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# LUTEOLIN 7-*O*-(6″-*O*-MALONYL)-β-D-GLUCOSIDE AND *TRANS*-CHLOROGENIC ACID: OVIPOSITION STIMULANTS FOR THE BLACK SWALLOWTAIL BUTTERFLY

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**Key Word Index**—*Daucus carota*; Umbelliferae, wild carrot, *Papilio polyxenes*; Papilionidae; black swallowtail, oviposition stimulant, flavone malonate, chlorogenic acid, luteolin 7-*O*-(6″-*O*-malonyl)-β-D-glucopyranoside.

**Abstract**—Oviposition by females of the black swallowtail butterfly, *Papilio polyxenes*, was stimulated by tarsal contact with ethanolic extracts of carrot foliage, *Daucus carota*. Two of the stimulants were identified as *trans*-chlorogenic acid and luteolin 7-*O*-(6″-*O*-malonyl)-β-D-glucopyranoside. These were inactive alone but in combination accounted for about 70% of the response to the parent extract. Ready loss of stimulant activity in carrot extracts was associated with loss of malonic acid from the flavone malonate, yielding inactive luteolin 7-β-D-glucoside. On the basis of NMR spectroscopy of the free acid and its anion, a tertiary structure is proposed for the side-chain of luteolin 7-*O*-(6″-*O*-malonyl)-β-D-glucopyranoside, the first for a flavonoid malonate. The structure depicts a 10-membered boat–chair–boat ring, formed by hydrogen-bonding of the carbonyl group of the malonyl residue to the 4″-OH group of the glucose.

## INTRODUCTION

The host plants of related species of plant-feeding insects commonly share secondary compounds of the same chemical classes even though the host plants themselves may not be close taxonomic relatives of one another [1]. Dethier [2] and Jermy [3] have proposed that such patterns result from behavioural facilitation of host shifts by the insects' colonization of novel host plants will be more likely if such hosts contain compounds already used as host-finding cues The present study lays some of the groundwork for a test of this hypothesis. If host-shifts by insects have been catalysed by responses to behavioural cues, then at least some of the compounds shared by the host plants of related insects should be among those that are used by the insects for host recognition.

This work is part of a longer-term study [4] of the roles of plant chemistry in the evolution of the swallowtail butterflies (family Papilionidae), a family whose 550 or so species feed, between them, on more than 30 plant families of several subclasses. Though many of these families are unrelated to one another, they share various combinations of secondary compounds, including essential oils, furanocoumarins, and alkaloids [1, 5]

Dethier [2] first formulated the behavioural-facilitation hypothesis as a result of experiments with larvae of the black swallowtail, *Papilio polyxenes* Fabr., which feed primarily on plants of the family Umbelliferae. He reported that the larvae are attracted by certain essential oil components that occur both in the Umbelliferae and in certain species of Rutaceae on which *P. polyxenes* occasionally feeds. We chose to study the role of chemistry in oviposition behaviour by swallowtails since it seems likely that oviposition 'mistakes' by females provide the most probable route to colonization of novel host plants.

Both visual and volatile chemical cues influence the decision of female black swallowtails to land on potential host plants [6]. Once they have alighted on a leaf, however, females decide whether or not to oviposit on the basis of compounds perceived by contact chemoreceptors located on their tarsi [5] In earlier work, we found that *P. polyxenes* females could be stimulated to curl their abdomens and lay eggs on filter paper treated with ethanolic extracts of carrot foliage, *Daucus carota* L The contact stimulants contained in such extracts were polar and remained in the aqueous phase after extraction with organic solvents [5] Isolation of stimulants by chromatography was hampered by separation of synergistic ingredients from one another and by progressive degradation of at least one of the active compounds. One stimulant was tentatively identified as *trans*-chlorogenic acid on the basis of chromatographic behaviour, the formation of caffeic and quinic acids on hydrolysis, and weak but consistent activity of the standard compound in bioassay [5]. Here we confirm the activity of chlorogenic acid and describe the isolation and identification of a flavonoid oviposition stimulant for *P. polyxenes*

## RESULTS

### *Initial fractionation of activity*

Comparison of the stimulant activity of an initial ethanolic extract of carrot leaves before and after extraction with $Et_2O$, $CHCl_3$, EtOAc and *n*-BuOH confirmed earlier findings that most activity remained in the aq. phase. This 'post-BuOH' aq fraction stimulated abdomen-curling down to a concentration of 0 001 g leaf equivalents ('gle', see Experimental) and was as active as the original extract. Little activity, by contrast, was detected in the combined organic phases (Table 1).

The post-BuOH aq. fraction was separated preparatively on an open C-18 column into five fractions, eluted

Table 1 Dose responses of *P polyxenes* females to (a) ethanolic extract of carrot leaves before and after extraction with organic solvents and (b) post-BuOH aq fraction before and after extraction into EtOAc at pH 2 5 ($N = 20$–30 butterflies per trial)

| Fraction | Per cent female response* † | | | | | |
|---|---|---|---|---|---|---|
| | 0 0001 gle | 0 001 gle | 0 01 gle | 0 1 gle | 1 0 gle | 4 0 gle |
| (a) | | | | | | |
| Ethanolic carrot extract | | 21 | **62** | **67** | **60** | **69** |
| Post-BuOH aq fraction | 11 | **36** | **68** | **64** | **68** | **72** |
| Combined organic fractions | | 2 | **37** | 19 | 30 | 24 |
| (b) | | | | | | |
| Post-BuOH aq fraction | 11 | **36** | **68** | **64** | **68** | **72** |
| Acid EtOAc extract | | 0 | **69** | **78** | **81** | **73** |
| Residual aq layer | | 4 | 4 | 14 | **35** | 27 |

*Concentrations of tested extracts given as gram leaf equivalents (gle) per bioassay paper

†Responses in bold face indicate significant difference from response to distilled water control ($p < 0.05$, $\chi^2$ test)

in water (fraction A), 1% HOAc (fraction B), 1% HOAc/20–100% MeOH (fractions C, D and E, see Experimental) Fractions A, B and D exhibited moderate stimulant activity at a dose of 0 1 gle (Table 2) Mixtures of A with either B or D increased activity markedly and a synergistic interaction was also evident between fractions B and D (Table 2) Fractions C and E appeared to have little activity

From further elutions of the same C-18 column, fraction D was collected as two equal subfractions, D1 and D2 Bioassays with fraction B showed activity to be present in D2 but not D1 (Table 2) Fraction B was also collected as two equal subfractions, B1 and B2 Bioassays revealed that both B1 and B2 were active in combination with fraction D or D2, though neither enhanced the activity of D as well as did a combination of both (Table 2)

*Isolation of compounds from fraction D*

Analysis of D2 by HPLC (system 1, see Experimental) revealed only one major UV-absorbing peak (compound **1**), whereas analysis of (inactive) D1 revealed major and minor components (compounds **2** and **3**, respectively) HPLC also showed that **1**, trapped from injections of fraction D, disappeared with time and yielded increasing quantities of material that eluted at the same retention time as that of **3** The changes were associated with loss of activity of fraction D2 to butterflies

TLC of **1** and **2** in 5% HOAc (system A) revealed, in each case, only a yellow-fluorescing spot near the origin, as did an authentic sample of luteolin 7-*O*-*β*-D-glucoside When run in water (system B), however, **1** and **2** gave rise to yellow-fluorescing comet-like spots with variable $R_f$ values, whereas luteolin 7-glucoside remained near the origin Mobility in water, but not in 5% HOAc, indicated that both **1** and **2** were acids

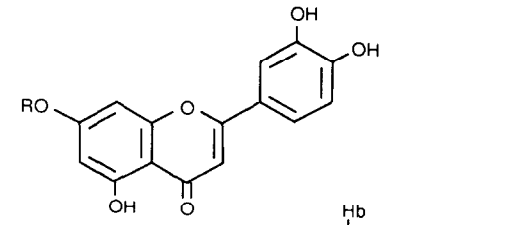
Confirmation that at least one of the active compounds was acidic in nature was obtained by bioassay of an EtOAc extract of an aliquot of the post-BuOH aq fraction that had been acidified to pH 2 5 This EtOAc

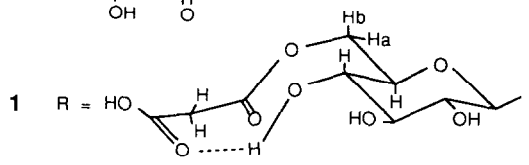
Table 2 Contact oviposition responses of *P polyxenes* females to fractions and subfractions of carrot extract (post-BuOH aq phase after extraction with organic solvents), eluted from C-18 open column

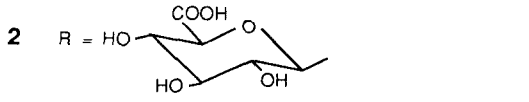
| Extract or fraction(s) (Dose = 0 1 gle) | Number of females | % response* |
|---|---|---|
| Post-BuOH carrot extract | 33 | **82** |
| Fraction A | 20 | **50** |
| Fraction A | 26 | **62** |
| Fraction A | 27 | **78** |
| Fraction A | 20 | **95** |
| Fraction B† | 21 | **48** |
| Fraction C | 21 | 33 |
| Fraction D† | 20 | **45** |
| Fraction E | 23 | 17 |
| Fractions B + C | 20 | **45** |
| Fractions B + D† | 25 | **72** |
| Fractions C + D | 21 | **48** |
| Fractions A + B | 26 | **92** |
| Fractions A + D | 27 | **89** |
| Fractions A + B + D | 21 | **90** |
| Fractions B + D1 | 27 | 19 |
| Fractions B + D2 | 28 | **75** |
| Fractions B + D2 | 29 | **76** |
| Fraction B1 | 25 | 20 |
| Fractions B1 + D | 21 | **52** |
| Fractions B2 + D | 21 | 38 |
| Fractions B1 + D2 | 20 | **75** |
| Fractions B2 + D2 | 20 | **50** |
| Fraction D2 | 24 | 17 |
| Fractions B1 + B2 + D2 | 33 | **79** |
| Fraction B + Compound **1** | 20 | **75** |

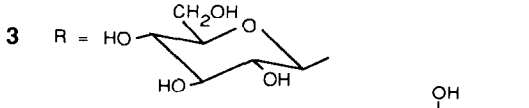
*Responses in bold face indicate significant difference from assumed response of 12% to distilled water ($\chi^2$ test, $p < 0.05$)

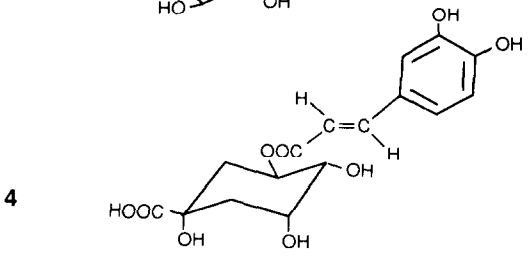
†When tested at 0 01 gle/strip. Fractions B and D were inactive alone (23 and 10%, respectively) but active when combined (57%)











extract was almost as active as the parent post-BuOH aq fraction (Table 1). HPLC (system 1) revealed the dominant UV-absorbing peaks to be those corresponding to **1**, **2** and a compound **4** that had been noticed earlier in HPLC traces of fractions A, B and C

Larger amounts of **1–3** were trapped individually from the post-BuOH aq. fraction by HPLC (system 2, see Experimental) (Fig 1) Compound **1** was purified by further HPLC and by extraction into EtOAc after acidification. The UV spectra of **1**, using diagnostic shift reagents [7], showed the presence of the 7-*O*-substituted luteolin nucleus IR bands at 1732 and 1738 $cm^{-1}$ indicated the presence of acid and/or ester group(s). Alkaline hydrolysis yielded malonic acid and luteolin 7-*O*-*β*-D-glucoside The site of esterification was discovered by high-resolution NMR spectroscopy. The complete $^1H$ NMR assignment (Table 3) was acquired from a combination of 1D and 2D spectra Phase-sensitive homonuclear correlation spectroscopy (COSYPS) [8] was used for $^1H$–$^1H$ correlation, leading to unambiguous assignment of all signals (Fig. 2). Noteworthy is the full assignment of glucosyl protons which would have been difficult with the 1D technique alone. The coupling constants of the multiplets were derived from Homonuclear 2D *J*-resolved spectroscopy (HOM2DJ) (Fig. 3) [9] and were in agreement with the 6-substituted glucopyranose moiety in its $^4C_1$ conformation, *β*-linked to the luteolin nucleus

The full assignment of the $^1H$ NMR spectrum facilitated interpretation of the $^{13}C$ NMR spectrum (Table 4) using a $^{13}C$–$^1H$ heteronuclear chemical shift correlation (HETCOR) technique [10]. These assignments were supported by the results of distortionless enhancement of polarization transfer (DEPT) [8, 11]. In the $^1H$ NMR spectrum the non-equivalence of protons at C-6″ indicated that the malonyl group was substituted at this position This was confirmed by the $^{13}C$ NMR spectrum as the chemical shift of C-6″ appeared 2-3 ppm downfield and that of C-5″ 2–3 ppm upfield from reported values for D-glucose [12]

On addition of 10% $D_2O$ (v/v) to the pyridine-$d_5$ soln of **1**, the two doublets corresponding to the -$CH_2$ of the malonyl moiety at $\delta$3.85 and 3.94, respectively, disappeared completely due to deuterium exchange with the solvent. This exchange also accounts for the low intensity of the carbon signal at $\delta$43 0 attributable to its coupling with deuterium, longer spin-lattice relaxation time and to quadrupolar broadening of the signal.

Matern *et al.* [13] deduced the existence of intramolecular hydrogen bonding in apigenin 7-*O*-(6″-*O*-malonylglucoside) but were uncertain as to its site To examine the tertiary structure of **1**, we compared the $^1H$ NMR spectrum of its salt form (see Experimental) in pyridine-$d_5$ with that of the free acid. We observed that the signals of H-6″a and H-5″ in the free acid were shifted upfield by 0 19 and 0.07 ppm, respectively, and those of H-6″b and H-4″ were shifted downfield by 0.17 and 0.10, respectively, compared to the corresponding signals in the salt form (Table 3). We studied molecular models of **1** with the malonyl chain hydrogen-bonded to the glucose unit at various sites and found that H-bonding between the -COOH and 4″-OH resulted in the least strained 10-membered ring in its distorted boat–chair–boat conformation The upfield and downfield shifts of the H-4″, H-5″ and H-6″ protons can be explained by the fact that pyridine forms a complex with the carbonyl function, thereby inducing a positive or negative shift according to the 'carbonyl plane rule' [14] In the salt form, where there is no hydrogen bonding between the ester chain and the sugar moiety, the sugar protons would remain almost unaffected by the solvent-induced effect. In the unionized (i e. intramolecularly H-bonded) form, by contrast, the ester carbonyl would be oriented such that H-6″ and H-5″ would fall behind the plane defined according to the carbonyl plane rule, and H-6″b and H-4″ in front of it.

Thus **1** was characterized as luteolin 7-*O*-(6″-*O*-malonyl)-*β*-D-glucopyranoside This is the first report of this compound from higher plants, though it has been reported recently from a bryophyte [15]. Compound **2** was purified by HPLC (system 2) On the basis of TLC, spectral data (UV, IR, $^1H$ NMR, FABMS) and enzyme hydrolysis, it was identified as luteolin 7-*O*-*β*-D-glucuronide, reported previously from carrot [16]. After purification by HPLC and precipitation from EtOAc–MeOH, **3** was identified as luteolin 7-*O*-*β*-D-glucoside on the basis of chromatographic behaviour, co-chromatography, co-HPLC, and UV spectroscopy. It has been reported previously from carrot leaves [16, 17]. The generation of **3** from **1** with time, noted earlier, can now be interpreted simply as the loss of malonic acid from **1** Malonyl esters of flavonoid glycosides are known to be quite labile [18]. Since most of the luteolin 7-glucoside in our original carrot extract was extracted by EtOAc and *n*-BuOH, its presence in the post-BuOH aqueous fraction probably

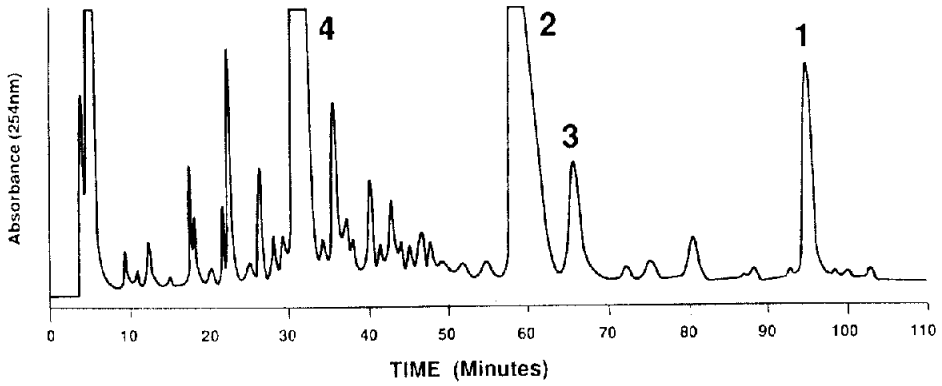



Fig. 1. HPLC trace (system 2) of post-BuOH aq. fraction of carrot-leaf extract showing retention times of compounds **1**–**4**.

Table 3. $^1$H NMR spectral data (400 MHz)* for compound **1**

| Proton | **1** in *pyridine-$d_5$* | **1** in *pyridine-$d_5$* + $D_2O$† | **1**(salt) in *pyridine-$d_5$* + $D_2O$† |
|---|---|---|---|
| 3 | 6.91*s* | 6.86*s* | 6.72*s* |
| 6 | 6.83*d* | 6.77*d* | 6.67*d* |
| | (2.4) | (2.2) | (2) |
| 8 | 6.97*d* | 6.94*d* | 7.09*d* |
| | (2.4) | (2.2) | (2) |
| 2′ | 7.95*d* | 7.96*d* | 8.25*d* |
| | (2.4) | (2.4) | (2.3) |
| 5′ | 7.34*d* | 7.37*d* | 7.29*d* |
| | (8.4) | (8.4) | (8.6) |
| 6′ | 7.57*dd* | 7.52*dd* | 7.41*dd* |
| | (2.4, 8.4) | (2.4, 8.4) | (2.3, 8.6) |
| 1″ | 5.72*d* | 5.68*d* | 5.66*d* |
| | (7.7) | (7.7) | (7.6) |
| 2″ | 4.36*m* | 4.28*dd*‡ | 4.23*dd* |
| | | (7.7, 8.8) | (7.6, 10) |
| 3″ | 4.36*m* | 4.40*t* | 4.36*dd*‡·§ |
| | | (8.8) | (8, 10) |
| 4″ | 4.16*dd* | 4.12*dd* | 4.02*t* |
| | (8.1, 10.4) | (8.8, 10.34) | (10) |
| 5″ | 4.36*m* | 4.31*dd*‡ | 4.38*dd*‡·§ |
| | | (1.9, 7.0, 10.34) | (8, 10) |
| 6″a | 5.11*dd* | 5.07*dd* | 5.26*dd* |
| | (2.2, 11.8) | (1.9, 11.8) | (2.9, 12.3) |
| 6″b | 4.85*dd* | 4.67*dd* | 4.50*dd* |
| | (7.1, 11.8) | (7.0, 11.8) | (8, 12.3) |
| $-CH_2$ | 3.85*d* | —¶ | —¶ |
| | (15.7) | | |
| | 3.94*d* | —¶ | —¶ |

*Chemical shifts in ppm, *J* values in Hz given in parentheses.

†*J* values obtained by HOM2DJ spectroscopy.

‡H-2″ and H-5″ signals (column 2) and H-3″ and H-5″ signals (column 3) were not resolved by HOM2DJ. Coupling constants given are from the values of neighbouring signals.

§Appeared as a triplet in HOM2DJ and did not show the small interaction, $J = 2.9$.

¶No signals, due to proton exchange with deuterium.

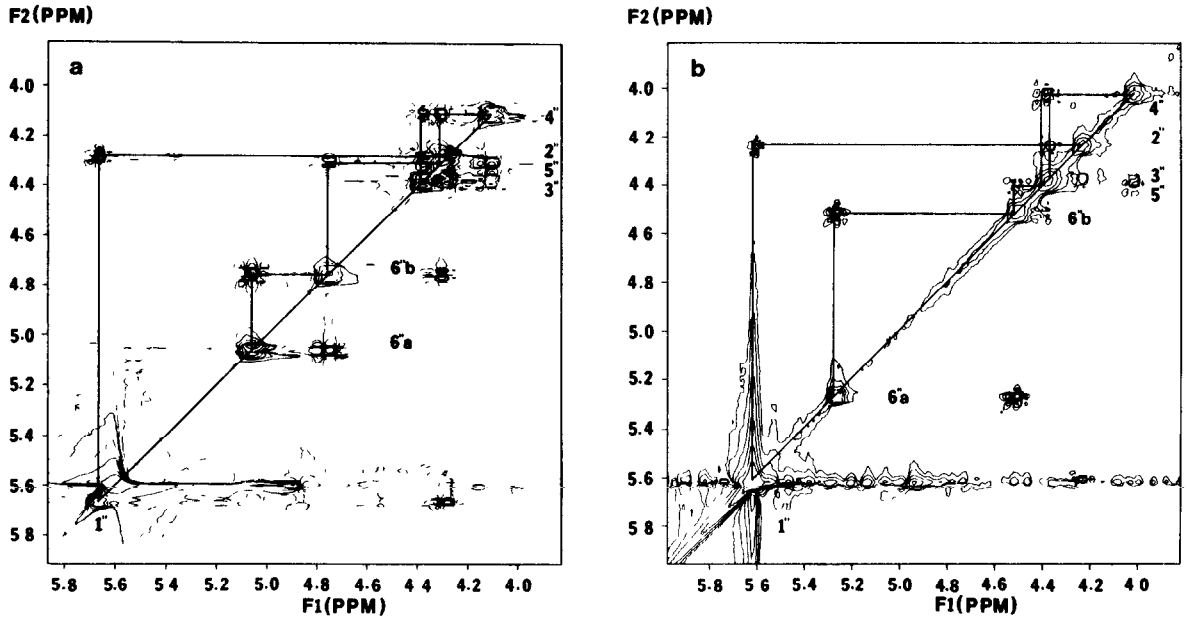



Fig. 2 A section of the contour plot of the COSYPS spectrum of **1** in pyridine-$d_5$–$D_2O$ (a) the free acid, (b) the acid anion (salt).

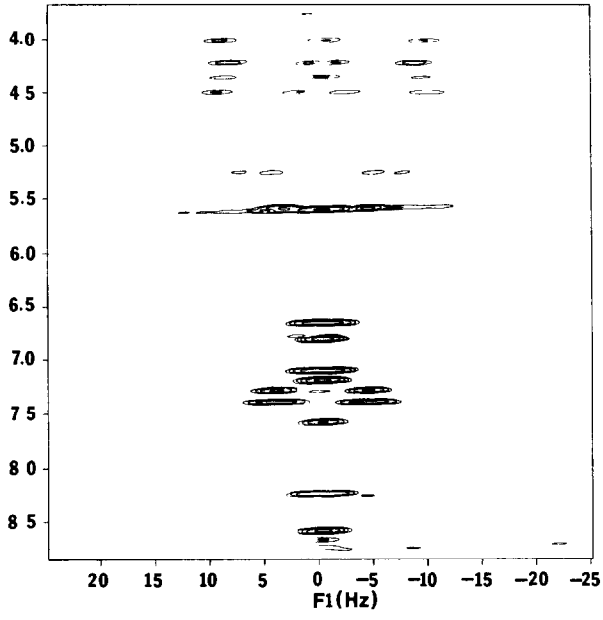



Fig. 3 Contour plot of HOM2DJ spectrum of **1** (ionized form) in pyridine-$d_5$–$D_2O$.

resulted largely from degradation of the malonyl ester.

*Isolation of active compound from fraction B*

Attention was focused on fraction B1, which was more active than B2 (Tables 2 and 5). HPLC (system 2) revealed that compound **4** was the major UV-absorbing component of B1. This compound, trapped from HPLC injections of B1, proved active in bioassay with **1**, whereas the remaining components of B1, collected as separate fractions and recombined, proved to be less active (Table 5). The activity of fraction B1 was restored by readdition of **4** (Table 5).

Larger quantities of **4** were isolated directly from HPLC injections of the post-BuOH aq. fraction (Fig. 1). After further purification by HPLC, **4** was identified definitively as a caffeoyl ester of quinic acid by com-

Table 4. $^{13}C$ NMR spectrum of compound **1** (pyridine-$d_5$ + $D_2O$)

| C | Chemical shift (ppm) | |
|---|---|---|
| 2 | 164.255* | |
| 3 | 104.480 | |
| 4 | 183.338 | |
| 5 | 162.504 | |
| 6 | 101.027 | |
| 7 | 166 029* | |
| 8 | 96 211 | |
| 9 | 158 262 | |
| 10 | 106 984 | |
| 1′ | 123 024 | |
| 2′ | 115 002 | |
| 3′ | 147 947 | |
| 4′ | 152 081 | |
| 5′ | 117 399 | |
| 6′ | 120 316 | |
| 1″ | 102 039 | |
| 2″ | 74.890 | |
| 3″ | 78 186 | |
| 4″ | 71 488 | |
| 5″ | 75 952 | |
| 6″ | 65.676 | |
| Carbonyls of malonyl group | 168.887 | 170.442 |
| $-CH_2$ | 43 000 (weak) | |

*Values may be interchanged.

Table 5 Responses of *P polyxenes* females to components of Fraction B of post-BuOH aq extract of carrot leaves and to various combinations of compound **1** and authentic standards (CA = *trans*-chlorogenic acid)

| Fraction or compound (Dose, gle)* † | No of females | Per cent response‡ |
|---|---|---|
| Fraction B1 (0 5)+**1** (0 2) | 20 | 80[a] |
| Fraction B2 (0 5)+**1** (0 2) | 21 | 6[b] |
| **1** (0 2) | 22 | 0[a] |
| Fraction B1 (0 5) | 26 | 50[b] |
| Fraction B1 (0 5)+**1** (0 2) | 19 | 89[c] |
| **4** from B1 (0 5) | 25 | 28[a] |
| **4** from B1 (0 5)+**1** (0 2) | 26 | 80[b] |
| Fraction B1 without **4** (0 5)§ | 25 | 12[a] |
| Fraction B1 without **4** (0 5)§+**1** (0 2) | 25 | 36[b] |
| Fraction B1 without **4** (0 5)§+**1** (0 2)+**4** (0 5) | 25 | 72[c] |
| CA (0 01) | 16 | 0[a] |
| **1** (0 01) | 14 | 14[a] |
| **1** (0 01)+CA (0 01) | 14 | 0[a] |
| CA (0 1) | 20 | 5[a] |
| **1** (0 1) | 20 | 5[a] |
| **1** (0 1)+CA (0 1) | 22 | 40[b] |
| CA (0 5) | 18 | 0[a] |
| **1** (0 5) | 20 | 20[a] |
| **1** (0 5)+CA (0 5) | 20 | 65[b] |
| CA (1 0) | 16 | 12[a] |
| **1** (1 0) | 14 | 36[a, b] |
| **1** (1 0)+CA (1 0) | 14 | 64[b] |
| **1** (0 2) | 20 | 0[a] |
| CA (0 1) | 20 | 15[a] |
| **1** (0 2)+CA (0 1) | 22 | 77[b] |
| CA (0 5) | 25 | 24[a] |
| CA (0 5)+luteolin 7-*O*-β-D-glucoside (*ca* 0 5) | 15 | 27[a] |
| CA (0 5)+luteolin 7-*O*-β-D-glucuronide (*ca* 0 5) | 17 | 24[a] |

*Grouped assays carried out with females from same rearing batch on same day

†1 gle of ethanolic carrot extract was estimated to contain 440 μg of **1** 3 3 −3 7 mg of **4**, and *ca* 800 μg each of **2** and **3**

‡Within each bioassay group, responses denoted by different superscripts differed significantly ($p<0\,05$, $\chi^2$ test)

§B1 without **4**=collected material off HPLC recombined but without the trapped peak **4**

parison of its UV and $^1$H NMR spectra, HPLC retention times and PC and TLC $R_f$ values and colour responses with those of a *trans*-chlorogenic acid standard The GC retention time and mass fragmentation pattern of its TMSi derivative were also identical to the corresponding data from an authentic sample of *trans*-chlorogenic acid. Acid and base hydrolysis of **4** yielded quinic acid and caffeic acid, respectively, identified by GC-MS of their TMSi derivatives in comparison with those of standard compounds

Isomers of chlorogenic acid were generated by the method of Nagels *et al* [19] and were resolved and identified by HPLC (system 3, see experimental) with reference to the data of Möller and Herrmann [20] The identity of **4** as *trans*-3-caffeoyl quinic acid was confirmed by its retention time (46 0 min). Chlorogenic acid has been reported previously from the leaves of cultivated carrot [21] Crude **4** also appeared to contain smaller amounts of *trans*-4-caffeoyl quinic acid ($R_t$ = 44 4 min) Chlorogenic acid was also found to be present in fractions A and C from the C-18 open column. Its distribution over three fractions presumably resulted from shifting equilibrium between free acid and salt forms during elution in water and 1% HOAc

*Stimulant activity of purified components*

When bioassayed alone, neither **1** nor authentic *trans*-chlorogenic acid was active to butterflies Mixtures of the two compounds, however, stimulated oviposition behaviour significantly Activity increased with dose up to 0 5 gle and varied somewhat with the ratio of the two

Table 1. Contact oviposition responses of *P. polyxenes* females to standard samples of flavonoid glycosides

| Compound* | Number of females | % response† |
|---|---|---|
| Luteolin 7-glucoside (10 μg) | 20 | 30 |
| Luteolin 7-glucoside (20 μg) | 20 | 25 |
| Apigenin 7-rutinoside (30 μg) | 25 | 28 |
| Apiin (30 μg) | 21 | 38 |
| Rutin (30 μg) | 25 | 16 |
| Naringin (20 μg) | 26 | 12 |
| Hesperidin (20 μg) | 26 | 15 |
| Narirutin (20 μg) | 24 | 29 |
| Narirutin (30 μg) | 20 | 10 |

*All compounds bioassayed in combination with fraction B (0·1 gle)

†No responses differed significantly from assumed response of 12% to distilled water ($\chi^2$ test, $p < 0.05$)

components (Table 5). When bioassayed with fraction B, purified **1** gave a similar response to that of fractions D and D2 at the same dosage (Table 2). Most or all of the activity in fraction D can therefore be attributed to **1**.

Neither **2** nor **3** stimulated oviposition responses in combination with chlorogenic acid (Table 5). When authentic samples of several flavonoid glycosides, known from the Umbelliferae or Rutaceae, were bioassayed with fraction B at doses similar to those used by Nishida *et al.* [22, 23] for flavonoid stimulants of *P. xuthus* (see discussion), none was found to be active (Table 6).

While chlorogenic acid undoubtedly contributes to the activity of fractions A and B, the facts that fraction A was always active alone and that fraction B was more active than chlorogenic acid in combination with **1** (Tables 2 and 5) indicate that both A and B contain additional stimulant compounds.

## DISCUSSION

From methanolic extracts of one of its host plants, *Citrus unshiu*, Nishida and colleagues [22, 23] have identified several oviposition stimulants for the swallowtail *Papilio xuthus*. Four of the active compounds, identified as vicenin-2, hesperidin, narirutin and rutin, are flavonoids. These were only weakly active on their own or as a flavonoid mixture. They evoked responses of up to 100%, however, when mixed with either of two bases (adenosine and 5-hydroxy-*Nω*-methyltryptamine), which were likewise inactive by themselves [23]. T. Ohsugi, R. Nishida and H. Fukami (personal communication) have recently isolated four further stimulants, namely bufotenine, stachydrine, (−)-synephrine and D-*chiro*-(+)-inositol. The complete mixture accounts for the activity of *C. unshiu* extracts to *P. xuthus* females.

From the epicarp of *Citrus natsudaidai*, Honda [24] isolated two flavonoid glycosides, hesperidin and naringin, that stimulated oviposition by *Papilio protenor*, another Rutaceae-feeding swallowtail. The compounds were inactive alone, but were active when combined with a more polar subfraction of the parent extract. The butterflies responded only to flavanones tested, and did not respond to flavones or flavonols or their glycosides (including rutin). More recently, K. Honda (personal communication) has isolated several more active compounds, namely L-proline, L-stachydrine, (−)-quinic acid, (−)-synephrine and chlorogenic acid.

Carrot plants are reported to contain a variety of flavonoid glycosides, including some of those found to be stimulants for *P. xuthus* and *P. protenor* (see above). In addition to luteolin 7-glucoside (compound **3**) and luteolin 7-glucuronide (compound **2**), the leaves contain other glycosides of luteolin (7-rutinoside, 4′-glucoside) as well as glycosides of apigenin (7-glucoside, 7-rutinoside) and chrysoeriol (7-glucoside) [16]. Additional flavone glycosides reported from carrot fruits include luteolin 7-diglucoside, luteolin 4′-diglucoside, apigenin 6,8-di-*C*-glucoside (vicenin-2), and diosmetin 7-glucoside [25, 26]. There are, in addition, unconfirmed reports of apiin and luteolin galactoside in carrot leaves and of rutin in the flowers [16]. The flavonol glycosides quercetin 3-glucoside, kaempferol 3-glucoside and kaempferol 3-diglucoside have been reported from fruits or flowers [25, 27]. Though we have not eliminated the possibility that some of these non-acylated compounds are stimulants for *P. polyxenes*, our results indicate that the major flavonoid stimulant for this butterfly in carrot foliage is the 6″-*O*-malonyl ester of luteolin 7-glucoside. Non-malonylated flavonoids, such as those found to stimulate *P. xuthus* and *P. protenor*, appear to be less significant as oviposition stimulants for *P. polyxenes*.

Though flavonoid malonates have apparently not been reported previously from carrot, their presence in the leaves of parsley, *Petroselinum crispum* (= *P. hortense*), another umbellifer host plant of *P. polyxenes*, has been known since 1972 [28]. Of 24 flavone glycosides isolated from illuminated cell suspensions of parsley, half were found to be malonates [18]. Apiin, apigenin 7-glucoside and luteolin 7-glucoside were among several standard glycosides that were malonylated by malonyltransferase preparations from these cell suspensions, the site of malonylation being assigned to the 6-position of the glucose [13, 29]. Malonylation represents the final stage of biosynthesis of flavonoids in parsley and, by implication, in many other plants [30]. Because malonates are easily deacylated when plant material is processed, flavonoid malonates may be much more widespread than is currently realized.

As chlorogenic acid is widely distributed [21, 31, 32] and the distribution of flavone malonates is relatively unknown, it is not yet possible to assess the extent to which the combination of chlorogenic acid and luteolin 7-(6″-*O*-malonyl)-glucoside accounts for the preference of *P. polyxenes* females to lay eggs on plants of the Umbelliferae. Suggestive are the observations that chlorogenic acid is virtually universal in leaves of umbelliferous plants [33], that luteolin 7-glucoside is the most widespread flavone glycoside in the family [33], and that umbellifers tend to store large amounts of malonic acid [34]. Current studies (L. Rosenberry, K. Sachdev and P. Feeny, unpublished results) show that the females are stimulated by additional compounds, including at least two bases in fraction B and a sugar or inositol in fraction A. Probably, as in the cases of *P. xuthus* and *P. protenor*, it is the complete profile of compounds that provides the basis for specificity [23, 24].

The response patterns of three different *Papilio* species, two of them restricted to the Rutaceae and the other primarily to the Umbelliferae, are sufficiently similar to provide tentative support for the behavioural-facilitation

hypothesis of Dethier [2] and Jermy [3]. Though the stimulants identified so far do not belong to classes expected from earlier phytochemical surveys of host plants [1, 5, 35], they indicate an underlying conservatism in oviposition response to mixtures containing similar classes of ingredients (flavonoid glycosides, cyclitols, hydroxycinnamic acids and simple bases). Superimposed on the basic pattern, however, are apparent differences in specificity to particular compounds within the active classes. These differences may represent adaptations for more accurate recognition of the particular sets of host plants used by each species in the field.

## EXPERIMENTAL

*Insects.* Females of *P. polyxenes* were taken from our year-round laboratory culture [36]. For bioassay, females were allowed to walk up narrow strips (2.54 cm wide) of Whatman #1 filter paper to which a narrow band (1 cm) of test soln had been applied and misted with water immediately before bioassay. A positive response was recorded if the female, upon encountering the treated zone with her forelegs, curled her abdomen in preparation for oviposition [5]. The activity of each test soln was recorded as the percentage of females exhibiting a positive response. Any single female was never used for more than one test per day. On a bioassay day, females were fed individually between 8 and 9 a.m. [36] and then deprived of oviposition plants until used for experiments, always conducted between 12 noon and 2 p.m. in a controlled-environment chamber (27 ± 1°, 70–85% R.H., light intensity 20000 lux). The females were then caged with carrot plants until the following bioassay day.

In early experiments (Table 1), the responses of females to test solns were compared with those of an equal number of different females, selected randomly from the same group, that were exposed to filter paper strips treated only with dist. $H_2O$. Differences in response frequencies to experimental and control treatments were compared by a $\chi^2$ test. Since the mean positive response of females to the dist. $H_2O$ control was 12 ± 8% ($\bar{x}$ ± s.d.) in 67 trials with >2500 insects, the control treatments were omitted in later experiments (Tables 2 and 6) so that more insects could be used for experimental trials. For purposes of statistical comparisons, the response to distilled water was assumed to be 12%. In the most recent experiments (Table 5) the procedure was further modified. Each test female was first exposed to dist. $H_2O$ and those that responded positively were eliminated from further consideration. Any females that failed to respond positively to live carrot foliage immediately after a trial were also eliminated from consideration. For the remaining females, responses to a test substrate were compared with responses of other females from the same group to other substrates tested on the same day.

Voucher specimens of the insects used in this research have been deposited in the Cornell University Insect Collection (Lot No. 1023, Sublot No. 12).

*Plant material.* Foliage of carrot, *D. carota*, was taken fresh from the field in the vicinity of Ithaca, NY, and transported to the laboratory in coolers containing crushed ice. Leaves, 50 g fr. wt at a time, were immediately blended in boiling 95% EtOH (500 ml) for 5–10 min and the extract filtered. After removal of the EtOH by evapn *in vacuo* at <40°, the resulting aq. suspension was centrifuged and then extracted sequentially (each 3×) with equal vols of $Et_2O$, $CHCl_3$, EtOAc and *n*-BuOH. The *n*-BuOH fraction was washed once with water and this backwash was mixed with the aq. fraction, henceforth referred to as the post-BuOH aq. fraction.

Voucher specimens of *D. carota*, as used in this research, have been deposited in the L. H. Bailey Hortorium, Cornell University.

*Dose-responses to initial extracts.* For bioassay, aliquots of the parent ethanolic carrot extract, of the post-BuOH aq. fraction, and of the recombined organic phases were adjusted in volume by rotary evaporation *in vacuo* or by serial dilution to a range of concs expressed as g leaf equivalents/ml (1 gle = amount of material extracted from 1 g fr. wt of carrot leaves).

*Preparative fractionation of post-*BuOH *aq. fraction.* This was carried out on an open column (2.5 cm i.d.), packed with 67.5 g ODS (Bonded Phase, Baker; 40 μ particle size). For each run, 5 ml (100 gle) of post-BuOH aq. fraction was applied to the column and eluted successively with 400 ml $H_2O$ (fraction A), 1000 ml 1% HOAc in $H_2O$ (fraction B), 1000 ml 20% MeOH in 1% aq. HOAc (fraction C), 1200 ml 40% MeOH in 1% aq. HOAc (fraction D), and 500 ml pure MeOH (fraction E). Fractions were concd *in vacuo* at <40° and adjusted with $H_2O$ to the initial conc of 20 gle/ml. *Acid extraction of the post-*BuOH *aq. fraction.* Post-BuOH extract (500 gle in 50 ml $H_2O$) was acidified with 2 M HCl to pH 2.5 and extracted with EtOAc (3 × 100 ml). The EtOAc fraction was backwashed with $H_2O$ (100 ml), concd *in vacuo* and taken up into $H_2O$ (50 ml).

*HPLC.* System 1: Fractionations were carried out on a Waters isocratic system (M6000 pump, U6K injector), monitored at 254 nm (Waters Model 440 dual-channel absorbance detector). The semi-prep. column (IBM 10 × 250 mm 5μ C-18) was eluted at a flow rate of 3.0 ml/min using a stepped gradient: 2% HOAc (initial), 2% HOAc–MeOH (80:20, 15 min), 2% HOAc–MeOH (60:40, 15 min), 2% HOAc–MeOH (40:60, 10 min), 2% HOAc–MeOH (20:80, 10 min), to MeOH (20 min). System 2: Fractionations were performed on a Waters 600 Multisolvent Delivery System equipped with a Waters model U6K LC injector, a Waters 490 Programmable Multiwavelength Detector and a Waters 730 Data Module. Elution from the reverse-phase C-18 column (Phenomenex IB-sil, 10 × 250 mm, 5μ), monitored at 254 and 320 nm, was carried out at a flow rate of 3 ml/min according to the following programme:

| Time (min) | 1% HOAc | MeOH | Curve |
|---|---|---|---|
| 0 | 100 | 0 | * |
| 15 | 80 | 20 | 6 |
| 40 | 65 | 35 | 6 |
| 90 | 55 | 45 | 9 |
| 100 | 55 | 45 | 6 |
| 110 | 0 | 100 | 6 |

Gradient curve shapes '6' (linear) and '9' (concave) are described in the manual for the Waters Multisolvent Delivery System (Millipore Corp., Milford, MA). System 3: Using the same instrument and column as in system 2, elution in a gradient of aq. HOAc–MeOH at 3.0 ml/min followed the programme of Möller and Herrmann [20] except that 1% HOAc was used instead of 2% HOAc.

*TLC.* TLC was carried out on HPTLC cellulose plates (10 × 10 cm, Merck) using the following solvent systems: (A) HOAc (5% aq.), (B) $H_2O$, (C) *n*-BuOH–HOAc–$H_2O$ (4:1:2.2), (D) *n*-PrOH-1 M $NH_4OH$ (7:3). Plates were examined under UV light (254 and 360 nm), before and after fuming with $NH_3$.

*PC.* Two-dimensional PC was conducted on Whatman No. 1 sheets (25.4 × 25.4 cm.) in an 'Universal Apparatus' (Shandon Scientific Co., London) [37]. Chromatograms were developed ascendingly at room temp. (18–21°) first in TBA (3:1:1) and then in 15% HOAc [7]. They were examined under UV light (365 nm), before and after fuming with $NH_3$.

*Spectral techniques* TMSi derivatives were separated by GC on a Perkin Elmer Model 3920 GC, equipped with a FID detector and a glass column of 3% OV-101 (180 × 0.6 cm O D and 0 2 cm I D ) supported on Chromosorb W-HP (mesh range 100–120) Injector and detector temps = 280°, $N_2$ flow rate = 40 ml/min The programme was (1) 100° for 4 min, (2) 100–305° at 8°/min, (3) hold for 20 min EI mass spectra of TMSi derivatives Finnigan Model 3300 instrument (70 eV), equipped with a wide-bore capillary column (Supelco) coated with SPB-1 FAB/MS 50 (Kratos Ltd) sample dissolved in glycerol/thioglycerol on a copper probe tip and inserted into the source at $10^{-5}$ torr pressure The sample was bombarded with Xe atoms at 8 kV energy

*Compound* **1** From repeated HPLC (system 2) of aq post-BuOH extract, the peak at $R_t$ = 96.5 min was collected and MeOH blown off with $N_2$ After adjusting the pH to 3 with 2 M HCl, the compound was extracted into EtOAc The fraction was concd, dissolved in $H_2O$ and the compound allowed to ppt. overnight in the refrigerator The ppt thus obtained was further purified by crystallization from MeOH It yielded fine yellow needles, mp 228°, HPLC $R_t$ = 61.5 (system 1), 96 5 (system 2) TLC Yellow (vis, UV), bright yellow (UV + $NH_3$), $R_f$ = 0 08 (system A), 0 70, variable (system B), 0 63 (system C) PC $R_f$ = 0.39/0 22 (TBA/HOAc) IR $\nu_{max}^{MeOH}$ $cm^{-1}$ 3259 (OH), 1732 and 1728 (ester and acid), 1658 (4-CO), UV $\lambda_{max}^{MeOH}$ nm (log $\varepsilon$). 350 (4 03), 267sh (3 99), 256 (4 04), + $AlCl_3$ 415 (4 18), 296sh (3 72), 272 (4 09), + $AlCl_3$–HCl 388 (4 07), 360 (4.01), 296sh (3 81), 273 (4 06), + NaOAc: 410 (3 68), 354 (4 00), 266sh (4 01), 257 (4 04), + NaOAc–$H_3BO_3$ 373 (4 13), 259 (4 16), + NaOMe 404 (4 19), 261 (4.07), NMR see Tables 3 and 4, FAB-MS (positive ion, thioglycerol) *m/z* 535 $[M+H]^+$, 448 $[M+H-COCH_2COOH]^+$, 257 [luteolin + H]$^+$, FAB-MS (negative ion, diethanolamine) *m/z* 533 $[M-H]^-$, 489 $[M-H-CO_2]^-$, 447 $[M-COCH_2COOH]^-$, 285 [luteolin − H]$^-$

Direct concentration of the HPLC fraction without acidification and solvent extraction yielded **1** in its salt form, presumably due to cationic impurities in the $H_2O$

For alkaline hydrolysis, **1** (2 9 mg) was dissolved 0 2 M NaOH (300 $\mu$l) and stirred for 45 min under an argon atmosphere It was then acidified to pH 3 0 with 1 M HCl and extracted with $Et_2O$ (4 × 300 $\mu$l) followed by EtOAc (4 × 300 $\mu$l) *Identification of the acid*: A portion of the $Et_2O$ layer (150 $\mu$g) was heated with Sil-Prep (100 $\mu$l, Alltech Associates) at 70–80° for 20 min GC of the derivative was conducted using the following programme. initial temp 60° for 4 min, then increasing at 8°/min to 250° The single intense peak had a $R_t$ of 11 7 min, identical to that of the TMSi derivative of a standard sample of malonic acid The acid was also compared with the standard compound by co-TLC on cellulose, using solvent system D ($R_f$ = 0 27)

*Identification of deacylated* **1** Purification of the deacylated material was conducted by HPLC using system **2**. The major peak at $R_t$ = 67 min was identified as luteolin 7-*O*-$\beta$-D-glucoside (UV, MS, co-TLC, HPLC)

*Compound* **2** From repeated HPLC (system 2) of aq post-BuOH extract, the peak at $R_t$ = 61 0 min was collected and MeOH blown off with $N_2$ After adjusting the pH to 3 with 2 M HCl, the compound was extracted into EtOAc This fraction contained some impurities that were removed by pptn of the compound from $H_2O$ HPLC $R_t$ = 54.5 (system 1), 61 0 (system 2) TLC: Yellow (vis, UV), bright yellow (UV + $NH_3$), $R_f$ = 0 08 (system A), 0 50, variable (system B), 0 50 (system C) Its spectral data (UV, IR) and enzyme hydrolysis with $\beta$-glucuronidase containing phosphate buffer (Sigma Type VII Source *E coli*) revealed it to be luteolin 7-*O*-$\beta$-D-glucuronide [15, 38] $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$2 4–3.57 (*m*, H-2″, H-3″, H-4″, H-5″), 5 05 (*d*, *J* = 6 7 Hz, H-1″), 6 40 (*d*, *J* = 3 9 Hz, H-6), 6 74 (*s*, H-3), 6 76 (*d*, *J* = 3 9 Hz, H-8), 6 88 (*d*, *J* = 9 3 Hz, H-5′), 7 41 (*d*, *J* = 3 7 Hz, H-2′) and 7 43 (*dd*, *J* = 3.7 and 9 3 Hz, H-6′) This identification was further confirmed by comparison with the standard compound FABMS (positive ion, thioglycerol), *m/z* 485 $[M+Na]^+$, 463 $[M+H]^+$, 419 $[M+H-CO_2]^+$, 287 [luteolin + H]$^+$, FABMS (negative ion, diethanolamine), *m/z* 483 $[M-2H+Na]^-$, 461 $[M-H]^-$, 285 [luteolin − H]$^-$.

*Compound* **3** HPLC $R_t$ = 55 5 min (system 1), 67.0 min (system 2) **3** was pptd from EtOAc–MeOH It was identified as luteolin 7-*O*-$\beta$-D-glucoside by TLC, PC and HPLC in comparison with an authentic sample HPLC $R_t$ = 55 5 (system 1), 67 0 (system 2) TLC Yellow (vis, UV), bright yellow (UV + $NH_3$) $R_f$ = 0 02 (system A), 0 01 (system B), 0 58 (system C) PC $R_f$ = 0 38/0 13 (TBA/HOAc)

*Compound* **4** The HPLC peak at $R_t$ = 32 0 min (system 2) was trapped directly from injections of fraction B1 or of the parent post-BuOH aq fraction The major component, purified by further HPLC (system 3), yielded colourless crystals upon concentration followed by crystallization from MeOH It was identified as *trans*-chlorogenic acid (*trans*-3-*O*-caffeoylquinic acid) on the basis of its UV and $^1$H NMR spectrum [39] and also by TLC and HPLC comparison with the authentic sample UV $\lambda_{max}^{MeOH/H_2O}$ nm 243, 299 (*s*), 328 MS (TMSi), *m/z* 787 $[M+H]^+$. HPLC $R_t$ = 38 0 min (system 1), 32 0 min (system 2), 46 0 min (system 3) TLC Blue (UV), bright turquoise (UV + $NH_3$), $R_f$ = 0 59 (system A, 10% HOAc), 1 0 (system B), 0 72 (system C) PC $R_f$ = 0 64/0 77 (TBA/HOAc) Acid hydrolysis the compound was refluxed with 2 M HCl at 100° for 2 hr and the hydrolysate extracted with $Et_2O$–EtOAc (1 1) The aq fraction was concd *in vacuo* at 50° to remove HCl The residue, transferred to a reacti-vial, was dried under $N_2$ and reacted with MSTFA (Pierce Chemical Co ) at 110° for 1 hr The GC retention time of the major peak corresponded to that of the TMSi derivative of a quinic acid standard, as did its MS spectrum Quinic acid, TMSi, GC/MS, 70 eV, *m/z* 537 $[M-15]^+$, *Base Hydrolysis* The compound was hydrolysed with 4 M NaOH under $N_2$ for 4 hr at room temp [40] The hydrolysate, adjusted to pH 1 with 4 M HCl, was extracted with $Et_2O$–EtOAc (1 1) Solvent was removed from the organic layer under $N_2$ and the aglycone dissolved in MeOH The presence of caffeic acid was confirmed by co-TLC (system 3, silica gel plates, $R_f$ = 0 76) and by GC–GC/MS of the TMSi derivative alongside the TMSi derivative of a standard sample Caffeic acid, TMSi, GC/MS 70 eV, *m/z* 396 $[M]^+$

For comparison with minor components, *trans*-4-caffeoyl- and *trans*-5-caffeoylquinic acids were generated by treating *trans*-chlorogenic acid with a satd soln of $NaHCO_3$ at 90° for 30 min [19] The soln was then adjusted to pH 1 5 with 1 M $H_2SO_4$, and extracted with EtOAc. The organic phase was concd to dryness *in vacuo* and the residue dissolved in MeOH–$H_2O$

*Quantitative estimation* Carrot leaves (5 g fr wt, 2 replicates) were extracted in 95% EtOH. The soln was concentrated *in vacuo* and extracted with $Et_2O$, $CHCl_3$, EtOAc and *n*-BuOH HPLC (System 2) was used to estimate **1** and **4** in the post-BuOH aqueous fraction, comparing peak heights with those from standard solutions (**4**) or purified material (**1**) Approximate estimates of **2** and **3** in the EtOAc, *n*-BuOH and post-BuOH aqueous fractions were obtained by similar means

*Sources of standard compounds* Chlorogenic acid, D(−)-quinic acid, rutin, naringin and hesperidin (Sigma), caffeic acid (Aldrich), luteolin 7-*O*-$\beta$-D-glucoside, luteolin, apiin and narirutin (Roth) Luteolin 7-*O*-glucuronide and apigenin 7-*O*-rutinoside were gifts from Prof. Dr Karl Herrmann

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