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EXTERNAL FLAVONES IN SWEET BASIL, *OCIMUM BASILICUM*, AND RELATED TAXA

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Key Word Index—*Ocimum basilicum*; *O.* × *citriodorum*; *O. minimum*; Lamiaceae; basil; surface flavonoid composition; nevadensin; salvigenin; chemotaxonomy.

Abstract—Sixteen accessions of *Ocimum basilicum* L. belonging to different cultivars and varieties, and one accession each of *O.* × *citriodorum* Vis. and *O. minimum* L., were examined for their external leaf flavonoids. The *O. basilicum* samples showed the presence of one or two major flavone aglycones, which were identified as salvigenin and nevadensin, and up to 10 minor ones: cirsileol, cirsilineol, eupatorin, apigenin, acacetin, genkwanin, apigenin 7,4′-dimethyl ether, cirsimaritin, ladanein and gardenin B. No trace was found of xanthomicrol, the only lipophilic flavone so far reported from *O. basilicum*. There were appreciable differences amongst the accessions in total flavonoid concentration and in their nevadensin / salvigenin ratios, which, on the whole, were not correlated with morphological characters. The surface flavonoid profile of *O.* × *citriodorum* was very similar to those of the *O. basilicum* plants studied, but that of *O. minimum* was significantly different. Only 4′-methyl ethers of apigenin derivatives were detected in the latter taxon, whereas apigenin and luteolin derivatives in which the 4′-hydroxyls are free were absent. Furthermore, the ratio of nevadensin / salvigenin was much higher in *O. minimum* than in *O. basilicum* or *O.* × *citriodorum*. These differences support the treatment of *O. minimum* as a separate species rather than as a variety of *O. basilicum*.

INTRODUCTION

A characteristic chemical feature of many species belonging to the plant family Lamiaceae is the secretion of lipophilic flavonoid aglycones on the leaf surface, e.g. in the genera *Teucrium, Sideritis, Scutellaria, Mentha, Salvia* and *Thymus* [1, 2]. These external or surface flavonoids are especially common in species which grow in the wild in arid and semi-arid regions, presumably to give the plant protection against harmful UV radiation [3].

Additionally, lipophilic flavonoids may protect plants against infection by microorganisms, as several have been shown to have antibacterial [4] or antifungal activities [5, 6]. So far only a few surface flavonoid studies have been carried out on the genus *Ocimum* L. (basil), e.g. the flavones nevadensin, salvigenin and hispidulin have been reported from *O. canum* Sims (= *O. americanum* L.) [1, 7] and xanthomicrol from *O. basilicum* L. [8]. The latter species has been used for a long time as a medicinal plant, culinary herb and insect-controlling agent and is an important essential oil crop in some tropical and subtropical countries, e.g. India. It is very variable in morphology, chromosome numbers [9] and essential oil chemistry because of centuries of selection and crossing for desirable smell and taste characteristics, and many different essential oil chemotypes have been described [10–13]. During a chemotaxonomic survey of the genus *Ocimum*, we also found that essential oil compositions in *O. basilicum* are extremely variable. Even varieties were not homogeneous in this respect. For instance, five different essential oil chemotypes were found within *O. basilicum* var. *basilicum*, and three different ones within var. *purpurascens* Benth. [14]. In order to establish whether surface flavonoid profiles in this group of plants are equally variable or more characteristic of the species or varieties, we examined the same accessions of *O. basilicum* for their external flavonoids. Additionally, one accession each of *O.* x *citriodorum* Vis. and *O. minimum* L. was studied. *O.* x *citriodorum* is thought to be a hybrid between *O. basilicum* and the related *O. americanum* [9]. *Ocimum minimum* is considered by some to be a variety of *O. basilicum*, whereas others treat it as separate species because there are clear morphological differences between the two taxa and no viable seed is produced on

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crossing *O. minimum* with *O. basilicum* [9]. Extracts of all accessions were investigated for surface flavonoids by means of HPLC with photodiode array detection.

RESULTS AND DISCUSSION

Flavonoids present in dichloromethane extracts prepared from fresh leaves of *O. basilicum* plants were truly external because the leaves had been washed by the solvent for only 20 sec and the extracts thus obtained were still colourless, i.e. no chlorophyll and hence presumably no internal constituents were extracted in this short time. HPLC with photodiode array detection of the dichloromethane extracts revealed the presence of one or two major and some 10 minor peaks in most samples which showed flavone-type UV spectra [15]. The two major constituents, **1** and **2**, were isolated from bulk extracts of accessions NY B 145 (var. *basilicum*) and NY B 203 (var. *difforme* Benth.) by means of preparative TLC and column chromatography. Comparison of the UV absorption spectra of **1** and **2** with those of a range of flavones found in Lamiaceae [16] suggested that **1** was an apigenin derivative methoxylated in the 6- and 8-positions, and **2** was a derivative of scutellarein 6-methyl ether. As to compound **1**, two 6,8-methoxylated flavones have been reported so far from *Ocimum* species, xanthomicrol (5,4'-dihydroxy-6,7,8-trimethoxyflavone) from *O. basilicum* [8] and nevadensin (5,7-dihydroxy-6,8,4'-trimethoxyflavone) from *O. americanum* [7]. Chromatographic (TLC and HPLC) comparison of **1** with authentic nevadensin and xanthomicrol standards showed that **1** is the former compound. The UV spectrophotometric data after addition of the shift reagent NaOH [15] confirmed that **1** is nevadensin rather than xanthomicrol (band III at 301 nm, indicating that 7-hydroxyl is free; decrease of band I, indicating that 4'-hydroxyl is substituted). In addition, analysis of the ^{1}H NMR spectrum of **1** supported its identification as nevadensin, as the chemical shift values matched those given previously for this compound [7], with the exception of the 5-OH resonance at δ 12.77, which was presumed to be incorrect in the earlier report. In a similar fashion **2** was identified authoritatively by means of ^{1}H NMR and UV spectroscopic and chromatographic comparison with an authentic standard as salvigenin (5-hydroxy-6,7,4'-trimethoxyflavone), a compound also reported from *O. americanum* [7]. The remaining flavones **3–12** were not obtained pure, but were all identified in purified fractions by means of comparative HPLC (retention times and photodiode array UV spectra, see Table 1) and sometimes also by comparative TLC with a range of authentic samples as **3**: cirsileol (5,3',4'-trihydroxy-6,7-dimethoxyflavone); **4**: cirsilineol (5,4'-dihydroxy-6,7,3'-trimethoxyflavone); **5**: eupatorin (5,3'-dihydroxy-6,7,4'-trimethoxyflavone); **6**: apigenin (5,7,4'-trihydroxyflavone); **7**: acacetin (5,7-dihydroxy-4'-methoxyflavone); **8**: genkwanin (5,4'-dihydroxy-7-methoxyflavone); **9**: apigenin 7,4'-dimethyl ether (5-hydroxy-7,4'-dihydroxyflavone); **10**: cirsimaritin (5,4'-dihydroxy-6,7-dimethoxyflavone); **11**: ladanein (5,6-dihydroxy-7,4'-dimethoxyflavone); and **12**: gardenin B (5-hydroxy-6,7,8,4'-tetramethoxyflavone). No trace of xanthomicrol was found in any of the accessions examined, not even as a minor component, despite the report of its occurrence in *O. basilicum* [8].

Although there were only few qualitative differences in the flavone profiles among the different accessions of *O. basilicum* (e.g. some of the minor constituents could not be detected in all samples), there were appreciable quantitative differences among them. For instance, the ratio between **1** and **2** varied considerably and the total external flavonoid concentration seemed much higher in

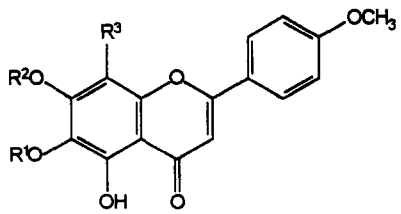
Table 1. Chromatographic and UV spectral properties of *Ocimum* flavones and their identification

Flavone	TLC* R_f(×100)	HPLC* R_t(min)	UV spectral maxima (nm)†	Identification
1	48	19.8	282, 295sh, 333	Nevadensin
2	67	21.6	276, 333	Salvigenin
3	—‡	15.8	255, 273, 348	Cirsileol
4	—‡	17.8	252, 274, 346	Cirsilineol
5	45	17.9	253, 274, 344	Eupatorin
6	15	16.3	267, 295sh, 339	Apigenin
7	36	20.5	268, 297sh, 333	Acacetin
8	30	20.7	267, 338	Genkwanin
9	69	24.8	269, 331	Apigenin 7,4'-diMe
10	30	17.7	275, 336	Cirsimaritin
11	—‡	18.7	286, 335	Ladanein
12	67	23.0	283sh, 292, 331	Gardenin B
xanthomicrol standard	34	19.1	282, 295sh, 333	—

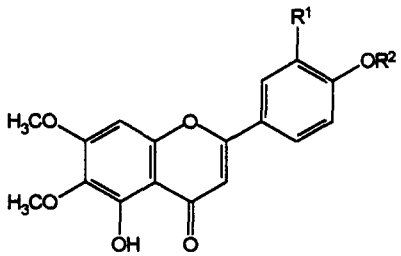
* For details of TLC and HPLC solvents: see Experimental.

† The UV maxima in this column are those recorded by the photodiode array detector in the slightly acidic HPLC solvent used. UV maxima of the pure compounds in a neutral methanol solution may be different.

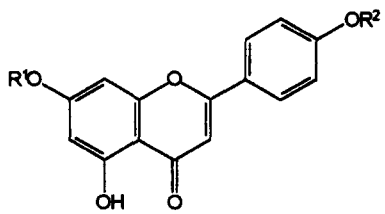
‡ The amounts of **3**, **4** and **11** isolated were too low for TLC detection. The R_f values of the cirsileol, cirsilineol and ladanein standards were 16, 44 and 43, respectively.



1 $R^1 = CH_3$, $R^2 = H$, $R^3 = OCH_3$
12 $R^1 = R^2 = CH_3$, $R^3 = OCH_3$
11 $R^1 = R^3 = H$, $R^2 = CH_3$



2 $R^1 = H$, $R^2 = CH_3$
3 $R^1 = OH$, $R^2 = H$
4 $R^1 = OCH_3$, $R^2 = H$
5 $R^1 = OH$, $R^2 = CH_3$
10 $R^1 = R^2 = H$



6 $R^1 = R^2 = H$
7 $R^1 = H$, $R^2 = CH_3$
8 $R^1 = CH_3$, $R^2 = H$
9 $R^1 = R^2 = CH_3$

Scheme 1. **1**$R^1 = CH_3$, $R^2 = H$, $R^3 = OCH_3$, **12**$R^1 = R^2 = CH_3$, $R^3 = OCH_3$, **11**$R^1 = R^3 = H$, $R^2 = CH_3$, **2**$R^1 = H$, $R^2 = CH_3$, **3**$R^1 = OH$, $R^2 = H$, **4**$R^1 = OCH_3$, $R^2 = H$, **5**$R^1 = OH$, $R^2 = CH_3$, **10**$R^1 = R^2 = H$, **6**$R^1 = R^2 = H$, **7**$R^1 = H$, $R^2 = CH_3$, **8**$R^1 = CH_3$, $R^2 = H$, **9**$R^1 = R^2 = CH_3$.

some plant samples than in others (higher absorbance of the peaks in the HPLC traces). In order to see whether these quantitative differences had been caused by the short and non-exhaustive extraction method or whether on the contrary they reflected genuine chemical differences between the accessions, weighed freeze-dried leaves were exhaustively extracted with diethyl ether. HPLC analysis of these extracts gave very similar results to those obtained from the dichloromethane washings of fresh leaves. The results for the diethyl ether extracts, i.e. total flavone concentration, **1/2** ratios and flavone compositions for each accession, are presented in Table 2. In this table, total flavone concentration is expressed as the sum of the peak areas measured at 335 nm of all HPLC flavone peaks after a 40 μl injection (corresponding to 40 mg of dried leaves). The **1/2** ratios and the proportions of each of the compounds **1–12** in this total were also calculated from HPLC peak areas measured at 335 nm. This gives a reasonable representation of the flavonoid composition, although one which is not completely accurate because not all the flavones have the same extinction coefficient at 335 nm for a given concentration. The three luteolin derivatives (**3–5**) may be slightly underrepresented in relation to the apigenin derivatives, because their maximum absorbance is not at 335 nm, but between 344 and 348 nm. Compounds **4** and **5** did not separate during HPLC runs of crude extracts, so their combined value is given in Table 2. However, they did give separate peaks when a purified TLC fraction of the NY B 145 extract was analysed by HPLC.

The results in Table 2 show that the total flavone concentration varied considerably within *O. basilicum* (more than by a factor of 20; from 2.54 units in accession NY B 100Y to 59.53 in NY B 145), but even within some of the varieties there was a strong variation, especially in var. *basilicum*. The **1/2** ratio was equally variable, ranging from 0.09 to 1.89 within the whole species, from 0.09 to 1.16 in var. *basilicum* and from 0.10 to 0.84 in var. *purpurascens*. On the other hand, the three convex-leaved accessions of var. *basilicum* showed quite similar flavone patterns, both qualitatively and quantitatively. The same applied to the two accessions studied for cv. Dark Opal. In this respect it is interesting that the essential oil profiles and chromosome numbers give the same picture (see Table 3): much variation within var. *basilicum* (flat leaves) and *purpurascens* and little difference among the accessions of var. *basilicum* (convex leaves) and cv. Dark Opal. However, in two cases, accessions within var. *basilicum* (flat leaves) also showed strong similarities in chemical profiles. These are accessions NY B 122 and 130W (low in total flavones, very low **1/2** ratio, high concentrations of **12**, absence of **11** and same essential oil chemotype) and accessions NY B 145 and 147/97 (very high total flavone, high **1/2** ratio, same order of percentages for most other flavones, and an essential oil profile in which phenylpropanoids dominate; additionally a high chromosome number, see Table 3). There are also similarities among accessions belonging to different taxa. For instance, accession NY B 130P, which looks like an intermediate between var. *basilicum* and var. *purpurascens*, is chemically very similar to NY B 130W and 122 which both belong to var. *basilicum*, whereas accession NY B 197 (*O.* × *citriodorum*) shows chemical similarities to NY B 145 and 147/97 of var. *basilicum*.

Table 2. External flavone composition (%) of freeze-dried *Ocimum* leaves

Taxon and accession	Total flavone*	Ratio 1/2	Flavone composition (%) (HPLC peak area at 335nm in relation to total peak area)										
			1	**2**	**3**	**4 and 5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
O. basilicum var. *basilicum* (flat leaves)													
NY B 17	10.26	1.16	44.5	38.4	1.4	3.1	0.9	1.3	1.6	3.8	3.2	1.8	t
NY B 122	3.21	0.24	14.6	61.6	t	4.4	-	t	t	4.8	3.7	-	10.9
NY B 130W	3.15	0.09	6.4	70.1	1.9	-	3.8	1.9	-	2.5	7.0	-	6.4
NY B 145	59.53	1.02	39.4	38.8	1.2	3.8	-	1.3	1.1	1.8	4.4	2.9	3.3
NY B 147/97	46.58	0.92	37.0	40.1	1.4	4.9	1.0	1.2	1.4	2.2	4.4	2.9	3.3
NY B SW	4.50	0.62	28.9	46.7	t	5.8	-	2.8	2.8	2.8	3.0	4.3	2.8
O. basilicum var. *basilicum* (convex leaves)													
NY B R-1	43.81	0.38	24.4	64.1	0.9	2.7	0.8	0.9	1.2	0.9	3.2	-	0.8
NY B R-3	30.61	0.56	30.2	53.8	0.9	2.1	2.5	0.7	2.0	1.6	5.9	-	0.2
NY B US	13.83	0.49	26.9	55.0	2.5	2.7	-	0.9	1.7	3.8	5.9	-	0.5
Intermediate var. *bas.*/ var. *purp.*													
NY B 130P	5.20	0.14	9.6	66.3	0.8	2.5	1.0	1.4	-	5.5	4.9	-	8.0
O. basilicum var. *purpurascens*													
NY B 74	8.00	0.10	5.6	54.0	t	5.5	1.4	1.9	2.6	4.7	3.8	4.7	15.9
NY B 76	4.70	0.84	32.7	38.8	6.5	6.5	t	t	t	2.6	3.5	9.3	t
NY B 147/13	20.99	0.43	26.4	60.7	1.3	3.6	-	0.8	0.9	3.3	1.7	-	7.3
O. basilicum var. *difforme*													
NY B 203	12.70	1.32	48.0	36.4	1.4	0.9	1.3	1.1	1.4	2.8	1.8	3.6	1.3
O. basilicum cv. *Dark Opal*													
NY B 100R	4.20	1.89	53.0	28.0	-	5.5	-	1.7	2.4	6.5	2.9	-	-
NY B 100Y	2.54	1.40	51.7	37.0	-	5.0	-	t	t	2.1	4.2	-	-
O. × *citriodorum*													
NY B 197	52.17	0.92	38.7	42.0	-	3.3	-	0.5	1.6	3.7	3.4	3.2	3.6
O. minimum													
NY B 209	8.90	2.89	58.4	20.2	-	-	-	11.2	-	6.7	-	1.1	2.2

* Total flavone HPLC peak area (Vsec) at 335 nm detection for a 40 μl injection.
t = traces.

Table 3. Details of *Ocimum* accessions used for chemical studies

Species and variety	Accession	Origin	Chromosome no. (2*n*) [9]	Essential oil chemotype [14]
O. basilicum var. *basilicum*	NY B 17	Italy	56	Methyl chavicol/linalool
(flat leaves)	NY B 122	Holland	56	Linalool
	NY B 130W	India	52	Linalool
	NY B 145	Yemen	72	Methyl chavicol
	NY B 147/97	U.S.A.	74	Methyl chavicol/ methyl eugenol
	NY B SW	Brazil	52	Linalool/eugenol
O. basilicum var. *basilicum*	NY B R-1	U.S.A.	52	Methyl chavicol/linalool
(convex leaves)	NY B R-3	U.S.A.	52	Methyl chavicol/linalool
	NY B US	U.S.A.	52	Methyl chavicol/linalool
O. basilicum var. *purpurascens*	NY B 74	Thailand	52	Methyl chavicol
	NY B 76	Israel	52	Linalool/eugenol
	NY B 147/13	Unknown	53	Methyl chavicol/linalool/geraniol
basilic./purpur. intermediate	NY B 130P	India	52	Linalool
O. basilicum var. *difforme*	NY B 203	U.K.	56	Methyl chavicol/linalool
O. basilicum cv. Dark Opal	NY B 100R	U.K.	52	Linalool/eugenol
	NY B 100Y	U.K.	52	Linalool/eugenol
O. × *citriodorum*	NY B 197	Thailand	64	Geranial/neral
O. minimum	NY B 209	Turkey	76	Not determined

Furthermore, NY B 17 of var. *basilicum* has chemical characteristics in common with NY B 203 of var. *difforme* (medium total flavone, high **1/2** ratio, similar percentages for most other flavones, same essential oil chemotype, and the same chromosome number 2n = 56). All the chemical and cytological data indicate that varieties *basilicum* and *purpurascens* are unlikely to be monophyletic taxa and that chemical characters together with chromosome counts and characters from other disciplines should be used in a revaluation of the relationships within *O. basilicum*. Relationships with *O.* x *citriodorum*, the flavonoid profile of which fell within the range of those encountered in *O. basilicum* accessions, should be reassessed in the same way.

From both a biochemical and chemotaxonomic point of view, the flavone substitution patterns in *Ocimum* are interesting and informative. In common with most external flavones in the Lamiaceae the 5-position of the *Ocimum* flavones was always found to be hydroxylated; 5-methoxylated flavones in this family seem to be restricted to the genus *Orthosiphon* [1]. However, there appears to be a substantial variation of substitution patterns at positions 6, 7 and 8 of the flavone A-ring in *O. basilicum*: 7-hydroxyl (in **6** and **7**), 7-methoxyl (**8** and **9**), 6-hydroxyl and 7-methoxyl (**11**), 6- and 7-methoxyl (**2–5** and **10**), 6-methoxyl, 7-hydroxyl and 8-methoxyl (**1**) and 6-, 7- and 8-methoxyl (**12**). Additionally, five different B-ring substitution patterns were encountered: 3′,4′-dihydroxyl (**3**), 3′-hydroxyl and 4′-methoxyl (**5**), 4′-hydroxyl and 3′-methoxyl (**4**), 4′-hydroxyl (**6**, **8** and **10**) and 4′-methoxyl (**1**, **2**, **7**, **9**, **11** and **12**). Thus, a variety of different enzymes appear to be operating in *O. basilicum* involved in various different biogenetic pathways. In this respect it is interesting that in *O. minimum* only one B-ring substitution pattern was found (4′-methoxyl), whereas the A-ring substitution patterns were the same as in *O. basilicum*. The apigenin derivatives hydroxylated in the 4′-position (**6**, **8** and **10**) and all luteolin derivatives (**3**, **4** and **5**) were absent from the accession investigated for *O. minimum*. Thus only compounds **1**, **2**, **7**, **9**, **11** and **12** were present in this species. It is conceivable that the difference in flavonoid profile with *O. basilicum* is caused by environmental factors, as the plant material used for the extraction of *O. minimum* was grown in a greenhouse in the winter and the plant had not received any UV radiation from the sun like the other accessions. This point merits further investigation. However, aside from environmental factors, the differences in flavonoid profiles noted here indicate that *O. minimum* lacks the enzymes necessary for the biosynthesis of the luteolin and 4′-hydroxylated apigenin derivatives present in *O. basilicum* (genes coding for these enzymes may be absent or not switched on) and that it is biochemically distinct from the latter taxon. The much higher nevadensin / salvigenin ratio found in *O. minimum* further substantiates this premise. *O. minimum* has been treated as a variety of *O. basilicum* by some taxonomists, but other authors treat it as a separate species because there are clear morphological differences between the two taxa and no viable seed is produced on crossing *O. minimum* with *O. basilicum* [9]. The present flavonoid results support the treatment of *O. minimum* as a separate species.

EXPERIMENTAL

Plant material. Plants were raised from seeds and grown in pots in a heated greenhouse. All plants apart from NY B 209 (*O. minimum*) were allowed to grow outside for 1 month in the summer before being collected for chemical analysis or freeze-drying. Voucher specimens have been deposited at the Kew Herbarium. The species and variety of each plant, accession number, origin, chromosome number and essential oil profile of each accession are given in Table 3.

General. NMR spectra were recorded at 270 MHz for ^1H in $CDCl_3$ at 30°.

Extraction and isolation. Leaf washes of each cv. were prepared by rinsing 5 g fresh leaves in 10 ml $CH_2Cl_2$for 20 sec. Each extract (4 ml) was evap. to dryness and the residue dissolved in 1.0 ml 80% aq. MeOH. A 40 μl portion of this extract was injected for HPLC analysis (see below). For quantitative analysis of surface flavonoids, 0.5 g ground freeze-dried leaves were extracted ×2 at room temp. for 24 hr with Et_2O (10 and 5 ml, respectively). The combined Et_2O extracts were evap. and the residue dissolved in 0.5 ml 80% MeOH. A 40 μl portion of this extract was used for HPLC analysis. Bulk extracts were prep. from 7.8 g ground dried material of NY B 145 and from 26 g fresh leaves of NY B 203 for isolation of flavones. This isolation was carried out by prep. TLC (silica gel; solvent: 4 or 5% MeOH in $CHCl_3$) and further purification by CC over Sephadex LH-20.

Nevadensin (5,7 - *Dihydroxy* - 6,8,4′ - *trimethoxy* - *flavone*) (**1**). UV λ_{max}^{MeoH} nm: 283, 333; (+ NaOH) 283, 301sh, 370 (decreased intensity of band I); (+ NaOAc) 283, 302sh, 377. ^1H NMR ($CDCl_3$): δ 12.77 (1H, *s*, OH-5), 7.88 (2H, *d*, J = 8.8 Hz, H-2′,6′), 7.03 (2H, *d*, J = 8.8 Hz, H-3′,5′), 6.57 (1H, *s*, H-3), 4.04, 4.01, 3.89 (3 x 3H, *s*, OMe-6,8,4′). TLC and HPLC: see Table 1.

Salvigenin (5-*Hydroxy*-6,7,4′-*trimethoxyflavone*) (**2**). UV: λ_{max}^{MeoH} nm: 276, 329; (+ NaOH) 295, 368 (decreased intensity of band I); (+ NaOAc) 276, 329. ^1H NMR ($CDCl_3$): δ 12.76 (1H, *s*, OH-5), 7.84 (2H, *d*, J = 8.8 Hz, H-2′,6′), 7.01 (2H, *d*, J = 8.8 Hz, H-3′,5′), 6.58 (1H, *s*, H-8), 6.54 (1H, *s*, H-3), 3.96, 3.92, 3.89 (3 x 3H, *s*, OMe-6,7,4′). TLC and HPLC: see Table 1.

Identification of remaining flavones. Flavones **3–12** were tentatively identified by chromatographic (TLC, HPLC) and UV comparison with a range of authentic standards. The R_ts, R_fs, and UV maxima given by the photodiode array detector of these *Ocimum* flavones and their tentative identification are given in Table 1. Analyt. TLC was carried out on silica gel in 5% MeOH in $CHCl_3$. HPLC was performed on a reverse phase 100RP-18 column (4.0 mm i.d. x 25 cm). The solvent system was a linear gradient of A (2% HOAc) and B (MeOH–HOAc–H_2O, 18:1:1), starting at 60% A and

40% B, which changed to 0% A and 100% B over 20 min, and was then kept at 100% B for 5 min. Flow rate: 1.0 ml min^{-1}; column temp.: 25°; UV detection at 335 nm.

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