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The chemotaxonomic significance of two bioactive caffeic acid esters, nepetoidins A and B, in the Lamiaceae

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

A survey of leaf surface constituents in the family Lamiaceae using HPLC with diode array detection revealed the presence of two characteristic phenolic compounds in many species. The distribution of these phenolics in the Lamiaceae was found to be of taxonomic significance, as they were present in the great majority of species investigated for the subfamily Nepetoideae, including representatives of the well-known genera of culinary herbs, mint, rosemary, sage, thyme and basil. In contrast, they were absent from species of the other subfamilies of Lamiaceae studied and from the related families Verbenaceae, Scrophulariaceae, Acanthaceae and Buddlejaceae. The compounds were isolated from *Plectranthus crassus* and identified by NMR spectroscopy as the known caffeic acid esters (*Z,E*)-[2-(3,5-dihydroxyphenyl)ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate and (*Z,E*)-[2-(3,4-dihydroxyphenyl)-ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate, for which the trivial names nepetoidins A and B are proposed. The presence of this pair of caffeic acid esters adds another character to the chemical, palynological and embryological features distinguishing the Nepetoideae from the other subfamilies of Lamiaceae and related families, and supports the view that the Nepetoideae are a specialised and monophyletic group within the family. Nepetoidin B was shown to have a greater antioxidant activity than gallic, rosmarinic and caffeic acids, and showed activity as an insect phagostimulant. Both compounds were antifungal.

Keywords: Lamiaceae; Nepetoideae; (*Z,E*)-[2-(3,4-dihydroxyphenyl)ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate; (*Z,E*)-[2-(3,5-dihydroxyphenyl)-ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate; Caffeic acid esters; Chemosystematics; Free radical scavenging activity; Antifungal; Insect phagostimulant

1. Introduction

The Nepetoideae with ca. 3400 species in 105 genera is the largest and most characteristic of the seven subfamilies recognised by Harley et al. (in press) in the family Lamiaceae, which comprises ca. 7200 species and 236 genera. Many taxonomic studies, including those based on DNA sequence data (e.g. Wagstaff et al., 1998), have revealed that this is a monophyletic and natural group. The Nepetoideae comprise the majority of the essential oil-rich genera of the Lamiaceae, such as the well-known culinary and medicinal herbs mint, sage, rosemary, thyme and basil. Bentham (1876) placed all the essential oil genera into four tribes, which he arranged together on an intuitive basis, and classified the remaining genera of the family into four further tribes. However, Briquet (1895–1897) distributed the essential oil and non-essential oil genera into the four subfamilies he recognised. His classification has been followed extensively in floras and botanical books until the present day, despite the fact that subsequent research has shown that the presence of monoterpenoid-rich essential oils is an important phylogenetic character in the Lamiaceae, correlated with characters from several different disciplines. Leitner (1942) suggested that Briquet's system required revision, because it did not correlate with pollen characters. Erdtman (1945) took this further and divided the family into two subfamilies, the Lamioideae with tricolpate, 2-nucleate pollen and the

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Nepetoideae with hexacolpate, 3-nucleate pollen. Wunderlich (1967) and El-Gazzar and Watson (1968, 1970) found several more characters correlated to pollen type that distinguished the Nepetoideae from the Lamioideae sensu Erdtman, including differences in seed development, types of stomata and susceptibility to the pathogen *Puccinia menthae*. As to chemical differences between the Lamioideae and Nepetoideae, representatives of the former subfamily are characterised by the production of iridoid glycosides (Hegnauer, 1973) and caffeoyl phenylethanoid glycosides such as verbascoside (=acteoside) (Hegnauer, 1989; Pedersen, 2000), which are largely absent from the Nepetoideae. In turn, the Nepetoideae tend to accumulate rosmarinic acid and monoterpenoid-rich essential oils (Harborne, 1966; Hegnauer, 1973), constituents which are largely absent from the Lamioideae (Pedersen, 2000). The chemistry of related families, such as the Verbenaceae and Scrophulariaceae, is very similar to that of the Lamioideae, but different to that of the Nepetoideae (Hegnauer, 1989).

During HPLC surveys of the surface flavonoids in a number of genera belonging to the Lamiaceae (e.g. Grayer et al., 1996, 2001; Jamzad et al., in press), two related non-flavonoid phenolics (**1** and **2**) were detected that appeared to represent a third chemical character distinguishing the Nepetoideae from the Lamioideae. To test this hypothesis, a large survey was carried out to study the distribution of these compounds in the Lamiaceae and related families.

In the latest classification of the family, Harley et al. (in press) recognise seven subfamilies in the Lamiaceae, the Symphorematoideae, Viticoideae, Ajugoideae, Prostantheroideae, Scutellarioideae, Lamioideae and Nepetoideae. The Symphorematoideae and Prostantheroideae were formerly separate families, whereas the Viticoideae and part of the Ajugoideae used to belong to the family Verbenaceae. However, all these taxa were transferred to the Lamiaceae by Cantino et al. (1992) on the basis of a number of characters from different disciplines. The Lamioideae sensu Erdtman (1945) comprise the present Scutellarioideae, Lamioideae and part of the Ajugoideae. Representatives of all subfamilies sensu Harley et al. (in press), apart from the Symphorematoideae, were surveyed for the presence of **1** and **2**. Furthermore, 22 species belonging to 19 genera of related families (Scrophulariaceae, Verbenaceae, Buddlejaceae and Acanthaceae) were also investigated. The compounds were isolated in order to identify their chemical structures and to provide material for biological activity assays.

2. Results and discussion

2.1. Distribution of *1* and *2* in the Lamiaceae and related families

Non-polar extracts from fresh leaves of 166 species belonging to 78 genera of Lamiaceae and related families were analysed by HPLC with diode array detection. The presence of **1** and **2** in the HPLC traces of the extracts was easily established from their relatively short retention times for diethyl ether-extractable compounds and characteristic UV absorption spectra. Although similar to flavonoids, the spectra showed an unusual shoulder at 305 nm, which was more pronounced in **2** than in **1** (Fig. 1). The results of the analyses are presented in Table 1.

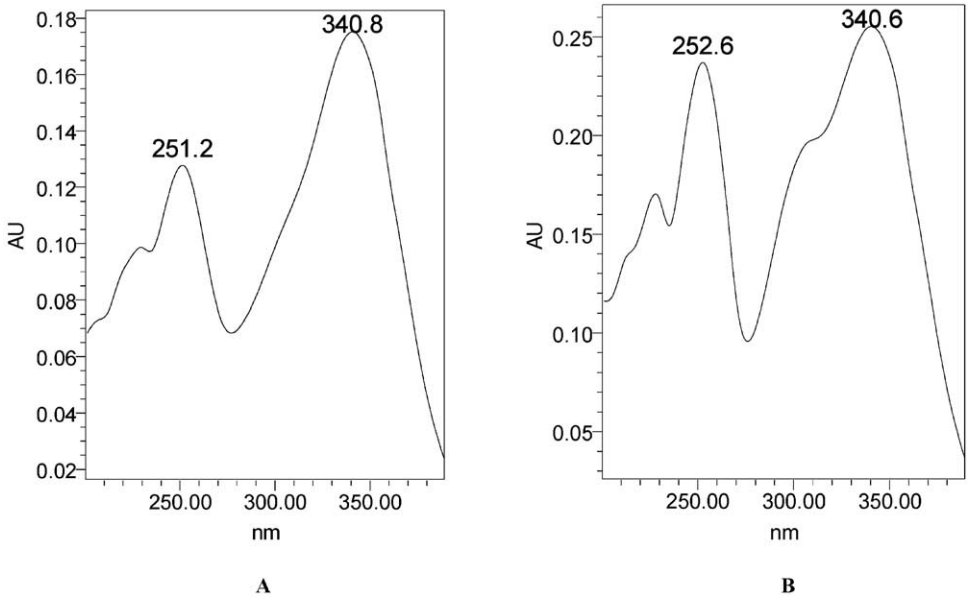


Fig. 1. UV spectra of nepetoidins A (**1**) and B (**2**).

Table 1
Distribution of Nepetoidins A and B in the Lamiaceae and related families (classification of the Lamiaceae according to Harley et al., in press)

Family, subfamily, tribe or subtribe	Species	Kew Accession no.	Nepetoidins	
			A	B
Family Lamiaceae				
Subfamily Nepetoideae				
Tribe Mentheae				
Subtribe Salviinae				
	Perovskia atriplicifolia	1969–19321	+	+ +
	Rosmarinus officinalis	BI 7152	+	+ +
	Salvia digitaloides	1998–3374	+	+ +
	S. nilotica	1970–4361	+	+ + +
	S. officinalis	BI 7151	+	+ +
Subtribe Menthinae				
	Clinopodium vulgare	1979–4649	+	+
	Hyssopus officinalis	1940–3801	+	+
	Lycopus exaltatus	1969–19292	+	+ +
	Mentha aquatica	1998–692	+	+
	Mentha longifolia	1996–4601	+	+
	M.×villosa	1998–691	+	+
	Monarda fistulosa	1990–1477	+	+
	Origanum syriacum	1995–3817	+ +	+
	O. vulgare	BI 7153	+	+ +
	Prunella hyssopifolia	1998–4384	+	+ +
	Pycnanthemum linifolium	1952–6432	+ +	+ +
	Thymus serpyllum	1969–19395	+ +	+
Subtribe Nepetinae				
	Agastache urticifolia	1993–108	–	–
	Cedronella canariensis	1974–3274	+	+
	Dracocephalum grandiflorum	1996–1159	–	–
	D. prattii	1969–19274	–	–
	Glechoma hederacea	BI 7973	+	+
	Meehania fargesii	1991–1268	+	+
	Nepeta cataria	BI 7154	+	+ +
Incertae sedis				
	Melissa officinalis	1969–19289	+	+ +
Tribe Ocimeae				
Subtribe Lavandulinae				
	Lavandula×allardii	1996–4408	+	+
	L. angustifolia ssp. *delphinensis*	2000–3658	+	+
	L. aristibracteata	1996–702	+ +	+ +
	L. bipinnata	2000–3634	+	+
	L. brevidens	1996–2457	+	+
	L. buchii var. *gracile*	2000–3630	+	+
	L. canariensis	1999–2174	+	+
	L. christiana	2000–3652	+	+
	L. coronopifolia	2000–3631	+	+
	L. dentata var. *dentata*	1997–6577	+	+
	L. dhofarensis var. *dhofarensis*	1996–730	+ +	+ +
	L.×heterophylla	2000–3654	+ +	+ +
	L. lanata	1996–704	+	+
	L. latifolia	2000–3628	+	+
	L. mairei var. *mairei*	1998–2589	+	+
	L. maroccana	1998–2588	+	+
	L. minutolii var. *minutolii*	2000–3640	+	+
	L. multifida	1996–717	+	+
	L. pinnata ssp. *pinnata*	2000–3661	+	+
	L. pubescens	1996–715	+	+
	L. rotundifolia	2000–3632	+	+
	L. stoechas ssp. *luisieri*	1999–2177	+ +	+ +
	L. tenuisecta	1996–699	+	+
	L. viridis	2000–3633	+ +	+ +

(*continued on next page*)

Table 1 (*continued*)

Family, subfamily, tribe or subtribe	Species	Kew Accession no.	Nepetoidins	
			A	B
Subtribe Hyptidinae				
	Eriope macrostachya	1974–762	+	+
	E. salvifolia	1977–1400	+	+ +
	Hypenia brachystachys	1996–1823	+	+ +
	Hyptis eriocephala	1979–1648	+ +	+ +
	H. lanceolata	1993–1076	+	+
	H. ramosa	1991–1732	+	+ +
	H. umbrosa	1991–1549	+ +	+ +
	H. urticoides	1977–2082	+ +	+ +
	Rhaphiodon echinus	1993–1074	+	+ +
Subtribe Ociminae				
	Catoferia chiapensis	1983–661	+ +	+ +
	Dauphinea brevilabra	1998–2417	+	+
	Endostemon obtusifolius	1997–4733	+	+
	Ocimum basilicum 'Purple Ruffles'	2000–794	+	+ +
	O. gratissimum	1996–1455	+	+ +
	O. selloi	1987–2003	+	+ +
	Orthosiphon serratus	1999–2424	–	–
Subtribe Plectranthinae				
	Aeollanthus buchnerianus	1970–2734	+	+
	A. densiflorus	1970–3760	–	–
	Perrierastrum oreophilum	1978–4488	+	+ +
	Plectranthus ambiguus	1996–2728	+	+
	P. argentatus	1999–10	+	+ +
	P. argentifolius	1993–3101	+	+
	P. asirensis	1978–1972	+ +	+ +
	P. barbatus	1982–5914	+	+
	P. buchananii	1970–4920	+ +	+ +
	P. ciliatus	1999–16	+ +	+ +
	P. coeruleus	1955–42604	+	+ +
	P. comosus	1975–4486	+	+
	P. crassus	1970–2059	+ +	+ +
	P. aff. cyaneus	1970–3766	+	+ +
	P. cylindraceus	1996–1453	+	+
	P. ecklonii	1996–2733	+ +	+ +
	P. elegans	1970–3417	+	+
	P. ernstii	1998–3516	+	+
	P. forsteri 'marginatus'	1999–11	+	+
	P. frederici	1999–15	+	+ +
	P. gracilis	1993–3264	+	+
	P. grandis	1999–14	+ +	+ +
	P. hadiensis	1977–2634	+	+
	P. hilliardiae	1996–2726	+	+
	P. hyemalis	1977–1639	+ +	+ +
	P. igniarius	1970–3784	+	+
	P. kivuensis	1994–120	+ +	+ +
	P. lanuginosus	1973–1207	+	+
	P. madagascariensis	1980–3446	+	+
	P. mutabilis	1955–47101	+	+
	P. neochilus	1980–3448	+	+
	P. njassae	1963–16901	+	+
	P. oertendahlii	1996–2731	+	+
	P. ovatus	1977–2631	+ +	+ +
	P. parviflorus	1961–66401	+	+
	P. petiolaris	1996–2729	+	+
	P. pseudomarrubioides	1972–2774	+	+
	P. purpuratus	1965–31901	+	+
	P. saccatus	1996–2730	+	+
	P. sanguineus	1970–2072	+ +	+ +
	P. aff. spicatus	1980–3450	+	+
	P. strigosus	1967–41108	+	+

(*continued on next page*)

Table 1 (*continued*)

Family, subfamily, tribe or subtribe	Species	Kew Accession no.	Nepetoidins	
			A	B
	P. tenuiflorus	1993–3100	+	+
	P. xerophilus	1989–1322	+	++
	P. zuluensis	1992–2850	++	++
	Pycnostachys umbrosa	1970–3755	+	+
	Solenostemon latifolius	1992–450	++	++
	S. shirensis	1970–3550	++	++
	Tetradenia nervosa	1993–3116	+	++
	Thorncroftia media	1993–3115	+	+
Subtribe Hanceolinae				
	Isodon pharicus	1953–208	–	–
Subfamily Viticoideae				
	Vitex agnus-castus	1995–13	–	–
Subfamily Ajugoideae				
	Ajuga reptans	BI 7923	–	–
	Caryopteris incana	1968–22905	–	–
	Clerodendrum bungei	1973–12319	–	–
	C. colebrookianum	1995–4111	–	–
	Karomia speciosa	1990–1266	–	–
	Teucridium parvifolium	1992–1553	–	–
	Teucrium abutiloides	1995–3339	–	–
	T. botrys	1970–1200	–	–
	T. flavum	1970–904	–	–
	T. heterophyllum	1995–3341	–	–
Subfamily Prostantheroideae				
	Prostanthera ovalifolia	1949–39004	–	–
Subfamily Scutellarioideae				
	Holmskioldia sanguinea	1991–1363	–	–
	Scutellaria racemosa	1990–611	–	–
Subfamily Lamioideae (*sensu stricto*)				
	Ballota nigra	1972–4087	–	–
	Colquhounia coccinea	1961–4711	–	–
	Galeopsis pyrenaica	1972–21041	–	–
	Lamium garganicum	1969–19285	–	–
	L. maculatum	1969–19284	–	–
	L. orvala	1961–6501	–	–
	Leonotis ocymifolia	1991–1269	–	–
	Leonurus cardiaca	1970–991	–	–
	Marrubium peregrinum	1969–19287	–	–
	Phlomis russeliana	1982–465	–	–
	P. tuberosa	1979–1737	–	–
	P. umbrosa	1949–4801	–	–
	Pogostemon cablin	1986–280	–	–
	P. plectranthoides	1983–660	–	–
	Rostrinucula dependens	1985–4257	–	–
	Sideritis macrostachya	1991–589	–	–
	Stachys alopecuros	1998–760	–	–
	S. macrantha	1940–2511	–	–
	S. officinalis	1970–683	–	–
	S. recta	1995–422	–	–
Family Scrophulariaceae				
	Alonsoa caulialata	1969–50437	–	–
	Calceolaria mexicana	1969–50441	–	–
	Digitalis ferruginea	1998–1962	–	–
	Gratiola officinalis	1969–50446	–	–
	Kickxia spuria	1975–01820	–	–
	Linaria genistifolia	1995–2170	–	–
	Penstemon kunthii	1977–03509	–	–
	Scrophularia glabrata	1998–1384	–	–
	Verbascum phlomoides	1969–50523	–	–
	Veronica officinalis	1978–01342	–	–
	Veronicastrum sibiricum	1969–50549	–	–

(*continued on next page*)

Table 1 (*continued*)

Family, subfamily, tribe or subtribe	Species	Kew Accession no.	Nepetoidins	
			A	B
Family Verbenaceae				
	Citharexylum berlandieri	1973–12402	–	–
	C. illicifolium	1973–12321	–	–
	Duranta repens	1963–45503	–	–
	Lantana camara	1952–14801	–	–
	Phyla canescens	1957–75902	–	–
	Rhaphithamnus spinosus	1985–8074	–	–
	R. venustus	1970–653	–	–
	Verbena lasiostachys	1970–1291	–	–
	V. officinalis	1969–50669	–	–
Family Buddlejaceae				
	Buddleja nivea	1982–8608	–	–
Family Acanthaceae				
	Pseudaechmanthera glutinosa	1969–50088	–	–

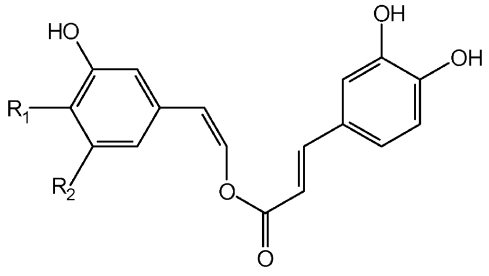
The UV absorbance was measured at 335 nm for a 10 μl injection from a 1.0 ml extract corresponding to 1.0 g of fresh leaf material.
−, not detected; +, UV absorbance of peak lower than 0.1 AU; ++, UV absorbance of peak between 0.1 and 1.0 AU; +++, UV absorbance of peak above 1.0 AU.

The presence of **1** and **2** was confirmed in 110 out of the 116 species and in 33 out of the 37 genera of Nepetoideae studied. In contrast, none of the species of the other subfamilies of Lamiaceae investigated, nor those of related families, contained the substances. Although only a small proportion of the total number of species present in the Nepetoideae was studied for **1** and **2**, species belonging to more than one third of the genera were analysed (37 out of 105), which constitutes a good representation. The fact that they were present in 95% of the species investigated for this subfamily and not in the other subfamilies suggests that the presence of **1** and **2** constitutes the most important chemotaxonomic character established to date distinguishing the Nepetoideae from the Lamioideae *sensu lato*. Some other important chemotaxonomic characters, such as the presence of essential oils and rosmarinic acid, are not completely confined to the Nepetoideae, but occasionally occur in the other subfamilies.

2.2. Isolation and identification of *1* and *2*

Samples of both compounds were isolated by semi-preparative HPLC from a diethyl ether extract of fresh leaves of *Plectranthus crassus*. APCI-MS gave $[M+H]^+$ at *m/z* 315 for **1** and **2** and in MS/MS analysis this ion generated product ions at *m/z* 161, 163 and 205 for both compounds. The product ion at *m/z* 163 is typical of caffeic acid esters. ^1H and ^{13}C NMR spectroscopic data for both compounds obtained in DMSO-d_6 were identical to those published previously for two caffeic acid esters isolated from another species belonging to the Nepetoideae, *Perilla frutescens* var. *acuta* (Nakanishi et al., 1990). These authors identified the compounds as (*Z,E*)-[2-(3,5-dihydroxyphenyl)ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate and (*Z,E*)-[2-(3,4-dihydroxyphenyl)ethenyl] 3-(3,4-dihydroxyphenyl)-2-propenoate (Fig. 2). Because of the apparent restriction in the occurrence of **1** and **2** to the Nepetoideae, we propose to give these compounds the trivial names of nepetoidins A and B.

Although the presence of **1** has only been confirmed in *P. frutescens*, **2** has been reported more frequently. It was first isolated from the leaf glands of another species in the Nepetoideae, *Plectranthus caninus* (Arihara et al., 1975). Ten years later Banthorpe et al. (1985) found high concentrations of **2** in the secretion of callus cultures of *Lavandula angustifolia* ssp. *angustifolia* (=*L. officinalis*), again a representative of the Nepetoideae, but the constituent could not be detected in the parent plant. These authors also reported a second caffeic acid ester in the callus culture, which was described as the (*E,E*)-isomer of **2**. Subsequently, leaves and suspension cultures of eight species of Lamiaceae were investigated



	R_1	R_2
1	H	OH
2	OH	H

Fig. 2. Structures of **1** and **2**.

for these caffeic acid esters. Most cultures and some leaf material of these taxa contained the compounds, but they were absent from *Teucrium fruticans*, which does not belong to the Nepetoideae (Banthorpe et al., 1989). Therefore, Brown and Banthorpe (1992) suggested that the presence of **2** and its (*E*,*E*)-isomer might be useful as a chemotaxonomic marker for the Lamiaceae.

The reason why **1** and **2** have not been reported more frequently from this well-studied group of plants, although they occur in nearly every species of this subfamily, is probably because they do not appear to survive drying of the plant material very well. We could not detect them in extracts prepared from fragments of herbarium specimens and even in freeze-dried material the amounts were always much lower than in fresh material of the same species. Perhaps the compounds are unstable and are oxidised easily, or perhaps active enzymes are present in the plants which hydrolyse the nepetoidins during drying of the plant material. A related caffeic acid conjugate, rosmarinic acid, also tends to decrease in quantity during drying of the plants, but is usually still detectable in herbarium specimens. Even in fresh leaf surface extracts of Lamiaceae species the nepetoidins appear to have been overlooked, probably because this material is traditionally investigated for essential oils and extracted by hydrodistillation. As the nepetoidins are non-volatile, they will not be obtained using this procedure. Nevertheless, according to Arihara et al. (1975), nepetoidins A and B are constituents of the leaf surface glands that also contain the essential oils. The present results confirm the fact that the nepetoidins are exudate compounds and not vacuolar substances like rosmarinic acid, as they were extracted by washing fresh intact leaves with an organic solvent for a short time.

2.3. Chemotaxonomic and ecological considerations

According to the analysis of molecular characters (sequences of the *rbc*L, *atp*B and 18S rDNA genes), the family Lamiaceae is closely related to the Scrophulariaceae, Verbenaceae, Orobanchaceae, Bignoniaceae, Buddlejaceae, Oleaceae, Globulariaceae, Plantaginaceae, Acanthaceae and some smaller families (Angiosperm Phylogeny Group, 1998). This group of families is usually referred to as the order Lamiales. The close relationship among the families within the Lamiales suggested by molecular characters is also reflected in their secondary chemistry, as they are chemically characterised by the tendency to produce iridoids and caffeoyl phenylethanoid glycosides (Grayer et al., 1999). Nearly all the subfamilies of the Lamiaceae show this "primitive" chemical profile that is characteristic of the order Lamiales as a whole. However, in the Nepetoideae a different chemical profile has evolved, which is found in nearly every species of that subfamily. This supports the view based on palynological, molecular and other characters that the Nepetoideae is a specialised and monophyletic group. The terpenoid chemistry has been altered to produce monoterpenoid essential oils instead of monoterpenoid iridoid glycosides, whereas the phenylpropanoid chemistry has been modified to produce the caffeic acid esters rosmarinic acid and nepetoidins A and B instead of the more polar caffeoyl phenylethanoid glycosides. More than 35 years ago, Harborne (1966) predicted that caffeoyl conjugates would represent useful characters for the classification of the Tubiflorae, which includes the order Lamiales. This prediction is supported by the present results.

It is possible that the change in secondary chemistry that took place in the Nepetoideae offered some ecological advantages, as this group is the most species-rich subfamily of the Lamiaceae, comprising 47% of all the species in this large family. Rosmarinic acid and essential oils occurring in Nepetoideae have a wide range of biological activities, particularly towards micro-organisms and insects (Harborne et al., 1999), which could explain the ecological success of this plant group. Nepetoidin B has been shown to have antifungal activity towards *Cladosporium herbarum* (Banthorpe et al., 1989). We have tested the antifungal activity of both **1** and **2** against *Aspergillus niger* and the effect of the compounds on the feeding behaviour of *Spodoptera littoralis*. The results of these assays are reported below, together with an assessment of the free radical scavenging activity of the compounds.

Another property of **1** and **2** was demonstrated by Nakanishi et al. (1990). These authors reported potent inhibition of xanthine oxidase by both compounds and particularly **2**, suggesting that this substance could have the potential for the control of hyperuricemia in human gout.

2.4. Free radical scavenging activity of *1* and *2*

The free radical scavenging activity was determined for nepetoidin B (**2**) and three known antioxidants (gallic acid, rosmarinic acid and caffeic acid) using the coloured free radical DPPH (see Section 3) as a reagent. The antioxidant activity was expressed as the EC_{50}, which is the concentration of the test solution required to give a 50% decrease in absorbance from that of the control solution (Lamaison et al., 1991). The EC_{50} of nepetoidin B (2.6 μM) was smaller than that of gallic (4.7 μM), rosmarinic (5.2 μM) and caffeic (11.1 μM) acids. This means that the free radical scavenging activity of nepetoidin B was greater than that of the test substances, including gallic acid, which has been used commercially as an antioxidant. Insufficient amounts of pure nepetoidin A (**1**) were available to carry out the required tests to determine its EC_{50} accurately. However, low concentrations of the substance gave considerable

loss of the colour of a DPPH solution, so that it is likely that nepetoidin A also exhibits a strong antioxidant activity.

2.5. Antifungal activity of *1* and *2*

The crude *Plectranthus* extract and 10 μg of pure solutions of **1** (nepetoidin A) and **2** (nepetoidin B) were applied as spots on a TLC plate. After development in chloroform-acetone, the plate showed yellow spots at R_f 0.08 for each of the three applied samples. The spots were blue in long wave UV light (350 nm) and after spraying of the plate with a spore suspension of *A. niger* and incubation, these spots appeared to be free of fungal growth, whereas the remainder of the plate was covered with mycelia. Therefore, both nepetoidin A and B showed antifungal activity towards *A. niger*.

2.6. Activity of *1* and *2* on insect feeding behaviour

When tested for activity against larvae of *Spodoptera littoralis* (see Section 3), nepetoidin B (**2**) stimulated feeding, giving a dose-dependent response over a range of concentrations from 1 to 1000 ppm. At 100 ppm the Feeding Index was -42.4 ± 6.89 (mean ± S.E.M., $P < 0.05$). Therefore, nepetoidin B shows some insect phagostimulant activity. Nepetoidin A (**1**) was inactive when tested over the same range of concentrations, with a Feeding Index of 6.4 ± 21.49 at 100 ppm.

3. Experimental

3.1. General

^{1}H NMR and ^{13}C NMR spectra were acquired using a Varian 500 MHz instrument. Samples were dried carefully, dissolved in DMSO-d_6 and referenced to the residual solvent resonance at δ_H 2.50 ppm or δ_C 39.5 ppm as appropriate. All experiments were carried out at 37 °C. Positive ion APCI-mass spectra were obtained with a quadrupole ion-trap instrument (Finnigan LCQ) using a vaporiser temperature of 550 °C, sheath and auxiliary nitrogen pressures of 80 and 20 psi, respectively, a needle current of 5 μA and a capillary temperature of 150 °C. Samples were introduced via an HPLC fitted with a Merck LiChrospher 250×4.0 (i.d.) mm (5 μm particle size) C_{18} column using a 20 min linear gradient of 25–100% MeOH in 1% aq HOAc at 1 ml/min. For the antioxidant assay a Molecular Devices OPTImax microplate reader was used. Incubator temperature was set to 30 °C and absorbance was measured at a wavelength of 517 nm. DPPH reagent, gallic acid and caffeic acid standards were purchased from Sigma and rosmarinic acid from Apin. Flat-bottom microtitre plates, were from Fisher (96–wells of 300 μl).

3.2. Plant material

All the plant material used was obtained from the Living Collections of the Royal Botanic Gardens, Kew, and for all species the garden accession numbers are given in Table 1. The identifications of the plant species were verified by two of the authors (A.J.P. and P.D.M).

3.3. Extraction

For each plant accession, fresh leaves (1.0 g) were rinsed briefly in a vial containing diethyl ether (10 ml). The extract was filtered and evaporated in a fume cupboard to dryness. Prior to HPLC analysis, the residue was dissolved in 80% aq. MeOH solution (1 ml), which was filtered through a nylon Acrodisc 13 syringe filter (Gelman Science, pore size 0.45 μm) into a 1 ml autosampler HPLC vial. For the HPLC analysis, 10 μl of extract was injected. For the isolation of **1** and **2**, fresh leaves (130.0 g) of *Plectranthus crassus*, RBG Kew accession number 1970–2059, were extracted for 20 min in diethyl ether (1 l), the solvent was poured off and the leaves were re-extracted with 0.5 l of diethyl ether. The combined ether extracts were left to evaporate in a fume cupboard after a brown precipitate (consisting of quinonoid diterpenoids) was removed. The dried residue was taken up in 4×1.0 ml of 80% aq MeOH. This was used after filtration through an Acrodisc 13 syringe filter for semiprep. HPLC.

3.4. HPLC analysis of fresh leaf extracts

The HPLC system consisted of a Waters LC 600 pump with a 996 photodiode array detector controlled by Millennium software. Merck Lichrospher 100RP-18 columns were used, 250×4.0 mm (i.d.), 5 μ particle size, for analytical work, and 250×10.0 mm (i.d.) for semi-preparative isolations. The column temperature was 30 °C. All solvents used were HPLC grade. For analytical work (the survey of **1** and **2**), elutions took place with a linear gradient of 2% aq. HOAc (solvent A) and MeOH, HOAc, H_2O (18:1:1, solvent B). The initial composition of the solvent system was 40% A, 60% B, changing to 100% B at $t = 15$ min. The program continued with 5 min of isocratic elution using 100% B, before returning to the initial conditions at $t = 21$ min. The next injection (by autosampler) was at $t = 30$ min to allow time for equilibration of the column. The flow rate was 1 ml min^{-1}. The retention times of the compounds when this system was used were 5.6 min for **1** and 6.7 min for **2**. For semiprep. HPLC the solvents were H_2O (solvent A) and MeOH (solvent B) and the elution program was as follows: $t = 0$ min, 60% B; $t = 10$ min, 70% B; $t = 15$ min, 100% B; $t = 20$ min, 100% B; $t = 21$ min, 60% B. The flow rate was 4.5 ml min^{-1}.

3.5. Free radical scavenging activity

The method used to determine the free radical scavenging activity was adapted from that described by Lamaison et al. (1991). Prior to the test a fresh solution of 100 μM 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a coloured free radical, was prepared, and also stock solutions of nepetoidin B (**2**) and three known antioxidants (gallic, rosmarinic and caffeic acids). MeOH (250 μl) in designated wells on the microtitre plate was used as blanks, and 100 μM solutions (250 μl) of DPPH as controls. For the tests, standard or test solutions (50 μl) in various concentrations were added to wells containing DPPH solution (200 μl) and each concentration was tested in triplicate. Six dilutions of both test and standard solutions in MeOH were prepared, with the following final concentrations of each substance: 20, 10, 5, 2.5, 1.25 and 0.625 μM. The plates were shaken and the DPPH left to react with the various test substances for 30 min before the absorbance of each solution in the wells was read at 517 nm. The antioxidant activity EC_{50}, which is the concentration of the test solution required to give a 50% decrease in absorbance from that of the control solution (Lamaison et al., 1991), was determined for each compound from a graph in which concentration and absorbance were plotted against each other.

3.6. Antifungal activity

The TLC bioautography method of Homans and Fuchs (1970) for testing antifungal activity of compounds was adapted as follows. Solutions of nepetoidins A and B (**1** and **2**) were spotted onto two precoated silica gel TLC plates (Merck 1.05554, thickness 0.2 mm), so that the final amount of compound in the spot was 10 μg for both **1** and **2**. The plates were developed in $CHCl_3$–Me_2CO (24:1, v/v), dried thoroughly and viewed under UV light (both short and long wave). Any coloured or dark absorbing spots were marked on the plate. One plate was sprayed with a spore suspension of *A. niger* in 2% malt extract–1% glucose solution. After incubation at 25±1 °C for 3 days at 100% relative humidity, the growth of the fungus was examined visually and the degree of growth inhibition was recorded. The second TLC plate was used as a reference.

3.7. Activity on insect feeding behaviour

A binary choice bioassay using sucrose treated glass-fibre discs (Whatman 2.1 cm diameter) was used to investigate the activity of the compounds on the feeding behaviour of final stadium larvae of *Spodoptera littoralis* (Simmonds et al., 1990). The amount eaten of the compound treated (T) and control (C) discs were used to calculate the Feeding Index [(C−T)/(C+T)]%. In this Index, a negative value indicates a feeding stimulant. The compounds were tested at a range of concentrations from 1 to 1000 ppm to establish whether the compounds gave dose-dependent responses. The Feeding Index at 100 ppm is presented in the results in order to enable comparisons to be made with the activity of other compounds. The Wilcoxon matched-pairs test was used to analyse the data.

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