythraline (11) led to different increases in the activities of superoxide dismutase, catalase and glutathione *S*-transferase.

CONCLUSION: The study demonstrated that the *Erythrina* alkaloids erysodine (9), erysovine (10), erysotrine (8) and erythraline (11) have potential use as botanical aphicides for commercial application, or as templates for the development of new insecticides.

Supporting information may be found in the online version of this article.

Keywords: *Erythrina crista-galli* L.; *Erythrina* alkaloids; aphicidal activity; *Aphis gossypii* Glover

1 INTRODUCTION

Animal pests, most of them insects, potentially account for tremendous economic losses in worldwide agricultural production.[1,2] Among these insects, aphids (Hemiptera: Aphidoidea) are one of the most invasive pests of temperate agriculture.[3] Aphids feeding on phloem sap lead to crop losses and quality deterioration, as a result of their excretion of honeydew in which harmful sooty molds consequently breed, and transmit plant viruses.[4,5] Indeed, more than half of all insect-vectored plant viruses are transmitted by aphids.[6] Historically, the approach employed to control aphids and other hemipteran pests has depended on the use of specific sets of systemic synthetic insecticides.[7] However, long-lasting application of these synthetic aphicides over past decades has led to the development of pesticide resistance in several aphid species.[8,9] In coping with resistant strains, the continued use of insecticides poses substantial deleterious hazards to the environment, including pesticide residues and toxicity to non-target organisms, such as mammals and beneficial insects (natural predators, pollinators, and parasitoids).[10–12] Therefore, there is a great demand for efficient and environmentally friendly pest control approaches as alternatives to the synthetic pesticides used currently.

Botanical aphicidal secondary metabolites are a promising alternative to synthetic pesticides, due to their easy biodegradation and low residuals, relatively low toxicity to mammals and other non-target organisms, and different mode of action compared with the currently available commercial insecticides.[13–15] Bioactive phytochemicals with unique structures, such as lignans, flavonoids, terpenoids and alkaloids, are far better than man-made compounds. They mainly exert defensive functions to protect plants from predators such as insects, pathogenic microorganisms and other herbivores, and their mode of action is the result of millions of years' coevolution between plants and their predators.[16] This is why many botanical phytochemicals have long been used as insecticides and have served extensively as templates for a vast array of commercial synthetic insecticides on the market today.[15]

Erythrina crista-galli L., often known as the cockspur coral tree, is distributed widely in tropical and subtropical areas.[17] The genus *Erythrina* (Fabaceae) has more than 100 species that used in

* *Correspondence to: H Yan, Research & Development Center of Biorational Pesticide, Northwest A&F University, Yangling 712100, Shaanxi, China. E-mail: yh-run@163.com*

Research & Development Center of Biorational Pesticide, Northwest A&F University, Yangling, Shaanxi, China

traditional medicine to treat human diseases, such as insomnia, asthma and toothache.[17,18] Many different types of bioactive phytochemicals such as alkaloids, flavonoids, polyphenols and terpenes have been isolated from *Erythrina*;[17] of these, the alkaloids have generated the greatest interest because of their useful biological profiles and characteristically unique spirocyclic structure, which is a target template in organic synthesis methodology.[18–20] Nevertheless, their insecticidal activity has been little studied to date. Here, we used the bioassay-guided method to isolate insecticidal *Erythrina* alkaloids from the seeds of *E. crista-galli* L., and verified their aphicidal potential against *Aphis gossypii* Glover, an extraordinarily crucial pest mainly feeding on industrial crop cotton. It is expected that the seeds of *E. crista-galli* L. produced annually could sustainably supply the bioactive phytochemicals for pest management.

2 MATERIALS AND METHODS

2.1 General apparatus and chemicals

UV–visual analysis was carried out using a Shimadzu UV-2700 spectrophotometer. A Bruker AMX-500 spectrometer was used to record ^{1}H NMR and ^{13}C NMR, distortionless enhancement by polarization transfer (DEPT), correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum coherence (HMQC) spectra (solvent CD_3OD) with tetramethylsilane (TMS) as an internal standard at room temperature. Compounds with KBr pellets were studied in a Nicolet iS 50FT-IR spectrometer for infrared (IR) analysis. High resolution electrospray ionisation mass spectrometry (HR-ESI-MS) analyses were carried out using an API QSTAR Pular-1 mass spectrometer. A Shimadzu LC20A HPLC apparatus with a YMC-Pack ODS (YMC, 250 × 20 mm) chromatographic column was used for semi-preparative reversed-phase chromatography (UV detector, flow rate at 4 ml min^{-1}). Column chromatography (CC) was carried out on silica gel (200–300 mesh, Qingdao Haiyang Chemical Inc., Qingdao, China), and Lichroprep RP-18 gel (40-63 mm, Merck, Darmstadt, Germany). Precoated GF_{254} silica gel glass plates (Qingdao Haiyang Chemical Inc.) were used to monitor isolation. Imidacloprid (≥ 98%) was purchased from Shanghai Aladdin Reagent Co. Ltd. (China).

2.2 Plant material

Seeds of *E. crista-galli* L. were collected in September 2012 in Guangzhou, China, and its voucher sample (No. 20121012) was preserved in the Research & Development Center of Biorational Pesticide, Northwest A&F University. The plant *E. crista-galli* L. was identified by Professor Li Yan of Northwest A&F University.

2.3 Test insects

Aphis gossypii Glover (Hemiptera: Aphidoidea) of clonal lineages used in the subsequent aphicidal tests was initially gathered from cotton plants (*Gossypium barbadense* Linn.) infested with a wild population of *A. gossypii* in the experimental fields of Northwest A&F University. The collected aphids were reared on the foliage of cotton plants at the seedling stage kept in inside-vented plastic cages (100 × 50 × 50 cm) in a greenhouse at the Research & Development Center of Biorational Pesticide. Aphids were kept at 23 ± 2 °C under a 16: 8 h light/dark photoperiod and 55 ± 5% relative humidity (RH). For the bioassays, identical-sized wingless (apterous) adult aphids were used after collection between June and August in 2013–2016.

2.4 Bioassay-guided isolation of aphicidal alkaloids

Dried seeds of *E. crista-galli* L. (9.70 kg) were crushed and immediately extracted with 95% methanol (CH_3OH) (25 L; 3 × 3 days). The filtrate was concentrated using a rotary evaporator (Shanghai SENCO Technology Co., Ltd., Shanghai, China) at 45 °C. After evaporation, a yellowish-brown residue (613.2 g) was obtained and stored at 4 °C. The obtained residue was suspended in 3% aqueous tartaric acid and then partitioned between water and EtOAc. After concentration, the EtOAc phase afforded a non-alkaloidal portion (181.7 g). The aqueous phase was adjusted to pH 9.0 using ammonia water (wt%, 25–28%), and was partitioned with EtOAc to give an alkaloidal portion (29.8 g). The contact aphicidal activity of the methanolic extract, non-alkaloidal and alkaloidal portions against *A. gossypii* was tested before further isolation and the results are summarized in Table 1. The alkaloidal portion with aphicidal activity was chromatographed on a silica gel column (10 × 150 cm, 500 g) eluted successively with $CHCl_3/CH_3OH$ (99:1, 85:15, 80:20, 50:50 and 1:99, each 7.0 L, v/v) to yield five fractions $^{1-5}$. Fractions 2 and 4, which showed aphicidal activity (Table 1), were followed by further isolations. Fraction 2 (3.9 g) was loaded onto a silica gel column and eluted using petroleum ether (boiling range: 60–90 °C)/acetone (100:0, 30:70, 50:50, 70:30 and 0:100 v/v) to give six subfractions (2a–2f). Aphicidal subfractions 2b and 2c were subjected to the following isolations (Table 1). For subfraction 2b, Sephadex LH-20 gels were used in further purification eluting with a $CHCl_3/CH_3OH$ mixture (1:1 v/v) to give compounds **1** (23 mg), **6** (17 mg) and **10** (74 mg). For subfraction 2c, preparative HPLC was performed for further purification eluting with a CH_3OH/H_2O mixture (60:40 v/v) to give compounds **2** (11 mg), **7** (42 mg) and **8** (4 mg). Fraction 4 (3.1 g) was chromatographed on ODS HPLC and eluted with a CH_3OH/water mixture of increasing polarity (10:90, 30:70, 50:50, 70:30, 90:10 and 100:0 v/v) to give six subfractions (4a–4f). Aphicidal subfractions 4c and 4d were used for the following isolations (Table 1). Further purification of subfraction 4c was carried out on a Sephadex LH-20 chromatograph eluted with a $CHCl_3/CH_3OH$ mixture (1:1 v/v) to give compounds **3** (11 mg), **4** (23 mg), **11** (19 mg) and **12** (19 mg). Similar treatment of subfraction 4d yielded compounds **5** (17 mg), **9** (36 mg), **13** (26 mg) and **14** (74 mg).

Cristanines C (**1**): Yellow solid; $[\alpha]_D^{31}$ +176.3 (~ 0.20, methanol); UV (methanol) λ_{max} (logε): 207 (4.74), 237 (4.00), 259 nm (3.80); IR (KBr) ν_{max}: 3412, 1677, 1609, 1513, 1461 cm^{-1}; HRESIMS *m/z* 366.1314 $[M + Na]^+$ (calculated for $C_{19}H_{21}NO_5Na$, 366.1317); ^{1}H and ^{13}C NMR spectroscopic data (Table 2).

Cristanines D (**4**): Bright white powder; $[\alpha]_D^{31}$ +183.5 (~ 0.20, methanol); UV (methanol) λ_{max} (logε): 204 (4.70), 238 (3.78), 292 nm (3.76); IR (KBr) ν_{max}: 3475, 1617, 1504, 1482 cm^{-1}; HRESIMS *m/z* 354.1314 $[M + Na]^+$ (calculated for $C_{18}H_{21}NO_5Na$, 354.1317); ^{1}H and ^{13}C NMR spectroscopic data (Table 2).

Cristanines E (**7**): Brownish red solid; $[\alpha]_D^{31}$ +124.9 (*c* 0.20, methanol); UV (methanol) λ_{max} (logε): 204 (4.70), 238 (3.78), 292 nm (3.76); IR (KBr) ν_{max}: 3415, 1610, 1514, 1460 cm^{-1}; HRESIMS *m/z* 370.1627 $[M + Na]^+$ (calculated for $C_{19}H_{25}NO_5Na$, 370.1630); ^{1}H and ^{13}C NMR spectroscopic data (Table 2).

2.5 Contact aphicidal assay

Contact aphicidal activity against *A. gossypii* was tested using the reported topical application method[21,22] with some modifications. A microcapillary with a calibrated volume of 0.053 µl (Department of Entomology, Nanjing Agricultural University, China) was used for in the topical application. Test samples were diluted to the

Table 1. Contact aphicidal activity of crude extract, alkaloidal fraction, non-alkaloidal fraction, fractions, subfractions, and pure isolated compounds **1**–**14** against *Aphis gossypii* at 24 h after treatment

Sample	$LD_{50} \pm SEM$ (ng aphid^{-1})	Toxicity regression equation ($y = a + bx$)	χ^2	df	*P*
Crude extract	372.50 ± 56.51	0.71 + 1.67*x*	2.17	3	0.53
Alkaloidal fraction	162.96 ± 37.92	2.57 + 1.09*x*	0.28	3	0.96
Non-alkaloidal fraction	> 1000	–	–	–	–
Fraction 2	77.60 ± 10.75	1.25 + 1.98*x*	1.67	3	0.64
Fraction 4	50.65 ± 8.03	2.15 + 1.67*x*	1.35	3	0.71
2b	34.39 ± 5.02	2.27 + 1.77*x*	1.50	3	0.68
2c	50.02 ± 6.03	1.19 + 2.24*x*	0.71	3	0.87
4c	42.36 ± 5.77	1.96 + 1.86*x*	3.16	3	0.36
4d	29.57 ± 3.56	1.40 + 2.44*x*	5.31	3	0.15
1–**7**, **12**–**14**	> 530	–	–	–	–
8	5.13 ± 1.10	4.03 + 1.35*x*	0.90	3	0.82
9	7.48 ± 1.55	3.79 + 1.37*x*	0.79	3	0.85
10	6.68 ± 1.16	3.66 + 1.62*x*	0.31	3	0.62
11	4.67 ± 0.73	3.87 + 1.68*x*	2.97	3	0.39
Imidacloprid[a]	1.84 ± 0.40	4.68 + 1.20*x*	0.78	3	0.85

[a] Commercial aphicide imidacloprid as positive control.

Table 2. NMR data for compounds **1**, **4** and **7** (δ in ppm and *J* in Hz)[ab]

	1		**4**		**7**	
No.	δ_H (*J*)	δ_C	δ_H (*J*)	δ_C	δ_H (*J*)	δ_C
1	6.96 dd (10.2, 2.5)	123.4	5.63 m	122.9	5.90 m	127.7
2	6.40 d (10.2)	138.0	4.21 m	72.3	4.33 m	71.3
3	3.75 m	74.8	3.55 m	80.3	3.70 m	80.6
4	2.97 dd (11.8, 4.4)	40.9	2.23 dd (12.2, 4.1),	38.5	2.76 overlap	32.3
	1.89 dd (11.7, 10.3)		1.59 t (12.2).		2.03 dd (11.5, 4.6)	
5		67.5		64.9		80.9
6		157.7		143.3		137.5
7	6.06 s	119.5	2.40 m	26.3	2.81 overlap	24.5
			2.27 m		2.43 m	
8		172.2	3.11 overlap	48.7	3.75 overlap	61.8
			3.04 m		3.40 overlap	
10	5.67 (dd, 4.3, 1.7)	74.0	4.59 dd (4.7, 1.8)	62.1	3.73 overlap	56.0
11	3.32 overlap	34.7	3.66 dd (15.1, 4.7)	49.3	3.25 m	25.6
	3.20 overlap		3.16 dd (15.1, 1.8)			
12		125.8		128.7		122.0
13		129.1		129.6		126.4
14	6.79 s	107.9	6.84 s	106.9	6.85 s	110.8
15		147.4		147.0		147.8
16		148.7		147.3		149.6
17	6.82 s	112.6	7.03 s	108.7	6.86 s	111.9
dioxy-methylene			5.96 s	101.1		
3-OCH_3	3.36 s	56.1	3.33 s	56.3	3.35 s	55.1
15-OCH_3	3.81 s	56.2			3.85 s	55.5
16-OCH_3	3.92 s	56.4			3.83 s	56.3

[a] Measured at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C) in CD_3OD.
[b] Assignments were based on HSQC, HMBC and ^{1}H–^{1}H COSY experiments.

desired concentrations with 75% acetone/water, which gave ~10–90% mortality. The diluent was used as the blank control and imidacloprid was used as the insecticide control. Each dose was applied to the dorsum of wingless adult aphids. For each dose, 30 aphids were treated and each treatment was conducted in triplicate. After treatment, each replicate was placed in a plastic Petri dish (5.7 cm diameter), in which a filter paper holding a fresh cotton leaf disc (2.7 cm diameter) was placed. The plates were then transferred to a box containing a moist towel, and the covered box was put in an artificial climate incubator under controlled conditions (22 ± 2 °C, 16: 8 h L/D, 55 ± 5% RH). Aphids were monitored under a dissecting microscope. If aphids did not move at all after poking with a dissecting needle, death or moribundity was identified.

2.6 Potter spray tower assay

The Potter spray tower assay was conducted using the reported method.[23] For direct contact exposure, three Petri dishes (9.0 cm diameter) without lids, each containing 30 aphids, were placed in a Potter tower (Burkard Manufacturing Co. Ltd., UK) and sprayed at 78 kPa with 2 ml of the prepared solutions. After spraying, treated aphids in one dish were transferred using a soft brush to a clean Petri dish (5.7 cm diameter) in which a filter paper with a fresh cotton leaf disc (2.7 cm diameter) was placed. The plates were then transferred to a box containing a moist towel, and the covered box was put in an artificial climate incubator under controlled conditions (22 ± 2 °C, 16: 8 h L/D, 55 ± 5% RH). The aphids were monitored under a dissecting microscope. If aphids did not move at all after poking with a dissecting needle, death or moribundity was identified.

2.7 Enzyme preparation and activity assays

The most potent compound, erythraline (**11**), was chosen to investigate the effect on enzyme activities in *A. gossypii* at 58.80 μg ml^{-1} (LC_{25}), 112.78 μg ml^{-1} (LC_{50}) and 216.40 μg ml^{-1} (LC_{75}). Aphids were treated with the above concentrations of erythraline (**11**) by Potter spray tower as described above. After 24 h, living insects were weighed and homogenized in 0.1 M ice-cold phosphate buffer at pH 7.4 (1.5 ml). The obtained extracts were then centrifuged (10 000 ***g***) for 20 min at 4 °C. The final supernatants after centrifugation were transferred to Eppendorf tubes, and used to determine enzyme activities and protein concentrations. Five replicates were carried out for each sample.

The activities of antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), and detoxification enzyme, glutathione *S*-transferase (GST) in *A. gossypii* were determined by spectrophotometry. The enzyme activity assay for SOD used a tetrazolium salt to detect superoxide radicals generated by xanthine and the xanthine oxidase system, and the detection was recorded at 550 nm.[24] SOD activity was expressed in units per milligram of protein. One unit of SOD activity was defined following the reported literature.[25] For the CAT activity assay, the Beutler method was used to monitor H_2O_2 (10 mM) hydrolysis and the decrease in its absorbance at 240 nm (ΔA_{240}).[26] CAT activity was defined as ΔA_{240} min^{-1} mg protein^{-1}. GST activity was tested by following GST catalysis of the reaction of 1-chloro-2, 4-dinitrobenzene (CDNB) (25 mM) with the SH groups of glutathione.[27] GST activity was calculated as the rate of change in absorbance at 340 nm per mg protein (ΔA_{340} min^{-1} mg protein^{-1}). Total protein content was calculated following the Bradford method.[28]

2.8 Statistical analysis

Mortality rates were calculated from the percentage of dead aphids at 24 h, and were corrected using Abbott's formula. Calculation of LD_{50} (or LC_{50}) values and analysis of variance (ANOVA) with respect to least significant difference test (LSD, $P < 0.05$) were performed using SPSS software, version 18.0.

3 RESULTS AND DISCUSSION

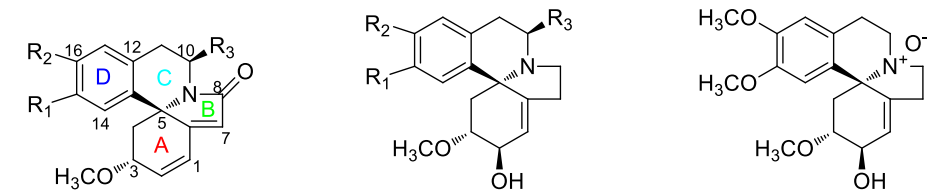
3.1 Contact aphicidal activity of the crude methanol extract, alkaloidal and non-alkaloidal portions, fractions and subfractions

During the bioassay-guided isolation, the contact aphicidal activity of the crude extract, alkaloidal and non-alkaloidal portions, fractions 1–6, and subfractions 2a–2f and 4a–4f was evaluated against *A. gossypii* after 24 h. The results are shown in Table 1. As can be seen, the methanol extract and alkaloidal portion showed aphicidal activity with LD_{50} values of 372.50 and 162.96 ng aphid^{-1}, respectively; the non-alkaloidal portion had no potency. Only fractions 2 and 4 had obvious aphicidal activity (77.60 and 50.65 ng aphid^{-1}, respectively), therefore leading to further isolation to give the corresponding subfractions. Among the subfractions of 2, 2b exhibited a remarkable activity with an LD_{50} value of 34.39 ng aphid^{-1}, followed by subfraction 2c with an LD_{50} value of 50.02 ng aphid^{-1}. In the subfractions of 4, both 4c and 4d displayed significant aphicidal activity (42.36 and 29.57 ng aphid^{-1}, respectively). No aphicidal activity was observed for the other subfractions of 2 and 4 (data not shown).

3.2 Structural elucidation of isolated alkaloids

In this study, seeds of *E. crista-galli* were collected, dried and extracted with 95% CH_3OH. The alkaloidal portion was concentrated after acidic and alkaline treatments, and repeatedly chromatographed on silica gel, Sephadex LH-20 and ODS HPLC following the bioassay-guided isolation method to give three new *Erythrina* alkaloids, and 11 known congeners (Fig. 1).

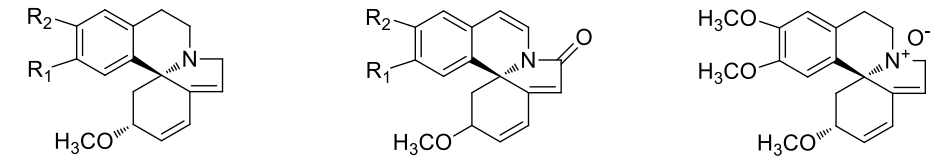
Compound **1** is an amorphous yellow solid. The molecular formula was established as $C_{19}H_{21}NO_5$ according to HR-ESI-MS (*m/z* 366.1314 $[M + Na]^+$), being responsible for 10 degrees of unsaturation. The ^1H, ^{13}C NMR and DEPT data for compound **1** indicated the presence of three methoxyls (-OCH_3), two methylenes, seven methines (including two oxy-methines, two aromatic methines and three olefins methines) and seven non-protonated carbons (including four aromatic quaternary carbons). Three methoxy groups were attached to C-3, C-15 and C-16, as deduced from the HMBC correlations of H_3-3-OMe, H_3-15-OMe and H_3-16-OMe, to each methoxy-bearing carbon, respectively. Comparison of its ^1H and ^{13}C NMR data (Table 2) with those of erysotramidine (**2**) indicated that they shared the same skeleton.[29] The only difference is the C-10 site which contains an additional oxymethine group. This was confirmed by the downfield shifted signal ($\delta_{C\text{-}10}$ 74.0) in the ^{13}C NMR spectrum and downfield chemical shift of $\delta_{H\text{-}10}$ 5.67 in the ^1H NMR (Table 2). The positive specific rotation $[\alpha]_D^{31}$ +183.6 (*c* 0.20, CH_3OH) of **1** suggested that its C-5 position presented an *S* configuration.[30,31]NOESY correlations (Fig. 2) of H-3/H-14 suggested that the methoxy group at C-3 had an α-configuration. Other NOESY correlations are satisfied by the configuration of the new compound **1** illustrated in Fig. 2. In addition, biogenetic considerations on *Erythrina* alkaloids[20,32,33] and the identical chemical shift of C-3 with known compounds[34–37] suggested that **1** has an *R* configuration at C-3. The absolute configuration of C-10 was assigned as *R* from the



1 $R_1 = R_2 = OCH_3$, $R_3 = OH$
2 $R_1 = R_2 = OCH_3$, $R_3 = H$
3 R_1-OCH_2O-R_2, $R_3 = H$

4 R_1-OCH_2O-R_2, $R_3 = OH$
5 $R_1 = R_2 = OCH_3$, $R_3 = H$
6 R_1-OCH_2O-R_2, $R_3 = H$

7

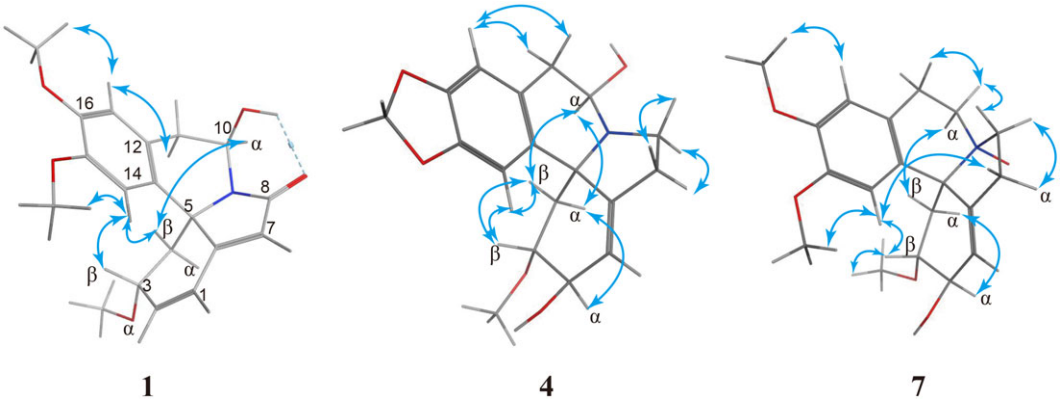


8 $R_1 = OCH_3$, $R_2 = OCH_3$
9 $R_1 = OCH_3$, $R_2 = OH$
10 $R_1 = OH$, $R_2 = OCH_3$
11 R_1-OCH_2O-R_2

12 R_1-OCH_2O-R_2
13 $R_1 = R_2 = OCH_3$

14

Figure 1. Chemical structures of isolated compounds **1**–**14** from *Erythrina crista-galli* L.



1 **4** **7**

Figure 2. Selected key NOESY correlations of compounds **1**, **4** and **7**.

NOESY, which indicated that there was an interaction between H-10 and H-4β (Fig. 2). This interaction was identical to that of the reported compound (3*R*,5*S*,10*R*)-10-hydroxy-11-oxoerysotrine, in which an α-configuration of the hydroxyl (-OH) was identified at C-10.[38] Therefore, compound **1** was characterized as (3*R*,5*S*,10*R*)-10-hydroxy-erysotramidine, and named cristanines C. This compound is only the third *Erythrina* alkaloid with an -OH group at C-10.[38,39]

Compound **4** is a bright white powder. The molecular formula was established as $C_{18}H_{21}NO_5$ according to its HR-ESI-MS (*m/z* 354.1314 $[M + Na]^+$). The ^1H and ^{13}C NMR and HSQC data for compound **4** indicate that it had one double bond (δ_H 5.63 m, δ_C 122.9, C-1; δ_C 143.3, C-6) with three substituents, two secondary -OH groups (δ_H 4.21 m, δ_C 72.3, C-2; δ_H 4.59 dd, δ_C 62.1, C-10), one $-OCH_3$ group (δ_H 3.33 s, δ_C 56.3, C-3-OMe), and one methylenedioxy group (δ_H 5.96 s, δ_C 101.1). It was supposed that compound **4** had an erythrinan skeleton with a $-OCH_3$ group at C-3, two -OH groups at C-2 and C-10, and a $\Delta^{1,6}$-olefin, which was deduced from the sequences of ^1H–^1H COSY cross-peaks (C-1 to C-4, C-7 to C-8) and the following HMBC correlations: H-dioxy-methylene to C-15 and 16; H_3-3-OCH_3 to C-3; H-17/C-11, 12 and 16; H-14/C-5, 13 and 15; H-11/C-10, 12, 13 and 17; H-7/C-1, 5, 6 and 8; H-1/C-2, 3, 5, 6 and 7; H-2/C-1 and 3; H-3/C-4 and 5; and H-4/C-3, 5 and 6 (Fig. 3). These data (Table 2) suggested that the structure of compound **4** was similar to that of erythratine (**6**) except that the methylene signal at the C-10 position was replaced by an oxymethine signal which displayed the downfield shifted signal (δ_C 62.1).[40] In the ^1H NMR spectrum, the chemical shift of this moiety appeared downfield (δ_H 4.59). The positive specific rotation value $[\alpha]_D^{31}$ +183.6 (*c* 0.20, CH_3OH) of **4** suggested an *S* configuration at C-5.[30,31] The interaction between H-10 and H-4β in the NOESY indicated that compound **4** had a *R* absolute configuration at C-10 (Fig. 2). Compound **4**, therefore, was determined to be (3*R*,5*S*,10*R*)-10-hydroxy-erythratine, and named cristanines D.

Compound **7** is a brownish red solid. It had a molecular formula of $C_{19}H_{25}NO_5$ deduced from its HR-ESI-MS (*m/z* 370.1627

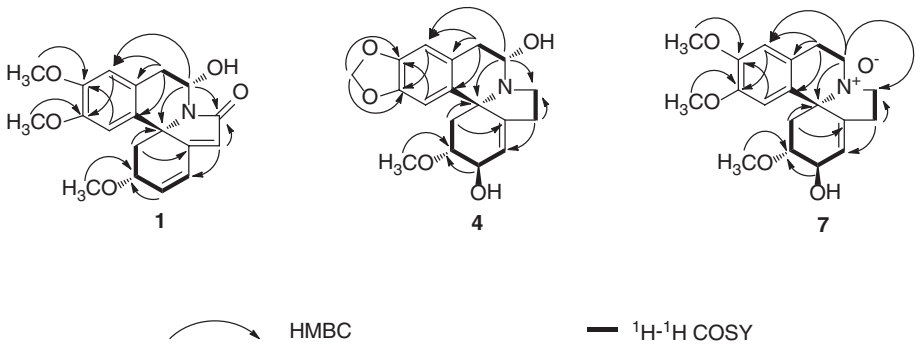


Figure 3. Selected key ^{1}H – ^{1}H COSY and HMBC correlations of compounds **1**, **4** and **7**.

Table 3. Potter spray tower assay of compounds **8**, **9**, **10** and **11** against *Aphis gossypii* at 24 h after treatment

Compound	$LC_{50} \pm SEM$ [a] (μg ml^{-1})	Toxicity regression equation ($y = a + bx$)	χ^2	df	*P*
8	163.74 ± 22.67	1.00 + 1.81*x*	2.53	3	0.46
9	186.81 ± 25.49	0.87 + 1.82*x*	2.96	3	0.39
10	165.35 ± 14.12	−1.52 + 2.94*x*	2.18	3	0.53
11	112.78 ± 11.95	0.11 + 2.38*x*	0.60	3	0.89
Imidacloprid[a]	45.40 ± 10.18	3.19 + 1.09*x*	1.51	3	0.67

[a] Commercial aphicide imidacloprid as positive control.

$[M + Na]^+$). The ^{1}H, ^{13}C NMR, DEPT and HSQC data for compound **7** indicated that it had three methoxyls (-OCH_3), five methylenes, five methines (including two oxy-methines, two aromatic methines and one olefin methines) and six non-protonated carbons (including four aromatic quaternary carbons). In light of the data, the structure of **7** was shown to be similar to that of erythratidine (**5**).[41] The positive specific rotation $[\alpha]_D^{31}$ +124.9 (*c* 0.20, CH_3OH) of **7** suggested that it has as an *S* configuration at C-5.[30,31] Additionally, there was one more oxygen atom in compound **7** compared with erythratidine (**5**). Analysis of differences in the ^{13}C NMR spectra between erythratidine and compound **7**, showed that the adjacent atoms C-5, C-6, C-8 and C-10 of compound **7** were markedly deshielded, which revealed that *N*-9 was oxygenated. Therefore, the relative configuration of **7** was assigned to erythraline *N*-oxide (Fig. 3), and named cristanines E.

The other 11 known compounds were established as erysotramidine (**2**),[29] 8-*oxo*-erythraline (**3**),[42] erythratidine (**5**),[41] erythratine (**6**),[40] erysotrine (**8**),[43] erysodine (**9**),[44] erysovine (**10**),[45] erythraline (11),[46] crystamidine (**12**),[38] erytharbine (**13**)[47] and erythrinan (**14**)[48] by comparing their spectroscopic data with those in the literature.

3.3 Aphicidal assay and structure–activity analysis

The aphicidal activity of the isolated compound was preliminarily assessed using the reported topical application method[21,22] and the results are given in Table 1. The new compounds **1**, **4** and **7** showed an absence of aphicidal activity at a dose of 530 ng aphid^{-1}. Among the known compounds, erythraline (**11**) displayed the highest aphicidal activity with an LD_{50} value of 4.67 ng aphid^{-1}. Its potency was slightly lower than that of imidacloprid (1.84 ng/aphid), a commercial systemic aphicide. Three other active compounds erysotrine (**8**), erysodine (**9**) and erysovine (**10**) showed moderate aphicidal activity (LD_{50} values of 5.13, 7.48 and 6.68 ng aphid^{-1}, respectively) in comparison with imidacloprid. The Potter spray tower assay under controlled experimental conditions is routinely used to simulate the spray effects of insecticides in field. The results of the Potter spray tower assay for compounds **8**, **9**, **10** and **11** were summarized in Table 3. Erythraline (**11**) was the most potent compared with the other three aphicidal compounds, although its potency (LC_{50}, 112.78 μg ml^{-1}) was obviously lower than that of imidacloprid (45.40 μg ml^{-1}).

Comparison of the structures of between bioactive and inactive compounds shows some interesting structure–activity relationships (Fig. 4). Generally, the four bioactive compounds **8**, **9**, **10** and **11** have the same substructure comprising rings A, B and C, which includes an sp^3 methylene at C-8 and a conjugate dienes group ($\Delta^{1,2}$ and $\Delta^{6,7}$). First, the substituent on C-8 had a significant influence on the activity, which could be explained by comparisons of compounds **2** and **8**. Specifically, replacement of H atoms at C-8 (**8**) by a carbonyl group resulted in the loss of potency for compound **2**. Second, the conjugate dienes group ($\Delta^{1,2}$ and $\Delta^{6,7}$) was also a critical factor in the activity, as seen from the inactive compounds **4**, **5** and **6** in which an olefin group $\Delta^{1,6}$ was presented. In addition, the non-oxygenated state of N-9 was necessary for the activity. Compared with bioactive compound **8**, compound **13** with an oxygenated state of N-9 was void of activity. Additionally, with substituents on ring D varying for compounds **8**, **9** and **10**, the aphicidal activity was mostly reserved. Taken together, the unique substructures of rings A, B and C are pivotal in the aphicidal activity of *Erythrina* alkaloids, whereas the D ring could serve as a modified site for improvement of the potency of the bioactive scaffold (Fig. 4).

3.4 Effect of erythraline on enzyme activity in *A. gossypii*

The activities of SOD, CAT and GST in *A. gossypii* after treatment with the most potent compound erythraline at concentrations of 58.80 μg ml^{-1} (LC_{25}), 112.78 μg ml^{-1} (LC_{50}) and 216.40 μg ml^{-1}

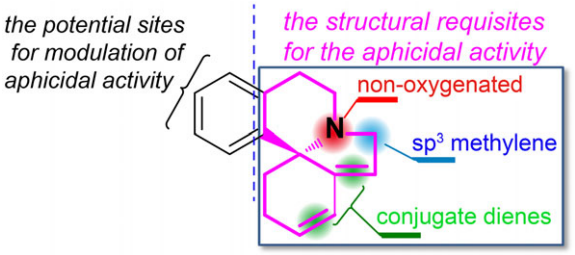


Figure 4. Structure–activity analysis of the isolated *Erythrina* alkaloids.

(LC_{75}) were assessed at 24 h (Fig. 5). The treated groups showed significant elevation in SOD and CAT activity at LC_{50} and LC_{75} (one-way ANOVA, $P < 0.05$). At LC_{25}, the treated groups and controls showed no significant differences in SOD and CAT activity, respectively. GST activity was significantly greater than in the control following treatment with erythraline at doses of LC_{25}, LC_{50} and LC_{75} (one-way ANOVA, $P < 0.05$). Meanwhile, a positive correlation was observed between erythraline concentration and GST activity in *A. gossypii* ($N = 4$, $r = 0.9951$).

Insects are depend on their antioxidant enzymes, including SOD and CAT, responding concatenately to endogenous and exogenous toxic compounds. Exogenous toxic chemicals such as insecticides activate these antioxidant enzymes, which are associated with insect resistance to these chemicals.[49,50] Many botanical alkaloids are known for their toxicity to insects and are resourcefully used as natural insecticides. It has been demonstrated that these biological alkaloids induce oxidative stress and activate the antioxidant systems in some phytophagous aphids.[51–53] Our study showed that the activities of SOD and CAT increased significantly at LC_{50} and LC_{75} doses of erythraline, indicating that both SOD and CAT in *A. gossypii* are involved in erythraline metabolism when aphids are exposed to erythraline above a threshold concentration. By contrast, detoxification enzymes, such as GST, are closely involved in the detoxification of various xenobiotics in insects, which makes pests resistant to many insecticides.[54] GST could catalyze the antioxidant defensive reactions of glutathione to the electrophilic centers of natural and synthetic exogenous xenobiotics and thus negotiate the resistance of insects to many commercial insecticides. It has been shown that natural xenobiotic alkaloids increase GST activity in many phytophagous pests.[55–57] In our study, erythraline significantly increased GST activity in *A. gossypii*, and the increase was positively correlated with erythraline concentration, suggesting that GST takes part in erythraline detoxification.

Owing to their structures and diverse biological activities, *Erythrina* alkaloids have attracted great interest. Systematic

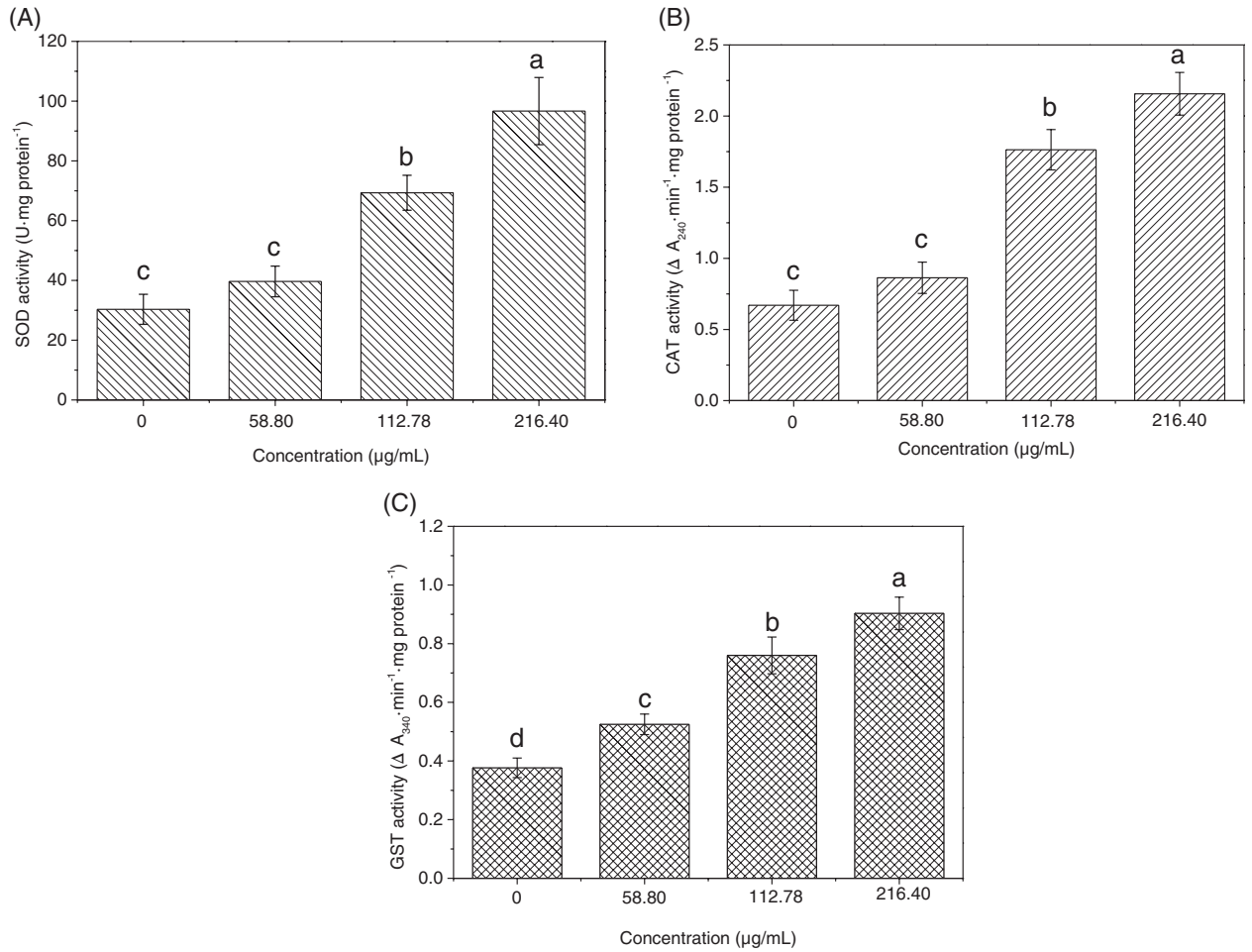


Figure 5. Effect of erythraline on activities (mean ± SD) of superoxide dismutase (SOD) (A), catalase (CAT) (B) and glutathione *S*-transferase (GST) (C) in *A. gossypii* with different concentrations of 58.80 μg ml^{-1} (LC_{25}), 112.78 μg ml^{-1} (LC_{50}) and 216.40 μg ml^{-1} (LC_{75}) at 24 h after treatment. Values marked with different letters indicate significant differences between groups (least significant difference test, $P < 0.05$).

studies have shown that some *Erythrina* alkaloids, including dihydro-β-erythroidine, erysodine (**9**), erysotrine (**8**), epierythratidine *O*-acetylerysodine and erysopine, are potent antagonists of neuronal nicotinic acetylcholine receptors (nAChRs), and display selectivity for the $\alpha4\beta2$ subtype of nAChRs.[58–60] The binding sites of *Erythrina* alkaloids on nAChRs are located at the interfaces between the α- and β-subunits in heteromeric receptors of $\alpha4\beta2$ subtype, which differs greatly from the binding sites of neonicotinoids, the agonists of nAChRs.[61,62] Therefore, aphicidal *Erythrina* alkaloids might have great potential in the management of aphids that are resistant to neonicotinoids.

4 CONCLUSION

This is the first study investigating the aphicidal activity of *E. crista-galli* L. seed extracts and isolated *Erythrina* alkaloids. A crude methanol extract of *E. crista-galli* L. seed was subjected to the isolation principle of the bioassay guided method. Three novel alkaloids were isolated and identified as cristanines C (**1**), cristanines D (**4**) and cristanines E (**7**). Among the isolated compounds, erysodine (**9**), erysovine (**10**), erysotrine (**8**) and erythraline (**11**) exhibited moderate to high aphicidal activity. Based on the results of aphicidal tests, several structure–activity relationships were identified, which may be helpful in further exploring these bioactive compounds. The most potent compound erythraline (**11**) led to significant elevation in SOD and CAT activity in *A. gossypii* at LC_{50} and LC_{75} doses. GST activity in *A. gossypii* showed a significant increase that was positively correlated with the erythraline concentration. We are undertaking further studies to investigate the mechanisms underlying the observed aphicidal activity of the bioactive compounds and elucidate the potential of those bioactive compounds for commercial application.

ACKNOWLEDGEMENTS

This work was financially supported by National Natural Science Foundation (No. 31401776), Science and Technology Innovation Training Program (No. 201510712006), and National Key Research and Development Program of China (2017YFD0201203).

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Oerke EC, Crop losses to pests. *J Agric Sci* **144**:31–43 (2005).
2 Oerke EC and Dehne HW, Safeguarding production – losses in major crops and the role of crop protection. *Crop Prot* **23**:275–285 (2004).
3 Pal N, Yamamoto T, King GF, Waine C and Bonning B, Aphicidal efficacy of scorpion- and spider-derived neurotoxins. *Toxicon* **70**:114–122 (2013).
4 Fokkema NJ, Riphagen I, Poot RJ and de Jong C, Aphid honeydew, a potential stimulant of *Cochliobolus sativus* and *Septoria nodorum* and the competitive role of saprophytic mycoflora. *Trans Brit Mycol Soc* **81**:355–363 (1983).
5 Ng JCK and Perry KL, Transmission of plant viruses by aphid vectors. *Mol Plant Pathol* **5**:505–511 (2004).
6 Emden HFv and Harrington R, *Aphids as Crop Pests*. CABI Publishing, London, UK (2007).
7 Jacobson RM and Thriugnanam M, New selective systemic aphicides, In *Synthesis and Chemistry of Agrochemicals II*, ed. by Don RB, Joseph GF and William KM. American Chemical Society, Washington, DC, pp. 322–339 (1991).
8 Bass C, Puinean AM, Zimmer CT, Denholm I, Field LM, Foster SP *et al.*, The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*. *Insect Biochem Mol Biol* **51**:41–51 (2014).
9 Bhatia V, Uniyal PL and Bhattacharya R, Aphid resistance in Brassica crops: challenges, biotechnological progress and emerging possibilities. *Biotechnol Adv* **29**:879–888 (2011).
10 Pisa LW, Amaral-Rogers V, Belzunces LP, Bonmatin JM, Downs CA, Goulson D *et al.*, Effects of neonicotinoids and fipronil on non-target invertebrates. *Environ Sci Pollut Res* **22**:68–102 (2015).
11 Uhl P, Bucher R, Schäfer RB and Entling MH, Sublethal effects of imidacloprid on interactions in a tritrophic system of non-target species. *Chemosphere* **132**:152–158 (2015).
12 Döker İ, Pappas ML, Samaras K, Triantafyllou A, Kazak C and Broufas GD, Compatibility of reduced-risk insecticides with the non-target predatory mite *Iphiseius degenerans* (Acari: Phytoseiidae). *Pest Manag Sci* **71**:1267–1273 (2015).
13 Sparks TC, Crouse GD and Durst G, Natural products as insecticides: the biology, biochemistry and quantitative structure–activity relationships of spinosyns and spinosoids. *Pest Manag Sci* **57**:896–905 (2001).
14 Cantrell CL, Dayan FE and Duke SO, Natural products as sources for new pesticides. *J Nat Prod* **75**:1231–1242 (2012).
15 Dayan FE, Cantrell CL and Duke SO, Natural products in crop protection. *Bioorg Med Chem* **17**:4022–4034 (2009).
16 Berenbaum MR, Postgenomic chemical ecology: from genetic code to ecological interactions. *J Chem Ecol* **28**:873–896 (2002).
17 Ashmawy SN, Ashour LM, Wink M, El-Shazly M, Chang F-R, Swilam N *et al.*, Polyphenols from *Erythrina crista-galli*: structures, molecular docking and phytoestrogenic activity. *Molecules* **21**:726 (2016).
18 Parsons AF and Palframan MJ, *Erythrina* and related alkaloids, In *The Alkaloids: Chemistry and Biology*, ed. by Geoffrey AC. Elsevier, San Diego, CA, pp. 39–81 (2010).
19 Jackson AH, *Erythrina* alkaloids, In *The Chemistry and Biology of Isoquinoline Alkaloids*, ed. by Phillipson JD, Roberts MF and Zenk MH. Springer, Berlin, Germany, pp. 62–78 (1985).
20 Dyke SF and Quessy SN, *Erythrina* and related alkaloids. In *The Alkaloids: Chemistry and Physiology*, ed. by Rodrigo RGA, Vol. 18. Academic Press, New York, NY pp. 1–98, (1981).
21 Hewlett PS and Lloyd CJ, Topical application of mobile liquids to insects by means of micro-capillary tubes. *Ann Appl Biol* **48**:125–133 (1960).
22 Smith SC, Harris EG and Wilson K, Effect of temperature regime on the toxicity of endosulfan and deltamethrin to tsetse flies, *Glossina morsitans morsitans*. *Trop Sci* **34**:391–400 (1994).
23 Faraone N, Hillier NK and Cutler GC, Plant essential oils synergize and antagonize toxicity of different conventional insecticides against *Myzus persicae* (Hemiptera: Aphididae). *PLOS ONE* **10**:e0127774 (2015).
24 Fridovich I, Superoxide and superoxide dismutases. *Free Radical Biol Med* **15**:472 (1993).
25 Zhang K, Li Z, Zhu S and Weng Q, ^{60}Co-γ irradiation affects the enzymatic antioxidant system of the citrus red mite *Panonychus citri* (Acari: Tetranychidae). *Molecules* **19**:6382–6392 (2014).
26 Aebi H, Catalase *in vitro*. *Methods Enzymol* **105**:121 (1984).
27 Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem* **249**:7130–7139 (1974).
28 Bradford M, Rapid and sensitive method for quantification of microgram quantities of protein utilizing principle of protein-dye-binding. *Anal Biochem* **25**:248–256 (1976).
29 Ito K, Furukawa H and Haruna M, [Studies on the erythrina alkaloids. VI. Alkaloids of *Erythrina arborescens* Roxb. 1. Extraction and isolation of alkaloids (author's transl)]. *Yakugaku zasshi: J Pharm Soc Japan* **93**:1611–1616 (1973).
30 Rukachaisirikul T, Innok P and Suksamrarn A, *Erythrina* alkaloids and a pterocarpan from the bark of *Erythrina subumbrans*. *J Nat Prod* **71**:156–158 (2008).
31 Amer ME, Shamma M and Freyer AJ, The tetracyclic *Erythrina* alkaloids. *J Nat Prod* **54**:329–363 (1991).
32 Tsuda Y and Sano T, *Erythrina* and related alkaloids. *The Alkaloids: Chemistry and Pharmacology*, ed. by Cordell GA, Vol. 48. Academic Press, New York, NY pp. 249–337, (1996).
33 Maier UH, Rödl W, Deus-Neumann B and Zenk MH, Biosynthesis of *Erythrina* alkaloids in *Erythrina crista-galli*. *Phytochemistry* **52**:373–382 (1999).
34 Umihara H, Yoshino T, Shimokawa J, Kitamura M and Fukuyama T, Development of a divergent synthetic route to the erythrina

alkaloids: asymmetric syntheses of 8-*oxo*-erythrinine, crystamidine, 8-*oxo*-erythraline, and erythraline. *Ange Chem Int Ed* **55**:6915–6918 (2016).
35 He M, Qu C, Ding B, Chen H, Li Y, Qiu G *et al.*, Total synthesis of (±)-8-*oxo*-erythrinine, (±)-8-*oxo*-erythraline, and (±)-clivonine. *Eur J Org Chem* **2015**:3240–3250 (2015).
36 L'Homme C, Ménard M-A and Canesi S, Synthesis of the *Erythrina* alkaloid erysotramidine. *J Org Chem* **79**:8481–8485 (2014).
37 Paladino M, Zaifman J and Ciufolini MA, Total synthesis of (+)-3-demethoxyerythratidinone and (+)-erysotramidine via the oxidative amidation of a phenol. *Org Lett* **17**:3422–3425 (2015).
38 Tanaka H, Hattori H, Tanaka T, Sakai E, Tanaka N, Kulkarni A *et al.*, A new *Erythrina* alkaloid from *Erythrina herbacea*. *J Nat Med* **62**:228–231 (2008).
39 Tanaka H, Tanaka T and Etoh H, *Erythrinan* alkaloid from *Erythrina* x *bidwillii*. *Phytochemistry* **48**:1461–1463 (1998).
40 Amer ME, Kassem FF, El-Masry S, Shamma M and Freyer A, NMR spectral analysis of five alkaloids from *Erythrina caffra*. *Alex J Pharm Sci* **7**:28–30 (1993).
41 Venancio D, Argentine plants. XII. Erythratidine, a new alkaloid from *Erythrina falcata*. *Chem Berichte* **85**:620–623 (1952).
42 Chawla AS, Chunchatprasert S and Jackson AH, Studies of *Erythrina* alkaloids: VII – ^{13}C NMR spectral studies of some *Erythina* alkaloids. *Org Magn Reson* **21**:39–41 (1983).
43 Redemann CE, Wisegarver BB and Alles GA, Characterization of certain alkaloids from *Fagara coco*. *J Am Chem Soc* **71**:1030–1034 (1949).
44 Wanjala CCW and Majinda RRT, Two novel glucodienoid alkaloids from *Erythrina latissima* seeds. *J Nat Prod* **63**:871–873 (2000).
45 Gentile RA and Labriola R, Studies on Argentine plants. IV. Alkaloids from *Erythrina* species. *J Org Chem* **7**:136–139 (1942).
46 Ozawa M, Etoh T, Hayashi M, Komiyama K, Kishida A and Ohsaki A, TRAIL-enhancing activity of Erythrinan alkaloids from *Erythrina velutina*. *Bioorg Med Chem Lett* **19**:234–236 (2009).
47 Ito K, Haruna M, Jinno Y and Furukawa H, Studies on the *Erythrina* alkaloids. XI. Alkaloids of *Erythrina crystagalli* LINN. Structure of a new alkaloid, crystamidine. *Chem Pharm Bull* **24**:52–55 (1976).
48 Juma BF and Majinda RRT, Erythrinaline alkaloids from the flowers and pods of *Erythrina lysistemon* and their DPPH radical scavenging properties. *Phytochemistry* **65**:1397–1404 (2004).
49 Felton GW, Oxidative stress of vertebrates and invertebrates, In *Oxidative Stress and Antioxidant Defenses in Biology*, ed. by Ahmad S. Springer, Boston, MA, pp. 356–434 (1995).
50 Ahmad S, Biochemical defence of pro-oxidant plant allelochemicals by herbivorous insects. *Biochem Syst Ecol* **20**:269–296 (1992).
51 Lukasik I, Changes in activity of superoxide dismutase and catalase within cereal aphids in response to plant *o*-dihydroxyphenols. *J Appl Entomol* **131**:209–214 (2007).
52 Saikkonen K, Gundel PE and Helander M, Chemical ecology mediated by fungal endophytes in grasses. *J Chem Ecol* **39**:962–968 (2013).
53 Bi JL and Felton GW, Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J Chem Ecol* **21**:1511–1530 (1995).
54 Li X, Schuler MA and Berenbaum MR, Molecular mechanisms of metabolic resistance to synthetic and natural xenobiotics. *Annu Rev Entomol* **52**:231–253 (2007).
55 Khosravi R, Sendi JJ, Ghadamyari M and Yezdani E, Effect of sweet wormwood *Artemisia annua* crude leaf extracts on some biological and physiological characteristics of the lesser mulberry pyralid, *Glyphodes pyloalis*. *J Insect Sci* **11**:1–13 (2011).
56 Després L, David J-P and Gallet C, The evolutionary ecology of insect resistance to plant chemicals. *Trend Ecol Evol* **22**:298–307 (2007).
57 Cai Q-N, Han Y, Cao Y-Z, Hu Y, Zhao X and Bi J-L, Detoxification of gramine by the cereal aphid *Sitobion avenae*. *J Chem Ecol* **35**:320–325 (2009).
58 Decker MW, Anderson DJ, Brioni JD, Donnelly-Roberts DL, Kang CH, O'Neill AB *et al.*, Erysodine, a competitive antagonist at neuronal nicotinic acetylcholine receptors. *Eur J Pharmacol* **280**:79–89 (1995).
59 Williams M and Robinson JL, Binding of the nicotinic cholinergic antagonist, dihydro-*beta*- erythroidine, to rat brain tissue. *J Neurosci* **4**:2906 (1984).
60 Jepsen TH, Jensen AA, Lund MH, Glibstrup E and Kristensen JL, Synthesis and pharmacological evaluation of DHβE analogues as neuronal nicotinic acetylcholine receptor antagonists. *ACS Med Chem Lett* **5**:766–770 (2014).
61 Iturriaga-Vásquez P, Carbone A, García-Beltrán O, Livingstone PD, Biggin PC, Cassels BK *et al.*, Molecular determinants for competitive inhibition of $\alpha4\beta2$ nicotinic acetylcholine receptors. *Mol Pharmacol* **78**:366 (2010).
62 Hansen CP, Jensen AA, Christensen JK, Balle T, Liljefors T and Frølund B, Novel acetylcholine and carbamoylcholine analogues: development of a functionally selective $\alpha4\beta2$ nicotinic acetylcholine receptor agonist. *J Med Chem* **51**:7380–7395 (2008).