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Identification of tyrosinase inhibitors from *Marrubium velutinum* and *Marrubium cylleneum*

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Abstract—Tyrosinase is a key enzyme in the production of melanins in plants and animals. Forty-five secondary metabolites isolated from *Marrubium velutinum* and *Marrubium cylleneum* belonging to the classes of flavonoids, phenylethanoid glycosides, phenolic acids and lignan glycosides were screened for their inhibitory activity against mushroom tyrosinase. Flavonoids and phenylethanoid glycosides showed moderate inhibitory activity, while phenolic acids were less active than phenylethanoid glycosides, suggesting that both phenolic groups are important for the activity.

1. Introduction

Tyrosinase, also known as polyphenol oxidase (PPO), is a copper containing enzyme, which is widely distributed in microorganisms, animals and plants. It catalyzes the oxidation of monophenols, *o*-diphenols and *o*-quinones.[1] Tyrosinase is known to be a key enzyme for melanin biosynthesis and is responsible for melanization in animals and browning in plants. In food industry, tyrosinase is responsible for the enzymatic browning reactions in damaged fruits during post-harvest handling and processing. Controlling enzymatic browning is essential during fruit pulp manufacturing process.[2,3]

Many tyrosinase inhibitors find applications in cosmetic products[4] for whitening and depigmentation after sunburn[5] and in the treatment of dermatological disorders related to melanin hyperpigmentation. Some of the most traditionally used skin whitening products are hydroquinone, corticosteroids and mercury containing products. However, during the last years their use has been prohibited, because of serious health concerns, such as irreversible cutaneous damage, ochronosis, accumulation of mercury in the body and poisoning. In contrast, naturally derived whitening agents are free of any harmful side effects. Lately, a broad spectrum of natural compounds have been described to inhibit tyrosinase. Among them phenolic compounds seem to be potent agents,[6–10] activity which is attributed to their structural resemblance to L-DOPA and tyrosine, natural substrates of tyrosinase.[3,11]

Marrubium sp. are rich in polyphenols.[12–14] Preliminary assays on the methanolic extracts of both plants showed strong tyrosinase inhibitory activity. In previous communications we reported the isolation and structural elucidation of several flavonoids and phenylethanoid glycosides isolated from *Marrubium velutinum*.[15–17] In this paper, we report on the isolation of further secondary metabolites from *M. velutinum* and *Marrubium cylleneum*, and the evaluation of their inhibitory activity against mushroom tyrosinase.

The genus *Marrubium* (Lamiaceae) comprises around 30 species, indigenous in Europe, the Mediterranean and Asia.[18] *M. velutinum* is an endemic herb of central and southern Greece growing in dry rocky places in pastures. *M. cylleneum* is an endemic herb of southern Greece.

2. Results and discussion

From *M. velutinum* (both methanol and dichloromethane extracts) 15 flavonoids were isolated (**1**, **2**, **4**–**6**, **10**–**15**, **17**, **18**, **20**, **21**), belonging to the groups of

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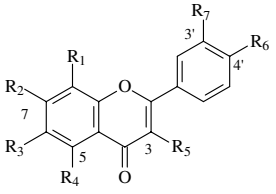
flavones, acylated glycosides of flavones and flavonols and flavonol glycosides, as well as 15 phenylethanoid glycosides (**22**–**36**), belonging to disaccharides, trisaccharides and tetrasaccharides. Further studies on the methanol extract of this species afforded three lignan glycosides (**42**–**45**) and the phenolic acids chlorogenic (**38**) and phaselic (2-*O*-caffeoyl-L-malate) (**39**). Phytochemical investigations on *M. cylleneum* afforded 7 flavonoids (**3**, **7**–**9**, **15**, **16**, **19**), the phenylethanoid trisaccharide **26** and phenolic acids *E*-p-coumaric acid (**37**), *E*-ferulic acid (**40**) and *E*-isoferulic acid (**41**). Furthermore, both plants yielded in large amounts stachydrine (**45**), a simple alkaloid, chemotaxonomic index for Lamiaceae sp.[19] (see Figs. 1–3).

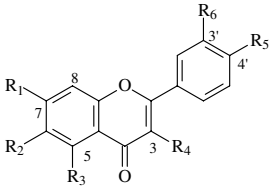
On the basis of ^{1}H and ^{13}C NMR and UV spectral analyses compounds **3**, **7**–**9**, **11**, **15**, **16**, **19**, **37**–**39**, **42**–**45** were identified as 5,6,7,8,4′-pentahydroxyflavone (**3**),[20] quercetin (**7**),[21] luteolin-7-*O*-glucoside (**8**),[22] kaempferol-3-*O*-glucoside (**9**),[23] kaempferol-3-*O*-β-D-rutinoside (**11**),[24] kaempferol-3-*O*-(6″-*E*-pCm)-glucoside (**15**),[25] quercetin 3-O-(6″-*O*-*E*-p-coumaroyl)-β-D-glucopyranoside (**16**),[26] apigenin-7-*O*-(6″-*E*-pCm)-glucoside (**19**),[27] *E*-p-coumaric acid (**37**),[28] chlorogenic acid (**38**),[29] phaselic acid (**39**),[30] 4-*O*-lariciresinol-glucoside (**42**),[31] 4′-*O*-lariciresinol-glucoside (**43**),[31] 4,4′-*O*-lariciresinol-bis-glucoside (**44**)[32] and stachydrine (**45**).[33]

For compounds **1**–**3**, **5**–**8**, **12**, **13**, **15**–**17**, **20**, **21**, **23**–**40**, see our previous studies.[15–17]

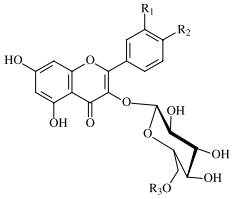
Tyrosinase catalyzes two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity), both using molecular oxygen.[34] The tested compounds were assayed for the diphenolase inhibitory activity which demands L-DOPA as substrate. The results of the effect of the isolates on tyrosinase are summarized in Tables 1–3. Flavonoids and phenylethanoid glycosides showed moderate inhibitory activity against tyrosinase (almost 2–3 times weaker than kojic acid). Previous studies in the literature focus mainly on genines. This is the first report on the tyrosinase inhibitory activity of flavoinoid derivatives, such as methoxylated flavones and acylated flavonoid glycosides. Methoxylation of the hydroxyl groups decreases the activity. Methoxylated flavones, like the methylethers of scutellarein (**4**–**6**), showed 10 times lower inhibitory activity than kojic acid. Previous studies on flavonoid antityrosinase activity[8] have shown that substitution of the 3-OH group reduces the activity of the compounds. In our case any further testing of flavones and flavonols was impossible, as the plants afforded small quantities of this class of compounds. It seems however, that in smaller concentrations flavones

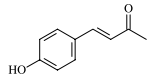
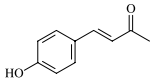
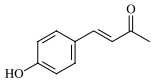


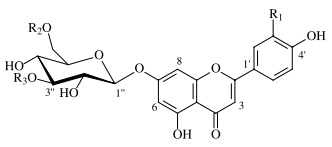
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
apigenin (**1**)	H	OH	H	OH	H	OH	H
chrysoeriol (**2**)	H	OH	H	OH	H	OH	OMe
5,6,7,8,4′-pentahydroxyflavone (**3**)	OH	OH	OH	OH	H	OH	H
5,6,7,4′-tetramethylscutellarein (**4**)	H	OMe	OMe	OMe	H	OMe	H
5,7,4′-trimethylscutellarein (**5**)	H	OMe	OH	OMe	H	OMe	H
ladanein (**6**)	H	OMe	OH	OH	H	OMe	H
quercetin (**7**)	H	OH	H	OH	OH	OH	OH



	R_1	R_2	R_3	R_4	R_5	R_6
luteolin-7-*O*-glucoside (**8**)	OGlu	H	OH	H	OH	OH
kaempferol-3-*O*-glucoside (**9**)	OH	H	OH	OGlu	OH	H
isorhamnetin-3-*O*-glucoside (**10**)	OH	H	OH	OGlu	OH	OMe
nikotiflorin (**11**)	OH	H	OH	ORut	OH	H
isorhamnetin-3-*O*-rutinoside (**12**)	OH	H	OH	ORut	OH	OMe
quercetin-3-*O*-rutinoside (**13**)	OH	H	OH	ORut	OH	OH
6-OH-kaempferol-3-*O*-rutinoside (**14**)	OH	OH	OH	ORut	OH	H



	R_1	R_2	R_3
kaempferol-3-*O*-(6′-*E*-pCm)-glucoside (**15**)	H	OH	
quercetin-3-*O*-(6′-*E*-pCm)-glucoside (**16**)	OH	OH	
isorhamnetin-3-*O*-(6′-OAc)-glucoside (**17**)	OMe	OH	OAc
isorhamnetin-3-*O*-(6′-*E*-pCm)-glucoside (**18**)	OMe	OH	



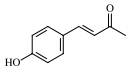
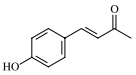
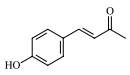
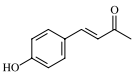
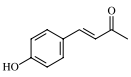
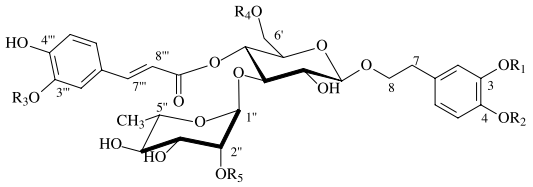
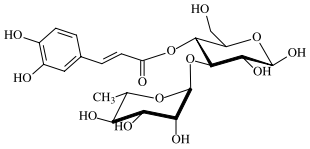
	R_1	R_2	R_3
apigenin-7-*O*-(3″-*E*-pCm)-glucoside (**19**)	H	H	
apigenin-7-*O*-(3″, 6″-di-*E*-pCm)-glucoside (**20**)	H		
chrysoeriol -7-*O*-(3″, 6″-di-*E*-pCm)-glucoside (**21**)	OCH_3		

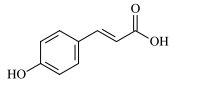
Figure 1. Chemical structures of flavonoids **1**–**21**.



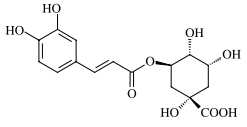
	R_1	R_2	R_3	R_4	R_5
acteoside (**22**)	H	H	H	H	H
leucosceptoside A (**23**)	H	H	Me	H	H
martynoside (**24**)	H	Me	Me	H	H
echinacoside (**25**)	H	H	H	Glu	H
6'-glucosyl-martynoside (**26**)	H	Me	Me	Glu	H
forsythoside B (**27**)	H	H	H	Api	H
alyssonoside (**28**)	H	Me	Me	Api	H
lavandulifolioside (**29**)	H	H	H	H	Ara
stachysoside D (**30**)	H	Me	Me	H	Ara
lamiophlomiside A (**31**)	Me	H	Me	Api	H
velutinoside IV (**32**)	Me	H	Me	H	Ara
velutinoside I (**33**)	H	H	H	Glu	Ara
velutinoside II (**34**)	H	H	Me	Glu	Ara
velutinoside III (**35**)	H	Me	Me	Glu	Ara



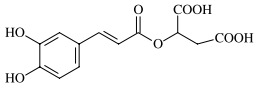
cistanoside F (**36**)



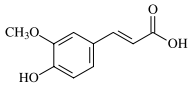
E-p-coumaric acid (**37**)



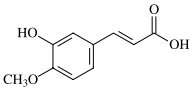
chlorogenic acid (**38**)



phaselic acid (**39**)



E-ferulic acid (**40**)



E-isoferulic acid (**41**)

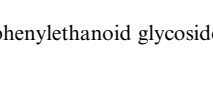
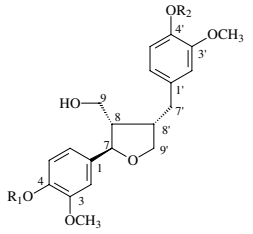
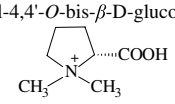


Figure 2. Chemical structures of phenylethanoid glycosides **22**–**36** and phenolic acids **37**–**41**.

and flavonols are more active than the corresponding flavonol glycosides, whereas in higher concentrations glycosides react better. This could be explained in terms of solubility. It also explains the extremely low activity of methoxylated flavones. In the latter case, besides the lack of free hydroxyl groups which help to bind to the active site of the enzyme, flavones are hardly soluble in the water based test solution which makes any kind of interaction with the enzyme difficult. Increase in the



	R_1	R_2
7*S*, 8*R*, 8'*R*-(-)-lariciresinol-4-*O*-*β*-D-glucopyranoside (**42**)	Glu	H
7*S*, 8*R*, 8'*R*-(-)-lariciresinol-4'-*O*-*β*-D-glucopyranoside (**43**)	H	Glu
7*S*, 8*R*, 8'*R*-(-)-lariciresinol-4,4'-*O*-bis-*β*-D-glucopyranoside (**44**)	Glu	Glu



stachydrine (**45**)

Figure 3. Chemical structures of lignan glycosides **42**–**44** and stachydrine **45**.

Table 1. Inhibitory activity of crude extracts of *M. velutinum* and *M. cylleneum*

	C (mg/mL)	100% inhibition
M. velutinum	1.00 (initial) 0.033 (final)	30.65 ± 2.44
M. cylleneum	1.00 (initial) 0.033 (final)	35.44 ± 3.11

number of sugars attached to position 3 reduces the activity, with the exception of compound **14**, which carries an extra OH– group on carbon 6 of the flavonoid nucleus. The increase in the activity could be explained by the presence of many ortho-hydroxyl groups which favors the chelate formation with copper in the enzyme. In contrast to mono- and di-glycosides, the presence of acyl groups on the sugar moiety favors the inhibitory activity. Low solubility of the substances in the medium did not permit us to assay them at appropriate concentrations to establish their IC_{50} values. In general at lower concentrations activity descends as:

flavonols > flavones > acylated monoglycosides > monoglycosides > diglycosides.

Concerning phenylethanoid glycosides this is the first time that this class of compounds is tested for antityrosinase activity. In general they showed moderate inhibitory activity with the exception of diglycosides which were two times less active than kojic acid. The inhibitory activity of phenylethanoid glycosides could be attributed to the presence of ortho-hydroxyls on the phenolic rings, which give them the property to chelate with metals. To support this hypothesis we measured the UV bathochromic shift of the band of phenylethanoid glycosides in the presence of Cu^{+2} ions. In the UV–vis spectra of phenylethanoid glycosides with ortho-hydroxyl groups (acteoside, echinacoside, forsythoside B, lavandulifolioside) (Table 4) bathochromic shifts (approximately 330 → 390 nm) were observed by adding $CuSO_4$. The shift was not observed in the case of methylated phenylethanoid glycosides such as leucosceptoside A. Increase in the number of sugars

Table 2. Inhibitory activity of compounds **1**–**21**

	Compounds	C (mM)	100% inhibition
	Kojic acid	0.118	80.02 ± 1.05
		0.047	53.87 ± 0.76
	Flavones/flavonols		
1	Apigenin	0.093	13.98 ± 3.82
2	Chrysoeriol	0.083	16.42 ± 0.75
3	5,6,7,8,4′-Pentahydroxyflavone	0.055	5.21 ± 4.23
4	5,6,7,4′-Tetramethylscutellarein	0.049	3.23 ± 2.85
5	5,7,4′-Trimethylscutellarein	0.050	4.24 ± 2.24
6	Ladanein	0.053	2.78 ± 0.98
7	Quercetin	0.051	49.67 ± 1.16
	Flavone/flavonol glucosides		
8	Luteolin-7-*O*-glucoside	0.074	27.35 ± 1.24
9	Kaempferol-3-*O*-glucoside	0.074	24.20 ± 4.84
10	Isorhamnetin-3-*O*-glucoside	0.070	24.22 ± 1.02
11	Nikotiflorin (kaempferol-3-*O*-rutinoside)	0.056	16.05 ± 1.01
12	Isorhamnetin-3-*O*-rutinoside	0.053	13.13 ± 1.56
13	Rutin	0.055	12.65 ± 0.78
14	6-OH-kaempferol-3-*O*-rutinoside	0.055	27.11 ± 1.12
	Flavone/flavonol acylated glucosides		
15	Tiliroside	0.056	30.19 ± 9.60
	Kaempferol-3-*O*-(6″-pCm)-glucoside	0.011	14.69 ± 1.50
16	Quercetin-3-*O*-(6″-pCm)-glucoside	0.055	21.86 ± 1.56
17	Isorhamnetin-3-*O*-(6″-OAc)-glucoside	0.064	23.31 ± 1.58
18	Isorhamnetin-7-*O*-(6″-pCm)-glucoside	0.053	21.10 ± 2.11
19	Apigenin-7-*O*-(6″-pCm)-glucoside	0.058	17.66 ± 2.64
20	Apigenin-7-*O*-(3″,6″-di-pCm)-glucoside	0.046	20.69 ± 2.25
21	Chrysoeriol-7-*O*-(3″,6″-di-pCm)-glucoside	0.044	15.59 ± 3.23

Table 3. Inhibitory activity of compounds **22**–**45**

	Compounds	C (mM)	100% inhibition
	Phenylethanoid glycosides		
22	Acteoside	0.053	25.78 ± 4.16
23	Leucosceptoside A	0.051	21.65 ± 1.38
24	Martynoside	0.052	20.55 ± 4.12
25	Echinacoside	0.042	17.52 ± 0.82
26	6′-Glucosyl-martynoside	0.041	22.65 ± 3.35
27	Forsythoside B	—	—
28	Alyssonoside	—	—
29	Lavandulifolioside	0.044	12.00 ± 2.44
30	Stachysoside D	0.042	9.48 ± 1.86
31	Lamiophlomiside	0.042	12.73 ± 3.55
32	Velutinoside IV	0.042	17.29 ± 1.22
33	Velutinoside I	0.036	12.36 ± 1.17
34	Velutinoside II	0.036	15.49 ± 2.45
35	Velutinoside III	0.035	13.21 ± 2.38
36	Cistanoside F	0.034	10.92 ± 1.00
	Phenolic acids		
37	p-Coumaric acid	0.041	8.87 ± 1.75
38	Chlorogenic acid	0.049	7.78 ± 2.14
39	Phaselic acid	0.056	4.62 ± 2.26
40	Ferulic	0.035	6.68 ± 3.47
41	i-Ferulic	0.035	7.90 ± 1.95
	Lignan glucosides		
42	4-*O*-Lariciresinol-glucoside	0.064	17.74 ± 4.36
43	4′-*O*-Lariciresinol-glucoside	0.064	12.27 ± 2.11
44	4,4′-*O*-Lariciresinol-bis-glucoside	0.049	11.06 ± 1.55
45	Stachydrine	0.231	11.08 ± 3.16

leads to lower activity probably because the sugars cover the OH groups and hinder their approach to the active site in the enzyme. This is better shown when comparing the activity of tetrasaccharides (**33**–**35**) with cistanoside F (**36**). They exhibit almost the same activity although they contain one more ortho-hydroxyl system in their structure. Moreover, phenolic acids were less active than phenylethanoid glycosides (10 times less active than kojic acid, 2–3 times less active than phenylethanoid glycosides), suggesting that both phenolic groups are important for the activity.

Lignan glycosides showed moderate inhibitory activity against tyrosinase (4–5 times lower than kojic acid). The lignan diglucoside (**45**) showed the lowest activity propably because of the complete lack of any free hydroxyl group.

Table 4. Bathochromic shift of selected phenylethanoid glycosides (0.01 mM) in the presence of $CuSO_4$ (0.05 mM)

Compounds	Shift by Cu^{+2}
Acteoside	329.5 → 383
Echinacoside	331 → 393
Forsythoside B	331 → 390
Lavandulifolioside	330 → 392
Leucosceptoside A	[a]
6′-Glu-martynoside	[a]
Velutinoside II	[a]

[a] Did not shift.

Taken all together, the tested flavonoids and phenylethanoid glycosides from *M. velutinum* and *M. cylleneum* showed moderate activity. However, the fact that the methanolic extracts of both plants showed higher activity at the same concentration (1 mg/mL) as the isolates suggests possible synergistic effects. The possible synergistic effect of different compounds could allow application of mixtures containing lower concentrations of each agent possibly reducing adverse effects.[11]

Kojic acid is an excellent skin whitening agent but has been accused of serious side effects, such as cytotoxicity, skin cancer, dermatitis, and has been banned in cosmetics in many countries.[4] Flavonoids and phenylethanoid glycosides are milder and safer tyrosinase inhibitors, whereas they are well documented to exhibit strong antioxidant and anti-inflammatory properties.[35–38] Compounds with redox properties or scavenging reactive oxygen species seem to prevent melanin biosynthesis via different ways and therefore substances acting at different steps of pigmentation should be advantageous depigmenting agents.[11]

3. Experimental

3.1. General experimental procedures

^{1}H, ^{13}C and 2D NMR spectra were recorded in CD_4O on Bruker DRX 400 and Bruker AC 200 (50.3 MHz for ^{13}C NMR) instruments at 295 K.

Vacuum liquid chromatography (VLC): silica gel 60H (Merck, Art. 7736); HPLC: CE 1100 Liquid Chromatography Pump. Column: Kromasil C_{18} (250 × 10 mm) Column chromatography (CC): silica gel 60 (SDS, 40–63 μm), gradient elution with the solvent mixtures indicated in each case; Sephadex LH-20 (Pharmacia). TLC: Merck siliga gel 60 F_{254} (Art. 5554). Detection: UV-light, spray reagent [vanillin-H_2SO_4 on silica gel].

3.2. Plant material

Aerial parts of *M. velutinum* Sibth. & Sm. were collected from Kellaria–Parnassos mountain (Sterea Hellas) in July 1998 and of *M. cylleneum* were collected from Mainalon mountain (Peloponnese) in May 1999. Voucher specimens have been kept in the Herbarium of Patras University (UPA) under the numbers Skaltsa and Lazari 114 and Skaltsa and Lazari 111b-99.

3.3. Extraction and isolation

The air-dried powdered aerial parts of *M. cylleneum* (1.33 kg) were successively extracted at room temperature with petroleum ether, ether, EtOAc and MeOH (2 L of each solvent, twice, 48 h). The dried MeOH extract (49.7 g) was subjected to VLC over silica gel (10 × 8 cm) with CH_2Cl_2–MeOH mixtures of increasing polarity to yield eight fractions (A–J) of 500 mL. Fraction B (1.61 g; eluted with CH_2Cl_2–MeOH 85:15) was further purified by CC over Sephadex LH-20 (MeOH) and afforded fractions (BA–BF). Fraction BF (2.2 mg) was identified as quercetin (**7**). Fraction BC (63.2 mg) was further purified by CC over silica gel and afforded ferulic (**40**) (12.3 mg) and isoferulic acids (**41**) (4.7 mg). Part of fraction BD (35.0 mg) was subjected to RP-18 HPLC (MeOH/AcOH 5% 60:40) and afforded compounds **3** (5.4 mg; t_R 46.4 min), **19** (4.5 mg; t_R = 70.0 min) and **37** (3.6 mg; t_R = 79.1 min). Fraction F (9.04 g; eluted with CH_2Cl_2–MeOH 40:60) was further purified by CC over Sephadex LH-20 (MeOH) and afforded fractions (FA–FG). Fraction FE was applied to CC over silica gel gel with mixtures of CH_2Cl_2–MeOH–H_2O of increasing polarity (98:2:0.2–30:7:0.7) and afforded 13 fractions (FEA–FEM). Fraction FEH (74.3 mg; eluted with CH_2Cl_2–MeOH–H_2O 87:13:1.3) was subjected to RP-HPLC (MeOH–H_2O 60:40) and yielded compounds **9** (6.2 mg; t_R = 11.5 min), **15** (19.8 mg; t_R = 18.0 min) and **16** (5.9 mg; t_R = 12.5 min). Fraction FEJ was combined with fraction FEK (154.0 mg; eluted with CH_2Cl_2–MeOH–H_2O 85:15:1.5-82:18:1.8) which was further purified by CC over silica gel to give eight fractions (FEKA–FEKH). Fraction FEKE (40.1 mg; eluted with CH_2Cl_2–MeOH–H_2O 80:20:2-76:24:2.4) was subjected to RP-HPLC (MeOH–H_2O 60:40) and yielded compounds **8** (1.2 mg; t_R = 9.0 min), **11** (5.9 mg; t_R = 11.8 min). Fraction FC (2.29 gr) was subjected to subsequent CC over Sephadex LH-20 and RP-MPLC (RP-18 silica gel) and yielded compounds (**26**) (3.6 mg) and **45** (216.6 mg).

The air-dried powdered aerial parts of *M. velutinum* (0.63 kg) were successively extracted at room temperature with petroleum ether, ether, EtOAc and MeOH (2 L of each solvent, twice, 48 h). The dried MeOH extract (77.0 g) was subjected to VLC over silica gel (10 × 8 cm) with CH_2Cl_2–MeOH mixtures of increasing polarity to yield eight fractions (A–H) of 500 mL. Part of fraction G (1.0 g; eluted with MeOH/H_2O 8:2) was further applied to RP-18 VLC using mixtures of MeOH:H_2O of decreasing polarity to yield 14 fractions. Fraction G_4 (40.0 mg; eluted with MeOH/H_2O 1:9) was further purified by prep. TLC (EtOAc/MeOH/HCOOH 5:1:1) and afforded chlorogenic acid (**38**) (10.0 mg) and phaselic acid (**39**) (5.2 mg). Fraction E (13.4 g; eluted with CH_2Cl_2–MeOH 5:5–4:6) was further applied to VLC over silica gel using CH_2Cl_2–MeOH and yielded four fractions (A_1–D_1). Fraction D_1 (3.32 g; eluted with CH_2Cl_2–MeOH 55:45–MeOH 100%) was fractionated by CC over Sephadex LH-20 and yielded seven fractions (A_3–G_3). Fraction D_3 was subjected to RP-MPLC (RP-18 silica gel) and yielded compounds (**25**) (27.3 mg), (**27**) (11.0 mg)[15] and **44** (2.4 mg). Fraction C_1 (6.49 g; eluted with CH_2Cl_2–MeOH 65:35–60:40) was applied to VLC over silica gel using mixtures of CH_2Cl_2–MeOH (9:1–3:7) and afforded six fractions (A_4–F_4). Fraction D_4 (2.31 g; eluted with CH_2Cl_2–MeOH 7:3) was applied to CC on silica gel with mixtures of CH_2Cl_2–MeOH–H_2O (97:3:0.3–30:7:0.7) and afforded six fractions (A_5–F_5). Fraction D_5 was subjected to CC over Sephadex LH-20 (MeOH) to yield nine fractions (A_6–I_6). Part of fraction E_6 (34.2 mg) was subjected to RP-18 HPLC (ACN/H_2O 85:15) and afforded compounds **42** (3.0 mg; t_R = 36.2 min) and **44** (1.5 mg; t_R = 29.9 min). The isola-

tion of compounds **1**, **2**, **4–6**, **10–15**, **17**, **18**, **20–36** from *M. velutinum* has been previously described.[15–17]

3.4. Enzyme assay

The enzyme assay was performed according to Masamoto[39] with slight modifications. The mushroom tyrosinase and L-DOPA used for the bioassay were purchased from Sigma Chemical Co. Although mushroom tyrosinase differs somewhat from other sources it is widely used by researchers[3] as it is commercially available and inexpensive. Inhibitors in this paper are inhibitors of diphenolase activity of mushroom tyrosinase. All samples were first dissolved in DMSO and used at 30 times dilution (final concentration of DMSO 0.03%). All the tested samples were preliminary assayed at 0.033 μg/mL. It should be noted, however, that some of the flavones and acylated flavone glycosides are hardly soluble in the medium at this concentration. First 0.1 mL of mushroom tyrosinase solution (1375 U/mL, Sigma) was mixed with 1.8 mL of phosphate buffer (pH 6.8) and 0.1 mL of the test solution in the same buffer and incubated at 25 °C for 10 min. Then 1 mL of 0.03% L-DOPA in buffer was added and incubated for 2 min at 25 °C. After incubation the amount of dopachrome in the reaction mixture was measured at 475 nm. The percent inhibition of tyrosinase reaction was calculated as follows:

$$\%\text{Inhibition} = \{[(A-B)-(C-D)]/(A-B)\} \times 100$$

A: optical density at 475 nm without test sample
B: optical density at 475 nm without test sample and enzyme
C: optical density at 475 nm with test sample
D: optical density at 475 nm with test sample, but without enzyme.

Kojic acid was used as positive control. All measurements were carried out in triplicate. The bathochromic shift of phenylethanoid glycosides (0.01 mM) was monitored by adding 0.05 mM $CuSO_4$.

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