PERGAMON

Phytochemistry 55 (2000) 411–417

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Benzylbenzoate and norlignan glucosides from *Curculigo pilosa*: structural analysis and in vitro vascular activity

Giovanna Palazzino [a], Corrado Galeffi [a,*], Elena Federici [a], Franco Delle Monache [b], Maria Francesca Cometa [c], Maura Palmery [c]

[a] *Laboratorio di Chimica del Farmaco, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy*
[b] *CNR, Centro per la Chimica dei Recettori e delle Molecole Biologicamnete attive, Istituto di Chimica, Università Cattolica, L.go F. Vito 1, 00168 Rome, Italy*
[c] *Dipartimento di Farmacologia delle Sostanze Naturali e Fisiologia Generale, Università "La Sapienza", P.le A. Moro 5, 00185 Rome, Italy*

Received 5 August 1999; received in revised form 20 June 2000

Abstract

From the rhizomes of *Curculigo pilosa,* two benzylbenzoate diglucosides, piloside A and piloside B, and a glucosyl-fused norlignan, pilosidine, previously obtained only as the tetra-*O*-methyl derivative, were isolated. Pilosidine showed facilitating effect on adrenaline evoked contractions in rabbit aorta isolated preparations. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Curculigo pilosa*; Hypoxidaceae; Benzylbenzoate glucosides; Norlignan glucosides; Piloside A; Piloside B; Pilosidine; Vascular activity

1. Introduction

Our previous studies on plants of the genera *Hypoxis* and *Curculigo* (Hypoxidaceae) resulted in the isolation of some glucosides with aglycone skeletons Ph–C_5–Ph and Ph–C_3(C_2)–Ph (Nicoletti et al., 1992). They can be considered as norlignans generated by the coupling of two Ph–C_3 units (cinnamic acid and cinnamyl alcohol), in position β-γ′ and α-β′, respectively, with the loss of the terminal carbon atom of the side chain (Whiting, 1985). Thus, hypoxoside, successfully used as an oral pro-drug for cancer therapy (Smit et al., 1995) and patented as antitumoural and to treat viral infections (Liebenberg et al., 1997), was isolated from *Hypoxis obtusa* (Marini-Bettolo et al., 1982), nyasoside from *H. nyasica* (Marini-Bettolo et al., 1985), nyasicoside from *Curculigo recurvata* (Chifundera et al., 1991), interjectin from *H. interjecta* and *H. multiceps* (Marini-Bettolo et al., 1991) and, more recently, curculigine from *C. recurvata* (Chifundera et al., 1994) and shipamanine from *H. obtusa* (Galeffi et al., 1997).

This study examines the constituents of the rhizomes of *Curculigo pilosa*, which was the first African species to be described of the *Curculigo* genus, established by Gaertner (Zimudzi, 1994). The vernacular name of the plant in Benin is "ayoglèn" and it is used to treat epilepsy, sterility, meteorism, stypsis and drepanocytosis (Rossi, 1998).

Two new diglucosides of substituted benzylbenzoates, piloside A, **1**, and piloside B, **2**, and a known monoglucoside, curculigoside, **3**, (Kubo et al., 1983) were isolated from the rhizomes of *C. pilosa,* together with nyasicoside, **4**, curculigine, **5**, and pilosidine, **6**, a new glucosyl-fused norlignan. The physical-chemical data of its tetra-*O*-methyl derivative, **7**, are practically identical to those of tetramethylcurcapicycloside, the name given to the methyl derivative of a substance considered unstable and therefore neither isolated nor characterised as such, occurring in *Curculigo capitulata* (Chang et al., 1999).

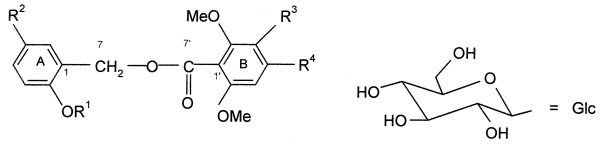
The in vitro vascular activities of the total methanolic extract, the butanolic fraction and the substances isolated from the rhizomes of *C. pilosa* were studied.

2. Results and discussion

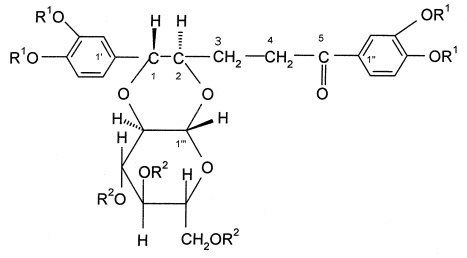
Structure **1** was assigned to piloside A, $C_{28}H_{36}O_{16}$ (ICRMS, m/z 651.18987 $[M+Na]^+$, calcd 651.18955), by 2D NMR spectroscopy. The NMR data, reported in

* Corresponding author. Fax: +39-6-49387100.
E-mail address: galeffi@iss.it (C. Galeffi).

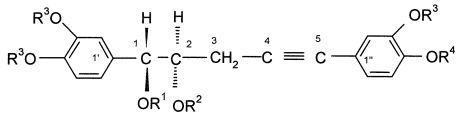
PII: S0031-9422(00)00256-9



	R^1	R^2	R^3	R^4	
1	Glc"	H	OGlc"'	H	piloside A
2	Glc"	H	OGlc"'	OH	piloside B
3	Glc"	OH	H	H	curculigoside
8	Glc"	H	OGlc"'	OMe	*O*-methylpiloside B



	R^1	R^2	
6	H	H	pilosidine
7	Me	H	tetra-*O*-methylpilosidine
9	Me	Ac	



	R^1	R^2	R^3	R^4	
4	H	Glc	H	H	nyasicoside
10	-C(Me)$_2$-		Me	Me	
11	*p*-Br-benzoyl	*p*-Br-benzoyl	Me	Me	
12	H	*p*-OH-cinnamoyl→2Glc	H	Glc	interjectin
5	H	Glc	H	H	-C^{4}H$_2$-C^{5}O- curculigine

Table 1
NMR data of piloside A, **1**[a,b]

Position	δ_C	δ_H (J in Hz)	LR HETCOR	INEPT
1	125.3		H_2-7	
2	155.3			
3	115.2	7.27, *br d* (8.4)		
4	129.5	7.41, *dd* (7.5;8.4)		
5	122.2	7.16, *t* (7.5)		C(1)
6	128.5	7.51, *br d* (7.5)		C(2)
7	62.1	5.52, *s*		C(1), C(2), C(7′)
1′	118.9		H-5′	
2′	147.0			
3′	144.6		H-5′	
4′	119.3	7.37, *d* (9.3)		
5′	107.2	6.87, *d* (9.3)		C(1′), C(3′)
6′	151.2			
7′	165.5		H_2-7	
1″	101.4	4.97, *d* (7.5)		
2″	73.7*	[c]		
3″	77.4**	[c]		
4″	70.0	[c]		
5″	76.8	[c]		
6″	61.1	3.7–3.9		
1‴	102.1	4.91, *d* (7.5)		C(3′)
2‴	73.6*	[c]		
3‴	77.0**	[c]		
4‴	70.0	[c]		
5‴	76.8	[c]		
6‴	61.1	3.7–3.9		
OCH_3(2′)	61.8	3.91, *s*		C(2′)
OCH_3(6′)	56.4	3.86, *s*		C(6′)

[a] In DMSO-d_6.
[b] The values with the same number of asterisks are interchangeable.
[c] In the range δ 3.2–3.6.

Table 1, are the result of the HETCOR spectrum for the protonated carbons and long range HETCOR and selective INEPT experiments which established the C–H and H–C long-range correlations, respectively. The examination of the data suggested the presence of two aromatic rings, of which one, ring A, is *ortho*-disubstituted and the other, ring B, is tetrasubstituted with the two aromatic protons in *ortho* relationship (δ 7.37 and δ 6.87, doublets, J=9.3 Hz). In addition, the ^{13}C NMR spectrum of **1** exhibited signals for an ester (δ_{CO} 165.5), for two glucose units ($\delta_{C\text{anom}}$ 101.4 and 102.1) and for two methoxy groups, of which one, δ_C 61.8, is *ortho, ortho*-disubstituted.

The 2′, 3′, 4′ oxygenation pattern for ring B was discarded because of the absence of any signal in the range 130–135 ppm of the ^{13}C NMR spectrum, as should be expected for an oxygenated carbon on position 3′. The assigned structure for **1** (which is not methylable by diazometane) was confirmed by the hydrolysis with saponification by β-glucosidase which yielded D-glucose, 2-hydroxybenzyl alcohol (saligenin) and 2,6-dimethoxy-3-hydroxy benzoic acid. In agreement with structure **1**, the selective irradiation of the anomeric proton at δ 4.91 by INEPT experiments gave response on the resonance at δ_C 144.6 attributed to C(3′).

Structure **2** was assigned to piloside B, $C_{28}H_{36}O_{17}$ (ICRMS, m/z 667.18498 $[M+Na]^+$, calcd 667.18447), on the basis of the following considerations. Its NMR data (Table 2) are practically identical to those of **1** for ring A (likewise, **2** gave 2-hydroxybenzyl alcohol and glucose by hydrolysis with β-glucosidase) and further suggest the presence of an additional hydroxy group on ring B. Accordingly, by methylation of **2** with diazomethane, a mono-methyl derivative (OMe δ 56.9 or 57.0), **8**, was obtained. The alternative 2′, 3′, 4′, 5′ or 2′, 3′, 5′, 6′ oxygenation patterns for ring B were discarded since the methine (δ 96.2) and one of the four oxygen-bearing carbon (δ 131.8) of **2** are both *ortho,ortho*-dioxygen substituted. A long-range connectivity with the latter carbon, C(3′), was shown by the anomeric hydrogen at δ 4.69 in the selective INEPT experiments. The same hydrogen in *O*-methylpiloside B, **8**, was shifted to δ 4.87 by the sterically close methoxy group. Therefore, piloside B, **2**, is 2-*O*-β-D-glucopyranosyl-benzyl-2,6-dimethoxy-3-*O*-β-D-glucopyranosyl-4-hydroxy-benzoate.

Together with piloside A, **1**, and piloside B, **2**, a known benzylbenzoate monoglucoside, curculigoside, **3**, (Kubo et al., 1983), was isolated from *Curculigo pilosa*. The last substance is the characteristic constituent of the Chinese vegetable drug *C. orchioides* Gaertner (Yamasaki et al., 1994) and its ^{13}C NMR data are first reported.

Likewise three norlignan monoglucosides, pilosidine, **6**, nyasicoside, **4**, (Chifundera et al., 1991) and curculigine, **5**, (Chifundera et al., 1994) and protocatechuic acid were isolated from *C. pilosa*.

Pilosidine, **6**, has molecular formula $C_{23}H_{26}O_{11}$ on the basis of the ICRMS (*m/z* 479.15500 $[M+H]^+$, calcd 479.15479) also in agreement with the number of signals in the ^{13}C proton noise-decoupled NMR spectrum. The ^1H NMR spectrum showed the presence of two 3,4-disubstituted aromatic rings, in one of which H-2 and H-6 are shifted downfield (δ 7.40 and 7.39, respectively) by an *ortho* carbonyl group (IR ν_{co} 1659 cm^{-1} and δ_{CO} 200.9), in agreement with the bathochromic effect on the aromatic UV absorption (see Section 3). By selective hydrogen-hydrogen decoupling, the norlignan sequence Ph–CH(O)–CH(O)–CH_2–CH_2–CO–Ph was established. The remaining carbons presumably belong to an hexose unit (δ_{Canom} 97.3), however, both pilosidine and its tetra-*O*-methyl derivative, **7**, resisted any attempt of enzymatic hydrolysis. The NMR data of the latter substance, as well as the other spectroscopic data, are practically identical to those reported for tetramethylcurcapicycloside, i.e., the methyl derivative of a substance considered unstable, occurring in *Curculigo capitulata* (Chang et al., 1999). The NMR experiments on **7** and its triacetyl derivative, **9**, gave a full account of their structure.

Pilosidine, **6**, at variance with **7**, has shown a facilitating effect on adrenaline evoked contractions and a dose dependent vasoconstricting effect on rabbit aorta (see below).

In the aforementioned paper (Chang et al., 1999), the C(2) stereochemistry of nyasicoside, **4**, has been revised as the irradiation of either methyl group of the 1-2 acetonide of the tetra-*O*-methyl aglucone of nyasicoside, **10**, enhanced the signals of H-1 and H-2, respectively.

Table 2
NMR data of piloside B, **2**[a,b]

Position	δ_C of **2**	δ_H of **2** (*J* in Hz)	LR HETCOR	INEPT
1	125.2		H_2-7	
2	155.1			
3	115.0	7.27, *br d* (8.4)		
4	129.3	7.41, *t* (8.1)		
5	122.0	7.16, *t* (7.2)		
6	128.4	7.50, *br d* (6.6)		
7	61.7	5.50, *d* (12); 5.44, *d* (12)		C(1), C(2)
1′	109.5		H-5′	
2′	151.2		OMe-2′	
3′	131.8		H-5′	
4′	153.4			
5′	96.2	6.48, *s*		C(1′),C(3′), C(4′),C(6′)
6′	152.9		H-5′	
7′	165.4		H_2-7	
1″	101.4	4.97, *d* (7.2)		
2″	73.9*	[c]		
3″	77.4**	[c]		
4″	69.8***	[c]		
5″	76.6****	[c]		
6″	60.9	3.7–3.9		
1‴	105.6	4.69, *d* (7.5)		C(3′)
2‴	73.4*	[c]		
3‴	77.3**	[c]		
4‴	69.7***	[c]		
5‴	76.2****	[c]		
6‴	60.9	3.7–3.9		
OCH_3(2′)	62.2	3.91, *s*		C(2′)
OCH_3(6′)	56.1	3.83, *s*		C(6′)

[a] In DMSO-d_6
[b] The values with the same number of asterisks are interchangeable.
[c] In the range δ 3.2–3.6.

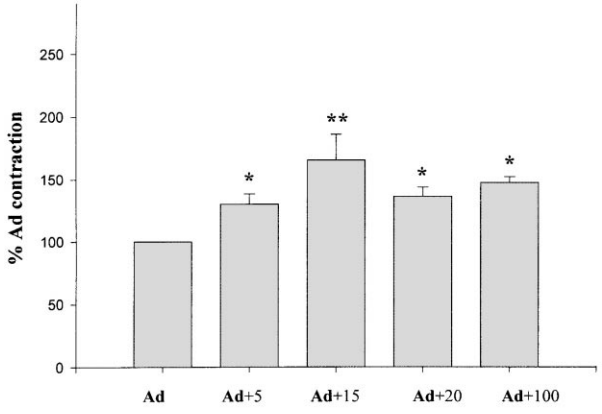


Fig. 1. Facilitating effect of the total extract on adrenaline (**Ad**, $EC_{50} = 1.09 \times 10^{-1}$ μM) induced contractions in rabbit aorta strips. Concentractions are expressed as μg/ml. The values are the mean ±S.E. $*P < 0.1$ $**P < 0.01$ ($n = 10$).

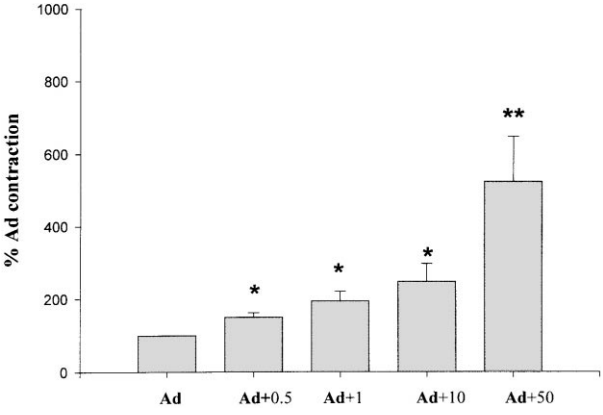


Fig. 2. Facilitating effect of the butanolic fraction on adrenaline (**Ad**, $EC_{50} = 1.09 \times 10^{-1}$ μM) induced contractions in rabbit aorta strips. Concentractions are expressed as μg/ml. The values are the mean ± S.E. $*P < 0.1$ $**P < 0.01$ ($n = 10$).

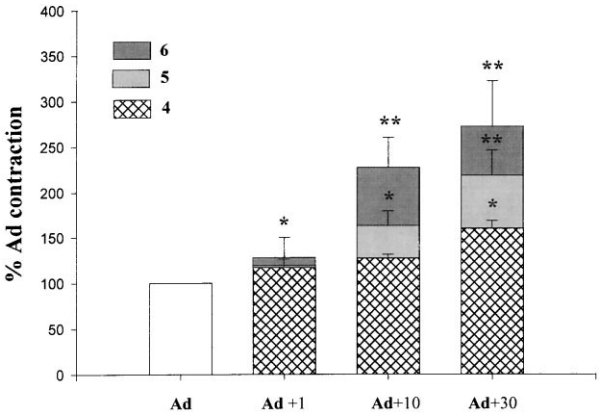


Fig. 3. Facilitating effect of **4**, **5**, **6** on adrenaline (**Ad**, $EC_{50} = 1.09\times10^{-1}$ μM) induced contractions in rabbit aorta strips. Concentrations are expressed as μM. The values are the mean ± S.E. $^{*}P<0.1$ $^{**}P<0.01$ ($n=10$).

This NOE effect is in fact consistent with the *trans*-relationship between H-1 and H-2 and, therefore, with the 1*R*,2*R* configuration, being the 1*R* configuration previously established by circular dichroism. The wrong *S* assignment by one of us (C.G.) for the chirality at C(2) (Chifundera et al., 1991) was due to misunderstanding of the coupling constant $J_{1,2}$ (7.5 Hz) of the bis-*p*-bromobenzoate of the tetra-*O*-methyl aglucone of nyasicoside, **11**, interpreted as a H-1/H-2 *cis*-relationship. This ruled out the very *trans* conformation consistent with the strong negative exciton chirality and the conformational preference for the *gauche* oxygen functions in the di-esters of the 1-phenyl-1,2-butandiols.

The 1*R*,2*R* configuration of nyasicoside, **4**, which must be likewise assigned to interjectin, **12**, (Marini-Bettolo et al., 1991), is, however, identical to that first assigned by us to the biogenetically correlated curculigine, **5**, (Chifundera et al., 1994). They co-occur in *C. recurvata*, *C. pilosa* and *C. capitulata* (Chang et al., 1999).

2.1. In vitro vascular activity

Rhizomes of C*urculigo pilosa* displayed a reversible facilitating action on rabbit aorta strips.

In particular the total extract (at doses from 5 to 100 μg/ml) (Fig. 1), the butanolic fraction (0.5–50 μg/ml) (Fig. 2) and the norlignan glucosides, pilosidine, **6**, nyasicoside, **4**, and curculigine, **5**, (1–30 μM) induced a facilitating effect on adrenaline evoked contractions (Fig. 3). This effect was reversed by the prior administration of nifedipine (5.8×10^{-2} μM), thus suggesting an involvement of Ca^{2+} channels.

Moreover only the butanolic fraction (from 50 to 120 μg/ml) and pilosidine (30–62 μM) showed a dose dependent vasoconstricting effect on rabbit aorta strips, characterised by a delayed onset and long-lasting duration, while the total extract (up to 400 μg/ml), nyasicoside and curculigine (up to 1 mM) did not evoke any direct contraction. In any case the effect is reversible and no phenomenon of tachiphylaxis was observed.

The tetra-*O*-methyl derivative of pilosidine, **7**, previously obtained also from *C. capitulata* (Chang et al., 1999), was quite an inactive in vitro test.

The benzylbenzoate glucosides, piloside A, **1**, piloside B, **2**, and curculigoside, **3**, (1–30 μM), induced a weak and not significative facilitating effect on adrenaline induced contractions, while high doses (up to 10 mM) evoked an irreversible block of contractions (data not shown).

Therefore, as the bioactive norlignan glucosides from *C. capitulata* (Chang et al., 1997), also the norlignan glucosides from *C. pilosa* contribute to the total extract activity on the cardiovascular system, thus suggesting an important role of their cathecol moiety that might possess biological activity related to the structurally similar adrenaline. However, the activity of these glucosides is different in potency and efficacy, pilosidine being the most active among them, thus allowing to envisage a structure–activity relationship. On the contrary, the benzylbenzoate glucosides counteract the total extract activity inducing, at high concentrations, a block of adrenaline contractions.

However, further experiments are needed to ascertain the possible mechanism of action involved on these effects on rabbit aorta.

3. Experimental

A Craig-Post apparatus (200 stages, 10:10 ml, upper and lower phase) was used for the separations performed by counter-current distribution (CCD). Separations were monitored by TLC (silica gel F_{254}, *n*-BuOH:HOAc:H_2O = 4:1:1, system *a*, and EtOAc:HOAc:H_2O = 75:8:8, system *b*; detection by fluorescence quenching and/or by Folin-Ciocalteu). ^{1}H and ^{13}C NMR spectra were recorded on a Varian Gemini 300, observing ^{1}H and ^{13}C at 300 and 75 MHz, respectively. The ^{1}H and ^{13}C chemical shifts are given in ppm relative to TMS (internal standard). ICRMS (ion cyclotron resonance mass spectrometry) spectra were obtained by electrospray ionisation with instrument APEX II 4.7 (Bruker Daltonics, MA, USA), whereas FABMS spectra were obtained with instrument VG 7070 EQ-HF using *m*-nitrobenzyl alcohol as matrix. CD curves were recorded on a Jasco 710. β-Glucosidase is β-glucosidase from sweet almonds, SERVA.

3.1. Plant material

Rhizomes of *Curculigo pilosa* (Schum. et Thonn.) Engl. et Drude were first purchased in the market of Cotonou (Benin) by S. Rossi (Turin) and subsequently provided by Clarisses Capucines, Donaten (Cotonou). The plant was identified in a local herbarium and a

voucher specimen is deposited at Dipartimento di Biologia Vegetale, Università "La Sapienza", Rome.

3.2. Extraction and separation

Air-dried, ground rhizomes (550 g) were extracted three times with MeOH. The residue (47 g) from evaporation of the solutions was dissolved in H_2O (250 ml) and extracted with *n*-BuOH (3×150 ml). The residue from the combined organic phases (18.5 g) was submitted to CCD at portions of 6 g with the biphase system H_2O:EtOAc:*n*-BuOH changing discontinuously the polarity of the upper phase from 10:10:0 to 10:5:5. Five fractions, A–E, were collected. Fraction A was submitted to CCD with the solvent system H_2O:EtOH:EtOAc:cyclohexane from the initial ratio 10:4:4:7 to the final one, 10:4:12:2 (TLC, system *b*). Protocatechuic acid (4 mg), curculigoside, **3**, (40 mg) and pilosidine, **6**, (52 mg) were obtained in the order. Fractions B and C, separately submitted to CCD with the solvent system H_2O:EtOAc:*n*-BuOH (10:8.5:1.5; TLC, system *a*) afforded as pure substances nyasicoside, **4**, (79 mg) and curculigine, **5**, (95 mg), respectively. Fraction D was submitted to CCD with recycling with system H_2O:EtOAc:*n*-BuOH (10:5:5; TLC, system *a*) and piloside A, **1**, $K_r = 0.28$ (240 mg), and piloside B, **2**, $K_r = 0.55$ (80 mg), were obtained. Fraction E was not examined.

Nyasicoside, **4**, curculigine, **5**, and protocatechuic acid were identified by direct comparison.

3.3. Piloside A (*1*)

Crystals from MeOH, mp 150–152°C. $[\alpha]_D^{20} = -50.2$ (MeOH; *c* 0.6). IR (KBr), ν_{co} 1722 cm^{-1}. UV (MeOH), λ_{max} (log ε): 209 (4.37), 279 (3.52), 289 (3.50). Molecular formula $C_{28}H_{36}O_{16}$, ICRMS, *m/z*: 651.18987 $[M+Na]^+$, calcd 651.18955; 489.1444 $[M+Na\text{-}C_6H_{10}O_5]^+$, calcd 489.13673; 383.0971 [M + Na- 2-hydroxybenzyl moiety]$^+$, calcd 383.09486; 221.0442 [M + Na- 2-hydroxybenzyl moiety-$C_6H_{10}O_5]^+$, calcd 221.04204. The substance is negative to Folin-Ciocalteu and is not methylable with CH_2N_2. NMR data in Tab. 1.

3.4. Enzymatic hydrolysis with saponification of piloside A (*1*)

β-Glucosidase (10 mg) was added to a soln of **1** (110 mg) in acetate buffer, pH 5.5 (20 ml). The soln was allowed to stand at 36°C, monitored by TLC with solvent system *b*. After 4 days, the soln was added with few drops of HOAc and extracted with *n*-BuOH. The residue of the organic phase was submitted to CCD with the solvent system H_2O:EtOH:EtOAc:cyclohexane (10:4:7:7) and two fractions positive to Folin-Ciocalteu were isolated and identified as 2-hydroxybenzyl alcohol, 18 mg [^{13}C NMR ($CDCl_3$): 156.1 (C-2), 129.5 (C-6), 127.8 (C-4), 124.5 (C-1), 120.0 (C-5), 116.6 (C-3), 64.7 (CH_2)] and 2,6-dimethoxy-3-hydroxy-benzoic acid, 21 mg [^{1}H NMR (acetone-d_6): 6.89 (1H, *d*, *J* = 8.9 Hz, H-4), 6.66 (1H, *d*, *J* = 8.9 Hz, H-5), 3.83, 3.74 (OMe x 2). ^{13}C NMR (acetone-d_6): 166.9 (COOH), 150.4 (C-6), 145.8 (C-2), 144.8 (C-3), 121.0 (C-1), 117.8 (C-4), 108.3 (C-5), 61.5 (2-OMe), 56.8 (6-OMe)]. The aqueous phase, after evaporation of *n*-BuOH, was percolated through a column of Dowex 50 W(H^+). In the residue, D-glucose was identified by TLC and by the positive test with Glucotest sticks.

3.5. Piloside B (*2*)

Amorphous powder, mp 132–136°C. $[\alpha]_D^{20} = -39.3$ (MeOH, *c* 0.34). IR: ν_{CO} 1724 cm^{-1}. UV (MeOH), λ_{max} (log ε): 215 (4.43), 278 (3.73). Molecular formula $C_{28}H_{36}O_{17}$, ICRMS, *m/z*: 667.18498 $[M+Na]^+$, calcd 667.18447; 645.20266 $[M+H]^+$, calcd 645.20252; 505.1339 $[M+Na\text{-}C_6H_{10}O_5]^+$, calcd 505.13164; 399.0908 [M + Na- 2-hydroxybenzyl moiety]$^+$, calcd 399.08978; 237.0378 [M + Na- 2-hydroxybenzyl moiety- $C_6H_{10}O_5]^+$, calcd 237.03695. The substance is positive to Folin-Ciocalteu reagent. NMR data in Table 2.

3.6. Enzymatic hydrolysis with saponification of piloside B (*2*)

The procedure was identical to that reported for piloside A. 2-Hydroxy-benzyl alcohol and D-glucose were identified. The isolation of the aromatic acid was impossible in this case.

3.7. O-Methylpiloside B (*8*)

Piloside B dissolved in MeOH was added with an ethereal solution of CH_2N_2 (TLC, solvent system *a*). A spot detectable by fluorescence quenching at R_f value lower than **2** was obtained. The substance **8** was purified by CCD with the system H_2O:EtOAc:*n*-BuOH (10:5:5). Amorphous powder, mp 96–98°C. $[\alpha]_D^{20} = -33.7$ (MeOH, *c* 0.5). FABMS (negative ion mode) *m/z* :657 $[M\text{-}H]^-$, corresponding to molecular formula $C_{29}H_{38}O_{17}$. ^{1}H NMR (MeOH-d_4) δ: 7.42 (1H, *br d*, *J* = 7.3 Hz, H-6); 7.30 (1H, *dd*, *J* = 8.2, 7.3 Hz, H-4); 7.21 (1H, *br d*, *J* = 8.2 Hz, H-3); 7.04 (1H, *t*, *J* = 7.3 Hz, H-5); 6.50 (1H, *s*, H-5′); 5.44 (2H, *q*, *J* = 12 Hz, H_2-7); 4.95 (1H, *d*, *J* = 7.1 Hz, H-1″); 4.87 (1H, *d*, *J* = 7.5 Hz, H-1‴); 3.89, 3.83, 3.81 (3H×3, OMe×3); 3.8-3.2 (12H, H-2″-H-5″, H_2-6″, H-2‴-H-5‴, H_2-6‴). ^{13}C NMR (MeOH-d_4) δ: 167.6 (C-7′); 156.8, 156.5, 155.2 (C-2, C-2′, C-4′); 152.6 (C-6′); 133.5 (C-3′); 130.7, 130.6 (C-4, C-6); 126.7 (C-1); 123.3 (C-5); 116.4 (C-3); 108.0 (C-1′); 104.8 (C-1‴); 102.6 (C-1″); 94.6 (C-5′); 78.2, 78.1 (C-3″, C-3‴); 78.0, 77.8 (C-5″, C-5‴); 75.6, 74.9 (C-2″, C-2‴); 71.3 (C-4″, C-4‴); 63.5 (C-7); 63.0 (2′-OMe); 62.5, 62.4 (C-6″, C-6‴); 57.0; 56.9 (4′-OMe, 6′-OMe).

3.8. Pilosidine (**6**)

Crystals from EtOAc, mp 165–167°C. $[\alpha]_D^{20} = +43.2$ (MeOH, *c* 0.84). IR (KBr): ν_{CO} 1659 cm^{-1}. UV(MeOH), λ_{max} (log ε): 230 (4.27), 278 (4.05), 307 (3.84). Molecular formula $C_{23}H_{26}O_{11}$, ICRMS, *m/z*: 479.15500 $[M+H]^+$, calcd 479.15479; 317.10212 $[M+H\text{-}C_6H_{10}O_5]^+$, calcd 317.10196. ^{1}H NMR (DMSO-d_6) δ: 7.40 (1H, *d*, *J* = 1.9 Hz, H-2″); 7.39 (1H, *dd*, *J* = 6.6, 1.9 Hz, H-6″); 6.94 (1H, *d*, *J* = 2.0 Hz, H-2″); 6.81 (2H, *m*, H-6′, H-5″); 6.75 (1H, *d*, *J* = 8.1 Hz, H-5′); 4.79 (1H, *d*, *J* = 8.3 Hz, H-1‴); 4.56 (1H, *d*, *J* = 6.0 Hz, H-1); 4.49 (1H, *ddd*, *J* = 13, 6.0, 3.5 Hz, H-2); 3.87 (1H, *dd*, *J* = 11.8, 1.6 Hz, H_a-6‴); 3.69 (1H, *dd*, *J* = 11.8, 5.0 Hz, H_b-6‴); 3.61 (1H, *dd*, *J* = 9.2, 8.5 Hz, H-3‴); 3.51 (1H, *dd*, *J* = 9.2, 8.1 Hz, H-4‴); 3.4–3.3 (2H, *m*, H-2‴, H-5‴); 3.02 (2H, *m*, H_2-4); 2.05 (1H, *m*, H_b-3); 1.78 (1H, *m*, H_a-3). ^{13}C NMR (DMSO-d_6) δ: 200.9 (C-5); 152.0 (C-4″); 146.4 (C-3″); 146.3 (C-3′); 146.2 (C-4′); 131.8 (C-1′); 130.4 (C-1″);123.0 (C-6″); 120.7 (C-6′); 116.2, 116.1 (C-2′, C-5′); 115.9, 115.8 (C-2″, C-5″); 97.3 (C-1‴); 79.8 (C-2); 79.4 (C-1); 75.9 (C-3‴); 75.5 (C-5‴); 73.7 (C-2‴); 71.8 (C-4‴); 62.5 (C-6‴); 34.6 (C-4); 27.5 (C-3). CD (MeOH): λ nm ($\Delta\varepsilon$): 275 (−0.56), 232 (+2.55).

3.9. Tetra-*O*-methylpilosidine (*7*)

Crystals from MeOH/EtOAc, mp 166–168°C. $[\alpha]_D^{20} = +58.4$ ($CHCl_3$:MeOH = 1:1, *c* 0.5). IR (KBr): ν_{co} 1664 cm^{-1}, UV(MeOH), λ_{max} (log ε): 230 (4.31), 277 (4.04), 307 (3.82). FABMS (negative ion mode) *m/z* 533 [M-H$^-$], corresponding to molecular formula $C_{27}H_{34}O_{11}$. ^{1}H NMR (DMSO-d_6) δ: 7.61 (1H, *dd*, *J* = 8.5, 1.9 Hz, H-6″); 7.41(1H, *d*, *J* = 1.9 Hz, H-2″); 7.06 (1H, *d*, *J* = 8.5 Hz, H-5″); 7.01 (1H, *d*, *J* = 1.9 Hz, H-2′); 6.93 (1H, *d*, *J* = 7.1 Hz, H-5′); 6.70 (1H, *dd*, *J* = 7.1, 1.9 Hz, H-6′); 4.70 (1H, *d*, *J* = 8.2 Hz, H-1‴); 4.66 (1H, *d*, *J* = 5.7 Hz, H-1); 4.51 (1H, *ddd*, *J* = 9.7, 5.7, 4.1 Hz, H-2); 3.84 (overlapped, H_a-6‴); 3.84, 3.80; 3.76, 3.75 (3H×4, OMe×4); 3.7 (1H, *m*, H_b-6‴); 3.6–3.3 (4H, H-2‴-H-5‴); 3.4–3.2 (1H, *m*, H_a-4); 3.0–3.1 (1H, *m*, H_b-4); 1.9–2.3 (1H, *m*, H_a-3); 1.7–1.8 (1H, *m*, H_b-3). ^{13}C NMR (DMSO-d_6) δ: 198.1 (C-5); 153.1 (C-4″); 148.8, 148.7, 148.6 (C-3′, C-3″; C-4′); 132.0 (C-1′); 129.7 (C-1″); 122.7 (C-6″); 120.0 (C-6′); 111.7, 111.6, 111.0, 110.2 (C-2′, C-2″, C-5′, C-5″); 95.7 (C-1‴); 78.6, (C-2); 77.0 (C-1); 74.2 (C-3‴); 73.4 (C-5‴); 72.8 (C-2‴); 70.6 (C-4‴); 61.0 (C-6‴); 55.9, 55.8 (OMe x 2), 55.7 (OMe x 2); 33.5 (C-4); 26.1 (C-3). CD (MeOH): λ nm ($\Delta\varepsilon$): 275 (−1.36), 235 (+2.66).

Pilosidine, **6**, and tetra-*O*-methylpilosidine, **7**, resisted any attempt of hydrolysis with β-glucosidase and cellulase.

3.10. Curculigoside (*3*)

Crystals from EtOAc, mp 158–159°C. $[\alpha]_D^{20} = -26.7$ (MeOH, *c* 0.6). ^{13}C NMR (DMSO-d_6) δ: 165.7 (C-7′); 156.8 (C-2′ and C-6′); 152.5 (C-2); 147.7 (C-5); 131.5 (C-1); 126.9 (C-4′); 117.4 (C-4); 114.9 (C-3); 114.6 (C-6); 115.4 (C-1′); 104.5 (C-3′ and C-5′); 102.8 (C-1″); 77.2 (C-3″); 76.8 (C-5″); 73.6 (C-2″); 70.0 (C-4″); 61.6 (C-7); 61.1 (C-6″); 56.5 (2′-OMe and 6′-OMe).

3.11. Rabbit aorta preparation

New Zealand white rabbits (2–3 kg) were killed by a blow to the back of the head. The descending thoracic aorta was prepared according to Garcia et al. (1985). The tissue was allowed to equilibrate under 5 g basal tension for two hours before isotonic contractions were recorded with a displacement transducer (Ugo Basile 7006) coupled to a pen recorder (Ugo Basile 7050). The Krebs' solution contained NaCl, 104; KCl, 4.7; KH_2PO_4, 1.8; $MgSO_47H_2O$, 1.17; $CaCl_22H_2O$, 2.5; $NaHCO_3$, 25; and Glucose, 5.5 (mM) at pH = 7.4.

3.12. Tested drugs and solutions

(-) Epinephrine [= (-)adrenaline, **Ad**] bitartrate, from Sigma Chemical Co., USA, and the total extract were dissolved in a 50% ethanol stock solution and diluted in distilled water before each experiment.

The butanolic fraction and the glucosides **4**, **5**, **6**, were dissolved in freshly distilled water on the day of experiments.

3.13. Vascular activity determination

Isolated rabbit aorta strips were exposed to concentrations of adrenaline ($EC_{50} = 1.09\times10^{-1}$ μM). Control curves for this mediator were obtained at the beginning of the experiments until two consecutive curves were almost identical. Tissues were then exposed to the total extract or the butanolic fraction or the glucosides for 5 min before the addition of adrenaline to the bath. The results of all experiments are expressed as a percentage of the maximal control contractions. Values are expressed as the mean ± S.E. and the significance between groups were calculated by one way analysis of variance and Student–Newman–Keuls method. The differences between control and experimental values were considered significant at $^*P < 0.1$ and $^{**}P < 0.01$.

Acknowledgements

The authors are greatly indebted to Mr. Silvio Rossi (Turin) for the suggestion of this research and the supplying of the vegetable material, to Dr. G. Baykut and Dr. M. Witt (Bruker Daltonik GmbH, Bremen) for the registration of electrospray ionisation spectra and to Mr. C. De Sena for the recording of the CD curves. Part

of this study was the subject of the thesis in Pharmacy of Miss I. Perugini (24/7/1998).

References

Chang, W.L., Su, M.J., Lee, S.S., 1997. Bioactive norlignan glucosides from *Curculigo capitulata*. Journal of Natural Products 60, 76–80.

Chang, W.L., Chen, C.H., Lee, S.S., 1999. Three novel constituents from *Curculigo capitulata* and revision of C-2 stereochemistry in nyasicoside. Journal of Natural Products 62, 734–739.

Chifundera, K., Messana, I., Galeffi, C., DeVicente, Y., 1991. Research on African medicinal plants. XXV. The (1*R*,2*S*) absolute configuration of nysicoside. Its occurrence in *Curculigo recurvata*. Tetrahedron 47, 4369–4374.

Chifundera, K., Palazzino, G., Messana, I., Liu, P., Galeffi, C., Cannarsa, G., 1994. Research on African medicinal plants. Part 32. Norlignan glucosides from *Curculigo recurvata*. Phytochemistry 35, 1343–1348.

Galeffi, C., Federici, E., Palazzino, G., Nicoletti, M., 1997. Further norlignans from *Hypoxis obtusa* Burch. Gazzetta Chimica Italiana 127, 501–504.

Garcia, R., Thicault, G., Seidah, N.G., Lazure, C., Cantin, M., Genest, J., Chrétien, M., 1985. Structure–activity relationship of atrial natriuretic factor (ANF). II. Effect of chain length modifications on vascular reactivity. Biochemical and Biophysical Research Communications 126, 179–185.

Kubo, M., Namba, K., Nagamoto, N., Nagao, T., Nakanishi, J., Uno, H., Nishimura, H., 1983. A new phenolic glucoside, curculigoside, from rhizomes of *Curculigo orchioides*. Planta Medica 47, 52–55.

Liebenberg, R., Kruger, P.B., Bouic, P.J.D., De Vos Albrech C.F., 1997. Method of treating viral infections and reducing the rate of decrease in CD4 lymphocyte counts with compounds isolated from Hypoxidaceae. US Patent 5609874 A, 11/03/1997.

Marini Bettolo, G.B., Patamia, M., Nicoletti, M., Galeffi, C., Messana, I., 1985. Research in African medicinal plants. II. Hypoxoside, a new glycoside of uncommon structure from *Hypoxis obtusa* Bush. Tetrahedron 38, 1683–1687.

Marini Bettolo, G.B., Nicoletti, M., Messana, I., Galeffi, C., Msonthi, J.D., Chapya, W.A., l985. Research on African medicinal plants. X. Glucosides of *Hypoxis nyasica* Bak. The structure of nyasoside, a new glucoside biologically related to hypoxoside. Tetrahedron 41, 665–670.

Marini Bettolo, G.B., Galeffi, C., Multari, G., Palazzino, G., Messana, I., 1991. Research on African medicinal plants. XXVII. Interjectin, a derivative of nyasicoside from *Hypoxis interjecta* and *Hypoxis multiceps*. Tetrahedron 47, 6717–6724.

Nicoletti, M., Galeffi, C., Messana, I., Marini-Bettolo, G.B., 1992. Hypoxidaceae. Medicinal uses and the norlignan constituents. Journal of Ethnopharmacology 36, 95–101.

Rossi, S., Turin, 1998. Personal communication.

Smit, B.J., Albrecht, C.F., Liebenberg, R.W., Kruger, P.B., Freestone, M., Gouws, L., Theron, E., Bouic, P.J.D., Etsebeth, S., van Jaarsveld, P.P., 1995. A phase I trial of hypoxoside as an oral pro-drug for cancer therapy-absence of toxicity. South African Medical Journal 85, 865–870.

Yamasaki, K., Hashimoti, A., Kokusenya, Y., Miyamoto, T., Matsuo, M., Sato, T., 1994. Determination of curculigoside in curculiginis rhizoma by high-performance liquid chromatography. Chemical and Pharmaceutical Bulletin 42, 395–397.

Whiting, D.A., 1985. Lignans and neolignans. Natural Product Reports 2, 191–211.

Zimudzi, C., 1994. Revision of the genus *Curculigo* (Hypoxidaceae) in the flora Zambesiaca area. Nordic Journal of Botany 14, 311–314.